Study of the role of SSAO/VAP-1 in OGD conditions using SSAO/VAP-1-expressing HUVEC and human brain endothelial cells (hCMEC/D3) as experimental models of ischemic stroke, and its possible nexus with Alzheimer´s disease

Ping Sun

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Universitat Autònoma de Barcelona
Departament de Bioquímica i Biologia Molecular
Facultat de Medicina

Mercedes Unzeta López, professor at Universitat Autònoma de Barcelona, and Montserrat Solé Piñol, postdoctoral researcher at Universitat Autònoma de Barcelona,

CERTIFY:

That the thesis **"Study of the role of SSAO/VAP-1 in OGD conditions using SSAO/VAP-1-expressing HUVEC and human brain endothelial cells (hCMEC/D3) as experimental models of ischemic stroke, and its possible nexus with Alzheimer´s disease"** presented by Ping Sun to obtain the PhD degree in Neurosciences from Universitat Autònoma de Barcelona, has been conducted under the direction of the Institute of Neurosciences and the Department of Biochemistry and Molecular Biology at Universitat Autònoma de Barcelona, which is able to be reviewed and evaluated by the academic committee.

PhD student	Thesis supervisor	Thesis supervisor
Ping Sun	Mercedes Unzeta López	Montserrat Solé Piñol

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INDEX

ABBREVIATIONS

2-BEA	2-bromoethylamine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADMET	Absorption, distribution, metabolism, excretion, toxicity
AIF	Apoptosis inducing factor
AMPA	D, L-α-amino-3-hydroxy-5-methyl-isoxazolpropionic acid
AOC3	Amine oxidase copper-containing 3
AOs	Amine oxidases
Apaf-1	Apoptotic protein-activating factor-1
apoE	Apolipoprotein E
APP	Amyloid precursor protein
Apr	Aprotinin
ASICS	Acid-sensing ion channels
ATCC	American Type Culture Collection
ATF-4 and -6	Activating transcription 4 and 6
ATP	Adenosine triphosphate
Aβ	β-amyloid
$A\beta_{1-40}D$	Dutch-mutated $A\beta_{1-40}$
BACE1	β-site APP-cleaving enzyme 1
BAL	Bronchoalveolar lavage
BB-94	Batimastat
BBB	Blood-brain barrier
Bcl-2	B-cell leukemia/lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Fibroblast Growth Factor-basic
Bid	Bcl-2 interacting domain
BuChE	Butyrylcholinesterase
CAA	Cerebral amyloid angiopathy
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAMs	Cellular adhesion molecules
CAO	Copper containing amine oxidases
CBF	Cerebral blood flow
CHOP	CCAAT/enhancer binding protein homologous protein
CINC	Cytokine-induced neutrophil chemoattractant
CL	Contralateral hemisphere
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CTF	C-terminal fragment

Ctrl	Control
Cytc	Cytochrome c
DAO	Diamine oxidases
Dep	Deprenyl
DISC	Death inducing signalling complex
DMSO	Dimethylsulfoxide
DPH	Donepezil + propargylamine + 8-hydroxyquinoline hybrids
ECE	Endothelium converting enzyme
eIF2α	Eukaryotic initiation factor 2α
eMCAO	embolic MCAO model
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FAD	Flavin adenine dinucleotide
FADD	Fas-associated death domain protein
FasL	Fas ligand
FasR/Fas	Fas receptor
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FLA336	Amiflamine
G418	Geneticine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRP78	Glucose-regulated protein 78
HBSS	Hanks' Balanced Salt Solution
hCMEC/D3	human Cerebral Microvascular Endothelial Cells/D3
HEV	High endothelial venules
HFIP	1,1,1,3,3,3-hexa-fluoro-2-propanol
HIF-1α	Hypoxia inducible factor 1α
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HRP	Horseradish peroxidase
HSECs	Hepatic sinusoidal endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IDE	Insulin-degrading enzyme
IGF-1	Insulin-like growth factor 1
IP	Ipsilateral hemisphere
IRE1	Inositol requiring enzyme 1
IRF1	Interferon regulatory factor 1

IRS-1 and -3	Insulin receptor substrate-1 and -3
JNK	C-Jun amino-terminal kinase
KPI	Kunitz-type protease inhibitor
LDH	Lactate dehydrogenase
LOX	Lysyl oxidases
LPS	Lipopolysaccharide
LRP-1	Low-density lipoprotein receptor protein-1
LTP	Long-term potentiation
LTQ	Lysine tryosylquinone
MA	Methylamine
mAbs	Monoclonal antibodies
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MAO-A and -B	Monoamine oxidase A and B
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral arterial occlusion
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MTDL	Multitarget-directed ligands
MTP	Mitochondrial transition pores
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NEP	Neprilysin
NMDA	N-methyl-D-aspartate
nNOS	Neuronal NOS
Norm	Normoxia
NOS	Nitric oxide synthase
NXY-059	Disodium 2, 4-disulphophenyl-N-tert-butylnitrone
OGD	Oxygen and Glucose Deprivation
PAO	Polyamine oxidases
PARP	Poly ADP ribose polymerase
PBN	N-*tert*-butylnitrone
PBS	Phosphate-buffered saline
PERK	Protein kinase-like endoplasmic reticulum kinase
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3 kinase
PLZ	Phenelzine
PMNs	Polymorphonucluear leukocytes
PMSF	Phenylmethanesulfonylfluoride
PSEN1 and 2	Presenilin 1 and 2
RAGE	Receptor for advanced glycation end products
RBC	Red blood cells

Reox	Reoxygenation
ROS	Reactive oxygen species
rt-PA	Recombinant tissue plasminogen activator
rVAP-1	Recombinant VAP-1
sAPP	Soluble APP fragment
SC	Semicarbazide
sICAM	soluble ICAM-1
Simva	Simvastatin
SMS	Smooth muscle cells
SOD	Superoxide dismutase
SSAO	Semicarbazide sensitive amine oxidase
STAT3	Signal transducer and activator of transcription 3
sVAP-1	Soluble vascular adhesion protein 1
tBid	Truncated Bcl-2 interacting domain
TBN	2-[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-Trimethylpyrazine
Tf Rec	Transferrin receptor
TGF-β	Transforming growth factor beta
TIA	Transient ischemic attack
TJs	Tight junctions
tMCAO	intraluminal transient MCAO model
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor-α
TPQ	2,4,5-trihydroxyphenylalanine quinone
TRAF2	Tumour necrosis factor receptor associated factor 2
TTC	2,3,5- triphenyltetrazolium chloride
UPR	Unfolded protein response
VAP-1	Vascular adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
VD	Vascular dementia
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WT	Wild type
XBP1	X-box-binding protein 1

RESUMEN

La proteína de adhesión vascular 1 (VAP-1) es una proteína pro-inflamatoria que facilita el reclutamiento leucocitario a través de su actividad amino oxidasa sensible a semicarbazida (SSAO, E.C 1.4.3.21). La SSAO plasmática incrementa en pacientes con infarto cerebral o "stroke" isquémico y hemorrágico, y su actividad predice la aparición de hemorragias parenquimales después del tratamiento con tPA en pacientes con stroke isquémico. Además, la SSAO/VAP-1 se encuentra también incrementada en el plasma y tejido cerebral de pacientes con enfermedad de Alzheimer (EA). Así pues, creemos que la SSAO/VAP-1 puede contribuir al daño vascular en ambos stroke y EA. Sin embargo, los mecanismos moleculares por los que la SSAO/VAP-1 participa en el stroke, así como su posible contribución al nexo entre el stroke y la EA no han sido estudiados en detalle.

En este trabajo se ha puesto a punto un modelo de isquemia sencillo usando células endoteliales periféricas que expresan la proteína SSAO/VAP-1 humana (HUVEC hSSAO/VAP-1) en condiciones de deprivación de oxígeno y glucosa (OGD). Gracias a este modelo encontramos que la expresión de la SSAO/VAP-1 incrementa la susceptibilidad de las células endoteliales a la OGD, y que la oxidación de sus sustratos por su actividad enzimática aumenta el daño en células vasculares. Las caspasas 3 y 8 se activan durante esta muerte celular. Además, la OGD constituye un estímulo para la liberación de la SSAO/VAP-1 soluble, que depende parcialmente del corte ejercido por la metaloproteinasa 2. También una OGD corta induce la unión de leucocitos al endotelio dependiente de la SSAO/VAP-1, que es parcialmente mediada por su actividad enzimática.

Con el fin de evaluar mejor los efectos beneficiosos de nuevos compuestos farmacéuticos inhibidores de la actividad SSAO/VAP-1 en condiciones de isquemia cerebral, generamos una línea celular endotelial cerebral humana que expresa la SSAO/VAP-1 humana (hCMEC/D3 hSSAO/VAP-1), como modelo de barrera hematoencefálica (BHE). Las condiciones óptimas de OGD fueron establecidas también con estas células. Mediante el uso de las células HUVEC y hCMEC/D3 que expresan la SSAO/VAP-1 probamos que el DPH-4, un nuevo compuesto multidiana diseñado para la terapia de la EA, es capaz de proteger ambas células endoteliales y de disminuir la adhesión leucocitaria dependiente

de SSAO en condiciones de OGD con reoxigenación. El DPH-4 también fue efectivo contra el daño inducido por la OGD con reoxigenación en presencia de beta amiloide, como modelo de patología de EA.

Para determinar los mecanismos moleculares subyacentes a los efectos beneficiosos de la simvastatina en el stroke isquémico, utilizamos las células hCMEC/D3 que expresan la SSAO/VAP-1 en condiciones de OGD, así como dos modelos animales de oclusión de la arteria cerebral media (MCAO). Los resultados revelaron que la simvastatina es capaz de impedir la liberación de la SSAO/VAP-1 soluble al plasma o al medio de cultivo celular, la cual induce la expresión de las moléculas de adhesión E-selectina y VCAM-1, y amplifica la inflamación y el daño subsiguiente en el cerebro infartado.

Finalmente, las células hCMEC/D3 que expresan la hSSAO/VAP-1 fueron usadas en el estudio del posible papel de la SSAO/VAP-1 en el nexo existente entre el stroke y la EA. Resultados preliminares mostraron que, en las células que expresan la SSAO/VAP-1 sometidas a OGD y reoxigenación, se induce la expresión de BACE1 así como una disminución de LRP-1, y que el sustrato de la SSAO/VAP-1 es capaz de incrementar los niveles de APP en una situación de OGD con reoxigenación. Además, el metabolismo de la metilamina por la actividad SSAO/VAP-1 induce una muerte celular adicional cuando se co-trata con $A\beta_{1\text{-}40}D$, en condiciones de OGD y reoxigenación.

En resumen, de estos resultados se concluye que la expresión de la SSAO/VAP-1 en células endoteliales puede incrementar el daño celular asociado a la OGD, y que la OGD induce la liberación de la SSAO/VAP-1 soluble. También, que la oxidación de su sustrato media parte del daño tisular y que la adhesión leucocitaria dependiente de la actividad SSAO/VAP-1 agrava la progresión de la patología aumentando la inflamación en la isquemia cerebral. La inhibición de la actividad SSAO/VAP-1 por el DPH-4 puede aportar un beneficio terapéutico para el retraso y/o prevención del stroke isquémico, así como para su progresión a la EA. La modulación de los niveles de SSAO/VAP-1 media parte de los efectos beneficiosos de la simvastatina en la isquemia cerebral. Además, la presencia de SSAO/VAP-1 en el endotelio cerebral puede facilitar la generación de β-amiloide, incrementando así el riesgo y el empeoramiento neurológico de la EA.

ABSTRACT

Vascular adhesion protein 1 (VAP-1) is a pro-inflammatory protein that mediates leukocyte recruitment through its semicarbazide-sensitive amine oxidase (SSAO, E.C 1.4.3.21) activity. Plasmatic SSAO increases in ischemic and in hemorrhagic stroke patients, and its activity predicts the appearance of parenchymal hemorrhages after tPA treatment in ischemic stroke patients. Moreover, SSAO/VAP-1 is also increased in AD patients' plasma and brain tissue. Hence, we believe that SSAO/VAP-1 could contribute to the vascular damage in both stroke and AD. However, the molecular mechanisms of SSAO/VAP-1 in stroke and its possible contribution to the nexus of ischemic stroke and AD have not been studied in detail.

In this work, an easy ischemic model was set up by using peripheral endothelial cells expressing the human SSAO/VAP-1 protein (HUVEC hSSAO/VAP-1) under oxygen-glucose deprivation (OGD) conditions. Based on this model, it was found that SSAO/VAP-1 expression increases the susceptibility of endothelial cells to OGD, and that its substrates oxidation through its enzymatic activity increases the vascular cell damage. Caspase-3 and caspase-8 are activated during the death process. In addition, OGD constitutes a stimulus for the soluble SSAO/VAP-1 release, partly mediated by metalloproteinase-2-dependent shedding. Also, short-time OGD induces SSAO/VAP-1-dependent leukocyte binding on endothelial cells, which is partly dependent on its enzymatic activity.

In order to better evaluate the beneficial effects of new pharmaceutical compounds by SSAO/VAP-1 activity inhibition under cerebral ischemia conditions, a human brain endothelial cell line expressing the human SSAO/VAP-1 (hCMEC/D3 hSSAO/VAP-1) was further generated as a model of the brain blood barrier (BBB). OGD conditions were established with these cells as well. By using hSSAO/VAP-1 HUVEC and hCMEC/D3 cells, a novel multitarget-directed ligand (MTDL) DPH-4, designed for AD therapy, was proved able to protect both endothelial cells, as well as to decrease the SSAO-dependent leukocyte adhesion under OGD with reoxygenation. DPH-4 was also effective

against the damage induced by OGD and reoxygenation in the presence of beta amyloid as a model of AD pathology.

With regard to determine the molecular mechanisms underlying the beneficial effect of simvastatin on ischemic stroke, hCMEC/D3 hSSAO/VAP-1 cells subjected to OGD conditions and two middle cerebral arterial occlusion (MCAO) rat models were used. Results revealed that simvastatin could suppress the release of soluble SSAO/VAP-1 into the plasma or cell culture media, which induces the expression of the adhesion molecules E-selectin and VCAM-1, and amplifies the inflammation and the consequent damage in the infarcted brain.

At last, hCMEC/D3 hSSAO/VAP-1 cells were used so as to study the possible role of SSAO/VAP-1 in the nexus between ischemic stroke and AD. Preliminary results showed that in SSAO/VAP-1-expressing cells, OGD with reoxygenation induces the expression of BACE1 and decreases the expression of LRP-1, and that the substrate of SSAO/VAP-1 can further up-regulate the levels of APP under OGD with reoxygenation. Furthermore, the metabolism of methylamine by SSAO/VAP-1 activity induces additional cell death when co-treated with $A\beta_{1-40}D$ under OGD with reoxygenation.

In summary, these results conclude that the expression of SSAO/VAP-1 in endothelial cells can increase the OGD-associated cell damage. OGD induces soluble SSAO/VAP-1 release. The oxidation of its substrate mediates part of the tissue damage. SSAO/VAP-1 activity-dependent leukocyte binding further exacerbates the disease progression by augmenting inflammation in cerebral ischemia. The inhibition of SSAO/VAP-1 activity by DPH-4 can provide a therapeutic benefit to the delay and/or prevention of ischemic stroke as well as its progression to AD. The modulation of the SSAO/VAP-1 levels mediates part of the beneficial effect of simvastatin on cerebral ischemia. In addition of ischemic condition, the presence of SSAO/VAP-1 in brain endothelium may facilitate the generation of β-amyloid, hence increasing the risk and neurological worsening of AD.

I. INTRODUCTION

I. INTRODUCTION

1. Semicarbazide-sensitive amine oxidase (SSAO)

1.1 The semicarbazide-sensitive amine oxidases family

The amine oxidases (AOs) from mammalian tissues are a family of enzymes that catalyses the deamination of a wide range of endogenous and xenobiotic monoamines, diamines or polyamines by their oxidative activities. Based on their chemical nature of the attached cofactors, the amine oxidases can be divided into two main groups: the flavin adenine dinucleotide (FAD)-containing enzymes (EC 1.4.3.4) and the quinone-containing enzymes (EC 1.4.3.6). The FAD-containing AOs include monoamine oxidase A and B (MAO-A and -B) and the intracellular form of polyamine oxidase (PAO) (Gong *et al.*, 2006). MAO-A and MAO-B are located in the outer mitochondrial membrane and are involved in the metabolism of neurotransmitters and other biogenic amines (Lyles, 1996; McGuirl *et al.*, 1999; Jalkanen *et al.*, 2001). PAO contributes to the maintenance of polyamine homeostasis and possibly regulates the cell growth (Seiler, 1995). For the second class of amine oxidases, instead of containing FAD cofactors, they possess copper and one or more carbonyl groups, named 2,4,5-trihydroxyphenylalanine quinone (TPQ) or lysine tryosylquinone (LTQ) groups (Finney *et al.*, 2014), making them sensitive to be inhibited by carbonyl reagents such as semicarbazide. Thus, these enzymes can be together labelled as semicarbazide-sensitive amine oxidases (SSAO) (Jalkanen *et al.*, 2001; Gong *et al.*, 2006).

The quinone-containing SSAOs, or copper-containing amine oxidases (CAOs) include diamine oxidase (DAO), lysyl oxidase (LOX) and plasma membrane and soluble semicarbazide-sensitive amine oxidase (SSAO). All SSAOs are able to catalyse the deamination of primary amines, according to the following reaction (Klinman *et al.*, 1994; Wilmot *et al.*, 1999):

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2.$$

DAO is an intracellular enzyme metabolizing putrescine and cadaverine as preferred diamines. This enzyme can also deaminate histamine, being thus involved in regulating inflammation and allergic reactions (Lyles, 1996; Jalkanen *et al.*, 2001). LOX possess lysine tryosylquinone (LTQ) as the cofactor, which metabolizes the lysine residues in the side chain amino groups in collagen and elastin, participating in the peptide crosslinking and in the extracellular matrix formation of connective tissue (Lyles, 1996).

The rest of the SSAOs are extracellular enzymes mainly present on the cell surface or in a soluble form in most mammals with diverse biological functions. In humans, the most abundant of these SSAOs, also named as amine oxidase copper-containing 3 (AOC3), vascular adhesion protein-1 (VAP-1) (EC 1.4.3.21) (Smith *et al.*, 1998; O'Sullivan *et al.*, 2004), or more recently, primary amino oxidase (PRAO) (Finney *et al.*, 2014), has been the most extensively studied enzyme among three human CAOs. A concise classification of semicarbazide-sensitive amine oxidases family has been shown as follows (see Fig 1). Although DAO and LOX also belong to the SSAO family, this work is focused on the primary amine oxidase SSAO/VAP-1.

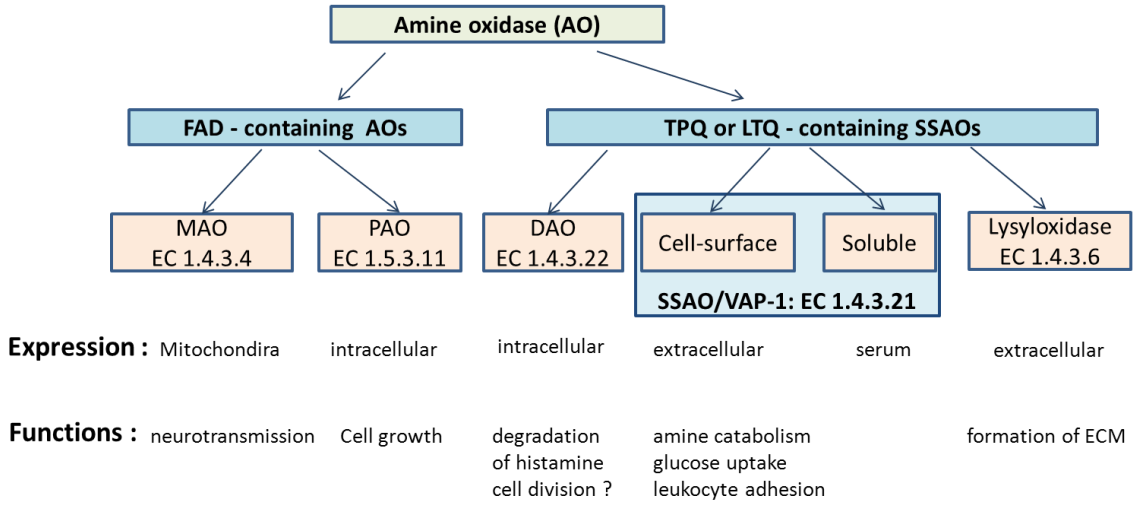


Fig 1. The classification of amine oxidases (AO) and semicarbazide-sensitive amine oxidases (SSAO) families. Abbreviations: FAD, flavin adenine dinucleotide, TPQ, 2,4,5-

trihydroxyphenylalanine quinone, LTQ, lysine tryosylquinone, MAO, monoamine oxidase, PAO, polyamine oxidase, DAO, diamine oxidase (Jalkanen *et al.*, 2001).

1.2 Substrates of SSAO

SSAO can metabolize primary aliphatic and aromatic amines, including physiological amines (endogenous) and non-physiological amines (xenobiotic). A number of SSAO substrates and their specificities are shown as follows (see Fig 2).

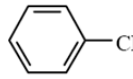
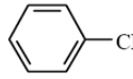
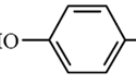
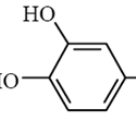
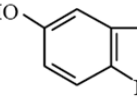
SUBSTRATES	COMMENTS
CH_3NH_2 Methylamine	Endogenous and xenobiotic. Not a substrate for MAO
$CH_2{=}CHCH_2NH_2$ Allylamine	Xenobiotic. Not a substrate for MAO. Highly toxic product
$CH_3{-}\overset{\overset{O}{\|}}{C}C{-}H_2NH_2$ Aminoacetone	Endogenous. Not a substrate for MAO
$CH_3(CH_2)_3CH_2NH_2$ *n*-Pentylamine	Xenobiotic. Also MAO-B substrate
 CH_2NH_2 Benzylamine	Xenobiotic Also MAO-B substrate
 $CH_2CH_2NH_2$ 2-Phenethylamine	Trace amine Also MAO-B substrate
HO  $CH_2CH_2NH_2$ Tyramine	Endogenous & xenobiotic Also MAO A & B substrate
HO HO  $CH_2CH_2NH_2$ Dopamine	Endogenous Also MAO A & B substrate
HO  $CH_2CH_2NH_2$ 5-Hydroxytryptamine	Substrate in dental pulp. MAO-A substrate
$CH_2CH_2NH_2$ Mescaline CH_3O OCH_3 OCH_3	Xenobiotic. Also MAO substrate
CH_3 $NHCH(CH_2)_3NH_2$ Primaquine CH_3O	Xenobiotic. Also MAO substrate

Fig 2. SSAO substrates with their structures and properties (O'Sullivan *et al.*, 2004).

Endogenous SSAO substrates include methylamine, aminoacetone, 2-phenylethylamine, tyramine and dopamine, etc. (O'Sullivan *et al.*, 2004). Many SSAO substrates can be also oxidatively deaminated by MAO, but the physiological ones methylamine and amiocetone are not MAO substrates. With the oxidative deamination of SSAO, methylamine and aminoacetone are catalysed into formaldehyde and methylglyoxal, respectively, as well as hydrogen peroxide and ammonia. In the human body, methylamine is found in large amounts in urine, and it is present in blood and tissues as well (Yu *et al.*, 2003). Methylamine can be derived from the metabolism of epinephrine (Schayer *et al.*, 1952), adrenaline (Dar *et al.*, 1985), creatine, creatinine (Jones *et al.*, 1975; Yu *et al.*, 2000), sarcosine and choline (Zeisel *et al.*, 1983; Precious *et al.*, 1988; Yu *et al.*, 2003). Methylamine can be also originated from the digestion of food and drink or inhaled cigarette smoke (Yu *et al.*, 1996b). Aminoacetone is a product of glycine and threonine metabolism (Bird *et al.*, 1984). Since its physiological substrates are still under dispute, it has been predicted that a free amino group of an amino acid might also serve as substrate of SSAO (Salmi *et al.*, 2001a). In this context, siglec-9 present on the surface of granulocytes and siglec-10 present on the surface of lymphocytes are the first identified ligands that could serve as endothelial SSAO substrates participating in the SSAO/VAP-1-mediated transmigration of leukocytes during inflammation (Kivi *et al.*, 2009; Aalto *et al.*, 2011).

Xenobiotic SSAO substrates include allylamine, n-phetylamine and benzylamine etc. (Tipton *et al.*, 2001). Although benzylamine is a non-physiological amine and can be also metabolized by MAO-B, it is a particular good substrate for SSAO. Therefore, the determinations of SSAO activity are also based on the ability of these enzymes to convert benzylamine to benzaldehyde, ammonia and hydrogen peroxide with the addition of specific MAO-B inhibitors, with the radiochemical method by using [^{14}C]-benzylamine hydrochloride as substrate (Gella *et al.*, 2013). There are also sensitive and efficient fluorometric determinations of SSAO activity by using HPLC (van Dijk *et al.*, 1995).

1.3 Inhibitors of SSAO

To elucidate the functions of SSAO, the inhibition of its activity is an ideal way to follow. However, the main problem to do that has been the lack of selective and specific inhibitors with excellent potency, because many SSAO inhibitors can also inhibit other amine oxidases, but the acetylenic inhibitors, such as clorgyline and L-deprenyl, used for MAO inhibition show insensitivity for SSAO. There are a high number of compounds that exhibit both SSAO and MAO inhibition properties. α-methyl substituted monoamines, like mexiletine or amphetamine are reversible and competitive inhibitors for SSAO, which are also good MAO (A and B) inhibitors (Kinemuchi *et al.*, 2004). Amiflamine (FLA 336) and its metabolites [FLA 788(+) and FLA 668(+)] inhibit SSAO activity, but they are selective MAO-A inhibitors (Kinemuchi *et al.*, 1983; Morikawa *et al.*, 1986; Lyles, 1996); MD 780236 is also SSAO inhibitor, but it is a selective MAO-B inhibitor as well (Strolin-Benedetti *et al.*, 1982). The hydrazine derivative semicarbazide is a sensitive inhibitor for SSAOs that distinguishes the FAD-containing amine oxidases, but with a narrow sense of definition, it could as well interact with DAO and LOX. Other hydrazine derivatives including phenelzine, phenylhydrazine, hydrallazzine, benzerazide and carbidopa are also SSAO inhibitors (Kinemuchi *et al.*, 2004), but some of them are MAO inhibitors as well. The haloallylamine derivative, such as MDL72145, has shown to be an irreversible SSAO inhibitor in spite of its selective and irreversible inhibition against MAO-B (Zreika *et al.*, 1984; Lyles *et al.*, 1987b; Kinemuchi *et al.*, 2004).

There are also some compounds which show relatively more selectivity and competitiveness against SSAO than MAO activities. Semicarbazide is a selective inhibitor for SSAOs, as previously described. Propargylamine and hydroxylamine can selectively inhibit SSAO activity at relatively low concentrations compared to MAO activity (Kinemuchi *et al.*, 2004). A haloamine 2-bromoethylamine (2-BEA) has been found to be a highly selective, potent and irreversible SSAO inhibitor with no inhibitory effect on both MAO-A and -B (Kinemuchi *et al.*, 2000). The drugs procarbazide and monomethylhydrazine seem to be reversible and

selective inhibitors of SSAO *in vitro* and *in vivo*, but there may have different sensitivities of SSAO inhibition among different species and tissues (Kinemuchi *et al.*, 2004; O'Sullivan *et al.*, 2004).

Since the deamination of SSAO substrates can generate potentially hazard products, the reduction of SSAO activity may be beneficial in some pathological conditions. Therefore, a highly selective and potent SSAO inhibitor, which is yet to be discovered, could be helpful for the SSAO-related research and medical application.

1.4 Distribution of SSAO

SSAO is present in a membrane-bound form in a variety of mammalian tissues as well as in a soluble form in the plasma (Lyles, 1996).

1.4.1 Membrane-bound SSAO

High enzyme activity of SSAO has been associated with the existence of the membrane-bound SSAO in the blood vessels and there, it has been proved to exist in smooth muscle cells in tunica media (Lewinsohn, 1983) and also in endothelial cells (Salmi *et al.*, 1993). It is also present in non-vascular smooth muscle cells in uterus, ureter, vas deferens (LEWINSOHN, 1981), and other tissues such as white and brown adipocyte (Barrand *et al.*, 1984), lung (O'Sullivan *et al.*, 2004), liver and others. Notably, in human brain, although SSAO is absent from the neurons and glial cells in the central nervous system (CNS), it has been confirmed to be present in cerebral meningeal vessels and microvessels (Castillo *et al.*, 1998a), hence its presence there may be related to the blood-brain barrier (BBB) function. However, the distribution of the membrane-bound SSAO in other species may be different from that in humans. Chondrocytes (Lyles *et al.*, 1987a) and odontoblasts (Norqvist *et al.*, 1989), for example, express SSAO in some animals, but it is absent in those from humans (Jalkanen *et al.*, 2001). In humans, molecular modelling studies have proved that SSAO/VAP-1 is a 180 kDa homodimeric glycoprotein comprising two identical monomeric subunits of 90 kDa, and this glycoprotein is constituted by a short

membrane-spinning domain and three extracellular copper-containing amine oxidase domains with the active catalytic site (Salminen *et al.*, 1998). The structure of SSAO/VAP-1 has been identified and it is shown as follows (see Fig 3).

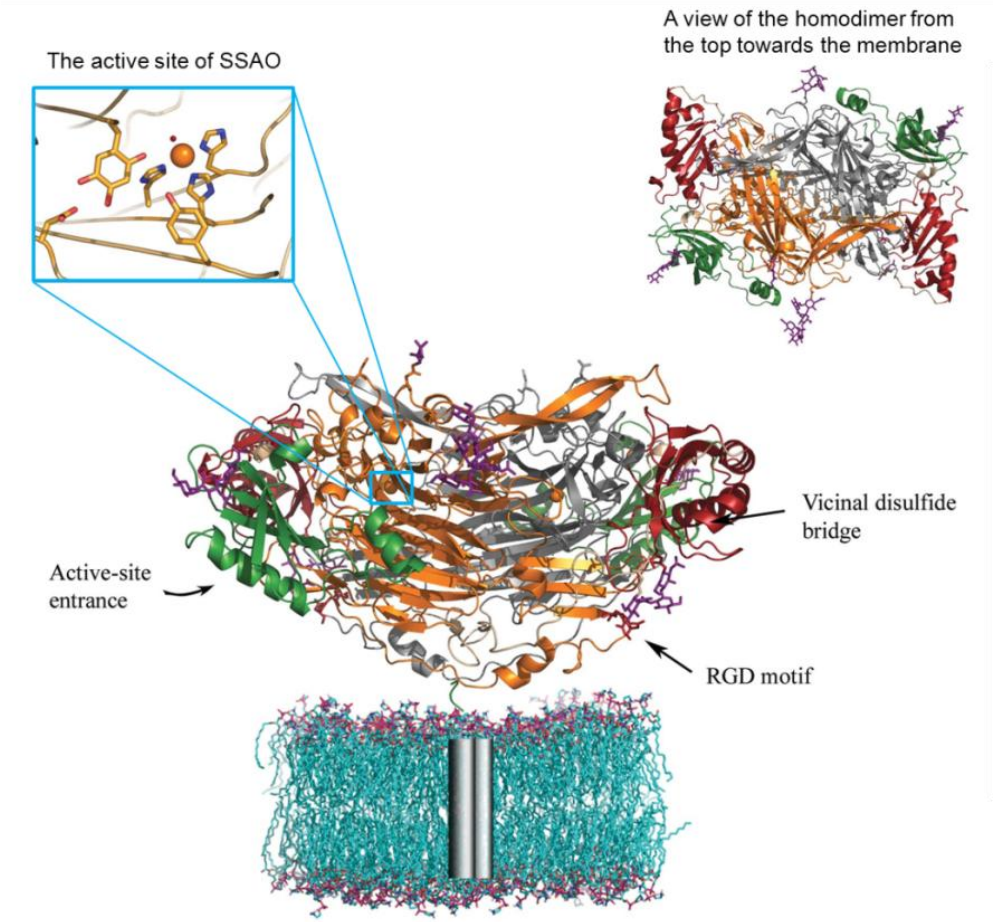


Fig 3. Structure of human SSAO/VAP-1. Domains D2, and D3 are shown in green and red, respectively. Domain D4 is shown in grey (monomer A) or orange (monomer B), and carbohydrates are shown as purple sticks. The membrane-bound helices are shown as the grey cylinders inside the lipid bilayer (Jakobsson *et al.*, 2005).

1.4.2 Soluble SSAO

The presence of serum SSAO activity was first defined in horse (Bergeret *et al.*, 1957), and soluble SSAO has been found to exist in various species of

mammalian plasma (Lyles, 1996). Soluble SSAO activity varies considerably between species (Boomsma *et al.*, 2000), and soluble SSAO enzymes exist among different species are also different, for example, there are four kinds of porcine SSAO enzymes encoded by four different AOC genes, AOC1 encodes diamine oxidase (DAO), AOC2 retina-specific amine oxidase (RAO), AOC3 SSAO/VAP-1 and AOC4 a VAP-1 homologue expressed mainly in liver. However, humans and rodents lack a functional AOC4 gene, thus low levels of this type of amine oxidase may probably derived from partial proteolytic release of membrane-bound SSAO/VAP-1 (Schwelberger, 2007). In humans, soluble SSAO exists in the serum of healthy adults, and it is believed to be formed by a proteolytic cleavage from the membrane-bound SSAO, because the N-terminal amino acid sequence of soluble SSAO is identical to the proximal membrane-spanning region of the membrane-bound SSAO (Kurkijärvi *et al.*, 1998). However, there is also the possibility that an independent gene encodes the soluble SSAO/VAP-1. Although there is firm evidence that liver is a major source of soluble SSAO (Kurkijärvi *et al.*, 1998), it has also been suggested that, at least in part, SSAO in the blood stream may be derived from bone tissue (Ekblom *et al.*, 1999). Molecular characterization revealed also that soluble SSAO/VAP-1 accounts for most of, if not all, the soluble species of SSAO in the human serum (Kurkijärvi *et al.*, 2000). Latterly, by using a transgenic mouse model created with overexpressing the full-length human VAP-1 expressed on smooth muscle cells, endothelial or adipose tissues, and VAP-1 knockout mice, researchers demonstrated that VAP-1 is the only source of serum SSAO. Thus, circulating SSAO/VAP-1 can be derived from the human VAP-1-encoding gene, and the endothelium is a major source of circulating SSAO under physiological conditions, whereas serum SSAO/VAP-1 can be originated from smooth muscle cells (Gokturk *et al.*, 2003), endothelial cells and adipocytes (Stolen *et al.*, 2004) under pathological conditions.

Further experiments gave evidences that soluble SSAO/VAP-1 is released by the shedding of the membrane-bound form by a matrix metalloproteinase (MMP)-dependent process, since a broad-spectrum metalloproteinase inhibitor,

batimastat, could block the release of soluble SSAO/VAP-1 (Abella *et al.*, 2004). However, given the fact that a various cellular source, such as brain endothelium, astrocytes, neurons and inflammation-activated neutrophils, can secrete different types of MMPs, which or which kinds of MMPs participate in the soluble SSAO shedding process is still to be known.

1.5 Functions of SSAO in physiological conditions

Although the precise functions of SSAO have not yet been well elucidated, there are some functions that have been described in the literature under physiological conditions, related with the protection against endogenous/ xenobiotic amines, the local generation of the signalling molecule hydrogen peroxide, glucose transportation, protein cross-linkage and leucocyte trafficking (Boomsma *et al.*, 2003).

1.5.1 Metabolic functions

As the first described enzymatic function of SSAO, it is involved in the removal or the scavenging of physiologically active endogenous and xenobiotic amines in pharmacological concentrations. Since the endogenous levels of some SSAO substrates are altered under different pathological conditions, the physiological roles of them may be changed as well. Methylamine has been reported to target the voltage-operated neuronal potassium channel, thus it probably modulates the release of neurotransmitters (Pirisino *et al.*, 2005). Its plasma and urinary levels, for example, increase in some pathological disorders such as diabetes, typhoid fever, liver or renal insufficiency. The deamination of methylamine by SSAO can reduce its biological activity, however, this action could result in the overproduction of formaldehyde in tissues with high SSAO activity, especially in blood vessels (Yu *et al.*, 1996a; Matyus *et al.*, 2004). Moreover, it has been suggested that SSAO contributes to the elimination of the inhaled volatile aliphatic amines (Lizcano *et al.*, 1996).

All the products of SSAO catalytic deamination are biologically active, and although they are potentially toxic at higher concentrations, they may have some

important roles at more physiological concentrations. In this regard, probably the most interesting product is hydrogen peroxide (H_2O_2), since it possesses some signalling roles at low concentrations. It can act as intracellular second messenger to participate in the ligand stimulation, regulate cell growth and death, and activate NF-κB signalling pathways. Consequently, the activation of NF-κB would modulate the expression of many other genes, including chemokines, cytokines, metalloproteinases and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) (Kunsch *et al.*, 1999; Jalkanen *et al.*, 2001). It has also been demonstrated that H_2O_2 can activate mitogen-activated protein kinase (MAPK) as well as the c-Jun amino-terminal kinase (JNK) (Finkel, 1998). Furthermore, immunohistochemical studies indicate that in most human peripheral tissues, SSAO localizes together with the oxidative deamination of monoamines and therefore, SSAO might participate in the regulation of physiological processes through H_2O_2 generation (Andrés *et al.*, 2001).

SSAO activity has also been reported to play an important role in the differentiation and maturation of some cell types like adipocytes. Methylamine, or other SSAO substrates can promote, in a time- and dose-dependent manner, the maturation of 3Y3-L1 pre-adipocytes, which do not express SSAO at the immature state. SSAO inhibitor semicarbazide can antagonize the maturation of these adipocytes. Treatment of catalase reverted the maturation triggered by substrates indicating the important role of hydrogen peroxide generated by SSAO activity (Mercier *et al.*, 2001).

1.5.2 Glucose uptake

In mammals, membrane-bound SSAO has been found abundantly expressed in adipocytes, vascular smooth muscles cells and endothelial cells from a subgroup of vessels like skin, brain, kidney, liver and heart (Salmi *et al.*, 1993). Immunoisolation and immunotitration analysis indicated that SSAO co-localizes with intracellular glucose transporter type 4 (GLUT4), the most effective glucose transporter in adipocytes (Fischer *et al.*, 1997), and in the endosomal vesicles of rat adipocytes as well. The SSAO substrate benzylamine could stimulate the

glucose transport in isolated rat adipocytes in the presence of vanadate, and SSAO inhibitor semicarbazide could totally abolish this stimulation. This implicates the role of SSAO activity in glucose transport (Enrique-Tarancón *et al.*, 1998). Further detailed investigations revealed that the combination of benzylamine and vanadate induced the recruitment of GLUT4 from the intracellular storage pool to the plasma membrane, and hydrogen peroxide produced during the SSAO catalytic reaction regulates the GLUT4 trafficking, hence enhancing the glucose uptake in an insulin-simulated way. Later on it was found that, besides benzylamine, other SSAO substrates could stimulate glucose transportation as well when combined with vanadate, through a potent tyrosine phosphorylation of insulin receptor substrate-1, -3 (IRS-1 and IRS-3) and phosphoinositide 3-kinase activation in rat adipocytes, without altering the plasma levels of SSAO (Enrique-Tarancon *et al.*, 2000). Afterwards, in isolated human adipocytes without the presence of vanadate, SSAO oxidation of benzylamine and methylamine could dose-dependently stimulate glucose transport in a semicarbazide-sensitive manner (Morin *et al.*, 2001). A similar action was also found for vascular smooth muscle cells derived from rat aortic media (El Hadri *et al.*, 2002).

In fact, SSAO-mediated stimulation of glucose transport could also be achieved by MAO catalysed deamination, since the hydrogen peroxide generated during its catalytic reaction plays the central role for glucose uptake. However, because of the abundant existence of SSAO in adipocytes compared with other MAOs, together with fact that it can as well colocalize with GLUT4 in the intracellular endosomal vesicles, it may indicate a more important role of this enzyme in this regard (O'Sullivan *et al.*, 2004).

1.5.3 Cell adhesion function

Despite the functions mediated by the traditional SSAO enzymatic activity, another investigation surprisingly found that SSAO behaves as an adhesion protein, which is involved in the leukocyte trafficking function. The extravasation process of leukocytes has been well defined by a multistep adhesion cascade

(Springer, 1994; Butcher *et al.*, 1996; Salmi *et al.*, 1997a). By receiving the appropriate activation signals, the leukocytes would adhere to the endothelium after tethering and rolling along the endothelial luminal surface, and ultimately transmigrate through the endothelium to the inflamed tissue and execute the immune functions. Multiple adhesion and signalling molecules are involved in the extravasation cascade (see Fig 4), including vascular adhesion protein-1 (VAP-1).

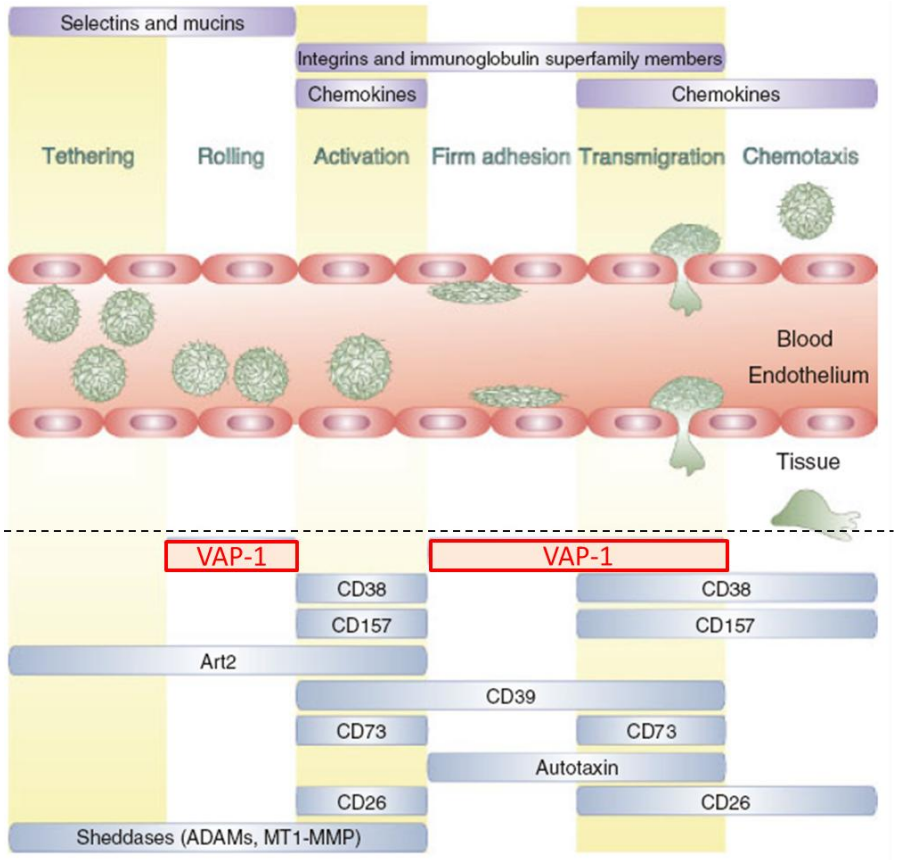


Fig 4. The molecules involved in the multistep adhesion cascade of leukocyte extravasation. The different phases of the cascade that mediate the emigration of leukocytes from the blood into the tissue, and the involved major superfamilies of adhesion and activation molecules are shown in the above panel. The well-characterized ectoenzymes that are involved in each cascade step are shown in the below panel. Vascular adhesion protein-1 (VAP-1) is involved at many steps at leukocyte rolling, firm adhesion and transmigration (Salmi *et al.*, 2012).

1.5.3.1 Inflammation induces VAP-1

By using human monoclonal antibodies (mAbs), a novel cell adhesion molecule, endothelial vascular adhesion protein-1 was defined, which could mediate the leukocyte binding to high endothelial venules (HEVs) in peripheral lymph node, tonsils and inflamed synovia in humans (Salmi *et al.*, 1992). In spite of its presence in endothelial cells under normal conditions, mostly in HEVs of lymphatic tissues, the expression of VAP-1 was also found increased at the sites of inflammation, and acting as a functional molecule in recirculation of lymphocytes and controlling the entry of leukocytes into the sites of inflammation, such as in inflammatory bowel diseases and chronic skin inflammation in humans (Salmi *et al.*, 1993). Notably, using confocal microscopy and granular staining, researchers demonstrated that VAP-1 is stored in intracellular granules in the resting endothelial cells of HEVs, however, it translocates to the luminal surface of endothelial cells under the elicitation of inflammation (Salmi *et al.*, 1993; Jaakkola *et al.*, 2000; Salmi *et al.*, 2001a), see Fig 5.

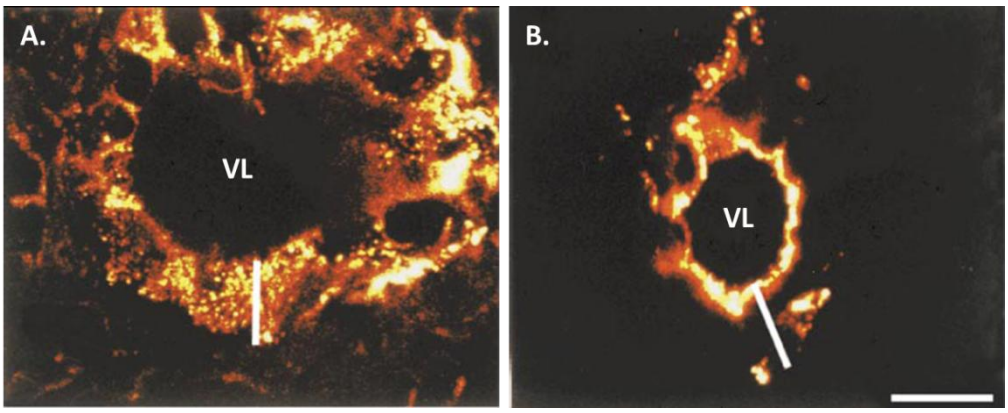


Fig 5. Location of VAP-1 in high endothelial venules (HEVs). A. VAP-1 is stored within intracellular granules in resting endothelial cells, whereas (B.) it translocates to the luminal surface during inflammation. The white line indicates the size of the endothelial cell. VL, vesicle lumen. Confocal micrograph scale bar = 20 μM (Salmi *et al.*, 2001a).

1.5.3.2 VAP-1 mediates leukocyte rolling and extravasation

VAP-1-mediated leukocyte adhesion function was mostly characterized with the help of anti-VAP-1 mAbs of the protein. The mAbs could block the lymphocyte binding and reduce the rolling and adherent leukocytes on VAP-1-positive endothelial cells under experimental conditions (Salmi *et al.*, 1992; Salmi *et al.*, 2001b; Lalor *et al.*, 2002).

Glycosidase digestion of VAP-1 discovered that the sialic acids present in the protein surface were indispensable for the lymphocyte-binding function of VAP-1, and L-selectin was not essential for lymphocyte binding to VAP-1 under shear stress (Salmi *et al.*, 1996). Surprisingly, the cloning of VAP-1 cDNA revealed that VAP-1 had significant identity to copper-containing amine oxidase and also had SSAO activity. Since then, VAP-1 was proposed to be a novel type of adhesion molecule with dual function, the cell adhesion function and the SSAO enzymatic function, hereinafter named SSAO/VAP-1 (Smith *et al.*, 1998). SSAO/VAP-1 also belongs to ectoenzymes, which are cell surface-expressed enzymes with catalytic domains outside the plasma membrane, and make crucial contribution to the leukocyte trafficking cascade (Salmi *et al.*, 2005).

In human lymph nodes HEVs, evidences indicated first that VAP-1 mediated only a subtype-specific binding of CD-8 positive T cells and naturel killer cells to human endothelium, and that this adhesion was independent of the lymphocyte receptors- L-, P-selectin glucoprotein ligand 1 and α4 integrins (Salmi *et al.*, 1997b). By recombining human VAP-1 into a non-lymphocyte binding endothelial cell line, researchers further demonstrated that, among different leukocytes, VAP-1 preferably bound lymphocytes especially for T-killer cells than monocytes, and showed no specific granulocyte adherence under shear. This binding could be partially supressed by VAP-1 monoclonal antibodies, which do not interfere the enzymatic activity of the protein, and be enhanced by CD44 ligation on lymphocytes (Salmi *et al.*, 2000). However, an *in vivo* study suggested that VAP-1 is as well involved in granulocyte extravasation at sites of inflammation, since VAP-1 blockade increased the granulocytes rolling velocity and reduced the firm

adhesion and transmigration of leukocytes (TOHKA *et al.*, 2001). Moreover, the transfectant experiments showed that VAP-1-transfected non-endothelial cells didn't exhibit lymphocyte binding abilities, suggesting that although VAP-1 contributes to the multistep adhesion cascade when the activation of selectins, chemokines, immunoglobulin superfamily molecular and integrins had taken place, VAP-1 is not the unique participant in this process (Salmi *et al.*, 2000).

The mechanism elucidated by "in vitro" models

With enzymatically active VAP-1 expression from primary endothelial cells, firm evidences showed that the SSAO activity of VAP-1 directly regulated lymphocyte adhesion at rolling step (Salmi *et al.*, 2001b). Under *in vitro* flow chamber assay, anti-VAP-1 monoclonal antibodies inhibited lymphocyte rolling and firm adhesion in VAP-1-expressing endothelial cells, when neither affecting tethering nor firmness of binding, nor have any effect on SSAO oxidative activity. Strikingly, the inhibition of SSAO activity by inhibitors effectively reduced the 50% of the lymphocyte rolling and firm adhesion. However, SSAO reaction products such as hydrogen and benzaldehyde were ineffective in modulating the leukocyte endothelial cell adhesion. SSAO substrate benzylamine even significantly reduced the firm adhesion rather than amplified lymphocyte adherence, which suggested that benzylamine may compete with lymphocyte surface-bound molecules for binding to VAP-1 during rolling. By peptide synthesis and molecular modelling, it was demonstrated that lymphocyte could provide surface-bound amines, such as N-terminal sites of proteins, NH_2-containing amino acid side chains or amino sugars for VAP-1, resulting in a transient covalent binding between the lymphocyte and the endothelial cells (Jalkanen *et al.*, 2001; Salmi *et al.*, 2001b). By using an enzymatically inactive SSAO/VAP-1 mutant, specific SSAO inhibitors and anti-VAP-1 mAbs, it was revealed that the oxidase activity of SSAO/VAP-1 as well regulates the polymorphonucluear leukocytes (PMNs) transmigration through the endothelium (Koskinen *et al.*, 2004). VAP-1 was also found to be critical for the adhesion and transmigration of $CD16^+$ monocytes across hepatic sinusoidal endothelial cells (HSECs) in the liver (Aspinall *et al.*,

2010). Most notably, the enzyme-inactive SSAO/VAP-1 or the enzyme-specific inhibitor both resulted in abolished rolling, firm adhesion and transmigration of leukocytes (Salmi *et al.*, 2001b; Koskinen *et al.*, 2004).

The consistent implication by "In vivo" models

Multiple *in vivo* experiments also confirmed the contribution of VAP-1 in leukocyte-endothelium adhesion. VAP-1 deficient mice and other disease models revealed that VAP-1 is needed for normal leukocyte extravasation both under normal conditions and in inflammation, and the absence of VAP-1 could lead to abnormal leukocyte traffic *in vivo* (Stolen *et al.*, 2005). VAP-1 deficient mice also have shown the reduction of leukocyte migration in inflammatory response in peritonitis, obesity, arthritis, ischemia-reperfusion injury and in tumour models (Stolen *et al.*, 2005; Koskinen *et al.*, 2007; Kiss *et al.*, 2008; Bour *et al.*, 2009; Marttila-Ichihara *et al.*, 2009). The anti-VAP-1 mAbs and SSAO inhibitors could alleviate the inflammatory actions in various animal models, including peritonitis, arthritis, diabetes, ischemia-reperfusion injury, lymph- and angiogenic diseases, lung and liver inflammation (Merinen *et al.*, 2005; Marttila-Ichihara *et al.*, 2006; Noda *et al.*, 2008; O'Rourke *et al.*, 2008; Nakao *et al.*, 2011; Salmi *et al.*, 2012; Foot *et al.*, 2013; Weston *et al.*, 2014).

Although anti-VAP-1 mAbs, SSAO inhibitors and enzymatically inactive SSAO/VAP-1 mutant exhibited the same levels of leukocyte-endothelial cell adhesion, a combination of mAbs with inhibitor or mAbs with enzymatically inactive SSAO/VAP-1 didn't show more blockage (Salmi *et al.*, 2001b; Koskinen *et al.*, 2004). Hence, VAP-1 has been proposed to be a molecule with dual adhesion function (Salmi *et al.*, 2005; Jalkanen *et al.*, 2008). First, through the anti-VAP-1 mAb-defined epitopes, VAP-1 binds lymphocytes. Second, by using the SSAO enzymatic activity of VAP-1, a reaction between a cell surface-bound amine or substrate and VAP-1 is carried out. This also indicated a transient link between the enzyme and its substrate, which predictably located on the surface of leukocyte, should be formed under physiological stress. A SSAO/VAP-1-mediated leukocytes-endothelial cell adhesion model can be seen in Fig 6.

Although the physiological substrates of VAP-1/SSAO, which are the soluble primary amines, still need to be extensively clarified, methylamine and aminoacetone have been demonstrated to be endogenous substrates (see above SSAO substrates part). Furthermore, methylamine has been proved to be involved the inflammatory action mediated by SSAO/VAP-1. Inhalation of methylamine can increase the bronchoalveolar lavage (BAL) neutrophil counts in lipopolysaccharide-induced pulmonary inflammation (Peter *et al.*, 2006). Methylamine treatment also increased the THP-1 monocyte adhesion to the human umbilical vascular endothelial cells stably transfected with human SSAO/VAP-1 under experimental conditions (Sole *et al.*, 2011). Moreover, VAP-1 could also interact with the leukocytes surface-bound ligands, such as N-termini of proteins, NH_2-containing amino acid side chains, and amino sugars, during the cell adhesion process. Siglec-10 was the first identified ligand on lymphocytes, which could interact with VAP-1 and mediate lymphocyte adhesion to endothelium (Kivi *et al.*, 2009). Seglec-9 was another candidate counter-receptor expressed on granulocytes contributing to the binding to the endothelium (Aalto *et al.*, 2011).

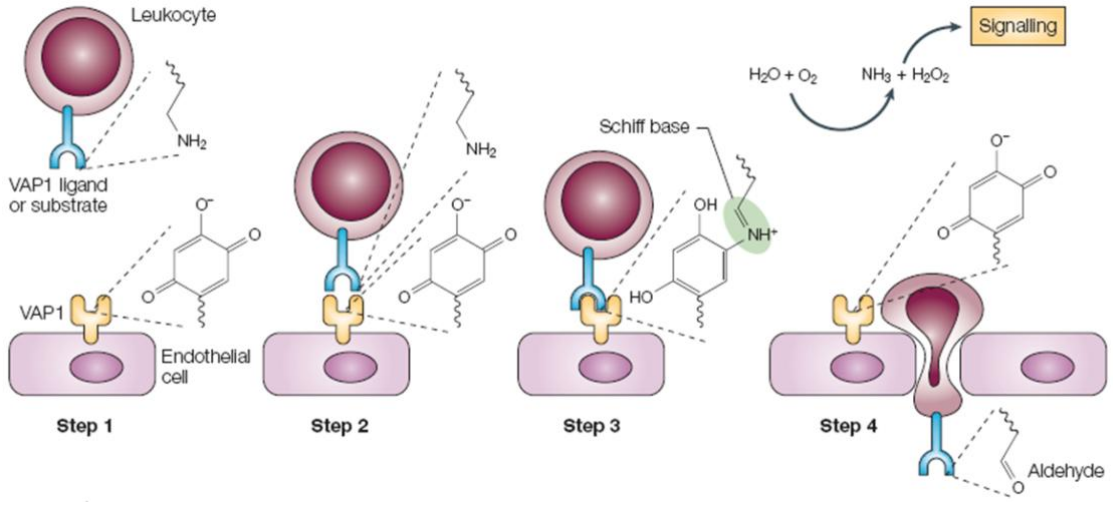


Fig 6. Functions of vascular adhesion protein 1 (VAP-1) during leukocyte-endothelial cell adhesion. A model consisting of four step actions of VAP-1 in the interaction between leukocyte and endothelial cell is shown. Step 1, VAP-1 is present at the surface of the endothelial cells and VAP-1 ligand or substrate is present on the leukocyte. Step 2, VAP-

1 binds leukocyte by a cell-surface epitope. Step 3, the interaction guides leukocyte substrate contact with the catalytic centre of SSAO/VAP-1, a transient Schiff base is formed in the presence of H_2O and O_2. Step 4, the catalytic activity leads to the formation of aldehyde of leukocyte substrate and the release of hydrogen peroxide (H_2O_2) and ammonia (NH_3) (Salmi *et al.*, 2005).

Additionally, the SSAO-catalysed end-products are biologically active compounds as well, such as hydrogen peroxide and formaldehyde (products from methylamine), and they may also contribute to this cell adhesion process indirectly or at later stage (see below) (Salmi *et al.*, 2005), although it has been controversial with the ineffective modulation of them on lymphocyte adhesion during some *in vitro* assays (Salmi *et al.*, 2001b).

1.5.3.3 VAP-1 modulates other cell adhesion molecules

Through the SSAO catalytic activity, VAP-1 can also modulate the expression of other molecules during the leukocyte adhesion cascade, since all the end products derived from the deamination reaction are biologically active. Probably the major contributor of the products is hydrogen peroxide, which can act as a signalling molecule and induce the adhesion-related proteins in the local endothelial microenvironment, thereby increasing cell migration (Salmi *et al.*, 2005). Hydrogen peroxide can play an important role in the initiation and amplification of signalling at the antigen receptor on lymphocytes by inhibiting protein tyrosine phosphatases (Reth, 2002). An increased oxygen tension can stimulate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on micro- and macrovascular endothelial cells, which facilitates the leukocyte adhesion and transmigration through endothelium (Willam *et al.*, 1999). Reactive oxygen species can also increase the chemokine-receptors expression, which account for the activation step of leukocyte adhesion cascade mediated by chemokines (Saccani *et al.*, 2000). Additionally, endothelial P-selectin and E-selectin can be induced by the enzymatic activity of VAP-1 *in vitro*, which is presumably mediated by the generation of hydrogen peroxide through SSAO activity. SSAO/VAP-1 activity

also accounts for the increased P-selectin-dependent lymphocytes binding to endothelial cells *in vivo* (Jalkanen *et al.*, 2007). Moreover, SSAO/VAP-1 is able as well to up-regulate E-selectin, ICAM-1, VCAM-1 and chemokine CXCL8, through the NF-κB, phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, resulting in enhanced lymphocyte adhesion on human hepatic endothelial cells (Lalor *et al.*, 2007). Furthermore, recent studies also demonstrated that the deamination of methylamine by SSAO/VAP-1 would induce mucosal adressin cell adhesion molecule 1 (MAdCAM-1) in hepatic endothelial cells, contributing to the recruitment of mucosal effector lymphocytes to the liver (Liaskou *et al.*, 2011).

1.5.4 Other functions

Despite the above functions that SSAO/VAP-1 can mediate, some studies also suggest that SSAO/VAP-1 might have a drug binding property, and may be involved in the lipid transport in the mature adipocytes with an unidentified mechanism (O'Sullivan *et al.*, 2004).

Furthermore, SSAO activity also exerts a role in the connective tissue matrix development, and in the maintenance of collagen and elastin architecture within blood vessel walls (Langford *et al.*, 1999). Moreover, it has been suggested that SSAO could play a modulating role by regulating extracellular matrix deposition by vascular smooth muscle cells (Langford *et al.*, 2002).

Notably, lysyl oxidase (LO), which also belongs to SSAO but distinct from VAP-1, can metabolize the lysine residues in the side-chain amino groups from collagen and elastin by an well-established mechanism, contributing to the cross-linking collagen and elastin during the formation of extracellular matrix (Lyles, 1996).

2. SSAO/VAP-1 in pathological conditions

During the past decades, the role of SSAO/VAP-1 has been substantially studied, and there have been investigated its physiological functions mainly including the metabolic deamination of primary amines, the regulation of glucose metabolism

and the regulation of leukocyte trafficking in the endothelial cells. There are also considerable evidences that VAP-1/SSAO may participate and play an important role under pathological conditions.

In mammalians, the implications of SSAO/VAP-1 under pathological conditions have been focused on the deamination of endogenous and xenobiotic primary amines for a long time. Although the pathological importance of this metabolism needs further elucidation, high levels of the toxic products generated may be responsible, at least in part, for the protein cross-linkage, oxidative stress and cytotoxicity, and for the advanced protein aggregation and the vascular damage related to vascular disorders, such as diabetic complications, atherosclerosis and aged-related diseases (Yu *et al.*, 2003). For instance, the endogenous substrates methylamine and aminoacetone can be catalysed by both the membrane-bound SSAO and the soluble SSAO, resulting in the production of formaldehyde and methylglyoxal, hydrogen peroxide and ammonia, respectively. Formaldehyde is extremely cytotoxic and potentially carcinogenic, it can produce cross-linked complexes with proteins and with single-stranded DNA (Bolt, 1987; Heck *et al.*, 1990). Given the fact that serum does not contain glutathione-dependent formaldehyde dehydrogenase, formaldehyde can't be metabolized unless being transported into erythrocytes in the blood, which may be important regarding the toxic effects to blood vessels (Yu *et al.*, 2003). Methylglyoxal is as well toxic and has similar effects on proteins cross-linking and contributing to the aging-related advanced protein aggregation (Nagaraj *et al.*, 1996). In addition, hydrogen peroxide is a well-known reactive oxygen specie, leading to oxidative stress at relatively high levels by itself or by generation of other more toxic hydroxyl free radicals (Sies, 1997; Aruoma, 1998), impairing DNA, lipids, proteins and other molecules, damaging the cells and tissues. Indeed, the toxicity of methylamine has shown to be attenuated by SSAO inhibitors by various *in vitro* and *in vivo* approaches (Yu *et al.*, 1993; Yu, 1998; Solé *et al.*, 2008; Sole *et al.*, 2011).

On the other hand, due to the ability of regulating leukocyte trafficking, SSAO/VAP-1 may also play an important role at sites of inflammation under

different pathologies, since the inhibition of SSAO mAbs-defined epitope or activity could alleviate the inflammatory action in multiple models including diabetes, peritonitis, colitis, chronic skin inflammation, ischemia-reperfusion injury, sepsis, allergic encephalomyelitis, arthritis, lung and liver inflammation (Koskinen *et al.*, 2004; Merinen *et al.*, 2005; Salter-Cid *et al.*, 2005; Marttila-Ichihara *et al.*, 2006; Xu *et al.*, 2006; Noda *et al.*, 2008; O'Rourke *et al.*, 2008; Nakao *et al.*, 2011; Salmi *et al.*, 2012; Foot *et al.*, 2013; Weston *et al.*, 2014). The role of SSAO/VAP-1 in mediating leukocyte adhesion on endothelium has been fully described during the previous chapter.

2.1 SSAO/VAP-1 alteration in pathologies

Nowadays, increasing evidences have found that the expression and activity of SSAO/VAP-1, mostly in plasma, is altered in a high number of pathologies. There are some cases where plasma SSAO activity is found to be decreased, such as in severely burnt patients, or in patients with cancer (Lewinsohn, 1977), while there are more considerable investigations exhibiting increased levels of soluble serum SSAO activity in some special pathological conditions, such as diabetes mellitus (both type 1 and 2) (Boomsma *et al.*; Boomsma *et al.*, 1999), diabetic retinopathy and atherosclerosis (Meszaros *et al.*, 1999; Grönvall-Nordquist *et al.*, 2001; Karádi *et al.*, 2002), congestive heart failure (Boomsma *et al.*, 1997), non-diabetic morbidity obesity (Weiss *et al.*, 2003), malignant hypertension (Boomsma *et al.*, 2003), inflammatory diseases (cirrhotic liver inflammation) (Kurkijärvi *et al.*, 1998), Alzheimer's disease (AD) (del Mar Hernandez *et al.*, 2005), ischemic and hemorrhagic stroke (Hernandez-Guillamon *et al.*, 2010; Hernandez-Guillamon *et al.*, 2012) and others. For congestive heart failure and AD, the increase of plasma SSAO is correlated with the severity of the disease. In AD and CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), the membrane-bound SSAO expression is also increased in cerebral blood vessels (Ferrer *et al.*, 2002a).

The mechanisms underlying these increased soluble plasmatic SSAO presence and activity are still unclear. Since it has been proposed that soluble SSAO/VAP-

1 is derived from the membrane-bound enzyme, the increased plasma SSAO activity in diabetic patients or AD may be a result from the elevated expression of SSAO/VAP-1 (Yu *et al.*, 2003), since it can be up-regulated in response to inflammation (Kurkijärvi *et al.*, 1998).

Notably, the soluble plasma SSAO/VAP-1 could augment the lymphocyte binding to endothelial cells as well, presumably by triggering the up-regulation of other functional adhesion molecules on lymphocytes (Kurkijärvi *et al.*, 1998), rather than functioning as inhibitor of the cell adhesion cascade by competing with their membrane-bound adhesion molecules, such as soluble L-selectin and ICAM-1 (Schleiffenbaum *et al.*, 1992; Meyer *et al.*, 1995).

2.2 Stroke

Stroke is the second most common cause of death after ischemic heart disease, and the major cause of disability worldwide, which accounts for 9% of all deaths around the world (Donnan *et al.*, 2008). In western countries, the proportion of deaths caused by stroke is 10-12%, and 12% of these deaths are in people less than 65 years old (Bonita, 1992). Epidemiological studies indicate that many risk factors may accelerate the onset of stroke. Age is the strongest risk factor for stroke, while other risk factors include hypertension, high total cholesterol, diabetes mellitus, female gender, cigarette smoking, alcohol excess, heredity, race and drug treatments, etc. (Markus, 2008).

A stroke occurs when the oxygen-rich blood flow inside the blood vessel is blocked to a portion of the brain, resulting in an immediate loss of oxygen and glucose to part of the cerebral tissue, inducing the brain cells death. Sudden bleeding in the brain also causes a stroke if it brings about damage of brain cells, which is hemorrhagic stroke. Consequently, symptoms would appear in parts of the body controlled by those brain cells, which include partial paralysis (face, arms or legs), difficulties with memory, thinking, language and movements (Lakhan *et al.*, 2009).

Thus, there are two main types of stroke, ischemic stroke and hemorrhagic stroke, but ischemic stroke consists in 85-90% of all them. Another condition that is similar to stroke is transient ischemic attack (TIA), which is a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia (loss of blood flow), without acute infarction (Easton *et al.*, 2009), hence the damage of the brain cells is not permanent. The symptoms can be restored within a few minutes or 24 hours in this case.

2.2.1 Ischemic stroke and hemorrhagic stroke

An ischemic stroke occurs if there is a blockage of a blood vessel that supplies oxygen-rich blood to the brain, leading to the dysfunction of the brain tissue in that area. There are mainly two types of ischemic stroke: thrombotic and embolic stroke (Johns Hopkins Medicine Health Library, 2015).

Thrombotic stroke is caused by a thrombus (blood clot) that develops in the blood vessels locally inside the brain. This type of stroke is usually happens to elderly especially with high cholesterol levels, atherosclerosis or diabetes. Embolic stroke is due to the obstruction of the blood vessels inside the brain by an embolus (blood clot or other substance, such as plaque debris and fatty material) that develops elsewhere in the body and travels through the bloodstream to the brain. This type of stroke often associates with heart disease or heart surgery. Both type of ischemic stroke can block the blood flow rich in oxygen and glucose in the blood vessels, resulting in brain tissue damage.

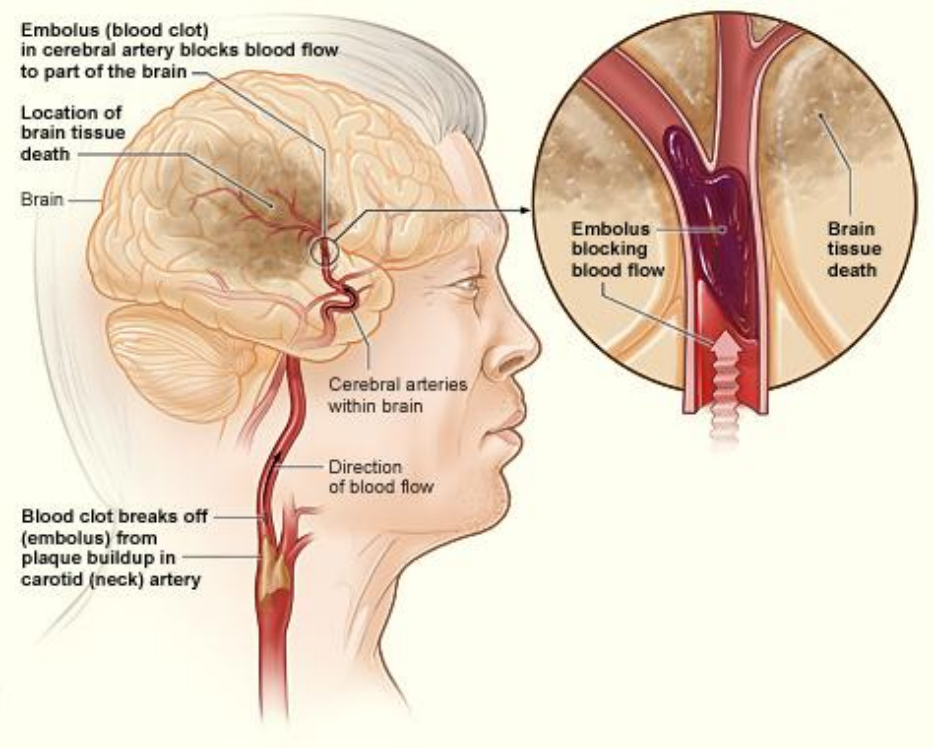


Fig 7. Ischemic stroke. Thrombus formed inside the cerebral blood vessels or embolus breaks away form a plaque buildup in a carotid artery can travel to and lodge in an artery in the brain, which can block blood flow to a portion of brain and induce brain tissue damage (National Institutes of Health, 2014).

