PHD THESIS

Defective sarcoplasmic reticulum-mitochondria communication in aged heart and its effect on ischemia and reperfusion injury

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"Je suis de ceux qui pensent que la science est d'une grande beauté. Un scientifique dans son laboratoire est non seulement un technicien: il est aussi un enfant placé devant des phénomènes naturels qui l'impressionnent comme des contes de fées."

Marie Skłodowska-Curie

"Ève Curie, Marie Curie, éd. Da Capo Series in Science"

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Los agradecimientos de esta tesis van en castellano porque ya vale de escribir en inglés.

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Nomenclature

ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
Bcl 2	B-cell lymphoma 2
bpm	Beats per minute
CaMKII	Calmodulin kinasa II
CK	Creatine kinase
CMTA	Charcot-Marie-Tooth neuropathy
CRU	Calcium-release unit
CsA	Cyclosporine A
CSQ2	Calsequestrin 2
CVD	Cardiovascular disease
CyP-D	Cyclophilin D
Cys	Cysteine
DNA	Deoxyribonucleic acid
δPKC	Protein kinase C
ΔΨm	Mitochondrial membrane potential
EC coupling	Excitation–contraction coupling
EDLVvol	End-diastolic left ventricular volume
EGTA	Ethylene glycol tetra acetic acid
ER	Endoplasmic reticulum
ER	Endoplasmic reticulum
FADH	Flavin adenine dinucleotide
GPX	Glutathione peroxidase
Grp 75	Glucose-regulated protein 75

GTP	Guanosine triphosphate
HCR	Histidine-rich calcium binding protein
HK	Hexokinase
HR	Heart rate
IFM	Interfibrillar mitochondria
IP3R	Inositol-P_3 receptor
LDH	Lactate dehydrogenase
LV	Left ventricular
LVdevP	Left ventricular developed pressure
LVEDD	Left ventricular end-diastolic diameter
LVEDDIN	LVEDD indexed by weight
LVEDP	Left ventricular end-diastolic pressure
LVEF	Left ventricular ejection fraction
LVm	Left ventricular mass
MAM	Mitochondria-associated membranes
MAM	Mitochondria-associated membrane
MCU	Mitochondrial calcium uniporter
Mfn-2	Mitofusin 2
mNCX	Mitochondrial Na^+/Ca^{2+} exchanger
MnSOD	Manganese superoxide dismutase
mPTP	Mitochondrial permeability transition pore
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NADH/NAD+	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NXC	Na^+/Ca^{2+} exchanger
OxPhos	Oxidative phosphorylation
PCr	Phosphocreatine
PiC	Pi carrier
PKA	Protein kinase A
PLA	Proximity ligation assay
PLB	Phospholamban
PNM	Perinuclear mitochondria

PP1	Phosphatase 1
PRX	Peroxiredoxin
PWT	Posterior wall thickness
RAGEs	Receptors for advanced glycation end products
ROS	Radical oxygen species
Ru360	Ruthenium 360
RyR	Ryanodine Receptors
SEM	Scanning electron microscope
SERCA	SR calcium ATPase
SR	Sarcoplasmic reticulum
SSM	Subsarcolemmal mitochondria
SWT	Septum wall thickness
TRX	Thioredoxin
VDAC	Voltage dependent anion channel

Summary

Mitochondrial alterations are critically involved in the increased vulnerability to disease during aging. On the other hand, aging is a major determinant of the incidence and severity of ischemic heart disease. Preclinical information suggests the existence of intrinsic cellular alterations that contribute to ischemic susceptibility in senescent myocardium, by mechanisms not well established.

The first part of this thesis investigates the contribution of mitochondria-sarcoplasmic reticulum (SR) communication in the functional decline of cardiomyocyte during aging. Echochardiographic analysis of aging mice (>20 months) showed a rather preserved cardiac contractile function in resting conditions respect to young mice (5-6 months). Similarly, ATP/phosphocreatine were preserved in hearts from old mice as quantified by RMN spectroscopy. In isolated mitochondria from young and old mouse hearts, mitochondrial membrane potential and resting O_2 consumption were similar in both groups. However, stimulation of O_2 consumption after the addition of saturating concentrations of ADP resulted in a partial failure of interfibrillar mitochondria from aged hearts to achieve maximal respiratory rate, whereas subsarcolemmal mitochondria were not altered by age. Second generation proteomics disclosed an increase of mitochondrial protein oxidation in advanced age. Because both energy production and oxidative status are regulated by mitochondrial calcium, this work further investigated the effect of age on mitochondrial calcium uptake. While no age-dependent differences were found in calcium uptake kinetics in isolated mitochondria, in which the contribution of other organelles and sarcolemma is absent, mitochondrial calcium uptake secondary to SR calcium release was significantly reduced in cardiomyocytes from old hearts. Reduced mitochondrial calcium uptake in aging cardiomyocytes was associated with decreased NAD(P)H regeneration and a concomitant increase of mitochondrial ROS production manifested only when cells were exposed to high frequency electrical stimulation. Immunofluorescence and proximity ligation assay identified defective communication between mitochondrial voltage dependent anion channel (VDAC) and SR ryanodine receptor (RyR) in cardiomyocytes from aged hearts. Functional analysis of calcium handling in fluo-4 loaded cardiomyocytes disclosed an altered pattern of RyR gating properties. The observed defects in SR calcium transfer and in calcium handling could be reproduced in young cardiomyocytes after interorganelle disruption with colchicine, at concentrations that had no significant effect in aged cardiomyocytes or isolated mitochondria.

The second part of this work investigates the potential impact of the altered mitochondrial function in the adverse effect of aging on myocardial ischemia and reperfusion (IR) injury. Isolated perfused hearts from old mice submitted to transient IR displayed an increase in hypercontracture, sarcolemmal rupture (LDH release) and infarct size, as compared to hearts from young mice, despite a paradoxical delay ischemic rigor contracture onset. In isolated cardiomyocytes from aging hearts submitted to IR there was a faster decline of mitochondrial membrane potential ($\Delta\Psi m$) in comparison with young ones, but ischemic rigor shortening was also delayed. Transient recovery of $\Delta\Psi m$ observed during ischemia, secondary to the reversal of mitochondrial FoF1 ATP synthase to ATPase mode, was markedly reduced in aging cardiomyocytes. Proteomic analysis demonstrated an increased oxidation of different subunits of FoF1 ATP synthase. Altered bionergetics in aging cells was associated with reduced mitochondrial calcium uptake and more severe cytosolic calcium overload during both ischemia and reperfusion. Despite attenuated mitochondrial calcium overload, the occurrence of mitochondrial permeability transition pore (mPTP) opening (as determined by mitochondrial calcein release), hypercontracture and cell death were increased during reperfusion in cardiomyocytes from old mice. In vitro studies demonstrated a significantly reduced calcium retention capacity in interfibrillar mitochondria from aging hearts.

Thus, defective SR-mitochondria communication underlies inefficient interorganelle calcium exchange that contributes to energy demand/supply mismatch and oxidative stress in the aged heart. The **FoF1 ATP** synthase oxidation is associated with an **altered function** of the enzyme and an **increased sensitivity of mitochondria to undergo mPTP opening**. Because ATP synthase has been proposed to conform mPTP, it is tempting to hypothesize that oxidation of the FoF1 ATP synthase is related with the increased susceptibility of the aged myocardium to IR injury.

Resumen

Las alteraciones mitocondriales están vinculadas a la mayor vulnerabilidad de padecer enfermedades durante el envejecimiento. Por otro lado, la edad avanzada es un factor determinante de la incidencia y gravedad de la cardiopatía isquémica. Estudios preclínicos sugieren la existencia de un daño celular intrínseco, por mecanismos no del todo establecidos, que contribuye a un incremento de la susceptibilidad del miocardio senescente al daño isquémico.

Esta tesis investiga el papel de la comunicación mitocondria-retículo sarcoplásmico (RS) en el deterioro funcional de los cardiomiocitos durante el envejecimiento. El estudios ecocardiográfico ha demostrado que la función cardiaca en el reposo se mantiene preservada en los animales de edad avanzada (>20 meses) con respecto a los jóvenes (5-6 meses). El cociente ATP/fosfocreatina cuantificado mediante espectroscopía de RMN no ha revelado cambios asociados a la edad. Tampoco se han observado cambios debidos a la edad en el potencial de membrana mitocondrial ni en el consumo de oxígeno en condiciones de reposo en mitocondrias aisladas de corazones de ratón. Sin embargo, el consumo de oxígeno inducido por concentraciones saturantes de ADP ha revelado que las mitocondrias interfibrilares de corazones de ratones viejos no alcanzan el nivel respiratorio máximo. Este defecto no se observa en las mitocondrias subsarcolemales. El análisis proteómico de segunda generación ha demostrado un aumento de la oxidación de proteínas mitocondriales relacionado con el envejecimiento. Debido a que la producción de energía y el estado oxidativo están regulados por el calcio mitocondrial, esta tesis ha investigado el posible efecto de la edad sobre la capacidad de las mitocondrias para captar calcio. No se han observado diferencias asociadas a la edad en las cinéticas de captación de calcio en mitocondrias aisladas (un modelo sin contribución del sarcolema u otros orgánulos). En cambio, en cardiomiocitos la captación mitocondrial del calcio procedente del RS se ha visto reducida de forma significativa en el envejecimiento. Esta disminución de la captación de calcio mitocondrial se asoció a una reducida capacidad de regeneración de NAD(P)H y a un incremento de la producción de ROS mitocondriales en cardiomiocitos viejos estimulados a altas frecuencias eléctricas. Ensayos de inmunofluorescencia y de ligación por proximidad (PLA) han revelado una comunicación defectuosa entre el canal dependiente de voltaje (VDAC) mitocondrial y el receptor de rianodina (RyR) del RS en cardiomiocitos de corazones senescentes. El análisis funcional del manejo del calcio en cardiomiocitos marcados con fluo-4 ha desvelado una alteración en las propiedades de apertura del RyR asociado al envejecimiento. La desestructuración de las uniones entre el RS y

la mitocondria con colchicina fue capaz de reproducir el efecto de la edad sobre las alteraciones en el manejo/transferencia de calcio entre ambos orgánulos en cardiomiocitos jóvenes. La dosis de colchicina utilizada no tuvo efecto sobre las mitocondrias aisladas ni los cardiomiocitos senescentes.