A hemorrhagic stroke occurs when a blood vessel in the brain ruptures and bleeds. There are mainly two types, intracerebral hemorrhage and subarachnoid hemorrhage. The former one occurs when bleeding within the brain and the later one happens when bleeding in the subarachnoid space. The most common cause of hemorrhagic stroke is uncontrolled hypertension.

When hemorrhagic stroke takes place, brain cells and tissues after the rupture can't receive sufficient oxygen and nutrients. Then, pressure and irritation build up surronding the tissues can bring about further more brain damage (Johns Hopkins Medicine Health Library, 2015).

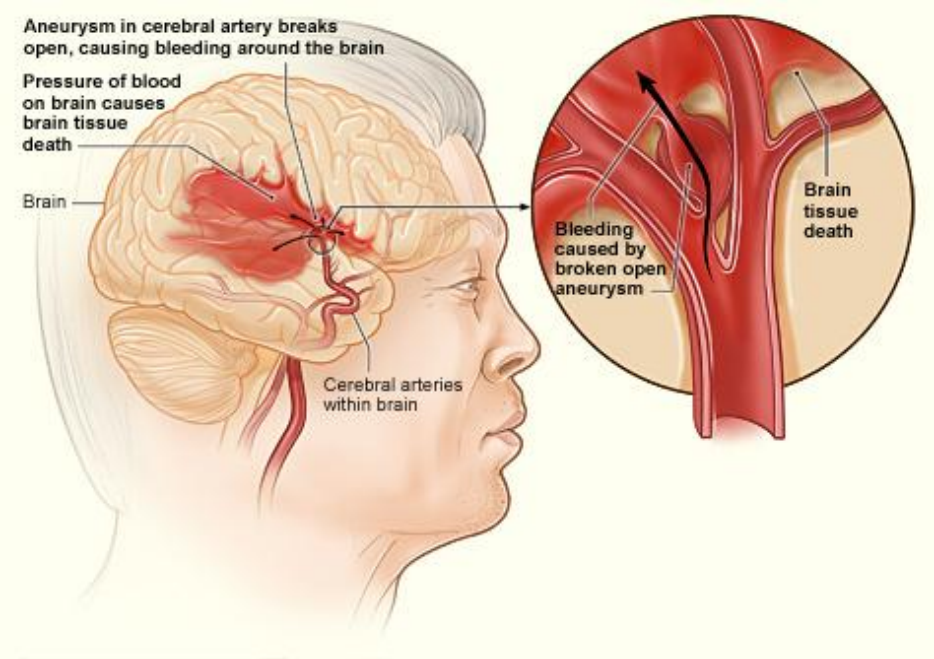


Fig 8. Hemorrhagic stroke (intracerebral hemorrhage). An anerurysm in a cerebral artery breaks open, which casues bleeding within the brain. Lack of oxygen and nutrients, the pressure, irritation and swelling from blood lead to the damage of bleeding area in the brain (National Institutes of Health, 2014).

2.2.2 Molecular mechanisms underlying ischemic stroke

Ischemic stroke could be caused by a thrombosis, an embolism or systemic hypo-perfusion, resulting in a restriction of blood flow to the brain, leading to the failure of oxygen and glucose supply to support brain cellular homeostasis. This ultimately causes brain injury with multiple processes including excitotoxicity, ionic imbalance, oxidative and nitrative stress, inflammation and apoptosis among others (Doyle *et al.*, 2008). In the centre or core of the ischemic territory, where blood flow is severely deficient, excitotoxic and necrotic cell death may progress in minutes. While in the peripheral zones, the ischemic penumbra, the residual perfusion from collateral blood vessels may slow down the cell death process, apoptosis and inflammation may occur gradually (Gonzalez, 2006).

2.2.2.1 Ischemic cascade

The ischemic cascade comprises a series of subsequent biochemical events that are initiated in the brain within seconds to minutes after the loss of blood flow to a brain region. The ischemic cascade can be generally seen as follows (see Fig 9):

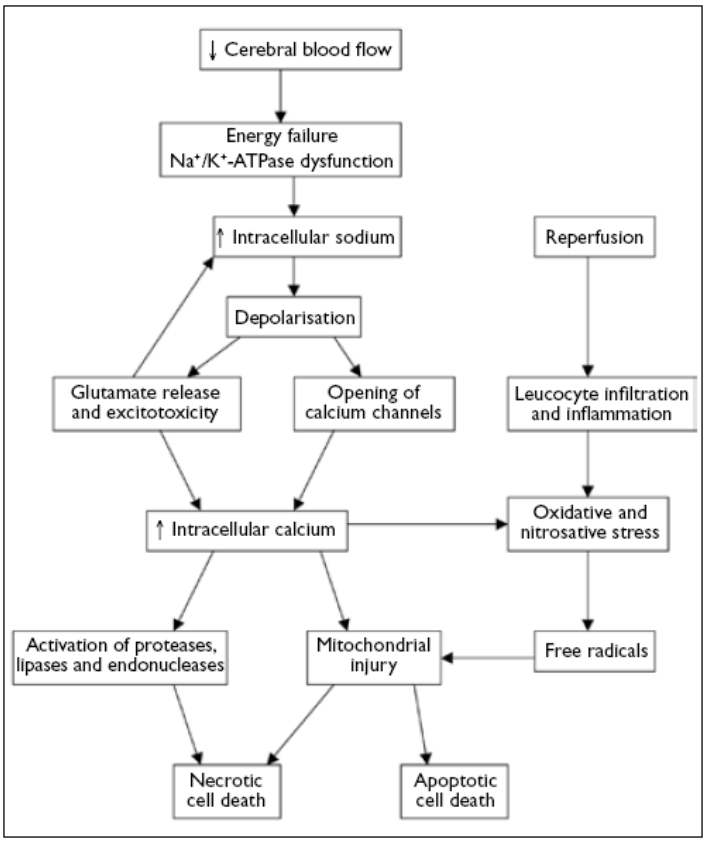


Fig 9. Ischemic cascade in acute ischemic stroke (Venketasubramanian *et al.*, 2011).

The ischemic cascade involves extensive mediators and overlapping interactions between them. After the onset of ischemia, energy deficits lead to ionic imbalance, glutamate excitotoxicity and augment of intracellular calcium. Meanwhile, the lack of energy supply leads to mitochondrial dysfunction and generation of oxidative and nitrosative stress. The post-ischemic inflammation is also initiated after the injury to the cells and tissues where reperfusion is introduced. All of these events exacerbate the initial injury and lead to the cell death of endothelial cells, neurons and glial cells (Lo *et al.*, 2003; Lakhan *et al.*, 2009).

2.2.2.2 Ionic imbalance and excitotoxicity

Under normal condition, the brain needs large amounts of adenosine triphosphate (ATP) to maintain and restore ionic gradients, Na^+/K^+ ATPase plays an important role in this process and maintains high intracellular levels of K^+ and low intracellular levels of Na^+. The exhaustion of ATP resulted from ischemic conditions causes cell membrane depolarization, resulting in Na^+/K^+ ATPase dysfunction and release of K^+ into the extracellular space and entry of Na^+ into the cells. Meanwhile, Ca^{2+} ATPase is also affected, leading to influx of excessive Ca^{2+}, which triggers an array of downstream DNAases, phospholipases and proteases that degrade membranes and proteins of the cell (Doyle *et al.*, 2008). Membrane depolarization and accumulation of Na^+ inside the cell also lead to neurotransmitters release such as glutamate, which further exacerbates the membrane depolarization and the intracellular calcium overload (excitotoxicity) (Olney, 1969), ultimately resulting in cell damage.

2.2.2.3 Oxidative stress

Oxidative stress is an important mediator of tissue injury in acute ischemic stroke. During ischemia, excessive superoxide (O_2^-) is produced during the electron transport. The free radicals can inhibit the mitochondrial electron transport, leading to even more oxygen radicals production (Fiskum *et al.*, 1999) (Chan, 2001). More free radicals can be formed from the primary radical O_2^-, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$). High levels of intracellular Ca^{2+}, Na^+ and ADP also stimulate mitochondria to produce excessive reactive oxygen species (ROS). Oxygen radicals are also produced during enzymatic reactions such as cyclooxygenase (COX)-dependent conversion of arachidonic acid, or the generation of nitric oxide (NO) in ischemia by nitric oxide synthase (NOS). Following reperfusion, there is another wave of production of O_2^-, NO and peroxynitrite, which play an important role in the reperfusion-induced injury (Doyle *et al.*, 2008). Moreover, oxygen radicals as well are generated during the post-ischemic inflammatory response (Lo *et al.*, 2003).

The reactive oxygen species can directly damage lipids, proteins, nucleic acids and carbohydrates, resulting in cell death induction. These radicals can also activate matrix metallo-proteinases (MMPs), which contribute to the disruption of the vascular wall and increase the permeability of BBB by degrading collagen and laminins (Doyle *et al.*, 2008). Oxidative stress can also trigger the expression of numerous pro-inflammatory genes by including the synthesis of transcription factors, such as NF-κB, hypoxia inducible factor 1α (HIF-1α), interferon regulator factor 1 and STAT3, then consequently up-regulating the expression of adhesion molecules including ICAM-1, P-selectin and E-selectin, enhancing leukocytes recruitment and migration to the cerebral vasculature (Yilmaz *et al.*, 2008). The enzymes released by leukocytes can further exacerbate the lamina degradation and increase vascular permeability, facilitating the transformation of parenchymal hemorrhage, vasogenic brain edema and neutrophil infiltration into the brain (Crack *et al.*, 2005).

Oxidative stress is relatively more harmful to the brain cells compared to other cell types, since endogenous levels of antioxidant enzymes and antioxidant vitamins are not high enough here to match the excessive radicals formation (Lo *et al.*, 2003). Antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPx), antioxidant vitamins such as tocopherol (vitamin E) and ascorbic acid (vitamin C).

2.2.2.4 Post-ischemic inflammation

Inflammation contributes additionally to the stroke-related injury and the expansion of the ischemic lesion. This inflammatory response involves many different types of cells, inflammatory mediators and extracellular receptors.

Among different cell types, inflammatory cells like microglial cells and astrocytes participate in tissue remodelling after injury in the brain. Ischemia can activate and increase the number of microglial cells (Arumugam *et al.*, 2009), which then release a variety of cytotoxic and/or cytoprotective substances. It is well evidenced that activated microglial cells have the potential to release pro-

inflammatory cytokines such as TNF- α, IL-1β, IL-6 as well as other cytotoxic molecules including NO and ROS (Lucas *et al.*, 2006). However, microglial cells can also produce neuroprotective proteins such as insulin-like growth factor 1 (IGF-1). Astrocytes can also secret inflammatory factors such as cytokines, chemokine and NO (Swanson *et al.*, 2004).

Stroke can cause an increase in the number of neutrophils and monocytes in the brain. Neutrophils are the earliest leukocyte subtype to show substantial up-regulation and to infiltrate the ischemic area (Lakhan *et al.*, 2009). When neutrophils transmigrate into the cerebral parenchymal, they cause tissue damage by releasing oxygen radicals and proteolytic enzymes. Lymphocytes also participate in the post-ischemic brain inflammation (Schroeter *et al.*, 1994), although this happens later than neutrophils, but they contribute to ischemia-induced damage as well.

Among inflammatory mediators, cytokines and chemokines play an important pro-inflammatory action and contribute to the brain injury under ischemic stroke (Gong *et al.*, 1998). During ischemia, a variety of cells, including endothelial cells, neurons, microglia, astrocytes, platelets, leukocytes and fibroblasts, can be activated and secret cytokines, such as IL-1β, IL-6, IL-10, TNF-α, Transforming growth factor beta (TGF-β) and chemokines such as monocyte chemoattractant protein 1 (MCP-1) and cytokine-induced neutrophil chemoattractant (CINC) (Huang *et al.*, 2006). Among cytokines, IL-1β and TNF-α appear to exacerbate brain injury, while IL-10 and TGF-β may be neuroprotective (Lakhan *et al.*, 2009). IL-1β has demonstrated to possess deleterious effects in fever, arachidonic acid release, excitotoxicity and stimulation of NO synthesis. IL-1β may be also involved in the recruitment of neutrophils and in the formation of brain edema, since IL-1β can induce the up-regulation of E-selectin, ICAM-1, ICAM-2 and VCAM-1 on cerebral endothelial cells (Yamasaki *et al.*, 1997; Huang *et al.*, 2006). IL-6 is also up-regulated under ischemia, and it has similar effects as IL-1, suggesting a pro-inflammatory effect in stroke from human studies (Huang *et al.*, 2006). TNF-α can also indicate the ischemic injury (Zaremba *et al.*, 2001), it is

able as well to induce adhesion molecules expression and to promote neutrophil accumulation and transmigration. Chemokines, such as MCP-1, can guide the migration of neutrophils and macrophages towards the source of chemokines, so they also play an important role in cellular communication and inflammatory cells recruitment (Lakhan *et al.*, 2009).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes which play a central role in brain development, as they modulate the extracellular matrix to allow neurite outgrowth and cell migration (Lo *et al.*, 2003). MMPs levels are increased in experimental models of ischemia and haemorrhage. MMPs levels are as well increased in the brain and plasma of stroke patients (Lo *et al.*, 2003). Excessive MMPs activity could cause cell death and inflammation in the brain (Anthony *et al.*, 1998). On the other hand, MMPs inhibitors may reduce infarction and edema (Rosenberg *et al.*, 1998; Asahi *et al.*, 2001b). MMP-2 knockout mice are not resistant to focal ischemia (Asahi *et al.*, 2001a), but MMP-9 knockout mice are protected against cerebral ischemia (Asahi *et al.*, 2001b). However, both MMP-2 and MMP-9 have been associated to cerebral ischemia under various conditions.

Cellular adhesion molecules (CAMs) are a very important group of members activated during the inflammation process under ischemic stroke, since they are responsible for the leukocyte adhesion and extraversion, which are mediated by three classes of CAMs: the selectins, the integrins and the immunoglobulins, (see cell adhesion part). Moreover, CAMs can also be up-regulated by various cytokines (see above). It has been proved that P-selectin and E-selectin can be enhanced and mediate leukocyte rolling and recruitment during the early stage of ischemia (Zhang *et al.*, 1998). Among immunoglobulins, ICAM-1 increases its expression upon stimulation of cytokines within hours after the onset of stroke (Lindsberg *et al.*, 1996). Soluble ICAM-1 (sICAM-1) is also found to increase with acute ischemic stroke patients, and sICAM-1 levels are significantly higher in patients who died than those who survived, therefore indicating the severity of the stroke (Rallidis *et al.*, 2009). Recently, the soluble form of vascular adhesion

protein 1 (sVAP-1) has also been found to increase its protein level and activity after ischemic and hemorrhagic stroke (Hernandez-Guillamon *et al.*, 2010; Hernandez-Guillamon *et al.*, 2012). Since VAP-1 plays an important role during leukocyte adhesion to endothelial cells, sVAP-1 may be another indicator involved in ischemic stroke-associated inflammation.

The described post-ischemic inflammatory actions leading to dysfunction of the BBB, cerebral edema and finally neuronal cell death have been summarized as follows, see Fig 10.

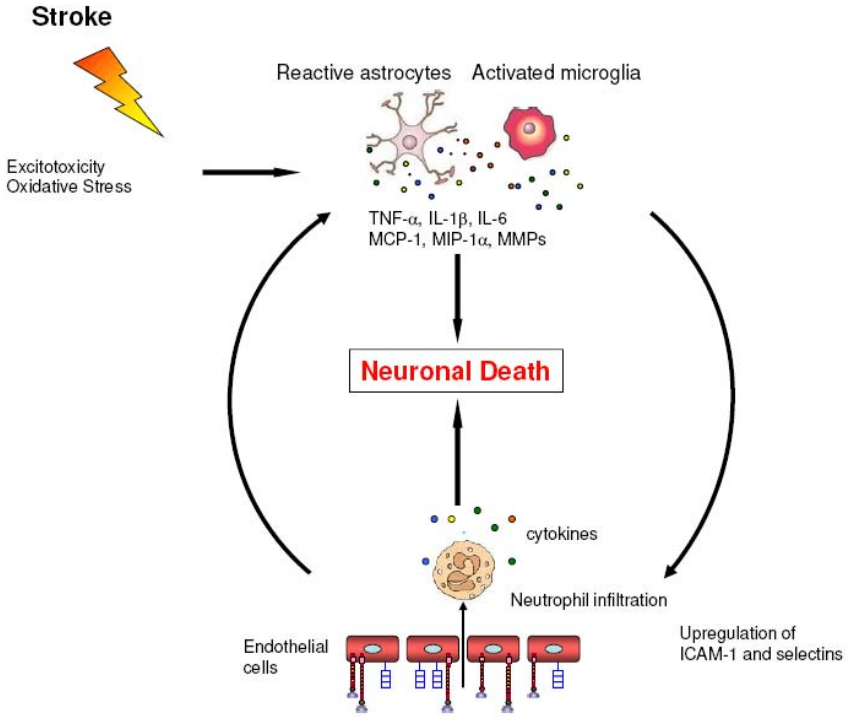


Fig 10. Scheme of post-ischemic inflammatory responses. Excitotoxicity and oxidative stress generated during the ischemic cascade activate microglia and astrocytes, which consequently secret cytokines, chemokines and matrix metalloproteinases (MMPs). These mediators lead to up-regulation of cell adhesion molecules on endothelial cells, facilitating leukocytes, mainly neutrophils infiltration to the ischemic brain region. Neutrophils also secrete cytokines that then further activate glial cells. All of these procedures ultimately result in neuronal death and ischemic brain injury (Lakhan *et al.*, 2009).

2.2.2.5 The principle apoptotic cell death pathways

Within minutes of onset of the occlusion, the core of the ischemic territory suffers severe blood flow deficits, low ATP levels, ionic disruption and metabolic failure, and cells undergo necrotic death. Whereas the peripheral zones surrounded by the core, known as the ischemic penumbra, are less severely affected and suffer milder insults due to residual perfusion from the collateral blood vessels (Lo *et al.*, 2003), cells there undergo apoptotic-like mechanisms rather than necrosis (Gonzalez, 2006; Doyle *et al.*, 2008; Broughton *et al.*, 2009). The triggers of apoptosis include ionic imbalance, oxygen free radicals, death receptor ligation, DNA damage and protease activation (Doyle *et al.*, 2008).

Intrinsic apoptotic pathway

During the intrinsic apoptotic pathway, a cytotoxic accumulation of intracellular calcium is thought to initiate a series of cytoplasmic and nuclear events. Under ischemic stroke, the release of neurotransmitters such as glutamate, stimulates the increase of cytosolic calcium by binding to ionotropic NMDA (N-methyl-D-aspartate) and AMPA (D, L-α-amino-3-hydroxy-5-methyl-isoxazolpropionic acid) receptors. Ischemia-induced acidosis can also activate ASICs (acid-sensing ion channels). All these processes result in the accumulation of intracellular calcium. Increased calcium activates calpains and results in the cleavage of Bcl-2 interacting domain (Bid) to its truncated active form (tBid) (Love, 2003; Culmsee *et al.*, 2005). tBid targets the outer mitochondrial membrane and interacts with pro-apoptotic proteins, such as Bak, Bax, Bad and Bcl-XS (Sugawara *et al.*, 2004), which can be prevented by anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2 or Bcl-xL. After tBid heterodimerization with pro-apoptotic proteins, mitochondrial transition pores (MTP) are opened and release cytochrome c (Cytc) or the apoptosis-inducing factor (AIF). Cytc binds apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an apoptosome, activating caspase-9 and -3, leading to DNA damage and apoptosis. AIF can translocate into the nucleus and mediate apoptosis in a caspase-independent manner (Cho *et al.*, 2008). Phosphorylation and activation of tumour suppressor

transcription factor p53 also happens in response to DNA damage, which further increases Bax expression and leads to apoptosis. Furthermore, the free radical oxygen species such as superoxide anions can cause DNA damage directly (Broughton *et al.*, 2009). The intrinsic signalling cascade of apoptosis is as follows, see Fig 11.

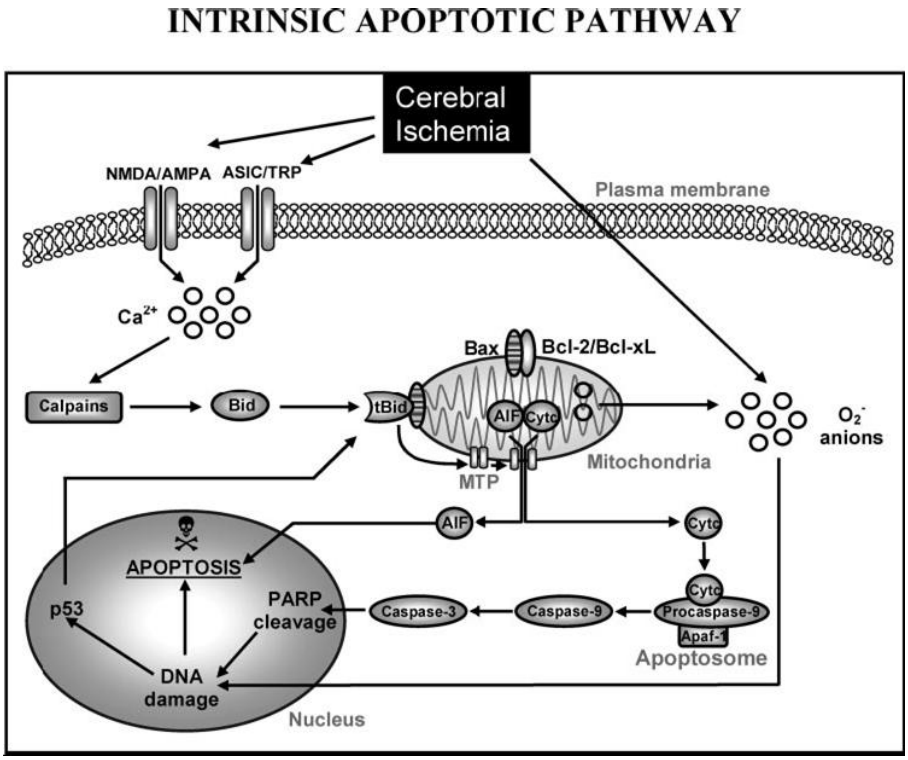


Fig 11. Intrinsic signalling cascade of apoptosis after cerebral ischemia (for details, see text) (Broughton *et al.*, 2009).

Extrinsic apoptotic pathway

Cerebral ischemia can also trigger the forkhead family transcription factor, such as Forkhead 1, stimulating the expression of Fas ligand (FasL) (Sugawara *et al.*, 2004), and initiating the extrinsic apoptosis by binding to Fas receptor (FasR, or Fas), which is a death domain-containing member that belongs to tumour necrosis factor receptor (TNFR) superfamily. The interaction between FasL and FasR triggers the recruitment of the cytoplasmic adaptor protein Fas-associated

death domain protein (FADD) (Broughton *et al.*, 2009). FADD further binds to procaspase-8 by interacting with its death effector domain (Love, 2003), allowing the formation of death inducing signalling complex (DISC), which now contains FasL, FasR, FADD and procaspase-8. DISC subsequently activates procaspase-8 and releases caspase-8 into cytoplasm, either triggering the cleavage of Bid into tBid, enhancing the mitochondria-mediated apoptosis (see above), or activating the cleavage of caspase-3 directly. Cleaved caspase-3 metabolizes many substrate proteins, such as the DNA-repairing enzymes poly ADP ribose polymerase (PARP) (Endres *et al.*, 1997; Namura *et al.*, 1998), leading to DNA injury and apoptotic cell death (Broughton *et al.*, 2009).

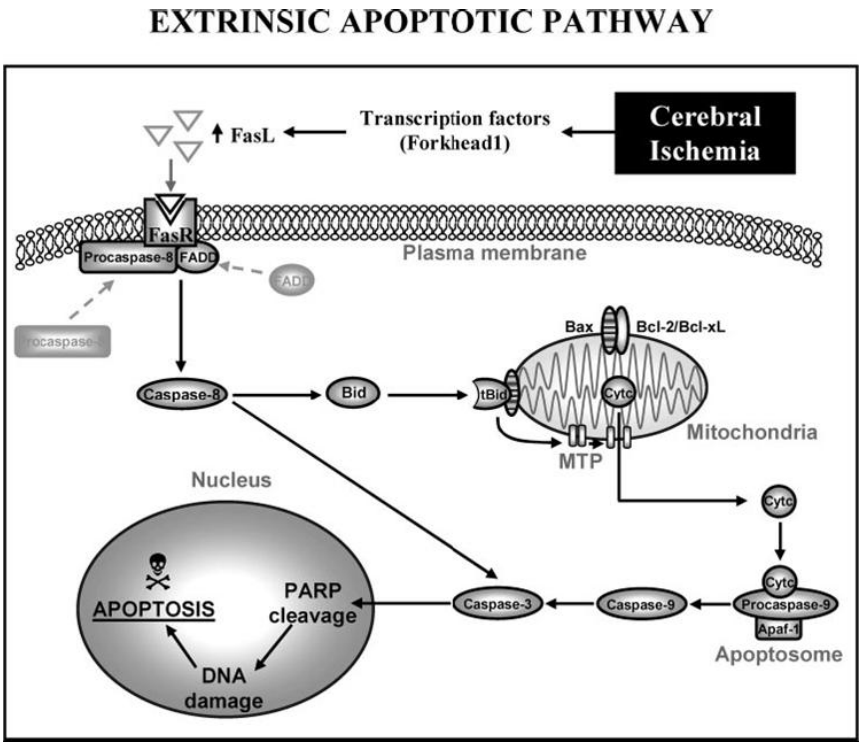


Fig 12. Extrinsic signalling cascade of apoptosis after cerebral ischemia (for details, see text above) (Broughton *et al.*, 2009).

Endoplasmic reticulum stress apoptotic pathway

The endoplasmic reticulum (ER) is an important subcellular organelle that is responsible for the production of functional and mature proteins, calcium homeostasis and lipid biosynthesis (Pizzo *et al.*, 2007; Anelli *et al.*, 2008).

However, a number of pathological insults can lead to the accumulation of unfolded proteins in the ER and induce ER stress. Recently, there are increasing evidences revealing that ER stress also plays a crucial role in cerebral ischemia-induced cell dysfunctions (Kumar *et al.*, 2001; DeGracia *et al.*, 2004; Azfer *et al.*, 2006; Nakka *et al.*, 2010). ER stress in cerebral ischemia is initiated by the overexpression and unfolded proteins, such as chaperone glucose-regulated protein (GRP78) (Lehotsky *et al.*, 2009), which are presumably induced by the overload of calcium and oxygen free radicals. Adverse ER stress during persistent ischemia can activate caspase-12, caspase-9 and caspase-3, resulting into the apoptotic cell death activation (Duan *et al.*, 2010).

During the ER stress, the accumulated unfolded proteins lead to the unfolded protein response (UPR) activation that modifies gene transcription and protein translation, and ER-associated protein degradation (ERAD), which up-regulates protein degradation. Both steps help to eliminate the unfolded or miss-folded proteins (Kaufman, 1999; Duan *et al.*, 2010; Pan *et al.*, 2012). As responses to UPR, several signalling pathways are initiated, including the activation of double-stranded RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK), the inositol requiring enzyme 1 (IRE1) and the transcription factor 6 (ATF6) (Wang *et al.*, 1998; Yoshida *et al.*, 1998; Harding *et al.*, 2000). Under physiological conditions, an ER GRP78 inhibits the activities of RERK, IRE1 and ATF6, while under ER dysfunctions, GRP78 dissociates from them, resulting in dimerization and phosphorylation of PERK and IRE1, and the cleavage of ATF6 (P90 to P50). All of these three pathways up-regulate the transcription factor CCAAT/enhancer binding protein homologous protein (CHOP), which can down-regulate the anti-apoptotic factor Bcl-2 and up-regulate the production of reactive oxygen species (ROS) (Tajiri *et al.*, 2004; Oida *et al.*, 2008; Xin *et al.*, 2014). Caspase-12 is also activated through the IRE1 pathway by dis-association with tumour necrosis factor receptor associated factor 2 (TRAF2), and subsequently activates caspase-9 and caspase-3 (Xin *et al.*, 2014). C-Jun N-terminal kinase (JNK) is activated as well through IRE1-ARTF2 pathway, which plays a pivotal role in stress reactions and cell death (Nickischer *et al.*, 2006). The induction of

CHOP and the activation of caspase-12 and JNK through ER stress finally lead to apoptotic cell death under the stimulation of ischemia insults. A scheme of cerebral ischemia-induced ER stress pathway is shown as follows, see Fig 13.

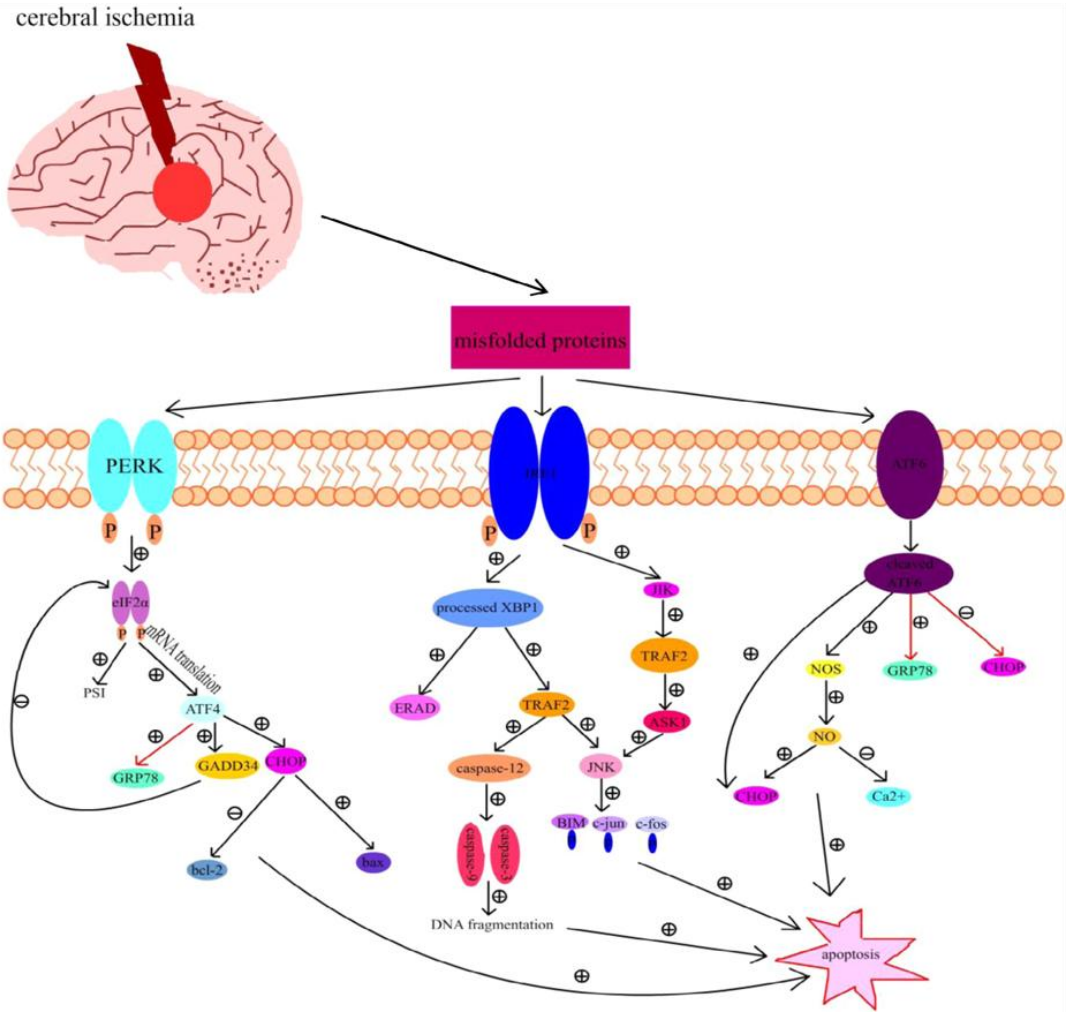


Fig 13. Scheme of cerebral ischemia-induced ER stress pathway (for details, see text above). Important abbreviations: PERK, double-stranded RNA dependent protein kinase-like endoplasmic reticulum kinase; IRE1, inositol requiring enzyme 1; ATF6, activating transcription factor 6; CHOP, CCAAT/enhancer binding protein homologous protein; JNK, c-Jun N-terminal kinase; ATF4, activating transcription factor 4; eIF2α, eukaryotic initiation factor 2α; GRP78, glucose regulated protein 78; XBP1, X-box-binding protein 1; TRAF2, tumour necrosis factor receptor associated factor 2 (Xin *et al.*, 2014).

2.2.3 New pharmaceutical approaches for ischemic stroke

Despite surgery and mechanical approaches providing effective pharmacological treatments immediately following an acute ischemic stroke to lessen the cerebral damage is promising, it still needs a long way to go. To date, the only one drug that is approved for clinical use is the thrombolytic recombinant tissue plasminogen activator (tPA, alteplase), however is only accessible to 4-5% of patients because it can only be administered in a short temporal window and in patients fulfilling very specific requirements.

The drugs for acute ischemic stroke under experimental development could be generally divided into two distinct groups: thrombolytics and neuroprotectors (Green, 2008). The former group contains compounds that restore the blood flow, including thrombolytics, antiplatelet and anticoagulant drugs. While the latter group encompasses a number of protective drugs including glutamate function modulators, metal chelators, free radical scavengers, immunomodulators, growth factors, 5-HT_{1A} agonists, statins, cytokines, citicoline, arundic acid, albumin and disodium 2,4-disulphophenyl-N-tert-butylnitrone (NXY-059) (Green, 2008; Mestre *et al.*, 2013).

2.2.3.1 tPA

Most ischemic strokes are resulted from thromboembolic occlusions of major arteries that supply blood flow to the brain. To lyse the clots inside the arteries and restore the perfusion to the ischemic brain constitutes the basis of the thrombolytic therapy. Thrombolysis using recombinant tissue plasminogen activator (tPA, alteplase) is one of the most biological effective treatments for acute ischemic stroke, indeed, it is the only therapy approved by the US Food and Drug Administration (FDA) (Lo *et al.*, 2003; Donnan *et al.*, 2008). However, the major adverse effect of thrombolysis is the transformation of symptomatic intracerebral hemorrhage, which increases with time, so the treatment is currently limited to 3-4.5-hour time window (Hacke *et al.*, 1998; Davis *et al.*,

2009). In fact, tPA is only used in about 4% of patients presenting after an acute ischemic stroke.

2.2.3.2 Statins

Nowadays, there are solid evidences showing that statins exhibit good perspectives of therapeutic efficacy towards cerebral ischemia. In addition, statins show a neuroprotective effect in neurodegenerative diseases such as Alzheimer´s disease, Parkinson´s disease, and Vascular Dementia as well (Wang *et al.*, 2011). These compounds are inhibitors of the HMG-CoA (3-hydroxy-3-methylglutaryl Coenzyme A) reductase, the rate-limiting enzyme in the synthesis pathway of endogenous cholesterol, and have been categorised in two types: lipophilic that cross the BBB such as lovastatin, simvastatin, atorvastatin and the hydrophilic ones that includes rosuvastatin and pravastatin among others (Vaughan *et al.*, 2004). In spite of their lipid profile improvement to reduce the risk of stroke, statins are also considered as “pleiotropic” molecules. It has been widely reported that their beneficial effects on the treatment of the ischemia, are related with different mechanisms such as the neuroprotection, anti-inflammatory effect, suppression of adhesion molecules activation, antioxidant effect and augmentation of endothelial nitric oxide synthase (eNOS) etc, see Fig 14. (Vaughan *et al.*, 1999; Montecucco *et al.*, 2012; Li *et al.*, 2014).

It has been demonstrated using different animal models of cerebral ischemia that the protective effect of statins in pre- and post-treatment increases cerebral blood flow, reduces the lesion volume by alleviating the tissue damage, improving neurological function and behaviour. Statins are able to increase of eNOS expression by both cholesterol-dependent and –independent mechanisms, increasing the release of beneficial NO, improving endothelial function, the vasodilatation and perfusion in the damaged brain (Cimino *et al.*, 2007). Moreover, statins show an antioxidant effect on reactive oxygen species (ROS) generated during ischemia and reperfusion that can oxidize proteins, lipids and nucleic acids damaging different cellular structures. Furthermore, statins exhibit anti-inflammatory and immunomodulatory properties by reducing the levels of a

number of inflammation molecule markers, such as NF-κB, iNOS, interleukins, cytokines and cell adhesion molecules including ICAM-1, P-selectin and E-selectin and VAP-1, which are activated by ischemia and contributing to the inflammatory response during ischemic stroke (Cimino *et al.*, 2007; Hernandez-Guillamon *et al.*, 2010). Additionally, statins can exert anti-thrombogenic ability by improving the platelet function and plaque stability.

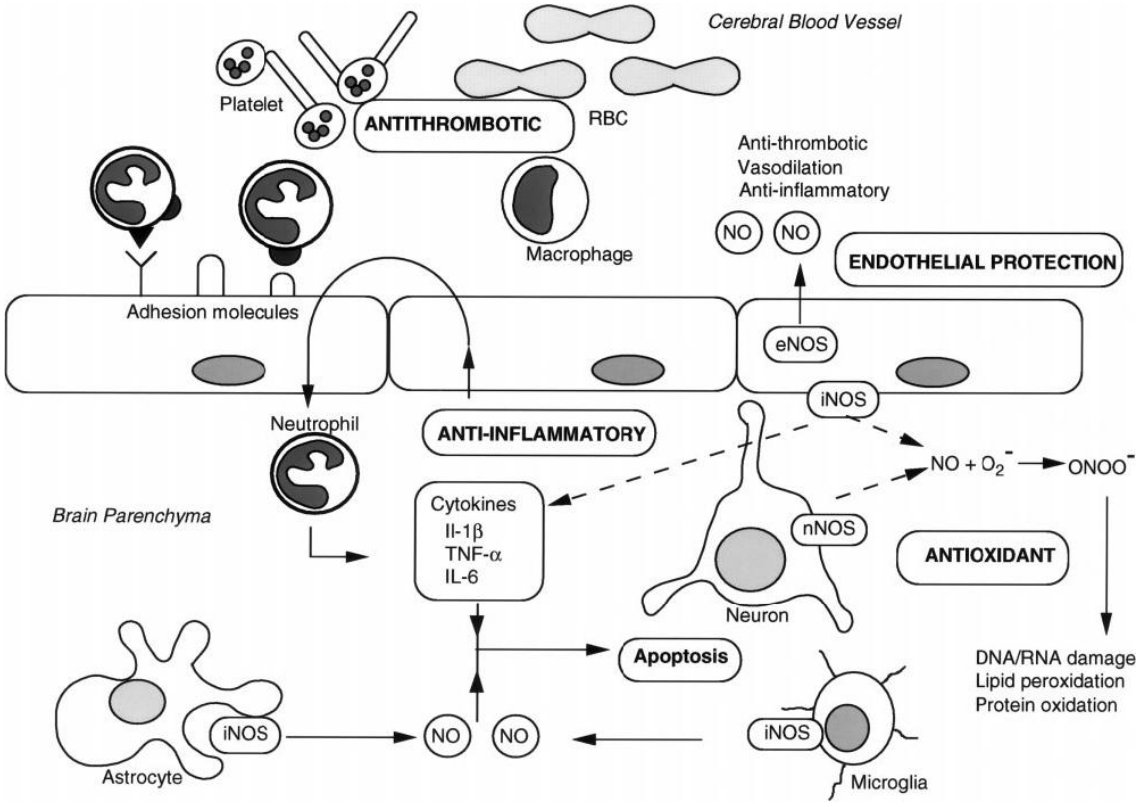


Fig 14. Neuroprotective properties of statins under cerebral ischemia. Statins maintain the endothelial function and exhibit anti-thrombotic, anti-inflammatory and antioxidant effects that may be neuroprotective under cerebral ischemia and reperfusion. Abbreviations: RBC, red blood cells; nNOS, neuronal NOS; O_2^-, superoxide anion; and $ONOO^-$, peroxynitrite (Vaughan *et al.*, 1999).

2.2.3.3 Nitrones

Oxidative stress response after the onset of cerebral ischemia and reperfusion is one of the most important molecular events for the cellular damage and death of cerebral tissue. In this regard, free radical scavengers, such as nitrones, constitute one of the neuroprotective approaches proved to bring beneficial outcomes in experimental cerebral ischemia. Additionally, nitrones exhibit as well neuroprotection on other experimental age-related disease models, such as hearing loss, retinal light damage and neuroinflammatory diseases (Floyd, 2006).

Nitrones are a series of organic compounds which have "X-CH=NO-Y" as a general structure. Because of this, nitrones have been originally used for radical trapping agents in free radical chemistry. Latterly, they were extensively investigated in biological systems for their protection in oxidative-related diseases including ischemic stroke. For instance, N-*tert*-butylnitrone (PBN) has been shown to reduce oxidative stress and lipids peroxidation (Kalyanaraman *et al.*, 1991), to ameliorate ischemic brain damage, and to reduce the infarct volume in animal models of ischemic stroke (Sun *et al.*, 2008). Another well-known PBN-related nitrone with good neuroprotective profile is Disodium 2,4-disulphophenyl-*N*-tert-butylnitrone (NXY-059), which exhibits free radical trapping properties (Maples *et al.*, 2001) and neuroprotection in clear dose-dependent manner both in transient (Kuroda *et al.*, 1999) and permanent (Zhao *et al.*, 2001) MCAO models of stroke. NXY-059 has also a wide window of opportunities producing significant neuroprotection, when given at 4-hour after permanent focal ischemia (Sydserff *et al.*, 2002) and at 5-hour after transient ischemia (Kuroda *et al.*, 1999). Besides its effectiveness in animal models, however, only unequivocal evidence of efficacy has been observed in advanced clinical studies (Macleod *et al.*, 2008). Recently, new multi-functional nitrones for the treatment of ischemic stroke have been reported. For example, 2-[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN) and a new pyrazine derivative (compound 21) possess both antiplatelet and antioxidant activities, and shows significant neuroprotective effects under *in vitro* and *in vivo* models of ischemic stroke (Sun *et al.*, 2008; Sun

et al., 2012). The (Z)-*α*-aryl and heteroaryl-*N*-alkyl nitrones also exhibit antioxidant and neuroprotective properties; in addition, they are anti-inflammatory and have the ability to cross the BBB. Therefore, these multi-functional nitrones targeting the concept of neuroprotection remain to be a good perspective and still could be considered as new therapeutic approaches for ischemic stroke treatments.

2.2.4 SSAO/VAP-1 possible involvement in stroke

To date, there are increasing evidences showing that SSAO/VAP-1 could probably play an important role after the onset of stroke. The soluble SSAO/VAP-1 protein and activity levels have been found increased in ischemic and hemorrhagic stroke patients (Airas *et al.*, 2008; Hernandez-Guillamon *et al.*, 2012), as well as in other pathologies involving inflammation (Kurkijärvi *et al.*, 1998; Ferrer *et al.*, 2002a; Boomsma *et al.*, 2003; del Mar Hernandez *et al.*, 2005; Airas *et al.*, 2006). Moreover, its plasmatic activity levels constitute a strong predictor of parenchymal hemorrhages after tPA treatment in ischemic stroke patients, also predicting the adverse neurological outcome (Hernandez-Guillamon *et al.*, 2010). Interestingly, the inhibition of SSAO/VAP-1 activity by SSAO inhibitors in different stroke animal models attenuates the adhesion molecules expression, the leukocyte adhesion and extravasation, down-regulates the inflammatory response, reduces the infarct volume and improves the neurological outcome (Hernandez-Guillamon *et al.*, 2010; Ma *et al.*, 2011; Watcharotayangul *et al.*, 2012; Xu *et al.*, 2014; Xu *et al.*, 2015). Similar anti-inflammatory effects and attenuated injury are also found in VAP-1-deficient or SSAO activity-suppressed animal models under ischemia-reperfusion treatment in lung (Kiss *et al.*, 2008) and heart (Yang *et al.*, 2011). Besides the benefits of the leukocyte adhesion blockade, the SSAO/VAP-1 activity inhibition also prevents the release of aldehydes, hydrogen peroxide and ammonia resulting from its enzymatic activity, which when overproduced can contribute to the oxidative stress and the disease progression (Conklin *et al.*, 1998; Yu *et al.*, 1998; Hernandez *et al.*, 2006; Solé *et al.*, 2008). In this context, by both the pro-

inflammatory and the metabolic actions, SSAO/VAP-1 could be possibly involved under stroke conditions, exacerbating the injury after the onset of stroke.

2.3 Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most common cause of dementia worldwide, accounting for 50-60% of all cases. According to the epidemiologic studies, the late onset AD accounts for approximately 90% of all cases at ages more than 60 years (Ferri *et al.*, 2006). The etiology of AD involves genetic, environmental, behavioural and developmental components (Anand *et al.*, 2014). The greatest risk factor is aging, while other factors may include positive family history, head trauma, female gender, previous depression, diabetes mellitus, hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity and stroke (Kivipelto *et al.*, 2001; Mayeux, 2003). From a genetic view, AD is a heterogeneous disorder with both familial and sporadic forms. In the familial AD, mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2), account for most cases of the disease of this type (Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995). While in the sporadic AD, aging, environmental and genetic reasons may act as important risk factors. The main genetic risk factor in sporadic AD is the inheritance of apolipoprotein E (apoE) ε4 allele (Corder *et al.*, 1993; Poirier *et al.*, 1993).

2.3.1 The pathophysiology of Alzheimer's neurodegeneration

AD is a progressive and neurodegenerative pathology and is characterized by the β-amyloid (Aβ)-formed plaques within the brain parenchyma and the Aβ accumulation in blood vessels, together with neurofibrillary tangles and the neurodegeneration (Duyckaerts *et al.*, 2009). When amyloid proteins accumulate in the walls of arteries, arterioles and capillaries and veins of the central nervous system, the term of cerebral amyloid angiopathy (CAA, or CAA-AD) is applied to describe the disorder (Revesz *et al.*, 2002). In fact, the pathological features of CAA occur in over 80% of Alzheimer disease brains (Ellis *et al.*, 1996). Both AD and CAA-AD would lead to cognitive impairment and dementia.

Several theories have been put forward on to elucidate the pathological mechanism of Alzheimer's disease, such as the amyloid beta cascade hypothesis (Hardy *et al.*, 1992), the cholinergic hypothesis (Francis *et al.*, 1999), the tau hypothesis (Mudher *et al.*, 2002), the oxidative stress hypothesis (Markesbery, 1997) and the inflammation hypothesis (Zotova *et al.*, 2010). Among these, the amyloid cascade hypothesis is the central one for the cause of Alzheimer's disease, which has been extensively studied during the last two decades.

According to the amyloid cascade hypothesis, the deposition of excessive β-amyloid (Aβ), the main component of the plaques, is the cause of Alzheimer's pathology, which latterly induces the neurofibrillary tangles, cell injury, vascular damage and ultimately dementia (Hardy *et al.*, 1992). Aβ peptides are generated by a sequential cleavage of APP by β-secretase and γ-secretase in the amyloidogenic pathway (Selkoe, 2001; Vassar, 2004). Nowadays, β-secretase is identified as a novel aspartyl protease, β-site APP-cleaving enzyme 1 (BACE1) (Hussain *et al.*, 1999; Sinha *et al.*, 1999). BACE1 cleavage of APP is a pre-requisite for Aβ generation. Cleavage by the γ-secretase can generate the majority of Aβ ended at amino acid 40 ($A\beta_{40}$), and a small proportion ended at amino acid 42 ($A\beta_{42}$) (Cole *et al.*, 2007b). While in the non-amyloidogenic pathway, APP is cleaved by α-secretase and γ-secretase, precluding the generation of Aβ, see Fig 15. Given that BACE1 is the initiating enzyme in the Aβ generation, and presumably rate-limiting, elevated in this disease, this enzyme can be considered as a prime drug target for inhibiting Aβ production in AD (Cole *et al.*, 2007a).

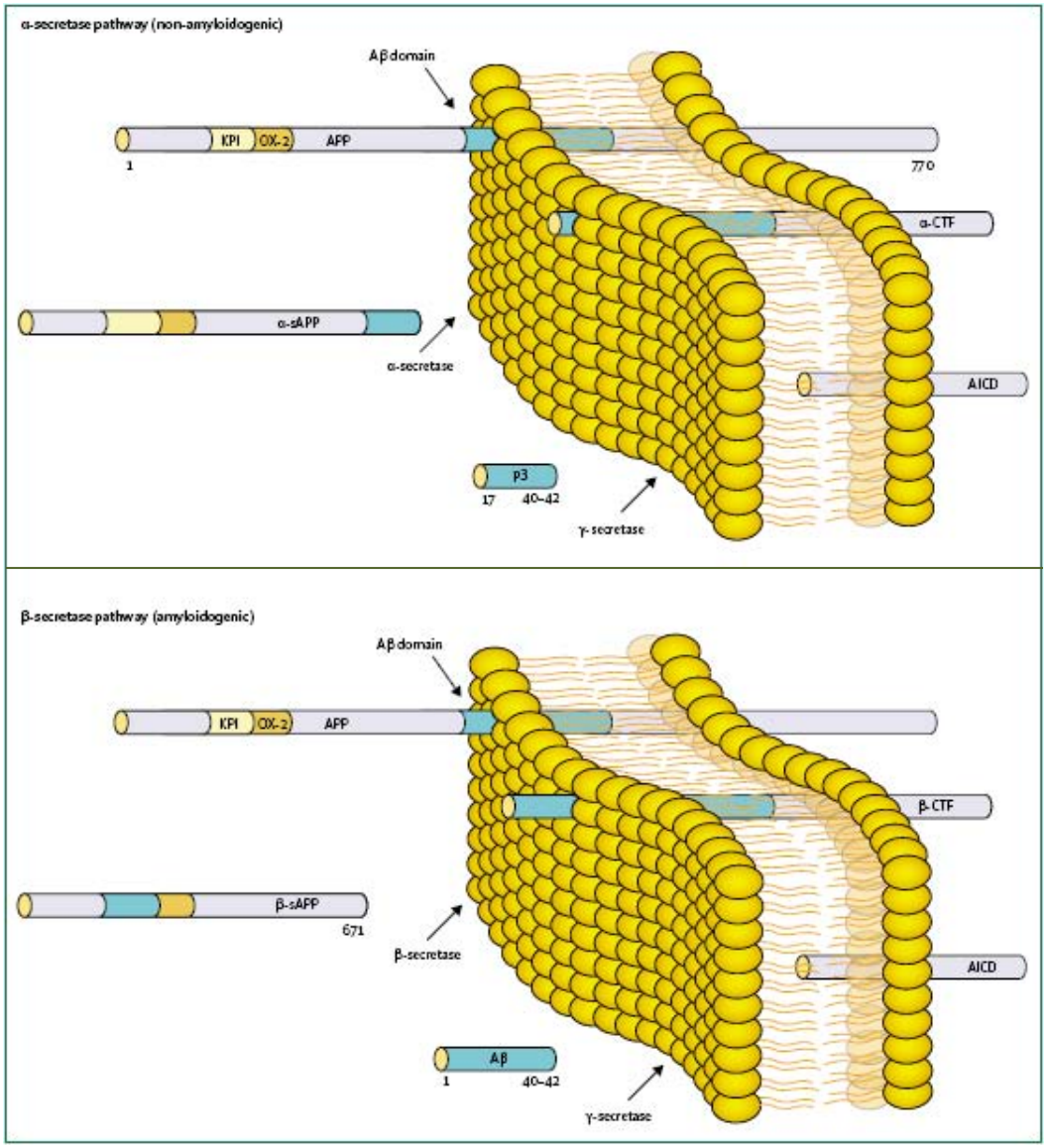


Fig 15. Metabolism of amyloid precursor protein (APP) by different secretases. APP is a trans-membrane protein with a large N-terminal extracellular tail. The full length form of APP is indicated, which contains the Kunitz-type protease inhibitor (KPI), the OX-2 antigen domain and Aβ domain. Aβ domain consists of 28 residues outside the plasma membrane and the first 12-14 residues inside the membrane. Under the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ domain, and releases the soluble APP fragment (α-sAPP). The remaining C-terminal fragment (CTF), α-CTF or C83 is cleaved by γ-secretase, releasing the short P3 fragment, hence without generating Aβ peptides. Under the amyloidogenic pathway, β-secretase (BACE1) firstly cleaves APP before Aβ domain, releasing the soluble βsAPP. The remaining CTF, β-

CTF or C99 is secondly cleaved by γ-secretase, releasing Aβ fragments with either 40 or 42 aminoacids. AICD is metabolized in the cytoplasm (Blennow *et al.*, 2006).

The imbalance between the production and clearance of Aβ is the initiating but central event of the amyloid hypothesis for the cause of Alzheimer's disease. Subsequently, the extracellular over-accumulated Aβ peptides ultimately lead to neuronal degeneration and dementia (Hardy *et al.*, 2002). The amyloid cascade hypothesis is shown as follows, see Fig 16.

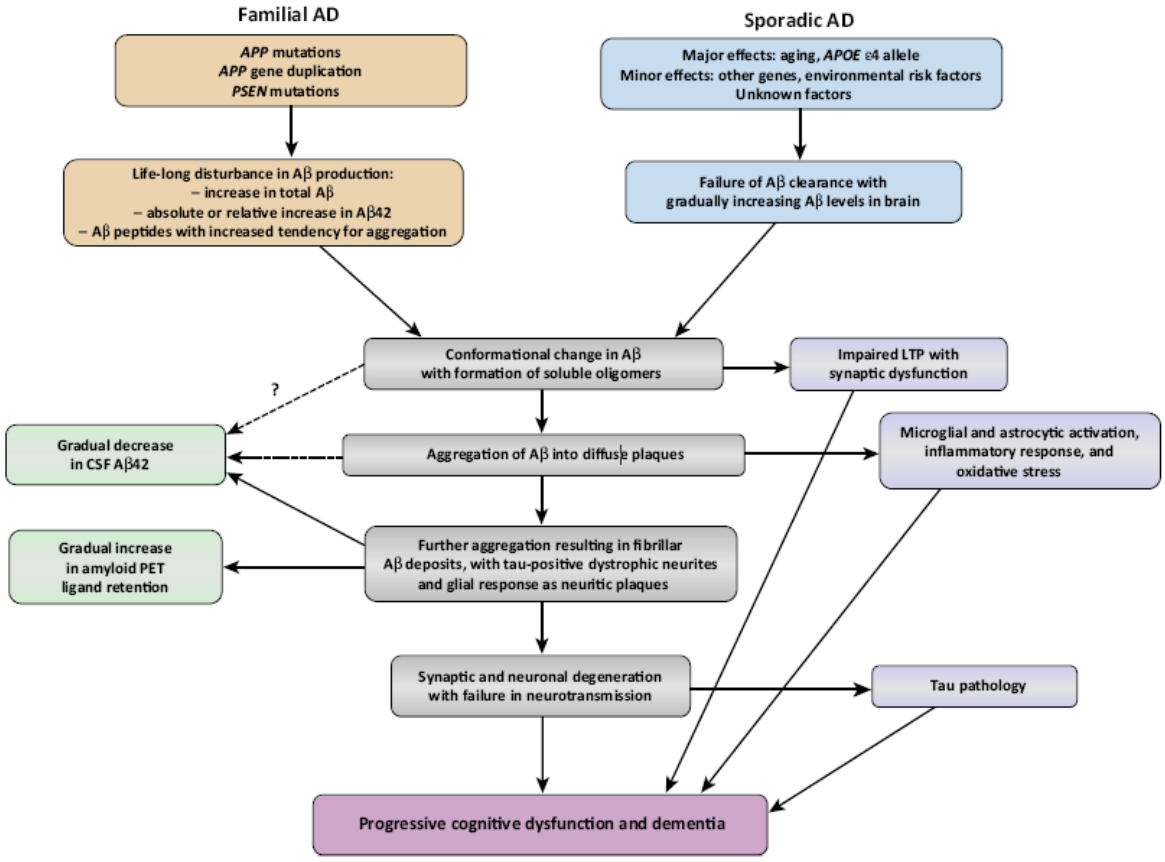


Fig 16. The amyloid cascade hypothesis. The central event of the hypothesis is the imbalance between Aβ production and its clearance. In familial AD, genetic variations induce a life-long disturbance in Aβ peptides production, or Aβ peptides are more inclined to aggregation. In sporadic AD, aging and apoE ε4 allele process main effects for increased Aβ production, together with the failure of Aβ clearance, increasing the risk for developing AD. The conformational change of Aβ peptides makes them more liable to aggregate, together with the initial formed soluble oligomers that then constitute larger

fibrils and accumulate into diffuse plaques, and finally neuritic plaques. Aβ oligomers impair hippocampal long-term potentiation (LTP) and synaptic function. The diffuse or deposited Aβ plaques activate inflammatory and oxidative stress responses. Aβ plaques and their responses can in addition injury neuronal and synaptic function, resulting in neurotransmitter deficits. Tau pathology with tangle formation is viewed as a downstream response, which could also contribute to the neuronal death and cognitive dysfunction. Abbreviations: CSF, cerebrospinal fluid; PET, positron emission tomography (Blennow *et al.*, 2006; Blennow *et al.*, 2015).

In the healthy brain, however, the levels of Aβ are rigorously regulated by its rate of production from APP and its clearance from the brain. The Aβ peptides in the brain can be degraded by a variety of proteolytic enzymes, including insulin-degrading enzyme (IDE), neprilysin (NEP), and endothelin-converting enzyme (Carson *et al.*, 2002). Aβ can also be cleared from the brain in a process balanced by the efflux and influx of Aβ across the BBB (Tanzi *et al.*, 2004). The main receptors responsible for the Aβ transportation from brain to blood and from blood to brain are the low-density lipoprotein receptor protein-1 (LRP-1) (Deane *et al.*, 2004) and the receptor for advanced glycation end products (RAGE) (Deane *et al.*, 2003), respectively. The pathways involved in the removal of cerebral Aβ are shown as follows, see Fig 17.

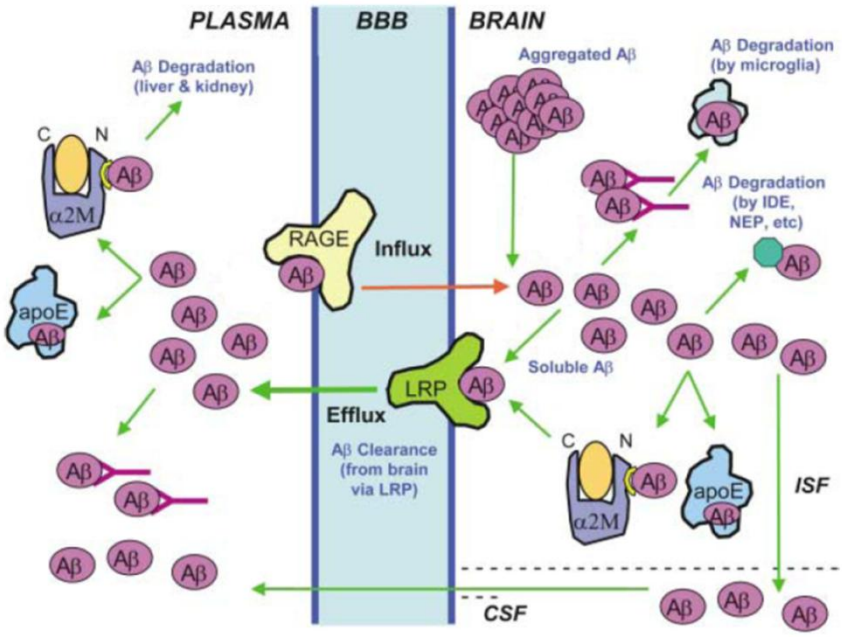


Fig 17. Scheme of the degradation of Aβ and its transportation across the blood-brain barrier (BBB), regulated by LRP1 and RAGE. On one hand, soluble Aβ can be removed from the brain via enzymatic degradation or receptor-mediated clearance. In the first case, Aβ can be degraded by activated microglia and specific peptidases, such as insulin-degrading enzyme (IDE) and neprilysin (NEP). In the second case, Aβ can be exported from BBB by directly binding to LRP or by first binding to LPR ligands/ Aβ chaperones apoE and α2M. On the other hand, Aβ present in the blood stream can be transported into the brain through RAGE or be delivered and degraded in peripheral organs (liver and kidney) by chaperones such as apoE and α2M (Tanzi *et al.*, 2004).

2.3.2 Therapeutic approaches towards AD

Since the description of Alzheimer's disease (AD), this complex central nervous system disorder has been existed for more than one century; however, the therapeutics of AD is still largely restricted. To date, the only available drugs that have been approved by the FDA are four acetylcholinesterase (AChE) inhibitors (tacrine, donepezil, galantamine, rivastigmine) and an N-methyl D-aspartate (NMDA) receptor antagonist (memantine) (Anand *et al.*, 2014), which mainly target the deficiency of the neurotransmission in AD. Among these, tacrine exhibits hepatotoxicity which leads to its limited utility (Watkins *et al.*, 1994). The AChE inhibitors can increase the availability of acetylcholine by inhibiting AChE, therefore increasing the cholinergic neurotransmission. While memantine is a non-competitive NMDA receptor antagonist, it can alleviate the glutamate-mediated cytotoxicity (Wilcock, 2003), since glutamatergic activity in AD is abnormally high, which impede the normal activation of NMDA receptors and their function for learning and memory (Areosa *et al.*, 2005). Although these drugs can provide a temporary symptomatic remittance, they cannot final re-establish the cognitive decline.

With the extensive investigation on the pathophysiology of the disease, more therapeutic strategies can be applied according to the different processes of the disease. Hence, except for the symptomatic treatment for AD, more approaches based on the molecular pathogenesis have emerged. These new approaches

include anti-Tau pathogenesis (Tau phosphorylation inhibition, microtubule stabilization, Tau oligomerization blockage, and Tau-based immunotherapy), anti-amyloid accumulation (decreasing Aβ production, modulating Aβ transportation, decreasing Aβ aggregation and Aβ-based immunotherapy), oxidative stress reduction (endogenous and exogenous antioxidants), anti-inflammation, modulation of mitochondrial dysfunction and calcium homeostasis, caspase inhibition, metal chelation, multi-target directed ligands, etc. (Anand *et al.*, 2014; Kumar *et al.*, 2015).

The fact that AD involves various complex mechanisms promotes research toward compounds that can interact with several potential targets. For this reason, the "one drug, multiple target" strategy, also called multitarget-directed ligand (MTDL) approach, might be more appropriated (Buccafusco *et al.*, 2000; Youdim *et al.*, 2005; Bolea *et al.*, 2013b). This strategy suggests the use of compounds with multiple activities for different biological targets (Cavalli *et al.*, 2008). In this concern, various compounds have been developed with multiple properties, such as AChE, Butyrylcholinesterase (BuChE) inhibition, monoamine oxidase-A (MAO-A), MAO-B inhibition, antioxidant properties, anti-inflammation, neuroprotection and metal chelation. For instance, M30 is one of such multifunctional compounds: it is brain selective MAO-A and MAO-B inhibitor, metal chelator, free radicals scavenger, Nrf2 activator responsible for antioxidant and anti-inflammatory effects, exhibits neuroprotective, neurotrophin and anti-glutamatergic effects (Zheng *et al.*, 2015). ASS234 is another multitarget-directed compound: it can inhibit AChE, BuChE, MAO-A and MAO-B activities as well as interfere in Aβ aggregation, being effective against Aβ-induced toxicity and oxidative stress (Bolea *et al.*, 2013a). Recently, another MTDL - DPH-6 (Donepezil + propargylamine + 8-hydroxyquinoline hybrid) has been synthesized and has demonstrated to be MAO-A, MAO-B, AChE and BuChE inhibitor, exhibits metal-chelating, moderate to good ADMET properties and brain penetration capacity as well as ameliorates scopolamine-induced learning deficits in mice (Wang *et al.*, 2014).

2.3.3 SSAO/VAP-1 involvement in AD/CAA

In Alzheimer's disease patients, the deposition of Aβ peptides in the cerebrovascular tissues constitutes a particular pathology, the cerebral amyloid angiopathy (CAA), which occurs in 80% of all the AD patients (Ellis *et al.*, 1996). The Aβ plaques accumulating in and around the cerebral blood vessels impair the vascular cells function, such as endothelial and smooth muscle cells, and accelerates the onset and progression of the cerebrovascular pathology (Kawai *et al.*, 1993; Van Nostrand *et al.*, 1996). Vascular adhesion protein-1 (VAP-1), as described before, is highly expressed in endothelial cells and in smooth muscle cells in the cerebrovascular tissues. During the SSAO catalytic reaction performed by the enzyme, products including aldehydes, hydrogen peroxide and ammonia are generated, which are potentially toxic at high levels, or also at low concentrations for a long-term affection on the microenvironment. SSAO/VAP-1 is also present in a soluble from shed from the membrane-bound form, in the blood plasma. The pro-inflammatory action and toxic products generated from both forms of SSAO/VAP-1 could probably contribute to the vascular damage and related tissues. These facts have promoted studies to investigate the possible association between SSAO/VAP-1 and Alzheimer's disease. It has been demonstrated that SSAO/VAP-1 is overexpressed in the cerebrovascular tissue in AD patients and AD-CAA (Ferrer *et al.*, 2002b; Unzeta *et al.*, 2007). Moreover, a strong expression of SSAO/VAP-1 co-localizes with Aβ deposits in cerebrovascular tissue of AD brains (Ferrer *et al.*, 2002b; Valente *et al.*, 2012). SSAO-mediated deamination increases as well the deposition of Aβ onto the blood vessel walls (Jiang *et al.*, 2008). Furthermore, the soluble SSAO/VAP-1, correlates with the overexpression of membrane-bound SSAO/VAP-1, and also exhibits elevated activity in plasma of moderate-severe and severe AD patients (del Mar Hernandez *et al.*, 2005; Unzeta *et al.*, 2007). Despite these human studies, it has also been proved that VAP-1/SSAO can induce Aβ aggregation and vascular cell damage, stimulating the Aβ deposition on the vascular walls *in vitro* (Chen *et al.*, 2006; Solé *et al.*, 2008; Sole *et al.*, 2011; Solé *et al.*, 2014), and in turn, the presence of vasculotropic Dutch-mutated $A\beta_{1\text{-}40}$ ($A\beta_{1\text{-}40}$ D)

increases the SSAO-dependent toxicity to the endothelium (Solé *et al.*, 2014). Therefore, SSAO/VAP-1 could be involved in the cerebrovascular dysfunction in AD and contribute to the progress and worsening of this disease.

3. Relationship between stroke and AD

AD and stroke are the most common forms of dementia in the elderly and both incidences increase with aging. Recent advances suggest that these two show considerable overlapping in risk factors and in pathological changes, indicating a strong association between stroke and AD (Kalaria, 2000; Zhu *et al.*, 2007). In addition, a deep meta-analysis recently reported that stroke significantly and independently increases the risk for AD and in turn, the risk of intracerebral hemorrhages (Zhou *et al.*, 2015). Given that both AD and stroke may share common pathogenic mechanisms, such as oxidative stress, inflammation, vasculature dysfunction and neurovascular unit compromise (Lucke-Wold *et al.*, 2015), and that both Aβ and ischemia-reperfusion injury can target the structure and function of the cerebrovasculature, resulting in the neurovascular dysfunction (Iadecola, 2004; Iadecola, 2010), the neurovascular unit plays an important role in maintaining the homeostasis of the brain's microenvironment (Iadecola, 2010).

3.1 Neurovascular unit and blood-brain barrier

The neurovascular unit consists of all the major cellular components of the brain including brain endothelium, vascular smooth muscle cells (VSMC), pericytes, astrocytes, neurons, microglia, and perivascular cells (Bell *et al.*, 2009). The communication between the cells of the neurovascular unit is very important and is responsible for regulating blood flow and controlling the exchange of substances across the BBB. Hence, it maintains the functional immune surveillance and provides energy and nutrients to the brain (Iadecola, 2010). BBB is mainly formed by the tightly sealed monolayer of brain endothelial cells in the capillary microvasculature, except for astrocyte end-feet and pericytes (Ballabh *et al.*, 2004). The tight junctions (TJs) between cerebral endothelial cells can form a diffusion barrier that selectively prevents the passive exchange of

solutes, regulates the trafficking of macromolecules, ions, amino acids, peptides, and signalling molecules between the blood and the brain (Abbott *et al.*, 2010). The structure and function of both neurovascular unit and BBB are largely impaired in a number of neurological disorders including stroke and AD, thus disrupting the homeostasis of the brain microenvironment and promoting the progression of both diseases (Iadecola, 2010).

3.2 Stroke and the risk for AD

Increasing evidences suggest that the vascular disorders such as cerebrovascular diseases and stroke in the elderly play an important role in the onset and progression of neurological disorders like Alzheimer's disease (AD) (Kalaria, 2000; Zlokovic, 2008; Grammas, 2011; Marchesi, 2014). In fact, 60%-90% of AD patients exhibit variable cerebrovascular pathology, such as cerebral amyloid angiopathy, endothelial degeneration and cerebral infarction (Kalaria, 2000). The fact that a high percentage of patients having suffered stroke subsequently develop AD, indicates that a strong link between these two pathologies exists. In this sense, some studies revealed that cerebral ischemia/stroke greatly increases the incidence of AD (Tatemichi *et al.*, 1994; Kokmen *et al.*, 1996; Kalaria, 2000) by about 2-fold among elderly patients (Schneider *et al.*, 2003; Altieri *et al.*, 2004), and this risk is even higher when stroke coexists with other vascular risk factors like atherosclerosis (Honig *et al.*, 2005).

There are also considerable evidences indicating that increased accumulation of APP is found at regions of ischemic stroke injury, such as focal cerebral ischemia and middle cerebral artery occlusion (MCAO), hence the cleavage of APP leading to amyloidogenic Aβ, may be up-regulated by ischemia (Stephenson *et al.*, 1992; Kalaria *et al.*, 1993; Shi *et al.*, 2000; Nihashi *et al.*, 2001; Badan *et al.*, 2004; van Groen *et al.*, 2005; Makinen *et al.*, 2008; Hiltunen *et al.*, 2009). Moreover, hypoxia and ischemic injury induce the up-regulation of BACE-1 transcription, expression and activity that increases the β-amyloid generation, Aβ deposition, neuritic plaque formation and potential memory deficits, facilitating

the pathogenesis of AD (Sun *et al.*, 2006; Zhang *et al.*, 2007; Guglielmotto *et al.*, 2009). The molecular mechanisms studied suggest that oxidative stress and hypoxia-inducible factor 1 (HIF-1) are responsible for the elevation of BACE1 expression at different stages following cerebral ischemia (Sun *et al.*, 2006; Zhang *et al.*, 2007; Guglielmotto *et al.*, 2009).

In the brain, the degradation of soluble Aβ can be carried out by activated microglia, or by several metalloproteinases such as insulin-degrading enzyme (IDE) and neprilysin (NEP) and endothelium converting enzyme (ECE). In this regard, ischemic conditions can also decrease the degradation of Aβ by down-regulating the levels of cerebral NEP and ECE-1 both *in vitro* and in *vivo* (Nalivaevaa *et al.*, 2004; Fisk *et al.*, 2007). Hypoxia/ischemia insult can also damage the BBB by various mechanisms, for example modifying the expression of LRP-1 that is a major Aβ clearance enzyme, hence impairing the clearance of Aβ from the brain (Zhang *et al.*, 2010). Moreover, the expression of the receptor of advanced glycation end products (RAGE), another important protein in the BBB that mediates the transport of Aβ across BBB, is stimulated in the mouse brain after experimental stroke and systematic hypoxia, thus decreasing the clearance of Aβ from the brain (Pichiule *et al.*, 2007).

Tau hyperphosphorylation in the neurons is a hallmark of AD, which can change the structure and conformation of tau, affecting its binding with tubulin and the capacity to promote microtubule assembly (Michaelis *et al.*, 2002). It has been found that transient hypoxia injury can also induce tau hyperphosphorylation in cortical neurons (Chen *et al.*, 2003; Wen *et al.*, 2004).

Therefore, there is strong association between cerebrovascular disease and AD, and through multiple pathways, the damage effects of stroke conditions such as ischemia could facilitate the progression and neurodegeneration, worsen dementia and the outcome of AD.

3.3 AD and the risk for stroke

Advanced age is the strongest risk factor for developing stroke, as it is for AD (Markus, 2008). Inflammation and oxidative stress accumulated during human aging negatively influence the vascular damage following a stroke incident (DiNapoli *et al.*, 2008), and the immune system may contribute to the infarct progression (Iadecola *et al.*, 2011). Recently, increasing investigations implicate that AD may be also associated with considerably increased risk of stroke development, although different studies may have some controversial results under different criterion and methodology. A population-based cohort study revealed that patients with AD had a higher risk of ischemic stroke and intracerebral hemorrhage than those without AD (Chi *et al.*, 2013). A follow-up study and a meta-analysis of cohort study showed also that patients with vascular dementia (VD) or baseline cognitive impairment have significantly higher risk for developing ischemic stroke than those without dementia, respectively (Imfeld *et al.*, 2013; Lee *et al.*, 2014). More investigations carried out by a register-based matched cohort study suggested that AD dementia patients, especially younger patients, have higher risk for developing hemorrhagic strokes (Tolppanen *et al.*, 2013). Similar results performed by a meta-analysis, as well showed that AD-like dementia is significantly associated with the risk of intracerebral hemorrhage (Zhou *et al.*, 2015). Moreover, molecular mechanisms studies established that both CAA and non-CAA developing amyloid precursor protein (APP) transgenic mice expressing elevated Aβ exhibit increased susceptibility to ischemic brain injury, develop larger infarct volumes and worse outcomes following middle cerebral artery occlusion (MCAO) treatments, by inducing cerebrovascular dysfunction, inflammation and greater cerebral blood flow (CBF) compromise (Zhang *et al.*, 1997a; Koistinaho *et al.*, 2002; Milner *et al.*, 2014). In this regard, it is confirmed that AD could also enhance the development and onset of stroke, increase the vulnerability and worsen the outcome following strokes.

3.4 Could SSAO/VAP-1 be the possible link between stroke and AD?

Stroke and AD are common diseases, which mostly happen in elderly people, and there are considerable studies establishing that there exists an interaction between each other, as it has been detailed above. On one hand, stroke-related processes occur in AD, precede AD and can enhance the risk for developing AD (Murray *et al.*, 2011), and on the other hand, AD can augment the onset of stroke (Zhou *et al.*, 2015) or increase the susceptibility to stroke injuries (Milner *et al.*, 2014). Among the overlaps shared by stroke and AD, the most notable common feature may be the dysfunction of cerebral blood vessels and the restricted brain perfusion which finally leads to neuronal injury and cognitive impairment (Attems *et al.*, 2014). Oxidative stress and inflammatory responses generated by the Aβ deposition in/ around the cerebral vascular wall or ischemia-reperfusion injuries contribute to the degeneration and dysfunction of the cerebral vasculature and the surrounding brain tissue. In this context, SSAO/VAP-1, which is highly expressed in the cerebral blood vesicles, more particularly in the endothelial and smooth muscles cells, mediates the rolling and transmigration of leukocytes partly through its SSAO activity, with aldehyde, hydrogen peroxide and ammonia as by-products, may be a possible link between stroke and AD, and involved in the pathogenesis of both diseases.

Evidences have revealed that SSAO/VAP-1 is overexpressed in the cerebrovascular tissue and co-localizes with Aβ plaques in AD patients (Ferrer *et al.*, 2002b; Unzeta *et al.*, 2007; Valente *et al.*, 2012). The soluble VAP-1/SSAO presents in the blood plasma, exhibits elevated protein and activity levels in both AD (del Mar Hernandez *et al.*, 2005; Unzeta *et al.*, 2007) and stroke patients (Airas *et al.*, 2006; Hernandez-Guillamon *et al.*, 2012), and correlates with the adverse neurological outcome. In addition, SSAO/VAP-1 can induce Aβ aggregation and vascular cell damage, stimulating the Aβ deposition on the vascular walls (Chen *et al.*, 2006; Jiang *et al.*, 2008; Solé *et al.*, 2008; Sole *et al.*, 2011; Solé *et al.*, 2014). VAP-1/SSAO can participate in the inflammatory response in multiple models of stroke, where the inhibition of its activity can

reduce leukocyte extravasation hence lessen the neurological dysfunction (Hernandez-Guillamon *et al.*, 2010; Watcharotayangul *et al.*, 2012; Xu *et al.*, 2015). Despite of SSAO/VAP-1 can mediate leukocyte-endothelium adhesion by itself during inflammatory conditions like AD and stroke, SSAO/VAP-1 can also up-regulate the expression of other adhesion molecules, such as ICAM-1, VCAM-1, P-selectin, E-selectin and chemokine receptors under different inflammation sites (Willam *et al.*, 1999; Saccani *et al.*, 2000; Jalkanen *et al.*, 2007; Lalor *et al.*, 2007), augmenting the leukocyte adhesion cascade, by producing biological active products such as hydrogen peroxide (Salmi *et al.*, 2005). Furthermore, products from SSAO catalytic reaction are harmful at relative high levels, which are responsible at least partly for the protein cross-linkage, oxidative stress and cytotoxicity, thus contributing to the advanced protein aggregation and cerebrovascular dysfunction. Taking into account the alteration and all the multiple effects of SSAO/VAP-1 on the brain microvasculature, we hypothesize that this protein could be a potential link and therapeutic target for both stroke and AD.

II. OBJECTIVES

II. OBJECTIVES

The broad objective of this work has been to study the molecular mechanisms by which SSAO/VAP-1 is involved in stroke, using experimental models of ischemic stroke, the therapeutic benefit of inhibiting its activity, and its possible nexus with Alzheimer's disease. To achieve this objective, we put forward the following specific objectives:

1. To study the possible involvement of SSAO/VAP-1 in oxygen-glucose deprivation (OGD)-mediated damage using endothelial hSSAO/VAP-1-expressing cells as experimental model of cerebral ischemia.

2. To assess the protective effect of new molecules with SSAO inhibitory capacity for the treatment of stroke in SSAO/VAP-1-expressing brain endothelial cells (hCMEC/D3) under OGD as a model of the blood-brain barrier in cerebral ischemia.

3. To determine by *in vitro* and *in vivo* approaches whether the beneficial effect of simvastatin observed in cerebral ischemia could be mediated by the modulation of the SSAO/VAP-1 levels.

4. To study the possible role of SSAO/VAP-1 in the nexus between ischemic stroke and Alzheimer's disease by using SSAO/VAP-1-expressing human brain endothelial cells (hCMEC/D3).

III. MATERIAL AND METHODS

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1. Biological materials

1.1. Cell culture

1.1.1. The HUVEC WT and HUVEC hSSAO/VAP-1 cell lines

The wild type Human Umbilical Vein Endothelial cell line (HUVEC WT) was a gift from Dr F.J. Muñoz (Universitat Pompeu Fabra, Barcelona, Spain). Human SSAO/VAP-1-transfected HUVECs (HUVEC hSSAO/VAP-1) were previously developed in our group (Sole *et al.*, 2011). HUVEC WT were cultured in M199 (Life technologies) supplemented with 2.2 g/L $NaHCO_3$ (Fluka), 2 mM glutamine (Invitrogen), 5% FBS (Foetal bovine serum, Gibco), 100 u/mL penicillin and 100 µg/mL streptomycin (PAN Biotech). HUVEC hSSAO/VAP-1 cells were cultured in M199 supplemented with 2.2 g/L $NaHCO_3$, 2 mM glutamine, 5% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin and 100 µg/mL geneticine (G418, Invitrogen), which was used to ensure the hSSAO/VAP-1 DNA maintenance. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and used until passage 40. The cells were subcultured when the confluence reached more than 95%, making a 1/50 dilution for WT and 1/40 for hSSAO/VAP-1-expressing HUVEC cells. This process was done after cells growing for one week, changing the media every 2-3 days.

1.1.2. The A7r5 hSSAO/VAP-1 cell line

The human smooth muscle cells (SMC) (A7r5 hSSAO/VAP-1) were previously developed in our group (Sole *et al.*, 2007), and cultured in DMEM (Dulbecco's Modified Eagle's Medium-high glucose, life technologies) supplemented with 2 mM glutamine, 10% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin and 100 µM G418, which was used to ensure the hSSAO/VAP-1 DNA maintenance. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and used until passage 25. The cells were subcultured when the confluence

reached more than 95%, making a 1/10 dilution after cells growing for 15 days, with changing the media every 2-3 days.

1.1.3. The hCMEC/D3 WT and hSSAO/VAP-1 cell lines

The human cerebral microvascular endothelial cell line hCMEC/D3 was obtained from Dr. Couraud's lab (Paris, France) (Weksler *et al.*, 2005) as a model of the BBB. hCMEC/D3 cells were cultured as recommended, on 150 µg/mL collagen type I (Rat Tail, Corning) –coated plates in EBM-2 (Lonza) medium supplemented with 5% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin, 1.4 µM Hydrocortisone (Sigma), 5 µg/mL Ascorbic Acid (Sigma), 1% Chemically Defined Lipid Concentrate (Life Technologies), 10 mM HEPES (Life Technologies) and 1 ng/mL human bFGF (Fibroblast Growth Factor-basic, Sigma). Plates were pre-coated with collagen for 1-3 hours and then washed with PBS 1X (Phosphate-buffered saline, life technologies) for one time before seeding. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and used until passage 40. Cells were subcultured once a week, when the confluence reached more than 95% at a 1/15 dilution, and the media was changed every 2-3 days.

In order to obtain the hCMEC/D3 cell line stably expressing the human SSAO/VAP-1, hCMEC/D3 WT cells were transfected with a PcDNA3.1(+) vector containing the human SSAO/VAP-1 cDNA (Sole *et al.*, 2007) using the Fugene® HD transfection reagent (Roche) according to the manufacturer conditions. After transfection, cells were selected by the addition of G418 antibiotic (800 µg/mL) for 1-2 months. Then, cells were diluted to allow the formation of monoclonal colonies in the presence of 200 µg/mL G418. This antibiotic concentration was used thereinafter for cell maintenance. Cell colonies were amplified and checked for SSAO/VAP-1 expression and activity before using; hCMEC/D3 hSSAO/VAP-1 cells were used until passage 40.

1.1.4. The THP-1 cell line

THP-1 monocytes were obtained from the American Type Culture Collection (ATCC: TIB-202) and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and 100 u/mL penicillin and 100 µg/mL streptomycin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were subcultured in new flask at concentrations between 2-4 x 10^5 cells/mL, and they were not allowed to overpass 1 x 10^6 cells/mL. THP-1 cells were used until passage 40.

1.2. Plasma samples and brain tissue from rats

The plasma samples and brain tissue from rats were prepared in the lab of Dr. Montaner from Institut de Recerca Vall d'Hebron (Barcelona). Briefly, after middle cerebral arterial occlusion (MCAO) treatments and before euthanasia, the blood samples from rats were drawn and collected in EDTA tubes, then immediately centrifuged at 1500 *g* for 15 minutes at 4℃ to obtain plasma supernatants, and stored at -80℃ until their use.

The brains of these rats were isolated, divided into ipsilateral hemisphere (IP) and contralateral hemisphere (CL), and homogenized with a lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN_3, 1% Triton X-100, phenylmethanesulfonyl fluoride solution (PMSF, Sigma-Aldrich), Aprotinin (Apr, Sigma-Aldrich). Each gram of tissue was homogenized in 2.8 ml of the aforementioned buffer and centrifuged at 12000 rpm for 12 minutes at 4℃ . Homogenates were stored at -80℃ until use.

1.3. Other materials

Recombinant VAP-1 (rVAP-1) was a kind gift from Dr. D.J. Smith and Biotie Therapies Corp (Turku, Finland). All other reagents were purchased from Sigma-Aldrich, unless specified.

2. *In vitro* and *in vivo* models of ischemic stroke

2.1. Combined Oxygen and Glucose Deprivation (OGD)

2.1.1. OGD conditions for HUVEC WT and hSSAO/VAP-1

Combined oxygen and glucose deprivation (OGD) and reoxygenation have been used as an experimental approach to ischemic stroke. In this part, OGD treatments were performed in glucose-free Hanks' Balanced Salt Solution (HBSS, 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 M NaH_2PO_4, 1.8 mM $CaCl_2$ and 26.2 mM $NaHCO_3$, pH 7.4), introducing cells into a temperature-controlled (37 ± 1°C) Invivo$_2$ hypoxia workstation (RUSKINN) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in the incubator under normoxia conditions (5% CO_2/95% air), in HBSS containing 5 mM glucose. In experiments performing reoxygenation, cells undergone OGD were returned to normoxia conditions by replacing HBSS with serum-free cell culture media (containing 5 mM glucose) and adding the same treatments present during OGD. In experiments analysing the soluble SSAO/VAP-1 release, HBSS was not replaced for the reoxygenation period, but glucose was added in OGD dishes.

2.1.2. OGD conditions for hCMEC/D3 WT and hSSAO/VAP-1

OGD treatments were performed in glucose-free DMEM (Life Technologies) after washing cells with glucose-free PBS, and then introducing the cells into the temperature-controlled (37±1°C) Invivo$_2$ hypoxia workstation as previously described. Control cells were maintained in DMEM (5 mM glucose) in the incubator under normoxia conditions (5% CO_2/95% air). In experiments including reoxygenation, cells that had undergone OGD were returned to normoxia conditions after replacing glucose-free DMEM by serum-free DMEM (5 mM glucose) and adding the same treatments present during OGD. In experiments analysing the soluble SSAO/VAP-1 release, DMEM was not replaced for the reoxygenation period, but glucose was added into OGD dishes.

2.2. Middle cerebral arterial occlusion (MCAO) models in rats

The male Wistar rats (270–300 g; Charles River Laboratories Inc., Wilmington, MA, USA) were used in all experiments, and rats were kept in a climate-controlled environment on a 12-hour light/12-hour dark cycle. Food and water were available *ad libitum*. Analgesia (Buprenorphine, 0.05 mg/Kg s.c; Divasa Farma-Vic S.A) was given to all rats every 24 h from the first until the last day of the experiment protocol to minimize their pain and discomfort.

The embolic MCAO (eMCAO) model and intraluminal transient MCAO (tMCAO) model were used in the study. The surgeries were performed in the lab of Dr. Montaner (Institut de Recerca Vall d'Hebron, Barcelona). In both models, animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery and body temperature was maintained at 37°C. The surgical exposure of the bifurcation of the external carotid artery and the internal carotid artery was performed on the right side.

The eMCAO was induced by a blood embolus placed at the origin of middle cerebral artery (MCA) through a middle neck incision (Zhang *et al.*, 1997b). One day before eMCAO surgery, arterial blood from a donor rat was withdrawn to form two identical clots (length: 1.5 cm; diameter: 0.3 mm, each) as described (Hernandez-Guillamon *et al.*, 2010).

The tMCAO was induced by introducing an intraluminal filament as described in detail (Garcia-Bonilla *et al.*, 2011). A silicone-coated nylon monofilament (Doccol Corporation, reference number: 403723PK10) was inserted to occlude the MCA.

Immediately after the occlusion, animals were allowed to recover from anesthesia. Sixty or eighty minutes after occlusion, reperfusion of blood flow in the MCA was induced and, to that end, animals were re-anaesthetized and the filament was removed from the artery. In both models, cranial trepanation was performed the day before MCAO to attach a laser-Doppler probe (Moor Instruments) and continuously monitor regional cerebral blood flow during the surgery. Only animals that exhibited a reduction > 75% in regional cerebral blood

flow during MCAO were included in the study. Sham-operated animals were submitted to the same processes except for the clot introduction in the eMCAO model, or the filament introduction in the tMCAO model. Three doses of analgesia were administered: after cranial trepanation, after MCAO surgery and at 24 h after it. All animals were euthanized 48 hours after the surgery.

3. Drug treatments for *in vitro* and *in vivo* ischemic stroke model

3.1. Drug treatments in OGD models

Depending on different experiment design, MA (methylamine; 1-3 mM), SC (semicarbazide; 1 mM), PLZ (phenelzine; 100 nM), batimastat (5 µM), MMP-2 inhibitor IV (Millipore; 0.1-10 µM), $A\beta_{1-40}D$ ($A\beta_{1-40}$ peptide containing the Dutch mutation, Bachem; 2.5-5 µM), DPH-4 (Wang *et al.,* 2014; 0.1-10 µM) or/and Simva (simvastatin; 100 nM) were added into HBSS or DMEM according to different cell types before OGD starting. Then introducing cells into a temperature-controlled (37 ± 1°C) $Invivo_2$ hypoxia workstation (RUSKINN) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in the incubator under normoxia conditions (5% CO_2/95% air), in HBSS or DMEM containing 5 mM glucose. In the reoxygenation process, the compounds were maintained at the same concentrations than during OGD.

To operate a pre-treatment of $A\beta_{1-40}D$ with hCMEC/D3 cells before OGD, EBM-2 media was changed to DMEM (5 mM glucose) after washing the plates with glucose-free PBS for 1 time, $A\beta_{1-40}D$ was added to the plates and cells were maintained at 37°C in a normal humidified incubator containing 5% CO_2. After the 24-hour pre-treatment, DMEM (5 mM glucose) was changed to glucose-free DMEM after washing the plates with glucose-free PBS again. $A\beta_{1-40}D$ and all other treatments were added to the plates before introducing the cells into the temperature-controlled (37±1°C) $Invivo_2$ hypoxia workstation to start OGD treatment. In the reoxygenation process, the treatments were maintained the same than during OGD.

For the preparation of $A\beta_{1-40}D$, it was pre-treated with 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP, Sigma-Aldrich) for more than 4h but less than 6h, then aliquoted, evaporated at room temperature, and stored at -80°C until using, then being dissolved in sterile PBS containing 0.1% ammonium hydroxide.

For the preparation of simvastatin, it should be activated by opening the lactone ring. In short, 52.9 mg simvastatin were dissolved with ethanol (32.4%), distilled H_2O (61.5%), 0.1 M NaOH (6%), neutralized to pH 7.2 by 2 M HCl and incubated at 60°C for 1 hour to obtain a 10 mM stock, then diluted quickly to the required concentration for treating the cells.

3.2. Drug treatments in MCAO models

Simvastatin was prepared by opening the lacton ring and activating it. In short, simvastatin was dissolved in vehicle [distilled H_2O (75%), absolute ethanol (10%), and 0.1 M NaOH (15%)], incubated at 50°C for 2 h and pH adjusted at 7.2.
After the occlusion for 15 minutes, 1 mL of simvastatin solution [20 mg/kg (diluted in vehicle)]; Uriach Laboratories, Barcelona, Spain) or vehicle [distilled H_2O (75%), absolute ethanol (10%) and 0.1 M NaOH (15%)] were subcutaneously injected into the rats in a blinded manner, and subsequently, rats were allowed to wake up.

4. Infarct volume evaluation

Infarct volumes were evaluated by an investigator blinded to the treatment. Infarct volumes were measured with thionin (eMCAO model) or 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) (tMCAO model). For the thionin staining, animals were killed and transcardially perfused with paraformaldehyde 4% through an infusion pump. Brains were fixed in paraformaldehyde 4% during 24 h at 4℃ , immersed in sucrose for another 24 h at 4℃ , embedded in OCT and placed at -80℃ . Coronal brain sections (30 µm) were cut in the cryostat and mounted on slides. Thionin staining was performed on slides: non-injured areas were stained in deep blue whereas ischemic regions

remained pale. Images were captured using a CanoScan 4200F scanner (Canon USA Inc.) and infarct size was quantified using an image analysis system (Scion Image v4.02, Scion Corporation). For the TTC staining, animals were killed and immediately perfused with heparin solution followed by saline solution, both injected with an infusion pump. Brains were removed and cut in sections (1 mm) and stained with TTC as described elsewhere (Bederson *et al.*, 1986). TTC images were captured using a CanoScan 4200F scanner and infarct volumes were measured using Image J software by integration of infarcted areas. Infarct volume data were expressed as percentage of the ipsilateral hemisphere. Brain slices from TTC staining were divided into ipsilateral (IP) and contralateral (CL) hemispheres and kept frozen until further use.

5. Neurological evaluation

Neurological status was assessed using a 9-point neurological deficit scale (modified Bederson test) in a blinded manner at 60-80 min, 24 h and 48h after MCAO. This scale consists of four consecutive tests, as previously described (Pérez-Asensio *et al.*, 2005). Four consecutive tests were conducted: (i) spontaneous activity (moving and exploring = 0, moving without exploring = 1, no moving or moving only when pulled by the tail = 2); (ii) left drifting during displacement (none = 0, drifting only when elevated by the tail and pushed or pulled = 1, spontaneous drifting = 2, circling without displacement, or spinning = 3), (iii) parachute reflex (symmetrical = 0, asymmetrical = 1, contralateral forelimb retracted = 2), and (iv) resistance to left forepaw stretching (stretching not allowed = 0, stretching allowed after some attempts = 1, no resistance = 2).

6. MTT reduction assay

HUVEC WT and hSSAO/VAP-1 were seeded at 40000 cells/mL and 53200 cells/mL, respectively. Both hCMEC WT and hSSAO/VAP-1 cells were seeded at 50000 cells/mL. Treatments were added after 48h grown, when the confluence reached 80% - 90%. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

bromide] reduction assay was employed to evaluate the cell viability. Briefly, at the end of the treatments, cells were incubated with 0.5 mg/mL MTT for 3 h in HUVECs and for 1.5 h in hCMECs at 37°C. The medium was then replaced by dimethylsulfoxide (DMSO) to dissolve the blue formazan precipitate, and it was spectophotometrically quantified at 560 nm and 620 nm in a microplate reader (Labsystems multiscan RC) (Plumb *et al.*, 1989).

7. LDH release assay

HUVEC WT and hSSAO/VAP-1 cells were seeded at 40000 cells/mL and 53200 cells/mL, respectively. Treatments were added after 48h growing, when the confluence reached 80% - 90%. The extent of lactate dehydrogenase (LDH) release into the medium was assessed using the TOX7 kit (Sigma-Aldrich), following the manufacturer instructions. Briefly, after the treatments, the culture media were collected after treatments, centrifuged at 800 *g* for 10 minutes at 4°C to eliminate dead cells. The supernatants were collected and directly used for LDH determination or stored at -80°C to be processed later. When performing LDH release assay, it was first prepared the Lactate Dehydrogenase Assay Mixture by mixing equal volumes of LDH Assay Substrate Solution, LDH Assay Dye Solution and 1X LDH Assay Cofactor Preparation. Then the sample media were transferred to a clean flat-bottom plate and Lactate Dehydrogenase Assay Mixture was added to each sample in a volume equal to twice the volume of the sample media. The plate was covered to protect it from light and incubated at room temperature for 20-30 minutes. The absorbance was spectrophotometrically measured at 490/690 nm in a microplate reader (Labsystems multiscan RC).

8. Adhesion assays

HUVEC WT and hSSAO/VAP-1 cells were seeded at 40000 cells/mL and 53200 cells/mL, respectively. Both hCMEC WT and hSSAO/VAP-1 cells were seeded at 50000 cells/mL. Treatments were added after 48h growing of the cells, when the confluence reached 80% - 90%. At the end of treatments, THP-1 monocytes

were labelled with 1 µM calcein-AM for 30 minutes. After washing the calcein-AM-labelled THP-1 monocytes for 1 time with FBS-free RPMI 1640 medium, they were added to HUVECs or hCMECs endothelial cells plates (2.5×10^5 per well in 24-well plates) and incubated for 30 minutes at 37°C. Then, unbound monocytes were removed by aspirating 80% of the media with pipette, or turning over the plates onto absorbent paper, then carefully adding FBS-free RPMI 1640 medium to the plates with an auto-pipette, and repeating the washing for at least three times. The fluorescence intensity was measured using a fluorescence microplate reader ($\lambda_{ex}/\lambda_{em}$: 495/530 nm). Results are represented as the percentage of fluorescence intensity, referring values to those obtained for the non-treated cells.

9. Hydrogen peroxide scavenging determination

Amplex ultrared reagent was applied to detect the hydrogen peroxide scavenging ability according to the manufacturer's instructions. Briefly, a series of concentrations (0-10 µM) of hydrogen peroxide (H_2O_2) were prepared, and RP18 was mixed with each concentration of H_2O_2 to a final concentration of 30 µM. Fifty µL of each sample were added into individual wells of a microplate. The reaction started by add 50 µL of Amplex UltraRed/HRP working solution (50 µL of 10 mM Amplex UltraRed reagent stock solution, 100 µL of 10 U/mL HRP and 4.85 mL of reaction buffer) to each microplate well containing samples. The plate was covered and incubated at room temperature for 30 minutes, protected from light. The fluorescence was measured at 530/590 nm in a microplate reader (Labsystems multiscan RC).

10. Cell lysates and concentrated culture medium

HUVEC WT and hSSAO/VAP-1 cells were seeded in dishes at 51000 cells/mL and 68000 cells/mL, respectively. Both hCMEC WT and hSSAO/VAP-1 cells were seeded in dishes at 60000 cells/mL, and all cells were grown to 80% - 90% confluence before the addition of treatments. After the treatments, cells were washed 1 time with cold PBS, collected and homogenized in 50 mmol/L Tris-HCl

(pH 7.5), containing 1% Triton X-100, 10 mmol/L EDTA and protease inhibitor cocktail (1:100) and were sonicated for 10 s. For SSAO activity determinations, the homogenization buffer was 100 mmol/L Tris-HCl, pH 9, and containing protease inhibitors. To obtain concentrated culture medium samples, culture media were collected after cell treatments and centrifuged at 4400 *g* for 10 minutes to eliminate dead cells and debris. Then media samples were dried out by evaporation in a Refrigerated CentriVap Concentrator (Labconco) and reconstituted in a smaller, known volume of distilled H_2O to obtain ten-fold concentrated culture medium.

11. The sub-cellular fractionations of hCMEC/D3 hSSAO/VAP-1

To obtain membrane-enriched fractions, cells were collected and homogenized in 10 mM HEPES, 1.5 mM $MgCl_2$ and 10 mM KCl buffer at pH 7.9, containing protease inhibitors cocktail. After a centrifugation at 2000 g for 15 min at 4°C, the obtained supernatant was ultracentrifuged at 100000 g (Sorvall Discorvery M120 SE) for 30 min at 4°C to separate the soluble cytosolic fraction from the pellet containing the membrane-enriched fraction.

To obtain lipid raft-enriched fractions, cells were scraped in PBS, and recovered through a 5 min at 800 g centrifugation. Then the pellet was reconstituted in 450 µL of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1% Brij 98 buffer at pH 7.2, containing protease inhibitors cocktail. After 15 min incubation at 37ºC under continuous agitation samples were centrifuged for 10 min at 2000 g to discard nuclei. The supernatants were mixed with 450 µL of 90% sucrose in Tris-HCl buffer to obtain a 45% sucrose fraction, which were deposited at the bottom of ultracentrifuge tubes. Two additional fractions of 35% (2 mL) and 5% (0.8 mL) sucrose were added to the former to generate a sucrose gradient, and then the samples were centrifuged for 19 h at 120000 g. Ten 370 µL-fractions were recovered and analysed by western blot to identify the lipid raft and soluble membrane-enriched fractions.

12. Antibodies and western blot analysis

The antibodies used were: mouse anti-Bcl-2 (BD Biosciences, 1:1000), rabbit anti-Bax (Cell Signaling, 1:1000), rabbit anti-cleaved caspase-3 (Cell Signaling, 1:1000), rabbit anti-VAP-1 (Abcam, 1:1000), rabbit anti-bovine SSAO (1:1000) (Lizcano *et al.*, 1998), rabbit anti-caspase-12 (Abcam, 1:1000), mouse-anti-GRP78 (BD Biosciences, 1:1000), mouse anti-caspase-8 (BD Biosciences, 1:1000), rabbit anti-IGF1-R (insulin-like growth factor 1 receptor) (Santa Cruz Biotechnology, 1:1000), mouse anti-Flotillin-1 (BD Biosciences, 1:1000), mouse anti-Tf Rec (ZYMED, 1:1000), rabbit anti-P-selectin (BioVision, 1:1000), rabbit anti-E-selectin (Santa Cruz, 1: 500), rabbit anti-VCAM-1 (Epitomics, 1:1000), rabbit anti-ICAM-1 (GeneTex, 1:1000), rabbit anti-NOS-2 (Santa Cruz, 1:1000), rabbit anti-COX-2 (Santa Cruz, 1:1000), goat anti-SOD-1 (Santa Cruz, 1:1000), rabbit anti-IκB-α (Santa Cruz, 1:1000), rabbit anti-BACE1(Abcam, 1:1000), rabbit anti-LRP-1 (Epitomics, 1:1000), rabbit anti-RAGE (Epitomics, 1:1000), mouse anti-APP 20.1 (W.E. Van Nostrand, Stony Brook University, NY, USA; 1:1000), mouse anti-*β*-actin (Sigma-Aldrich, 1:5000), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Ambion-Invitrogen, 1:20000), HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (BD Biosciences, 1:1000); HRP anti-mouse IgG (Dako, 1:1000), HRP anti-goat IgG (Pierce, 1: 2000). A complete relation of the antibodies used with their concentrations and reference numbers has been included as Annex I.

Equal amounts of protein (20 µg/lane) determined by the Bradford method, or equal volumes of media (45 µL/lane) were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with TBS/0.1%-Tween buffer containing 5% (w/v) non-fatty milk and incubated overnight at 4°C with the corresponding primary antibodies diluted in TBS/0.1%-Tween buffer containing 5% (w/v) non-fatty milk. After incubation with the secondary antibodies diluted in TBS/0.1%-Tween buffer containing 5% (w/v) non-fatty milk, blots were developed using ECL® Chemoluminiscent detection

reagents and High Performance Chemiluminiscence Films (GE Healthcare). The ImageJ software was used to quantify the western blot signals.

13. MAO-A and MAO-B enzymatic activity determination

For MAO-A and MAO-B activity determinations, cells were collected and homogenized in 50 mM potassium phosphate buffer, pH 7.4, and containing protease inhibitors cocktail. Enzymatic activities were determined radiochemically by using a modification of the Otsuka and Kobayashi method (Otsuka *et al.*, 1964). Briefly, [^{14}C]-Benzylamine hydrochloride (100 µM and 2 mCi/mmol, American Radiolabeled Chemicals) was used as substrate for MAO-B, and 1 mM semicarbazide (SC) was added to avoid SSAO activity interference. A 30-min inhibitory pre-treatment of samples was performed at 37°C with 1 mM SC. Moreover, 1 µM of deprenyl (Dep) was used to specifically inhibit MAO-B activity. For MAO-A activity determination, 5-[2-^{14}C]-hydroxytryptamine binoxalate (^{14}C-5-HT; 100 µM, 0.5mCi/mM; Perkin Elmer) was used as substrate and 1 µM of clorgyline (Clor) was used to specifically inhibit MAO-A activity. Reactions were performed at 37°C for 90 minutes (MAO-B) or 30 minutes (MAO-A) in a final volume of 200 µL of 50 mM potassium phosphate buffer, pH 7.4, and started by subsequently adding 25 µL of substrates to the 200 µL of reaction system. Two to three hundred µg of cell lysates were used in each reaction. Reaction was stopped by adding 100 µL of 2 M citric acid. The [^{14}C]-aldehyde products were extracted into 4 mL of toluene/ethylacetate (1:1, v/v) solution containing 0.6% (w/v) of diphenyloxazole per vial. The amount of [^{14}C]-aldehyde was quantified using a Tri-Carb 2810TR liquid scintillation counter (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer). Cell lysate activities are expressed as pmol/min per mg of protein.

14. SSAO enzymatic activity determination

For SSAO activity determinations, cells were collected and homogenized in 100 mM Tris-HCl, pH 9, and containing protease inhibitors cocktail. Enzymatic activity was determined radiochemically by using a modification of the Otsuka and

Kobayashi method (Otsuka *et al.*, 1964). Briefly, [^{14}C]-Benzylamine hydrochloride (100 µM and 2 mCi mmol^{-1}, American Radiolabeled Chemicals) was used as substrate, and 1 µM Dep (deprenyl) was added to avoid monoamine oxidase (MAO) B interference. For cell lysates, 80-100 µg of HUVEC hSSAO/VAP-1 or hCMEC/D3 hSSAO/VAP-1 cell lysates were used in each reaction and a 30-minutes inhibitory pre-treatment of samples was performed at 37°C with 1 µM Dep or Dep plus different concentrations of DPH-4. Especially, when analysed the inhibitory ability of simvastatin towards SSAO activity, 150 µg of HUVEC hSSAO/VAP-1 cell lysates were used in each reaction and a 1-hour inhibitory pre-treatment of samples was performed at 37°C with 1 µM Dep or Dep plus different concentrations of simvastatin. For media samples, 45 µL of ten-fold concentrated media were used in each reaction. Reactions were performed at 37°C for 120 minutes in 100 mM Tris-HCl buffer, pH 9.0, by adding 25 µL of substrate to the 200 µL of reaction. Reaction was stopped by adding 100 µL of 2 mol/L citric acid. The [^{14}C]-aldehyde products were extracted into 4 mL of toluene/ethylacetate (1:1, v/v) solution containing 0.6% (w/v) of diphenyloxazole. The amount of [^{14}C]-aldehyde was quantified using a Tri-Carb 2810TR liquid scintillation counter (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer). Cell culture media activities are expressed as *p*mol/min per mL of medium.

15. Statistical analysis

Results are given as means ± SEM for independent experiments. Statistical analyses were performed by one-way ANOVA and further Newman-Keuls multiple comparison test. For the rat brain tissue samples, statistical analyses were performed by two-way ANOVA and further Newman-Keuls multiple comparison test. For the correlation between infarct volume and soluble SSAO, Pearson correlation coefficients were computed and linear regression was calculated. $P<0.05$ was considered to be statistically significant. Statistical analyses and graphic representations were obtained by using the GraphPad Prism 3.0 or 6.0 software (Inc, San Diego, CA, USA).

IV. RESULTS

Chapter I:

"Involvement of SSAO/VAP-1 in Oxygen-glucose Deprivation-mediated Damage Using the Endothelial hSSAO/VAP-1-expressing Cells as an Experimental Model of Cerebral Ischemia

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Involvement of SSAO/VAP-1 in Oxygen-Glucose Deprivation-Mediated Damage Using the Endothelial hSSAO/VAP-1-Expressing Cells as an Experimental Model of Cerebral Ischemia

Ping Sun Montse Solé Mercedes Unzeta

Institut de Neurociències i Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Key Words
Adhesion proteins · Endothelial cells · Metalloproteinase activity · SSAO · VAP-1 · Stroke · Vascular damage

Abstract
Background: In the acute phase of ischemic stroke, endothelial cells are activated and induce the expression of adhesion molecules. Vascular adhesion protein 1 (VAP-1) is a proinflammatory protein that mediates leukocyte recruitment through its semicarbazide-sensitive amine oxidase (SSAO) activity (EC 1.4.3.21). Plasmatic SSAO activity predicts the appearance of parenchymal hemorrhages after tissue plasminogen activator treatment in ischemic stroke patients, and it is increased as well in hemorrhagic stroke patients. The aim of this study has been to elucidate the role of SSAO/VAP-1 present in endothelial cells during ischemic stroke conditions. ***Methods:*** Based on the use of endothelial cells expressing, or not expressing, the human SSAO/VAP-1 protein, we have set up an easy ischemic model using oxygen-glucose deprivation (OGD) as an experimental approach to the stroke process. Different OGD and reoxygenation conditions have been analyzed. Western blotting has been used to analyze the activated apoptotic pathways. Several metalloproteinase inhibitors were also used to determine their role in the SSAO/VAP-1 release from the membrane of endothelial cells to the culture media, as a soluble form. Adhesion assays were also performed in order to assess the SSAO/VAP-1-dependent leukocyte adhesion to the endothelia under different OGD and reoxygenation conditions. ***Results:*** Our results show that SSAO/VAP-1 expression increases the susceptibility of endothelial cells to OGD, and that its enzymatic activity, through specific substrate oxidation, increases vascular cell damage under these experimental conditions. Caspase-3 and caspase-8 are activated during the death process. In addition, OGD constitutes a stimulus for soluble SSAO/VAP-1 release, partly mediated by metalloproteinase-2-dependent shedding. Short-time OGD induces SSAO/VAP-1-dependent leukocyte binding on endothelial cells, which is partly dependent on its enzymatic activity. ***Conclusions:*** Our results show that SSAO/VAP-1 could participate in some of the processes occurring during stroke. Its expression in endothelial cells increases the OGD-associated cell damage. SSAO/VAP-1 mediates also part of the tissue damage during the reoxygenation process by oxidizing its known enzymatic substrate, methylamine. Also, OGD constitutes a stimulus for its soluble-form release, found elevated in many pathological conditions including stroke. OGD induces SSAO-dependent leukocyte-binding activity, which may have consequences in disease progression, since leukocyte infiltration has shown a determinant role in cerebral ischemia. For all the stroke-related processes in which SSAO/VAP-1 participates,

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E-Mail karger@karger.com
www.karger.com/ced

Mercedes Unzeta
Institut de Neurociències i Departament de Bioquímica i Biologia Molecular
Edifici M, Facultat de Medicina, Universitat Autònoma de Barcelona
ES–08193 Bellaterra, Barcelona (Spain)
E-Mail mercedes.unzeta@uab.es

it would be an interesting therapeutic target. Therefore, this model will be a very useful tool for the screening of new molecules as therapeutic agents for cerebral ischemia.

Introduction

Ischemic stroke occurs after a cerebral blood flow disruption, leading to cellular death and tissue damage due to the lack of glucose and oxygen supply [1]. Worldwide, stroke constitutes the second leading cause of death, with a higher incidence in old people. Inflammation and oxidative stress accumulated during human aging negatively influence the damages following a stroke incident [2]. The immune system may contribute to infarct progression [3]. Endothelial cells activated by hypoxia during stroke produce free radical species and induce the expression of adhesion molecules such as vascular adhesion protein 1 (VAP-1). These molecules mediate the recruitment of leukocytes, which infiltrate through the blood-brain barrier (BBB) to the injured tissue, inducing cell damage due, in part, to their cytokine release.

VAP-1 is a homodimeric glycoprotein with enzymatic function that binds leukocytes through its semicarbazide-sensitive amine oxidase (SSAO) activity (EC 1.4.3.21) [4, 5]. As an enzyme, SSAO/VAP-1 metabolizes primary amines producing aldehydes, hydrogen peroxide (H_2O_2) and ammonia. SSAO may also use free amino groups present on the surface of leukocytes as substrates, thereby mediating leukocyte recognition, rolling, adhesion and transmigration through the endothelium. H_2O_2 produced by SSAO activity induces expression of other pro-inflammatory proteins, and mediates other SSAO functions [6]. On the other hand, H_2O_2 and formaldehyde are able to induce cellular damage when overproduced [7].

SSAO/VAP-1 presence at the cell membrane or in soluble form, released into blood plasma, is altered in several human pathologies [8–14] including cerebral ischemia [15]. The mediators that induce these alterations in SSAO/VAP-1 levels are still unknown, but it is believed that increased SSAO/VAP-1 levels contribute to the physiopathology of these diseases by overproducing harmful agents [16–18]. In addition, human plasma SSAO activity is a strong predictor of parenchymal hemorrhages after tissue plasminogen activator treatment in ischemic stroke patients [19], and plasma SSAO activity, also elevated in hemorrhagic stroke patients, predicts their neurological outcome [20]. Despite this evidence, the role of SSAO/VAP-1 in stroke is still far from clear. Therefore, our aim has been to elucidate the molecular mechanisms in which SSAO/VAP-1 is involved during stroke in order to establish whether its inhibition could have a therapeutic benefit, as previously suggested [19]. SSAO/VAP-1 expression is lost in cultured cells, so we had previously set up immortalized cell lines stably expressing human SSAO/VAP-1 [21, 22], which allow an in-depth study of the involvement of this enzyme in stroke and other pathologies mentioned above. In this study, we describe that SSAO/VAP-1 expression and activity contribute to endothelial cell damage and to leukocyte binding to the endothelium during oxygen-glucose deprivation (OGD), used as an experimental model of ischemic stroke.

Material and Methods

Cell Culture and Treatments

The wild-type (WT) human umbilical vein endothelial cells (HUVEC) were a gift from Dr. F.J. Muñoz (Universitat Pompeu Fabra, Barcelona, Spain). Human SSAO/VAP-1-transfected HUVEC (hSSAO/VAP-1 HUVEC) were previously developed in our group [22]. WT HUVEC were used as control cells throughout this study, since they do not express SSAO/VAP-1, as proven by Solé and Unzeta [22]. HUVEC were cultured as detailed by Solé and Unzeta [22]. THP1 cells were obtained from the American Type Culture Collection and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. For cell viability and adhesion assays, HUVEC were seeded at 40,000 cells/ml (WT) and 53,200 cells/ml (hSSAO/VAP-1), adding treatments after 48 h. For immunoblot analyses, cells were seeded at 51,000 cells/ml (WT) and 68,000 cells/ml (hSSAO/VAP-1), and grown to 80–90% confluence before treatment. All reagents were purchased from Sigma-Aldrich, unless specified otherwise. Recombinant VAP-1 (rVAP-1) was a kind gift from Dr. D.J. Smith and Biotie Therapies Corp. (Turku, Finland).

Combined Oxygen and Glucose Deprivation

OGD treatment was performed in glucose-free Hank's balanced salt solution (HBSS; 116 mmol/l NaCl, 5.4 mmol/l KCl, 0.8 mmol/l $MgSO_4$, 1 mol/l NaH_2PO_4, 1.8 mmol/l $CaCl_2$ and 26.2 mmol/l $NaHCO_3$; pH 7.4), introducing cells into a temperature-controlled (37 ± 1°C) Invivo$_2$ hypoxia workstation (Ruskinn) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in the incubator under normoxic conditions (5% CO_2/95% air) in HBSS containing 5 mmol/l glucose. In experiments performing reoxygenation, cells undergoing OGD were returned to normoxic conditions, replacing HBSS by serum-free cell culture medium (containing glucose) and adding any treatment present during OGD. In experiments analyzing soluble SSAO/VAP-1 release, HBSS was not replaced for the reoxygenation period, but glucose was added to OGD plates.

Cell Viability Assay

Cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction method. Briefly, cells were incubated in 0.5 mg/ml MTT for 4 h at 37°C after treatment. The medium was then replaced by dimethyl sulf-

oxide to dissolve the blue formazan precipitate, and it was spectrophotometrically quantified at 560 and 620 nm in a microplate reader (LabSystems Multiskan RC) [23].

Lactate Dehydrogenase Release Assay

The extent of lactate dehydrogenase (LDH) release into the medium was assessed using the TOX7 kit, following the manufacturer's instructions. Briefly, culture media were collected after treatments, centrifuged at 800 *g* for 10 min at 4°C to eliminate dead cells, and directly used for LDH determination or stored at –80°C to be processed later.

Cell Lysates and Concentrated Culture Medium

Cells were homogenized in 50 mmol/l Tris-HCl (pH 7.5), containing 1% Triton X-100, 10 mmol/l EDTA and a protease inhibitor cocktail (1:100) and were sonicated for 10 s. For SSAO activity determination, the homogenization buffer was 100 mmol/l Tris-HCl (pH 9), containing protease inhibitors. For immunoblot analyses, the culture media were centrifuged at 4,400 *g* for 30 min to eliminate dead cells and debris, then dried out by evaporation in a Refrigerated CentriVap Concentrator (Labconco) and reconstituted in a smaller, known volume of distilled H_2O.

Immunoblot Analyses

Equal amounts of protein (20 μg/lane) determined by the Bradford method, or equal volumes of medium were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with TBS/0.1% Tween buffer plus 5% (w/v) nonfat dried milk and incubated overnight at 4°C with the corresponding primary antibodies. After incubation with the secondary antibodies, blots were developed using ECL® Chemoluminiscent detection reagents and High Performance Chemiluminiscence Films (GE Healthcare). The ImageJ software was used to quantify Western blot signals. The antibodies used were: mouse anti-Bcl-2 (1:1,000; BD Biosciences); rabbit anti-Bax (1:1,000; Cell Signaling); rabbit anti-cleaved caspase-3 (1:1,000; Cell Signaling); rabbit anti-VAP-1 (1:1,000; Abcam); mouse anti-β-actin (1:5,000; Sigma-Aldrich); mouse anti-flotillin (1:1,000; BD Biosciences); mouse anti-caspase-8 (1:1,000; BD Biosciences); HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (1:2,000; BD Biosciences); and HRP anti-mouse IgG (1:2,000; Dako).

Enzymatic Analyses

SSAO/VAP-1 enzymatic activity was determined radiochemically using a modification of the Otsuka and Kobayashi method [24]. [^{14}C]-benzylamine hydrochloride (100 μmol/l and 2 mCi/mmol; Amersham) was used as substrate, and 1 μmol/l deprenyl was added to avoid monoamine oxidase B interference. Reactions were performed at 37°C for 180 min in 100 mmol/l Tris-HCl buffer (pH 9.0), adding 25 μl of substrate to the 200 μl of reaction; 45 μl of 10-fold concentrated medium were used in each reaction. The reaction was stopped by adding 100 μl of 2 mol/l citric acid. The [^{14}C]-aldehyde products were extracted into 4 ml of toluene/ethyl acetate (1:1, v/v) solution containing 0.6% (w/v) of diphenyloxazole. The amount of [^{14}C]-aldehyde was quantified using a Tri-Carb 2810TR Liquid Scintillation counter (PerkinElmer) and the Quanta Smart 3.0 software (PerkinElmer). Cell culture medium activities are expressed as picomole per minute per milliliter of medium.

Adhesion Assays

After treatment, cells were incubated with 1 μmol/l calcein AM-labelled THP1 monocytes (2.5×10^5/24-well plate) for 30 min at 37°C. Then, unbound monocytes were removed by carefully washing the plates at least 3 times with FBS-free RPMI 1640 medium. Fluorescence intensity was measured using a fluorescence microplate reader ($\lambda_{ex}/\lambda_{em}$: 495/530 nm). Results are represented as the percentage of fluorescence units, referring values to those obtained for the nontreated WT HUVEC.

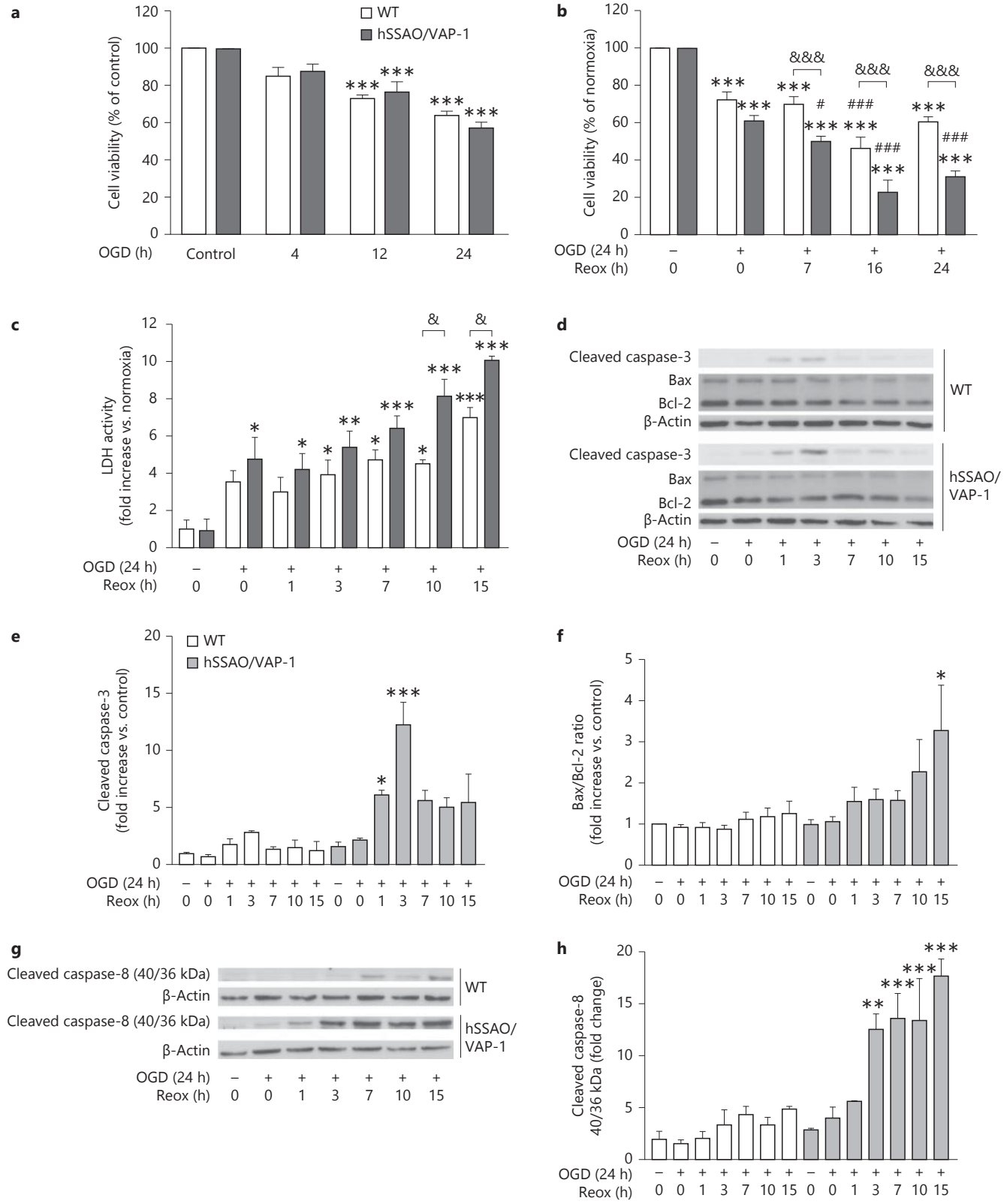
Statistical Analysis

Results are given as means ± SEM for independent experiments. Statistical analyses were performed by one-way ANOVA and, further, Newman-Keuls multiple comparison test. $p < 0.05$ was considered to be statistically significant. Statistical analyses and graphic representations were obtained by using the GraphPad Prism 3.0 program.

Results

OGD with Reoxygenation Induces Apoptosis in hSSAO/VAP-1-Expressing Cells, while WT Cells Remain More Resistant

Both HUVEC types underwent different OGD times (1–24 h). Cell viability, measured by MTT, progressively decreased with OGD time, although no differences were observed between the two cell types (fig. 1a). When different time periods of reoxygenation (0–24 h) were added to 24 h of OGD (fig. 1b), increased cell death was observed with longer reoxygenation times in SSAO/VAP-1-expressing cells. The MTT results were corroborated by LDH release measurement (fig. 1c). The presence of the caspase-3 active fragment in lysates was then determined to assess the possible activation of apoptotic cell death, as caspase-3 is one of the main executor caspases (fig. 1d, e). The immunoblots showed almost no caspase-3 cleavage in WT cells, while it was clearly observed in hSSAO/VAP-1-expressing cells. The Bax/Bcl-2 ratio was also measured as the mitochondrial pro-/anti-apoptotic balance, since the mitochondrial factors can induce caspase-3 activation (fig. 1d, f). The ratio increased after 24 h of OGD with 15-hour reoxygenation in hSSAO/VAP-1 cells, while WT cells remained unaltered. However, the maximum caspase-3 levels occurred earlier than the increase in Bax/Bcl-2 ratio. Reticular stress pathway activation was also studied by immunoblots of GRP78 and caspase-12, but their levels changed only with long-time treatment (data not shown). On the other hand, caspase-8 cleavage increased at times comparable with those at which caspase-3 cleavage occurred (fig. 1g, h).



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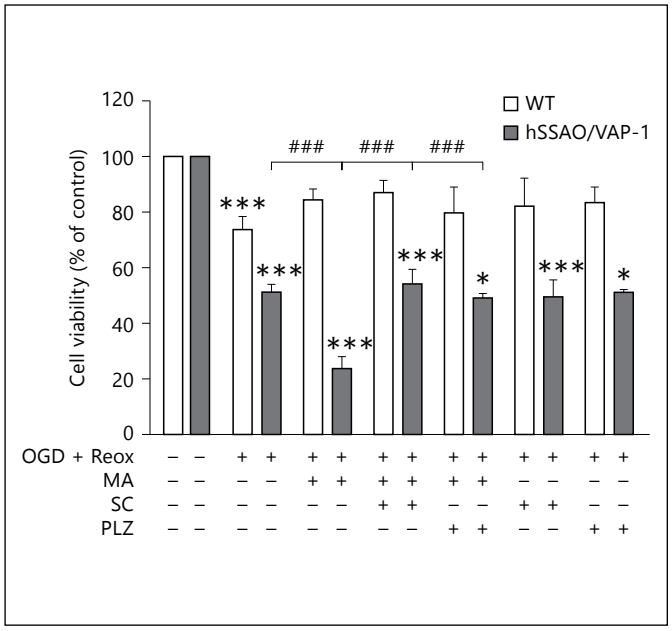

Fig. 2. Metabolism of MA in OGD with reoxygenation conditions induces a reduction in endothelial cell viability, prevented by SSAO activity inhibitors. WT HUVEC and hSSAO/VAP-1 HUVEC were subjected to OGD for 24 h, followed by 7 h of reoxygenation (OGD + Reox), and cell viability was assessed by MTT. MA (1 mmol/l) and the SSAO inhibitors SC (1 mmol/l) and phenelzine (PLZ; 100 nmol/l) were added before OGD and maintained during reoxygenation. Nontreated cells in normoxic conditions were used as control cells. Statistical analyses were performed by one-way ANOVA and the Newman-Keuls multiple comparison test. Data are expressed as means ± SEM from at least 3 independent experiments. * $p < 0.05$, *** $p < 0.001$, vs. control cells of the corresponding cell type; ### $p < 0.001$ vs. hSSAO/VAP-1 HUVEC treated with MA and subjected to OGD + Reox.

Fig. 1. Different duration of OGD or OGD with reoxygenation (Reox) induces WT and hSSAO/VAP-1 HUVEC death with apoptosis activation. MTT (**a**, **b**) and LDH release (**c**) were used to assess cell death under the different conditions assayed. Western blots (**d**) and their corresponding quantifications are shown for cleaved caspase-3 (**e**) and for the Bax/Bcl-2 ratio as a reference of mitochondrial pro-/antiapoptotic proteins (**f**). Western blots (**g**) and their quantifications are also shown for cleaved caspase-8 (**h**). Cells without OGD maintained under normoxia conditions for 24 h were considered control cells. Statistical analyses were performed by one-way ANOVA and Newman-Keuls multiple comparison test. Data are expressed as means ± SEM from at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control of the corresponding cell type; # $p < 0.05$, ### $p < 0.001$, vs. OGD 24 h without reoxygenation of the corresponding cell type; & $p < 0.05$, &&& $p < 0.001$, vs. WT HUVEC in the same experimental condition.

The Catalytic Activity of SSAO/VAP-1 Contributes to the Cell Death Observed in OGD with Reoxygenation Conditions

In order to elucidate whether SSAO/VAP-1 activity is involved in OGD-induced endothelial cell death, methylamine (MA; 1 mmol/l), as SSAO/VAP-1 substrate, was added to the cultures and cell viability was assessed (fig. 2). MA induced significant cell viability loss in hSSAO/VAP-1-expressing cells: a 25% decrease over the cell viability was observed in presence of the substrate. As expected, no effect was evident in WT cells. This effect was prevented by semicarbazide (SC; 1 mmol/l) or phenelzine (100 nmol/l), specific SSAO/VAP-1 inhibitors.

Endothelial Cells under OGD and Reoxygenation Release Soluble SSAO/VAP-1, and Matrix Metalloproteinase-2 Partly Mediates the Shedding

The soluble SSAO/VAP-1 form derives from shedding of the transmembrane protein, and this is elevated in pathologic conditions including stroke. In order to elucidate whether stroke constitutes a stimulus for releasing SSAO/VAP-1, its presence in culture medium was assessed after subjecting hSSAO/VAP-1 cells to OGD and reoxygenation (fig. 3a). A band at the expected molecular weight showed up when cells were subjected to 24 h of OGD with 7 h of reoxygenation. Flotillin-1 was used as a control of cell debris absence in the media. When the membrane-bound form was analyzed (fig. 3b), a progressive decrease in SSAO/VAP-1 presence was observed, correlating with the appearance of the soluble form in the medium and therefore corroborating the release of the protein. By adding rVAP-1 as an internal standard, the amount of SSAO/VAP-1 released could be quantified (fig. 3c). A value around 10 ng/ml was calculated to be present in the culture medium under the highest-releasing condition (24-hour OGD with 7-hour reoxygenation). SSAO enzymatic activity was also determined in the culture media (fig. 3d), which indicated the functionality of the soluble protein as it is found in blood plasma.

Matrix metalloproteinases (MMP) are activated and play an important role in extracellular matrix destruction in stroke [25]. Among them, MMP-2 seems to be more strongly associated with endothelial cells [26, 27]. On the other hand, batimastat (BB-94) is a broad-spectrum MMP inhibitor that has shown an effect in preventing the SSAO/VAP-1 release by adipocytes [28]. Given the closeness of all these players in the stroke condition, we analyzed the possible role of MMP in SSAO/VAP-1 shedding during stroke. Batimastat BB-94 (5 μmol/l) or a

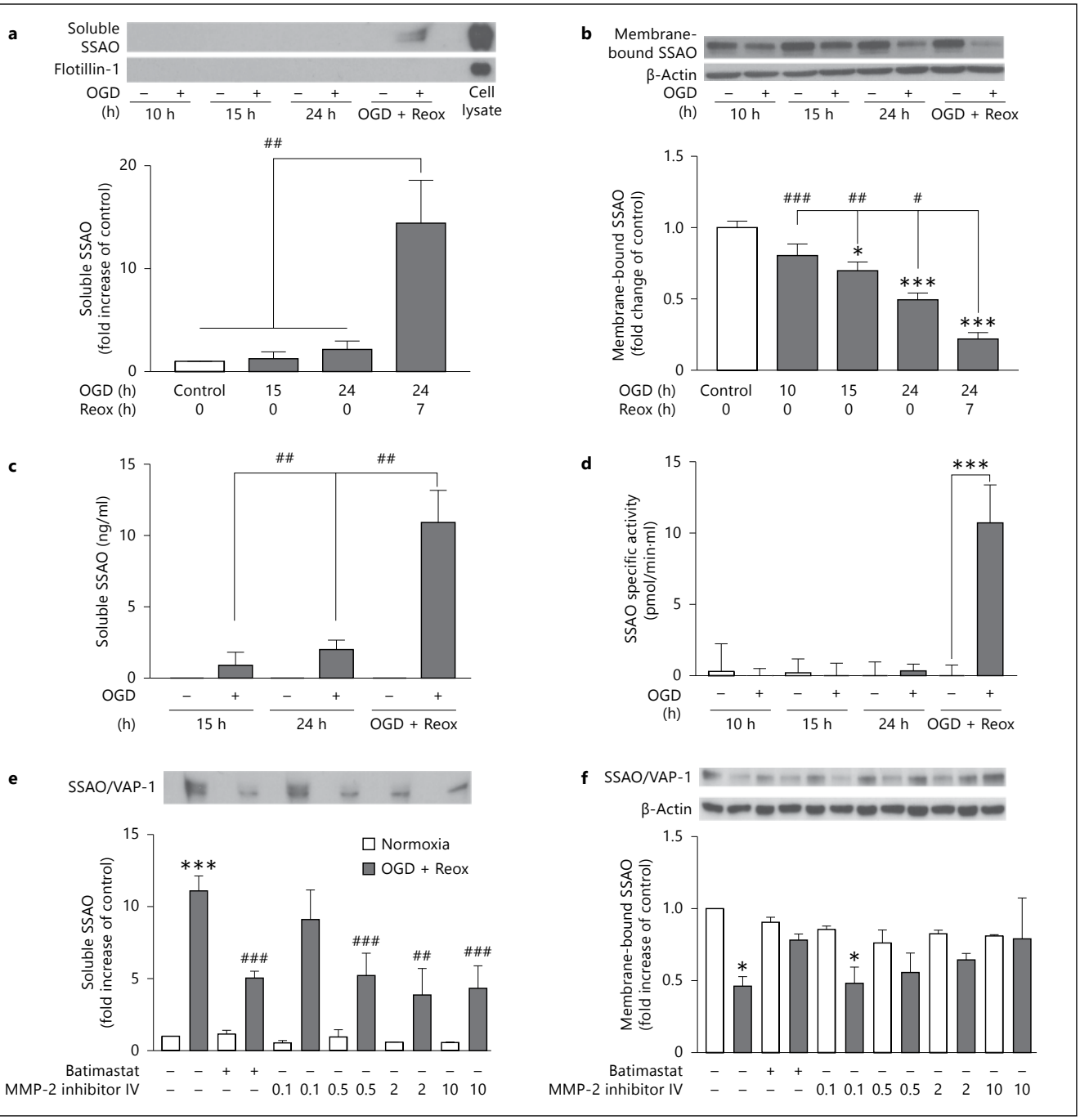

Fig. 3. MMP are involved in the release of soluble SSAO by hSSAO/VAP-1 endothelial cells subjected to 24-hour OGD with 7-hour reoxygenation (OGD + Reox). Data in graphs are expressed as means ± SEM and represent data obtained from at least 3 independent experiments. * $p < 0.05$, *** $p < 0.001$, vs. control cells (nontreated, normoxia); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. nontreated OGD + Reox cells, by one-way ANOVA and Newman-Keuls multiple comparison test. **a** Presence of soluble SSAO/VAP-1 in corresponding 10-fold-concentrated culture media under different OGD times and OGD + Reox. **b** Presence of membrane-bound SSAO/VAP-1 in hSSAO/VAP-1 HUVEC lysates under the same experimental conditions than in **a**. **c** Quantification of release of soluble SSAO in culture media. Addition of rVAP-1 was used as the reference for quantification. **d** Enzymatic activity by SSAO present in concentrated media obtained under the indicated OGD and reoxygenation times. SSAO activity was analyzed by radiometric method, using ^{14}C-benzylamine as a substrate. **e** SSAO/VAP-1 presence in 10-fold-concentrated culture media from cells subjected to OGD + Reox in the presence of 5 μmol/l batimastat or 0.1–10 μmol/l MMP-2 inhibitor IV. **f** Membrane-bound SSAO/VAP-1 levels in lysates from which media were previously analyzed. **b**, **f** The presence of membrane-bound SSAO was normalized to the β-actin levels.

specific MMP-2 inhibitor (MMP-2 inhibitor IV; 0.1–10 μmol/l; Millipore) was added to the hSSAO/VAP-1-expressing cells subjected to 24 h of OGD and 7 h of reoxygenation, and the presence of soluble SSAO/VAP-1 in cell culture media was determined (fig. 3e). Batimastat addition (IC_{50} = 20 nmol/l for MMPs) decreased SSAO/VAP-1 release by 45%, although it was not completely abolished. MMP-2 inhibitor IV (IC_{50} = 0.037 μmol/l for MMP-2, IC_{50} = 0.32 μmol/l for MMP-8 and IC_{50} > 1 μmol/l for MMP-9) [29] also led to a 45% reduction in the presence of 0.5 μmol/l of the inhibitor, not changing significantly at higher concentrations. The shedding pattern observed with the membrane-bound SSAO/VAP-1 was also complementary to that obtained for the soluble form (fig. 3f).

SSAO/VAP-1 Participates in Leukocyte Adhesion to the Endothelium under OGD

Binding of circulating inflammatory cells to endothelia for their infiltration into brain tissue after stroke, especially neutrophils, has been associated with BBB degradation and hemorrhage occurrence [30]. Given the ability of SSAO/VAP-1 to mediate polymorphonuclear cell extravasation [31], leukocyte binding was assessed in our experimental model. A shorter OGD time (5 h) was selected for these experiments, since longer OGD periods induce endothelial cell death. MA and SC were added since the SSAO enzymatic activity mediates its adhesion function. As figure 4 shows, SSAO/VAP-1-expressing cells bound a significantly high number of leukocytes under these conditions, while almost no THP1 adhesion is observed in WT cells. A slight increase in THP1 adhesion is also observed in MA treatment under normoxic conditions, and in both cases, SC prevents this proinflammatory effect. The OGD stimulus alone does not induce significant leukocyte adhesion, only a trend, which indicates the dependence of this effect on SSAO substrate metabolism. The presence of the adhesion proteins SSAO/VAP-1, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was also analyzed in these conditions, since SSAO/VAP-1 activity induces the expression of other adhesion molecules. No SSAO/VAP-1 or VCAM-1 changes were observed among the sequential OGD times studied in both HUVEC, while ICAM-1 progressively decreased in hSSAO/VAP-1-expressing cells after OGD (data not shown). VCAM-1 and ICAM-1 basal expression was higher in hSSAO/VAP-1 cells than in WT cells.

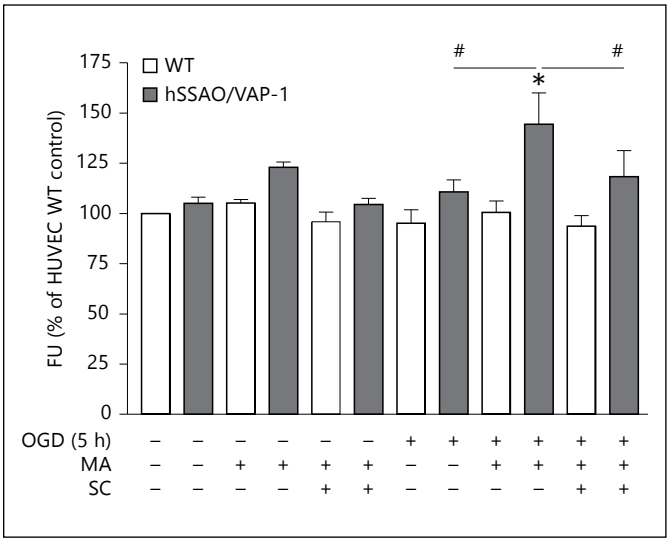

Fig. 4. OGD treatment induces leukocyte binding to hSSAO/VAP-1 HUVEC in the presence of SSAO substrate. Calcein AM-labelled THP1 leukocytes were incubated for 30 min with endothelial cells previously subjected to 5 h of OGD or normoxia in the presence of the SSAO substrate MA (3 mmol/l) or/and its inhibitor SC (1 mmol/l). After washing the plates to discard nonbound THP1 cells, the fluorescence of the remaining bound cells was measured at $\lambda_{ex}/\lambda_{em}$ of 495/530 nm. Nontreated WT cells under normoxic conditions were considered controls. Data are expressed as means ± SEM from at least 3 separate experiments. * $p < 0.05$ vs. hSSAO/VAP-1 HUVEC treated with MA under normoxia; # $p < 0.05$ vs. hSSAO/VAP-1 HUVEC treated with MA under OGD, by one-way ANOVA and Newman-Keuls multiple comparison test.