La segunda parte de este trabajo investiga el impacto potencial de las alteraciones de la función mitocondrial sobre los efectos adversos del envejecimiento en el daño por isquemia y reperfusión (IR). Los corazones aislados y perfundidos de ratones viejos sometidos a IR desarrollaron mayor rotura sarcolemal (liberación de LDH) e incremento del tamaño de infarto en comparación con los corazones jóvenes, a pesar de observarse un retraso significativo del desarrollo del rigor isquémico. Los cardiomiocitos viejos sometidos a isquemia simulada, desarrollaron una caída más rápida del potencial de membrana mitocondrial ($\Delta\Psi m$) junto con un retraso paradójico en la aparición del rigor. La tasa de recuperación transitoria del $\Delta\Psi m$ durante los primeros minutos de isquemia, debida a la actividad reversa de la FoF1 ATP sintasa mitocondrial, se encontró significativamente disminuida en cardiomiocitos de edad avanzada. El análisis proteómico ha demostrado un aumento de la oxidación de diferentes subunidades de la FoF1 ATP sintasa asociado al envejecimiento miocárdico. La alteración del $\Delta\Psi m$ observado en los cardiomiocitos viejos se asoció a una menor captación de calcio mitocondrial y a un agravamiento de la sobrecarga de calcio citosólico durante la IR. A pesar de la menor sobrecarga de calcio mitocondrial, el desarrollo de permeabilidad transitoria (mPT) (determinada por liberación de calceína) fue mayor en los cardiomiocitos senescentes y este efecto se correlacionó con una mayor hipercontractura y muerte celular durante la reperfusión. Las mitocondrias interfibrilares de corazones senescentes sometidas a sobrecarga de calcio *in vitro* demostraron una mayor susceptibilidad al mPT con respecto a las de corazones jóvenes.

Por lo tanto, el desarrollo de una comunicación defectuosa entre el RS y la mitocondria durante el envejecimiento produce un intercambio ineficiente de calcio entre ambos orgánulos, que contribuye al desajuste en la demanda/aporte de energía y a un aumento consiguiente del estrés oxidativo. La oxidación de la **FoF1 ATP sintasa** se asocia a una alteración de su **funcionamiento** y a un **incremento de la sensibilidad de la mitocondria para desarrollar mPT**. Debido al modelo recientemente propuesto según el cual la FoF1 ATP sintasa forma parte del mPTP, es posible especular que la oxidación de esta enzima está asociada al aumento del daño por IR en el miocardio senescente.

CHAPTER 1

Introduction

1.1 Aging of human population and cardiovascular diseases

In human population, the number of elderly people is expanding across the world without precedents in the history, due to the increase in the life expectancy together with a decline in the fertility and birth rates. So far, this phenomenon has the greatest impact on highly developed countries but the predictions are that aging of human population will experience an important increase in the next years, when other less developed countries follow the same demographic transition model (United Nations Population Fund, 2012).

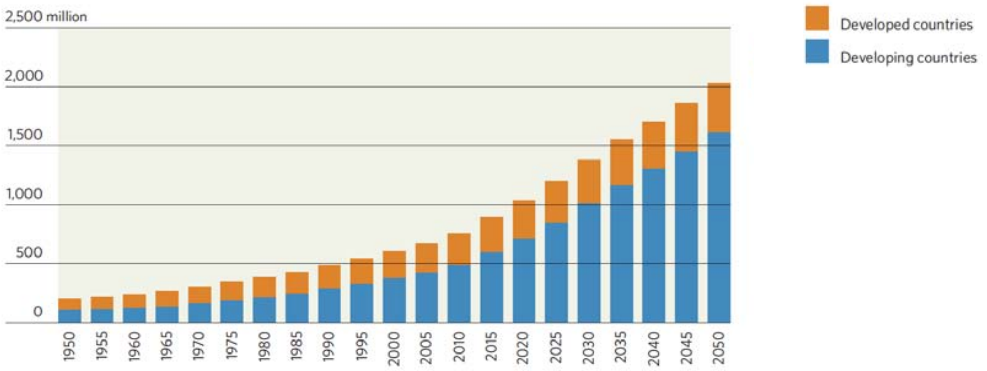


Figure 1.1: Number of people aged 60 or over: world, developed and developing countries, 1950-2050. "Developed countries" correspond to the "more developed regions" according to *The world population prospects: The 2010 Revision* and "developing countries" to the "less developed regions" of the same publication. Source: UNDESA, World population and aging 2011

In the last five years, the number of countries with a life expectancy over 80 has increased from 19 to 33. At present, Japan is the only country in which people aged more than 60 years old represent more than 30% of total population, but this percent-

age is expected to reach 64 countries by 2050 (United Nations Population Fund 2012). Whereas in 1950 only 205 million people aged 60 years or over, in 2012, people of this age represent around 810 million, and according to several estimations will reach 1 billion in less than 10 years and 2 billions by 2050 (United Nations Population Fund. 2012) (fig. 1.1). Moreover, the number of people living beyond the age of 100 years will cross the line of 1 million in the next decades in several countries, including China, United States, Japan, India and Brazil (United Nations, Department of Economic and Social Affairs, Population Division 2011).

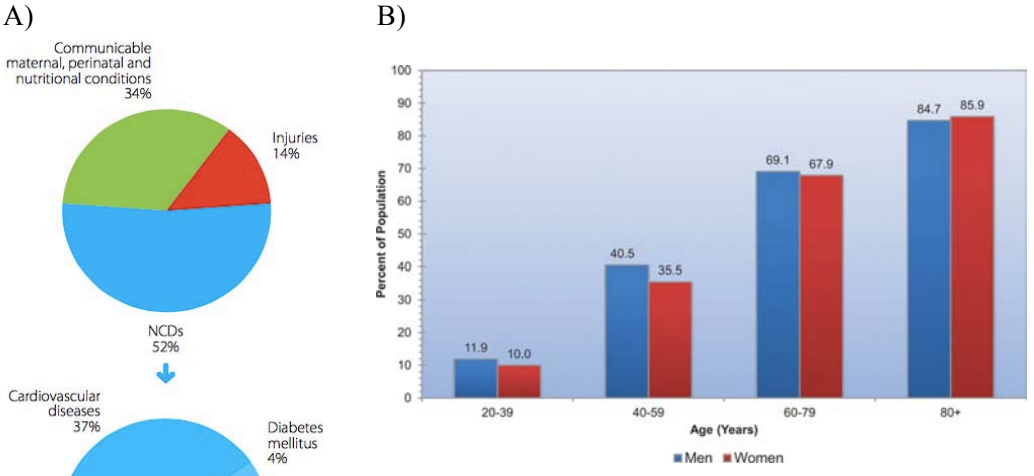


Figure 1.2: A) Proportion of global deaths under the age 70 years, by cause of death, comparable estimates (2012). Adapted from *World Health Organization 2014*. **B)** Prevalence of cardiovascular disease in adults ≥20 years of age by age and sex (National Health and Nutrition Examination Survey: 2009–2012). These data include coronary heart disease, heart failure, stroke, and hypertension. Source: National Center for Health Statistics and National Heart, Lung, and Blood Institute. Adapted from *Mozaffarian et al. 2015*

This demographic change has a profound medical, social and economical impact, due to an increased load in chronic conditions, treatment costs, dependencies and loss of quality of life. Remarkably, cardiovascular diseases (CVD) are already the leading cause of death in all countries (fig. 1.2), far ahead of cancer and degenerative diseases, and among them, ischemic heart disease reaches epidemic proportions in elderly people (fig. 1.3 A) (Lloyd-Jones et al. 2009, Cannon 2013, World Health Organization 2014, Mozaffarian et al. 2015).

Aging is related with increased incidence of myocardial infarction and concomitant aggravation of its clinical outcome. It has also been identified as the main

independent risk factor of the associated mortality in both genders (Dégano, Elosua, and Marrugat 2013) (fig 1.3 B). This may be due, in part, to increased load in comorbidities and extra-cardiac complications. Indeed, the higher load of associated comorbidities present in elderly patients, such as diabetes, atherosclerosis, hypertension and renal dysfunction, has been proposed to lower the threshold for clinical manifestations of cardiovascular disorders (Roger et al. 2011). However, data from preclinical studies and recent clinical observations demonstrate that the vulnerability to ischemic damage is constitutively increased in the senescent myocardium due to intrinsic physiological/structural changes in cardiomyocytes that significantly reduce their capacity to tolerate and adapt to an ischemic insult, even in the absence of other cardiovascular risk factors (Willems et al. 2005). Under similar severity of an ischemic insult aged heart develops an enhanced post-ischemic diastolic dysfunction and infarct size. The increased diastolic dysfunction is related with bigger areas of fibrotic tissue, thus limiting the myocardial elasticity and relaxation (Willems et al. 2005; Azhar et al. 1999). However, there are many other ionic and functional alterations present in the aged heart that can contribute to the increased vulnerability to ischemia. For example, aged cardiomyocytes display an impairment of calcium handling and decreased tolerance to calcium overload by mechanisms not fully elucidated (Cooper et al. 2013).