Discussion

Cerebrovascular diseases, and mainly stroke, affect 15 million people worldwide each year, leading to a high number of deaths and permanently disabled people and entailing huge economic costs. Contributions aimed at preventing or improving the effects of stroke are urgently needed. In this work we studied, at the molecular level, the role of the enzyme SSAO/VAP-1 in stroke physiopathology. We aimed to provide new knowledge about the mechanisms participating in stroke, as well as to propose SSAO/VAP-1 as a new therapeutic target to fight this disease.

The SSAO/VAP-1 presence in human cerebrovascular tissue [32–34] makes this enzyme a very interesting protein for study in brain vascular disorders, especially in stroke [15]. In addition, SSAO/VAP-1 mediates leukocyte adhesion and transmigration [31, 35], which partake in stroke pathology progression, and its activity is elevated in ischemic and hemorrhagic stroke.

Using our in vitro endothelial cellular models [22], we have standardized the OGD plus reoxygenation conditions to study cell viability loss. Our results show that cells expressing hSSAO/VAP-1 display a higher sensitivity to OGD plus reoxygenation-mediated cell damage than do WT cells, especially when reoxygenation is applied. A possible explanation for this higher susceptibility could be the metabolism of other endogenous, unknown SSAO substrates that are able to continuously generate potentially harmful agents. Actually, a similar effect was observed in livers of SSAO/VAP-1-overexpressing mice, which displayed an increased expression of oxidative-state sensor proteins [36]. More interestingly, when an SSAO substrate is added, cell death after OGD and reoxygenation significantly increases, while it does not occur under normoxia. These data confirm the involvement of SSAO activity in this experimental ischemic model, but also suggest that SSAO enzymatic activity could be punctually enhanced under ischemic conditions in humans, maximizing these effects. In the in vivo situation, it is reasonable to think that the formaldehyde and H_2O_2 generated by SSAO activity could contribute to oxidative stress, a key deleterious factor in brain ischemia and reperfusion [37], and directly contribute to apoptosis or necrosis [38].

When analyzing the observed cell death, an increase in caspase-3 cleavage can be determined, indicating activation of an apoptotic cell pathway. The mitochondria or reticular stress pathway do not seem to be relevant in this process, since proteins associated with these molecular pathways, such as Bax/Bcl2 or GRP78 and caspase-12, vary their levels a long time after caspase-3 activation occurs. On the other hand, caspase-8 cleavage was detected at times comparable with those at which cleaved caspase-3 increased, suggesting a possible contribution of the death receptor-associated pathway to the cell death observed in this model. This pathway could secondarily activate the mitochondria.

SSAO/VAP-1 exists in two isoforms, a membrane-bound and a soluble one, which are presumably originated by a shedding process in humans. Some authors have reported that 3T3-L1 adipocytes are able to release soluble SSAO by an MMP-dependent shedding mechanism [28], whereas batimastat, a broad-spectrum MMP inhibitor, avoided any SSAO release. In the periinfarcted region of stroke patients' brains, a decrease in the frequency of SSAO/VAP-1-containing vessels has been found [19, 20]. In this regard, it has been suggested that mediators present in cerebrovascular pathologies such as stroke could become a stimulus to activate SSAO/VAP-1 shedding. Consistent with this hypothesis, soluble SSAO/VAP-1 release was observed in our stroke experimental model after OGD with reoxygenation. Interestingly, the maximal increase in plasma SSAO activity in hemorrhagic stroke patients is observed 4–6 h after symptom onset [20]. In our experimental model, soluble SSAO/VAP-1 release was mainly observed after reoxygenation, at similar time points than in humans, and matching the strongest occurrence of endothelial cell death. In this context, SSAO/VAP-1 present in plasma is increased in some diseases associated with vascular complications, where both its proinflammatory action and its toxic catalytic products are related to vascular damage [16, 17, 39]. Therefore, the SSAO/VAP-1 released could not only locally enhance endothelial cell damage through its own catalytic action, but also bring it to other cerebral regions through the bloodstream, and contribute to a BBB breakdown.

Based on these previous findings and the knowledge that MMP are activated during stroke – where they are associated with the appearance of hemorrhagic transformation events – the involvement of MMP in SSAO/VAP-1 shedding under OGD conditions was assessed. The exact cellular source of MMP remains unknown; brain endothelium, astrocytes, neurons and inflammation-activated cells such as neutrophils may release different types of metalloproteinases [40]. However, MMP-2 seems to be more strongly associated with endothelial cells [27], so it was studied in particular. Identical results were obtained using different MMP inhibitors: the unspecific batimastat BB-94 and the specific MMP-2 inhibitor. These results point out that MMP-2 could be partly responsible for the SSAO/VAP-1 shedding in our experimental model. However, given the MMP-2 inhibitor concentrations used – close to the IC_{50} values for both MMP-2 and MMP-8 [29] – and the fact that SSAO/VAP-1 shedding is not completely abolished, it is reasonable not to rule out a possible involvement of both MMP, beside other different proteases, in the shedding of the SSAO/VAP-1 enzyme.

Polymorphonuclear leukocytes use MMP to digest the extracellular matrix and infiltrate inflamed tissue. This situation occurs in stroke, where peripheral inflammatory cells accumulate in blood vessels and induce BBB disruption and secondary tissue damage with a massive cytokine release [25]. In this context, inhibition of leukocyte adhesion and transmigration could have a beneficial effect on the postischemic damage. Our hSSAO/VAP-1 HUVEC increase THP1 leukocyte binding after 5 h of OGD in the presence of its substrate. These data introduce SSAO/VAP-1 as a new target to be considered in the prevention of leukocyte infiltration and subsequent damage in stroke.

Other inflammatory leukocyte endothelial cell adhesion molecules such as ICAM-1 or VCAM-1 play an important role as well in ischemic cerebrovascular pathology [41]. Although, in our model, a higher expression of both adhesion molecules was observed in basal conditions in cells expressing hSSAO/VAP-1 when compared with WT cells, no differences were observed during OGD. This basal increase in SSAO/VAP-1-expressing cells could be another indicator of a weaker status of these cells, related to the higher susceptibility of SSAO/VAP-1 to the OGD insult. On the other hand, the participation of other adhesion molecules cannot be excluded, especially E- and P-selectins, whose expression can be induced by SSAO activity [42].

In our study we propose for the first time a useful cellular experimental model of stroke in which the involvement of SSAO/VAP-1 is assessed. Our results allow us to conclude that SSAO/VAP-1 plays a role in leukocyte adhesion, inflammation and cell damage processes under ischemic conditions. Therefore, we propose this enzyme as a new therapeutic target for stroke treatment. This endothelial cell model has also become a useful experimental tool for studying the SSAO/VAP-1 release during stroke, although its physiopathological relevance in cerebrovascular damage will need further investigation. On the other hand, this model will provide additional valuable information helping to identify new SSAO/VAP-1 functions in stroke, and will allow the screening and evaluation of potential molecules as specific SSAO/VAP-1 inhibitors, aimed to induce a beneficial effect in the ischemic stroke conditions.

Acknowledgements

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Disclosure Statement

The authors declare that they have no conflict of interest.

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1. Annex chapter I.

1.1. Supplementary figure 1.

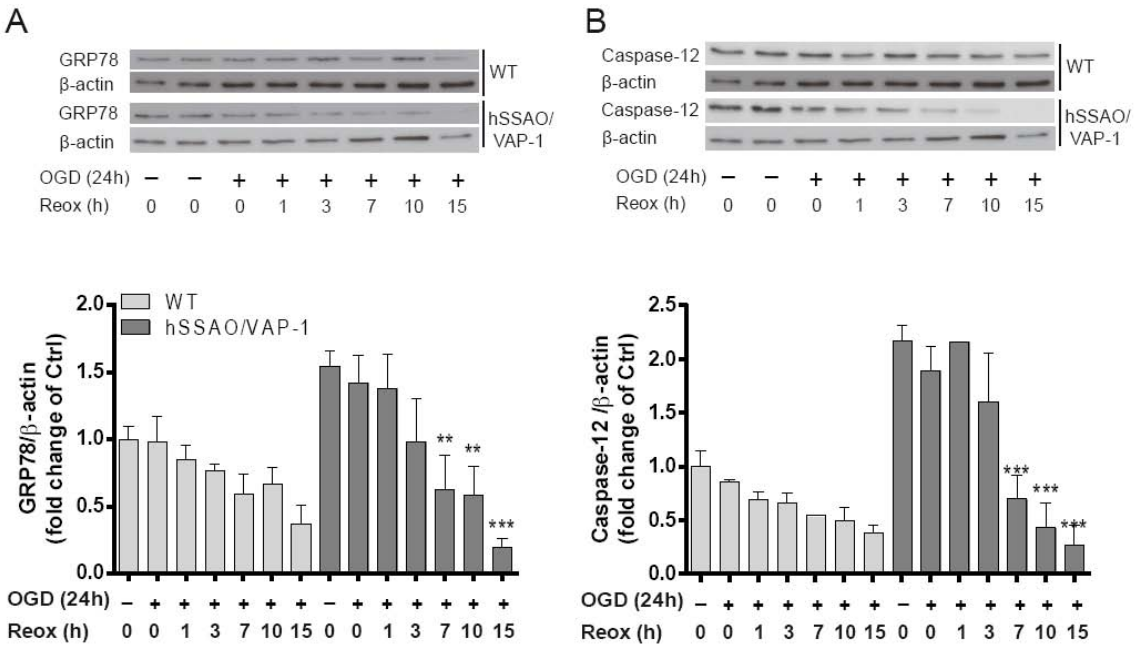

Fig S1. OGD 24-hour or OGD 24-hour with different duration of reoxygenation (Reox) alters the expression of endoplasmic reticulum (ER) stress related proteins in WT and hSSAO/VAP-1 HUVECs. Representative western blot images and quantifications of GRP78 (A) and caspase-12 (B) in WT and hSSAO/VAP-1 HUVECs under different OGD and Reox conditions. The presence of GRP78 and caspase-12 were normalized to the β-actin levels. WT HUVECs without OGD maintained under normoxia conditions for 24 hours were considered control cells (Ctrl). Statistical analyses were performed by one-way ANOVA and Newman-Keuls multiple comparison test. Data are expressed as mean ± SEM from at least 3 independent experiments. ***P*<0.01 and ****P*<0.001 versus hSSAO/VAP-1 HUVECs without OGD maintained under normoxia conditions for 24 hours.

1.2. Supplementary figure 2.

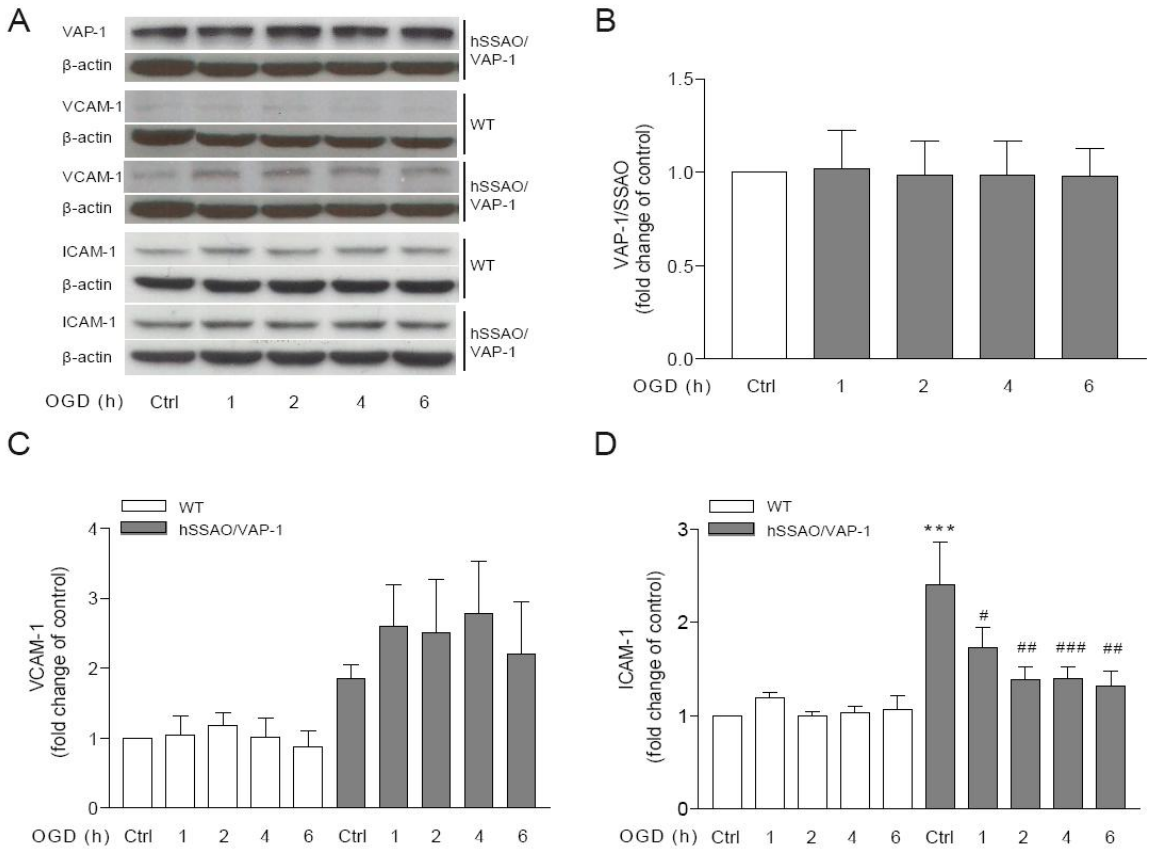

Fig S2. Influence of different duration of OGD on the expression of cell adhesion molecules in WT and hSSAO/VAP-1 HUVECs. (A) Representative western blot images, (B) quantifications of VAP-1/SSAO presents in hSSAO/VAP-1 HUVECs, (C) VCAM-1 and (D) ICAM-1 present in WT and hSSAO/VAP-1 HUVECs subjected to OGD 1-6 hours. The presence of VAP-1/SSAO, VCAM-1 and ICAM-1 were normalized to the β-actin levels. Cells under normoxia conditions in (B) and WT HUVECs without OGD maintained under normoxia conditions for 6 hours in (C) and (D) were considered as control (Ctrl). Statistical analyses were performed by one-way ANOVA and Newman-Keuls multiple comparison test. Data are expressed as mean ± SEM from 3 independent experiments. ***P<0.001 versus Ctrl; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 versus hSSAO/VAP-1 HUVECs without OGD maintained under normoxia conditions for 6 hours.

Chapter II:

"Protective effect of a novel multitarget compound on human SSAO/VAP-1-expressing HCMEC/D3 cells under OGD conditions as an experimental model of cerebral ischemia."

Protective effect of a novel multitarget compound on human SSAO/VAP-1-expressing HcMEC/D3 cells under OGD conditions as an experimental model of cerebral ischemia.

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Major area of pharmacology:	Neurodegeneration/neuroprotection
Cross-cutting area:	Blood brain barrier, Drug discovery/target validation
Additional area(s):	Endothelium, Neuroprotection

Protective effect of a novel multitarget compound on human SSAO/VAP-1-expressing HcMEC/D3 cells under OGD conditions as an experimental model of cerebral ischemia.

P. Sun, G. Esteban, M. Unzeta*, M. Solé*

Institut de Neurociències i Departament de Bioquímica i Biologia Molecular. Edifici M, Facultat de Medicina. Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona (Spain).

Short title: DPH-4 as multitarget drug in AD and stroke treatment

*** Corresponding authors:** Montse Solé and Mercedes Unzeta. Neurosciences Institute and Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra (Barcelona), Spain. Telephone: +34 93 581 1624; Fax: +34 93 581 1573. E-mail: montserrat.sole@uab.cat, mercedes.unzeta@uab.es

Authorship contributions:
P Sun and G Esteban performed research
M Unzeta and M Solé designed the research study
All authors contributed to data analysis and drafting the paper

Key Words: Multitarget-directed ligands (MTDLs); stroke; blood-brain barrier (BBB); semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1); inflammation.

Abstract

BACKGROUND AND PURPOSE
Stroke and Alzheimer's disease (AD) are related pathologies in which the cerebrovascular system is involved. Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1), increased in both stroke and AD patients' plasma, contributes to the vascular damage. During inflammation its enzymatic activity mediates the leukocytes recruitment into the injured tissue, inducing damage in the blood-brain barrier (BBB) and the neuronal tissue. We believe that through the alteration of the cerebrovascular function, SSAO/VAP-1 could play a role in the stroke-AD transition. Therefore, the protective effect on the BBB of the novel multitarget-directed ligand (MTDL) DPH-4, designed for AD therapy, was evaluated.

EXPERIMENTAL APPROACH
It was generated a human brain endothelial cell line expressing the human SSAO/VAP-1 (HcMEC/D3 hSSAO/VAP-1) because SSAO/VAP-1 expression is lost in cultured cells. To simulate the ischemic pathology, oxygen and glucose deprivation (OGD) conditions were established with these cells. The protective role of DPH-4 was then evaluated in the presence of methylamine as SSAO/VAP-1 substrate and/or beta amyloid.

KEY RESULTS
The presence DPH-4 was able to protect brain endothelial cells from the damage induced by OGD and reoxygenation as well as to decrease the SSAO-dependent leukocyte adhesion under these conditions. It was also effective against the damage induced by OGD and reoxygenation in the presence of beta amyloid as a model of AD pathology.

CONCLUSIONS AND IMPLICATIONS: These results allow us to conclude that the analogue DPH-4 might provide a therapeutic benefit to the delay and/or progression of these two linked neurological pathologies.

Abbreviations

Aβ, beta amyloid peptide; AD, Alzheimer's Disease; BBB, blood-brain barrier; bFGF, basic Fibroblast Growth Factor; Dep, deprenyl; DMSO, dimethylsulfoxide; FBS, foetal bovine serum; G418, Geneticine; HcMEC/D3, human cerebral microvascular endothelial cells/D3; HUVEC, human umbilical vein endothelial cells; MA, methylamine; MAO, monoamine oxidase; MTDL, multitarget-directed ligand; OGD, oxygen-glucose deprivation; PBS, phosphate-buffered saline; SC, semicarbazide; SMC, smooth muscle cells; SSAO/VAP-1, semicarbazide sensitive amine oxidase/vascular adhesion protein 1; WT, wild type.

Introduction

Increasing evidences suggest that the neurovasculature plays an important role in the onset and progression of neurological disorders like Alzheimer's disease (AD) (Zlokovic, 2008; Grammas, 2011; Marchesi, 2014). In this regard, the concept of "neurovascular unit", integrated by neurons, astrocytes and vascular cells constitutes a functional unit able to maintain the homeostasis of the brain's microenvironment (Iadecola, 2010). Stroke is a vascular disorder that constitutes the second leading cause of death worldwide with higher incidence in elderly people. Inflammation and oxidative stress accumulated during human aging negatively influence the vascular damage following a stroke incident (DiNapoli *et al.,* 2008), and the immune system may contribute to the infarct progression (Iadecola and Anrather, 2011). Actually, the fact that a high percentage of patients having suffered stroke subsequently developed AD, evidences that a strong link between these two pathologies exists. In this sense, hypoxia and ischemic injury induce the up-regulation of BACE-1 that increases the β-amyloid formation (Guglielmotto *et al.,* 2009). In addition, endothelial cells activated by hypoxia during stroke produce free radical species, which induce the expression of adhesion molecules such as the vascular adhesion protein 1 (VAP-1) that mediates the leukocytes recruitment, which infiltrate through the blood-brain barrier (BBB) into the injured tissue, inducing cell damage by their cytokines release that in turn, damage both the BBB and neuronal tissue.

VAP-1 is a homodimeric glycoprotein with enzymatic function that binds leukocytes through its semicarbazide-sensitive amine oxidase (SSAO, E.C 1.4.3.21) activity (Smith *et al.,* 1998; Jalkanen and Salmi, 2008). As enzyme, SSAO/VAP-1 metabolizes primary amines producing aldehydes, hydrogen peroxide (H_2O_2) and ammonia, which are able to induce cellular damage when overproduced (Yu and Deng, 1998). SSAO/VAP-1 present at the cell membrane or as soluble form, released into blood plasma, is altered in several human pathologies (Kurkijarvi *et al.,* 1998; Boomsma *et al.,* 2003) with special relevance to AD (Ferrer *et al.,* 2002; del Mar Hernandez *et al.,* 2005) and cerebral ischemia (Airas *et al.,* 2008). The mediators that induce these alterations in the SSAO/VAP-1 levels are still unknown, but it is believed that increased SSAO/VAP-1 levels may contribute to the physiopathology in these diseases (Conklin *et al.,* 1998; Solé *et al.,* 2008), constituting therefore a potential therapeutic target. Actually, human plasma SSAO activity is a strong predictor of parenchymal haemorrhages after tissue plasminogen activator (tPA) treatment in ischemic stroke patients (Hernandez-Guillamon *et al.,* 2010) and plasma SSAO activity, also elevated in haemorrhagic stroke patients, predicts their neurological outcome (Hernandez-Guillamon *et al.,* 2012). Considering all these data, SSAO/VAP-1 could mediate the link between stroke and the progression to AD through the alteration of the cerebrovascular tissue function.

Although the pathogenesis of AD is not yet fully understood, there is a clear consensus in describing it as a multifactorial disease caused by several agents including the BBB dysfunction. At present, it is widely accepted that a more effective therapy for the multifactorial nature of AD would result from the development of molecules aimed to interact with several or all the systems altered in this disorder. In this context, a novel series of derivatives based on the hybridisation of moieties from donepezil, propargylamine and 8-hydroxyquinoline (DPH) were designed, synthesised and pharmacologically evaluated for the potential prevention and treatment of AD (Wang *et al.,* 2014). Among them, the analogue DPH-4 emerged as an interesting compound able to dually inhibit cholinesterases and monoamine oxidases activities with metal chelating

properties and hence, showing a great therapeutic interest to be used in the AD treatment (Fig 1).

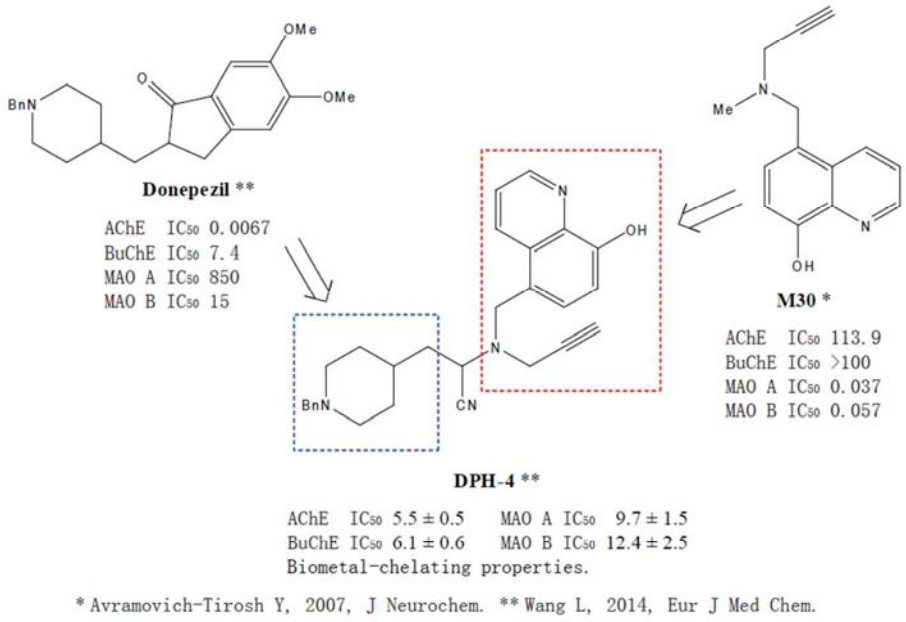

Figure 1. Design strategy of the multifunctional compound DPH-4. The N-benzylpiperidine moiety of donepezil was combined with the 8-hydroxyquinoline and propargylamine moieties of M30. IC_{50} values (μM) of donepezil, M30 and DPH-4 for AChE, BuChE, MAO (A and B), and biometal-chelating properties are indicated, as described in the corresponding publications.

Taking into account that stroke and AD are related pathologies and that one can trigger the progression of the other, the aim of this work was to analyse whether the beneficial effect of the novel multitarget-directed ligand (MTDL), DPH-4, previously observed in an experimental model of AD (Wang *et al.,* 2014), could also exhibit a protective effect on a new *in vitro* experimental model of cerebral ischemia that uses human cerebral microvascular endothelial cells as a model of BBB expressing the human SSAO/VAP-1 protein (HcMEC/D3 hSSAO/VAP-1). Obtaining SSAO/VAP-1-expressing cells was an essential preliminary step as the expression of this protein is lost in cultured cell, which would hinder its study.

Methods

Cell culture and transfection:

The human cerebral microvascular endothelial cell line HcMEC/D3 was obtained from Dr. Couraud's lab (Paris, France) (Weksler *et al.,* 2005) as a model of the BBB. HcMEC/D3 cells were cultured as recommended, on 150 μg mL^{-1} collagen type I (Rat Tail, Corning) –coated plates in EBM-2 (Lonza) medium supplemented with 5% FBS (Fetal Bovine Serum, Life Technologies), 1.4 μM Hydrocortisone (Sigma), 5 μg mL^{-1}

Ascorbic Acid (Sigma), 1 % Chemically Defined Lipid Concentrate (Life Technologies), 10 mM HEPES (Life Technologies) and 1 ng ml^{-1} human bFGF (Fibroblast Growth Factor-basic, Sigma). Human SSAO/VAP-1-expressing human umbilical vein endothelial cells (HUVEC hSSAO/VAP-1) and smooth muscle cells (SMC) (A7r5 hSSAO/VAP-1) were previously developed in our group, and cultured as described (Solé and Unzeta, 2011; Solé *et al.,* 2007). THP-1 monocytic cells were obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Geneticine (G418, 100 µg mL^{-1}; Invitrogen) was added to the culture medium of HUVEC and SMC cells to ensure the hSSAO/VAP-1 DNA maintenance.

In order to obtain the HcMEC/D3 cell line stably expressing the human SSAO/VAP-1, wild type (WT) HcMEC/D3 cells were transfected with a PcDNA3.1(+) vector containing the human SSAO/VAP-1 cDNA (Solé *et al.,* 2007) using the Fugene® HD transfection reagent (Roche) according to the manufacturer conditions. After transfection, cells were selected by the addition of G418 antibiotic (800 µg mL^{-1}) for 1-2 months. Then, cells were diluted to allow the formation of monoclonal colonies in the presence of 200 µg mL^{-1} G418, an antibiotic concentration that was used thereinafter for cell maintenance. Cell colonies were amplified and checked for SSAO/VAP-1 expression and activity before being frozen.

Cell lysates and concentrated culture medium:

Cells were collected and homogenized in 50 mM Tris-HCl (pH 7.5), containing 1% Triton X-100, 10 mM EDTA and protease inhibitors cocktail (Sigma) (1:100) and were sonicated for 10 seconds. To obtain concentrated culture medium samples, culture media were collected after cell treatments and centrifuged at 4400 *g* for 10 minutes to eliminate dead cells and debris. Then media samples were dried out by evaporation in a Refrigerated CentriVap Concentrator (Labconco) and reconstituted in a smaller, known volume of distilled H_2O to obtain ten-fold concentrated culture medium.

Sub-cellular fractionations:

Membrane-enriched preparations were obtained by cells homogenization in 10 mM HEPES, 1.5 mM $MgCl_2$ and 10 mM KCl buffer at pH 7.9, containing protease inhibitors cocktail. After a centrifugation at 2000 *g* for 15 min at 4ºC, the obtained supernatant was ultracentrifuged at 100000 *g* (Sorvall Discorvery M120 SE) for 30 min at 4ºC to separate the soluble cytosolic fraction from the pellet containing the membrane-enriched fraction.

Lipid raft-enriched fractions were obtained by scrapping the cells in PBS, recovering them through a 5 min at 800 *g* centrifugation and then reconstituting the pelled in 450 µL of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1% Brij 98 buffer at pH 7.2, containing protease inhibitors cocktail. After 15 min incubation at 37ºC under continuous agitation samples were centrifuged for 10 min at 2000 *g* to discard nuclei. The supernatants were mixed with 450 µL of 90% sucrose in Tris-HCl buffer to obtain a 45% sucrose fraction, which were deposited at the bottom of ultracentrifuge tubes. Two additional fractions of 35% (2 mL) and 5% (0.8 mL) sucrose were added to the former to generate a sucrose gradient, and then the samples were centrifuged for 19 h at 120000 *g*. Ten 370 µL-fractions were recovered and analysed by western blot to identify the lipid raft and soluble membrane-enriched fractions.

OGD model and cell treatment:

Combined oxygen and glucose deprivation (OGD) and reoxygenation have been used as an experimental approach to ischemic stroke. For HUVEC hSSAO/VAP-1 cells, ischemic condition was carried out by subjecting the cells to a 24-hour OGD followed by a 7-hour reoxygenation, as previously described (Sun *et al.,* 2014). For WT and hSSAO/VAP-1 HcMEC/D3 cells, the OGD treatment was performed in glucose-free DMEM (Life Technologies) after washing cells with glucose-free PBS, and then introducing the cells into a temperature-controlled (37±1°C) Invivo$_2$ hypoxia workstation (RUSKINN) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in DMEM (5 mM glucose) in the incubator under normoxia conditions (5% CO_2/95% air). In experiments including reoxygenation, cells that have undergone OGD were returned to normoxia conditions after replacing glucose-free DMEM by serum-free DMEM (5 mM glucose) and adding the same treatments present during OGD. In experiments analysing the soluble SSAO/VAP-1 release, DMEM was not replaced for the reoxygenation period, but glucose was added into OGD plates.

For cell viability and adhesion assays, HUVEC hSSAO/VAP-1 were seeded at 53200 cells mL^{-1}, HcMECs were seeded at 50000 cells/mL, and both were grown for 48h before the addition of treatments for other 24h. For immunoblot analysis, cells were seeded at 60000 cells mL^{-1}, and grown to 80% - 90% confluence before treatments. MA (methylamine), SC (semicarbazide), A$\beta_{1\text{-}40}$D (A$\beta_{1\text{-}40}$ peptide containing the Dutch mutation, Anaspec), or/and DPH-4 (Wang *et al.,* 2014) were added into DMEM before OGD starting. In the reoxygenation process, the compounds were maintained at the same concentrations than during OGD. For the preparation of A$\beta_{1\text{-}40}$D, it was pretreated with 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP, Sigma-Aldrich) for more than 4h but less than 6h, then aliquoted, evaporated at room temperature, and stored at -80°C until using, then being dissolved in sterile PBS containing 0.1% ammonium hydroxide.

Cell viability assay:

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay was employed to evaluate the cell viability. Briefly, at the end of the treatments, cells were incubated with 0.5 mg mL^{-1} MTT for 3 h in HUVECs and for 1.5 h in HcMECs at 37°C. The medium was then replaced by dimethylsulfoxide (DMSO) to dissolve the blue formazan precipitate, and it was spectophotometrically quantified at 560 nm and 620 nm in a microplate reader (Labsystems multiscan RC) (Plumb *et al.,* 1989).

Antibodies and western blot analysis:

The antibodies used were: rabbit anti-VAP-1 (abcam, 1:1000), rabbit anti-bovine SSAO (1:1000) (Lizcano *et al.,* 1998), rabbit anti-IGF1-R (insulin-like growth factor 1 receptor) (Santa Cruz Biotechnology, 1:1000), mouse anti-Flotillin (BD Biosciences, 1:1000), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Ambion-Invitrogen, 1:40000); mouse anti-Tf Rec (ZYMED, 1:1000), HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (BD Biosciences, 1:2000), HRP anti-mouse IgG (Dako, 1:2000). Equal amounts of protein (20 µg per lane) determined by the Bradford method, or equal volumes of media (45 µL per lane) were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with TBS/0.1%-Tween buffer containing 5% (w/v) non-fatty milk and incubated overnight at 4°C with the corresponding primary antibodies. After incubation with the secondary

antibodies, blots were developed using ECL® Chemoluminiscent detection reagents and High Performance Chemiluminiscence Films (GE Healthcare). The ImageJ software was used to quantify the western blot signals.

SSAO enzymatic activity determination:

For SSAO activity determinations, cells were collected and homogenized in 100 mM Tris-HCl, pH 9, and containing protease inhibitors cocktail. Enzymatic activity was determined radiochemically by using a modification of the Otsuka and Kobayashi method (Otsuka *et al.,* 1964). Briefly, [^{14}C]-Benzylamine hydrochloride (100 µM and 2 mCi mmol^{-1}, American Radiolabeled Chemicals) was used as substrate, and 1 µM Dep (deprenyl) was added to avoid monoamine oxidase (MAO) B interference. A 30-min inhibitory pre-treatment of samples was performed at 37°C with 1 µM Dep or Dep plus DPH-4. Reactions were performed at 37°C for 120 minutes in 100 mM Tris-HCl buffer, pH 9.0, adding 25 µL of substrate to the 200 µL of reaction. Eighty to a hundred µg of HUVEC hSSAO/VAP-1 or HcMEC/D3 hSSAO/VAP-1 cell lysates were used in each reaction. Reaction was stopped by adding 100 µL of 2 M citric acid. The [^{14}C]-aldehyde products were extracted into 4 mL of toluene/ethylacetate (1:1, v/v) solution containing 0.6% (w/v) of diphenyloxazole. The amount of [^{14}C]-aldehyde was quantified using a Tri-Carb 2810TR liquid scintillation counter (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer).

Adhesion assays:

THP-1 monocytes were labelled with 1 µM calcein-AM, and at the end of treatments, endothelial cells were incubated with the calcein-AM-labelled THP-1 cells (2.5×10^5 per well in 24-well plates) for 30 minutes at 37°C. Then, unbound monocytes were removed by turning over the plates onto absorbent paper, carefully adding FBS-free RPMI 1640 medium to the plates with an auto-pipette, and repeating the washing for at least three times. The fluorescence intensity was measured using a fluorescence microplate reader ($\lambda_{ex}/\lambda_{em}$: 495/530 nm). Results are represented as the percentage of fluorescence intensity, referring values to those obtained for the non-treated cells.

Analysis of Data:

Results are given as mean ± SEM of independent experiments. Statistical analysis was performed by one-way ANOVA and further Newman-Keuls multiple comparison test. $P<0.05$ was considered to be statistically significant. Statistical analyses and graphic representations were obtained with the Graph-Pad Prism 6.0 software.

Results

Generation and characterization of the human SSAO/VAP-1-expressing HcMEC/D3 cell line.

In order to obtain a brain endothelial cell model to study *in vitro* the role of SSAO/VAP-1 in cerebrovascular tissue, the immortalized human brain endothelial cell line HcMEC/D3 (Weksler *et al.,* 2005) was stably transfected with human SSAO/VAP-1 cDNA. This step was necessary because SSAO/VAP-1 expression is lost in cultured cells. After selection and amplification of G418-resistant cells, different clones were analysed for SSAO activity and expression (Fig. 2a). Clones 1 and 2 showed the highest

activity using benzylamine as specific SSAO substrate, as well as the highest protein expression by western blot analysis. The activity levels observed resulted significantly higher than those obtained with endothelial HUVECs (H) or A7r5 smooth muscle (SMC) transfected cell lines previously developed (Solé and Unzeta, 2011; Solé *et al.,* 2007).

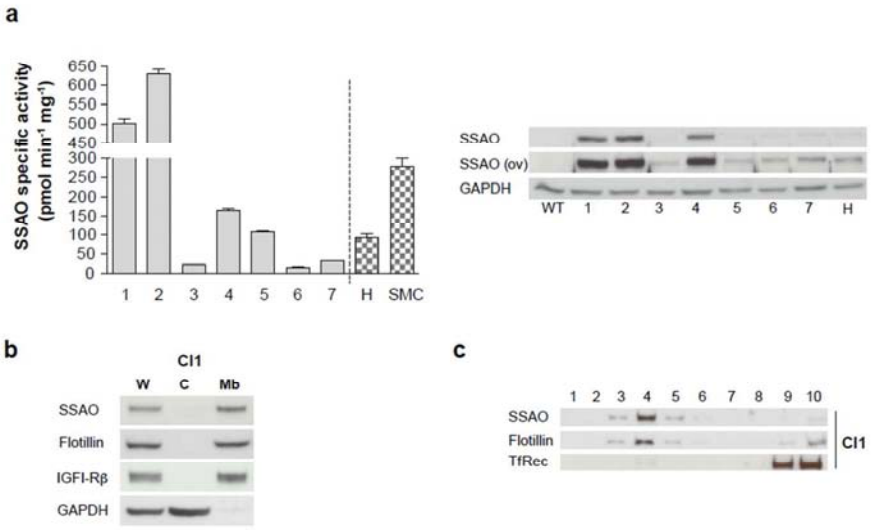

Figure 2. Characterization of the HcMEC/D3 cell line human SSAO/VAP-1-transfected. (**a**) SSAO specific activity (expressed as pmol min^{-1} mg^{-1} of protein) of the antibiotic-selected and amplified positive clones (1 - 7), with the corresponding protein visualization by western blot. HUVEC endothelial (H) or A7r5 smooth muscle (SMC) transfected cell lines are shown on comparative purposes. The overexposure (ov) of the SSAO western blot is shown to highlight the weaker bands. GAPDH was used as loading control. WT, wild type (non-transfected cells). (**b**) The transfected SSAO protein is located in the membrane fraction, and absent from the cytosol, clone 1 is showed as example. Flotillin and IGF1-Rβ were used as membrane-positive markers; GAPDH was used as cytosolic marker. W, whole fraction; C, cytosolic fraction; Mb, membrane fraction. (**c**) The transfected SSAO is located in the lipid raft fractions of the cell membrane; clone 1 is showed as example. Flotillin was used as raft-positive protein and transferrin receptor (Tf Rec) was used as marker of the soluble membrane fraction.

The correct sub-cellular localization of the expressed SSAO/VAP-1 protein was checked by two distinct cell fractionation procedures. On one hand, the membrane and cytosolic fractions were separated and their subsequent analysis by western blot showed that the protein expression is associated to the membrane fraction, while it is absent in the cytosol (Fig 2b). On the other hand, SSAO/VAP-1 was located in the lipid rafts regions as revealed by its presence in flotillin-containing fractions, a characteristic raft-positive protein (Fig 2c). The presence of other amine oxidases was also assessed, showing both WT and SSAO/VAP-1-expressing cells an absence of MAO B and a moderate MAO A activity with slightly increased levels in SSAO/VAP-1-expressing cells compared to WT (data not shown).

Effect of OGD and reoxygenation on the cell viability of WT and hSSAO/VAP-1-

expressing HcMEC/D3 cells, and influence of SSAO activity.

Both WT and hSSAO/VAP-1 HcMEC/D3 cells underwent OGD at different times (1 to 24h). The treatment induced a decrease in cell viability reaching 50% of cell death after 24h of OGD exposition. No differences between both cell types were observed at any of the analysed time-points (Fig 3a).

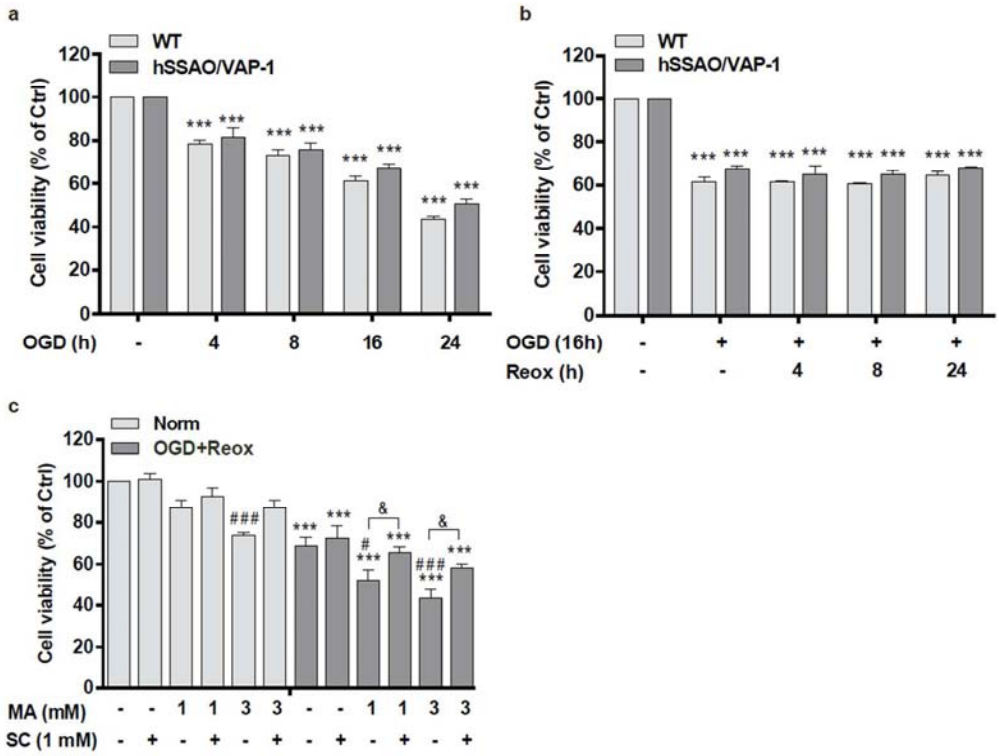

Figure 3. Effect of OGD, OGD with reoxygenation, and methylamine (MA) on cell viability of wild type (WT) and human SSAO/VAP-1-transfected HcMEC/D3 cells (HcMEC/D3 hSSAO/VAP-1). Different duration of OGD (**a**) or 16h-OGD with different duration of reoxygenation (**b**) induces WT and hSSAO/VAP-1-transfected HcMEC/D3 cell death. MTT reduction assay was used to assess cell viability under the different assayed conditions. Cells without OGD were maintained under normoxia conditions, and are considered control cells (Ctrl). The metabolism of MA under 16h-OGD plus 24h of reoxygenation condition (OGD+Reox) (**c**) induces a reduction of cell viability in hSSAO/VAP-1-expressing HcMEC/D3 cells, which is prevented by the SSAO activity inhibitor semicarbazide (SC). MA (1 mM or 3 mM) and SC (1 mM) were added before OGD and maintained during reoxygenation. Control cells are non-treated cells in normoxia (Norm). Data are expressed as mean ± SEM of at least 3 independent experiments. $^{***}P<0.001$ versus control of the corresponding cell type. $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus non-treated hSSAO/VAP-1-expressing HcMEC/D3 cells in the corresponding condition (Norm or OGD+Reox); $^{\&}P<0.05$ between the indicated treatments. Statistical analyses were performed by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

The 16h of OGD, inducing 40% cell death was selected to study the effect of different

reoxygenation times (1 to 24h) after OGD. Results showed in Fig 3b revealed that the addition of reoxygenation did not increase the cell death reached after the OGD treatment. In order to assess whether the SSAO catalytic activity versus methylamine (MA) as specific substrate could contribute to the vascular damage in these conditions, 16h OGD with 24h reoxygenation time was selected. SSAO/VAP-1-expressing cells were incubated in presence of MA (1 and 3 mM) and/or semicarbazide (SC, 1 mM) during 16h under OGD and 24h reoxygenation. Results displayed in Fig 3c indicate that the presence of MA enhanced the loss of cell viability induced by OGD with re-oxygenation in a dose-dependent manner, which was partially recovered by SC, a specific SSAO inhibitor. These results confirm that the SSAO catalytic activity enhances the cell death induced by OGD with reoxygenation conditions.

OGD with reoxygenation induces the release of soluble SSAO/VAP-1 to the culture media by HcMEC/D3 hSSAO/VAP-1 cells.

The soluble SSAO/VAP-1 form derives from the membrane-bound protein by a shedding process that may be activated under some pathologic conditions (Abella *et al.,* 2004; Sun *et al.,* 2014). In order to assess whether stroke can induce the SSAO/VAP-1 release in these cells, concentrated culture media of SSAO/VAP-1-expressing cells under different times of OGD and OGD with reoxygenation were analysed using TNF-α as positive control (Fig 4a).

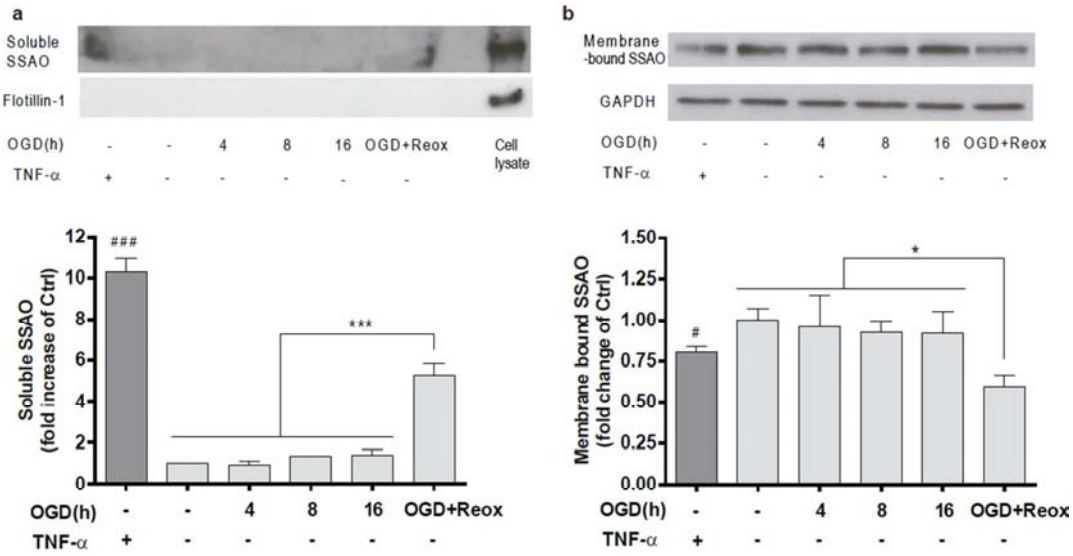

Figure 4. OGD with reoxygenation induces the release of soluble SSAO to the culture media by HcMEC/D3 hSSAO/VAP-1 cells. (**a**) Presence of soluble SSAO/VAP-1 in ten-fold concentrated culture media corresponding to TNF-α treatment (24h in normoxia, 100 ng mL^{-1}), different OGD times and 16h-OGD with 24h-reoxygenation (OGD+Reox). TNF-α was used as positive control of SSAO/VAP-1 release. Flotillin-1 was used as control of cell debris absence in the media. (**b**) Presence of membrane-bound SSAO/VAP-1 in HcMEC/D3 hSSAO/VAP-1 cell lysates under the same experimental conditions than in **a**. The presence of membrane-bound SSAO was normalized to the GAPDH levels. Non-treated cells or media under normoxia conditions were considered control samples (Ctrl). Data in graphs represent the western

blot quantifications and are expressed as mean ± SEM of data obtained from 3 independent experiments. $^{*}P<0.05$ and $^{***}P<0.001$ as indicated; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus control cells, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

The western blot results showed the highest soluble SSAO/VAP-1 release to the media after 16h OGD and 24h reoxygenation. This result correlated with the loss of signal observed in the membrane-bound form, obtained by analysing the cell lysates samples under the same experimental conditions (Fig 4b).

DPH-4 attenuates the cell death induced by the SSAO-mediated methylamine metabolism in both normoxia and OGD with reoxygenation conditions.

In order to determine a possible protective effect of DPH-4 in the stroke condition, the above established experimental model with hSSAO/VAP-1-expressing HcMEC/D3 cells was used compared with a peripheral endothelial model using hSSAO/VAP-1-expressing HUVEC cells previously described (Sun *et al.,* 2014). Both endothelial cell lines were pre-incubated with DPH-4 (1µM) before the addition of MA (3 mM) and subjected to OGD and re-oxygenation. A significant loss of cell viability was observed in presence of MA (Fig. 5a,b) in normoxia as well as an enhancement of cell toxicity induced by OGD and reoxygenation.

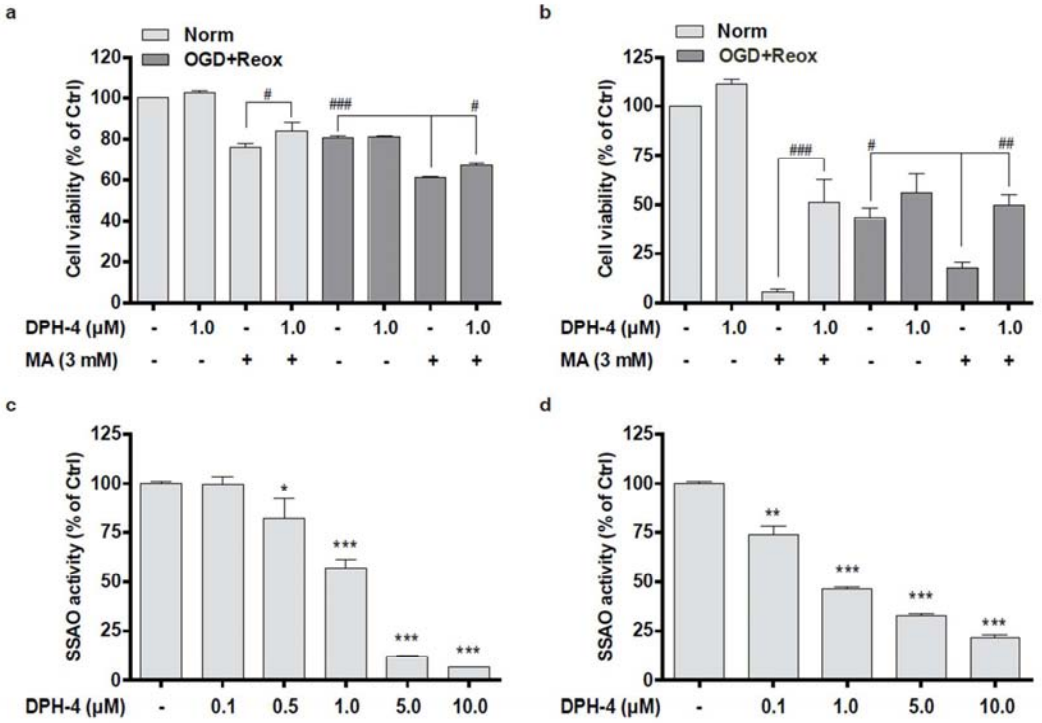

Figure 5. DPH-4 attenuates the cell death induced by the metabolism of methylamine in normoxia or in OGD with reoxygenation conditions in different endothelial cell types. MTT reduction assay was used to determine the cell viability of HcMEC/D3 hSSAO/VAP-1 cells subjected to 16h-OGD with 24h-reoxygenation (**a**), and HUVEC hSSAO/VAP-1 cells subjected to 24h-OGD with 7h-reoxygenation (**b**).

MA (3 mM) and DPH-4 (1 μM) were added before OGD and maintained during reoxygenation. Non-treated endothelial cells under normoxia were used as control (Ctrl) cells. The SSAO activity inhibiting capacity of DPH-4 was determined by investigating SSAO activity remaining in HcMEC/D3 hSSAO/VAP-1 cell lysates after being incubated with DPH-4 (0.1-10 μM) for 30 min (**c**), or by evaluating SSAO activity present in HUVEC hSSAO/VAP-1 cell lysates from DPH-4 (0.1-10 μM)-treated cells for 24 hours (**d**). SSAO activity was analyzed by radiometric method, using ^{14}C-benzylamine as substrate. A non-treated cell lysate was considered the control (Ctrl) sample. Data in graphs are expressed as mean ± SEM and represent data obtained from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus non-treated samples; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

The effect of MA was much more significant in HUVEC cells (Fig 5b) than in HcMEC/D3 (Fig 5a), revealing a greater resistance of the latter even showing higher SSAO/VAP-1 expression and activity levels (see Fig 2). Interestingly, the loss of cell viability was partially restored in both cell types in the presence of DPH-4, although the protective effect was surprisingly more significant in HUVECs (Fig 5b), reaching 50% recovery in normoxia and almost 100% under OGD with reoxygenation conditions, than in HcMEC/D3 (Fig 5a), which showed less cell toxicity with MA. These results confirmed the protective effect of DPH-4 on human brain endothelial cells expressing SSAO/VAP-1 in this experimental stroke model. The protective behaviour of DPH-4 might be explained by the SSAO activity inhibition. Thus, different concentrations of DPH-4 (0.1-10 μM) were used to determine the inhibition of SSAO activity using ^{14}C-benzylamine as substrate in both hSSAO/VAP-1-expressing HcMEC/D3 (Fig 5c) and HUVEC (Fig 5d) cells. Results revealed that DPH-4 inhibited SSAO activity with a rough IC_{50} value of 1μM in both endothelial cell types from different vascular origin.

DPH-4 shows an anti-inflammatory activity preventing the MA-induced leukocyte binding to hSSAO/VAP-1-expressing HcMEC/D3 cells subjected to OGD.

The BBB degradation and hemorrhage occurrence has been associated to the binding of inflammatory cells to endothelia for their infiltration to brain tissue after stroke. Because SSAO/VAP-1 mediates the leukocytes extravasation, the anti-inflammatory behaviour of DPH-4 was assessed in hSSAO/VAP-1-expressing HcMEC/D3 cells under normoxia and OGD conditions as well as in the presence of MA by quantifying the binding of calcein-AM-labelled THP-1 leukocytes to the endothelial cells (Fig 6). The presence of MA induced an increase of the leukocytes binding to the endothelium in hSSAO/VAP-1-expressing cells after short OGD (5h, Fig 6a), long OGD (16h, Fig 6b) and OGD with reoxygenation (16h + 24h, Fig 6c) conditions, while no effect was observed in WT cells, indicating that the inflammatory behaviour was induced by the catalytic action of SSAO with MA as substrate. This effect was prevented when cells were incubated in presence of DPH-4 in all the conditions assayed.

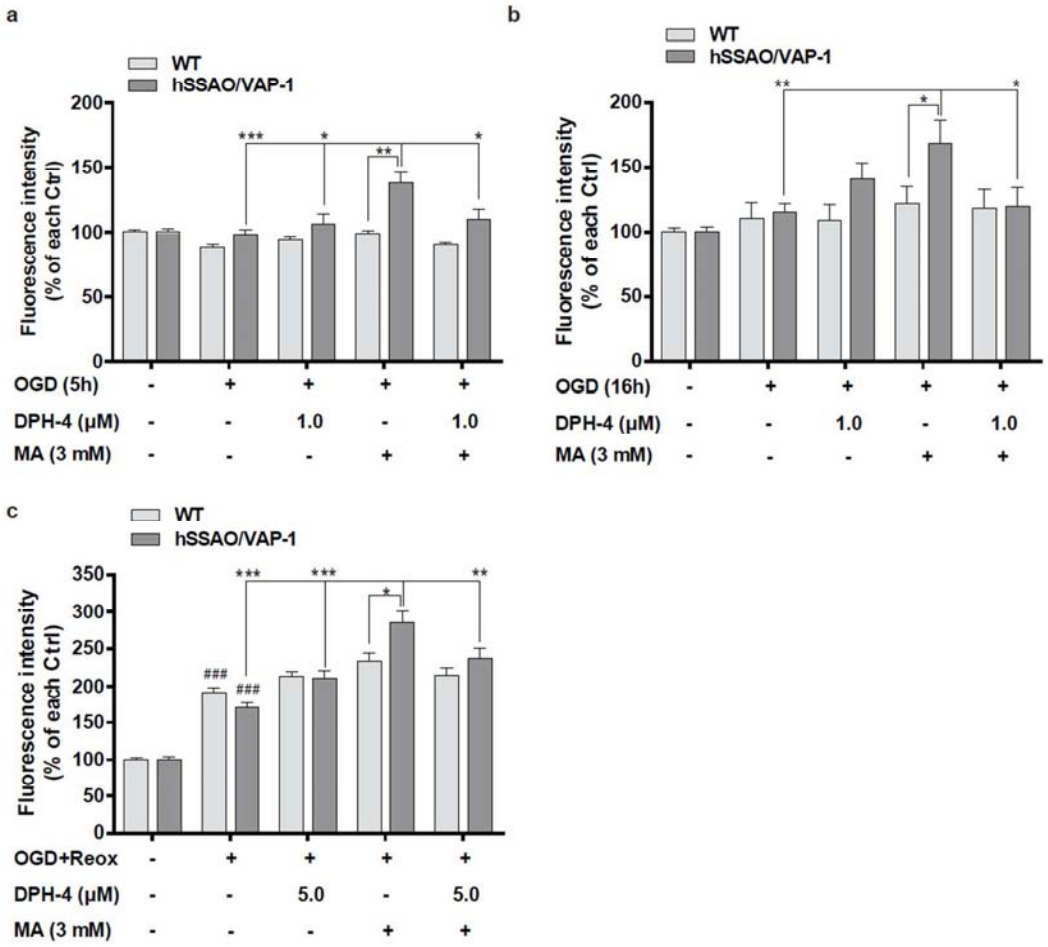

Figure 6. DPH-4 prevents the leukocyte binding to endothelial cells under different OGD conditions in presence of the SSAO substrate. Leukocyte-endothelium adhesion assay was performed to analyze the anti-inflammatory effect of DPH-4 against SSAO mediated pro-inflammatory action. WT and hSSAO/VAP-1-expressing HcMEC/D3 cells treated with MA (3 mM) or/and DPH-4 (1 or 5 µM) undergone 5h-OGD (**a**), 16h-OGD (**b**) or 16h-OGD with 24h-reoxygenation (OGD+Reox, **c**) and the binding of calcein-AM-labelled THP-1 leukocytes on these cells was quantified. Non-treated cells under normoxia conditions were considered as control (Ctrl) for each type of cells. Data are expressed as mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus HcMEC/D3 hSSAO/VAP-1 treated with MA under OGD conditions; $^{###}P<0.001$ versus control of the corresponding cell type, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

DPH-4 protects against cell death caused by the co-treatment of SSAO substrate methylamine (MA) and $A\beta_{1-40}$D in HcMEC/D3 hSSAO/VAP-1 cells under OGD with reoxygenation.

In order to confirm whether the presence of Aβ and SSAO/VAP-1 accelerates vascular damage under ischemic conditions, as we previously described in normoxia (Solé *et al.,*

2015), cells under normoxia and OGD with reoxygenation were incubated in the presence of $A\beta_{1\text{-}40}D$ and cell viability was determined. Both the co-treatment of MA with $A\beta_{1\text{-}40}D$ (Fig 7a), and the 24h pre-treatment with $A\beta_{1\text{-}40}D$ (Fig 7b) induced an increased loss of the cell viability compared with that produced separately by one of the two toxics under OGD with reoxygenation. The addition of DPH-4 in the presence of both toxics significantly increased cell viability; similar results were obtained when SC was used (data not shown).

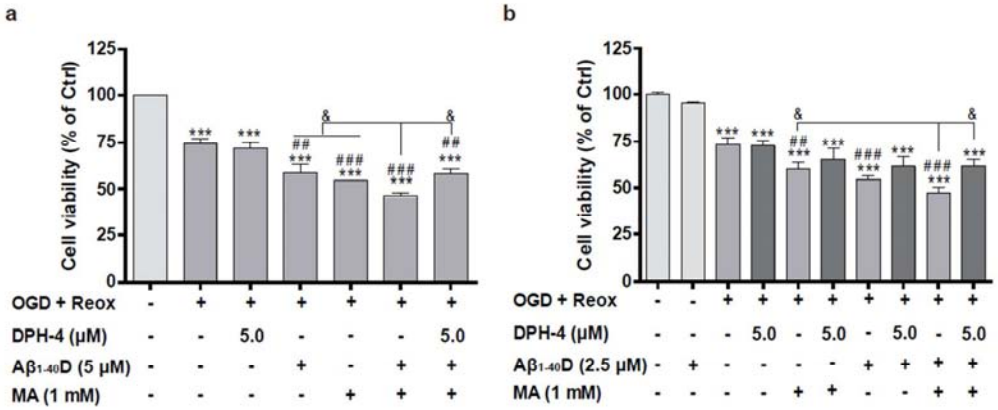

Figure 7. DPH-4 protects against cell death caused by the co-treatment of SSAO activity and $A\beta_{1\text{-}40}D$ in HcMEC/D3 hSSAO/VAP-1 cells under OGD with reoxygenation. MTT reduction assay was used to evaluate the cell viability of HcMEC/D3 hSSAO/VAP-1 cells subjected to 16h-OGD with 24h-reoxygenation (OGD+Reox) in the presence of $A\beta_{1\text{-}40}$ containing the Dutch mutation ($A\beta_{1\text{-}40}D$, 5 µM), MA (1 mM) or/and DPH-4 (5 µM) (**a**). $A\beta_{1\text{-}40}D$, MA and DPH-4 where added at the same time before OGD. (**b**) a 24h pre-treatment was performed with $A\beta_{1\text{-}40}D$ and cells were then subjected to 8h-OGD with 24h-reoxygenation. All treatments were maintained during reoxygenation. Non-treated cells under normoxia were considered as control (Ctrl). Data are expressed as mean ± SEM of three independent experiments. $^{***}P<0.001$ versus Ctrl; $^{\#\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus non-treated cells under OGD+Reox; $^{\&}P<0.05$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

Discussion & Conclusions

Cerebral hypoperfusion, atherosclerosis, oxidative stress, vascular β-amyloid deposition or a failure of its clearance are insults that can disturb the transport of nutrients across the BBB. These alterations can lead to an acute and big failure of the cerebrovascular function by way of a brain ischemia or haemorrhage, contributing to the cognitive decline and dementia. Moreover, a deep meta-analysis recently reported that stroke significantly and independently increases the risk for AD and in turn, the risk of intracerebral haemorrhage (Zhou *et al.,* 2015). Furthermore, both hypoxia and ischemic injury induce the up-regulation of BACE 1 and increase the β-amyloid generation (Guglielmotto *et al.,* 2009) confirming a link between AD and stroke.

AD is however a complex disease which therapy has been for long focused on one of its multiple symptoms: the depletion of basal forebrain cholinergic neurons and the

subsequent decrease in the cholinergic transmission (Perry *et al.,* 1977; Geula and Mesulam 1999). At present, no drug has been able to successfully prevent the neurodegenerative process of AD. The Food and Drug Administration (FDA)-approved drugs for the treatment of AD are based on the cholinergic hypothesis of AD and therefore, aimed to increase the cholinergic transmission to recover the cognitive function. To date, only five drugs have been approved by the FAD for the treatment of AD: rivastigmine, galantamine, tacrine, memantine and donepezil (Birks and Harvey, 2006). Among them, donepezil has showed some temporary efficacy, but not resolute to re-establish the cognitive decline. In the search for more effective therapies, the "one drug, multiple target" strategy, also called multitarget-directed ligand (MTDL) approach, might be more appropriated (Buccafusco and Terry, 2000; Youdim and Buccafusco, 2005; Bolea *et al.,* 2013). This strategy suggests the use of compounds with multiple activities for different biological targets (Cavalli *et al.,* 2008). In this concern, a family of novel multitarget-directed ligands (DPH) were synthesized and evaluated as dual inhibitors of cholinesterase (AChE and BuChE) and monoamine oxidase (MAO A and MAO B) activities, both altered in AD, for the potential prevention and treatment of this neurological disorder. Strong biometal-chelating properties versus Cu^{2+} and Fe^{2+}, ADMET properties and brain penetration capacity as well as a significantly decrease in scopolamine-induced learning deficits in healthy adult mice were also found with this compound (Wang *et al.,* 2014). Additionally, the analogue DPH-4 (see Fig 1) was also determined to inhibit bovine SSAO activity in low micromolar range (IC_{50} value of 2.8 ± 0.7 μM). Both the strong link between AD and the stroke condition and the important role of the cerebrovascular tissue in both pathologies prompted us to elucidate the potential protective effect of DPH-4 in a new experimental model of cerebral ischemia.

Because of the SSAO/VAP-1 alteration in AD (Ferrer *et al.,* 2002) and stroke (Hernandez-Guillamon *et al.,* 2010), and due to its high expression in cerebrovascular tissue, we hypothesized that this protein could be a potential link and therapeutic target for both pathologies. However, some protein expression is lost when cells are cultured, as it is the case for SSAO/VAP-1; therefore, herein we report by first time the preparation of a human brain endothelial-immortalized cell line derived from the HcMEC/D3 cell line, kindly supplied by Dr. Couraud (Paris) (Weksler *et al.,* 2005), that stably expresses the human SSAO/VAP-1 gene. The HcMEC/D3 hSSAO/VAP-1 cell line was characterized and compared with previously developed endothelial and smooth muscle cell lines expressing SSAO/VAP-1 (Solé and Unzeta, 2011; Solé, *et al.,* 2007). Then, we established a new experimental model of stroke consisting in subjecting these brain endothelial cells to OGD and reoxygenation conditions. The HcMEC/D3 cell line is the first stable well differentiated and well characterized human brain endothelial cell line (Weksler *et al.,* 2005) in terms of expression of endothelial markers, up-regulation of adhesion molecules in response to inflammatory cytokines, as well as BBB characteristics, so it was chosen as an accepted BBB cellular model instead of peripheral endothelial HUVEC cell line because genes expressed by cerebral endothelial cells are important and distinct in several processes as vasculo- and angiogenesis (VEGF), immunoregulation (decorin, IL6) or have growth-supporting properties (BDNF, transforming growth factor-β) (Kalmann *et al.,* 2002).

Cell viability under OGD with reoxygenation conditions was observed significantly decreased in the presence of MA, the main physiological SSAO/VAP-1 substrate, and it was restored when cells were pre-incubated in the presence of semicarbazide, a specific SSAO/VAP-1 inhibitor. These results confirmed that this enzyme plays an important

role enhancing the endothelial cell death under ischemia. Moreover, under these hypoxia conditions, the membrane-bound SSAO/VAP-1 is released due to an increase in its shedding, that could be explained by the activation of metalloproteinase 2 (MMP-2) (Sun *et al.,* 2014). This soluble form of the enzyme may be also able to contribute to the vascular cell damage through its catalytic activity, as previously described by our group (Hernandez *et al.,* 2006). At the concentrations assayed, DPH-4 inhibits about 50% of the SSAO/VAP-1 activity. DPH-4 pre-treatment shows a protective effect on hSSAO/VAP-1-expressing HcMEC/D3 cells in presence of MA in both normoxia and in OGD with reoxygenation conditions. This protection was found to be even more significant when the same experiment was carried out on hSSAO/VAP-1-expressing HUVEC cells. This different behaviour might be explained because cerebral endothelial cells constitute a firm barrier formed by tight junctions joining plasma membranes of neighbouring cells that may hinder DPH-4 accession to HcMEC/D3 cells. A different sensitivity of both cells to the toxics generated by SSAO activity cannot be ruled out. The beneficial effect of DPH-4 was also noticeable in terms of inflammation, since it significantly reduced the leukocyte adhesion to the endothelia subjected to different OGD conditions as a result of its inhibitory effect on the SSAO/VAP-1 activity. This anti-inflammatory effect may also contribute to the protective effect observed in this experimental model of stroke.

In addition, when $A\beta_{1\text{-}40}D$ treatment was introduced in this experimental model of ischemia simulating a pre-existing AD pathology, DPH-4 showed a protective effect on the synergic damage induced by MA and $A\beta_{1\text{-}40}D$. These results allow us concluding that $A\beta_{1\text{-}40}D$ together with the catalytic action of SSAO/VAP-1 induces more vascular damage under OGD with reoxygenation conditions, and that the protective effect of DPH-4 is higher than that observed using separately both toxics.

Herein we report by first time a new MTDL compound, as a donepezil+propargylamine+8-hydroxyquinoline hybrid, able to protect HcMEC/D3 hSSAO/VAP-1 cells under hypoxia conditions through its inhibitory and anti-inflammatory effect on SSAO/VAP-1. In the context of the close relationship between AD and stroke and the involvement of SSAO/VAP-1 in both diseases, DPH-4 could be considered as a promising drug with high therapeutic interest to be used in both pathologic conditions.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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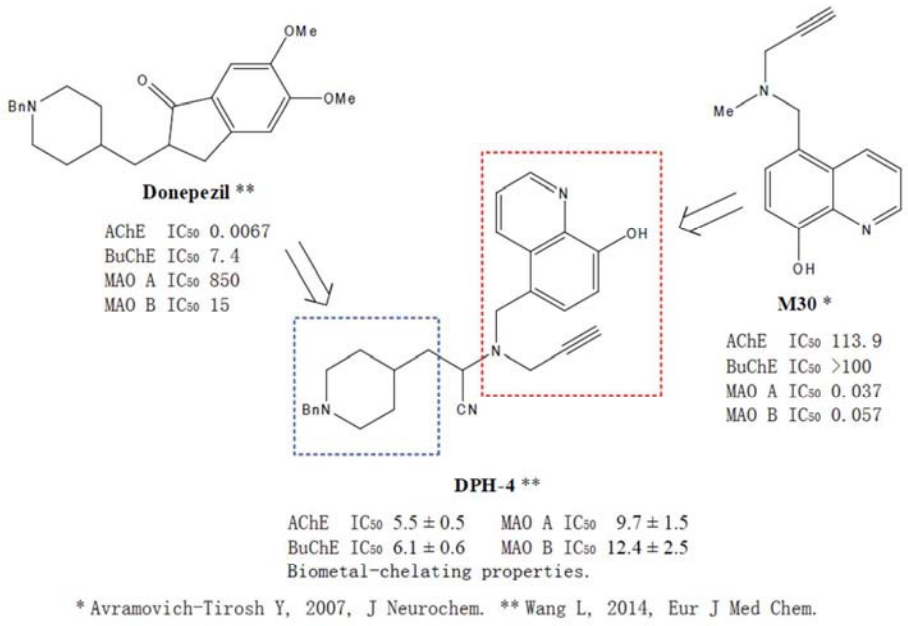


Figure 1. Design strategy of the multifunctional compound DPH-4. The N-benzylpiperidine moiety of donepezil was combined with the 8-hydroxyquinoline and propargylamine moieties of M30. IC_{50} values (μM) of donepezil, M30 and DPH-4 for AChE, BuChE, MAO (A and B), and biometal-chelating properties are indicated, as described in the corresponding publications.

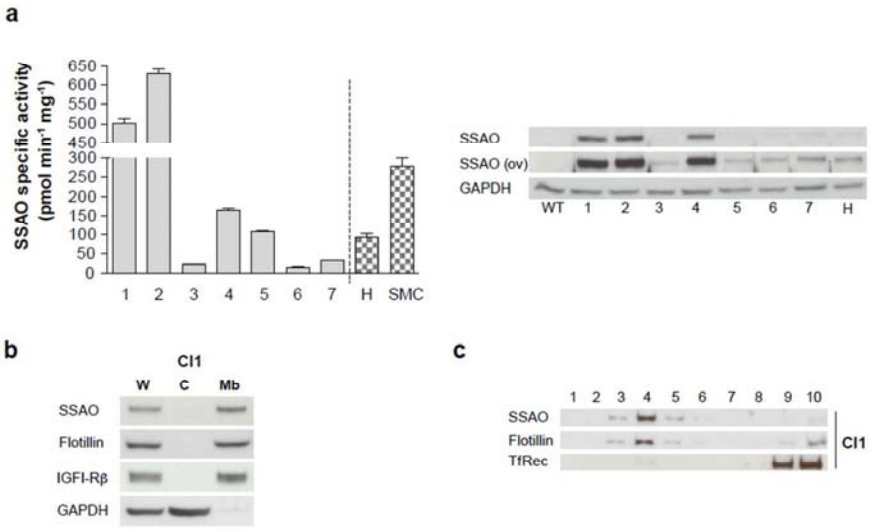


Figure 2. Characterization of the HcMEC/D3 cell line human SSAO/VAP-1-transfected. (**a**) SSAO specific activity (expressed as pmol min^{-1} mg^{-1} of protein) of the antibiotic-selected and amplified positive clones (1 - 7), with the corresponding protein visualization by western blot. HUVEC endothelial (H) or A7r5 smooth muscle (SMC) transfected cell lines are shown on comparative purposes. The overexposure (ov) of the SSAO western blot is shown to highlight the weaker bands. GAPDH was used as loading control. WT, wild type (non-transfected cells). (**b**) The transfected SSAO protein is located in the membrane fraction, and absent from the cytosol, clone 1 is showed as example. Flotillin and IGF1-Rβ were used as membrane-positive markers; GAPDH was used as cytosolic marker. W, whole fraction; C, cytosolic fraction; Mb, membrane fraction. (**c**) The transfected SSAO is located in the lipid raft fractions of the cell membrane; clone 1 is showed as example. Flotillin was used as raft-positive protein and transferrin receptor (Tf Rec) was used as marker of the soluble membrane fraction.

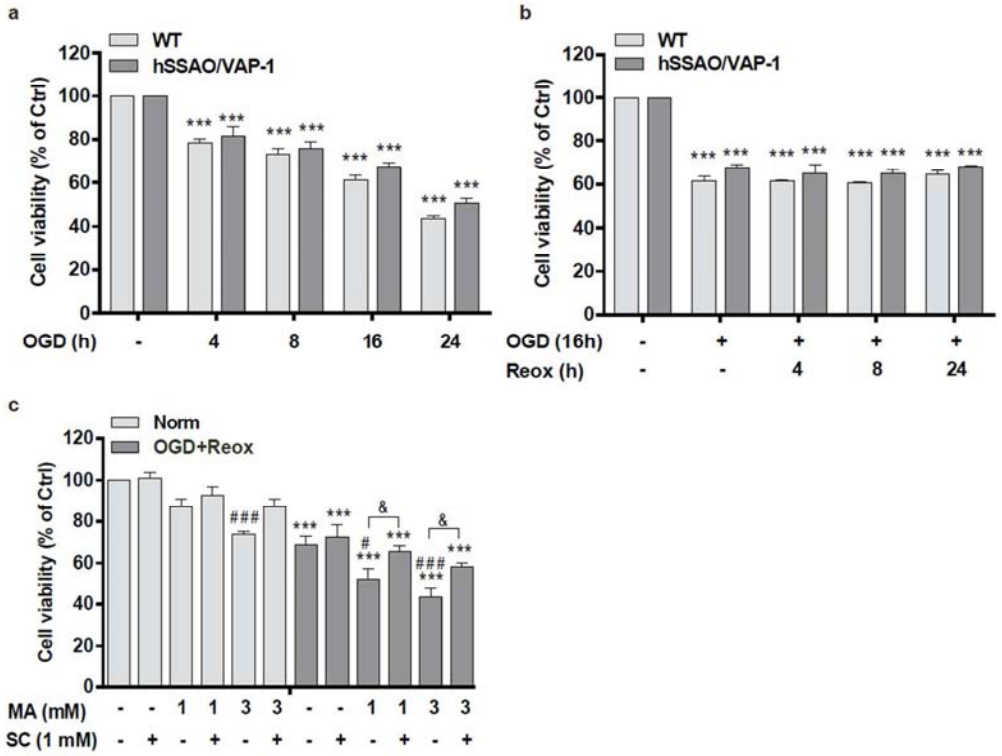


Figure 3. Effect of OGD, OGD with reoxygenation, and methylamine (MA) on cell viability of wild type (WT) and human SSAO/VAP-1-transfected HcMEC/D3 cells (HcMEC/D3 hSSAO/VAP-1). Different duration of OGD (**a**) or 16h-OGD with different duration of reoxygenation (**b**) induces WT and hSSAO/VAP-1-transfected HcMEC/D3 cell death. MTT reduction assay was used to assess cell viability under the different assayed conditions. Cells without OGD were maintained under normoxia conditions, and are considered control cells (Ctrl). The metabolism of MA under 16h-OGD plus 24h of reoxygenation condition (OGD+Reox) (**c**) induces a reduction of cell viability in hSSAO/VAP-1-expressing HcMEC/D3 cells, which is prevented by the SSAO activity inhibitor semicarbazide (SC). MA (1 mM or 3 mM) and SC (1 mM) were added before OGD and maintained during reoxygenation. Control cells are non-treated cells in normoxia (Norm). Data are expressed as mean ± SEM of at least 3 independent experiments. $^{***}P<0.001$ versus control of the corresponding cell type. $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus non-treated hSSAO/VAP-1-expressing HcMEC/D3 cells in the corresponding condition (Norm or OGD+Reox); $^{\&}P<0.05$ between the indicated treatments. Statistical analyses were performed by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

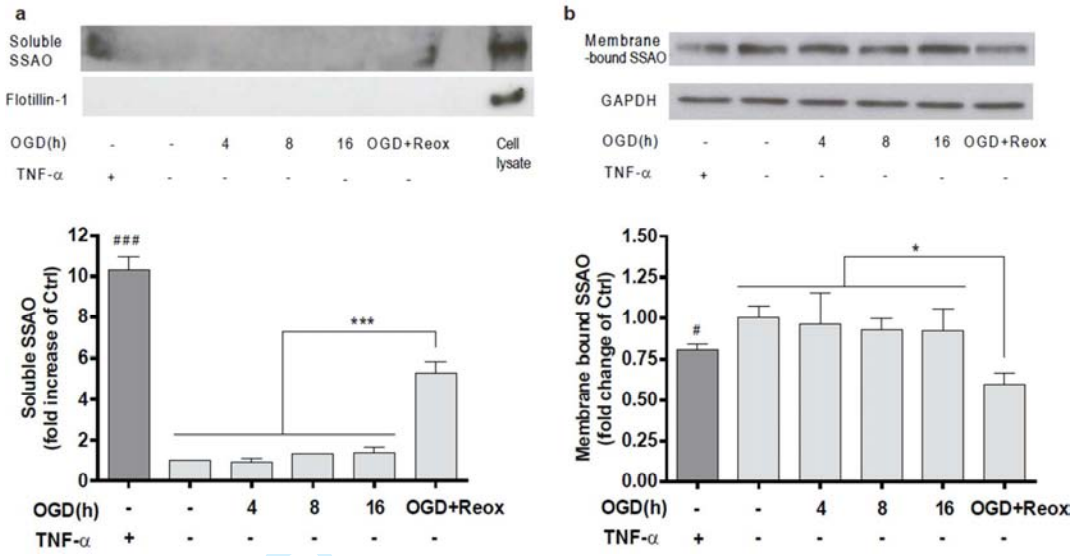


Figure 4. OGD with reoxygenation induces the release of soluble SSAO to the culture media by HcMEC/D3 hSSAO/VAP-1 cells. (**a**) Presence of soluble SSAO/VAP-1 in ten-fold concentrated culture media corresponding to TNF-α treatment (24h in normoxia, 100 ng mL^{-1}), different OGD times and 16h-OGD with 24h-reoxygenation (OGD+Reox). TNF-α was used as positive control of SSAO/VAP-1 release. Flotillin-1 was used as control of cell debris absence in the media. (**b**) Presence of membrane-bound SSAO/VAP-1 in HcMEC/D3 hSSAO/VAP-1 cell lysates under the same experimental conditions than in **a**. The presence of membrane-bound SSAO was normalized to the GAPDH levels. Non-treated cells or media under normoxia conditions were considered control samples (Ctrl). Data in graphs represent the western blot quantifications and are expressed as mean ± SEM of data obtained from 3 independent experiments. $^{*}P<0.05$ and $^{***}P<0.001$ as indicated; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus control cells, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

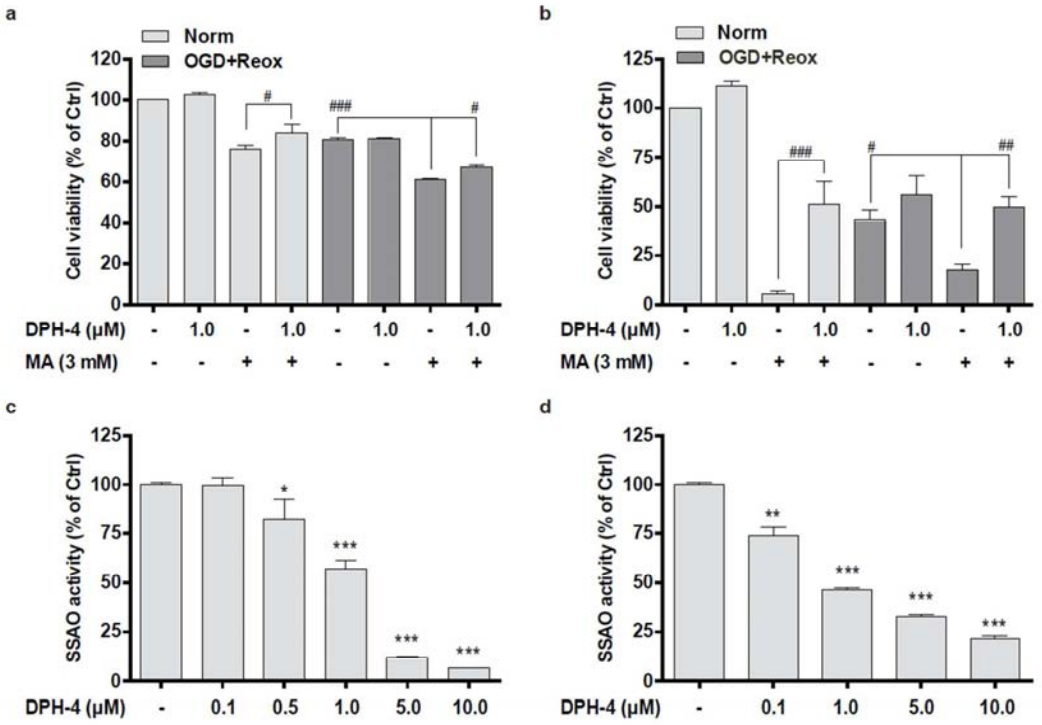


Figure 5. DPH-4 attenuates the cell death induced by the metabolism of methylamine in normoxia or in OGD with reoxygenation conditions in different endothelial cell types. MTT reduction assay was used to determine the cell viability of HcMEC/D3 hSSAO/VAP-1 cells subjected to 16h-OGD with 24h-reoxygenation (**a**), and HUVEC hSSAO/VAP-1 cells subjected to 24h-OGD with 7h-reoxygenation (**b**). MA (3 mM) and DPH-4 (1 µM) were added before OGD and maintained during reoxygenation. Non-treated endothelial cells under normoxia were used as control (Ctrl) cells. The SSAO activity inhibiting capacity of DPH-4 was determined by investigating SSAO activity remaining in HcMEC/D3 hSSAO/VAP-1 cell lysates after being incubated with DPH-4 (0.1-10 µM) for 30 min (**c**), or by evaluating SSAO activity present in HUVEC hSSAO/VAP-1 cell lysates from DPH-4 (0.1-10 µM)-treated cells for 24 hours (**d**). SSAO activity was analyzed by radiometric method, using ^{14}C-benzylamine as substrate. A non-treated cell lysate was considered the control (Ctrl) sample. Data in graphs are expressed as mean ± SEM and represent data obtained from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus non-treated samples; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

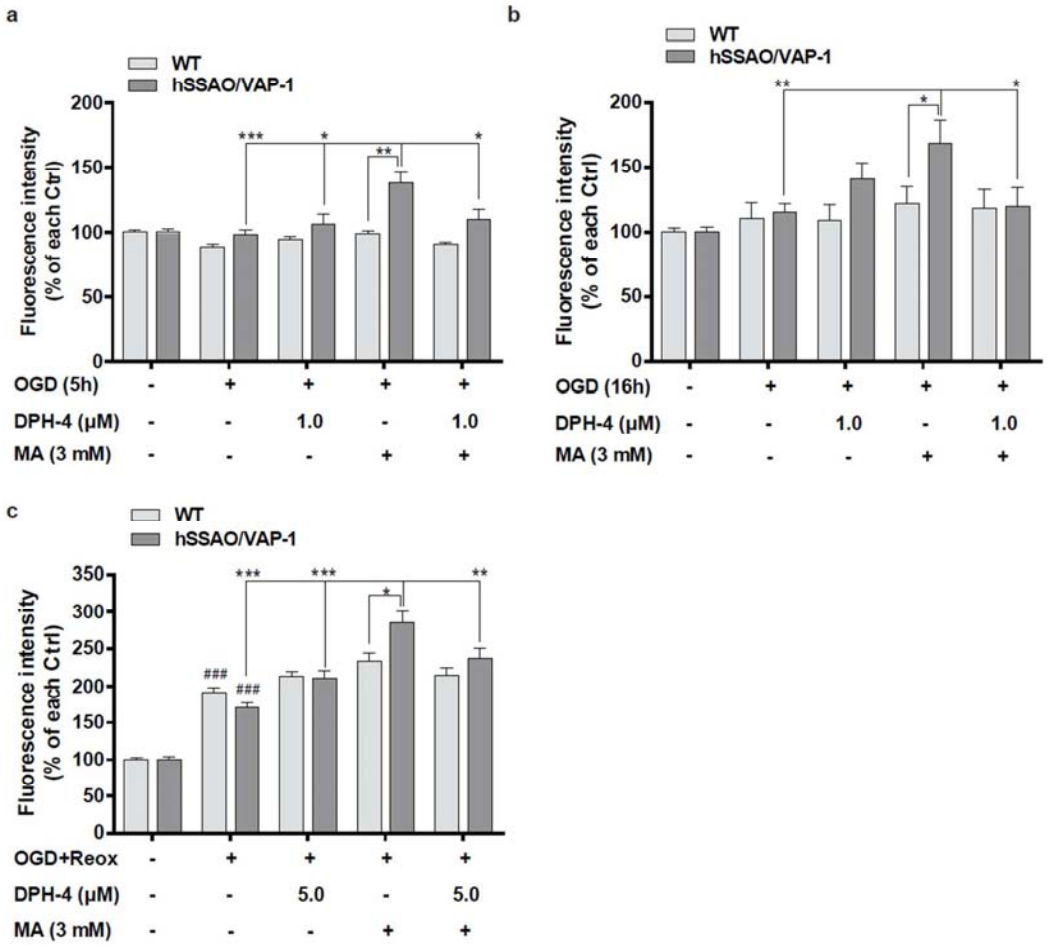


Figure 6. DPH-4 prevents the leukocyte binding to endothelial cells under different OGD conditions in presence of the SSAO substrate. Leukocyte-endothelium adhesion assay was performed to analyze the anti-inflammatory effect of DPH-4 against SSAO mediated pro-inflammatory action. WT and hSSAO/VAP-1-expressing HcMEC/D3 cells treated with MA (3 mM) or/and DPH-4 (1 or 5 µM) undergone 5h-OGD (**a**), 16h-OGD (**b**) or 16h-OGD with 24h-reoxygenation (OGD+Reox, **c**) and the binding of calcein-AM-labelled THP-1 leukocytes on these cells was quantified. Non-treated cells under normoxia conditions were considered as control (Ctrl) for each type of cells. Data are expressed as mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus HcMEC/D3 hSSAO/VAP-1 treated with MA under OGD conditions; $^{###}P<0.001$ versus control of the corresponding cell type, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

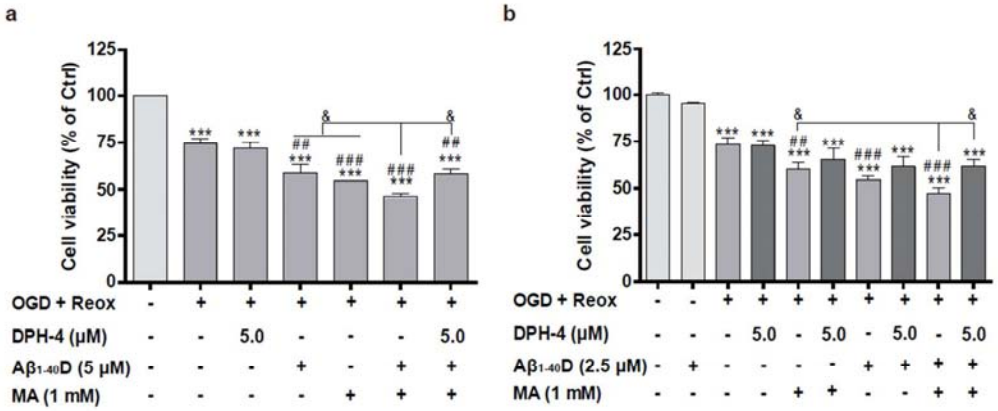
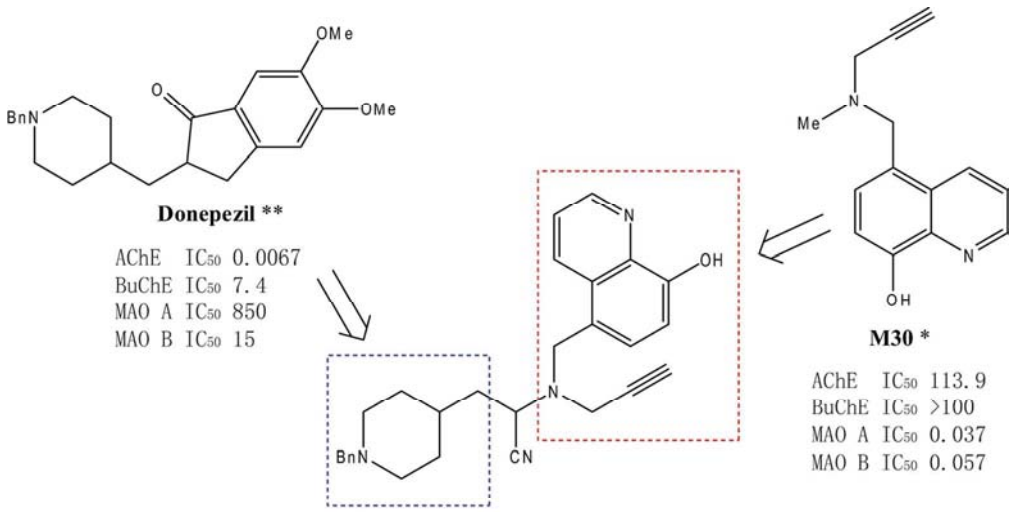


Figure 7. DPH-4 protects against cell death caused by the co-treatment of SSAO activity and $A\beta_{1-40}D$ in HcMEC/D3 hSSAO/VAP-1 cells under OGD with reoxygenation. MTT reduction assay was used to evaluate the cell viability of HcMEC/D3 hSSAO/VAP-1 cells subjected to 16h-OGD with 24h-reoxygenation (OGD+Reox) in the presence of $A\beta_{1-40}$ containing the Dutch mutation ($A\beta_{1-40}D$, 5 μM), MA (1 mM) or/and DPH-4 (5 μM) (**a**). $A\beta_{1-40}D$, MA and DPH-4 where added at the same time before OGD. (**b**) a 24h pre-treatment was performed with $A\beta_{1-40}D$ and cells were then subjected to 8h-OGD with 24h-reoxygenation. All treatments were maintained during reoxygenation. Non-treated cells under normoxia were considered as control (Ctrl). Data are expressed as mean ± SEM of three independent experiments. $^{***}P<0.001$ versus Ctrl; $^{\#\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus non-treated cells under OGD+Reox; $^{\&}P<0.05$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.



* Avramovich-Tirosh Y, 2007, J Neurochem. ** Wang L, 2014, Eur J Med Chem.

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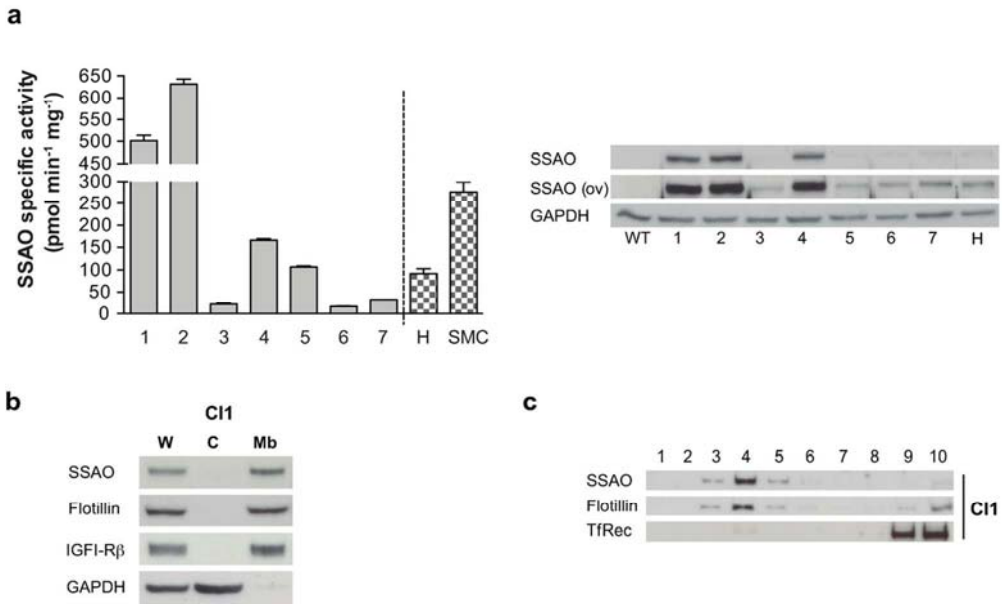

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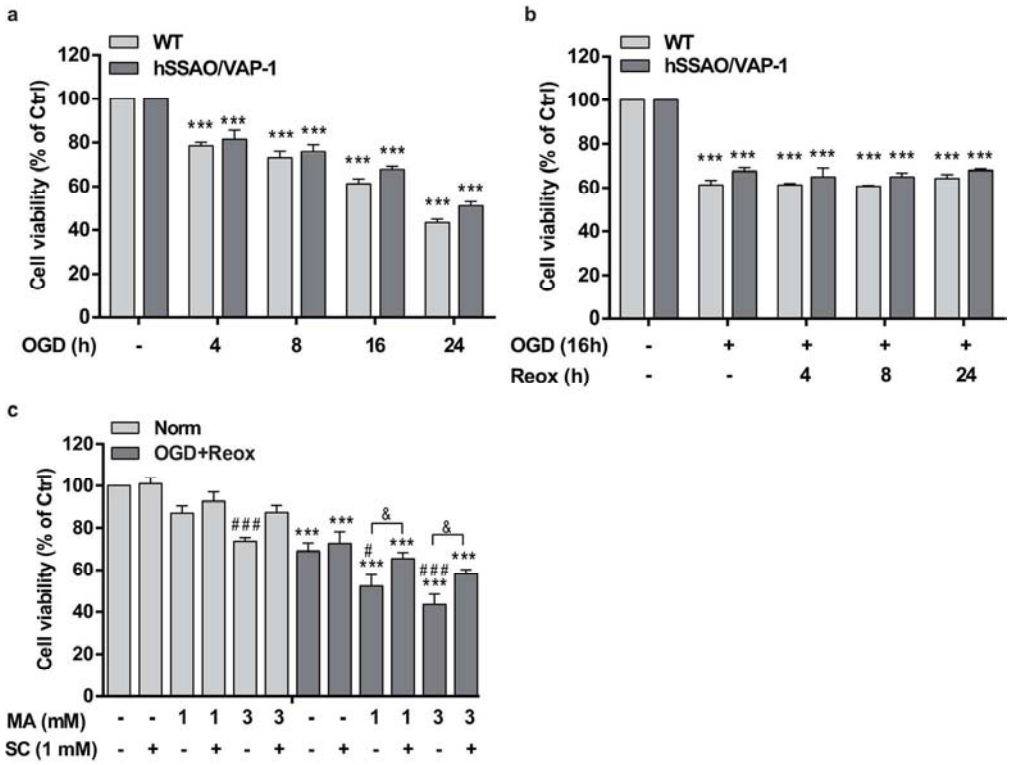

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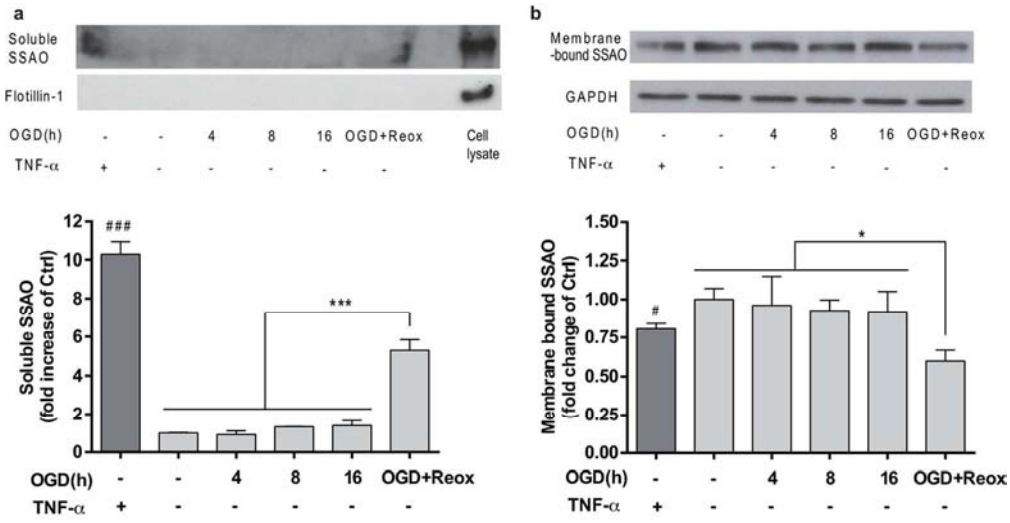

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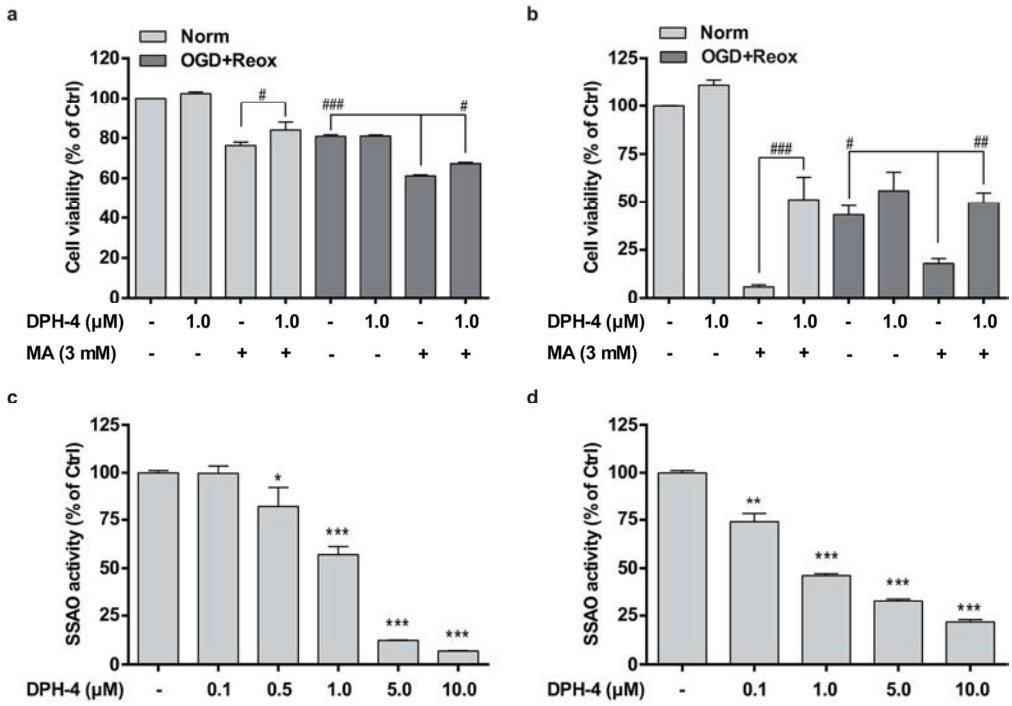

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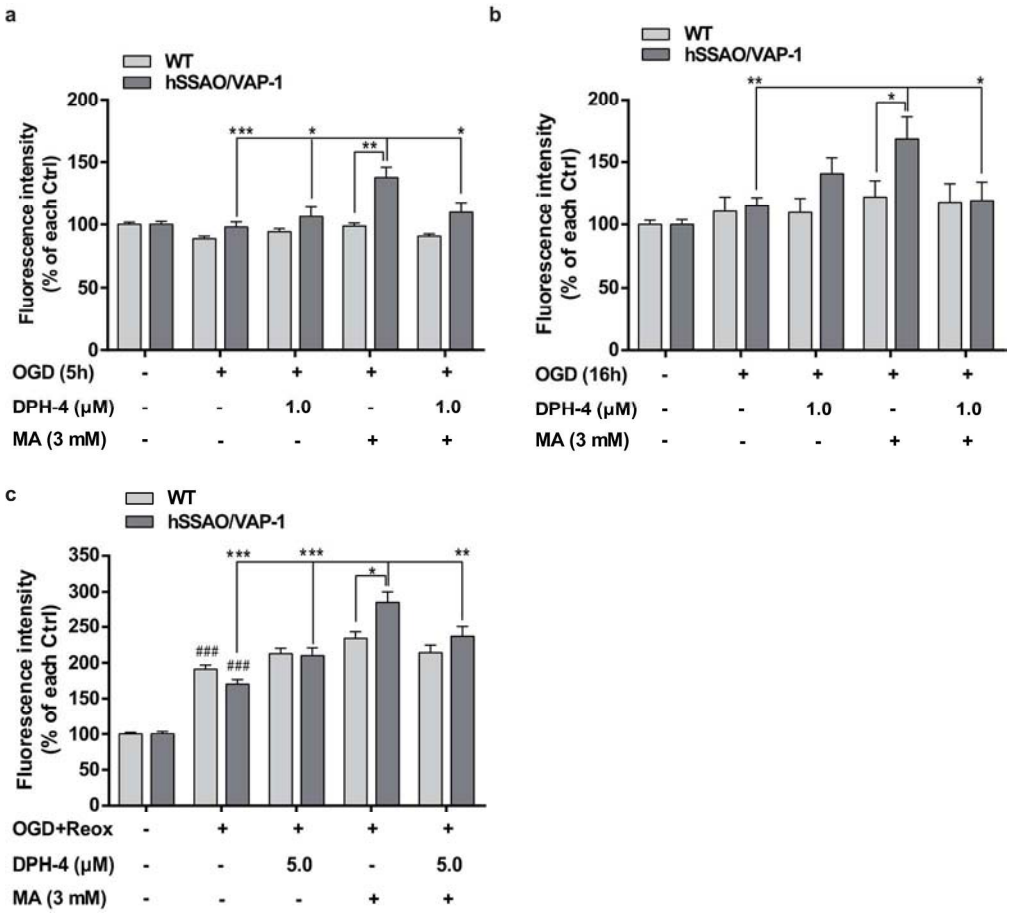

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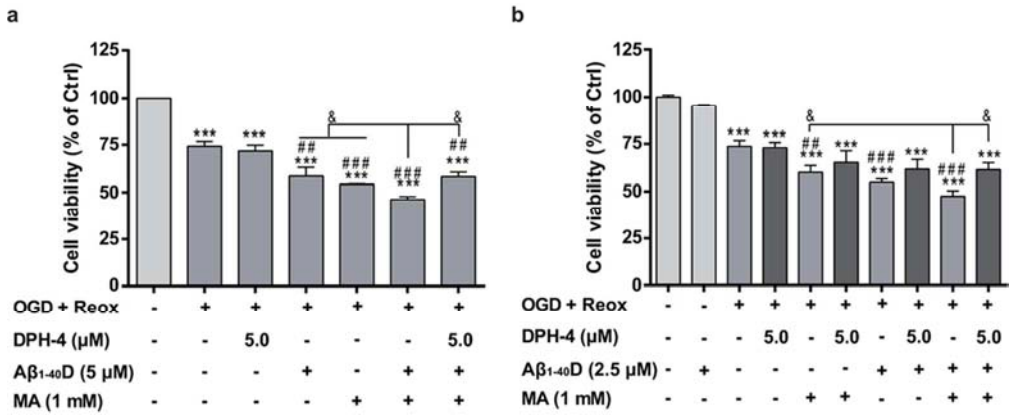

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1. Annex chapter II.

1.1. Supplementary figure 1

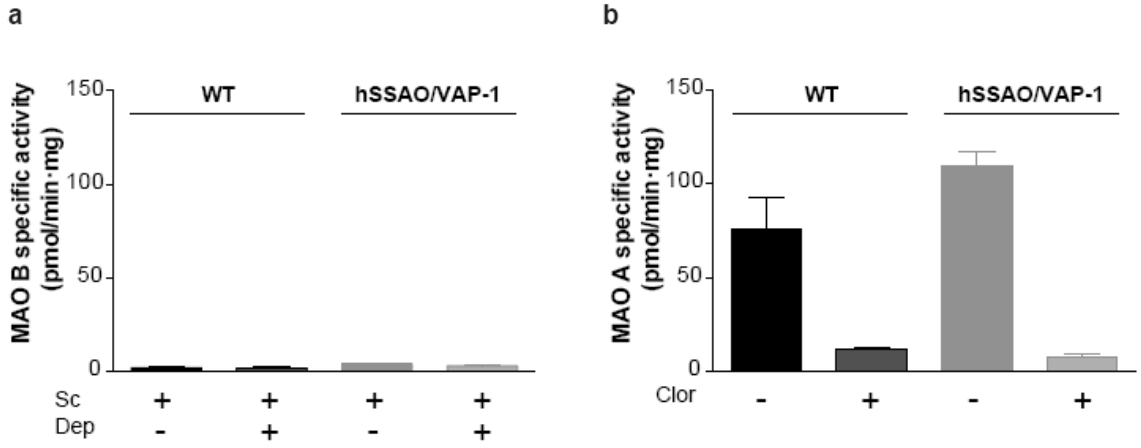


Fig S1. Enzymatic activity of MAO B and MAO A in hCMEC/D3 WT and hSSAO/VAP-1 cell lysates. (a) MAO B specific activity in WT and hSSAO/VAP-1 cell lysates was measured with 100 µM ^{14}C-Benzylamine. Inhibition of SSAO and MAO B activities were performed by 1 mM semicarbazide (SC) and 1 mM deprenyl (Dep), respectively. (b) MAO A specific activity in WT and hSSAO/VAP-1 cell lysates was measured with 100 µM ^{14}C-5-HT. Inhibition of MAO A activity was performed by 1 µM clorgyline (Clor). Data are expressed as mean ± SEM from 3 independent experiments.

1.2. Supplementary figure 2

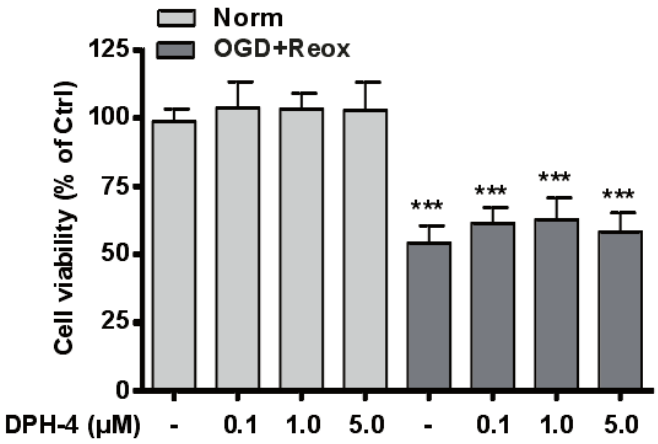


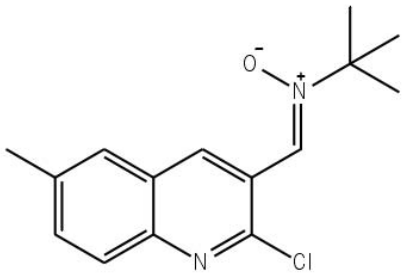
Fig S2. Effects of different concentrations of DPH-4 on the cell viability in HUVEC hSSAO/VAP-1 cells subjected to normoxia (Norm) and 24-hour OGD with 7-hour reoxygenation (OGD+Reox). MTT reduction assay was used to determine the cell viability. Different concentrations (0.1 µM, 1.0 µM and 5 µM) were added into the media before the starting of OGD and maintained the same concentrations during reoxygenation. Non-treated endothelial cells under normoxia were used as control (Ctrl) cells. Data in graphs are expressed as mean ± SEM and represent data obtained from 3 independent experiments. ***P<0.001 versus Ctrl, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

1.3. Protective effect of nitrone RP18 under OGD conditions on human SSAO/VAP-1-expressing HUVEC cells as an experimental model of cerebral ischemia

Oxidative stress response after the onset of cerebral ischemia and reperfusion is one of the most important molecular events for the cellular damage and death of cerebral tissue. In this regard, free radical scavengers, such as nitrones, constitute one of the neuroprotective approaches proved to bring beneficial outcomes in experimental cerebral ischemia.

Nowadays, nitrones are extensively investigated in biological systems for their protection in oxidative stress-related diseases including ischemic stroke. For instance, N-*tert*-butylnitrone (PBN) has been shown to reduce oxidative stress and lipids peroxidation (Kalyanaraman *et al.*, 1991), to ameliorate ischemic brain damage and reduce infarct volume in animal models of ischemic stroke (Sun *et al.*, 2008). 2-[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN) and a new pyrazine derivative (compound 21) possess both antiplatelet and antioxidant activities, and shows significant neuroprotective effects under *in vitro* and *in vivo* models of ischemic stroke (Sun *et al.*, 2008; Sun *et al.*, 2012). Among all the nitrones, probably the most studied one is disodium 2,4-disulphophenyl-*N*-tert-butylnitrone (NXY-059), which exhibits free radical trapping properties (Maples *et al.*, 2001), and neuroprotection in clear dose-dependent manner both in transient (Kuroda *et al.*, 1999) and permanent (Zhao *et al.*, 2001) MCAO (middle cerebral arterial occlusion) models of stroke. Besides its effectiveness in animal models, however, they showed only unequivocal evidence of efficacy in advanced clinical studies (Macleod *et al.*, 2008). In this context, new multi-functional nitrones targeting the concept of neuroprotection remain perspective and still could be considered as new therapeutic approaches for ischemic stroke treatments.

Recently, a new series of multi-functional nitrones for the treatment of ischemic stroke have been synthesised. The (Z)-*α*-aryl and heteroaryl-*N*-alkyl nitrones also exhibit antioxidant and neuroprotective properties; in addition, they are anti-inflammatory and have the ability to cross the blood-brain barrier (BBB) (Chioua *et al.*, 2011). Among these nitrones, RP18 was one of the best derivatives, exhibiting potent and balanced properties. Therefore, the protective effect of nitrone RP18 was investigated under OGD conditions on human SSAO/VAP-1-expressing HUVEC cells as an experimental model of cerebral ischemia.



RP18 *

* M Chioua *et al.*, J. Med. Chem. 2012

Fig 1. Chemical structure of nitrone RP18.

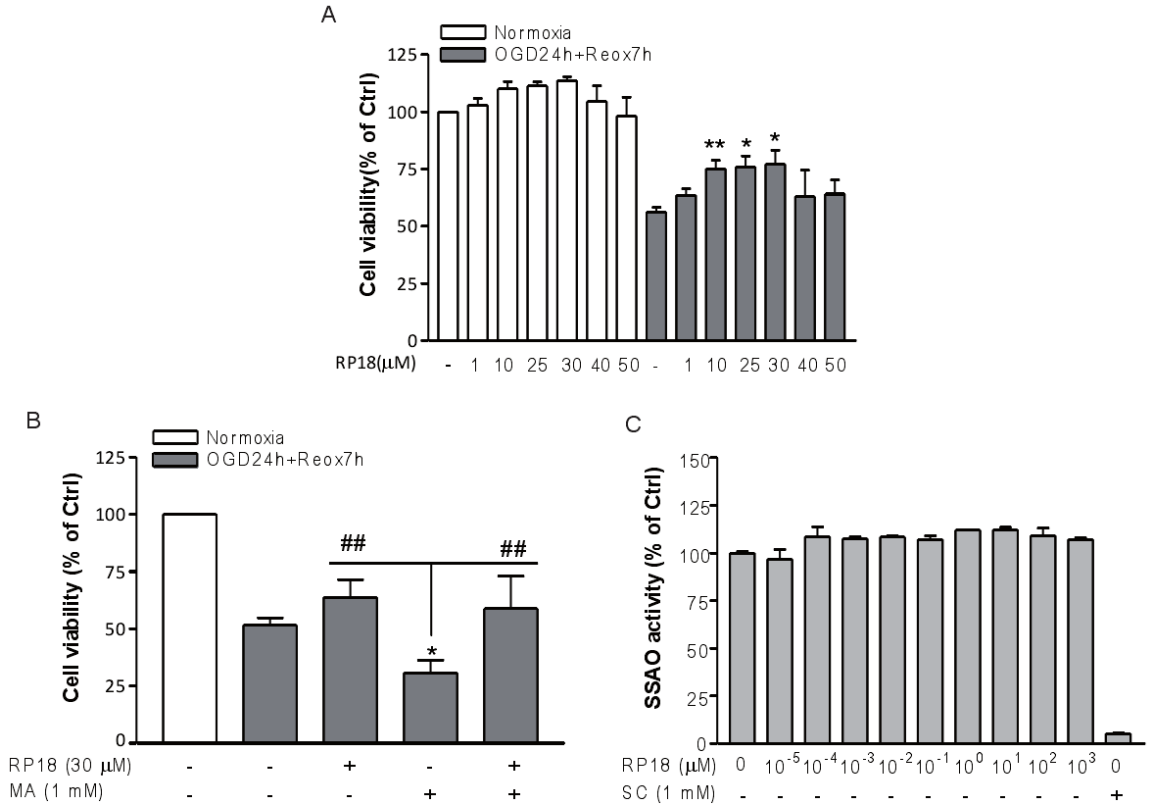


Fig 2. RP18 diminishes the cell death induced by the metabolism of methylamine (MA) under OGD with reoxygenation (Reox) conditions in HUVEC hSSAO/VAP-1 cells. MTT reduction assay was used to determine the cell viability of (A) HUVEC hSSAO/VAP-1 cells treated with 1-50 μM of RP18 subjected to normoxia or 24-hour OGD with 7-hour reoxygenation (OGD24h + Reox7h), (B) HUVEC hSSAO/VAP-1 cells treated with 30 μM RP18 or/and 1 mM MA subjected to normoxia or 24-hour OGD with 7-hour reoxygenation. RP18 and MA were added before OGD and maintained during reoxygenation. Non-treated endothelial cells under normoxia were used as control (Ctrl) cells. (C) The SSAO activity inhibiting capacity of RP18 was determined by investigating SSAO activity remaining in HUVEC hSSAO/VAP-1 cell lysates after being incubated with RP18 (10^{-5}-10^{3} μM) for 1 hour. SSAO activity was analyzed by radiometric method, using ^{14}C-benzylamine as substrate. A non-treated cell lysate was considered the control (Ctrl) sample. Data in graphs are expressed as mean ± SEM and represent data obtained from at least 3 independent experiments. *P<0.05 and **P<0.01 versus non-treated cells under OGD24h + Reox7h; $^{\#\#}P$<0.01 as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

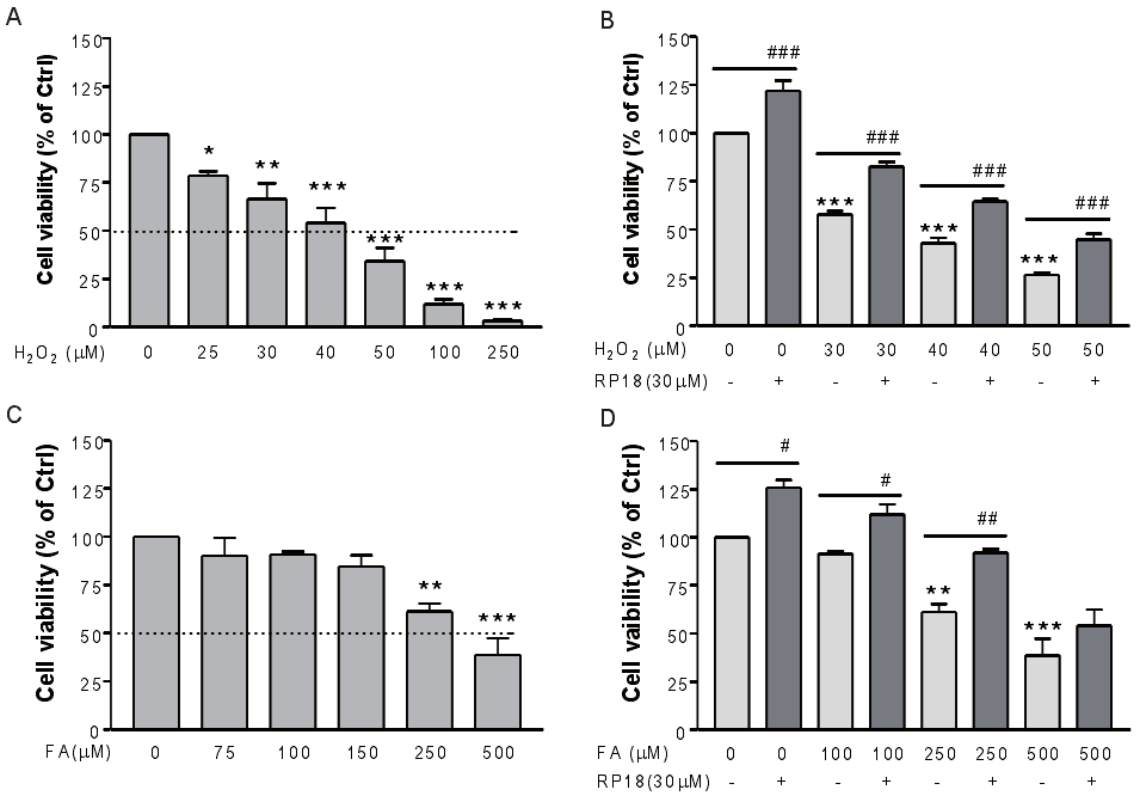


Fig 3. RP18 attenuates the cell death caused by hydrogen peroxide (H_2O_2) and formaldehyde (FA) in HUVEC hSSAO/VAP-1 cells. MTT reduction assay was carried out to determine the cell viability of HUVEC hSSAO/VAP-1 cells treated with (A) 25-250 µM H_2O_2, (B) H_2O_2 or/ and 30 µM RP18, (C) 75-500 µM formaldehyde and (D) formaldehyde or/and 30 µM RP18, under normoxia for 24 hours. Non-treated cells were considered control (Ctrl) cells. Data in graphs are expressed as mean ± SEM and represent data obtained from at least 3 independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ versus Ctrl; $^{\#}P<0.5$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

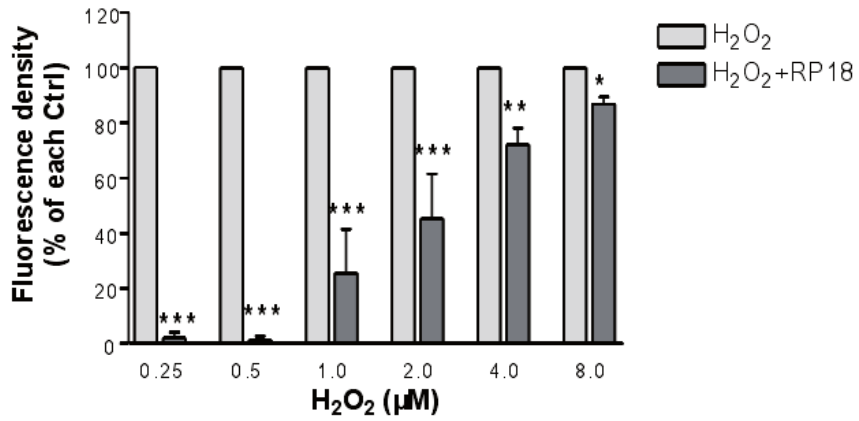


Fig 4. RP18 possesses hydrogen peroxide (H_2O_2) scavenging ability. Amplex red assay was performed to analyse the H_2O_2 scavenging ability of RP18 at 30 μM. 0.25-8 μM H_2O_2 incubated separately with 30 μM RP18, the remaining H_2O_2 was determined by measuring the fluorescence intensity at 530/590nm after adding the Amplex ultrared reagent. Fluorescence values of each concentration of H_2O_2 without adding RP18 were considered control (Ctrl). Data are expressed as mean ± SEM from three independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001 versus each Ctrl, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

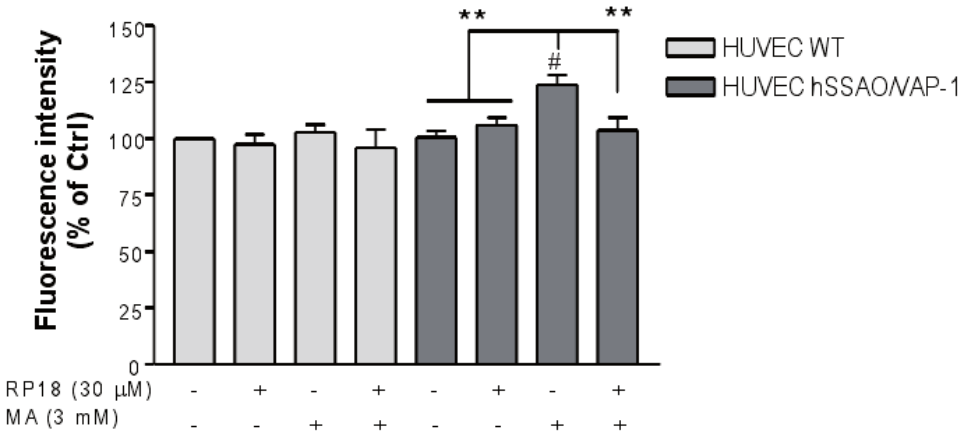


Fig 5. RP18 prevents the leukocyte binding to endothelial cells under short time OGD condition in presence of the SSAO substrate. Leukocyte-endothelium adhesion assay was performed to analyze the anti-inflammatory effect of RP18 against SSAO mediated pro-inflammatory action. WT and hSSAO/VAP-1-expressing HUVECs treated with MA (3 mM) or/and RP18 (30 μM) undergone 5h-OGD. Non-treated WT cells under normoxia

conditions were considered as control (Ctrl) cells. Data are expressed as mean ± SEM of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 versus HCMEC/D3 hSSAO/VAP-1 treated with MA under OGD conditions; $^{\#}P$<0.001 versus WT with the same treatment, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

Results and conclusions:

1. RP18 has a good protective effect against the damage caused by the insult of OGD24h + Reox7h, which is the condition selected to mimic ischemic stroke for HUVEC hSSAO/VAP-1 cells.

2. Although RP18 is not a SSAO inhibitor, it can alleviate the damage triggered by the addition of methylamine into hSSAO/VAP-1 expressing HUVECs under OGD + Reox conditions.

3. Since H_2O_2 and formaldehyde are the products of SSAO metabolism of MA, the toxicity by H_2O_2 and formaldehyde have been studied, and some protective effects have been observed by RP18, which could probably explained by its antioxidant and trophic effect.

4. RP18 showed the anti-inflammatory behaviour under OGD condition, which is not mediated by SSAO activity inhibition. And the mechanism of RP18 involved in attenuating the inflammatory action could to be further elucidated by analysing other SSAO-related cell adhesion molecules, such as ICAM-1, VCAM-1, P-selectin, E-selectin, ect., participating in the leukocyte-endothelium cell adhesion process.

Chapter III:

"The modulation of the soluble SSAO/VAP-1 levels mediates part of the beneficial effect of simvastatin in cerebral ischemia"

(Manuscript to be submitted for publication to Journal of Neurochemistry)

The modulation of the soluble SSAO/VAP-1 levels mediates part of the beneficial effect of simvastatin in cerebral ischemia

Ping Sun[1], Montse Solé[1]*, Mar Hernandez-Guillamón[2], Mireia Campos-Martorell[2], Alba Simats[2], Joan Montaner[2,3] and Mercedes Unzeta[1]*

[1]*Departament de Bioquímica i Biologia Molecular.Institut de Neurociències. Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain.*
[2]*Neurovascular Research Laboratory, Institut de RecercaValld'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain.*
[3]*Neurovascular Unit, Department of Neurology, Universitat Autònoma de Barcelona, Hospital Valld'Hebron, Barcelona, Spain.*

* Adress correspondence and reprint requests to Mercedes Unzeta, Departament de Bioquímica i Biologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona. 08193 Bellaterra, Spain. E-mail: mercedes.unzeta@uab.es; MontseSolé, Departament de Bioquímica I Biologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona. 08193 Bellaterra, Spain. E-mail: montserrat.sole@uab.cat.

Running title:SSAO/VAP-1 release amplifies stroke inflammation

Keywords:cerebral ischemia, SSAO/VAP-1, inflammation, simvastatin, blood-brain barrier, endothelial cells.

Abbreviations:

BBB, blood-brain barrier; CL, contralateral; COX-2, cyclooxygenase 2; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hCMEC, human cerebral microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule 1; IP, ipsilateral; MA, methylamine; MCAO, middle cerebral artery occlusion; MMP, metalloproteinase; NOS-2, nitric oxide synthase 2/inducible nitric oxide synthase; OGD, oxygen and glucose deprivation; PLZ, phenelzine; ROS, reactive-oxygen species; SOD-1, superoxide dismutase 1; SSAO/VAP-1, semicarbazide-sensitive amine oxidase/vascular adhesion protein 1; tPA, tissue-plasminogen activator; VCAM-1, vascular cell adhesion molecule 1.

Title

The modulation of the soluble SSAO/VAP-1 levels mediates part of the beneficial effect of simvastatin in cerebral ischemia

Abstract

Statins mediate their beneficial effects on stroke through pleiotropic effects. They have shown anti-inflammatory properties through different mechanisms, including the inhibition of the NF-κB transcriptional activity and the consequent increase and release of adhesion molecules. Here we describe another protein through which simvastatin may mediate its benefits on the stroke treatment. We report by first time that simvastatin blocks the soluble semicarbazide-sensitive amine oxidase/vascular adhesion protein 1 (SSAO/VAP-1) release to the circulation. This adhesion molecule has leukocyte-binding capacity by enzymatic activity-dependent and independent mechanisms. It also mediates the expression of other adhesion proteins through signaling molecules generated from its catalytic activity, which when overproduced contribute to the vascular damage. Our results indicate that the soluble SSAO/VAP-1 is released after an ischemic stimulus, and reaches non-infarcted brain areas through the blood circulation. There, it induces the expression of the adhesion molecules E-selectin and VCAM-1. This action amplifies the inflammation and the consequent damage in the infarcted brain. Simvastatin block its release, therefore preventing these pro-inflammatory-associated effects. The combined therapy with SSAO/VAP-1 inhibitors and statins would enhance the beneficial effects of both individual treatments on stroke patients.

Introduction

Cerebral ischemia, the most frequent cerebrovascular disease, constitutes nowadays the second cause of death and dementia, and the leading cause of long-term disability worldwide (Mozaffarian *et al.* 2015). The most common cause of an ischemic disease is the cerebral blood flow reduction due to an acute thrombotic or embolic occlusion of a cerebral artery. This blood flow decrease induces, as a consequence of the oxygen, glucose and nutrients absence, a cascade of events that leads to the cellular death (González *et al.* 2011). At present, the only available treatment for the acute phase of an ischemic stroke is the use of thrombolytic drugs as the tissue plasminogen activator (tPA) to restore brain perfusion. Despite its effectiveness, it has a limited therapeutic window and in some cases, it induces fatal haemorrhages as side effect (TNIoNDa 1995). Thus, the search for new treatments becomes really urgent. In this regard, there are solid evidences that statins pre- and post-treatment may improve both the incidence and outcome in acute ischemic stroke, showing thus good perspectives of therapeutic efficacy (Montecucco *et al.* 2012, Ni Chroinin *et al.* 2013). A neuroprotective effect of statins has also been described in neurodegenerative diseases such as Alzheimer´s disease, Parkinson´s disease, and Vascular Dementia (Wang *et al.* 2011). Statins are inhibitors of the HMG-CoA (3-hydroxy-3-methylglutaryl Coenzyme A) reductase, the rate-limiting enzyme in the synthesis pathway of endogenous cholesterol, but it has been described that their beneficial effect in stroke is independent from their cholesterol lowering effects (Li *et al.* 2014, Montecucco et al. 2012). Instead, simvastatin and other statins may act through pleiotropic effects such as reducing oxidative stress (Wagner *et al.* 2000, Nagotani *et al.* 2005, Campos-Martorell *et al.* 2014), improving platelet function (Vaughan *et al.* 2000), inducing an increase of endothelial nitric oxide synthase (eNOS) (Laufs *et al.* 1998), activating the Akt and CREB-dependent survival signalling pathways (Carloni *et al.* 2009) or reducing inflammation (Pahan *et al.* 1997).

The pro-inflammatory cascade after stroke involves the release of reactive-oxygen species (ROS) and cytokines such as TNF-α or IL-1from the injured neurons and glia (Amantea *et al.* 2009). These molecules induce an up-regulation of the transcription factor NF-κB, leading to the increase of the expression and release of endothelial cell adhesion molecules (ICAM-1, E-selectin, P-selectin and VAP-1) that mediate the recruitment of circulating inflammatory cells and their migration to the ischemic region (Frijns & Kappelle 2002, Yilmaz & Granger 2008, Hernandez-Guillamon *et al.* 2010). Then, both the activated endothelium and the recruited leukocytes amplify the tissue damage by activating metalloproteinases (MMPs) and generating ROS and

inflammatory mediators (COX-2, cytokines and chemokines) that lead to the blood-brain barrier (BBB) disruption, generate brain edema and induce neuronal death (Ishikawa *et al.* 2004, Jin *et al.* 2010). There have been described some targets of the anti-inflammatory action of statins including the modulation of MMPs imbalance (Wang *et al.* 2006), the inhibition of the NF-κB activity (Sironi *et al.* 2006, Lenglet *et al.* 2014) and the lowering of the endothelial adhesion molecules expression and release (Carloni *et al.* 2006, Miao *et al.* 2005) although more work needs to be done in order to know the exact mechanisms of action of statins.

Vascular adhesion protein 1 (VAP-1) is one of the adhesion molecules modulated during a stroke event. VAP-1 is a homodimeric glycoprotein that during inflammation facilitates the leukocyte recruitment through its semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.21) enzymatic activity, participating in the rolling and transmigration processes (Smith *et al.* 1998, Jalkanen & Salmi 2008). A soluble plasmatic form of the protein is shed from the cell membrane by a metalloproteinase-dependent activity (Abella *et al.* 2004, Sun *et al.* 2014). This soluble SSAO/VAP-1 has been found increased in ischemic and in hemorrhagic stroke patients (Airas *et al.* 2008, Hernandez-Guillamon *et al.* 2012b), as well as in other pathologies involving inflammation (Kurkijärvi *et al.* 1998, Ferrer *et al.* 2002, Boomsma *et al.* 2003, del Mar Hernandez *et al.* 2005, Airas *et al.* 2006), and it contributes to the tissue damage through the release of toxic products from its catalytic activity (Yu 1998). Moreover, its plasmatic activity levels constitute a strong predictor of parenchymal hemorrhages after tPA treatment in ischemic stroke patients, also predicting their neurological outcome (Hernandez-Guillamon *et al.* 2010). The soluble SSAO/VAP-1 participates as well in the leukocyte recruitment through the generation of signaling molecules like hydrogen peroxide from its catalytic activity, thus affecting inflammatory responses *in vivo* (Kurkijärvi et al. 1998, Salmi & Jalkanen 2001). Interestingly, the inhibition of SSAO/VAP-1 activity in stroke animal models reduces the infarct volume and improves the neurological outcome (Hernandez-Guillamon *et al.* 2010, Watcharotayangul *et al.* 2012). Molecular studies using cell lines have demonstrated the involvement of this protein in the endothelial activation and damage after OGD (Sun *et al.* 2014).

Given that the beneficial effects of statins include the reduction of the infarct volume, so does it the SSAO/VAP-1 inhibition, as well as the amelioration of the inflammation and the endothelial adhesion molecules activation, we wondered whether part of the beneficial effects of statins could be mediated by the modulation of SSAO/VAP-1 levels and/or activity. Among different statins, simvastatin was chosen because a meta-analysis study revealed that it induced the most potent effects in experimental stroke

models (Garcia-Bonilla *et al.* 2012, Campos-Martorell et al. 2014). Therefore, the protective effect of simvastatin was analyzed in two different animal models of stroke (eMCAO and tMCAO) and in human brain microvascular endothelial cells expressing human SSAO/VAP-1 subjected to oxygen and glucose deprivation (OGD).

Materials and methods

Animal experiments

All procedures were approved by the Ethics Committee of the Vall d'Hebron Research Institute and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union. Male Wistar rats (270-300 g; Charles River Laboratories Inc., Wilmington, MA, USA) were used in all experiments. Rats were kept in climate-controlled environment on a 12-hour light/12-hour dark cycle and food and water were available ad libitum. Analgesia (Buprenorphine, 0.05 mg/Kg s.c; Divasa Farma-Vic S.A) was given to all rats to minimize their discomfort.

MCAO models

Two different middle cerebral artery occlusion (MCAO) models were used in this study. In both protocols, animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories) in oxygen during the whole procedure and temperature was maintained at 37°C. The surgical exposure of the bifurcation of the external carotid artery and the internal carotid artery was performed on the right side. The embolic MCAO model (eMCAO) was induced through the injection of a clot as described previously (Zhang *et al.* 1997). Two clots (length: 1.5 cm; diameter: 0.3 mm) were inserted to occlude the MCA, and animals were allowed to recover from anesthesia immediately after the occlusion. The day before eMCAO surgery, arterial blood from a donor rat was withdrawn to form the clots. In the intraluminal transient MCAO model (tMCAO), infarction was induced by introducing an intraluminal filament as described in detail elsewhere (Garcia-Bonilla *et al.* 2011). A silicone-coated nylon monofilament (Doccol Corporation, reference number: 403723PK10) was inserted to occlude the MCA. Immediately after the occlusion, animals were allowed to recover from anesthesia. 90 minutes after occlusion, reperfusion of blood flow in the MCA was induced and, to that end, animals were re-anaesthetized and the filament was removed from the artery. In both models, cranial trepanation was performed the day before MCAO to attach a laser-Doppler probe (Moor Instruments) and continuously monitor regional cerebral blood flow during the surgery. Only animals who exhibited a reduction

of > 75% in cerebral blood flow during MCAO were included in the study. Sham-operated animals were submitted to the same processes except for the clot introduction in the eMCAO model, or the filament introduction in the tMCAO model. Three doses of analgesia were administered: after cranial trepanation, after MCAO surgery and at 24 h after it. All animals were euthanized 48 hours after the surgery.

Infarct volume evaluation

Infarct volumes were evaluated by an investigator blinded to the treatment. Infarct volumes were measured with thionin (eMCAO model) or 2,3,5- triphenyltetrazolium chloride (TTC, Sigma-Aldrich) (tMCAO model). For the thionin staining, animals were killed and transcardially perfused with paraformaldehyde 4% through an infusion pump. Brains were fixed in paraformaldehyde 4% during 24 h at 4°C, immersed in sucrose for another 24 h at 4°C, embedded in OCT and placed at -80°C. Coronal brain sections (30 μm) were cut in the cryostat and mounted on slides. Thionin staining was performed on slides: non-injured areas were stained in deep blue whereas ischemic regions remained pale. Images were captured using a CanoScan 4200F scanner (Canon USA Inc.,) and infarct size was quantified using an image analysis system (Scion Image v4.02, Scion Corporation). For the TTC staining, animals were killed and immediately perfused with heparin solution followed by saline solution, both injected with an infusion pump. Brains were removed and cut in sections (1 mm) and stained with TTC as described elsewhere (Bederson *et al.* 1986). TTC images were captured using a CanoScan 4200F scanner and infarct volumes were measured using Image J software by integration of infarcted areas. Infarct volume data were expressed as percentage of the ipsilateral hemisphere. Brain slices from TTC staining were divided into ipsilateral (IP) and contralateral (CL) hemispheres and kept frozen until further use.

Neurological evaluation

Rats were assessed using a 9-point neurological deficit scale (modified Bederson test), as previously described (Pérez-Asensio *et al.* 2005). Four consecutive tests were conducted: (i) spontaneous activity (moving and exploring = 0, moving without exploring = 1, no moving or moving only when pulled by the tail = 2); (ii) left drifting during displacement (none = 0, drifting only when elevated by the tail and pushed or pulled = 1; circling without displacement or spinning = 2); (iii) parachute reflex (symmetrical = 0; asymmetrical = 1; contralateral forelimb retracted = 2); (iv) resistance to left forepaw stretching (stretching not allowed = 0; stretching allowed after some attempts = 1; no resistance = 2). Neurological score was assessed in a blinded manner at 90 minutes, 24 and 48 h after MCAO.

Plasma and brain animal tissue samples

Frozen IP and CL hemispheres from each animal (tMCAO model) were homogenized separately with a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN_3, 1% Triton X-100, phenylmethanesulfonylflucride solution (PMSF, Sigma-Aldrich), Aprotinin (Apr, Sigma-Aldrich). Each gram of tissue was homogenized in 2.8 mL of the aforementioned buffer and centrifuged at 12 000 rpm for 12 minutes at 4°C. Homogenates were stored at -80°C until use. Blood samples were drawn and collected in EDTA tubes after MCAO treatments and before euthanasia, then immediately centrifuged at 1500 *g* for 15 minutes at 4ºC to obtain plasma supernatants, and stored at -80ºC until their use. The experimental group of eMCAO model was the same than that used in (Campos-Martorell et al. 2014), but only the plasma samples of these animals were available for using in the present study.

Cell culture

The human cerebral microvascular endothelial cell line hCMEC/D3 was obtained from Dr. Couraud's lab (Paris, France) (Weksler *et al.* 2005). The hCMEC/D3 cell line stably expressing the human SSAO/VAP-1 (hCMEC/D3 hSSAO/VAP-1) was generated as described (submitted manuscript). hCMEC/D3 cells were cultured as recommended, on 150 µg/mL collagen type I (Rat Tail, Corning) –coated plates in EBM-2 (Lonza) medium supplemented with 5% FBS (Fetal Bovine Serum, Life Technologies), 1.4 µM Hydrocortisone (Sigma), 5 µg/mL Ascorbic Acid (Sigma), 1% Chemically Defined Lipid Concentrate (Life Technologies), 10 mM HEPES (Life Technologies) and 1 ng/ml human bFGF (Fibroblast Growth Factor-basic, Sigma). Human SSAO/VAP-1-expressing human umbilical vein endothelial cells (HUVEC hSSAO/VAP-1) were previously developed in our group, and cultured as described (Sole & Unzeta 2011). THP-1 monocytic cells were obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell lysates and concentrated culture medium

Cells were collected and homogenized in 50 mM Tris-HCl (pH 7.5), containing 1% Triton X-100, 10 mM EDTA and protease inhibitors cocktail (Sigma) (1:100) and were sonicated for 10 seconds. To obtain concentrated culture medium samples, culture media were collected after cell treatments and centrifuged at 4400 *g* for 10 minutes to eliminate dead cells and debris. Then media samples were dried out by evaporation in

a Refrigerated CentriVap Concentrator (Labconco) and reconstituted in a smaller, known volume of distilled H_2O to obtain ten-fold concentrated culture medium.

OGD model and cell treatments

Combined oxygen and glucose deprivation (OGD) and reoxygenation have been used as an experimental approach to ischemic stroke. For HUVEC hSSAO/VAP-1 cells, OGD was carried out as previously described (Sun *et al.* 2014). For hCMEC/D3 hSSAO/VAP-1 cells, the OGD was performed in glucose-free DMEM (Life Technologies) after washing cells with glucose-free PBS, and then introducing the cells into a temperature-controlled (37±1°C) Invivo$_2$ hypoxia workstation (RUSKINN) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in DMEM (5 mM glucose) under normoxia conditions (5% CO_2/95% air). For reoxygenation treatment, cells that have undergone OGD were returned to normoxia conditions after replacing glucose-free DMEM by serum-free DMEM (5 mM glucose) and adding the same treatments present during OGD. In experiments analyzing the soluble SSAO/VAP-1 release, DMEM was not replaced for the reoxygenation period, but instead, glucose was added into OGD dishes.