A) **B)**

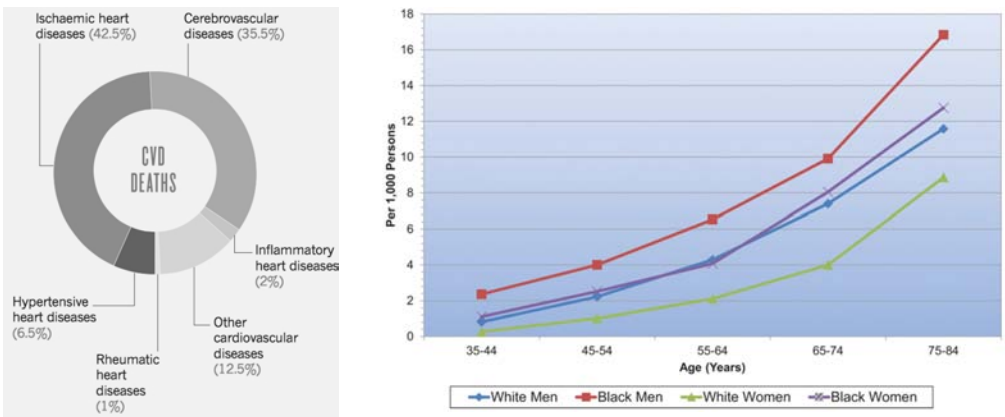


Figure 1.3: A) Among CVDs, ischaemic heart disease (failure to supply blood to the heart) is caused primarily by clogged arteries (atherosclerosis), it results in the most fatalities among men and women but also represents the largest disparity in heart-disease type between the sexes. Adapted from *Cannon 2013*. **B)** Incidence of heart attack or fatal coronary heart disease by age, sex, and race (Atherosclerosis Risk in Communities Surveillance: 2005–2011). Source: National Heart, Lung, and Blood Institute. Adapted from *Mozaffarian et al. 2015*.

Interestingly, aging or age-associated alterations result in a loss or attenuation of the efficiency of some well established cardioprotective interventions, like ischemic or pharmacological preconditioning as well as ischemic postconditioning (Ferdinandy, Schulz, and Baxter 2007; Boengler, Schulz, and Heusch 2009). This loss of cardioprotection results from an attenuated signal transduction, like for example reduced expression of the signal transducer activator of transcription 3 (STAT3) in elderly people (Boengler et al. 2008), which have been described to be involved in cardiac pre- and postconditioning cardioprotection (Bolli, Dawn, and Xuan 2003). Also, a reduction in the expression of connexin 43 –a protein involved in cell-to-cell communication, mitochondrial K^+ permeability and ischemic preconditioning protection- has been described to occur in animals and humans of advanced age (Boengler et al. 2007; Boengler, Schulz, and Heusch 2009).

1.2 Structural and functional changes of the aged heart

Advanced age has been related with structural, mechanical and electrical alterations of the heart. Senescent myocardium develops several cellular and extracellular changes, like reduced number of cardiomyocytes with a compensatory increase of their size and development of focal areas of fibrosis, as well as intracellular changes (oxidations and carbonylations of proteins, lipids and nucleotides) that have important functional and structural consequences (Dhalla, Rangi, Babick, et al. 2012; Fannin et al. 2014).

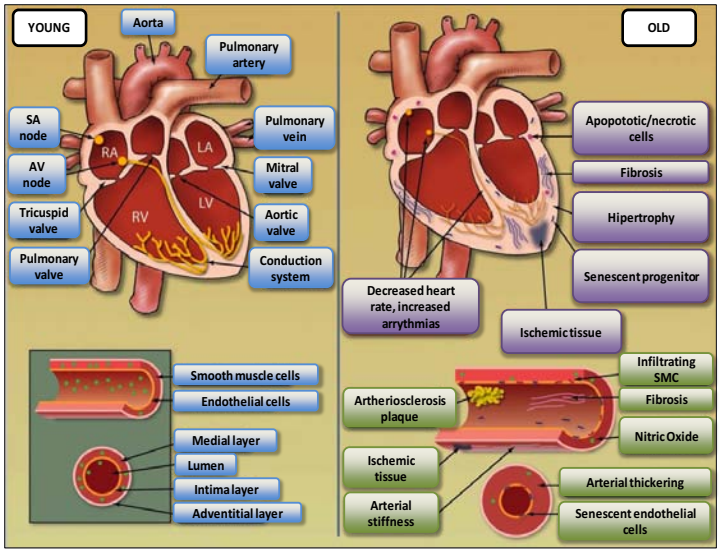


Figure 1.4: Aging is related with numerous alterations in cardiovascular tissues leading to an increase of cardiovascular diseases. Aged cardiovascular tissues show pathophysiological alterations like hypertrophy, arrhythmias, atherosclerotic plaques, arterial stiffness and thickening or fibrotic tissue. Modified from *North and Sinclair 2012*.

It has been demonstrated that aged mice develop structural changes similar to those seen in older humans (Boyle et al. 2011; Feridooni, Dibb, and Howlett 2014), including fibrosis, extracellular matrix remodelling, diminish cellular coupling in the cardiac muscle, as well as delayed activation and slowed velocity of the conduction system throughout both the ventricle and the His-Purkinje system. The alterations in the anisotropic conduction velocity provide a substrate for re-entrant arrhythmias and enforce a pro-arrhythmic effect by decreasing the threshold for ventricular fibrillation. This phenomenon is associated with reorientation of myofibrillar and myocardial sheet structures and contributes to myocardial wall thickening (fig.1.4) (Cooper et al. 2012; Spadaccio et al. 2015). At the vascular level, large elastic arteries develop dilatation and wall thickening, progressive atherosclerosis and reduction in distensibility (Lakatta and Levy 2003). Increased artery stiffness causes an elevation of the arterial pressure that in turn leads to compensatory mechanisms by the myocardium, like left ventricular wall thickening and hypertrophy (Lakatta and Levy 2003; North and Sinclair 2012).

At the functional level, aging heart is related with cardiac contractility impairment, increased left ventricular pressure and a concomitant decline in peak developed pressure indicative of systolic and diastolic dysfunction (Boyle et al. 2011, Qin et al. 2013; Feridooni, Dibb, and Howlett 2014). Some experimental data showed a reduction in the peak tension during aging, especially at higher heart rates, as well as a slowing of the maximum rate of tension (+dT/dt) and maximum rate of relaxation (Jiang, Moffat, and Narayanan 1993; Lim et al. 1999; Feridooni, Dibb, and Howlett 2014). It has been observed that aging is associated with decreased levels of the protein connexin 43 in the myocardium (Boengler et al. 2007)

Connexin 43 is present at the sarcolemma (gap junctions and hemichannels) and at the inner membrane of mitochondria (Rodríguez-Sinovas et al. 2010). Connexin 43 participates in the propagation of the electrical impulse, in the chemical coupling and in the cell permeability at the hemichannels. This last characteristic plays an important role in the calcium overload, cell swelling and cell death propagation during ischemia and reperfusion (Garcia-Dorado et al. 1997; Rodriguez-Sinovas et al. 2004). In mitochondria, connexin 43 has been suggested to form hemichannels that regulate K^+ permeability, respiration and radical oxygen species (ROS) formation, and it has been involved in the cardioprotection afforded by ischemic and pharmacological preconditioning (Miro-Casas et al. 2009; Boengler et al. 2012; Sánchez 2013). Reduction of connexin 43 levels at the gap junctions and mitochondria is associated with the loss of ischemic preconditioning cardioprotection (Boengler, Heusch, and Schulz 2006) and may also be related with impaired ROS generation and increased arrhythmogenesis observed in aged hearts (Jansen et al. 2010; Boulaksil et al. 2010; Rodríguez-Sinovas et al. 2010).

1.3 The aging cardiomyocyte

At the cellular level, biological aging is a multifactorial process characterized by distinct molecular changes, such as increased genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction and altered intercellular communication (López-Otín et al. 2013). Damaged molecules progressively accumulate within the cells, contributing to the loss of homeostasis and increasing the risk of disease and death. It is well accepted that the degree of molecular damage reflects the occurrence of stochastic processes that may be accelerated by environmental factors, whereas the rate of cell repairing mechanisms is genetically controlled, slows down during aging and ultimately determines the organism's longevity.

Importantly, cardiomyocytes are terminally differentiated post-mitotic cells and despite the efforts invested to demonstrate the existence of an active replication in adult cardiac cells, the consensus is that the renewal of human cardiomyocytes in adult age is extremely low (if any) and that most of the cardiac cells we have at the moment of birth remains up to end of our lives (Bergmann et al. 2009). Moreover, experimental evidences indicate that intrinsic (cytochrome c-dependent) apoptotic death is repressed in adult cardiomyocytes (Sanchis et al. 2003). Therefore, some of the phenotypic features of aging cardiomyocytes may reflect the development of progressive adaptive changes to age-induced modifications in the absence of an active cell suicide/cell replacement programme. However, it is presently not known what type of cell remodelling process triggers cell dysfunction or whether cell dysfunction itself favours the development of adaptive remodelling processes, although it seems clear that this type of reactive response may generate a vicious cycle of magnification of cell injury.