For adhesion and cell viability assays, HUVEC hSSAO/VAP-1 were seeded at 53200 cells/mL, hCMECs were seeded at 50000 cells/mL, and both were grown for 48h before the addition of treatments. For immunoblot analysis, cells were seeded at 60000 cells/mL, and grown to 80% - 90% confluence before treatments. MA (methylamine), PLZ (phenelzine), or/and simvastatin (Simva) were added before OGD starting. In the reoxygenation process there were maintained the same concentrations of compounds present during OGD. Simvastatin used for cells was previously activated by opening the lactone ring. In short, 52.9 mg simvastatin were dissolved in ethanol (32.4%), distilled H_2O (61.5%), 0.1 M NaOH (6%), neutralized to pH 7.2 by 2 M HCl and incubated at 60°C for 1 hour to obtain a 10 mM stock.

Western blot analysis

Equal amounts of protein (20 µg/lane) determined by the Bradford method, or equal volumes of media were separated by SDS/PAGE and transferred onto nitrocellulose membranes following the standard procedure, as described before (Sun *et al.* 2014). The ImageJ software was used to quantify the western blot signal. The antibodies used were: rabbit anti-VAP-1 (abcam, 1:1000), rabbit anti-bovine SSAO (1:1000) (Lizcano *et al.* 1998), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Ambion-Invitrogen, 1:20000), rabbit anti-P-selectin (BioVision, 1:1000), rabbit anti-E-selectin (Santa Cruz, 1: 500), rabbit anti-VCAM-1 (Epitomics, 1:1000), rabbit anti-ICAM-1

(GeneTex, 1:1000), rabbit anti-NOS-2 (Santa Cruz, 1:1000), rabbit anti-COX-2 (Santa Cruz, 1:1000), goat anti-SOD-1 (Santa Cruz, 1:1000), rabbit anti-IκB-α (Santa Cruz, 1:1000), mouse anti-*β*-actin (Sigma–Aldrich, 1:5000), HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (BD Biosciences, 1:1000); HRP anti-mouse IgG (Dako, 1:1000), HRP anti-goat IgG (Pierce, 1: 2000).

Adhesion assays

THP-1 monocytes were labeled with 1 μM calcein-AM, and at the end of treatments, endothelial cells were incubated with the calcein-AM-labeled THP-1 cells (2.5×10^5 per well in 24-well plates) for 30 minutes at 37°C. Then, unbound monocytes were removed by turning over the plates onto absorbent paper, carefully adding FBS-free RPMI 1640 medium to the plates with an auto-pipette, and repeating the washing for at least three times. The fluorescence intensity was measured using a fluorescence microplate reader ($\lambda_{ex}/\lambda_{em}$: 495/530 nm). Results are represented as the percentage of fluorescence intensity, referring values to those obtained for the non-treated cells.

Statistical analysis

Results are given as mean ± SEM of experimental data from animals or independent experiments. The Gaussian distribution of each variable was checked by the Kolmogorov-Smirnov test. Statistical analysis was performed by one or two-way ANOVA tests followed by Newman-Keuls multiple comparison test. Correlations were tested by the Pearson correlation coefficient. A $p<0.05$ was considered to be statistically significant. Statistical analyses and graphic representations were obtained with the Graph-Pad Prism 6.0 software.

Results

Effect of simvastatin on the infarct volume and the neurological score in rats subjected to MCAO

Animals subjected to the eMCAO model showed a reduction of the infarct lesion with the treatment of simvastatin, measured 48 h after the induction of the ischemia, which was accompanied by a significant improvement in neurological behavior at this time (Campos-Martorell et al. 2014). On the other hand, in animals undergone tMCAO, simvastatin treatment induced only a trend to reduce the infarct volume (58.29 ± 3.84 % for Veh versus 53.55 ± 2.81 % for Simva ($p = 0.3289$) (Fig. 1a). In the same way, simvastatin treatment of tMCAO-subjected animals did not induce significant differences in the neurological score, evaluated after 24 h or 48 h (Fig. 1b), although

the score mean values tended to be lower for simvastatin-treated groups at 48 h post-occlusion (4 ± 0.6 for Veh vs. 3.6 ± 0.5 for Simva, p = 0.7008).

Simvastatin suppresses the plasmatic release of SSAO/VAP-1 in rats subjected to two different MCAO models: eMCAO and tMCAO

It was analyzed whether simvastatin treatment could modify the release and/or the expression of SSAO/VAP-1 in rats subjected to transient MCAO inserting a nylon filament (tMCAO) or to embolic MCAO (eMCAO). Western blot analysis of SSAO/VAP-1 in plasma of vehicle-treated animals showed that both MCAO procedures induced an increase of the soluble SSAO/VAP-1 that was significant only in the eMCAO model (Fig. 2a). Interestingly, the simvastatin treatment induced a significant reduction in the plasmatic levels of SSAO/VAP-1 in both MCAO models without changes in the sham animals (Fig. 2a). However, neither the tMCAO surgery, nor the simvastatin treatment induced differences in the levels of the brain membrane-bound SSAO/VAP-1 (Fig. 2b), showing similar protein levels in all samples.

Soluble SSAO/VAP-1 levels in plasma of rats subjected to tMCAO or eMCAO correlate with infarct volume

The amount of soluble SSAO/VAP-1 present in plasma of rats undergone tMCAO (Fig. 3a) or eMCAO (Fig. 3b) shows a significant positive correlation with the infarct volume (Pearson's r=0,6031, p=0,0134*; Pearson's r=0,8084, p=0,0152*, respectively). Thus, when higher soluble SSAO/VAP-1 levels were observed in plasma, higher infarct volumes were detected in both MCAO models. In addition, when considering vehicle and simvastatin-treated animals from both MCAO models separately, the correlation between soluble SSAOVAP-1 and the infarct volume resulted to be significantly different only in case of vehicle-treated animals, and simvastatin treatment abolished this correlation.

Simvastatin treatment attenuates the expression of P-selectin and E-selectin in the brain of rats subjected to tMCAO

The adhesion molecules P- and E-selectin mediate the initial steps of the leukocyte adhesion cascade during inflammation, and their expression has been shown to be dependent on SSAO catalytic activity (Jalkanen *et al.* 2007). Therefore, these adhesion molecules were analyzed by western blot in brains of vehicle and simvastatin-treated sham and tMCAO-subjected rats (Fig. 4). Sham animals did not show changes in the P-selectin protein levels. However, overall trend to increase was observed in both brain sides aftert MCAO, where simvastatin treatment tended to lower, especially in the IP

brain side (Fig. 4b). E-selectin levels did not show significant differences among the treatments in sham animals. By contrast, E-selectin was increased in the CL side of the tMCAO-subjected brains without substantial variation in the occlusion side (IP). Simvastatin treatment showed a significant reduction of this tMCAO-induced E-selectin augment, maintaining a moderate difference between IP and IL brain sides in these animals (Fig. 4c). Two-way ANOVA analysis revealed a significant effect of both variables (tMCAO, $p<0.0001$***; simvastatin, $p=0.0015$**).

Effects of simvastatin on the release and protein levels of the inflammatory proteins VCAM-1 and ICAM-1 in MCAO-subjected animals

Other adhesion molecules such as the vascular cell adhesion molecule 1 (VCAM-1) and the intercellular adhesion molecule 1 (ICAM-1) play also an important role on the inflammation induced by a stroke insult. Together with VAP-1, they are responsible of the transendothelial migration of leukocytes to the injured tissues during inflammation. Both adhesion molecules are shed from the membrane, generating soluble forms that are correlated with several pathologies. The presence of soluble VCAM-1 in the plasma of rats undergone tMCAO or eMCAO was determined by western blot analysis. Results revealed a significant decrease of soluble VCAM-1 levels in simvastatin-treated animals undergone tMCAO, whereas no changes were observed in eMCAO-subjected animals (Fig. 5a). When the levels of membrane-bound VCAM-1 were analyzed in brain samples, it was observed a significant increase of this protein in the CL side of rats undergone tMCAO surgery, without changes in the infarcted side (Fig. 5b). This increase was prevented by the simvastatin treatment. Again, two-way ANOVA analysis revealed a significant effect of both variables (tMCAO, $p<0.0001$***; simvastatin, $p=0.0225$*). By contrast, neither the tMCAO surgery, nor the simvastatin treatment induced changes in the levels of the membrane-bound ICAM-1 protein.

Simvastatin down-regulates the levels of COX-2, NOS-2, SOD-1 and up-regulates the expression of IκB-α in brain of rats undergone tMCAO

One of the simvastatin-reported action mechanisms is the inhibition of the NF-κB pathway (Falcone *et al.* 2013). NF-κB regulates the expression of proteins involved in cell adhesion, immune and inflammatory responses. Cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (NOS-2) and superoxide dismutase (SOD-1), which are involved in inflammation and ROS generation under stroke conditions (Orrenius *et al.* 2003, Moro *et al.* 2004), are induced by NF-κB activity. Thus, these proteins were analyzed in tMCAO animals and those treated with simvastatin. No differences in any of these proteins were observed in sham animals treated with simvastatin. However, a

significant increase in COX-2 protein was detected in the stroked side of tMCAO-subjected animals, which was partially prevented with the simvastatin treatment (Fig. 6b). Similar trends were observed in NOS-2 (Fig. 6c) and SOD-1 (Fig. 6d) but changes detected in these proteins were less significant (Sham Veh IP, 0.731 ± 0.07 vs MCAO Veh IP 0.823 ± 0.03, p = 0.266 (n.s.); MCAO Veh IP 0.823 ± 0.03 vs MCAO Simva IP 0.668 ± 0.02, p = 0.0028**, for NOS-2)(Sham Veh IP, 0.992 ± 0.009 vs MCAO Veh IP 1.205 ± 0.06, p = 0.016*; MCAO Veh IP 1.205 ± 0.06 vs MCAO Simva IP 1.028 ± 0.03, p = 0.023*, for SOD-1). On the other hand, the levels of the NF-κB inhibitor IκB-α were determined in these animals, and results showed a significant increase of this protein in the IP brain side of tMCAO-undergone animals (Fig. 6e). This augment was even more significant in both brain sides of tMCAO simvastatin-treated rats (Sham Veh IP, 0.609 ± 0.11 vs MCAO Veh IP 1.005 ± 0.03, p = 0.002**; MCAO Veh IP 1.005 ± 0.03 vs MCAO Simva IP 1.117 ± 0.02, p = 0.033*), whereas no changes were observed in sham animals.

Simvastatin alleviates the OGD-induced soluble SSAO/VAP-1 release in brain endothelial SSAO/VAP-1-expressing cells.

In order to confirm that the vascular system was determinant in the effects of simvastatin observed in brains of rats subjected to MCAO, we studied also its effects in a brain endothelial cell model subjected to an *in vitro* experimental model of ischemia. We used a cellular model of human brain endothelial cells stably expressing SSAO/VAP-1 (hCMEC/D3 hSSAO/VAP-1) generated in our group (submitted manuscript) subjected to oxygen and glucose deprivation (OGD, 16 h) plus reoxygenation (Reox, 24 h). Under these experimental conditions, the levels of soluble and membrane- bound SSAO/VAP-1 were analyzed in the absence or presence of simvastatin. Results showed a significant increased levels of soluble SSAO/VAP-1 released to the culture media of cells undergone OGD+Reox compared to normoxic cells, whereas simvastatin treatment completely abolished this cell response (Fig. 7a). This effect was corroborated by the analysis of the membrane-bound protein in the same cells, where it was observed a reduced presence of membrane-bound SSAO/VAP-1 after OGD+Reox as a consequence of its release, which was partially prevented by the simvastatin treatment (Fig.7b).

Leukocyte binding to endothelial cells induced by OGD and SSAO/VAP-1 activity is prevented by simvastatin.

In order to determine whether the anti-inflammatory behavior of simvastatin had any effect on the SSAO/VAP-1-dependent leukocyte adhesion, this activity was analyzed in endothelial cells subjected to OGD in the presence of the SSAO physiological substrate, methylamine (MA). Two different endothelial models were used, the one from human brain origin (hCMEC/D3) and a human umbilical vein endothelial cell line (HUVEC) that was previously described (Sole & Unzeta 2011). Results showed that brain endothelial cells bound more leukocytes than HUVEC cells after 5 h of OGD (Fig. 8). Moreover, as previously described (Sun et al. 2014); submitted manuscript), the catalytic activity of SSAO/VAP-1 on methylamine as substrate induces the leukocyte binding to both endothelial cell types, and this effect is potentiated under OGD conditions. No significant differences were observed in wild type cells non-expressing the SSAO/VAP-1, indicating the relevance of this protein in the adhesion process. In addition, simvastatin treatment prevented this SSAO/VAP-1-mediated leukocyte adhesion induction both in normoxia and in OGD conditions. Given the fact that the catalytic activity of SSAO participates in its adhesion function (Salmi *et al.* 2001), the obtained results pointed to the possibility that simvastatin would act as SSAO enzymatic inhibitor. However, results indicated no inhibitory effect of simvastatin on SSAO enzymatic activity (supplementary material S1a. In the same line, simvastatin was not able to prevent the endothelial cell death induced by the SSAO-dependent methylamine metabolism under OGD conditions in HUVEC or brain endothelial cells (supplementary material S1b, c), or to act as a scavenger of the toxic products generated during its enzymatic activity (hydrogen peroxide, H_2O_2 or formaldehyde, FA) (supplementary material S1d, e).

Effects of simvastatin on the expression of inflammatory adhesion proteins in brain endothelial cells subjected to OGD with reoxygenation

In order to study whether besides SSAO/VAP-1, other adhesion molecules involved in the inflammation process could participate in the leukocyte adhesion induced by OGD and reoxygenation, and if they are as well affected by simvastatin, the levels of P- and E-selectins, VCAM-1 and ICAM-1 were determined in SSAO/VAP-1-expressing hCMEC/D3 cells subjected to OGD and reoxygenation. Results showed first that OGD and reoxygenation induced no change in P-selectin but reduced levels of E-selectin, VCAM-1 and ICAM-1 (Fig. 9). This reduction was more pronounced in the case of VCAM-1 and ICAM-1 proteins, in the same line that it was previously observed with SSAO/VAP-1 (Fig. 7). With the simvastatin treatment, P-selectin decreased after OGD+Reox, while no changes were observed without simvastatin (Fig. 9b). In the case of E-selectin, although the previously observed slight reduction induced by OGD+Reox

was maintained, the overall protein levels were diminished with the simvastatin treatment (Fig. 9c). The same behavior was detected for VCAM-1, which overall significantly decreased after simvastatin treatment but still maintaining the decrease induced by OGD+Reox (Fig. 9d). In case of ICAM-1, while OGD+Reox induced a reduction of the protein levels, no changes were observed as a response to OGD+Reox in the presence of simvastatin, which also significantly reduced the ICAM-1 levels in normoxia cells (Fig. 9e).

Simvastatin influences as well the levels of COX-2, NOS-2, SOD-1 and IκB-α in brain endothelial cells undergone OGD and reoxygenation.

In order to confirm the effects observed in animal brains subjected to tMCAO on some of the NF-κB expression-dependent proteins, COX-2, NOS-2 and SOD-1 were analyzed in our endothelial cell model. In SSAO/VAP-1-expressing hCMEC/D3 cells, OGD+Reox induced an increase in the levels of COX-2; this effect was maintained in the presence of simvastatin, although this treatment induced a significant reduction of COX-2 in both normoxia and OGD+Reox conditions (Fig. 10b). NOS-2 also increased after OGD+Reox, but this effect was completely prevented by the simvastatin treatment (Fig. 10c). By contrast, no significant changes were observed in the levels of SOD-1 after the OGD+Reox insult or by the simvastatin treatment (Fig. 10d). On the other hand, when the levels of the NF-κB inhibitor IκB-α were analyzed in endothelial cells, it was observed that OGD+Reox induced an increase of this protein. Simvastatin treatment in this case augmented the IκB-αlevels in normoxia conditions, and OGD+Reox induced a higher increase in this protein than that observed without the simvastatin presence.

Discussion

In this study we investigate whether the beneficial effects of simvastatin on stroke could be mediated by any modulation of the pro-inflammatory SSAO/VAP-1 activity or its levels by using "*in vitro*" and "*in vivo*" experimental approaches. In our results, the plasmatic form of SSAO/VAP-1 increased after the ischemia induction by two different models, as we previously observed in human samples (Hernandez-Guillamon et al. 2010), and it correlated as well with the infarct volume. The simvastatin-induced prevention of the soluble SSAO/VAP-1increase in MCAO animals and also in the OGD-subjected endothelial cells expressing SSAO/VAP-1 could be related with the described action of statins on the abolishment of the stroke-induced matrix

metalloproteinases (MMPs) up-regulation (Wang *et al.* 2006), which is associated to the BBB disruption cerebral ischemia (Montaner *et al.* 2003, Hernandez-Guillamon *et al.* 2012a). These variations are not shared in the same extent by the other adhesion molecules, but they seem specific for SSAO/VAP-1. In case of VCAM-1, for example, its soluble levels slightly decreased in simvastatin-treated tMCAO animals, while no changes were observed in the eMCAO model. By contrast to the results observed with the soluble SSAO/VAP-1, where even higher differences were detected in the eMCAO model, this may indicate a different pathway activation depending on the infarct insult or that VCAM-1 needs a stronger ischemic damage than SSAO/VAP-1 to be activated, since the infarct volumes induced by the tMCAO model are bigger than those induced by the eMCAO model.

Despite the decrease in the soluble SSAO/VAP-1 induced by simvastatin, no differences were observed on its membrane-bound form in animal samples. This may be explained because the levels of soluble SSAO/VAP-1 in plasma under physiological conditions are low, thus small amount differences are easier to detect there than in the highly present membrane-bound form. Alternatively, an increase of its expression *in vivo* that would compensate the loss of the released form cannot be ruled out. Actually, SSAO/VAP-1 expression can be regulated by NF-κB, which becomes activated after ischemia (Bono *et al.* 1998). In this case, the membrane-bound form reduction observed in the cell line would be explained by the lack of ability of this cell model to up-regulate the expression of the protein, in which a constitutive promoter induces its expression. Anyway, the effect of simvastatin would be beneficial in both cases by reducing the release of this enzyme that is related with inflammation processes. This is evidenced by the simvastatin-induced reduction of the leukocyte adhesion to the SSAO/VAP-1-expressing brain endothelial cells undergone OGD. In this regard, this anti-inflammatory effect may not be mediated by a direct action of simvastatin on the SSAO/VAP-1 function because it does not behave as inhibitor of its catalytic activity.

By contrast, it could be mediated by the inhibition of the SSAO/VAP-1-dependent expression of other adhesion molecules. It is known that the oxidase activity of SSAO/VAP-1 is able to induce the expression of other pro-inflammatory proteins and hence to promote leukocyte binding, as a cross-talk system between the adhesion molecules involved in tethering and rolling of leukocytes in inflammation. In this regard, the expression of E- and P-selectins, VCAM-1 and ICAM-1 can be modulated by SSAO/VAP-1 activity, although this effect on VCAM-1 and ICAM-1 has only been found in hepatic endothelial cells so far (Jalkanen *et al.* 2007, Lalor *et al.* 2007). Our results show similar results for E-selectin and VCAM-1, which expression increased in the

contralateral side of the tMCAO-subjected animals, while no changes were observed in the ischemic side. Simvastatin treatment prevented this contralateral protein increase as well as reduced the overall expression of these adhesion molecules. Similar trends were observed for P-selectin and ICAM-1, although changes were subtler in these proteins. These differences may be explained by a different regulation of these molecules, for example E-selectin is an inducible protein whereas P-selectin is constitutively present in membranes of endothelial cells (Frijns & Kappelle 2002). Interestingly, the changes observed in these molecules are strongly observed in the contralateral brain sides, especially for E-selectin and VCAM-1, which suggests us that this effect responds to a traveling signal from the infarcted area. These results together with the fact that these proteins are inducible by the SSAO/VAP-1 activity let us think that the release of SSAO could be the courier and booster of the ischemic inflammatory stimulus to the contralateral brain. Moreover, the up-regulation of these proteins in ischemia is prevented by the simvastatin treatment, which is compatible with the block of the SSAO/VAP-1 release to the circulation that would prevent the soluble molecule to reach the contralateral brain.

Considering the previous hypothesis, these changes would be difficult to measure in the cell culture system because it is all the tissue that receives the ischemic stimulus in this model, and there are no travelling signals towards a non-ischemic area. Actually, the alterations on the expression of these pro-inflammatory proteins are less evident in the endothelial cell line model. However, a simvastatin-mediated overall reduction of the expression of these adhesion molecules is also observed. The OGD and reoxygenation stimulus induces a reduction in the protein levels of E-selectin, VCAM-1 and ICAM-1 that is only prevented by simvastatin in case of ICAM-1. This decrease could result from the release of the protein to the culture medium, which is difficult to analyze in some cases due to the low expression levels of some of these proteins. In addition, differences in the regulation of the release and expression of these and other adhesion molecules that have been described among species may be a cause for these small differences in the effects observed between rat samples and human endothelial cells (Yao *et al.* 1999); for example, SSAO/VAP-1-dependent action in human endothelia increases the E-selectin release and the P-selectin expression, which are the effects that we observe in our cell line, but they occur in the opposite way in mice (Jalkanen et al. 2007). Other regulatory signals coming from neighboring cell types cannot be ruled out, and these ones could not be assessed using our cell model.

All the previous mentioned adhesion molecules are target genes of the transcription factor NF-κB, which simvastatin has shown to inhibit. In order to investigate the NF-κB-

dependent transcriptional activity and the simvastatin effects on it in our models, we analyzed others of its target genes. COX-2 is a pro-inflammatory mediator overproduced after ischemia that contributes to the oxidative stress by generating superoxide (Iadecola & Alexander 2001). In the same line, NOS-2 increases under stroke, and is the source of large amounts of NO in these conditions (Moro et al. 2004). On the other hand, the expression of the antioxidant SOD-1 trends to decrease at long times after ischemia (Ciancarelli *et al.* 2015). All them have been found modulated by statins action on NF-κB (Tu *et al.* 2014, Moro et al. 2004, Kumagai *et al.* 2004). In our results, these proteins behaved in a similar way, being up-regulated by ischemia and this effect was prevented by simvastatin. Interestingly, by contrast to the adhesion proteins previously analyzed, the up-regulation of these factors was observed in the IP brain side. In addition, the results observed with COX-2 and NOS-2 were much more consistent between the animal and the cell line models than those observed with VCAM-1 and the others previously analyzed. These results suggest us that changes of these proteins are more related to local signals, and therefore are observed in the cell line subjected to ischemia. Thus, the soluble SSAO/VAP-1 may not be related to them, although it could be its membrane-bound form.

Finally, the confirmation of the involvement of the NF-κB activity to the simvastatin-mediated actions in our models is derived from results obtained analyzing the IκB-α, an endogenous repressor of the NF-κB transcriptional activity. This molecule is up-regulated in the IP brain side as well as in endothelial cells after ischemia, probably as a response to the NF-κB activation, and simvastatin treatment increases its expression both in the basal levels and in response to ischemia, thus resulting in a major inhibition of the NF-κB pro-inflammatory signaling.

Taking together all these results, they suggest that part of the beneficial effect of simvastatin in both experimental models is mediated by a reduction of the inflammatory process. The coincidence between the results obtained from animal samples and those observed in cell lines highlights the relevance of the vascular system in the process. Also, it indicates that the lower levels of soluble SSAO/VAP-1 detected with the simvastatin-treated animals are not a result of a previous improvement induced by simvastatin but to a direct blocking of its shedding, as it is revealed from the studies with the isolated endothelial cells. Moreover, our results suggest that the inflammation after an ischemic stimulus is differently regulated in the local infarcted area or in the contralateral brain side. In this aspect, the major change that simvastatin induced on SSAO/VAP-1 protein was the inhibition of its release. This effect correlated with the

prevention in the increase of other adhesion proteins on the contralateral side that can be mediated by SSAO/VAP-1 catalytic activity. Therefore, we believe that upon stroke, SSAO/VAP-1 is released as a soluble form to travel along the circulation and reach non-infarcted brain areas, where it induces inflammation, amplifying the damage directly and locally induced by the ischemic process. Although simvastatin did not behave as inhibitor of the catalytic activity of SSAO/VAP-1, we cannot rule out that it could also interfere with its leukocyte adhesion function by other mechanisms, as it was observed in the endothelial cell model, for example obstructing the binding of SSAO/VAP-1 with Siglec-10, its counter-receptor on leukocytes (Kivi *et al.* 2009). We cannot discard also the participation of the membrane-bound SSAO/VAP-1, which deserves further studies, although this could be more related to the site of the infarct. Also, other simvastatin-mediated described mechanisms such as the inhibition of the NF-κB activity would contribute to its overall beneficial effects.

Inhibitors of SSAO/VAP-1 activity have shown already an improvement in animal stroke models (Hernandez-Guillamon et al. 2010, Ma *et al.* 2011, Watcharotayangul et al. 2012, Xu *et al.* 2015), where the SSAO/VAP-1 activity inhibition would not only prevent the leukocyte binding but also the release of aldehydes, hydrogen peroxide and ammonia resulting from its enzymatic activity, which when overproduced can contribute to the oxidative stress and the disease progression (Yu & Deng 1998, Conklin *et al.* 1998, Hernandez *et al.* 2006, Solé *et al.* 2008). Thus, a possible summation effect of both simvastatin and SSAO/VAP-1 inhibitors treatment could enhance their beneficial effects on the pathology development.

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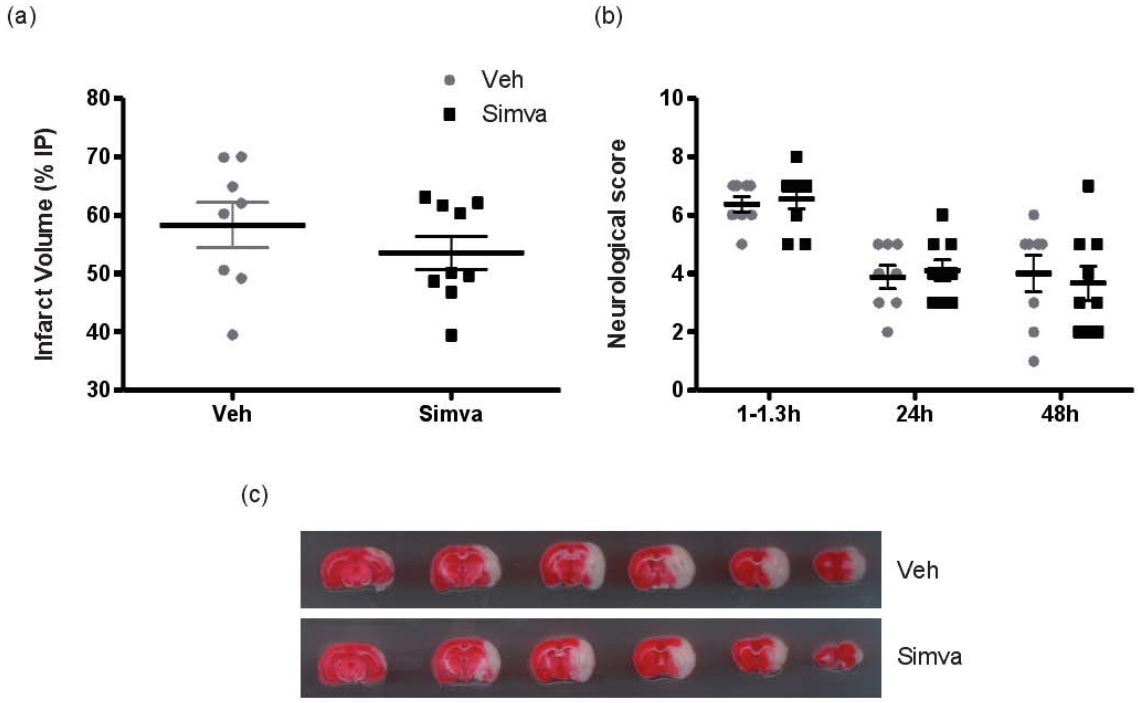


Fig. 1 Simvastatin effects on the infarct volume and neurological score of tMCAO animals. (a) Infarct volume of vehicle (Veh) and simvastatin (Simva) -treated animals represented as percentage of the ipsilateral brain side (% IP). (b) Neurological test assessment in tMCAO-subjected animals in the first 90 min (1-1.3 h), at 24 h and 48 h after the ischemia. Values are individually represented, with the mean ± SEM indicated for each group. (c) Representative images of TTC staining.

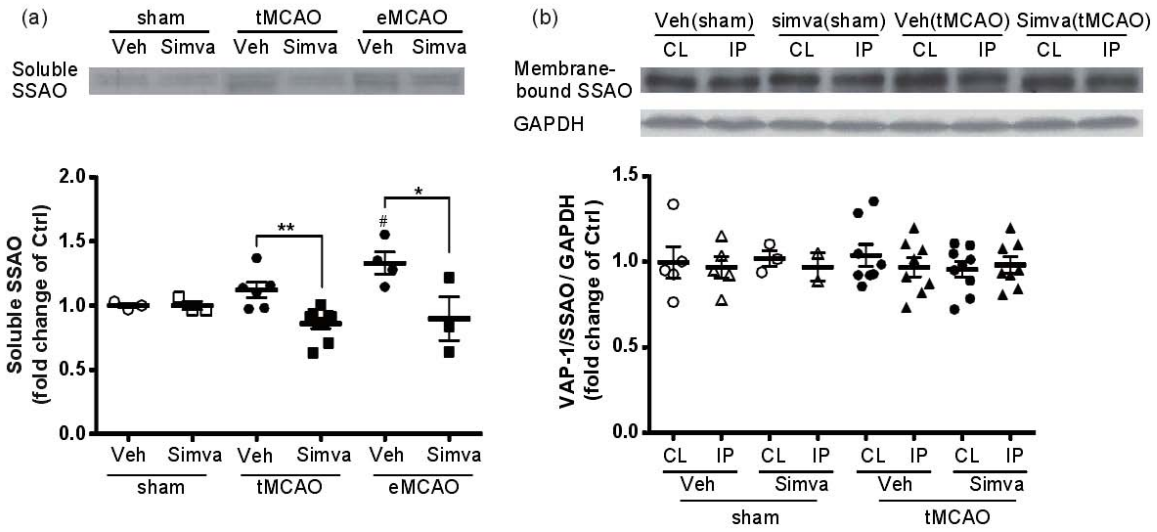


Fig. 2 Simvastatin suppresses the release of soluble SSAO/VAP1 into the blood plasma of rats subjected to tMCAO or eMCAO. (a) Soluble SSAO/VAP-1 levels in plasma of vehicle (Veh) and simvastatin (Simva) -treated rats subjected to tMCAO or eMCAO. (b) Levels of membrane-bound SSAO/VAP-1 in the contralateral (CL) and ipsilateral (IP) brain tissue sides of Veh and Simva-treated rats undergone tMCAO. The presence of membrane-bound SSAO/VAP-1 protein in tissue was normalized to the GAPDH levels. Veh-treated plasma samples and contralateral brains from sham animals were considered as control (Ctrl) for (a) and (b), respectively. Data in graphs represent the western blot quantifications and are expressed as mean ± SEM; Veh, n = 5 (3 for plasma) and Simva, n = 3 for the sham animals; Veh, n = 8 (6 for plasma) and Simva, n = 9 for animals subjected to tMCAO; Veh, n = 4 and Simva, n = 3 for animals subjected to eMCAO. *p<0.05 and **p<0.01 as indicated; $^{\#}p$<0.05 versus the corresponding sham group, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

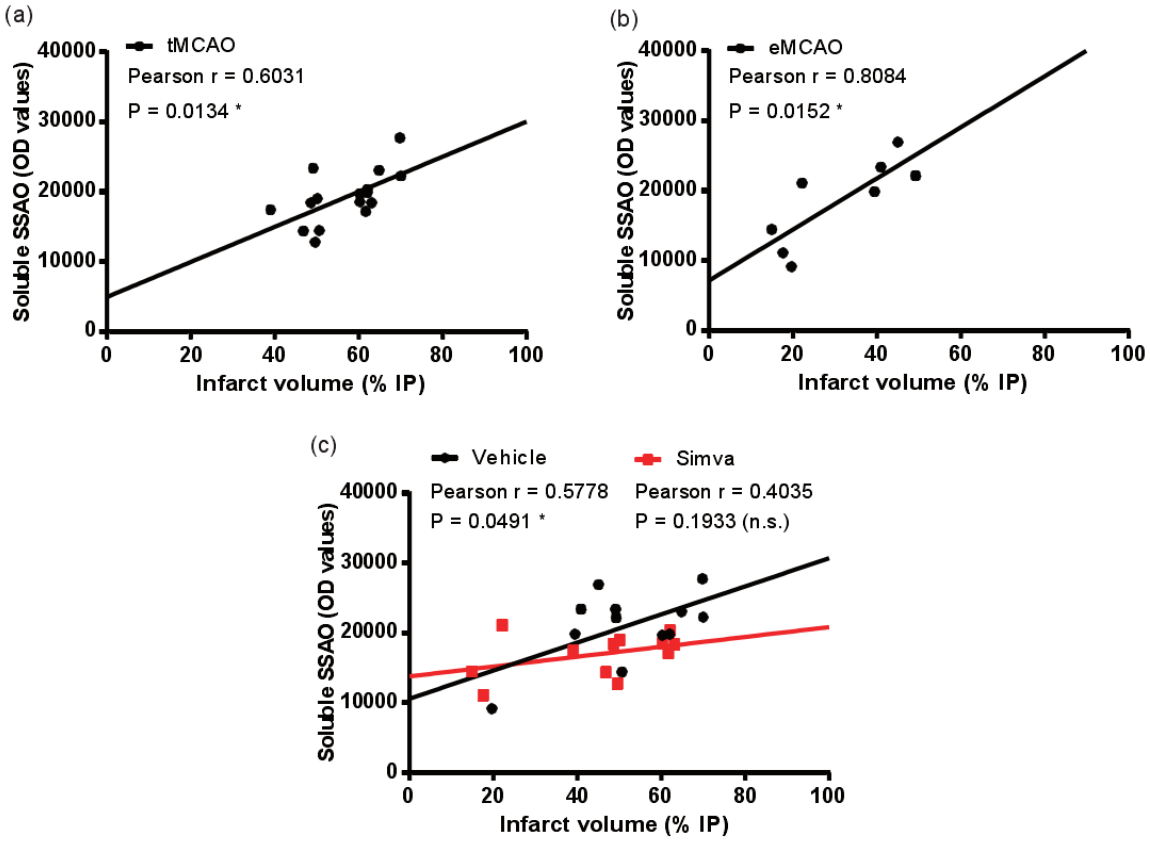


Fig. 3 Correlations between infarct volume and soluble SSAO/VAP-1 levels present in the blood plasma of rats subjected to MCAO. Correlations between infarct volume and soluble SSAO/VAP-1 in the (a) tMCAO model and (b) eMCAO model, and (c) vehicle or simvastatin-treated plasma samples of both tMCAO and eMCAO models. Data of soluble SSAO/VAP-1 is obtained from the western blot quantifications; n = 16 in (a); n = 8 in (b); vehicle, n = 12, simvastatin, n = 12 in (c). Pearson r coefficient and *p* value is shown for each correlation.

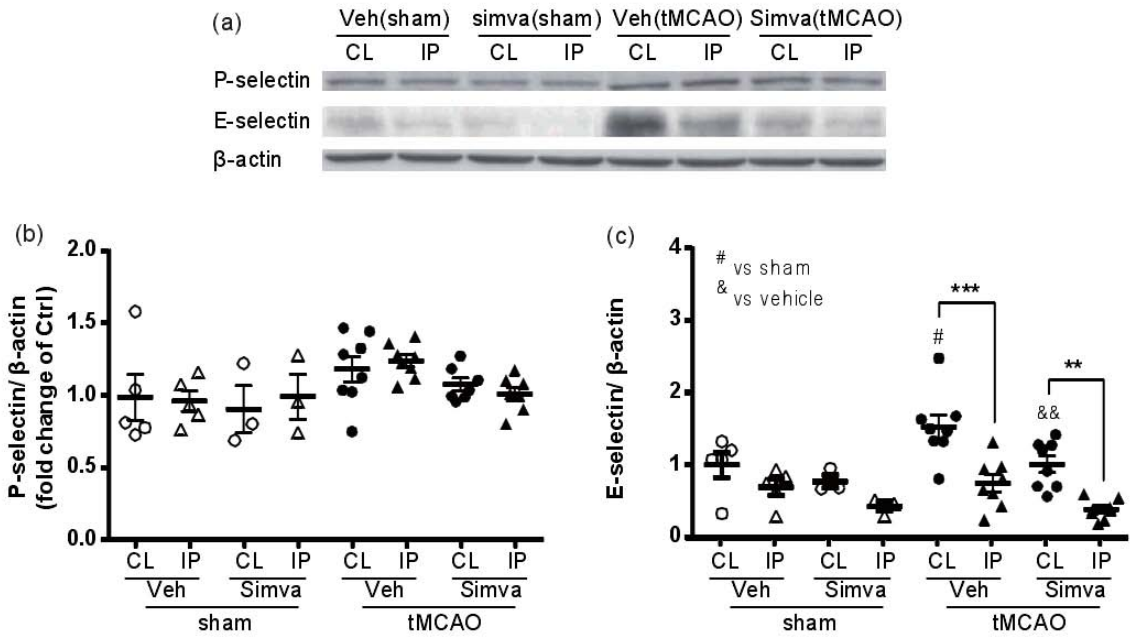


Fig. 4 Simvastatin reduces the levels of P- and E-selectins in brains of rats subjected tMCAO. (a) Representative western blot images of (b) P-selectin, and (c) E-selectin quantifications in the contralateral (CL) and ipsilateral (IP) brain sides of tMCAO-subjected rats treated with vehicle (Veh) or simvastatin (Simva). The presence of P- and E-selectin was normalized to the β-actin levels. Veh-treated CL brain sides from sham animals were considered controls (Ctrl). Data are plotted for each sample, and the mean ± SEM is indicated; Veh, n = 5, Simva, n = 3 for sham animals; Veh, n = 8, Simva, n = 8 for animals undergone tMCAO. $^{**}p<0.01$ and $^{***}p<0.001$ as indicated; $^{\#}p<0.05$ versus the homologous sham group; $^{\&\&}p<0.01$ versus the corresponding vehicle-treated group, by a two-way ANOVA (tMCAO and simvastatin treatment) and the addition of Newman-Keuls multiple comparison test.

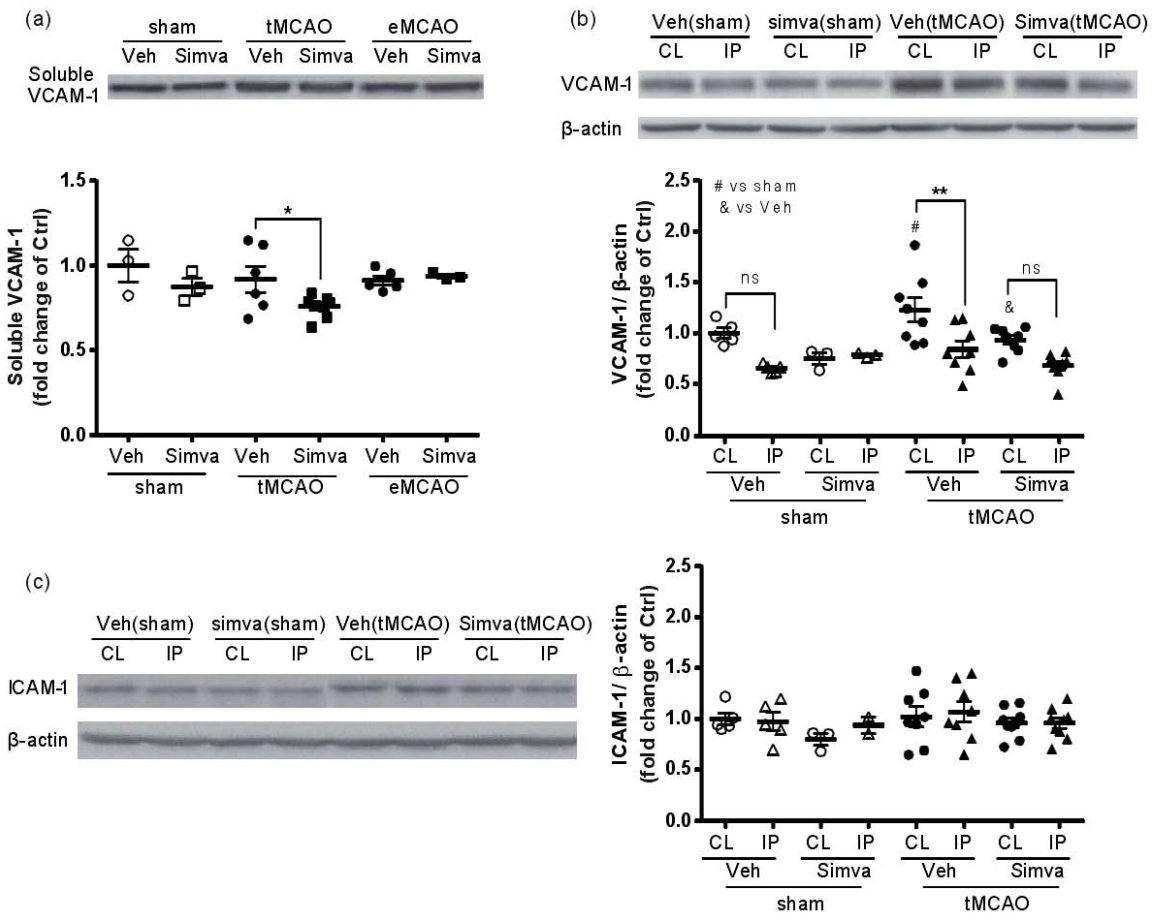


Fig. 5 Effects of simvastatin on the brain levels of VCAM-1 and ICAM-1, and the release of soluble VCAM-1 to the plasma, in rats subjected to MCAO. (a) Soluble VCAM-1 in the plasma of vehicle (Veh) and simvastatin (Simva)-treated rats undergone tMCAO or eMCAO. (b) VCAM-1 and (c) ICAM-1 protein levels in the CL and IP sides of Veh and Simva-treated rats brains subjected to tMCAO. VCAM-1 and ICAM-1 in the brains were normalized to the β-actin levels. Plasma samples from veh-treated rats and CL brain sides from veh-treated sham animals were considered as Ctrl for (a) and (b, c), respectively. Data in graphs represent the western blot quantifications. Values are plotted individually for each animal, and the mean ± SEM is indicated in each experimental group. Veh, n = 5 (3 for plasma), Simva, n = 3 for the sham animals; Veh, n = 8, Simva, n = 9 for tMCAO-subjected animals; Veh, n = 4, Simva, n = 3 for eMCAO-subjected animals. $^{**}p<0.01$ as indicated; $^{\#}p<0.05$ versus the corresponding sham group; $^{\&}p<0.05$ versus the homologous vehicle-treated group; ns, non significant, by a two-way ANOVA (MCAO and simvastatin) and the addition of Newman-Keuls multiple comparison test.

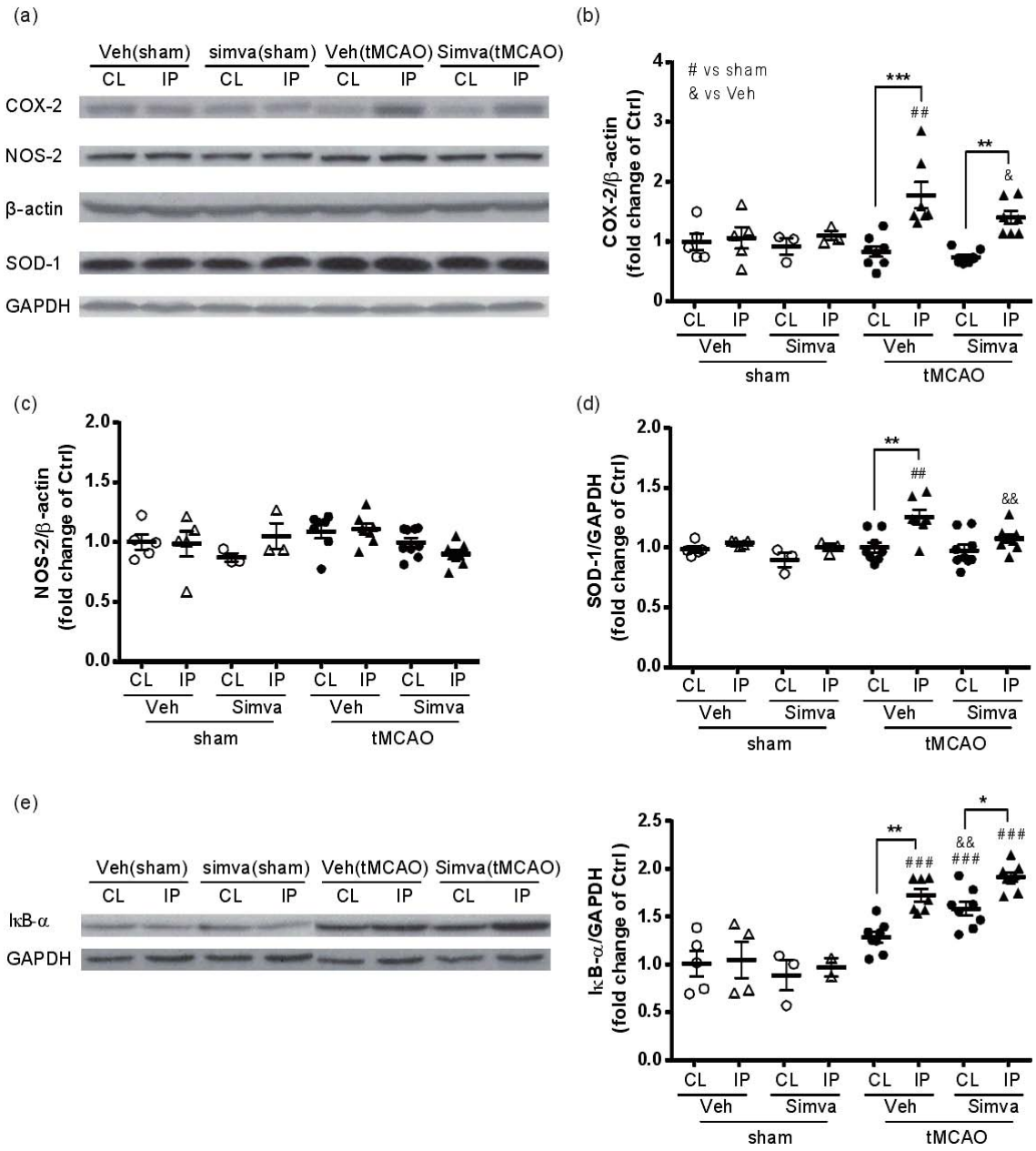


Fig. 6 Simvastatin down-regulates the levels of COX-2, NOS-2, SOD-1 and up-regulates the expression of IκB-α in the brains of rats subjected to tMCAO. (a) Representative western blot images of the analyzed proteins. (b) Quantifications of COX-2, (c) NOS-2 and (d) SOD-1 in the CL and IP sides of vehicle (Veh) and simvastatin (Simva)-treated rat brains subjected to tMCAO. (e) Western blot images and their quantifications are also shown for IκB-α under the same experimental conditions. COX-2 and NOS-2 in the brains were normalized to the β-actin levels. SOD-1 and IκB-α in the brains were normalized to the GAPDH levels. Veh-treated CL sides from sham animals were considered as Ctrl. Data are plotted individually showing the mean ± SEM for each experimental group; Veh, n = 5, Simva, n = 3 for sham animals; Veh, n = 8, Simva, n = 9 for tMCAO-undergone animals. *p<0.05, **p<0.01

and ***p<0.001 as indicated; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 versus the corresponding sham group; $^{\&}p$<0.05 and $^{\&\&}p$<0.01 versus the homologous vehicle-treated group, by a two-way ANOVA (MCAO and simvastatin) and the addition of Newman-Keuls multiple comparison test.

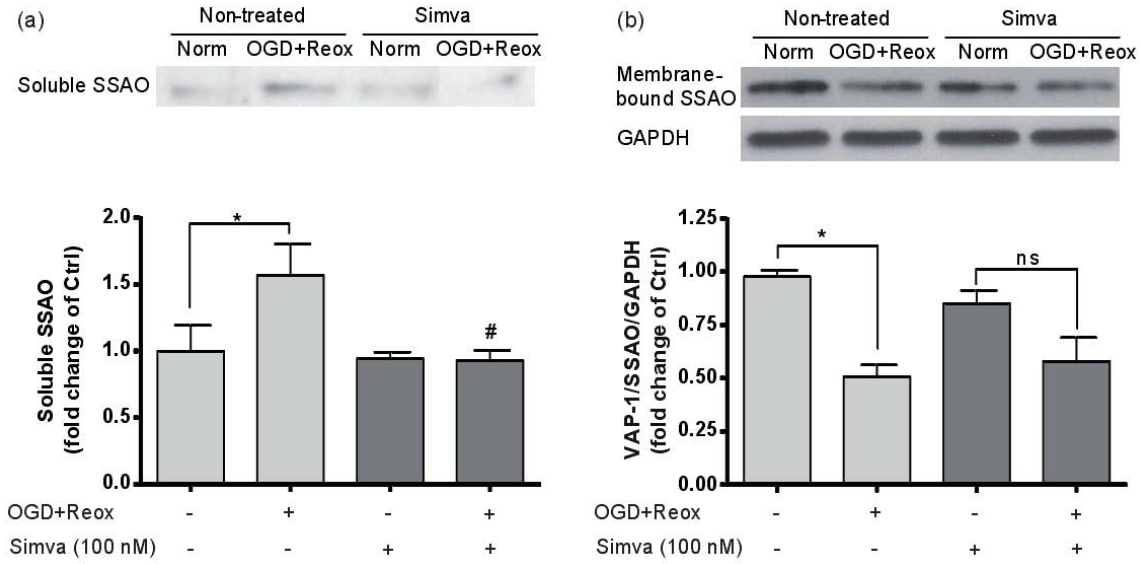


Fig. 7 Simvastatin alleviates the OGD-induced release of soluble SSAO/VAP-1 to the culture media by brain endothelial SSAO/VAP-1-expressing cells. (a) Presence of soluble SSAO/VAP-1 in ten-fold concentrated culture media of Simva (100 nM) -treated or not hCMEC/D3 hSSAO/VAP-1 cells under normoxia or oxygen-glucose deprivation (OGD, 16h) with reoxygenation (Reox, 24h) (OGD+Reox). (b) Presence of membrane-bound SSAO/VAP-1 in hCMEC/D3 hSSAO/VAP-1 cell lysates under the same previously analyzed experimental conditions. The presence of membrane-bound SSAO/VAP-1 was normalized to the GAPDH levels. Non-treated cells or media under normoxia conditions were considered as Ctrl. Data in graphs represent the western blot quantifications and are expressed as mean ± SEM from 3 independent experiments. *p<0.05 as indicated; $^{\#}p$<0.05 versus non-treated OGD+Reox media; ns, non significant, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

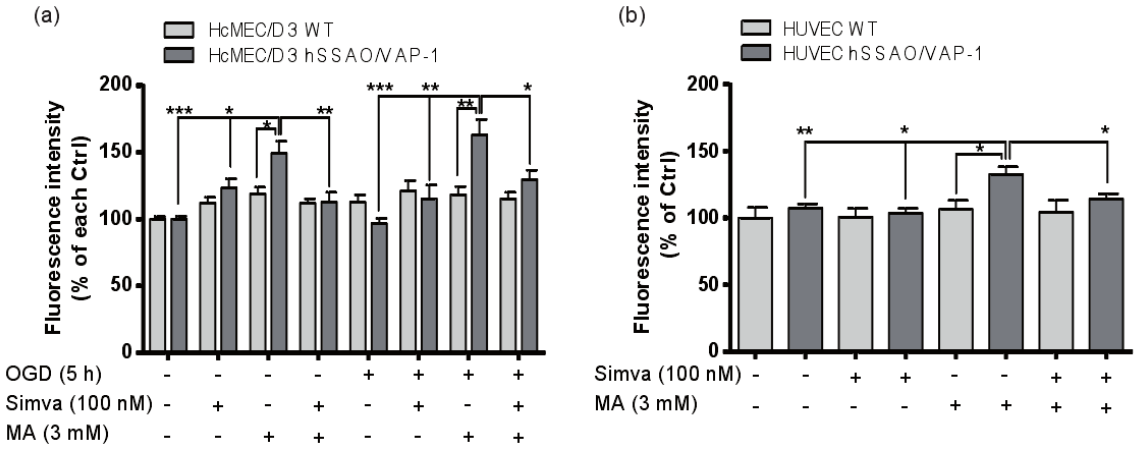


Fig. 8 Simvastatin prevents the leukocyte binding to endothelial cells induced by OGD in presence of the SSAO substrate. Leukocyte-endothelium adhesion assay was performed to analyze the anti-inflammatory effect of simvastatin against SSAO mediated pro-inflammatory action. It was quantified the binding of calcein-AM-labelled THP-1 leukocytes on (a) WT and hSSAO/VAP-1-expressing hCMEC/D3 and (b) hSSAO/VAP-1-expressing HUVEC cells treated with MA (3 mM) or/and Simva (100 nM) and subjected to OGD (5h). Non-treated cells under normoxia were considered as Ctrl for each type of cells in (a). Non-treated WT cells were considered as Ctrl in (b). Data are expressed as mean ± SEM from 4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 as indicated, by a one-way ANOVA and the addition of Newman-Keuls multiple comparison test.

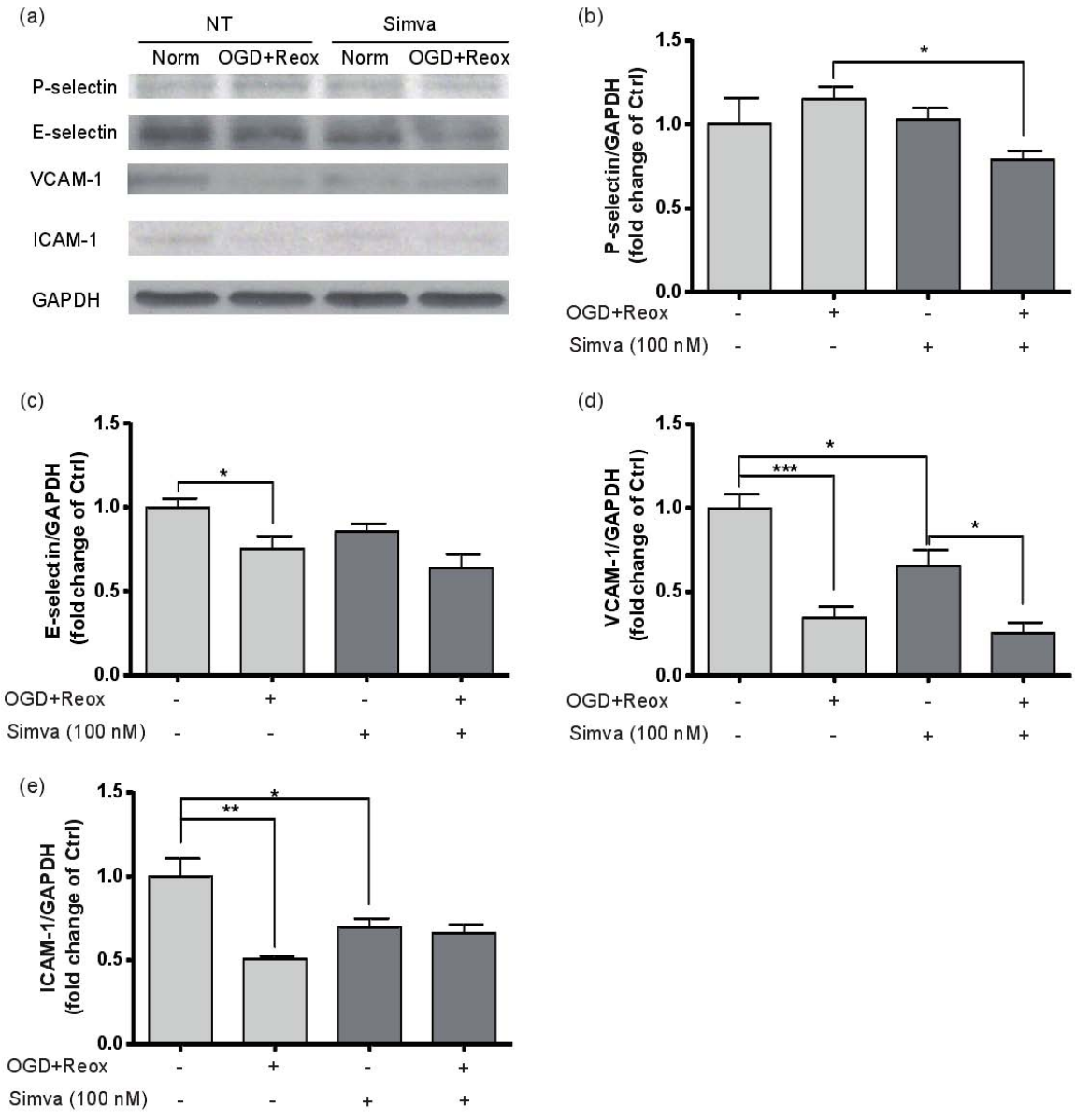


Fig. 9 Effects of simvastatin on the expression of the adhesion proteins P-selectin, E-selectin, VCAM-1 and ICAM-1 in brain endotelial cells after OGD with reoxygenation. (a) Representative western blot images of (b) quantification of P-selectin, (c) E-selectin, (d) VCAM-1 and (e) ICAM-1 levels in SSAO/VAP-1-expressing hCMEC/D3 cells treated with Simva (100 nM) or not, under normoxia or OGD (16h) with Reox (24h) (OGD+Reox). The presence of all proteins was normalized to GAPDH levels. Non-treated cells under normoxia conditions were considered as Ctrl. Data are expressed as mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

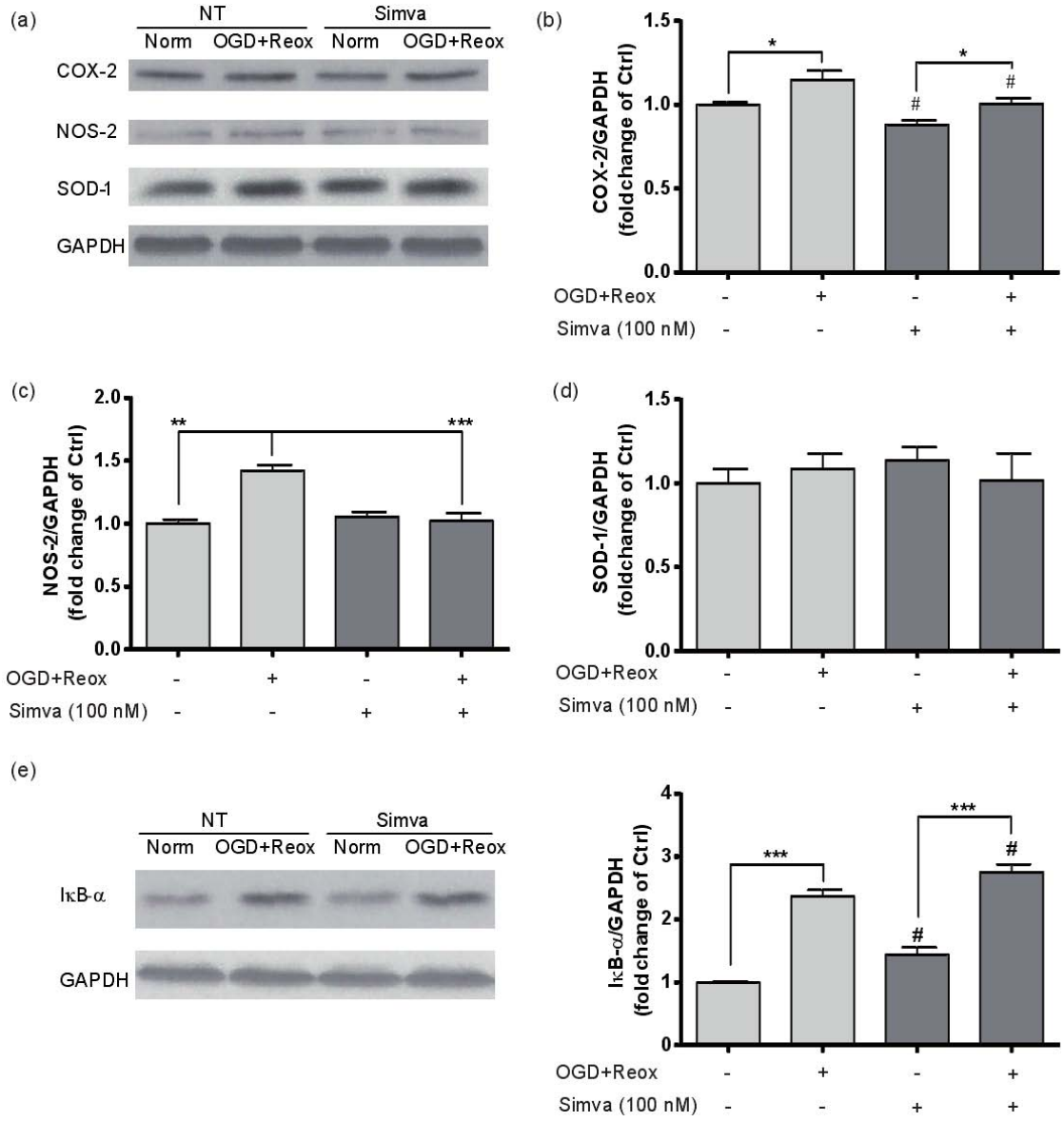


Fig. 10 Influences of simvastatin on the expression of COX-2, NOS-2, SOD-1 and IκB-α in endotelial cells subjected to OGD and reoxygenation. (a) Representative western blot images of (b) quantification of COX-2, (c) NOS-2 and (d) SOD-1 in hSSAO/VAP-1-expressing hCMEC/D3 cells treated with Simva (100 nM) or not, under normoxia or OGD (16h) with Reox (24h) (OGD+Reox). (e) Western blot images and their quantifications are also shown for IκB-α under the same experimental conditions. The presences of all proteins were normalized to GAPDH levels. Non-treated cells under normoxia conditions were considered Ctrl. Data are expressed as mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 as indicated; $^{\#}p$<0.05 versus the corresponding non-Simva treated cells, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

1. Annex chapter III.

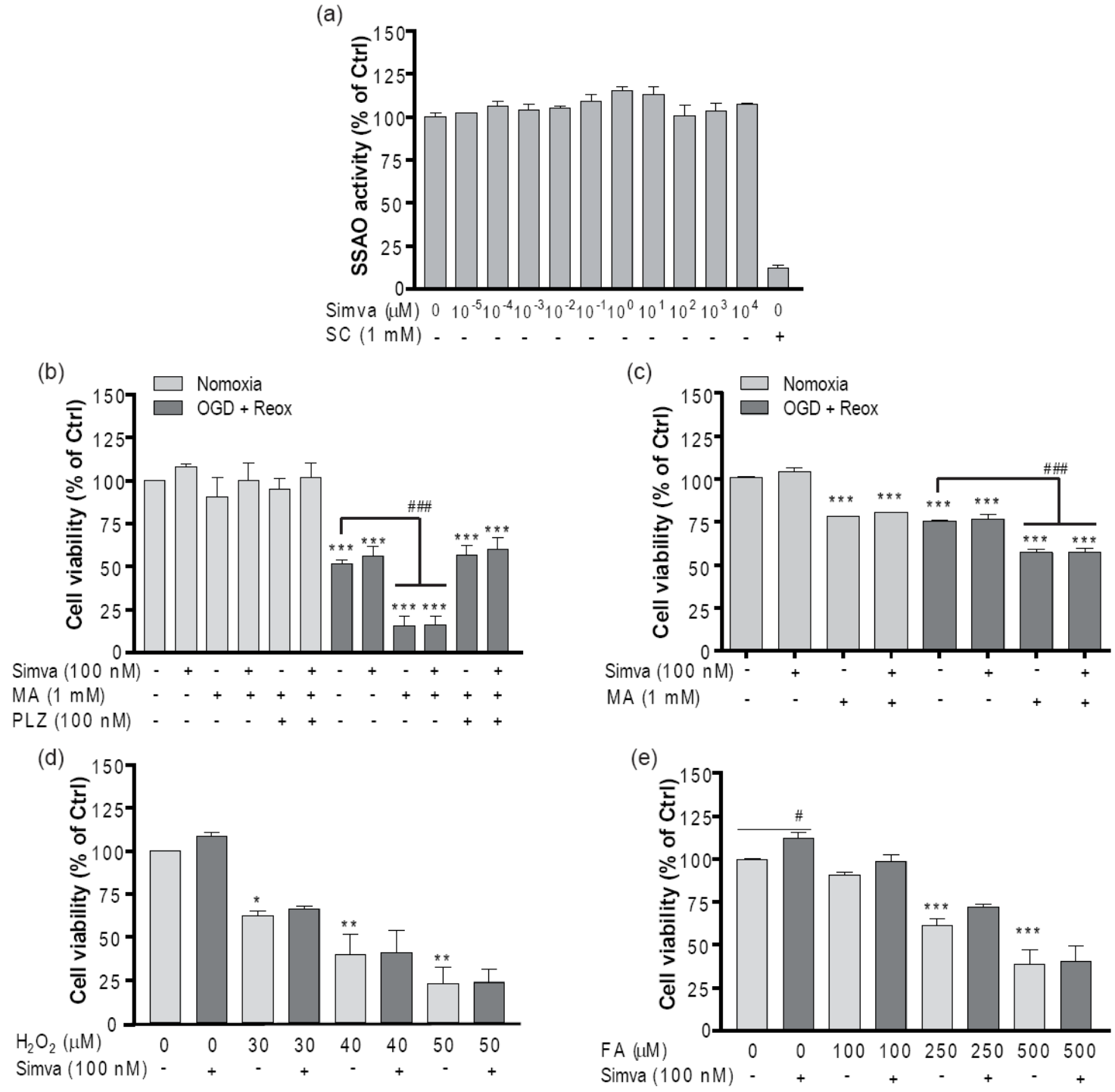
The modulation of the soluble SSAO/VAP-1 levels mediates part of the beneficial effect of simvastatin in cerebral ischemia

Supplementary Information

Ping Sun[1], Montse Solé[1]*, Mar Hernandez-Guillamón[2], Mireia Campos-Martorell[2], Alba Simats[2], Joan Montaner[2,3] and Mercedes Unzeta[1]*

*Corresponding authors:
Departament de Bioquímica i Biologia Molecular
Institut de Neurociències
Universitat Autònoma de Barcelona
08193, Bellaterra (Barcelona)
Spain
mercedes.unzeta@uab.es
montserrat.sole@uab.cat

Fig. S1 Simvastatin does not modulate the enzymatic activity of SSAO/VAP-1. (a) The SSAO inhibitory capacity of Simva was determined by measuring the SSAO activity remaining in HUVEC hSSAO/VAP-1 cell lysates after being incubated with Simva (10^{-5}-10^{4} µM) for 1 hour; semicarbazide (SC) was used as a positive control of inhibition. (b-c) Cell death induced by the metabolism of methylamine in normoxia or OGD-Reox conditions in different endothelial cell types. (b) Cell viability of HUVEC hSSAO/VAP-1 cells treated with MA (3 mM) or/and Simva (100 nM) subjected to 24h-OGD with 7h-reoxygenation (OGD+Reox). (c) The cell viability of hCMEC/D3 hSSAO/VAP-1 cells treated with MA (3 mM) or/and Simva (100 nM) subjected to 16h-OGD with 24h-reoxygenation (OGD+Reox) was also evaluated by MTT reduction assay. Non-treated cells under normoxia conditions were considered Ctrl. (d) The cell viability of HUVEC hSSAO/VAP-1 cells treated with H_2O_2 (30-50 µM) or/and Simva (100 nM), and (e) treated with formaldehyde (FA, 100-500 µM) or/and Simva (100 nM) for 24 hours. Data are expressed as mean ± SEM from 2-4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus Ctrl; $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.


(a)
SSAO activity (% of Ctrl)
Simva (μM) 0 10-5 10-4 10-3 10-2 10-1 100 101 102 103 104 0
SC (1 mM) - - - - - - - - - - - +
(b)
Nomoxia
OGD + Reox
Cell viability (% of Ctrl)
###
Simva (100 nM)
MA (1 mM)
PLZ (100 nM)
(c)
Nomoxia
OGD + Reox
Cell viability (% of Ctrl)
###
Simva (100 nM)
MA (1 mM)
(d)
Cell viability (% of Ctrl)
H2O2 (μM) 0 0 30 30 40 40 50 50
Simva (100 nM)
(e)
Cell viability (% of Ctrl)
#
FA (μM) 0 0 100 100 250 250 500 500
Simva (100 nM)

Supplementary Methods

Cell viability assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay was employed to evaluate the cell viability. Briefly, at the end of the treatments, cells were incubated with 0.5 mg/mL MTT for 3 h in HUVECs and for 1.5 h in hCMECs at 37°C. The medium was then replaced by dimethylsulfoxide (DMSO) to dissolve the blue formazan precipitate, and it was spectophotometrically quantified at 560 nm and 620 nm in a microplate reader (Labsystems multiscan RC) (Plumb *et al.,* 1989).

SSAO enzymatic activity determination

For SSAO activity determinations, cells were collected and homogenized in 100 mM Tris-HCl, pH 9, and containing protease inhibitors cocktail. Enzymatic activity was determined radiochemically by using a modification of the Otsuka and Kobayashi method (Otsuka *et al.,* 1964). Briefly, [^{14}C]-Benzylamine hydrochloride (100 µM and 2 mCi/mmol, American Radiolabeled Chemicals) was used as substrate, and 1 µM Dep (deprenyl) was added to avoid monoamine oxidase (MAO) B interference. A 1-hour inhibitory pre-treatment of samples was performed at 37°C with 1 µM Dep or Dep plus simvastatin. Reactions were performed at 37°C for 120 minutes in 100 mM Tris-HCl buffer, pH 9.0, adding 25 µL of substrate to the 200 µL of reaction. A hundred and fifty µg of HUVEC hSSAO/VAP-1 cell lysates were used in each reaction. Reaction was stopped by adding 100 µL of 2 M citric acid. The [^{14}C]-aldehyde products were extracted into 4 mL of toluene/ethylacetate (1:1, v/v) solution containing 0.6% (w/v) of diphenyloxazole. The amount of [^{14}C]-aldehyde was quantified using a Tri-Carb 2810TR liquid scintillation counter (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer).

References of the Supplementary Information

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Chapter IV:

"Study of the role of SSAO/VAP-1 as a possible nexus between ischemic stroke and Alzheimer's disease by using SSAO/VAP-1-expressing human brain endothelial cells (hCMEC/D3)"

Study of the role of SSAO/VAP-1 as a possible nexus between ischemic stroke and Alzheimer's disease by using SSAO/VAP-1-expressing human brain endothelial cells (hCMEC/D3)

Ping Sun, Montse Solé, Mercedes Unzeta

Institut de Neurociències i Departament de Bioquímica i Biologia Molecular. Edifici M, Facultat de Medicina. Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona (Spain).

Introduction

Ischemic stroke and Alzheimer's disease (AD) are common diseases which mostly affecting elderly people. Increasing evidences suggest that these two diseases are associated and that there is an interaction among each other. Stroke-related cerebrovascular disease occur in AD, precede AD and can enhance the risk for developing AD (Murray *et al.* 2011). Stroke-induced lesions co-exist with hallmarks of dementias, and subcortical infarcts are co-incidents with AD pathology. Moreover, injuries such as stroke or ischemia increase the risk of amyloid pathology, by increasing Aβ levels. During ischemia, amyloid precursor protein (APP) (Makinen *et al.* 2008, Hiltunen *et al.* 2009) and β-site amyloid precursor protein cleaving enzyme-1 (BACE-1) (Zhang *et al.* 2007, Guglielmotto *et al.* 2009) are elevated. Thus, this cerebrovascular disorder can increase the AD risk and to contribute to the neurodegenerative disease. On the other hand, AD is also able to augment the onset of stroke (Zhou *et al.* 2015) or increase the susceptibility to stroke injuries (Milner *et al.* 2014). Both the oxidative stress and inflammatory responses generated by the Aβ deposition in/ around the cerebral vascular wall and ischemia-reperfusion injuries contribute to the degeneration and dysfunction of the cerebral vasculature and the surrounding brain tissue. In this context, the neurovascular unit, consisting by glial, vascular and neuronal cells plays an important role in controlling the

exchange of molecules across the blood-brain barrier (BBB) and regulating the neuronal microenvironment homeostasis (Iadecola 2010). Disruption of the neurovascular unit and BBB occur in both stroke and AD, which result in the impairment of the microenvironment homeostasis and the exacerbation of outcome in both diseases.

Interestingly, vascular adhesion protein 1 (VAP-1), which is highly expressed in the cerebral blood vessels, has been found altered in both stroke and AD. It mediates the rolling and transmigration of leukocytes through its semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.21) activity (Smith *et al.* 1998, Jalkanen & Salmi 2008), generating aldehyde, hydrogen peroxide and ammonia as potential harmful by-products. In this regard, we hypothesize that SSAO/VAP-1 may be a possible link between stroke and AD promoting the progression from one of these disorders to the other, since we have demonstrated that it is involved in the pathogenesis of both diseases. We have considerable evidences proving that plasmatic SSAO/VAP-1 increases in ischemic and hemorrhagic stroke patients, and that its activity predicts the appearance of parenchymal hemorrhages after tPA treatment in ischemic stroke patients (Airas *et al.* 2006, Hernandez-Guillamon *et al.* 2012, Hernandez-Guillamon *et al.* 2010). Furthermore, SSAO/VAP-1 is increased as well in AD patients' plasma and cerebrovascular tissue (Ferrer *et al.* 2002, Unzeta *et al.* 2007, Valente *et al.* 2012, del Mar Hernandez *et al.* 2005).

Taking into account all the cerebral alterations in these two diseases and the effects of SSAO/VAP-1 on the brain microvasculature, we hypothesize that SSAO/VAP-1 may function as a link between ischemic stroke and AD. Therefore, the amyloidogenic-related proteins have been investigated in both wild type (WT) and hSSAO/VAP-1-expressing human cerebral microvascular endothelial cells (hCMEC/D3) treated with β-amyloid and subjected to OGD conditions as an experimental model of ischemic stroke.

Material and methods

Cell culture

The human cerebral microvascular endothelial cell line hCMEC/D3 was obtained from Dr. Couraud's lab (Paris, France) (Weksler *et al.* 2005). The hCMEC/D3 cell line stably expressing the human SSAO/VAP-1 (hCMEC/D3 hSSAO/VAP-1) was generated as described (submitted manuscript to BJP). hCMEC/D3 cells were cultured as recommended, on 150 µg/mL collagen type I (Rat Tail, Corning) – coated plates in EBM-2 (Lonza) medium supplemented with 5% FBS (Foetal Bovine Serum, Life Technologies), 1.4 µM Hydrocortisone (Sigma), 5 µg/mL Ascorbic Acid (Sigma), 1% Chemically Defined Lipid Concentrate (Life Technologies), 10 mM HEPES (Life Technologies) and 1 ng/ml human bFGF (Fibroblast Growth Factor-basic, Sigma). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

OGD models and cell treatments

Combined oxygen and glucose deprivation (OGD) and reoxygenation have been used as an experimental approach to ischemic stroke. For hCMEC/D3 cells, the OGD was performed in glucose-free DMEM (Life Technologies) after washing cells with glucose-free PBS, and then introducing the cells into a temperature-controlled (37±1°C) Invivo$_2$ hypoxia workstation (RUSKINN) containing a gas mixture composed of 5% CO_2, 95% N_2 and 0.5% O_2. Control cells were maintained in DMEM (5 mM glucose) under normoxia conditions (5% CO_2/95% air). For reoxygenation treatment, cells that have undergone OGD were returned to normoxia conditions after replacing glucose-free DMEM by serum-free DMEM (5 mM glucose) and adding the same treatments present during OGD. MA (methylamine), SC (semicarbazide) and A$\beta_{1\text{-}40}$D (A$\beta_{1\text{-}40}$ peptide containing the Dutch mutation, Bachem) were added into DMEM before OGD starting. In the reoxygenation process, the compounds were maintained at the same concentrations than during OGD. For the preparation of A$\beta_{1\text{-}40}$D, it was pretreated with 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP, Sigma-Aldrich) for

more than 4h but less than 6h, then aliquoted, evaporated at room temperature, and stored at -80°C until using, then being dissolved in sterile PBS containing 0.1% ammonium hydroxide.

Cell viability assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay was employed to evaluate the cell viability. Briefly, at the end of the treatments, cells were incubated with 0.5 mg/mL MTT for 1.5 hours at 37°C. The medium was then replaced by dimethylsulfoxide (DMSO) to dissolve the blue formazan precipitate, and it was spectophotometrically quantified at 560 nm and 620 nm in a microplate reader (Labsystems multiscan RC) (Plumb *et al.* 1989).

Western blot analysis

Cells were collected and homogenized in 50 mM Tris-HCl (pH 7.5), containing 1% Triton X-100, 10 mM EDTA and protease inhibitors cocktail (Sigma) (1:100) and were sonicated for 10 seconds. Equal amounts of protein (20 µg/lane) determined by the Bradford method, were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with TBS/0.1%-Tween buffer containing 5% (w/v) non-fatty milk and incubated overnight at 4°C with the corresponding primary antibodies. After incubation with the secondary antibodies, blots were developed using ECL® Chemoluminiscent detection reagents and High Performance Chemiluminiscence Films (GE Healthcare). The ImageJ software was used to quantify the western blot signal. The antibodies used were: rabbit anti-BACE1 (Abcam, 1:1000), rabbit anti-LRP-1 (Epitomics, 1:1000), rabbit anti-RAGE (Epitomics, 1:1000), mouse anti-APP 20.1 (W.E. Van Nostrand, Stony Brook University, NY, USA; 1:1000), mouse anti-*β*-actin (Sigma-Aldrich, 1:5000), mouse anti-GAPDH (Ambion-Invitrogen, 1:20000), HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (BD Biosciences, 1:1000); HRP anti-mouse IgG (Dako, 1:1000).

Statistical analysis

Results are given as mean ± SEM of independent experiments. Statistical analysis was performed by one-way ANOVA and further Newman-Keuls multiple comparison test. $P<0.05$ was considered to be statistically significant. Statistical analyses and graphic representations were obtained with the Graph-Pad Prism 6.0 software.

Results

OGD with reoxygenation induces the expression of BACE1 in hSSAO/VAP-1-expressing brain endothelial cells

In order to check whether the presence of SSAO/VAP-1 alters the amyloidogenic pathway-related proteins under different OGD conditions, WT and hSSAO/VAP-1-expressing human brain endothelial cells hCMEC/D3 were subject to 4-16 hours OGD and 16-hour OGD with 24-hour reoxygenation (OGD+Reox), and the expression of BACE1 was analysed by western blot (Fig 1). Results showed that neither OGD nor OGD with reoxygenation increased the protein levels of BACE1 in hCMEC/D3 WT cells. By contrast, in the hCMEC/D3 hSSAO/VAP-1 cells, OGD conditions progressively up-regulated the expression of BACE1 with the OGD treating time lasting, which was significantly higher than WT cells under the same treatment separately. Moreover, OGD with reoxygenation significantly increased the BACE1 protein levels compared with the rest of the treatments in hCMEC/D3 hSSAO/VAP-1 cells. Looking at these results, the fact that only the SSAO/VAP1-expressing cells induce an expression of BACE-1 comparing with wild type cells, suggests that SSAO/VAP-1 presence may be the responsible of this effect in OGD and OGD with reoxygenation conditions.

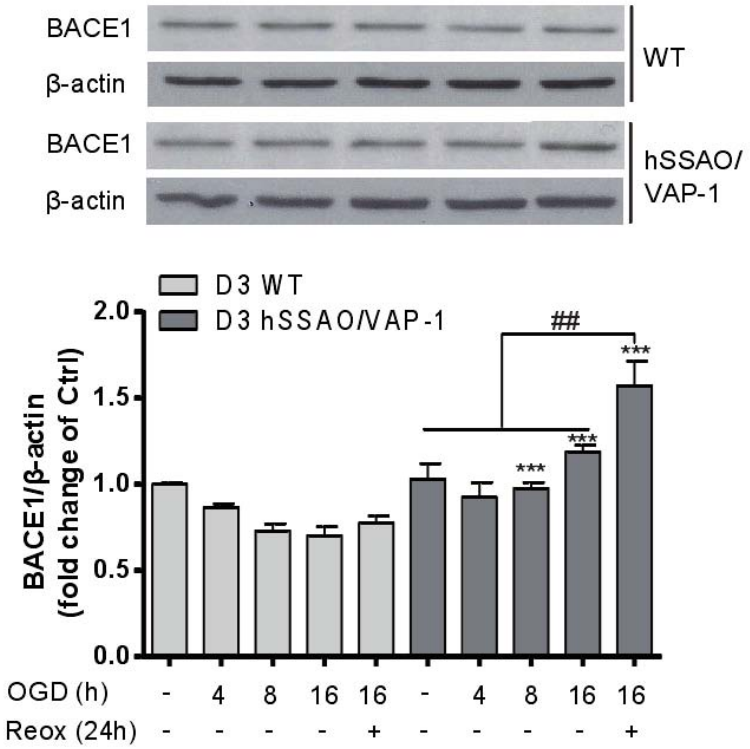

Fig 1. Oxygen and glucose deprivation (OGD) with reoxygenation (Reox) induces the expression of BACE1 in hSSAO/VAP-1-expressing hCMEC/D3 cells. Representative western blot images and quantification of BACE1 present in WT (wild type) and hSSAO/VAP-1-expressing hCMEC/D3 cells subjected to OGD 4-16 hours or 16-hour OGD with 24-hour reoxygenation (Reox). The presence of BACE1 was normalized to the β-actin levels. Cells without OGD treatment and under normoxia conditions for 16 hours were considered control (Ctrl). Data are expressed as mean ± SEM from 3 independent experiments. ***P<0.001 versus WT hCMEC/D3 cells with the same treatment; $^{\#\#}P$<0.01 as indicated, by one-way ANOVA and further Newman-Keuls multiple comparison test.

The influence of SSAO substrate and inhibitor on the expression of BACE1 under OGD with reoxygenation

With regard to assess whether the SSAO catalytic activity versus methylamine (MA) as specific substrate further contributes to the up-regulation of BACE1, the substrate effect under nomoxia and OGD with reoxygenation was analysed by western blot, and semicarbazide (SC) was used as a specific SSAO inhibitor (Fig 2). Since the oxidation of SSAO substrate can lead to the generation of hydrogen

peroxide, which is a major oxidative stress contributor, and oxidative stress under hypoxia can trigger the expression of BACE1 (Guglielmotto et al. 2009), we thought that the catalytic action of SSAO/VAP-1 may contribute to the BACE1 enhancement. As expected, there was not change in the WT cells in any of the treatments, while in the hSSAO/VAP-1-expressing hCMEC/D3 cells, the expression of BACE1 in all the treatments was higher than in WT hCMEC/D3 cells. Moreover, in hCMEC/D3 hSSAO/VAP-1 cells, the metabolism of methylamine (MA) tended to enhance the protein levels of BACE1 under normoxia, although SC didn't reduce this trend. Also, there was a high basal level of BACE1 in hCMEC/D3 hSSAO/VAP-1 cells under the insult of OGD with reoxygenation compared with nomoxia, however, neither MA or SC significantly changed significatively the BACE1 protein levels under this condition.

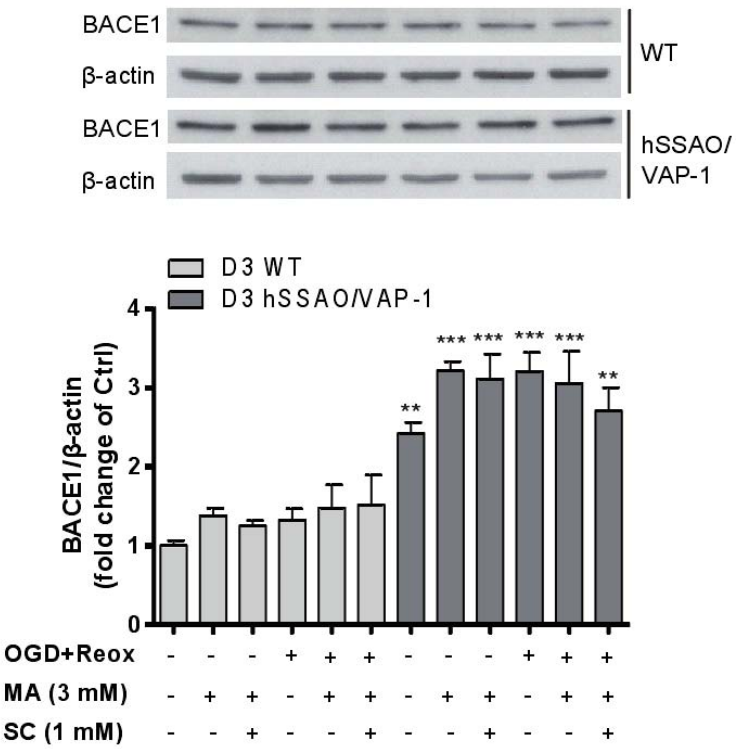

Fig 2. Effects of methylamine (MA) and semicarbazide (SC) on the expression of BACE1 in WT and hSSAO/VAP-1-expressing hCMEC/D3 cells under OGD with reoxygenation. Representative western blot images and quantification of BACE1 present in WT (wild type) and hSSAO/VAP-1-expressing hCMEC/D3 cells treated with 3 mM MA or/and 1 mM SC subjected to 16-hour OGD with 24-hour reoxygenation (OGD+Reox). The

presence of BACE1 was normalized to the β-actin levels. Cells without OGD treatment and under normoxia conditions for 40 hours were considered control (Ctrl). Data are expressed as mean ± SEM from 3 independent experiments. $^{**}P<0.01$ and $^{***}P<0.001$ versus WT cells with the same treatment, by one-way ANOVA and further Newman-Keuls multiple comparison test.

The metabolism of SSAO substrate up-regulates the protein levels of APP in hSSAO/VAP-1 hCMEC/D3 cells under OGD with reoxygenation

Since cerebral ischemia can increase the accumulation of APP at the region of ischemic injury (Hiltunen et al. 2009), it was also interesting to assess whether the expression of SSAO/VAP-1 or the catalytic activity of SSAO/VAP-1 could mediate this enhanced expression of APP under ischemic conditions. Hence, there were analysed the protein levels of APP in both WT and hSSAO/VAP-1-expressing hCMEC/D3 cells treated with MA and SC under OGD with reoxygenation conditions (Fig 3). Results revealed that there was no difference among all the treatments in WT cells, while the metabolism of MA significantly increased the expression of APP in hCMEC/D3 hSSAO/VAP-1 cells subjected to OGD with reoxygenation, compared with WT or with the non-treated hSSAO/VAP-1 hCMEC/D3 cells; SSAO inhibitor SC successfully suppressed this enhancement.

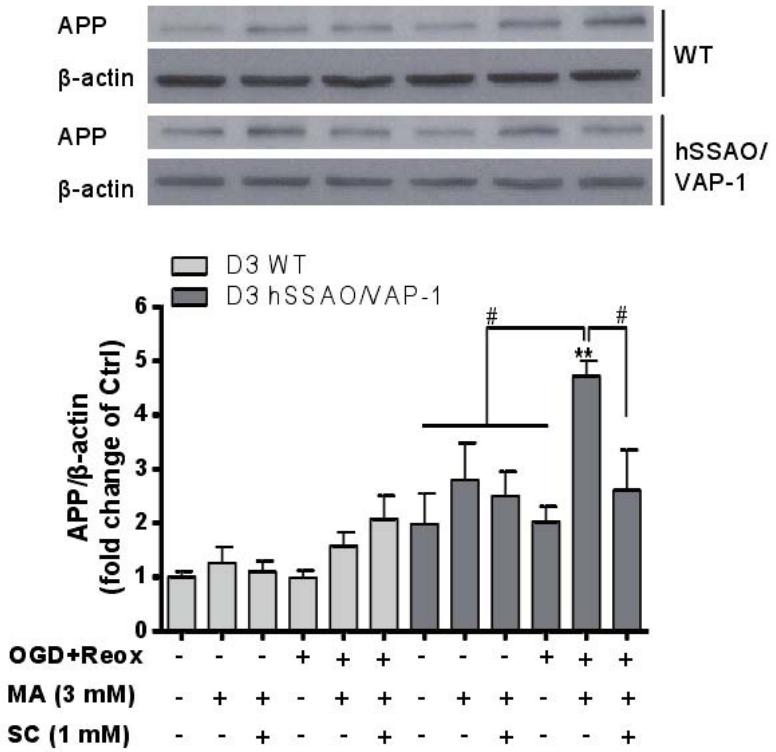

Fig 3. The metabolism of methylamine (MA) by SSAO/VAP-1 up-regulates the expression of APP in hSSAO/VAP-1-expressing hCMEC/D3 cells under OGD with reoxygenation. Representative western blot images and quantification of APP present in WT (wild type) and hSSAO/VAP-1-expressing hCMEC/D3 cells treated with 3 mM MA or/and 1 mM SC subjected to 16-hour OGD with 24-hour reoxygenation (OGD+Reox). The presence of APP was normalized to the β-actin levels. Cells without OGD treatment and under normoxia conditions for 40 hours were considered control (Ctrl). $^{**}P<0.01$ versus WT cells with the same treatment; $^{\#}P<0.05$ as indicated, by one-way ANOVA and further Newman-Keuls multiple comparison test.

The influence of SSAO substrate and inhibitor on the protein levels of LRP-1 and RAGE under OGD with reoxygenation

The clearance of β-amyloid across the blood-brain barrier (BBB) is also a crucial process for maintaining the homeostasis of β-amyloid in the brain. The accumulation of β-amyloid in the AD brain normally results from the imbalance of the production and clearance of Aβ peptides (Blennow *et al.* 2006). In this sense, LRP-1, the main receptor responsible for the Aβ transportation from brain to

blood, and RAGE, the main receptor responsible for the Aβ transportation from blood to the brain, were assessed under OGD with reoxygenation treated with MA or/and SC. Fig 4A showed that no changes were observed in WT cells again, while there were significantly higher expressions of LRP-1 in hSSAO/VAP-1-expressing hCMEC/D3 cells. Additionally, OGD with reoxygenation reduced the levels of LRP-1 compared with nomoxia conditions, but no differences were found by the addition of SSAO substrate or inhibitor. Moreover, as showed by Fig 4B, there was no difference of RAGE expression among all the treatments between WT and hSSAO/VAP-1-expressing hCMEC/D3 cells.

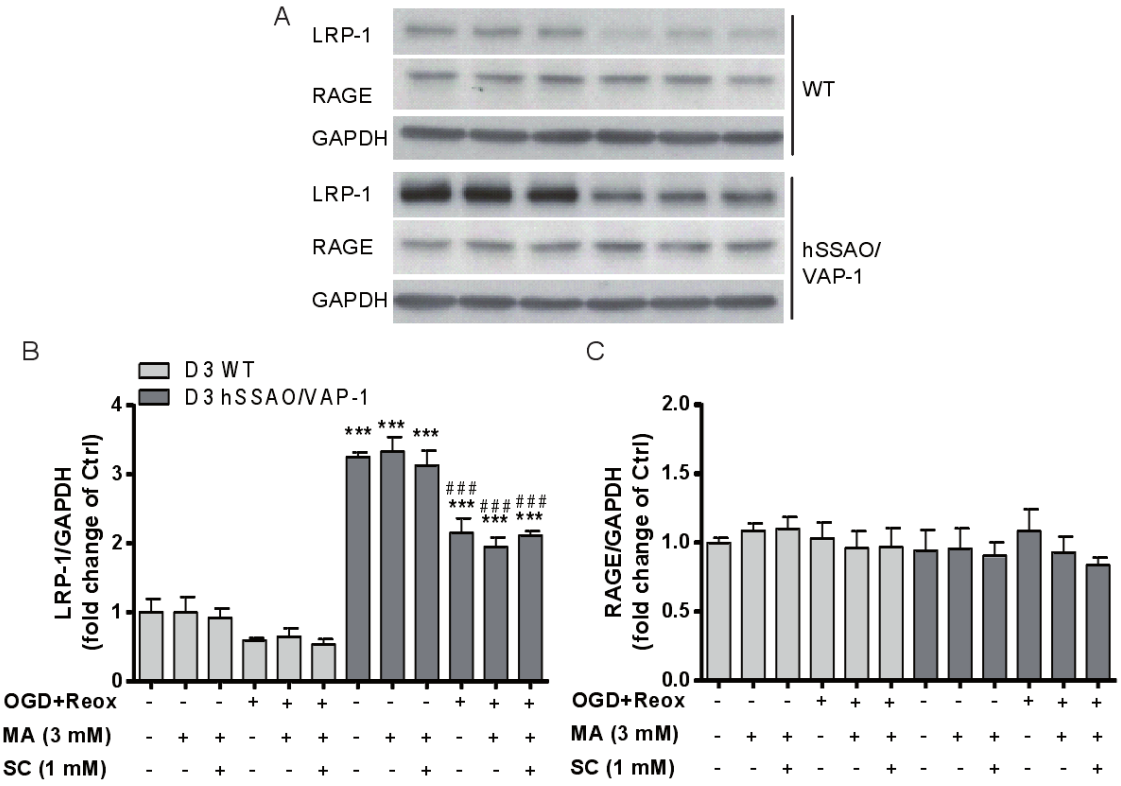

Fig 4. Methylamine (MA) processing or semicarbazide (SC) do not modulate the expression of LRP-1 or RAGE in WT and hSSAO/VAP-1 expressing hCMEC/D3 cells under OGD with reoxygenation. (A) Representative western blot images and (B) quantifications of LRP-1 and (C) RAGE present in WT and hSSAO/VAP-1 expressing hCMEC/D3 cells treated with 3 mM MA or/and 1 mM SC subjected to 16-hour OGD with 24-hour reoxygenation (OGD+Reox). The presence of LRP-1 and RAGE were normalized to the GAPDH levels. Cells without OGD treatment and under normoxia

conditions for 40 hours were considered as control (Ctrl). Data are expressed as mean ± SEM from 3 independent experiments. ***P<0.001 versus WT cells with the same treatment, ###P<0.001 versus hSSAO/VAP-1-expressing hCMEC/D3 cells under normoxia conditions for 40 hours with the same treatment, by one-way ANOVA and further Newman-Keuls multiple comparison test.

The metabolism of SSAO substrate induces additional cell death when co-treated with $A\beta_{1-40}D$ in hCMEC/D3 hSSAO/VAP-1 cells undergone OGD with reoxygenation

In order to confirm whether the presence of Aβ and SSAO/VAP-1 accelerates vascular damage under ischemic conditions, as we previously described in normoxia conditions (Solé *et al.* 2014), SSAO/VAP-1 expressing cells under normoxia and OGD with reoxygenation were incubated in the presence of $A\beta_{1-40}D$ and cell viability was determined (Fig 5). The co-treatment of MA with $A\beta_{1-40}D$ induced an increased loss of the cell viability compared with that produced separately by one of the two toxics under OGD with reoxygenation. The addition of SSAO inhibitor SC in the presence of both toxics significantly increased the cell viability.

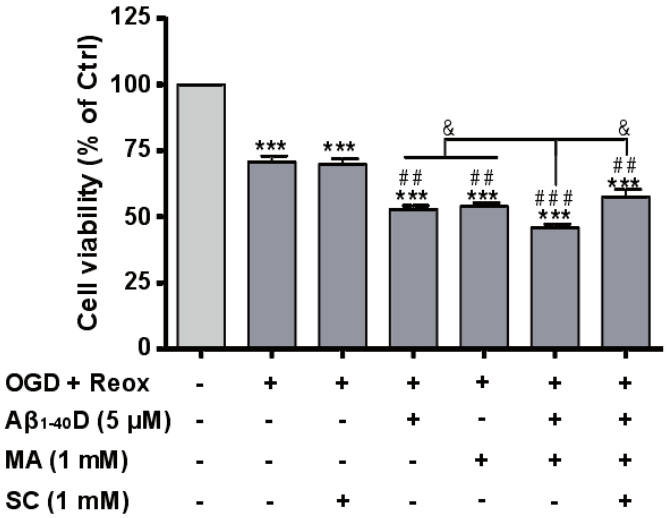

Fig 5. The metabolism of methylamine (MA) by SSAO/VAP-1 activity induces additional cell death when co-treated with $A\beta_{1-40}D$ in hCMEC/D3 hSSAO/VAP-1 cells under OGD with reoxygenation. MTT reduction assay was used to evaluate the cell viability of

HCMEC/D3 hSSAO/VAP-1 cells subjected to 16-hour OGD with 24-hour reoxygenation (OGD+Reox) in the presence of $A\beta_{1-40}$ containing the Dutch mutation ($A\beta_{1-40}D$, 5 μM), MA (1 mM) or/and SC (1 mM). $A\beta_{1-40}D$, MA and SC were added at the same time before OGD. All treatments were maintained during reoxygenation. Non-treated cells under normoxia were considered control (Ctrl). Data are expressed as mean ± SEM of three independent experiments. $^{***}P<0.001$ versus Ctrl; $^{\#\#}P<0.05$ and $^{\#\#\#}P<0.001$ versus non-treated cells under OGD+Reox; $^{\&}P<0.05$ as indicated, by a one-way ANOVA test and the addition of Newman-Keuls multiple comparison test.

Discussion

Increasing evidences reveal that ischemic stroke and Alzheimer's disease are related cerebral disorders. Ischemic injuries such as stroke play an important role in the onset and progression of neurological disorders like AD (Grammas 2011, Marchesi 2014), and in turn, AD can also increase the risk of intracerebral haemorrhages (Zhou et al. 2015) and the susceptibility to cerebral ischemic injury (Milner et al. 2014). The dysfunction of cerebral blood vessels and restricted brain perfusion constitute the most common features among all the overlaps between these two diseases, which ultimately lead to the neuronal injury and cognitive impairment (Attems & Jellinger 2014). Several studies have shown that hypoxia induces the up-regulation of BACE1 transcription, expression and activity, hence increasing the β-amyloid generation in non-endothelial cells or animal brain tissues (Guglielmotto et al. 2009, Sun *et al.* 2006, Zhang et al. 2007). In this work, by using human brain endothelial cells (hCMEC/D3), which proved to be a good model of BBB (Weksler *et al.* 2013), transfected with human SSAO/VAP-1, we were able to assess the possible contribution of the cerebrovascular-originated β-amyloid to different ischemic conditions, where the involvement of SSAO/VAP-1 was investigated. In our results, different OGD conditions didn't induce changes of BACE1 protein levels in WT cells, however, the SSAO/VAP-1-expressing cells exhibited high levels of BACE1 expression when subjected to OGD with reoxygenation. This may be related with the hypoxia inducible factor 1α (HIF-1α), since treatment of hypoxia can induce the

expression of BACE1 by HIF-1α activation (Guglielmotto et al. 2009, Zhang et al. 2007), although a possible underlying interaction between SSAO/VAP-1 and HIF-1α, if any, needs to be further elucidated. Also, we can't rule out the contribution of oxidative stress introduced by the reoxygenation process, which can up-regulate BACE1 as well under hypoxic conditions (Guglielmotto et al. 2009).

Since SSAO/VAP-1 can deaminate its endogenous substrates such as methylamine (MA), generate toxicity products such as formaldehyde and hydrogen peroxide, it was also necessary to investigate the substrate effect on the expression of BACE1. Results indicated that hCMEC/D3 SSAO/VAP-1 cells expressed significantly higher levels of BACE1 than WT, and MA treatment tended to exhibit more expression of BACE1 in nomoxia rather than in OGD with reoxygenation. Given that oxidative stress also contributes to the expression of BACE1 under hypoxia (Guglielmotto et al. 2009), elevated levels of BACE1 in normoxia condition could be triggered by the production of hydrogen peroxide. There was a high basal level of BACE1 protein under OGD with reoxygenation, and less time for the metabolism of MA (only in reoxygenation part) compared with nomoxia condition, which may explain why we didn't observe additional elevation of BACE1 when treated with MA. However, the ineffectiveness of SC in down-regulation of BACE1 under this special situation may need more investigations to carry out in order to understand the underlying mechanism.

In addition, the amyloid precursor protein (APP) evaluation revealed that the metabolism of MA by SSAO/VAP-1 catalytic activity can significantly enhance the protein levels of APP in the transfected cells under OGD with reoxygenation, and SC completely inhibited the overexpression of APP induced by MA. It is well implicated that focal cerebral ischemia and MCAO insult can stimulate the expression and accumulation of APP in the animal brain tissues (Makinen et al. 2008, van Groen *et al.* 2005). Here we found by the first time that the deamination of SSAO/VAP-1 substrate by its catalytic activity can lead to the overproduction of APP in the brain endothelial cells under OGD with reoxygenation condition, since its inhibitor can totally abolish this induction.

Taken together the previous results, that there are higher levels of BACE1 in OGD with reoxygenation conditions, there exists a high risk for the overproduction of Aβ peptides under this ischemic/reperfusion condition, compared with the WT or non-treated transfected endothelial cells. Considering the inhibition of APP overexpression by SC, a SSAO inhibitor therefore could contribute at least partially to the reduction of Aβ generation and to improve the neurological outcome for these two related diseases.

Moreover, endothelium constitutes one of the important continents of BBB which is responsible for the clearance of Aβ peptides and then to maintain the balance of Aβ in the brain microenvironment homeostasis (Deane *et al.* 2009). LRP-1 (Deane *et al.* 2004) and RAGE (Deane *et al.* 2003) are two main receptors in the control of the Aβ peptides efflux or influx across the BBB, respectively. In this sense, they were also evaluated under same condition as previous. Results indicated higher basal levels of LRP-1 in hSSAO/VAP-1-expressing hCMEC/D3 cells, which probably because of the presumably high levels of Aβ production into the media trigger the self-defence system to eliminate the overproduced Aβ. However, with the limitation of cell culture performance, Aβ cannot be transported out of the cells, which in turn could explain again the high expression of LRP-1 in this type of cells. In addition, OGD with reoxygenation treatment down-regulated the expression of LRP-1, which may result in less efflux of Aβ across the BBB under ischemic conditions, thus exacerbating the Aβ overproduction-induced neurological dysfunction. During this analysis, the substrate and inhibitor exhibited inefficacy in the modulation of LRP-1 in all the conditions. No change in the expression of RAGE in this ischemic stroke model between WT and hSSAO/VAP-1 hCMEC/D3 cells was observed.

Since cerebral amyloid angiopathy (CAA), characterized by beta amyloid (Aβ) deposits in the cerebrovascular tissue, occurs in the 80% of AD patients, we at last investigated the possible contribution of SSAO/VAP-1 on the cell damage when co-treated with Aβ under OGD with reoxygenation conditions. Results indicated that, when $A\beta_{1\text{-}40}D$ treatment was introduced in this experimental model

of ischemia simulating a pre-existing AD pathology, a synergic damage induced by MA and $A\beta_{1-40}D$ was observed, and SC significant enhanced the cell viability in the presence of both toxics. These results allow us concluding that $A\beta_{1-40}D$ together with the catalytic action of SSAO/VAP-1 induces more vascular damage under OGD with reoxygenation conditions, and that SSAO inhibitor can attenuate the cell damage caused by both toxics.

Taking into account all the results, we can conclude that, the presence of SSAO/VAP-1 in the brain endothelium under ischemic stimulation in addition to SSAO/VAP-1 substrate may facilitate the generation of β-amyloid, hence increasing the risk and neurological worsening of AD. It has been widely reported that the neurovascular unit plays a role in the AD pathology; in this context, the results herein described allow us to conclude that SSAO/VAP-1 could be a possible nexus between ischemic stroke and Alzheimer's disease.

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V. DISCUSSION

V. DISCUSSION

Cerebrovascular diseases and mainly stroke, affect each year 15 million people worldwide, leading to a high number of deaths and permanently disabled people, and implying large economic costs. Contributions aimed to prevent or improve its effects are urgently needed. Increasing evidences also reveal that stroke and Alzheimer's disease (AD) are related cerebral disorders. Ischemic injuries such as stroke play an important role in the onset and progression of neurological disorders like AD (Grammas, 2011; Marchesi, 2014), and in turn, AD can also increase the risk of intracerebral haemorrhages (Zhou *et al.*, 2015) and the susceptibility to cerebral ischemic injury (Milner *et al.*, 2014). The dysfunction of cerebral blood vessels and restricted brain perfusion constitute the most common features among all the overlaps between these two diseases, which ultimately lead to the neuronal injury and cognitive impairment (Attems *et al.*, 2014). In this context the SSAO/VAP-1, highly present in human cerebrovascular tissue (Castillo *et al.*, 1998b; Castillo *et al.*, 1999; Smeraldi *et al.*, 2001) makes this enzyme a very interesting protein to be studied in brain vascular disorders, such as stroke (Airas *et al.*, 2008) and AD (Ferrer *et al.*, 2002b). It has been described that SSAO/VAP-1 mediates leukocyte adhesion and transmigration (Salmi *et al.*, 2001b; Koskinen *et al.*, 2004; Stolen *et al.*, 2005) that can participate in the stroke and AD pathologies progression, and its activity is elevated in ischemic and hemorrhagic stroke (Hernandez-Guillamon *et al.*, 2010; Hernandez-Guillamon *et al.*, 2012), as well as in AD (Ferrer *et al.*, 2002b; del Mar Hernandez *et al.*, 2005; Unzeta *et al.*, 2007; Valente *et al.*, 2012).

In this context, the role of SSAO/VAP-1 was first studied at the molecular level in the stroke physiopathology, in order to provide new knowledge on the mechanisms for which SSAO/VAP-1 participates in stroke, as well as to propose it as a new therapeutic target to fight this disease. Moreover, it was studied the possibility of SSAO/VAP-1 being a link between stroke and its progression to AD. However, some proteins expression is lost when cells are cultured, as it is the case for SSAO/VAP-1 (Salmi *et al.*, 1995; Jaakkola *et al.*, 1999). Therefore,

human SSAO/VAP-1-expressing cells are ideal approaches to study the mechanisms of its involvement in vascular diseases. In this work, two hSSAO/VAP-1-expressing endothelial cells have been used, which are HUVEC hSSAO/VAP-1 (Sole *et al.*, 2011) and hCMEC/D3 hSSAO/VAP-1 (manuscript submitted to BJP).

First, by using WT cells, non-expressing the protein, and hSSAO/VAP-1-expressing HUVECs, the OGD with reoxygenation conditions were standardized. Results showed that HUVEC hSSAO/VAP-1 display a higher sensitivity to the OGD and reoxygenation-mediated cell damage compared to WT cells, especially when reoxygenation was applied. Notably, a similar effect was also observed in livers of SSAO/VAP-1-overexpressing mice, displaying increased expression of oxidative state-sensor proteins (Stolen *et al.*, 2004). More interestingly, when an SSAO substrate was added, the cell death after OGD and reoxygenation significantly increased, while it did not happen under normoxia. This data confirmed the SSAO activity involvement in this experimental ischemic condition, but also suggested that SSAO enzymatic activity could be punctually enhanced under ischemic conditions in humans, maximizing its effects. In the *in vivo* situation, it is reasonable to think that the formaldehyde and H_2O_2 generated by SSAO activity could contribute to the oxidative stress, a key deleterious factor in brain ischemia and reperfusion (Pradeep *et al.*, 2012), and directly contribute to the apoptosis or necrosis (Manzanero *et al.*, 2013). When analyzing cell death-activated pathways with HUVEC hSSAO/VAP-1, an increase in caspase-3 cleavage was observed, indicating the activation of an apoptotic cell pathway. The mitochondria or the reticular stress pathway seemed not to be relevant in the process, since proteins associated to these molecular pathways such as Bax/Bcl-2 ratio, or GRP78 and caspase-12 varied their levels at longer times after caspase-3 activation occurred. On the other hand, caspase-8 cleavage was detected at comparable times at which cleaved caspase-3 increased, suggesting a possible contribution of the death receptor-associated pathway to the cell death

observed in this model, which may secondarily activate the mitochondrial pathway.

Peripheral endothelial HUVECs cannot totally simulate the properties of cerebral endothelial cells, which possess important and distinct genes involved in several processes as vasculo- and angiogenesis (VEGF), immunoregulation (decorin, IL-6) or have growth-supporting properties (BDNF, transforming growth factor-β) (Kallmann *et al.*, 2002). Therefore, the hCMEC/D3 cell line was chosen for the transfection of human SSAO/VAP-1 gene, because it is the first stable, well differentiated and well characterized human brain endothelial cell line (Weksler *et al.*, 2005) in terms of expression of endothelial markers, up-regulation of adhesion molecules in response to inflammatory cytokines, as well as BBB characteristics. This hSSAO/VAP-1-expressing hCMEC/D3 cell line was characterized and compared with previously developed endothelial and smooth muscle cell lines expressing SSAO/VAP-1 described previously in our group (Sole *et al.*, 2007; Sole *et al.*, 2011). Then, a new experimental model of ischemic stroke by subjecting these brain endothelial cells to OGD and reoxygenation conditions was established. Cell viability under OGD with reoxygenation conditions was also observed significantly decreased in the presence of SSAO/VAP-1 substrate, and it was restored by SSAO/VAP-1 inhibitor. These results together with previous findings, confirm that this enzyme plays an important role enhancing the endothelial cell death under ischemia.

SSAO/VAP-1 exists in two isoforms, the membrane-bound and the soluble one that is presumably originated by a shedding process in humans. One study reported that 3T3-L1 adipocytes are able to release the soluble SSAO/VAP-1 by a metalloproteinase (MMP)-dependent shedding mechanism (Abella *et al.*, 2004), where batimastat, a broad spectrum MMP inhibitor, avoided the SSAO/VAP-1 release. In the peri-infarcted region of stroke patients' brains, a decrease in the frequency of SSAO/VAP-1-containing vessels has been found (Hernandez-Guillamon *et al.*, 2010; Hernandez-Guillamon *et al.*, 2012). In this regard, it has been suggested that mediators present in cerebrovascular

pathologies such as stroke could become a stimulus to activate the SSAO/VAP-1 shedding process from the membrane-bound form. Interestingly, the maximal plasma SSAO activity increase was observed in hemorrhagic stroke patients at 4-6 hours after the symptoms onset (Hernandez-Guillamon *et al.*, 2012). In the two hSSAO/VAP-1-expressing endothelial cell models used in this work, a soluble SSAO/VAP-1 release was also observed after reoxygenation, matching with the highest endothelial cell death. In this context, SSAO/VAP-1 present in plasma is increased in some diseases associated with vascular complications, where both its pro-inflammatory action and its toxic catalytic products can be related to vascular damage (Conklin *et al.*, 1998; Hernandez *et al.*, 2006; Unzeta *et al.*, 2007). Therefore, the released SSAO/VAP-1 may be not only able to contribute to the local vascular cell damage through its catalytic action, as previously described by our group (Hernandez *et al.*, 2006), but also bring it to other cerebral regions through the circulation, and contribute to the BBB breakdown.

By using HUVEC hSSAO/VAP-1 cells, the involvement of MMPs in the SSAO/VAP-1 shedding under OGD was assessed, since they can be activated during stroke, where they are associated with the appearance of hemorrhagic transformation events. The exact cellular source of MMPs remains unknown, however, MMP-2 seems to be more associated to endothelial cells (Reuter *et al.*, 2012), so it was particularly studied. Identical results were obtained using different MMP inhibitors, the unspecific batimastat-BB94, and the specific MMP-2 inhibitor, which points out that MMP-2 could be partly responsible of the SSAO/VAP-1 shedding in our experimental model. However, it is reasonable to not rule out the possible involvement of MMP-8 or others, given that the inhibitor concentrations used are close to the IC_{50} values for both MMP-2 and MMP-8, and the fact that SSAO/VAP-1 shedding was not completely abolished.

The BBB degradation and hemorrhage occurrence in stroke have been associated to the binding of inflammatory cells to endothelia for their infiltration to brain tissue after stroke (Rosell *et al.*, 2008). In this context, inhibition of the

leukocytes adhesion and transmigration could have a beneficial effect on the post-ischemic damage. Given that VAP-1 can mediate leukocyte extravasation through its SSAO activity, leukocyte binding assay was studied with hSSAO/VAP-1-expressing HUVEC and hCMEC/D3 cells under OGD conditions. Results showed an increased THP-1 leukocytes binding after 5h OGD in presence of its substrate, which was prevented by the SSAO inhibitor. Thus, this data introduces SSAO/VAP-1 as a new target to be considered in the prevention of the leukocyte infiltration and subsequent damage in stroke.

Thereby, two useful endothelial cell models of ischemic stroke have been established, where the involvement of SSAO/VAP-1 has been assessed in this work, which involved cell damage, inflammation and leukocyte adhesion processes under ischemic conditions. The SSAO/VAP-1-expressing endothelial cells can help to investigate new SSAO/VAP-1 functions, and the screening and evaluation of potential molecules as specific SSAO/VAP-1 inhibitors, aimed to obtain a beneficial effect on ischemic stroke or other pathological conditions. In this regard, the protective effects of two promising compounds, DPH-4 and simvastatin, have been studied in this work for the treatment of stroke.

DPH-4 belongs to the donepezil + propargylamine + 8-hydroxyquinoline (DPH) family, which is a novel multitarget-directed ligands (MTDL) family designed for the therapy of Alzheimer's disease (AD) (Wang *et al.*, 2014). This approach, based on the "one drug, multiple target" paradigm, has been suggested to be more appropriated for the treatment of AD, and we think it may be also helpful for other pathologies (Buccafusco *et al.*, 2000; Youdim *et al.*, 2005; Bolea *et al.*, 2013b). The derivatives of DPH family have been evaluated as inhibitors of cholinesterases (AChE and BuChE) and monoamine oxidases (MAO A and MAO B), as biometal-chelators versus Cu^{2+} and Fe^{2+}. DPH-4 shows ADMET properties, brain penetration capacity and it shows as well a significant protection against scopolamine-induced learning deficits in healthy adult mice (Wang *et al.*, 2014). Moreover, DPH-4 is able to inhibit bovine SSAO activity at low micromolar range

(IC_{50} value of 2.8 ± 0.7 µM) and it also inhibits human SSAO activity at around 1 µM.

With our cellular models of cerebral ischemia, at the concentrations assayed, DPH-4 pre-treatment showed a protective effect on hSSAO/VAP-1-expressing hCMEC/D3 cells in presence of MA in both normoxia and in OGD with reoxygenation conditions. This protection was found to be even more significant when the same experiment was carried out on hSSAO/VAP-1-expressing HUVEC cells. This different behaviour might be explained because cerebral endothelial cells constitute a firm barrier formed by tight junctions joining plasma membranes of neighbouring cells that may hinder DPH-4 access on hCMEC/D3 cells. The beneficial effect of DPH-4 was also noticeable in terms of inflammation, since it significantly reduced the leukocyte adhesion to the endothelia subjected to different OGD conditions as a result of its inhibitory effect on the SSAO/VAP-1 activity. This anti-inflammatory effect may also contribute to the protective effect observed in this experimental model of stroke. In addition, when $A\beta_{1-40}D$ treatment was performed in this experimental model of ischemia simulating a pre-existing AD pathology, DPH-4 showed a protective effect on the synergic damage induced by MA and $A\beta_{1-40}D$. These results allow us concluding that $A\beta_{1-40}D$ together with the catalytic action of SSAO/VAP-1 induces more vascular damage under OGD with reoxygenation conditions, and that the protective effect of DPH-4 is higher than that observed using separately both toxics.

Thus, DPH-4, as a donepezil + propargylamine + 8-hydroxyquinoline hybrid, able to protect both HUVEC hSSAO/VAP-1 and hCMEC/D3 hSSAO/VAP-1 cells under hypoxia conditions through its inhibitory and anti-inflammatory effect on SSAO/VAP-1, could be considered as a promising drug with high therapeutic interest to be used for both AD and ischemic stroke.

Another compound that has been studied in this work is simvastatin, one member of statins family, which has demonstrated to improve both the incidence and outcome in acute ischemic stroke, showing thus good perspectives of

therapeutic efficacy (Montecucco *et al.*, 2012; Ni Chroinin *et al.*, 2013). It was chosen in this work among all the statins because a meta-analysis study revealed that it induced the most potent effects in experimental stroke models (Garcia-Bonilla *et al.*, 2012; Campos-Martorell *et al.*, 2014). Since the beneficial effects of statins include the reduction of the infarct volume, so does it the SSAO/VAP-1 inhibition, as well as the amelioration of the inflammation and the endothelial adhesion molecules activation, we studied whether the beneficial effects of simvastatin on stroke could be mediated by the modulation of the SSAO/VAP-1 levels with the hSSAO/VAP-1-expressing hCMEC/D3 cells under OGD conditions and also with two different animal models of stroke (eMCAO and tMCAO).

Simvastatin treatment significantly suppressed the release of soluble SSAO/VAP-1, both observed in two MCAO animal models and in OGD with reoxygenation-insulted hSSAO/VAP-1-expressing hCMEC/D3 cells. This can bring about beneficial effects against ischemia since the ischemic condition can trigger the release of plasmatic SSAO/VAP-1, found in both this current work and in the previously observed results from human samples (Hernandez-Guillamon *et al.*, 2010), which significantly correlates with the infarct volume. Moreover, the suppression of the SSAO/VAP-1 release would also be beneficial since this protein is largely related with the inflammation process, and the soluble plasma SSAO/VAP-1 could augment the lymphocyte binding to endothelial cells as well, presumably by triggering the up-regulation of other functional adhesion molecules on lymphocytes (Jalkanen *et al.*, 2001).

In addition, we found that simvastatin anti-inflammatory action was partly mediated by the inhibition of the SSAO/VAP-1-dependent expression of other adhesion molecules, rather than through the inhibition of SSAO/VAP-1 enzymatic activity, because it doesn't behave as an inhibitor of SSAO. Other inflammatory leukocyte-endothelial cell adhesion molecules play as well an important role in ischemic cerebrovascular pathology such as ICAM-1 and VCAM-1 (Frijns *et al.*, 2002). In this regard, the oxidase activity of SSAO/VAP-1 is able to induce the

expression of E- and P-selectins, VCAM-1 and ICAM-1 (Jalkanen *et al.*, 2007; Lalor *et al.*, 2007), all of them contributing to the leukocyte adhesion cascade in inflammation. Our results showed that simvastatin treatment prevented the contralateral E-selectin and VCAM-1 enhancement as well as reduced the overall expression of these adhesion molecules. Similar trends were also observed for P-selectin and ICAM-1. The up-regulation of these adhesion molecules in the contralateral brain may be explained by the stimulation of soluble SSAO, which release is triggered by ischemia and can travel to the non-ischemic brain region. Simvastatin can inhibit the release of soluble SSAO to the circulation, hence attenuating the pro-inflammatory effects mentioned above. Furthermore, results also showed that simvastatin can reduce the overall expression of these adhesion molecules in the hSSAO/VAP-1-expressing hCMEC/D3 cells under normoxia and OGD conditions, which are concordant with simvastatin induced down-regulation of SSAO/VAP-1 mediated leukocyte adhesion on our brain endothelial cells under OGD condition. All these results allow us confirming that the anti-inflammatory effect of simvastatin can be partly mediated by the SSAO/VAP-1 and its associated adhesion molecules.

Additionally, our results also suggested that simvastatin can prevent the transcription factor NF-κB activity stimulated at ischemic brain region, which can induce by itself the expression of leukocyte adhesion molecules and other pro-inflammatory proteins, such as COX-2 or NOS-2 (Iadecola *et al.*, 2001; Moro *et al.*, 2004). The involvement of the NF-κB activity to the simvastatin-mediated actions was confirmed by the up-regulation of IκB-α, in both brain sides and in human brain endothelial cells after ischemia. This SSAO/VAP-1-independent effect of simvastatin is accordant with the reported inhibition action of statins on NF-κB activity (Kumagai *et al.*, 2004; Moro *et al.*, 2004; Tu *et al.*, 2014).

Therefore, all the simvastatin results from both experimental models suggest that the beneficial effect of simvastatin is partially mediated by a reduction of the inflammatory process. The major change that simvastatin introduced on SSAO/VAP-1 was the inhibition of its release, which correlated with the down-

regulation of other adhesion proteins on the contralateral side that could be presumably mediated by SSAO/VAP-1 catalytic activity. Therefore, we conclude that SSAO/VAP-1 is released as a soluble form by the stimuli of ischemia, and may travel along the circulation and reach non-infarcted brain areas, where it induces inflammation, amplifying the consequent damage in the infarcted brain.

Besides the role of SSAO/VAP-1 under ischemic conditions, the role of SSAO/VAP-1 as a possible nexus between ischemic stroke and AD was also investigated during this work, in consideration of the high relevance of SSAO/VAP-1 in both diseases, the high expression of SSAO/VAP-1 in the brain microvasculature and its functions under pathological conditions.

Firm evidences confirm that hypoxia can induce the up-regulation of BACE1 transcription, expression and activity, hence increasing the β-amyloid generation in non-endothelial cells or in animal brain tissues (Sun *et al.*, 2006; Zhang *et al.*, 2007; Guglielmotto *et al.*, 2009). However, the possible contribution of SSAO/VAP-1 to the production of the cerebrovascular-originated β-amyloid under different ischemic conditions had not been studied. By using hCMEC/D3 WT and hSSAO/VAP-1 cells, we found that the SSAO/VAP-1-expressing cells exhibited high levels of BACE1 expression when subjected to OGD with reoxygenation, compared with WT cells or cells in normoxic condition. MA treatment tended as well to induce more expression of BACE1 under nomoxia conditions in SSAO/VAP-1-expressing hCMEC/D3 cells. In both cases, the hypoxia inducible factor 1α (HIF-1α) and oxidative stress probably function as contributors for the up-regulation of BACE1 under hypoxic conditions (Zhang *et al.*, 2007; Guglielmotto *et al.*, 2009). HIF-1α induced overexpression of BACE1 maybe because hSSAO/VAP-1-expressing cells are more susceptible to OGD with reoxygenation insult. Oxidative stress induced overexpression of BACE1 by SSAO/VAP-1-expression cells can be mediated by H_2O_2, that is generated by the metabolic function of SSAO. This effect was found more clear under normoxia condition than OGD with reoxygenantion, which could be related with longer time oxidation of substrate by SSAO, hence more production of H_2O_2 than in OGD

conditions. However, more investigations should be done to confirm this explanation.

Moreover, SSAO/VAP-1 catalytic activity can mediate the overexpression of amyloid precursor protein (APP) under OGD with reoxygenation in the SSAO/VAP-1-expressing cells, since SSAO inhibitor completely inhibited the enhancement of APP induced by the metabolism of its substrate. Thus, there exists a high risk for the overproduction of Aβ peptides under this ischemic/reperfusion condition. It is well implicated that focal cerebral ischemia and MCAO insult can stimulate the expression and accumulation of APP in the animal brain tissues (van Groen *et al.*, 2005; Makinen *et al.*, 2008). Here we first describe that the metabolic function of SSAO/VAP-1 can also contribute to the Aβ generation under stroke condition, which may increase the risk of Aβ-related pathologies and exacerbate the worsening of the neurological outcome for both stroke and AD.

The neurovascular unit is responsible for regulating blood flow and controlling the exchange of substances across the BBB (Iadecola, 2010). Endothelial cells are essential components of both neurovascular unit and BBB, hence they also contribute to the clearance of Aβ peptides and then to maintain the balance of Aβ in the brain microenvironment homeostasis (Deane *et al.*, 2009) under pathophysiological conditions. Our results pointed out that OGD with reoxygenation treatment down-regulates the expression of LRP-1, which is a main receptor in the control of the Aβ peptides efflux across the BBB, thus it may also contribute to the Aβ over-accumulation induced under ischemia.

At last, when $A\beta_{1-40}D$ treatment was introduced in this ischemic experimental model simulating a pre-existing AD pathology, a synergic damage induced by MA and $A\beta_{1-40}D$ was further observed, and SSAO inhibitor significantly enhanced the cell viability in the presence of both toxics. Cerebral amyloid angiopathy (CAA), characterized by beta amyloid (Aβ) deposits in the cerebrovascular tissue, occurs in 80% of the AD patients (Ellis *et al.*, 1996). In this context, SSAO/VAP-1

present on the cerebrovascular tissue could induce more vascular damage by its catalytic activity under hypoperfusion or an ischemic attack, which actually often occur in AD pathology.

Therefore, the presence of SSAO/VAP-1 in the brain endothelium under ischemic stimulation in addition to the SSAO/VAP-1 substrate may facilitate the generation of β-amyloid, hence increasing the risk and neurological worsening of AD. It has been widely reported that the neurovascular unit plays a role in the AD pathology; in this context, the results herein described allow us to conclude that SSAO/VAP-1 could be a possible nexus between ischemic stroke and Alzheimer's disease. Taking toghter all the results of this work, we believe that SSAO inhibitors can be considered as new potential therapeutic approaches aimed to gain beneficial effects for both ischemic stroke and AD pathologies.

VI. CONCLUSIONS

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The results obtained in this work allow us to draw the following conclusions:

1. SSAO/VAP-1 expression increases the susceptibility of endothelial cells to OGD-induced damage, and the oxidation of its substrates through its enzymatic activity increases this vascular cell damage. Caspase-3 and caspase-8 are activated during the death process.

2. OGD constitutes a stimulus for the soluble SSAO/VAP-1 release *in vitro*, partly mediated by metalloproteinase-2-dependent shedding.

3. SSAO/VAP-1 activity-dependent leukocyte binding can further exacerbate the cell damage by augmenting inflammation in cerebral ischemia.

4. The inhibition of SSAO/VAP-1 activity by DPH-4 can protect both SSAO/VAP-1-expressing endothelial cells, decrease the SSAO-dependent leukocyte adhesion, and ameliorate the cell damage induced by OGD and reoxygenation in the presence of beta amyloid as a model of AD pathology. Therefore, it can provide a therapeutic benefit to the delay and/or prevention of ischemic stroke as well as its progression to AD.

5. Simvastatin can block the release the soluble SSAO/VAP-1 after an ischemic stimulus, thus preventing its pro-inflammatory-associated effects. Soluble SSAO/VAP-1 could reach non-infarcted brain areas through the blood circulation and induce the expression of the adhesion molecules E-selectin and VCAM-1, amplifying the inflammation and the consequent damage in the infarcted brain.

6. In the presence of SSAO/VAP-1, OGD with reoxygenation induces the expression of BACE1 and decreases the expression of LRP-1, and the substrate of SSAO/VAP-1 can further up-regulate the levels of APP, which

may facilitate the generation of β-amyloid and increase the risk and neurological worsening of AD.

7. Taken all together these results, we conclude that SSAO/VAP-1 could be the nexus of both AD and stroke pathologies reinforcing the important role that brain vasculature can play in these neurodegenerative diseases.

8. The design and synthesis of new specific SSAO/VAP-1 inhibitors, would allow us to go further in the elucidation of the important role of this enzyme in vascular disorders and to advance in the therapeutical treatment of AD and Stroke.

VII. BIBLIOGRAPHY

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VIII. ANNEXES

Annex I: Information of antibodies used in this work

Information of primary antibodies used in this work

Antigens	Hosts	Applications	Dilution	Companies	References
APP 20.1	mouse	WB	1:1000	W.E. Van Nostrand	-
BACE1	rabbit	WB	1:1000	Abcam	ab2077
Bax	rabbit	WB	1:1000	Cell Signaling	2772
Bcl-2	mouse	WB	1:1000	BD Biosciences	610538
bovine SSAO	rabbit	WB	1:1000	(Lizcano et al., 1998)	-
Caspase-12	rabbit	WB	1:1000	Abcam	ab62484
Caspase-8	mouse	WB	1:1000	BD Pharmingen	551242
Cleaved caspase-3	rabbit	WB	1:1000	Cell Signaling	9661
COX-2	rabbit	WB	1:1000	Santa Cruz	sc-1747R
E-selectin	rabbit	WB	1:500	Santa Cruz	sc-14011
Flotillin-1	mouse	WB	1:1000	BD Biosciences	610820
GAPDH	mouse	WB	1:20000	Ambion	4300
GRP78	mouse	WB	1:1000	BD Biosciences	610978
ICAM-1	rabbit	WB	1:1000	GeneTex	GTX100450
IGF1-R	rabbit	WB	1:1000	Santa Cruz	sc-713
IκB-α	rabbit	WB	1:1000	Santa Cruz	sc-371
LRP-1	rabbit	WB	1:1000	Epitomics	2703-S
NOS-2	rabbit	WB	1:1000	Santa Cruz	sc-651
P-selectin	rabbit	WB	1:1000	BioVision	3633R-100
RAGE	rabbit	WB	1:1000	Epitomics	T0769
SOD-1	goat	WB	1:1000	Santa Cruz	sc-8637
Tf Rec	mouse	WB	1:1000	ZYMED	136800
VAP-1	rabbit	WB	1:1000	Abcam	ab42885
VCAM-1	rabbit	WB	1:1000	Epitomics	3540-S
β-actin	mouse	WB	1:5000	Sigma-Aldrich	A1978

Information of secondary antibodies used in this work

Antigens	Conjugate	Applications	Dilution	Companies	References
goat IgG	HRP	WB	1:2000	Pierce	HB987316
mouse IgG	HRP	WB	1:1000	Dako	P0161
rabbit IgG	HRP	WB	1:1000	BD Biosciences	554021

Annex II: Other publications

Annex II. 1. Esteban G, Bolea I, **Sun P**, Solé M, Samadi A, Marco-Contelles J, Unzeta M. A therapeutic approach to cerebrovascular diseases based on indole substituted hydrazides and hydrazines able to interact with human vascular adhesion protein-1, monoamine oxidases (A and B), AChE and BuChE

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A therapeutic approach to cerebrovascular diseases based on indole substituted hydrazides and hydrazines able to interact with human vascular adhesion protein-1, monoamine oxidases (A and B), AChE and BuChE

Gerard Esteban · Irene Bolea · Ping Sun · Montse Solé · Abdelouahid Samadi · José Marco-Contelles · Mercedes Unzeta

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Abstract Herein, we report the biological evaluation of a series of indole substituted hydrazides and hydrazines throughout the assessment of their multipotent inhibitory potency towards monoamine oxidase (MAO) A and B, semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1), and the cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Hydrazine **JL72** (3-(3-hydrazinylpropyl)-1*H*-indole) showed a potent, reversible and non-time-dependent inhibition of MAO-A, which suggests its capacity in restoring serotoninergic neurotransmission being devoid of the side effects observed for classic MAO-A inhibitors. In addition, **JL72** behaved as a moderate BuChE inhibitor. Finally, both hydrazines and hydrazides derivatives showed high affinity towards SSAO/VAP-1. Among them, **JL72** behaved as a noncompetitive and the most potent inhibitor ($IC_{50} = 0.19 \pm 0.04$ μM), possessing also a significant anti-inflammatory activity. The combined inhibition of SSAO/VAP-1, MAO (A and B), AChE and BuChE appear as an important therapeutic target to be considered in the treatment of cerebrovascular and neurological disorders such as Alzheimer's disease.

Keywords Hydrazine · Hydrazide · Monoamine oxidase · Vascular adhesion protein-1 · Acetylcholinesterase · Butyrylcholinesterase

Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
BuChE	Butyrylcholinesterase
CAA	Cerebral amyloid angiopathy
DTNB	5,5′-Dithiobis-2-nitrobenzoic acid
5-HT	5-Hydroxytryptamine
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MA	Methylamine
SSAO/VAP-1	Semicarbazide sensitive amine oxidase/vascular adhesion protein-1

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the central nervous system, associated with cognitive impairment and dementia (Goedert and Spillantini 2006). β-Amyloid (Aβ) accumulation in the brain parenchyma produces senile plaques, a characteristic hallmark of AD and also produces the vascular deposits of cerebral amyloid angiopathy (CAA) (Castro et al. 2006). CAA is present in most cases of AD and it is characterised by the deposition of Aβ in the tunica media and adventitia of leptomeningeal vessels and intracortical microvessels, thus producing the degeneration of vascular smooth muscle

This manuscript is dedicated to the memory of Dr. Eldiberto M. Fernández-Álvarez (1928–1996).

G. Esteban · I. Bolea · P. Sun · M. Solé · M. Unzeta (✉)
Departament de Bioquímica i Biología Molecular,
Facultat de Medicina, Universitat Autònoma de Barcelona,
Bellaterra, 08193 Barcelona, Spain
e-mail: mercedes.unzeta@uab.cat

A. Samadi · J. Marco-Contelles
Laboratorio de Química Médica (IQOG, CSIC),
C/Juan de la Cierva 3, 28006 Madrid, Spain

cells and endothelial cells (Vinters et al. 1988). The fact that AD and cerebrovascular diseases share risk factors supports the common view that there is a link between vascular degeneration and AD. It has been suggested that the accumulation of Aβ in the vessel wall causes the functional deterioration of the blood brain barrier, which is essential for the correct transport and clearance of Aβ from parenchyma (Deane et al. 2004; Zlokovic 2005).

Semicarbazide-sensitive amine oxidase, also called vascular adhesion protein-1 (SSAO/VAP-1, E.C 1.4.3.21) is a multifunctional enzyme involved in the metabolic deamination of primary amines, the functions of which depend on the tissue where it is expressed (Jalkanen and Salmi 2008). SSAO/VAP-1 shows some overlap with monoamine oxidase [E.C.1.4.3.4], which is responsible of the primary, secondary and tertiary amine metabolism in human brain. MAO is present in most mammalian tissues and exists as two distinct enzymatic isoforms, MAO-A and MAO-B, based on their substrate and inhibitor specificities (Johnston 1968). SSAO/VAP-1 is a circulating and membrane-bound ectoenzyme, present in vascular tissue localised in two different types of cells such as smooth muscle cells and endothelial cells being these involved in the leukocyte and neutrophil recruitment through its SSAO catalytic activity (Koskinen et al. 2004). Alterations of SSAO/VAP-1 levels in human plasma have been related to several pathological situations, such as AD (Ferrer et al. 2002) and ischaemic stroke, where abnormally high plasma VAP-1/SSAO activity has been found in patients with certain vascular and/or inflammatory conditions with bad prognosis (Hernández-Guillamon et al. 2012, 2010). In this context, the inhibition of SSAO/VAP-1 and/or MAO-A and MAO-B activities, appears as an important therapeutic target to be used in the treatment of these cerebrovascular disorders. On the other hand, low levels of acetylcholine (ACh) (Geula and Mesulam 1999) are thought to play significant roles in the pathophysiology of AD (Perry et al. 1977). The cholinergic theory of AD suggests that the selective loss of cholinergic neurons in this disorder results in a deficit of ACh in specific brain regions that mediate learning and memory functions (Davies and Maloney 1976). However, alterations in other neurotransmitter systems, especially the serotoninergic and dopaminergic, are also thought to be responsible for the behavioral disturbances observed in patients with AD (García-Alloza et al. 2005; Terry et al. 2008). Thus, in addition to cholinesterase enzymes (acetylcholinesterase, AChE and butyrylcholinesterase, BuChE) and SSAO/VAP-1, monoamine oxidases also (MAO; EC 1.4.3.4) appear as an important target to be considered for the treatment of specific features of this complex and multifactorial disease.

Herein, we report the biological evaluation (MAO-A, MAO-B, SSAO/VAAP-1, AChE and BuChE inhibitory profile) of a number of indole substituted hydrazides and hydrazines. These compounds were designed and synthesized for the first time by Prof. Fernández-Álvarez's laboratory in the IQOG (CSIC, Madrid, Spain) as potential in vitro bovine MAO inhibitors (Alemany et al. 1966, 1975; Bernabé et al. 1971; Monge et al. 1985). In the present work, we have found that some of them and especially **JL72** (3-(3-hydrazinylpropyl)-1*H*-indole) are able to interact with human SSAO/VAP-1, MAO (A and B), as well as AChE and BuChE, showing an interesting pharmacological profile and suggesting their potential to be used in the therapy of cerebrovascular and cognitive decline disorders.

Materials and methods

HUVEC and HUVEC hSSAO/VAP-1 cell culture

These cell lines were prepared in our laboratory as previously described (Sole et al. 2011). Cells were cultured in M199 (Invitrogen), supplemented with 5 % FBS, 1.2 mM L-glutamine and antibiotics (50 U/ml penicillin/0.05 mg/ml streptomycin). The selected antibiotic geneticin (G418) was added at a final concentration of 100 μg/ml to maintain the SSAO/VAP-1 expression in HUVEC hSSAO/VAP-1 cells. Cells were maintained in an incubator at 37 °C, 5 % CO_2, and were subcultured every 5–7 days.

Chemistry

The indole substituted hydrazides and hydrazines **JL34**, **JL40**, **JL71**, **JL87**, **JL317**, **JL432**, **JL72**, **JL411**, and **JL418** (Fig. 1) were synthesized and purified according to the methods described in the literature (Alemany et al. 1966, 1975; Bernabé et al. 1971; Monge et al. 1985).

Inhibition experiments of MAO (A and B) and SSAO/VAP-1

Mitochondria from rat liver homogenates were used as source for MAO activities (Gómez et al. 1986). The inhibitory activity of the compounds towards MAO-A and MAO-B was determined as previously described (Fowler and Tipton 1981), using [^{14}C]-labelled substrates (Perkin Elmer, USA). For comparative purposes, **phenelzine** was used as reference compound. MAO-B activity was determined towards 20 μM [^{14}C]-phenylethylamine (PEA) (2.5 mCi/mmol) and MAO-A activity towards [^{14}C]-(5-hydroxy-triptamine) (5-HT) 100 μM (0.5 mCi/mmol). SSAO/VAP-1 activity was measured towards [^{14}C]-benzylamine 100 μM (2 mCi/mmol) using microsomes from bovine lung as source (Lizcano et al. 1996). Inhibition

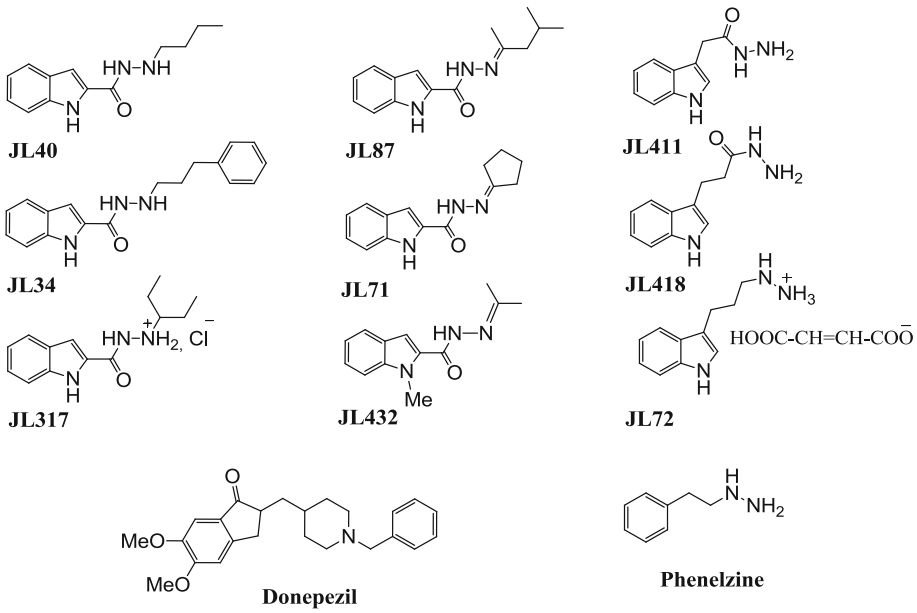

Fig. 1 Structures of the indole substituted hydrazides and hydrazines, and the reference compounds **donepezil** and **phenelzine**

curves were made by pre-incubating the enzyme with at least nine concentrations of each compound for 30 min in 50 mM phosphate buffer (pH 7.2). A sample without compound was always present to determine the 100 % enzyme activity. The reaction was carried out at 37 °C adding 25 µL of substrate in a final volume of 225 µL and stopped by the addition of 100 µL in 2 M citric acid. Radiolabelled aldehyde products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6 % (w/v) 2,5-diphenyloxazole before liquid scintillation counting (Tri-Carb 2810TR). The inhibition curves were expressed in percentage of total activity and the IC_{50} values were calculated by using the GraphPad Prism program (Prism 3.0, GraphPad Software Inc). Total protein was measured by the method of Bradford (1976) using bovine–serum albumin as standard. Data are the mean ± SEM of at least three different experiments realised in triplicate.