Because of the continuous contractile activity, both calcium control and ATP generation are tightly regulated in cardiomyocytes by the sarcoplasmic reticulum (SR) and mitochondria respectively, which make these organelles critical elements in the preservation of cell function. Indeed, disturbed calcium homeostasis and excitation–contraction coupling (EC coupling) (Terentyev et al. 2008), as well as deficient mitochondrial energetics (Rosca et al. 2008) and excessive mitochondrial ROS production (Sawyer et al. 2002) have been consistently reported both in heart failure and aging. These alterations along with many of the structural and functional changes developed by cardiomyocytes during senescence resemble those of failing cardiomyocytes (Strait and Lakatta 2012; North and Sinclair 2012).

1.3.1 The sarcoplasmic reticulum

The SR is a specialized form of endoplasmic reticulum present in cardiomyocytes whose main function is the storage and the release of calcium ions. Indeed, the SR is the main intracellular calcium reservoir of cardiomyocytes. To accomplish this function, it contains or interacts with several types of proteins: a) calcium release proteins (ryanodine receptor); b) calcium reuptake proteins (SR calcium ATPase); c) intraluminal calcium-binding proteins (calsequestrin, histidine-rich calcium-binding protein, sarcolumenin and calreticulin); and d) modulatory proteins involved in signalling pathways (calcium-calmodulin kinaseII, phosholamban, protein kinase A and others). The SR plays a major role in EC coupling.

The **EC coupling** is the conversion of an electrical stimulus (action potential) into a mechanical response (contraction) and is based on a synchronous and highly regulated calcium release from the SR.

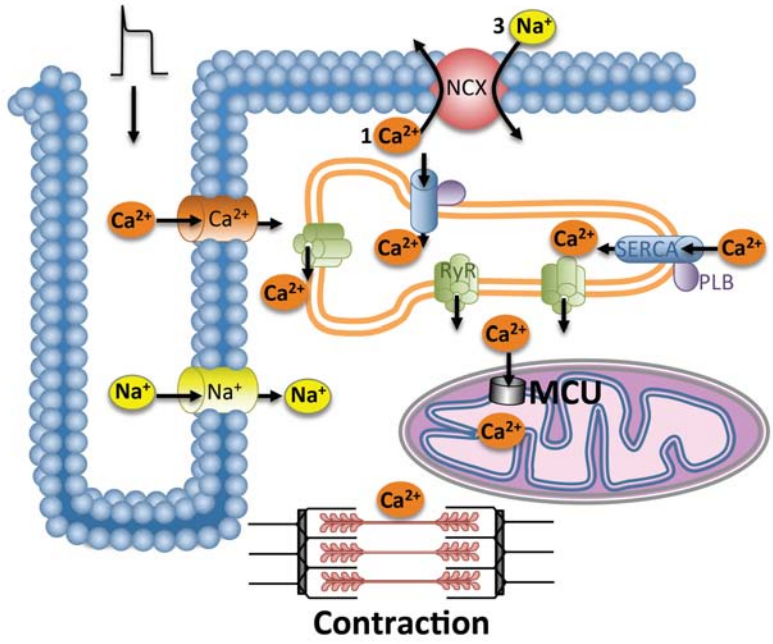


Figure 1.5: Calcium enters into the cells trough the voltage dependent L type calcium channels and triggers the calcium release from the sarcoplasmic reticulum (SR) trough the ryanodine receptors (RyR). Released calcium is mainly used for the cardiac muscle contraction. Nevertheless, part of this calcium is taken up by the mitochondria. Cytosolic calcium levels are normalised by the SR calcium ATPase (back into the SR) and the Na/Ca exchanger from the plasma membrane. Based on *Bers 2002*.

During normal action potential, each cardiomyocyte contraction is initiated by a small amount of calcium entry through L-type calcium channel that is activated in response to membrane depolarization. This small amount of calcium triggers the opening of ryanodine receptors (RyRs) at the SR and induces the subsequent release of calcium stored at the SR, elevating the resting cytosolic calcium concentration from ~100 nM to ~1-2 µM (calcium transient). The interplay between L-type calcium channel and RyRs is possible due to the apposition of both channels in the dyads, membranous structures where sarcolemmal t-tubes and SR are in close anatomical proximity. For a short period of time, calcium concentration around the site of release exceeds the concentrations present at the bulk cytosol by several orders of magnitude (Kohlhaas and Maack 2013). These calcium transients are restricted spatially and temporarily by the diffusion of calcium away from the sites of release to the rest of the cytosol. Most of the released calcium binds to myofilaments to initiate contraction, whereas a small fraction is transferred to mitochondria, where it stimulates ATP synthesis (Terentyev et al. 2008). Diastolic relaxation occurs when calcium is reabsorbed by the SR calcium ATPase (SERCA), whose activity is dependent on ATP availability and modulated by its endogenous inhibitor phospholamban (PLB). The excess of calcium that had entered through L-type channels is finally removed from the cell by the Na^{+}/Ca^{2+} exchanger (NCX) operating in its forward mode (fig. 1.5).

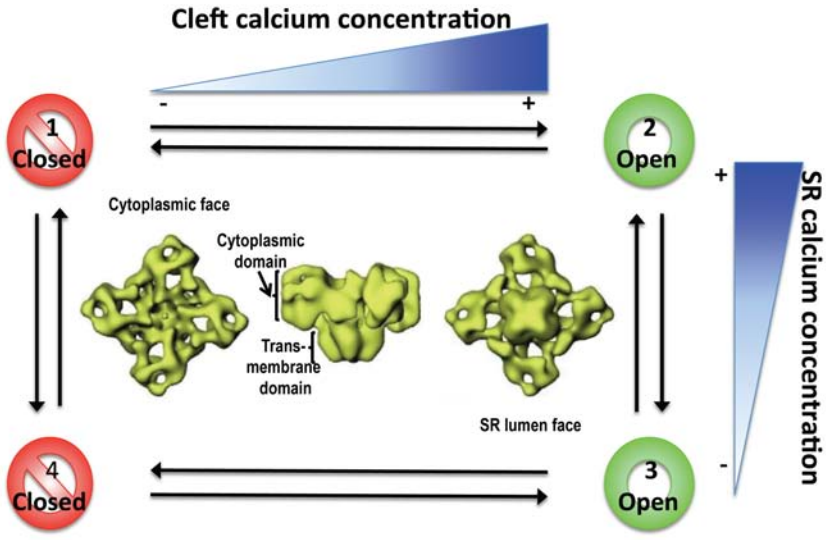


Figure 1.6: According to the probability gating scheme, the ryanodine receptor are more prone to open under high concentrations of calcium inside the sarcoplasmic reticulum and in the cleft.

Advanced age is associated with disturbances in EC coupling and contractile activity at the individual ventricular myocytes (Fares and Howlett 2010; Howlett 2010; Feridooni, Dibb, and Howlett 2014). Contractile dysfunction may arise in part because the ability of SR to handle calcium is adversely affected by the aging process (Feridooni, Dibb, and Howlett 2014). Rigorous control of calcium homeostasis in the cardiomyocyte is crucial, as disruption of any of the elements involved in calcium handling predisposes the aging heart towards the development of contractile dysfunction and heart failure. Among the several proteins and transporters of the SR that may be candidates to be affected by age, RyR and SERCA have been consistently proposed to play an important causative role (Zima et al. 2014).RyRs are intracellular calcium channels present in excitable cells that mediate the release of calcium from the SR in each contraction. Each RyR channel opens independently and stochastically. It is proposed as a four-state Markovian model regulated by the calcium concentration in the cleft and the SR calcium content (Sato and Bers 2011) (fig. 1.6).

RyR interacts with multiple proteins to form a large protein complex that regulate its function under physiological conditions. This protein interaction is also critical for the progression of cardiac dysfunction (Dulhunty et al. 2012). The SR luminal calcium buffering proteins calsequestrin 2 (CSQ2) and histidine-rich calcium binding protein (HRC) play an important role in the probability of transition from the closed to open state of the RyR in response to the luminal calcium concentration (Zhang, Waddell, and Jones 2015). Moreover, there are some molecular links directly connecting RyR with mitochondrial-related proteins like the glucose-regulated protein 75 (Grp 75) and the mitochondrial voltage dependent anion channel (VDAC) (Decuypere et al. 2011). Since the local calcium at the cleft also regulates RyRs, the calcium concentration in the vicinity of the mitochondria will determine the refilling of the SR and eventually the spatio-temporal characteristics of the subsequent calcium signals (Zhang, Waddell, and Jones 2015).

From the 3 different RyR isoforms, the RyR2 is the one primarily expressed in heart muscle. RyR2s are comprised of four identical subunits of ~560 KDa containing 364 cysteine residues, of which ~21 on each subunit are free (L. Xu et al. 1998), prone to be oxidised and acting as sensors of oxidative stress. Impaired activity of RyRs due to thiol oxidation of the cysteines is one of the causes that underlie the hyperactivity of these channels in failing cardiomyocytes (Bers 2006; Mazurek, Bovo, and Zima 2014). Because oxidative damage is increased during aging (Dai et al. 2012, Giordano 2005), the risk of thiol modifications and RyR2 dysfunction is expected to be increased (Terentyev et al. 2008; Andersson et al. 2011; Marx and Marks 2013; Mazurek, Bovo, and Zima 2014).

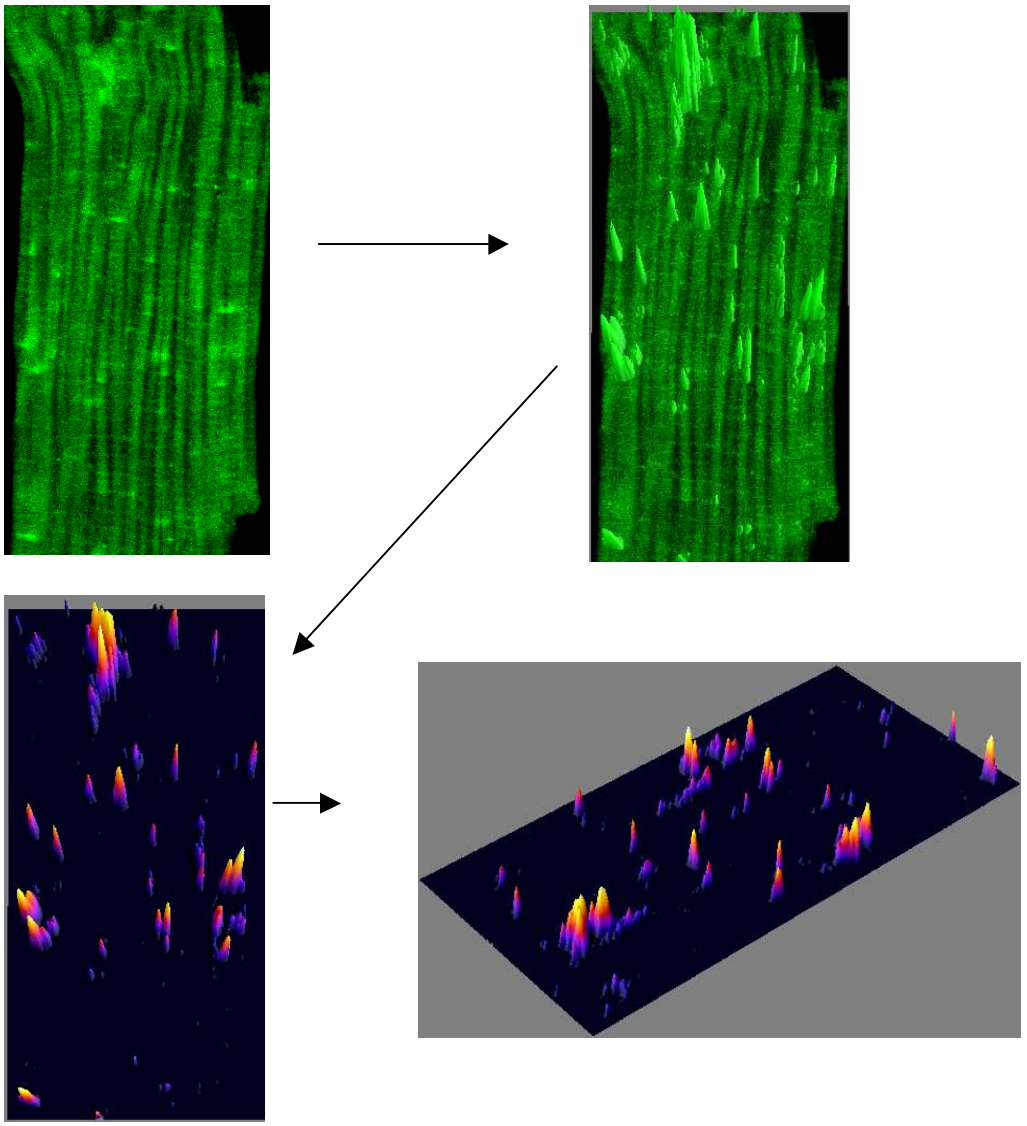


Figure 1.7: Quiescent cardiomyocyte loaded with fluo-4AM fluorescent probe and 3D surface plot (ImageJ). Red arrows and 3D images show the spontaneous calcium sparks: calcium release events causing a transient increase of cytosolic calcium concentration restricted to small areas all along the cell. Non-published data from our laboratory.

Together with RyR, **SERCA** is the most abundant protein of the cardiac SR. SERCA is a calcium ATPase that transfers calcium from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis, allowing contractile relaxation (diastole). It is responsible for the sequestration of most of the cytosolic calcium released

by RyR in each contraction, although the total amount of calcium taken up by SERCA depends on animal species. Thus, in rodents SERCA reuptakes 92% of cytosolic calcium and only the remaining 8% is extruded by the sarcolemmal Na^+/Ca^{2+} exchanger (Bassani, Bassani, and Bers 1994) while in humans SERCA and Na^+/Ca^{2+} exchanger account for 76% and 24% calcium normalization, respectively (Piacentino 2003). The affinity of SERCA for calcium is mainly controlled by the regulatory protein phospholamban (PLB). Thus, SERCA is normally inhibited by PLB, with whom it is closely associated. Phosphorylation of PLB at Ser-16 by protein kinase A (PKA) or by calcium dependent calmodulin kinasa II (CaMKII) at Thr-17 relieves its inhibition and increases the pumping rate by several folds (Simmerman et al. 1986; Zima et al. 2014). In addition to the PLB regulation, SERCA is highly sensitive to changes in the metabolic status of the cytosol, including the ATP/ADP ratio, pH and the redox potential (Zima et al. 2014), and it may interact with a wide array of proteins including HRC, phosphatase 1 (PP1), calreticulin and sarcolipin (Kranias and Hajjar 2012).The contribution of SERCA to the pathophysiology of heart failure and to functional decline during aging remains controversial. It has not yet been established whether its expression and function may be affected by these conditions, and current experimental data are somehow contradictory. While several studies showed no change in SERCA function in intact and permeabilized failing cardiomyocytes or in aged cells (Belevych et al. 2007; Belevych et al. 2011; Cooper et al. 2013) other data report a reduction in SR calcium uptake in heart failure (Jiang 2002, Lipskaia et al. 2014), as well as decreased calcium transient amplitude and diminished SR calcium content (Hobai and O'Rourke 2001; Jiang 2002; Piacentino 2003; Luo and Anderson 2013; Zima et al. 2014). Depressed SR calcium content and reduced calcium transient amplitude have also been described in aged human atrial cardiomyocytes, favouring the development of contractile dysfunction (Herraiz-Martínez et al. 2015). Overall, SERCA modifications and their role in heart failure and aging remain unclear.

1.3.2 The mitochondria

Due to the continuous contractile activity throughout life, the heart is the organ with the highest energy demand of the body (30 Kg ATP/day) (Skulachev 1999). To accomplish this mechanical function, an adult cardiomyocyte contains up to 7000 mitochondria (35-40% of total cell volume) that produce 90% of total cell ATP through oxidative phosphorylation (Dedkova and Blatter 2012). Most of the ATP is actively transferred to SR and to myofibrils for calcium handling and contractile activity, respectively. During oxidative phosphorylation, up to 1% of electrons escape their normal route and give rise to ROS (Chen and Zweier 2014). Although ROS can be gener-

ated in other cellular compartments by multiple enzymes, like NADH-oxidases at the plasma membrane, cyclo-oxygenases and xanthine-oxidase in the cytoplasm or lipid oxidation in the peroxisomes, mitochondria are the main cellular source of ROS (Dai, Rabinovitch, and Ungvari 2012).

Mitochondrial respiration and ROS production:

Mitochondrial respiratory complexes (complexes I to IV) are responsible for the oxidation of NADH or $FADH_2$ reducing equivalents, originated in different metabolic pathways (glycolysis, fatty acid oxidation or the Krebs cycle). Oxidation of NADH and $FADH_2$ is coupled to the pumping of protons into the intermembrane space.. NADH reducing equivalents enter the mitochondrial electron transport chain (ETC) through complex I, whereas $FADH_2$ reducing equivalents enter the mitochondrial ETC through complex II. The electrons are then transferred to coenzymeQ, and subsequently to complex III, cytochrome c, and complex IV, which passes them to oxygen as the final acceptor. ETC complexes organise in larger structures known as respiratory supercomplexes, which allow a more efficient transport of electrons. Finally the resulting proton gradient is used by the FoF1 ATP synthase (complex V) to generate ATP (Acin-Perez and Enriquez 2014) (fig. 1.8).

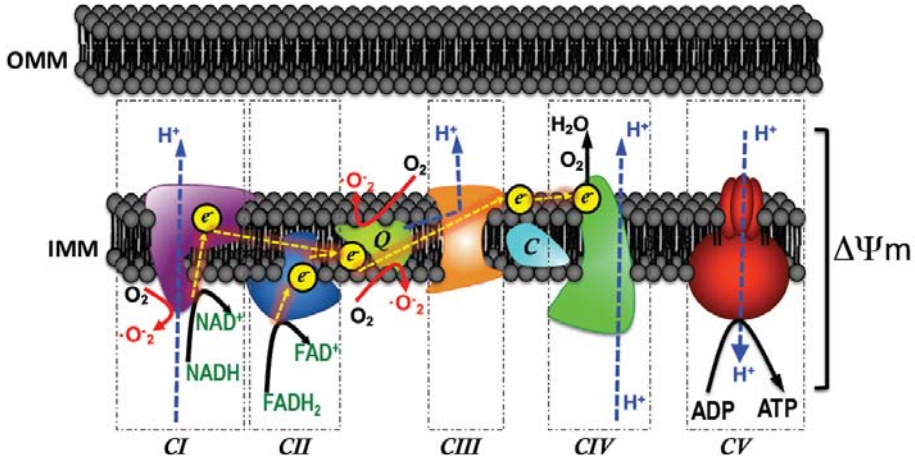


Figure 1.8: Schematic view of the electron transport chain complexes, electrochemical proton gradient generation, ROS production and ATP synthesis.