Inhibition experiments of AChE and BuChE

To assess the inhibitory activity of the compounds towards AChE (E.C.3.1.1.7) and BuChE (E.C.3.1.1.8), we followed a spectrophotometric method (Ellman et al. 1961), using purified AChE from *Electrophorus electricus* (Type V–S), or BuChE from equine serum (lyophilized powder) (Sigma-Aldrich, Madrid, Spain). For comparative purposes, **donepezil** was used as reference compound. The reaction took place in 96-well plates in a final volume of 300 µL in 0.1 M phosphate-buffered solution (pH 8.0) containing 0.035 U/ml AChE or 0.05 U/ml BuChE and 0.35 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich, Madrid, Spain). Inhibition curves were made by pre-incubating this mixture with serial dilutions of each compound for 20 min. The activity in the absence of compounds was always performed to determine the 100 % of enzyme activity. After the mentioned pre-incubation period, 0.35 mM acetyltiocholine iodide or 0.5 mM butyrylthiocholine iodide (Sigma-Aldrich) were added, allowing five more minutes of incubation, where the DTNB produces the yellow anion 5-thio-2-nitrobenzoic acid along with the enzymatic degradation of both substrates. Changes in absorbance were detected at 405 nm in a spectrophotometric plate reader (FluoStar OPTIMA, BMG Labtech). Compounds inhibiting AChE or BuChE activity would reduce the colour generation, thus, IC_{50} values were calculated as the concentration of compound that produces 50 % AChE or BuChE activity inhibition. Data are expressed as mean ± SEM of at least three different experiments in triplicate.

Reversibility and time-dependence inhibition studies

To study the nature of the MAO-A enzymatic inhibition exerted by **JL72**, the derivative with the most interesting pharmacological profile, the activity of the enzyme was determined in the presence and in the absence of the inhibitor before and after three consecutive washings with buffer. Enzyme samples were preincubated for 30 min at 37 °C with 100 nM **JL72** or 40 nM clorgyline, the latter used as control of irreversible inhibition. Samples were then washed with 50 mM phosphate buffer (pH 7.2) and centrifuged at 25,000*g* for 10 min at 4 °C consecutively three times. Finally, total protein was measured and MAO-A activity was determined as described above. We next evaluated the time-dependence inhibition of the reaction. Samples of enzyme plus **JL72** at the indicated concentration range (10^{-9} to

10^{-3} M), were preincubated for 0, 30, 60, 120, and 180 min before MAO-A activity determination as described above.

Kinetic analysis of MAO-A and SSAO/VAP-1 inhibition

To obtain estimates of the mechanism of action of **JL72** on MAO-A and SSAO/VAP-1, reciprocal plots of 1/V versus 1/[S] were constructed at different concentration of the substrate 5-HT (10–500 μM) for MAO-A and benzylamine (10–200 μM) for SSAO/VAP-1, as previously described (Fowler and Tipton 1981). The plots were assessed by a weighted least-squares analysis. Data analysis was performed with GraphPad Prism 3.0 software. Slopes of the reciprocal plots were then plotted against the concentration of **JL72** (0–1 μM for SSAO and 50–500 μM for MAO-A) to evaluate Ki data.

Leukocyte (THP-1)-endothelial (HUVEC) cell adhesion assay

HUVEC and HUVEC transfected hSSAO/VAP-1 monolayers were prepared, as previously reported (Sole et al. 2011). For leukocyte adhesion assays, cells were pre-treated with **JL72** or **phenelzine** at 10 μM for 1 h. Then, 1 mM methylamine (MA), a SSAO/VAP-1 specific substrate, was added to the media for the next 24 h. At the end of the treatment, calcein/AM-dyed THP-1 cells were added to the HUVEC monolayer, and were allowed to bind to the endothelial cells for 30 min at 37 °C. After this period, plates were successively washed in order to discard non-bound THP-1 cells. Leukocyte-binding ability was determined by the emitted fluorescence of bound THP-1 cells, measured at ex/em 495/530 nm. Non-treated cells were used as control.

Results and discussion

MAO-A, MAO-B and SSAO enzymatic inhibition

The indole substituted hydrazines and hydrazides were first evaluated as inhibitors of MAO-A and MAO-B (from rat liver mitochondria), and SSAO (from bovine lung microsomes). Values were compared with the inhibition exerted by the reference compound **phenelzine** (Table 1). Although compounds **JL87**, **JL71**, **JL432**, **JL411**, **JL418** were not active for MAO-A, compounds **JL40**, **JL34**, **JL317** showed a significant MAO-A inhibitory profile, acting in the micromolar range ($IC_{50} = 3.3 \pm 0.9$ to 80.3 ± 14.7 μM) (Table 1). Interestingly, **JL72** showed the highest potency ($IC_{50} = 0.12 \pm 0.03$ μM) and gave a similar value than that observed for the reference compound **phenelzine** ($IC_{50} = 0.13 \pm 0.02$ μM). Interestingly, we observed that only compounds **JL40** and **JL317** were able to interact with both MAO-A and MAO-B enzymes. Conversely, all analysed compounds showed high affinity towards SSAO/VAP-1 enzyme, showing IC_{50} values ranging from 0.19 ± 0.04 μM for **JL72** to 79.2 ± 10.5 μM for **JL40**.

From the structure–activity relationship (SAR) study some conclusions can be drawn. First of all, and comparing compounds bearing a substituent at the indole C2 position, it is clear that the *N*′-alkylidene-1*H*-indole-2-carbohydrazides **JL87**, **JL71**, and **JL432** are very weak towards MAO-A, but moderate towards MAO-B inhibitors, while the *N*′-alkyl-1*H*-indole-2-carbohydrazides **JL34** and **JL317** are moderate and selective MAO-A inhibitors. Also note that the substitution of a methyl by a phenyl group in **JL40** to render **JL34** increases the inhibitory effect on MAO-A, but strongly drops its inhibitory potency on MAO-B. Comparing with compounds bearing substituents at C3 position of the indole ring, such as **JL411** and **JL418** the presence of the carbonyl group loses the inhibitory activity drastically in both MAO-A and MAO-B isoforms. On the other hand, **JL72** is the only compound that does not have carbonyl group in its molecule. This could be the structural feature responsible for the facilitation of the interaction with the active centre showing its high inhibitory potency towards MAO-A and MAO- B comparing with the rest of molecules. It is worth to remark the high selectivity of **JL72** towards MAO-A, comparing with MAO-B.

Concerning the effects on SSAO inhibition, very surprisingly, the presence of such substituents on the C2 and C3 position does not affect the inhibitory activity of these compounds. Among them, the most potent are **JL34**, **JL411**, **JL418** and **JL72**, the more active being 3-(3-hydrazinylpropyl)-1*H*-indole (**JL72**). It is worth to remark that the presence of the indole ring in all these hydrazine analogs can induce the inhibitory interaction towards MAO-A because of the similarity towards serotonin, its specific substrate. Furthermore, they could act as potential pharmacological agents stimulating the monoaminergic and catecholaminergic transmission, showing the **JL72** the highest affinity towards MAO-A inhibition. The potency of **JL72** as SSAO inhibitor, suggests that this compound may also possess a good anti-inflammatory profile.

AChE and BuChE inhibition

To complete the study of the biological profile of the indole substituted hydrazides and hydrazines, the inhibitory activity against AChE (from *Electrophorus electricus,* EeAChE) and BuChE (from equine serum, eqBuChE) was determined by using Ellmans method (Ellman et al. 1961) and compared with that of the reference compound

Table 1 EeAChE, eqBuChE, rMAO-A, rMAO-B and bSSAO/VAP-1 Inhibitory activities of indole substituted hydrazides and hydrazines **JL34**, **JL40**, **JL71**, **JL87**, **JL317**, **JL432**, **JL72**, **JL411**, and **JL418**

Compound	IC_{50} (μM)[a]				
	rMAO-A	rMAO-B	bSSAO/VAP-1	*Ee*AChE	eqBuChE
JL40	80.3 ± 14.7	5.0	79.2 ± 10.5	>100	>100
JL34	3.3 ± 0.9	>100	10.6 ± 0.4	86.4 ± 15.8	>100
JL317	5.5 ± 1.5	33.2	45.8 ± 4.3	>100	16.1 ± 1.1
JL87	>100	47.1	31.6 ± 8.1	>100	>100
JL71	>100	84.0	29.7 ± 8.5	>100	49.2 ± 11.3
JL432	>100	54.3	37.2 ± 5.5	>100	>100
JL411	>100	>100	13.3 ± 2.9	>100	>100
JL418	>100	>100	17.2 ± 4.2	>100	>100
JL72	0.12 ± 0.03	>100	0.19 ± 0.04	46.3 ± 2.9	25.3 ± 6.5
Phenelzine	0.13 ± 0.02	2.3 ± 0.41	0.011 ± 0.002	>100	>100
Donepezil	>100	>100	–	0.022 ± 0.003	3.6 ± 0.53

[a] Values are expressed as the mean ± SEM of at least three independent experiments performed in triplicate

donepezil. The obtained IC_{50} values are summarised in Table 1. According to these values, the derivatives are poor inhibitors of *Ee*AChE, with the exception of **JL34** and **JL72** that showed activity in the micromolar range (IC_{50} = 86.4 ± 15.8 μM and 46.3 ± 2.9 μM, respectively). Nevertheless, these values are very far from the IC_{50} value determined for **donepezil** (Table 1), one of the most potent AChE inhibitors used in AD therapy. Similar trends were observed when the inhibitory potency was determined towards eqBuChE. In this case, only compounds **JL317**, **JL71** and **JL72** proved to be active in the micromolar range showing IC_{50} from 16.1 ± 1.0 μM to 49.2 ± 11.3 μM, being the most potent 3-(3-hydrazinylpropyl)-1*H*-indole (**JL 317**), almost equipotent with **donepezi**l. Note that, whereas compounds **JL317** and **JL71** are selective towards eqBuChE, **JL72** is almost equipotent for both enzymes.

Concerning the SAR study, the best inhibitor for both enzymes (*Ee*AChE and eqBuChE) is the indole hydrazine **JL72**, bearing the substituent at C3 position on the indole ring. Taking all these results into account, **JL72** (3-(3-hydrazinylpropyl)) was selected as the most interesting compound to follow up with its biological evaluation as an indole-2-carbohydrazyde able to interact with five different enzymatic systems, all of them involved in Alzheimer's disease pathology.

Reversibility and time-dependence inhibition

To study the type of inhibition of **JL72**, the most potent inhibitor of the series, towards MAO-A, we analysed the reversibility/irreversibility of the binding. We observed that **JL72** reversibly inhibited MAO-A, since the inhibition was significantly reverted (from 34.1 to 19.1 %) after three consecutive washings and centrifugations with buffer (Fig. 2a). Clorgyline was used under the same experimental conditions for comparative purposes and, in agreement with data reported in literature, behaves as an irreversible inhibitor. The reversibility of MAO-A inhibition by **JL72** was also confirmed by the time-dependent inhibition analysis (Fig. 2b), where identical inhibition was observed in spite of the different periods of incubation-time (0–180 min). The observed slight shift of the curve from zero time could be explained by the small delay in adding the substrate. This was considered not significant, since the time-independent inhibition was confirmed by finding similar inhibitory behavior at the other analysed times. Thus, these results allowed us to conclude that **JL72** behaves as a reversible and non time-dependent inhibitor of MAO-A.

In this regard, and because of the structural similarity with serotonin, a specific MAO**-**A substrate, **JL72** can be considered a potent inhibitor of this MAO isoform with potential benefit in restoring and enhancing the serotoninergic transmission altered in depression and other psychological disorders.

Kinetic analysis of SSAO and MAO-A

To gain further insight into the kinetic behavior of this family of compounds on SSAO and MAO-A, a kinetic study was carried out with **JL72**, the most promising compound investigated. Graphical analysis of the reciprocal to Lineweaver–Burk plots (Fig. 3), showed increased slopes (decreased V_{max}) and constant K_m values in both enzymes analysed, indicating that **JL72** behaves as a noncompetitive inhibitor and thus it binds to a different site from the active site in both MAO-A and SSAO enzymes.

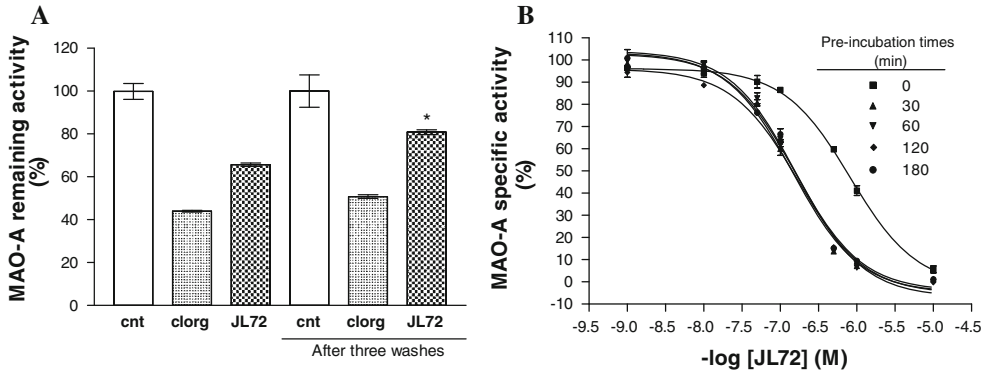

Fig. 2 Reversibility studies of MAO-A inhibition by **JL72**. **a** MAO-A was inhibited at 100 nM **JL72** and 40 nM clorgyline for 30 min. Then, three consecutive washings were performed with buffer. **b** Time-dependence inhibition was studied at several times of pre-incubation (0–180 min) of MAO-A with **JL72**. Data are the mean ± SEM of four independent experiments in triplicate. *$p < 0.05$ versus non-washed

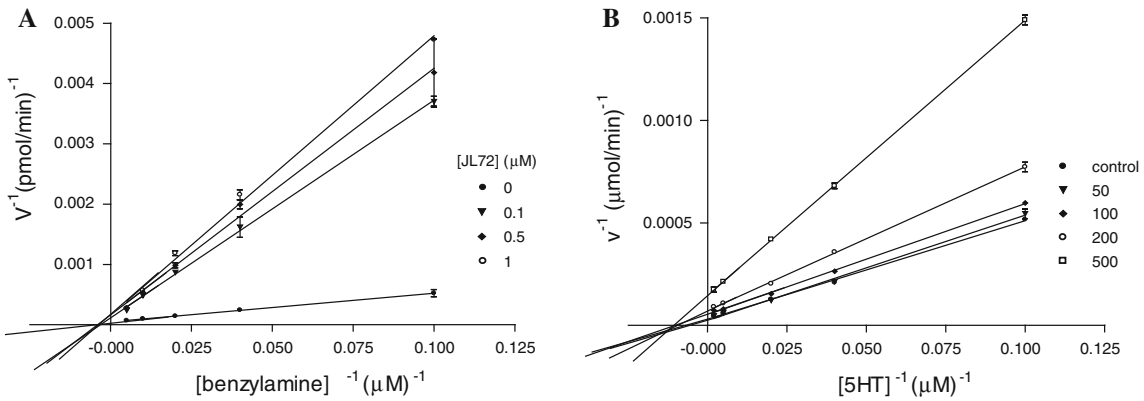

Fig. 3 Kinetic behavior of SSAO and MAO-A inhibition by **JL72**. **a** Double reciprocal plots (Lineweaver–Burk transformation) of SSAO inhibition by **JL72** towards (10–200 μM). **b** Double reciprocal plots (Lineweaver–Burk transformation) of MAO-A inhibition by **JL72** towards 5-HT as a substrate (10–500 μM). Data are the mean ± SEM of three different experiments

Replots of the slope versus concentration of **JL72**, gave an estimate of the inhibition constant Ki towards MAO-A of 163.2 ± 12.1 and 60.8 ± 12.5 nM towards SSAO. These results confirm the high inhibitory potency of **JL72** towards both MAO-A and SSAO.

Inhibition of SSAO activity in HUVEC hSSAO/VAP-1 cells by JL72 and phenelzine

The toxicity of **JL72** and **phenelzine** on HUVEC transfected hSSAO/VAP-1 cells was assessed as a previous step to determine their inhibitory behavior. Thus, the viability of cells treated with **JL72** and **phenelzine** at concentration range of (0.1–100 μM) was analysed after 30-min pre-incubation by the MTT method (Mosmann 1983). **Phenelzine** did not affect cell viability at all concentrations assayed, whereas 10 μM **JL72** induced a loss of cell viability of the 20 %. Thus, the concentrations used were always lower than 10 μM. Then, in order to determine the enzymatic inhibition of hSSAO/VAP-1 expressed in HUVEC cells, treatments with **JL72** and **phenelzine** at concentrations of 0.1, 1 and 10 μM for 30 min were carried out. SSAO/VAP-1 activity was determined in cell lysates radiometrically as previously stated in "Materials and methods", and expressed as percentage activity compared to non-treated cells. A complete inhibitory effect was observed at concentrations higher than 1 μM in both cases (Fig. 4), showing that both compounds behave as potent SSAO inhibitors on HUVEC human transfected hSSAO/VAP-1 cells model.

Leukocyte (THP-1)-endothelial (HUVEC) cell adhesion assay

Concerning cerebrovascular diseases, where the inflammation is one of the most significant factors involved, finding a molecule capable of inhibiting leukocyte adhesion to the endothelial wall has an important therapeutic value. The high inhibitory potency of **JL72** towards SSAO enzymatic activity ($IC_{50} = 0.19 \pm 0.04$ nM) led us to

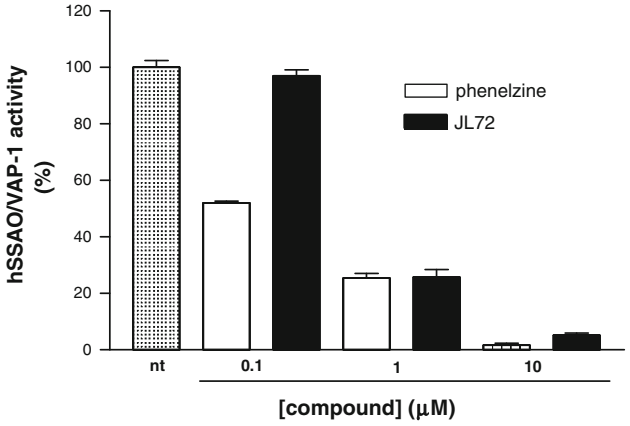

Fig. 4 HUVEC hSSAO/VAP-1 lysates were treated with **JL72** and **phenelzine** (0.1, 1 and 10 μM) for 30 min. SSAO activity was radiometrically measured and expressed as percentage activity compared to non-treated cells. Data are the mean ± SEM of three independent experiments

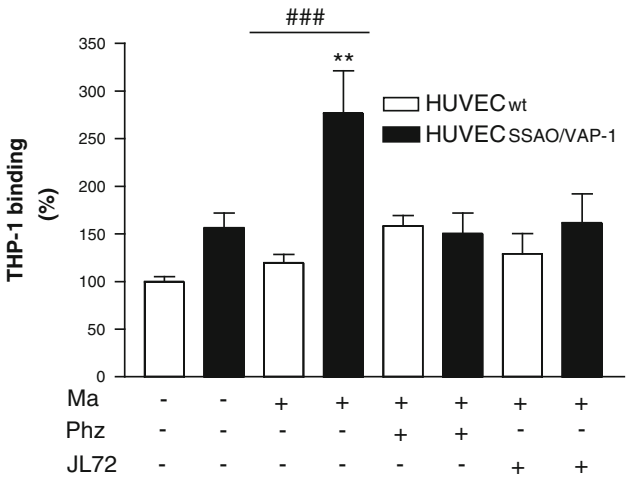

Fig. 5 Leukocyte (THP-1)-endothelial (HUVEC) cell adhesion assay. Higher THP-1 adhesion was observed in hSSAO/VAP-1 cells treated with MA compared to WT cells. As shown in the figure, both **phenelzine** and **JL72** significantly reduced THP-1 adhesion after MA addition in hSSAO/VAP-1 cells. Data are the mean ± SEM of five independent experiments $^{**}p < 0.01$ compared to non-treated hSSAO/VAP-1 cells, and $^{###}p < 0.001$ versus non-treated cells

consider whether this molecule can possess a potential anti-inflammatory effect. Consequently, endothelial HUVEC wild type cells, that do not express SSAO/VAP-1, and transfected hSSAO/VAP-1 cells were cultured as described in "Materials and methods". When cells were treated with methylamine (MA), a specific substrate of SSAO/VAP-1, the adhesion of leukocytes significantly increased when compared with the HUVEC wild type cells. One of the functions of this multifunctional enzyme through its catalytic action is to mediate the leukocyte-adhesion in inflammatory processes (Salmi et al. 1993). As expected, this effect disappears when transfected cells were pre-treated with **JL72** or **Phenelzine**, confirming the anti-inflammatory effect of these drugs on inhibiting the SSAO/VAP-1 activity (Fig. 5).

Conclusion

To sum up, some of the hydrazides and hydrazines studied here show a wide enzymatic inhibitory profile towards the two MAO isoforms, SSAO/VAP-1 and both AChE and BuChE, all them involved in Alzheimer's disease pathology. Among them, **JL34**, **JL317**, and **JL72**, are good and selective MAO-A inhibitors, thus possible modulators of the monoaminergic neurotransmission. In this regard, these molecules could act as potential antidepressant, and behavior enhancers, especially **JL72** a highly potent and selective MAO-A inhibitor. Then, **JL72**, may be able to stimulate the serotoninergic, catecholaminergic and noradrenergic transmission. Some other derivatives, such as **JL34**, **JL411**, **JL418**, and **JL72** were potent and selective inhibitors of SSAO/VAP-1. The involvement of this multifunctional enzyme in different pathologies, especially regarding Alzheimer and stroke (Ferrer et al. 2002; Hernandez-Guillamon et al. 2010, 2012), suggest that inhibitors of this enzyme can be a good therapeutic approach to solve these neurological disorders. Furthermore, the fact that **JL72** is able to block the leukocytes adhesion in human endothelial cells, transfected with human SSAO/VAP-1, confers to this molecule an additional anti-inflammatory profile. On the other hand, **JL317**, **JL71** and **JL72** were moderate and selective BuChE inhibitors. Only the hydrazine **JL72** showed a moderate non selective AChE inhibitory behavior. According to the Cholinergic hypothesis of Alzheimer's disease (Geula and Mesulam 1999), the cholinergic transmission is affected in this disorder. The IC_{50} values of **JL72**, herein, reported show that this compound could be able to restore cholinergic transmission by inhibiting AChE and at the same time to inhibit BuChE present in glia and both involved in Alzheimer's pathology. Recently it has been reported that the hydrazine type **phenelzine**, besides its antidepressant profile, it has a neuroprotective effect on neurons and astrocytes against formaldehyde-induced toxicity (Song et al. 2010). On the other hand **phenelzine** provided robust neuroprotection in the gerbil model of transient forebrain ischaemia (Wood et al. 2006).

Herein, we report a new hydrazine, **JL72**, that behaves as a multitarget ligand able to modulate monoaminergic transmission and besides showing an anti-inflammatory profile, both pathways known to be altered in neurological disorders. Further experimental work should be done to definitively confirm the neuroprotective effect of this novel hydrazine, **JL72**. The interesting pharmacological profile

of **JL72**, together with the observation of a significant anti-inflammatory activity, suggests that **JL72** is a promising lead compound for further development of drugs to be used in the therapy of cerebrovascular and neurological diseases.

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Annex II. 2. Fernández-Fernández L, Esteban G, Giralt M, Valente T, Bolea I, Solé M, **Sun P**, Benítez S, Morelló JR, Reguant J, Ramírez B, Hidalgo J, Unzeta M. Catecholaminergic and cholinergic systems of mouse brain are modulated by LMN diet, rich in theobromine, polyphenols and polyunsaturated fatty acids. Food Funct. 2015, 8;6(4):1251-60

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Catecholaminergic and cholinergic systems of mouse brain are modulated by LMN diet, rich in theobromine, polyphenols and polyunsaturated fatty acids

Laura Fernández-Fernández,†[a] Gerard Esteban,†[a] Mercedes Giralt,[b] Tony Valente,[a,c] Irene Bolea,[a] Montse Solé,[a] Ping Sun,[a] Susana Benítez,[a] José Ramón Morelló,[d] Jordi Reguant,[d] Bartolomé Ramírez,[d] Juan Hidalgo[b] and Mercedes Unzeta*[a]

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The possible modulatory effect of the functional LMN diet, rich in theobromine, polyphenols and polyunsaturated fatty acids, on the catecholaminergic and cholinergic neurotransmission, affecting cognition decline during aging has been studied. 129S1/SvlmJ mice were fed for 10, 20, 30 and 40 days with either LMN or control diets. The enzymes involved in catecholaminergic and cholinergic metabolism were determined by both immunohistological and western blot analyses. Noradrenalin, dopamine and other metabolites were quantified by HPLC analysis. Theobromine, present in cocoa, the main LMN diet component, was analysed in parallel using SH-SY5Y and PC12 cell lines. An enhanced modulatory effect on both cholinergic and catecholaminergic transmissions was observed on 20 day fed mice. Similar effect was observed with theobromine, besides its antioxidant capacity inducing SOD-1 and GPx expression. The enhancing effect of the LMN diet and theobromine on the levels of acetylcholine-related enzymes, dopamine and specially noradrenalin confirms the beneficial role of this diet on the "cognitive reserve" and hence a possible reducing effect on cognitive decline underlying aging and Alzheimer's disease.

Introduction

Aging is a highly complex process that can affect multiple organs and induce changes that will disturb the correct functioning of the organism over time, leading to different pathologies.[1] In this respect, neurological disorders such as Alzheimer's disease (AD), are deeply related to the aging process[2,3] and research in this area is becoming promising especially regarding the implications for both public health and social policies.

AD is a progressive neurodegenerative disorder characterized by memory loss and cognitive impairment. AD is a multifactorial disease in which oxidative stress, mitochondrial dysfunction, inflammation, metal dyshomeostasis, accumulation of miss-folded proteins, deficit of cholinergic transmission or apoptosis, among others play an important role.[4,5]

AD histological hallmarks such as the deposition of β-amyloid protein or the neurofibrillary tangle formation have been widely reported.[6,7] However, the correlation between the biological measurement of the pathology markers that are shown in the brain at middle age and the real loss of cognitive function that appears at advanced life stages has not yet been found. This gap between symptoms and pathology has been explained by the "cognitive reserve" theory, in which a set of variables such as education, training, intelligence or mental stimulation allow the brain to adapt or mask the pathology, maintaining the cognition in spite of the neuronal loss. It has been hypothesised that these key elements of "cognitive reserve" normalize the otherwise declining noradrenergic system during aging, and therefore the optimization of noradrenergic activity may reduce the risk of AD.[8]

The complexity of AD is corroborated by the fact that currently no drug is able to prevent this neurodegenerative process. To date, only five drugs have been approved by the Food and Drug Administration to enhance cholinergic trans-

[a]*Institute of Neurosciences, and Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain. E-mail: Mercedes.unzeta@uab.es; Fax: +34 93 581 3861; Tel: +34 93 581 1624*
[b]*Institute of Neurosciences, and Department of Cellular Biology, Physiology and Immunology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain*
[c]*Biomedical Research Institute of Barcelona (IIBB), Spanish National Research Council (CSIC), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Department of Brain Ischemia and Neurodegeneration, Barcelona, Spain*
[d]*La Morella Nuts SA, Reus, Tarragona, Spain*
† Both authors contributed equally.

mission related to cognitive deficits associated with AD,[9] leaving a vast majority of other potential AD targets nearly unaffected by current therapies.

Diet has been extensively reported to play an important role in cognition[10] and at present, there is mounting evidence that certain components of diet intake, especially those exhibiting antioxidant properties, could be beneficial for preventing and delaying neurodegenerative disorders such as AD.[11,12] Fish, vitamins and methionine-rich proteins have been identified to confer protection against AD.[13]

In this regard, polyphenolic compounds present in dry fruits, nuts and almonds, wine, tea, berry fruits, cocoa and fish oils, have shown anti-aging and neuroprotective effects.[14] All of them are potent antioxidant agents that could be useful as a nutritional approach against the oxidative stress and inflammation associated with AD.[15] Therefore, diet supplementation may offer an alternative or supplementary therapy to the use of acetylcholinesterase inhibitors.

It has been previously reported that LMN cream intake, [Patent ref WO2007063158 A2], based on cocoa, hazelnuts, polyphenols, vegetable oils rich in polyunsaturated fatty acids and flours rich in soluble fiber, is able to reduce the cardiovascular risk factors that are underlying AD.[16] Moreover, the LMN diet has been described as an inductor of neurogenesis in the adult mouse brain by promoting the proliferation and differentiation of neuronal cells in both the olfactory bulb and the hippocampus, being the latest one of the most highly affected brain regions in AD.[17]

Besides, the LMN diet is able to decrease the behavioural deterioration caused by aging in both wild type and in Tg2576 mice and diminish the Aβ plaque formation. LMN also reduces the Aβ (1–40 and 1–42) plasma levels in adult mice.[18] Taking all these results into account, the increasing importance of polyphenols as human dietary supplements playing a potential role in ameliorating the cognitive impairment during aging and neurological disorders is corroborated.

The objective of the present work was to evaluate the possible modulatory effect of the LMN diet on both cholinergic and catecholaminergic systems in 129S1/SvlmJ mice fed for 10, 20, 30 and 40 days by immunodetection and high-performance liquid chromatography (HPLC) approaches.

Results

AChE and ChAT immunohistochemical analysis in the striatum of LMN fed mice

The effect of the LMN diet on AChE and ChAT as the main enzymes involved in acetylcholine (ACh) metabolism has been studied since ACh is a depleted neurotransmitter in AD. Fig. 1 shows representative immunohistochemistry images for AChE and ChAT in basal ganglia (striatum) of 4-month old 129S1/SvImJ mice fed with the LMN diet for 10, 20, 30 and 40 days. The LMN diet induced a decrease of the AChE levels immunostaining in the striatum at early times, which was significantly different at 20 day feeding. However, AChE levels were reestablished at longer times. In contrast, ChAT levels showed an opposite pattern, being increased at 20 days and returning to normal values afterwards.

Immunohistochemical quantification of TH expression in basal ganglia of LMN fed mice

The effect of the diet on the TH expression, as a key enzyme in the synthetic pathways of catecholamines DA and NA was analysed. TH expression was determined in the catecholaminergic circuits of both striatum and substantia nigra as shown in Fig. 2. The corresponding quantification of the immunofluorescence signal showed a significant increase of TH expression in the catecholaminergic fibers of the striatum at 20 and 30 day feeding. In the substantia nigra, a progressive increase of the TH expression was observed in the neurons and fibers of catecholaminergic circuits. It was significant at 30 and 40 day feeding. The different periods of time observed on the expression of TH in the striatum and substantia nigra could be explained by the fact that different components of the LMN diet are probably not processed by the gastro-intestinal track with the same efficacy and furthermore, they can cross the blood–brain barrier with different accessibilities and hence to reach the selected cerebral locations at different rates.[19]

LMN cream modulates COMT expression in the hippocampus

The expression of Catechol-*O*-Methyl Transferase (COMT) was analysed in the hippocampus of 129S1/Svlmj mice fed with the LMN diet for 10, 20, 30 and 40 days. This brain region is involved in memory and learning processes and it is severely affected in AD. As shown in Fig. 3, the highest COMT expression was observed at 20 days while it progressively decreased at longer times.

HPLC analysis of the catecholaminergic neurotransmitters DA and NA in the striatum

The levels of DA, NA, their metabolites DOPAC and HVA, and 5HIAA were determined by HPLC in basal nuclei of 4-month old mice fed with the LMN diet for 10, 20, 30 or 40 days. Fig. 4 shows an increase in the levels of DA and NA at 20 day feeding. In the case of their metabolites DOPAC and HVA, the same trend was observed. However, no effect on the levels of the serotonin metabolite 5-HIAA was detected.

Theobromine, the main component of the LMN cream shows similar antioxidant effects on SH-SY5Y cells

It was also analyzed the antioxidant effect of theobromine (TBr) and compared to that of LMN cream. Theobromine is present in cocoa, the main component of LMN cream. Fig. 5a shows a similar protective effect of both the LMN cream and TBr on SH-SY5Y cells lesioned with 150 μM H_2O_2 for 24 h. Protection was observed in all doses, the highest effect being detected with 10 μg ml^{-1} LMN cream. An increase in the protein levels of the antioxidant enzymes Superoxide Dismutase-1 (SOD-1) (Fig. 5b) and Glutathione Peroxidase (GPx) (Fig. 5c) was observed after TBr and LMN cream treatment without H_2O_2 addition.

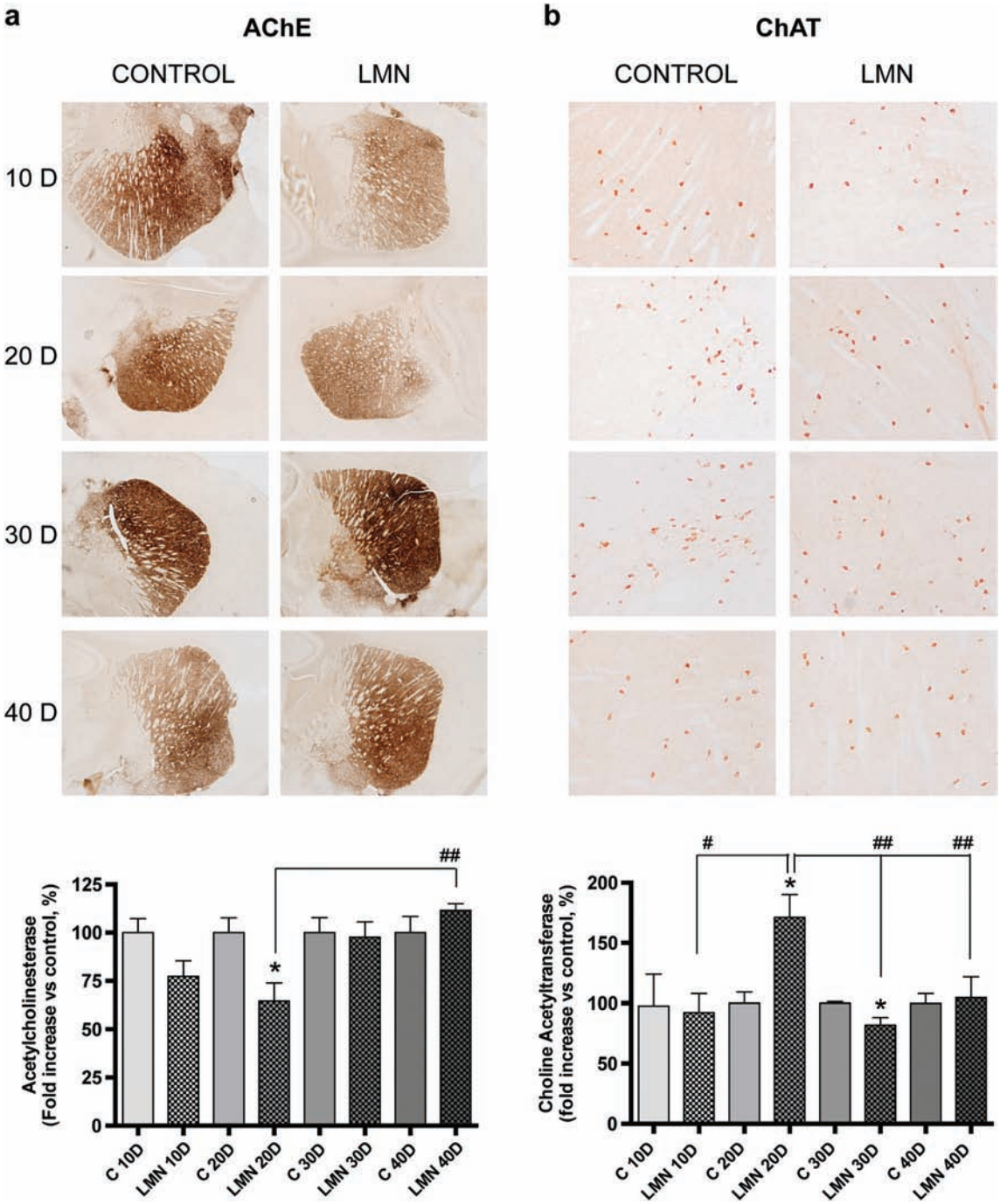

Fig. 1 Immunohistochemical analysis of AChE and ChAT in the striatum of 129S1/SvlmJ mice fed with the LMN diet. (a) Representative images for acetylcholinesterase (AChE, 2×) and (b) for choline acetyltransferase (ChAT, 10×) immunohistochemistry in basal nuclei of 4-month old 129S1/SvlmJ mice fed with the control or the LMN diet for 10 (n = 4), 20 (n = 4), 30 (n = 4) or 40 (n = 4) days. Graphs at the bottom show the IHC quantifications carried out in 10× images. Data are related to each representative control. *$p < 0.05$ *vs.* its own control (C), by Student's t-test; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ by one-way ANOVA and the addition of Newman–Keuls multiple comparison test.

In order to corroborate this antioxidant effect, Nrf2 activation, the transcription factor that activates the gene expression of these antioxidant enzymes, was determined after LMN or TBr treatments without the addition of H_2O_2. Fig. 5d shows representative images for Nrf2 immunofluorescence analysis in LMN or TBr-treated SH-SY5Y cells for 24 h. These results clearly show a translocation of Nrf2 to the nucleus as a response to LMN and TBr treatments.

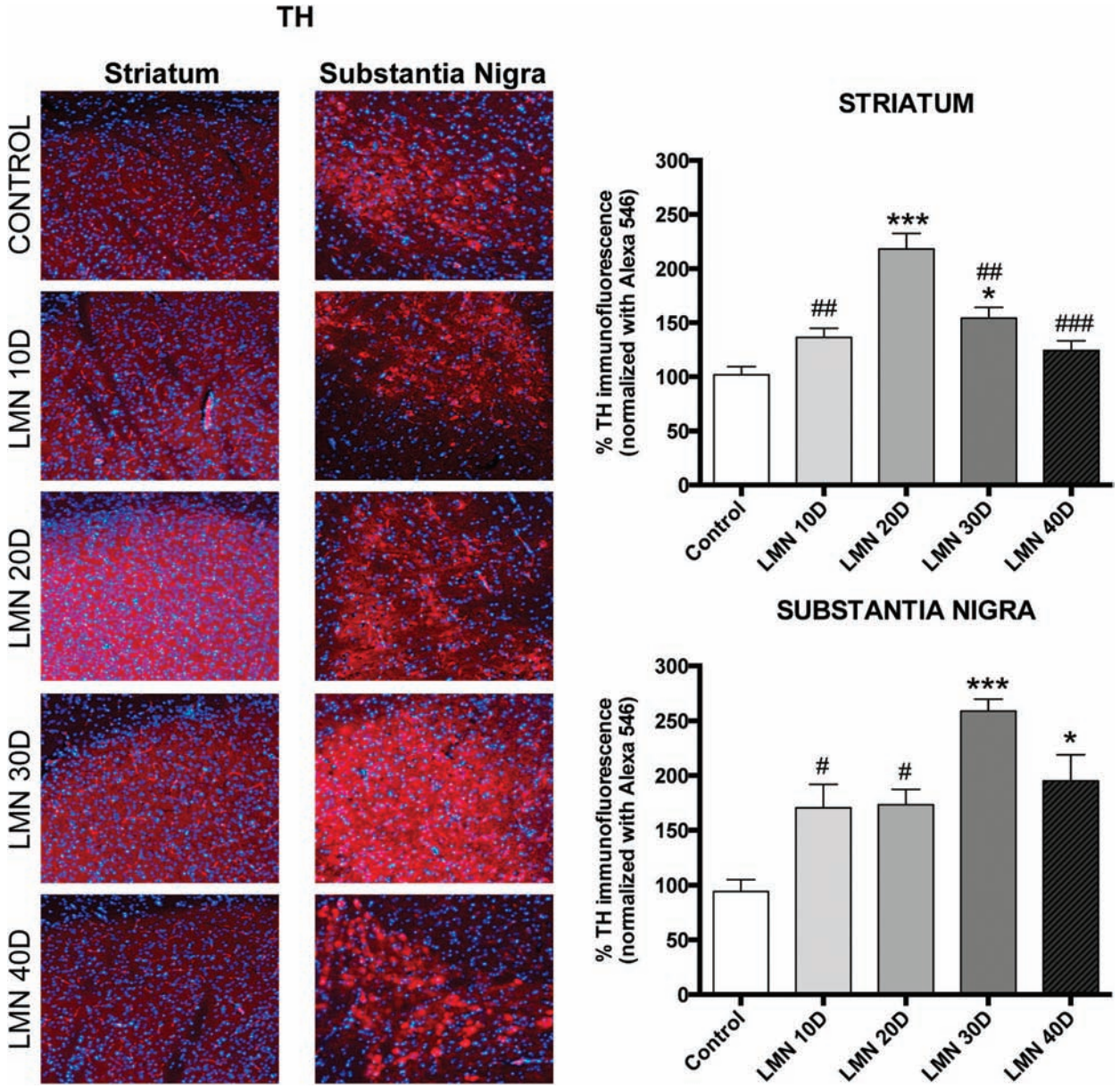

Fig. 2 Quantification of TH expression in nucleus basalis of the LMN fed mice. Representative images for tyrosine hydroxylase (TH) immunofluorescence in the striatum and substantia nigra of 4-month old 129S1/SvlmJ mice fed with the LMN diet for 10 (n = 4), 20 (n = 4), 30 (n = 4) or 40 (n = 4) days. Graphs show the quantification for each brain area. *p < 0.05, ***p < 0.001 *vs.* control diet fed animals; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 *vs.* LMN 20D in striatum or *vs.* LMN 30D in substantia nigra, by one-way ANOVA and the addition of Tukey's multiple comparison test.

Theobromine as a modulator of catecholaminergic metabolism in undifferentiated PC12 cells

Undifferentiated PC12 cells, derived from rat pheochromocytoma adrenal medulla, were treated with TBr (1–100 μM) for 24 h and catecholamine levels were subsequently quantified by HPLC-ED analysis. Broadly, the levels of NA, L-DOPA, DA and DOPAC increased after treatments with all concentrations of TBr used (Table 1). However, no changes were observed in 3-MT or HVA levels.

Significant increase in the levels of NA (60.6%), DOPAC (65.3%) and DA (78.5%) were only detected at 10 μM TBr *versus* non-treated cells. These results are in agreement with those previously determined in the mice fed with LMN for 20 days.

Discussion

The main component of the LMN cream is cocoa that contains a large amount of polyphenols, natural substances that are present in plants, coloured fruits and vegetables as well as in olive oil, tea and red wine. This wide family of natural products

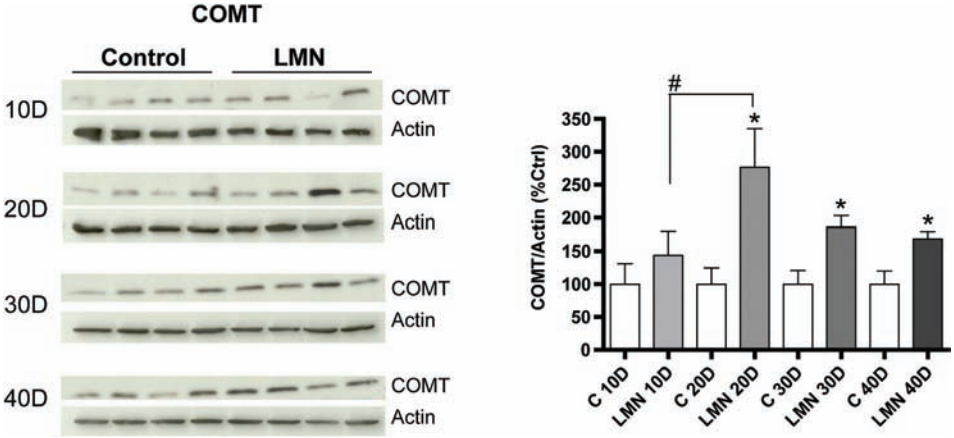

Fig. 3 Quantification of COMT in the hippocampus from LMN fed mice. Representative western blots for catechol-*O*-methyltransferase (COMT) in hippocampi from 4-month old mice fed with the control or the LMN diet for 10 (n = 3), 20 (n = 4), 30 (n = 4) or 40 (n = 4) days. Graph shows the data corresponding to the western blot densitometric analysis. *$p < 0.05$ *vs.* its respective control by Student's t-test. $^{\#}p < 0.05$ by one-way ANOVA and the addition of Newman–Keuls multiple comparison test.

contains flavonoids, the largest group of polyphenols including subclasses of flavones, isoflavones, flavonols, or flavans, among others. Because of their antioxidant properties, some of them are able to promote physiological benefits especially regarding the cognitive function and memory impairment.[20] The most abundant polyphenol present in green tea, the EGCG, also present in the LMN cream, has been reported to possess beneficial effects on cancer and cardiovascular function, with anti-inflammatory and antioxidant properties.[21] Furthermore, the importance of long-chain polyunsaturated fatty acids in neural development and neurodegeneration has been widely reported,[22] such as omega-3, present in dry fruits like hazelnuts, one of the LMN cream components.

According to the cholinergic hypothesis of AD, this neurological disorder is characterized by a loss of cholinergic neurons present in nucleus basalis, responsible for learning and memory functions.

Moreover, the existing gap between symptoms and pathology in AD has been explained by the concept of "cognitive reserve" in which both cognition and memory are maintained despite the progress in the pathology. Cognitive reserve has been hypothesized to help people to avoid greater brain pathology and it has been considered a preventive factor for dementia. Compensatory adjustments, such as an enhancement on the noradrenergic transmission, have been proposed to be involved.[8] Regarding nutritional aspects, some authors report that the Mediterranean diet does not support a beneficial effect on cognitive function, irrespective of educational level, which is the strongest indicator of cognitive reserve.[23] These results are in contrast with other studies which conclude that the Mediterranean diet is associated with lower risk of dementia.[24] In line with this, other authors have provided support for the hypothesis that cognitive reserve moderates the relationship between brain structure and cognition at middle age well before the onset of dementia.[25]

Neurotransmitters NA, DA and ACh are considered neuromodulators released by neurons, whose cell bodies are found in specific nuclei in the brainstem. The function of many brain regions can be influenced through their wide spread projections. Dopaminergic neurons innervate the striatum, prefrontal cortex and hippocampus. Noradrenalin has multiple effects on cellular excitability, intracellular cascades or synaptic plasticity of its target neurons. It is also able to enhance/block excitatory responses to glutamate depending on its concentration. The effect of NA on synaptic plasticity in the hippocampus promotes long-term potentiation (LTP) in both dentate gyrus through the action on β-receptors and in the prefrontal cortex improving working memory in primates.[26]

In this respect, the search for some natural products able to modulate cholinergic and noradrenergic systems could help delaying the loss of cognition. In this work, adult male 129S1/SvImJ mice were selected as a mid-age model in which strengthening of this neurotransmission could have beneficial effects during aging.

Basal nuclei, containing striatum, were selected as a rich area in both cholinergic as well as catecholaminergic terminals. The expression of both ChAT and AChE, the two main enzymes involved in the ACh metabolism, was modulated after LMN diet intake. The increase of ChAT expression indicates a stimulation of ACh synthesis, a neurotransmitter that is diminished in AD. Furthermore, the expression of AChE, the enzyme responsible for ACh degradation at the synaptic cleft, was significantly decreased after LMN feeding. Taken together, these results strongly suggest that the LMN diet shows a beneficial modulatory effect on the stimulation of cholinergic transmission.

The previous results were correlated with the increasing TH expression in the dopaminergic fibers of both striatum and substantia nigra. This is the key enzyme responsible for the regulation of the catecholamine synthesis pathway, which

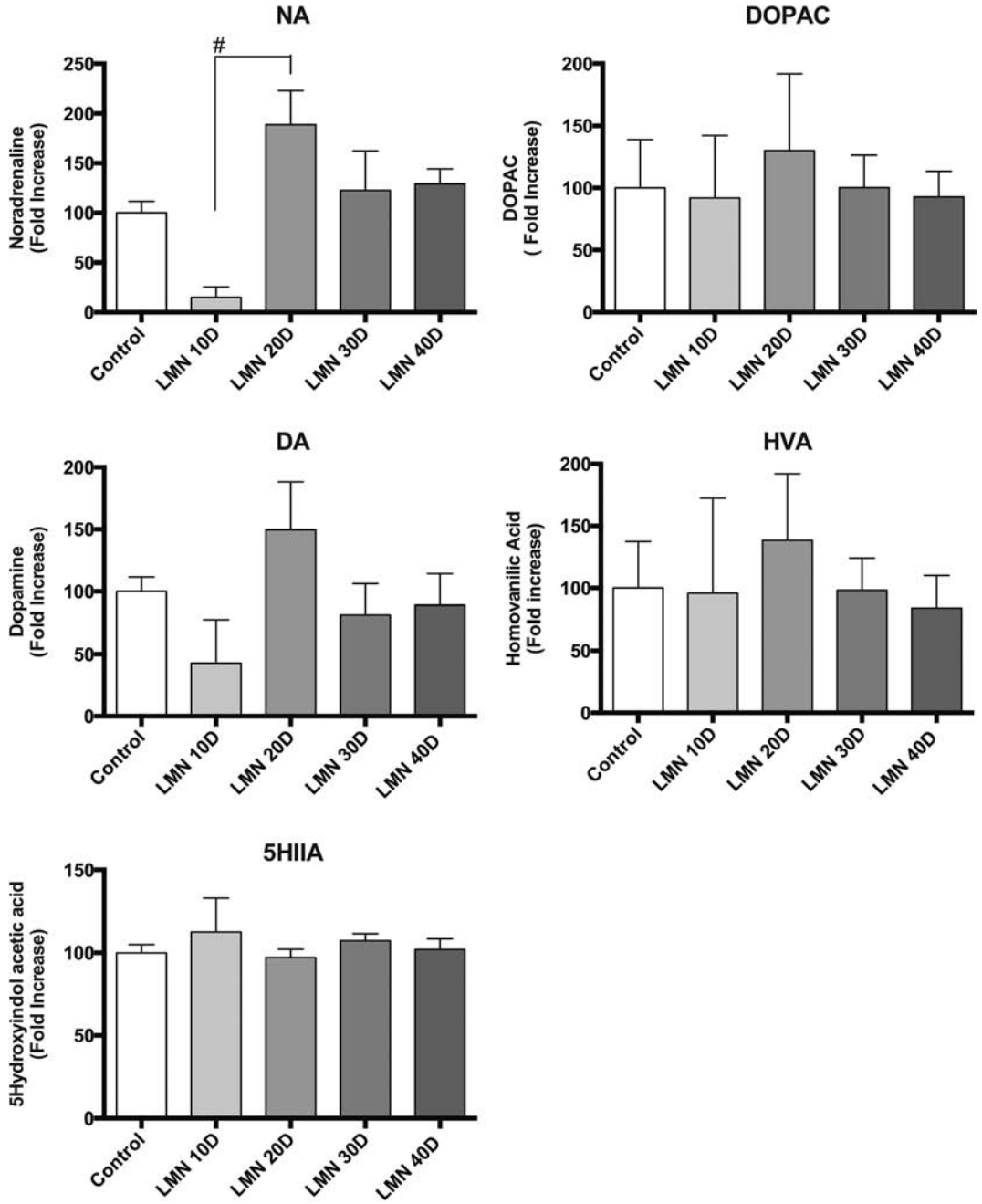

Fig. 4 HPLC analysis of catecholaminergic neurotransmitters: DA and NA in the striatum. Quantification of noradrenaline (NA), dopamine (DA), the metabolites DOPAC and HVA and 5-HIAA in basal nuclei of 4-month old mice fed with the LMN diet for 10 (n = 3), 20 (n = 4), 30 (n = 4) or 40 (n = 4) days. Data are related to each respective control. $^{\#}p < 0.05$ by one-way ANOVA and the addition of Bonferroni's multiple comparison test.

controls reward-induced neurotransmitter change in cognitive brain regions and learning processes.[27] Therefore, the LMN diet enhances the dopaminergic system and consequently, catecholamine production. COMT is the enzyme responsible for dopamine metabolism rendering HVA after its catalytic action on DOPAC, a metabolite of DA generated by both aldehyde dehydrogenase and monoamine oxidase activities. The increased expression of COMT in the hippocampus, the specific area related to memory and cognition, strongly confirms the stimulatory effect of the LMN diet on catecholamine metabolism. All these data obtained by immunohistological assays were corroborated by the metabolite quantification by HPLC analysis. The levels of NA, DA and their metabolites were also found to be increased in PC12 cells treated with TBr.

The antioxidant properties of the LMN cream previously reported[17,28] were also observed with TBr. This main component of cocoa is able to stimulate the Nrf2 activation, a transcription factor responsible for the expression of both SOD-1

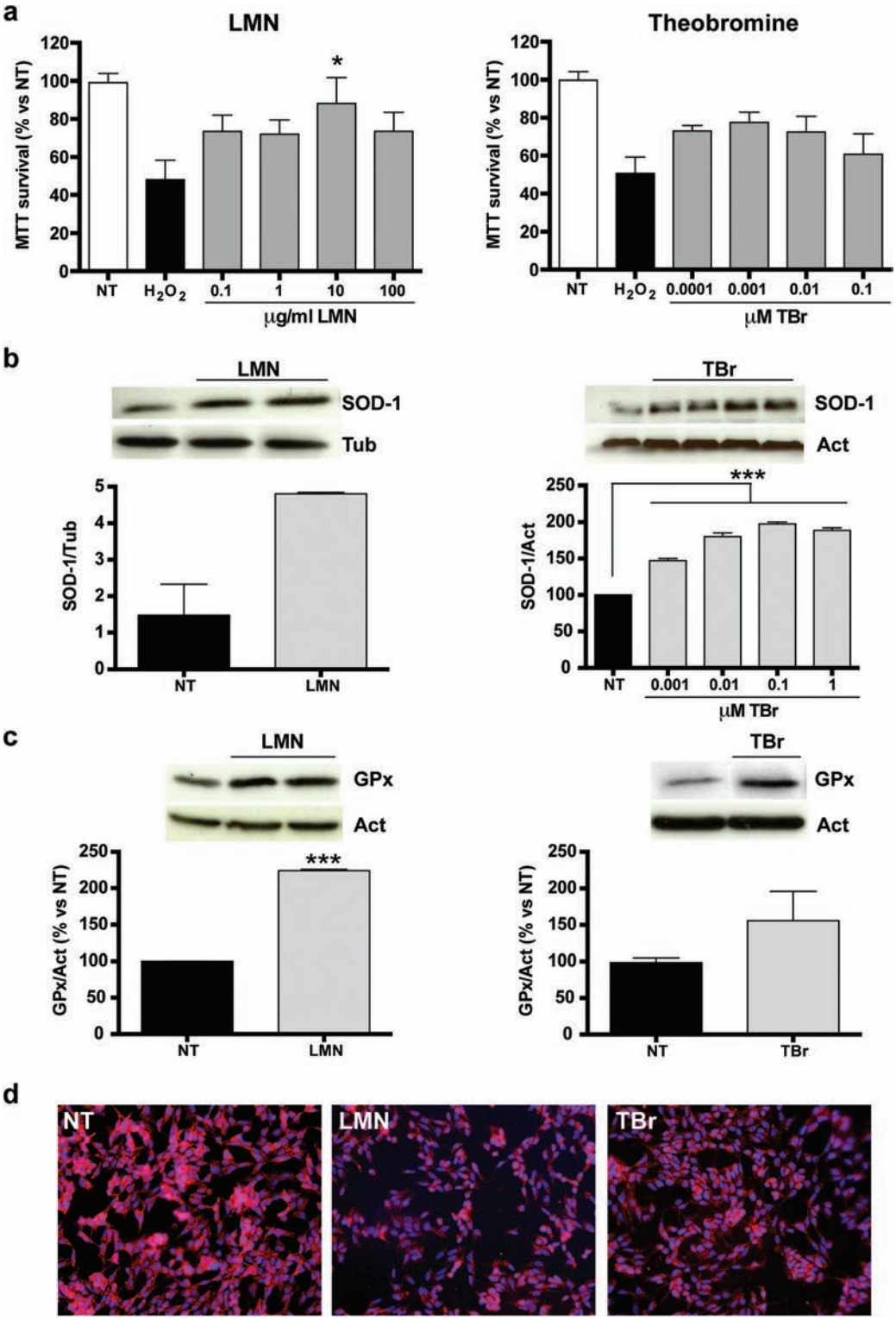

Fig. 5 Antioxidant effects of the LMN cream and its main component, theobromine, in SH-SY5Y cells. (a) SH-SY5Y cells were pretreated for 24 h with the LMN cream or with theobromine (TBr) and then lesioned with 150 μM hydrogen peroxide (H_2O_2) for 1 h. Cell viability was determined by the MTT method. Data are the mean ± S.E.M. of three independent experiments. *$p < 0.05$ *vs.* H_2O_2 by one-way ANOVA and the addition of Newman–Keuls multiple comparison test. (b, c) Effect of the LMN diet (0.1 μg ml^{-1}) or TBr (0.001–1 μM) on the expression of Superoxide Dismutase-1 (SOD-1, b) or Glutathione Peroxidase (GPx, c) in 72 h-treated SH-SY5Y. Tubulin (Tub) or β-actin (Act) was used as loading control. Graphs represent the western blot quantification of three independent experiments. ***$p < 0.001$ by one-way ANOVA and the addition of Tukey's multiple comparison test (TBr in SOD-1 analysis) or by Student's *t*-test (LMN treatment in GPx analysis). (d) Representative images for Nrf2 immunofluorescence analysis in LMN or TBr-treated SH-SY5Y cells for 24 h. Red staining represents Nrf2 whereas nuclei are stained in blue (DAPI).

Table 1 Modulation of the levels of catecholaminergic neurotransmitters in PC12 cell lysates treated with theobromine[a]

		[Theobromine] (μM)		
Metabolite	NT	1	10	100
Noradrenalin	100.0 ± 6.6	151.8 ± 15.5	160.6 ± 22.8*	138.2 ± 12.0
L-DOPA	100.0 ± 14.3	131.1 ± 7.9	135.8 ± 27.1	104.8 ± 9.7
DOPAC	100.0 ± 5.1	105.0 ± 11.4	165.3 ± 23.5*	120.6 ± 9.3
Dopamine	100.0 ± 4.3	142.6 ± 21.5	178.5 ± 35.0*	124.9 ± 16.8
HVA	100.0 ± 4.4	83.1 ± 4.3	92.7 ± 5.9	96.2 ± 6.2
3-MT	100.0 ± 6.7	123.8 ± 16.6	119.8 ± 10.5	113.8 ± 11.4

[a] PC12 cells were treated for 24 h with theobromine (TBr) (1–100 μM). The levels of the catecholamines were quantified by HPLC analysis. Values are expressed as the mean of the percentage increase from at least four independent experiments; *$p < 0.05$ *vs.* NT.

and GPx by themself, in the absence of any oxidant contaminant.

All these results suggest that TBr may be one of the main components responsible for the modulatory effects on catecholaminergic and cholinergic observed with the LMN cream. The effects observed only in a short-time window could be explained by the activation of compensatory mechanisms that need to be further elucidated.

Only male mice were used in this study due to their homogeneity. However, since females also experience aging and show higher AD prevalence, further studies carried out in females would also be interesting in order to confirm these results.

Experimental

Animals and diets

Mice were bred under controlled temperature and light conditions and were fed with control diet prior to the experiments. Animal care and experimental procedures were performed in accordance with European Community Council Directive 86/609/ECC. Four-month 129S1/SvImJ adult male mice were fed either with control diet (Harlan Global Diet 2014, Mucedola SRL, Milano, Italy) or control diet supplemented with the LMN cream (LMN diet, Harlan Global Diet 2014 containing 9.27% LMN cream, Mucedola SRL, Milano Italy) for 10, 20, 30 or 40 days. The LMN cream consists of a mixture containing at least one ingredient from each of the following four groups: dry fruits, cocoa, vegetable oils rich in unsaponifiable lipids and flours rich in soluble fibers [Patent ref WO2007063158 A2]. After predetermined feeding periods, all mice were killed by decapitation and their brains were immediately removed. Right hemispheres were dissected into regions and frozen in liquid nitrogen at −80 °C for subsequent western blot and HPLC analyses. Left hemispheres were fixed in 4% paraformaldehyde (PFA) for 24 hours. Then, tissue was cryoprotected in 30% sucrose/PBS solution for 48 hours at 4 °C, frozen in dry ice and cut into sagittal sequential 30 μm sections using a Leica cryostat. These sagittal slices were used for immunohistochemistry and histological techniques.

Cell lines and treatments

Human neuroblastoma SH-SY5Y cells were purchased and cultured as previously described.[18] PC12 cell line was purchased from the American Type Culture Collection (ATCC) and grown in DMEM containing 7% FBS, 7% foetal horse serum (FHS), 1.14 mM HEPES pH 6.8, 60 U ml^{-1} penicillin and 60 μg ml^{-1} streptomycin. Both cell lines were maintained at 37 °C under a saturating humidity atmosphere containing 5% CO_2.

For treatments, both SH-SY5Y and PC12 cells were seeded at a density of 2.5×10^4 cells mL^{-1} in full media onto collagen type I (BD Biosciences)-coated plates until 70–80% confluence was reached. Before treatments, full medium was replaced by 1% FBS-medium overnight except for HPLC analysis. Both theobromine (TBr) and the LMN cream were dissolved in PBS at 37 °C and added to the media for desired times. Non-toxic TBr concentrations (1, 10 or 100 μM) were used for 24 h. At the end of each treatment, cells were collected in PBS, centrifuged at 3000*g* for 5 minutes and the pellets resuspended in 100 μL of homogenization solution (0.25 M $HClO_4$, 100 μM sodium bisulfate and 250 μM EDTA). Samples were sonicated for 10 seconds on ice and lysates kept at −80 °C for at least 24 h prior to the HPLC analysis.

Histochemistry and immunohistochemistry

The brain sections were permeabilized in PBS-0.3% Triton X-100 for 30 min and blocked in PBS-Triton X-100 containing 10% FBS, 0.2 M glycine and 1% BSA for 10 minutes. Afterwards, the slides were incubated with goat anti-Choline Acetyltransferase (ChAT, 1 : 200, Millipore AB144P) overnight at 4 °C, washing with PBS-Triton X-100 and incubated with donkey anti-goat HRP (1 : 200; Thermo PA 1-28664) for 1 or 2 hours at room temperature. After washing, sections were incubated with the streptavidin–HRP (1 : 1250; Sigma, S5512) for one hour and developed with DAB/H_2O_2 (0.5 mg/0.22 μl ml^{-1}; Sigma). Acetylcholinesterase (AChE) histochemistry was performed according to the Geneser-Jensen procedure.[29]

Quantification of ChAT and AChE was carried out in the striatum. Brain sections from 4 animals per group were used and pictures were taken at 2× and 10× magnifications using the software ACT-1 version 2.70 (Nikon Corporation). They were examined with a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera. Three to five pictures per section were evaluated and quantified in 10× images using image analysis software ImageJ.

Immunofluorescence

For tyrosine hydroxylase (TH) immunohistofluorescence, the brain sections were rinsed in PBS, treated with 2% H_2O_2 in methanol for 15 minutes, rinsed in PBS-0.5% Triton X-100, blocked with 10% of normal goat serum in PBS-0.5% Triton X-100 and incubated overnight at 4 °C with polyclonal rabbit anti-TH (1 : 1000; Millipore). After rinsing in PBS-0.5% Triton X-100, sections were incubated for 2 h at room temperature

with goat anti-rabbit Alexa 546 (1 : 500; Molecular Probes). Finally, the sections were counterstained with DAPI and after being washed in PBS, were mounted in Mowiol medium (Sigma-Aldrich). For quantification of TH immunofluorescence, photographs of four sequential histological sections per animal were acquired with a Zeiss Axio observer Z1 microscope (Carl Zeiss) and a digital camera (QImaging Retiga Exi, QImaging). Using ImageJ software and Paxinos brain mouse atlas, the striatum and substantia nigra areas were delimited (striatum: lateral 1.32 to 2.04 mm; substantia nigra: lateral 0.84 to 1.68 mm) and relative TH immunofluorescence densitometry was obtained for each individual section, as well as for relative Alexa 546 immunofluorescence. The quantification of TH immunofluorescence was obtained by its normalization with Alexa 546 tissue background.

SH-SY5Y cells fixed in 4% PFA were incubated with the primary antibody against Nrf2 (1 : 100; Santa Cruz) diluted in PBS containing 0.2% gelatin, 0.1% Triton X-100, 20 mM glycine and 5% FBS overnight at 4 °C. Secondary anti-rabbit conjugated to Alexa Fluor® 594 (1 : 1000; Invitrogen) and 0.5 mg ml^{-1} Hoechst were then incubated for 1 h at room temperature and cells were mounted in Fluorescent Mounting Medium (Dako). Preparations were observed under a Nikon Eclipse TE 2000-E inverted fluorescence microscope with a Hamamatsu C-4742-80-12AG camera and Metamorph® Imaging System software.

Western blot analysis

SH-SY5Y cells were lysed in RIPA buffer, centrifuged at 3000*g* for 10 min at 4 °C and the supernatants were kept at −80 °C until their use. Protein concentration was determined using Bradford reagent (Bio-Rad). Twenty-five μg of total protein extract were resolved by SDS-PAGE according to standard protocols. Primary antibodies used were: Catechol-*O*-Methyl Transferase (COMT) (1 : 1000; Santa Cruz), Superoxide Dismutase-1 (SOD-1) (1 : 1000; Santa Cruz), Glutathione Peroxidase (GPx) (1 : 1000, Abcam), β-tubulin (1 : 50 000; Sigma) and β-actin (1 : 40 000; Sigma). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 2000; Dako) or goat anti-rabbit IgG (1 : 1000; BD Biosciences). Densitometry analyses were performed using Quantity One® software (Bio-Rad) following manufacturer's instructions.

Cell viability analysis

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. MTT solution was added at a final concentration of 0.5 mg ml^{-1} for 45 min. DMSO was used to dissolve the formazan blue precipitate formed, which was quantified at 560–620 nm using a microplate reader (Labsystems Multiskan RC).

Determination of catecholamine levels by HPLC

Catecholamine levels were determined by HPLC coupled to an electrochemical detector. The mobile phase consisting of 0.1 M citric acid, 0.05 mM EDTA and 1.2 mM sodium octylsulphate (SOS) was adjusted at pH 2.75 with triethylamine (TEA). Acetonitrile was added to reach 10% (v/v). Elution was performed at a flow rate of 1 mL per minute. A Coulochem 5100A Electrochemical Detector (ESA) with a Model 5011 dual-electrode analytical cell with porous graphite electrodes was used. The potential of the electrodes 1 and 2 was set at 70.05 V and +0.4 V, respectively.

PC12 cell lysates were thawed and centrifuged at 12 000*g* for 10 min at 4 °C. Next, 20 μL of each sample supernatant was injected into the HPLC system for analysis. Catecholamine analysis was performed at room temperature (20–25 °C). The level of metabolites was expressed in pg of metabolite per mg of protein.

Frozen samples from basal nuclei were homogenized in RIPA buffer. After that, samples were split into two parts and diluted (1 : 1) in homogenization solution containing 2000 pg ml^{-1} of 3,4-dihydroxybenzylamine (DHBA) as an internal standard for catecholamine determination and 4000 pg ml^{-1} of NW-5-met-5HT as an internal standard for serotonin determination. After homogenization, the samples were sonicated and centrifuged (15 000*g*, 10 min, 4 °C) and supernatants were injected into the HPLC system for analysis of 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenalin (NA), homovanillic acid (HVA), dopamine (DA), serotonin (5-HT) and serotonin metabolite 5-hydroxyindoleacetic acid (5HIAA). DHBA was used as an internal standard.

Statistics

Graphs were plotted and data were analysed using the GraphPad Prism 4.02 software. Values of $p < 0.05$ were considered to be statistically significant.

Conclusions

At present, there are few reports on the beneficial effect of nutrients on the stimulation of cholinergic and catecholaminergic transmission.[30] In this work, we report for the first time the enhancement of cholinergic and catecholaminergic transmissions, both of which are highly impaired in neurodegenerative disorders such as AD, by some natural nutrients present in the LMN cream. These results will give an insight into the possible contribution of the LMN cream in the "cognitive reserve"[8] and its beneficial effect on the cognitive decline related to aging and neurological disorders.

Acknowledgements

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