The mitochondrial FoF1 ATP synthase is a highly conserved molecular structure. In the heart, the mitochondrial it is responsible for production of more than 90% of the aerobic ATP (Bernardi et al. 2015). The whole complex has a molecular mass that varies between 540 and 585 kDa and is highly present in mammalian heart mito-

chondria (Schägger and Pfeiffer 2001). Favoured by the gradient generated by the ETC, the protons flow through membrane domain of the enzyme causing the rotation of the rotor assembly (subunits, and a ring of 10–15 copies of subunit c). Rotation of, integrated inside a cylinder, consisting on three copies each of alternating α and β subunits, changes the conformation of the catalytic nucleotide-binding sites on the three β subunits, so that bound ADP and Pi is converted to ATP and released (J. Weber 2010). A stator composed by the b, d, F6, and OSCP subunits connects the top of the F1 sector with the lateral portion of Fo sector (Jonas et al. 2015).

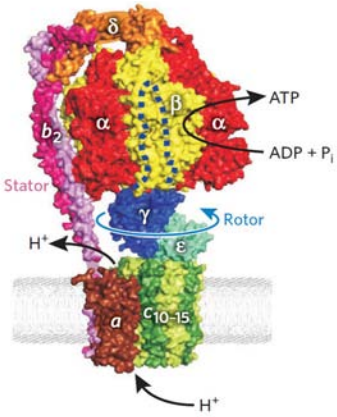


Figure 1.9: The model is assembled from several partial structures obtained by X-ray crystallography, NMR and modelling. Bacterial ATP synthase has a subunit stoichiometry of α3β3γδε*ab*2*c*. Proton flow through two half channels at the *a*–*c* interface drives rotation of the rotor (γε*c*), which in turn causes conformational changes in the catalytic sites on the β subunits, resulting in synthesis of ATP from ADP and Pi. Adapted from *Weber 2010*.

Some electrons escape their normal route along enzymatic complexes and give rise to ROS. Complexes I and III are the main responsible for mitochondrial ROS emission (net result of ROS production and elimination), of which 70-80% are released into the mitochondrial matrix and 20-30% diffuses to the intermembrane space (A. Navarro and Boveris 2007). Superoxide is transformed to H_2O_2 by mitochondrial manganese-dependent superoxide dehydrogenase. Glutathione peroxidase (GPX) and peroxiredoxin (PRX) transform H_2O_2 into water. GPX and PRX are subsequently regenerated by glutathione (GSH) and thioredoxin (TRX). Reduction of GSH and TRX is mediated by NADPH which is generated by 3 enzymes derived from the Krebs cycle: $NADP^+$ -dependent isocitrate, malic enzyme and nicotinamide nucleotide transhydrogenase. Krebs cycle is therefore the main source of the necessary reducing power for ATP production at the electron transport chain (NADH and $FADH_2$) and of the antioxidant capacity of the mitochondrial matrix through NADPH (Dorn and Maack 2012; Nickel, Kohlhaas, and Maack 2014).

Mitochondrial ROS production has been postulated to be causally linked to the aging process in what is known as the free radical theory of aging (Harman 1956). Although experimental data remains controversial, this theory has proved not to be

sufficient to explain the complexity of biological aging. Mitochondrial ROS have a dual role: on one hand, they participate in cardioprotective-promoting signalling pathways; on the other hand, they may have deleterious effects by acting on several cell targets, i.e., mitochondrial DNA, lipids and proteins -including the enzymes of the respiratory complexes-. Oxidative damage of respiratory complexes may further impair mitochondrial respiration and increase ROS production in a vicious cycle, eventually leading to the aggravation of energy deficit, decline in the number of mitochondria and cell death (Dai, Rabinovitch, and Ungvari 2012).

Experimental data confirm that aging is related with an increased ROS production in myocardial tissue (Judge et al. 2005) Furthermore, results obtained using a mouse model of impaired polymerase gamma proof reading support the concept that mitochondrial DNA mutations are directly associated with cardiomyopathy, cardiac hypertrophy and altered systolic and diastolic functions (Dai et al. 2009). These effects could partially be reversed by overexpressing the antioxidative enzyme catalase targeted to the mitochondria (Dai et al. 2009; Moslehi, DePinho, and Sahin 2012).

In mouse models, reduced antioxidant expression is sometimes related with an acceleration of the aging process (Edrey and Salmon 2014) while an increase in the antioxidant enzymes expression does not slow down aging (Pérez et al. 2009; Edrey and Salmon 2014). According to these findings, the causative role of mitochondrial ROS on age-induced cell dysfunction remains controversial and further investigation on this topic is required.

Mitochondrial calcium handling:

Mitochondria play an important role in cell biology as regulators of calcium signals. Together with SR, they are the main intracellular calcium storage organelle Calcium diffuses across the outer mitochondrial membrane using the non-specific VDAC and subsequently to the mitochondrial matrix through the low affinity mitochondrial calcium uniporter (MCU) (fig. 1.5) located at the inner mitochondrial membrane. Mitochondrial calcium is mainly extruded by the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) (Williams et al. 2013).

Transport of calcium through MCU is favoured by the electrochemical gradient (Kovács-Bogdán et al. 2014; Foskett and Philipson 2015). The MCU is a recently discovered 40 kDa channel protein (De Stefani et al. 2011) expressed in high density in the inner mitochondrial membrane (10-40 per μm^2). Its affinity for calcium is surprisingly low with half-maximal activation ($K_{0.5}$) in the range of high μM to low mM calcium concentration, remotely close to ~1 μM calcium concentration normally achieved in cardiomyocyte cytosol. Despite this low affinity, mitochondria are able to take up calcium in a beat-to-beat manner during the EC coupling, suggesting the existence of a

privileged communication between mitochondria and SR probably facilitated by "hot spots" calcium at the interface of both organelles (Dorn and Maack 2012). In fact, up to 90% of the calcium release units of ventricular SR are close to mitochondria (Sharma et al. 2000).

Mitochondrial calcium uptake is essential for short and long-term metabolic adaptations. Once inside the mitochondria, calcium regulates fatty acid oxidation, amino acid catabolism, aspartate and glutamate carriers, as well as the activity of the adenine-nucleotide translocase, Mn-SOD and FoF1 ATP synthase (Yamada and Huzel 1988; Decuypere et al. 2011). Moreover, calcium stimulates mitochondrial ATP production by regulating the activities of three dehydrogenases of the Krebs cycle: pyruvate dehydrogenase, isocitrate dehydrogenase and α- ketoglutarate dehydrogenase (Lawlis and Roche 1981; Decuypere et al. 2011; Dorn and Maack 2012). Because of the role of calcium in the control of Krebs cycle, tightly regulated mitochondrial calcium uptake is essential for matching energy demand with energy supply. By regulating Krebs cycle activity, mitochondrial calcium uptake modulates the generation of both electron donors (NADH and FADH) necessary for ATP synthesis and coenzymes (NADPH) necessary for the regeneration of antioxidant enzymes (TRX and GSH) (Dorn and Maack 2012; Nickel, Kohlhaas, and Maack 2014).

Maintaining calcium homeostasis has a major ATP cost for cardiomyocytes, being SERCA and the ATP dependent sarcolemmal ionic transporters (i.e. the Na^+/K^+ ATPase) the largest energy-consuming proteins (Luo and Anderson 2013). Hence, insufficient ATP production may trigger alterations in calcium handling and contractile activity (V. Saks et al. 2006; Kohlhaas and Maack 2011).Heart failure is related with abnormal energy metabolism, including decreased energy production and impaired energy utilization, which appear to adversely affect intracellular calcium homeostasis (Saks et al. 2006; Balaban 2002); Luo and Anderson 2013). Decreased ATP/ADP ratio, due to mitochondrial dysfunction, causes impaired function of SERCA in animal models of heart failure (Joubert et al. 2008). In this context, some validated clinical therapies for heart failure like β-adrenoreceptors antagonists and β-blockers have been demonstrated to improve myocardial energetics and normalize intracellular calcium homeostasis increasing contractile function in failing human hearts (Sabbah 2004). Thus, heart failure appears to be a condition that occurs, at least in part, by defects in mitochondrial calcium homeostasis (Luo and Anderson 2013).

Under certain pathological conditions, an excess of mitochondrial calcium may induce the irreversible opening of a mitochondrial permeability transition pore (mPTP). Mitochondrial PTP is a non-specific channel that allows the diffusion of molecules up to 1500 Da. Its opening is incompatible with cell life because it induces: 1) massive entry of protons and irreversible dissipation of the mitochondrial membrane

potential (ΔΨm), preventing oxidative phosphorylation and ATP synthesis; 2) leak of calcium and nucleotides, among other small molecules; 3) entry of water and solutes of molecular mass up to 1500 Da causing matrix swelling, unfolding of mitochondrial cristae and rupture of the outer mitochondrial membrane; and 4) release of cytocrome c and other pro-apoptotic molecules (eg., apoptosis inducing factor) able to activate cell death pathways (Muntean 2003; Ruiz-Meana 2012).

Although some reports point to an increased susceptibility to calcium-induced mPTP opening in heart mitochondria from aging rats (Petrosillo et al. 2010), the impact of aging on mitochondrial calcium handling in cardiomyocytes remains largely unknown. Moreover, mitochondria are not homogeneously distributed within the cardiomyocytes but specialized in different populations that differ in their location, biochemical properties and functional traits, including their ability to handle and tolerate calcium and susceptibility to undergo mPTP opening.

Subpopulations of cardiac mitochondria:

Cardiomyocytes have three spatially different mitochondrial populations: 1) the subsarcolemmal mitochondria (SSM), located immediately beneath the sarcolemma; 2) the interfibrillar mitochondria (IFM), located along the myofibrils; and 3) the perinuclear mitochondria (PNM), located around the nuclei (fig.1.10 A). These mitochondrial populations have differential distribution, morphology and function (Hoppel et al. 2009; Hollander, Thapa, and Shepherd 2014). This high subspecialisation of cardiac mitochondria indicates that they may be involved in different signalling pathways and may play specific physiological roles within the cardiomyocytes.

The IFM are the most abundant mitochondria in cardiac cells. They represent around 80% of total mitochondrial pool (Adhihetty et al. 2005). The IFM are typically more elongated and have tubular cristae. Because of their close proximity to myofibrils, they are probably the main source of ATP for myosin ATPases (Palmer, Tandler, and Hoppel 1985; Rosca and Hoppel 2013; Hollander, Thapa, and Shepherd 2014). The IFM have higher respiratory activity (around 1.4 to 1.7 times higher oxidative rates than SSM), suggesting a higher metabolic activity (Palmer, Tandler, and Hoppel 1985; Rosca and Hoppel 2013). As a consequence of a more active oxidative phosphorylation, the dissipation of the electrochemical gradient is more pronounced and they display lower membrane potential during respiration. However, the IFM have higher capacity to uptake and retain calcium and are much more resistant to undergo mPTP opening (Hofer et al. 2009), probably because they are physiologically exposed to higher calcium concentration.

By contrast, SSM have lamelliform cristae and are much more variable in shape and size (fig. 1.10 B) (Hoppel et al. 2009; Hollander, Thapa, and Shepherd

2014). The SSM are restricted to the surface of the cell in close contact with the sarcolemma. They are supposed to provide the ATP necessary to feed the sarcolemmal ionic transporters (i.e. the Na^+/K^+ ATPase) but they have probably other non-identified regulatory roles. It has been reported that connexin 43, a protein involved in the mitochondrial potassium permeability that plays a role in ischemic preconditioning protection, is expressed only at the inner membrane of SSM but it is absent in in the IFM (Boengler, Heusch, and Schulz 2006; Ruiz-Meana et al. 2014).

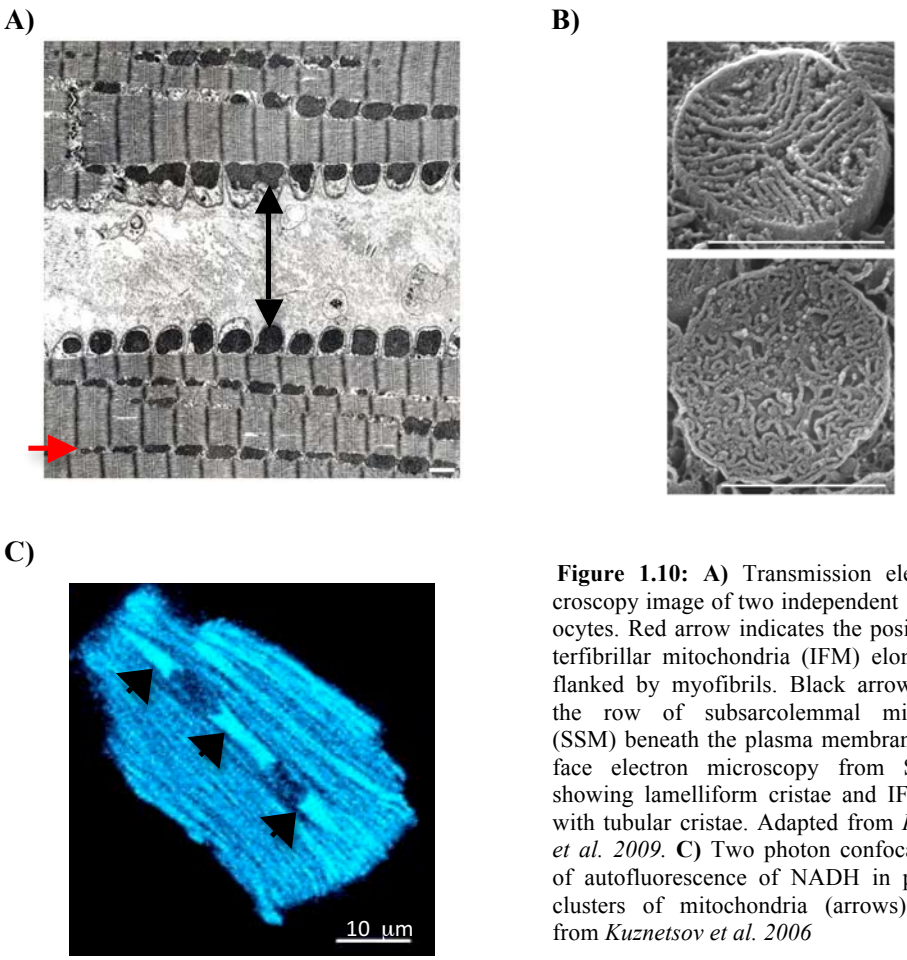


Figure 1.10: A) Transmission electron microscopy image of two independent cardiomyocytes. Red arrow indicates the position of interfibrillar mitochondria (IFM) elongated and flanked by myofibrils. Black arrows indicate the row of subsarcolemmal mitochondria (SSM) beneath the plasma membrane. **B)** Surface electron microscopy from SSM (up) showing lamelliform cristae and IFM (down) with tubular cristae. Adapted from *Hoppel CL et al. 2009.* **C)** Two photon confocal imaging of autofluorescence of NADH in perinuclear clusters of mitochondria (arrows). Adapted from *Kuznetsov et al. 2006*

The PNM are concentrated in clusters around the nuclei and display a more roundish morphology and smaller size than IFM. These perinuclear clusters are densely packed and show brighter autofluorescence as a result of higher concentration of mito-

chondrial NADH (Kuznetsov et al. 2006), (fig. 1.10 C). Such perinuclear mitochondrial clustering may serve to drive mitochondrial metabolism to generate ATP close to the nucleus (Bruce et al. 2004), according to a previously proposed concept of an integrated phosphotransfer network and energy channelling between mitochondria and nucleus (Dzeja et al. 2002). The physiological role of perinuclear mitochondria remains a mystery but it seem reasonable to speculate that they may play a role in the nuclear import machinery (Kuznetsov et al. 2006; Kuznetsov and Margreiter 2009; Hollander, Thapa, and Shepherd 2014).

The pathophysiological relevance of this mitochondrial specialization is only beginning to be deciphered. Thus, it has been demonstrated that while SSM are susceptible to be protected by ischemic preconditioning when submitted to transient ischemia-reperfusion, IFM are resistant to preconditioning protection (Ruiz-Meana et al. 2014). It is therefore conceivable that functional consequences of aging may be differentially manifested in mitochondrial populations. Indeed, aging process is related with decreased capacity to retain calcium (Hofer et al. 2009) and impaired respiratory rates in IFM, whereas SSM characteristics remain unaltered. The mechanisms by which age specifically impacts on the function of IFM remain unknown (Edward J Lesnefsky and Hoppel 2006) but impairment of IFM could be causally linked to the reduced contractile reserve of senescent cardiomyocytes (Judge et al. 2005).

Mitochondrial longevity pathways:

Mitochondria are critically involved in the longevity of the organisms and the mechanisms by which they may regulate the rate of aging are multiple. Although it was initially thought that this link was due to the deleterious effect of mitochondrial ROS on cell survival, the current view is that the relationship between mitochondria and longevity is much more complex. On one hand, mitochondria tightly communicate with nuclear genome (Kaniak-Golik and Skoneczna 2015), which ultimately determines the rate of aging (Moskalev et al. 2013). However, during normal aging the stability and integrity of DNA, both genomic and mitochondrial, are continuously challenged by exogenous physical, chemical and biological agents, as well as endogenous risk like DNA replication errors and ROS (Hoeijmakers, 2009). In consequence, the efficiency of the respiratory chain decreases with age, resulting in an increased electron leakage and a reduction of ATP production (Green, Galluzzi, and Kroemer 2011). Moreover, during aging there is a progressive imbalance between mitochondrial proliferation (biogenesis) and degradation process (mitophagy). This imbalance contributes to over-proliferation of damaged mitochondria and compromises cell resistance to stress (Palikaras K, Nature 2015). Dysfunctional mitochondria may impact in the cell signalling and calcium cross talk between ER/SR and mitochondria (Rafaello and Riz-

zuto 2011) impairing the regeneration of antioxidant enzymes necessary for the activity of the repairing systems.

Because aging is associated with an overall increase in the oxidative damage of mitochondria proteins, in part due to inefficient repairing systems, a destabilization of the macromolecular organization of respiratory chain super-complexes contributes to the impairment of mitochondrial bioenergetics in the senescent organism (López-Otín et al. 2013). This effect clearly impacts on cell functional decline during aging as it is well established that ATP levels and energetic status works as an internal clock for lifespan and aging rate in all organisms (Doonan et al. 2008; Wallace, Fan, and Procaccio 2010; Green, Galluzzi, and Kroemer 2011).

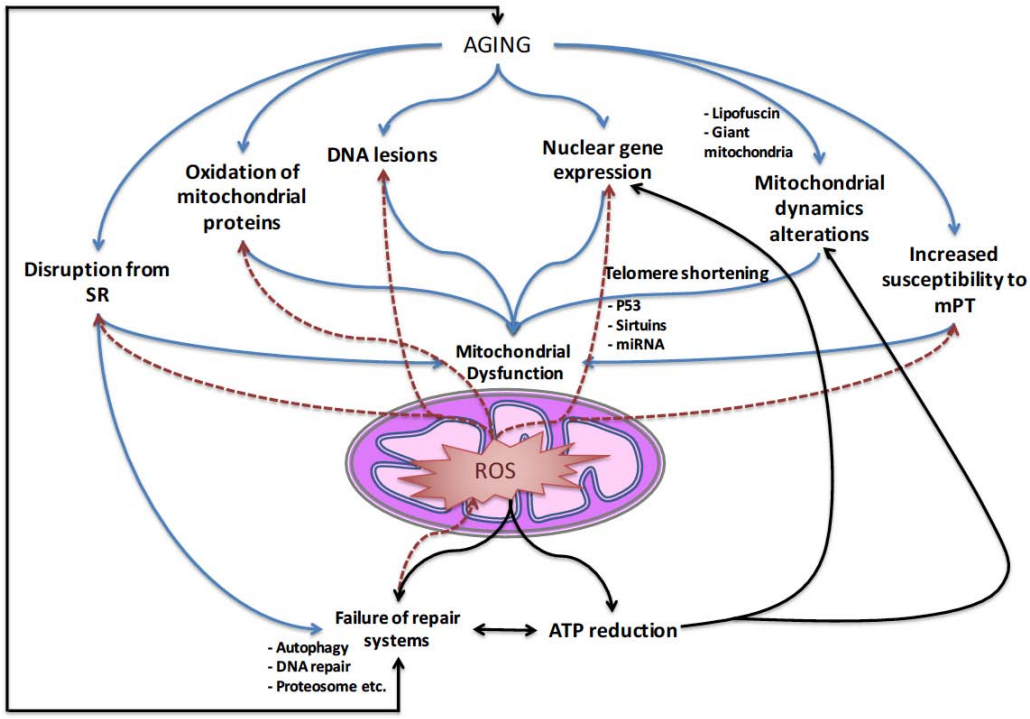


Figure 1.11: Summary of the complex pattern of mitochondrial dysfunction related with aging. Mitochondrial function becomes altered with aging resulting in an increase of ROS generation exacerbation the rate of aging by a vicious cycle.

Recent evidences indicate that mitochondrial longevity pathways can be exogenously regulated by some interventions like diet (North and Sinclair 2012). In this context, sirtuins - highly conserved proteins that function as NAD+ -dependent deacetylases- have been identified as one of the key players of the regulatory pathways

involved in the longevity programme of the cells (Haigis and Sinclair 2010; North and Sinclair 2012).Importantly, many of these pathways are interconnected by calcium signalling among mitochondria and the rest of the cell (Pinton et al. 2007)

In summary, mitochondria participate in the longevity of organisms modulating the rate of aging through myriads of pathways that only start to be identified. These pathways are interrelated following an intricate pattern (fig. 1.11) in which it is difficult to elucidate which player has a causative role or it is just a part of a more complex picture.

Mitochondrial turnover and dynamics

Mitochondria are semi-autonomous elements, the biogenesis of which depends on the coordinated expression of the genome located in the nucleus and the mitochondrial matrix. From the 1000-1200 mitochondrial proteins only around 1% are encoded by the mitochondrial DNA. The rest of the proteins are nuclear-encoded, synthesized in the cytoplasm and imported into the mitochondria where they are arranged and assemble into the mitochondrial subcompartments (Osiewacz and Bernhardt 2013). Mitochondria are dynamic organelles and may increase in number and mass as a result of fission and fusion of existing mitochondria, incorporation of newly synthesized molecules and selective degradation by mitophagy (Osiewacz and Bernhardt 2013). Fission may be an important component for excising severely damaged parts of an existing mitochondrion (Youle and van der Bliek 2012), whereas fusion is generally accepted to play a role in quality control by mixing damaged mitochondria with functional ones (Youle and van der Bliek 2012). In a healthy cell, mitochondrial fusion and fission processes occur in a constant and balanced manner and are, in turn, interrelated with the removal of dysfunctional/damaged mitochondria by mitophagy (Weber and Reichert 2010; Scorrano 2013).

Advanced age is associated with an impaired capacity of the cells to remove all damaged structures that gradually accumulate in the intracellular space and contribute to the functional deterioration. This process explains the presence of many dysfunctional and usually enlarged mitochondria, lipofuscin-loaded lysosomes and oxidatively modified cytosolic proteins and lipids in the aging cardiomyocytes (Squier 2001; Grune et al. 2004). The accumulation of biologic garbage is also related with certain toxic effects, such as increased ROS production by senescent mitochondria, or release of lysosomal enzymes by lipofuscin-loaded lysosomes (Wihlmark et al. 1997; Brunk and Terman 2002b; Terman et al. 2010).

A reduced mitochondrial turnover, secondary to decreased lysosomal degradative capacity (defective mitophagy), is the most likely cause of the increase in the number of damaged mitochondrial in the aged heart (Brunk and Terman 2002a). These

mitochondria show an aberrant structure (with reduction in the number of cristae and matrix swelling) and damaged membranes that can disturb fission process, which eventually results in the formation of giant mitochondria (Terman et al. 2003). Giant mitochondria are resistant to degradation and do not fuse with healthy ones. Since autophagy/mitophagy is an energy-dependent process, the degradation of large organelles like giant mitochondria is more energy demanding than that of small ones, impairing even more the energetic status of the cell (Terman and Brunk 2005). Primary changes in the mitochondria due to accumulative oxidative damage appear to be the trigger mechanisms involved in the sequence of alterations in mitochondrial turnover and dynamics during aging.

1.3.3 Mitochondria–Sarcoplasmic reticulum communication. Cross talk between close neighbours.

The physical relation between sarco/endoplasmic reticulum (SR/ER) and mitochondria was first reported in 1958 (Copeland and Dalton 1959). Thirty years later this cellular fraction, known as mitochondria-associated membranes (MAM) was isolated for the first time using differential centrifugation and Percoll gradient (Vance 1990). Some years later, the pioneering studies of Rizzuto and coworkers demonstrated the existence of calcium microdomains around SR-mitochondria subcellular regions in different cell types, with important functional consequences (Rizzuto et al. 1998).

Confocal microscopy imaging using mitochondria and endoplasmic reticulum (ER)-targeted spectral variants of green fluorescent protein allowed the visualization of the tethering structure connecting both organelles (Brito and Scorrano 2008). With this technical approach, the authors proposed an interorganelle distance below 270 nm (Brito and Scorrano 2008). Electron microscopy estimations disclosed an average distance between ER/SR and mitochondria on the range between 37 and 270 nm (Sharma et al. 2000). Electron tomography studies revealed a tethering pattern of clusters of six or more units in which the intermembrane distance was as low as 6-15 nm (Csordás et al. 2006). These estimations are coincident with more recent analysis of transmission electron micrographs in which the distance between the junctional SR and mitochondria averaged 15–20 nm (Chen et al. 2012; Kohlhaas and Maack 2013). Altogether, these observations are coincident in establishing the existence of subcellular anatomical structures in which SR and mitochondria are in close juxtaposition (fig. 1.12).

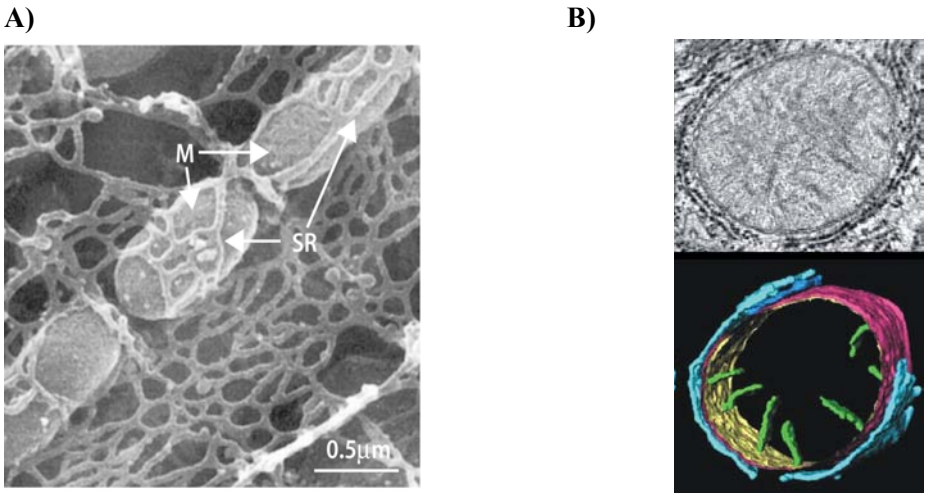


Figure 1.12: A) Mitochondria/ER interaction observed by field emission scanning electron microscope (SEM) from dog cardiomyocytes. *Adapted from Yoshikane H. J Submicrosc 1986*. **B)** Electron micrographs from a hepatocyte (up) and three-dimensional reconstruction (down) of the ER/mitochondria interaction where the close apposition of the ER (blue) and the outer mitochondrial membrane (pink) can be notice. The inner mitochondrial membrane is represented in yellow and the cristae in green in the three-dimensional reconstruction. *Adpated from Rizzuto and Pozzan 2006.*

So far, several proteins have been identified involved in both bridging/ structural stability and chemical communication (mainly calcium transfer) between SR and mitochondria (Wieckowski et al. 2009; Z. Liu et al. 2015) (fig. 1.13). Mitofusin 2 (Mfn-2), a dynamin-related GTPase, was the first identified protein providing both the physical connection between endoplasmatic reticulum (ER) and mitochondria in fibroblasts and HeLa cells and allowing the interorganelle calcium exchange (Brito and Scorrano 2008). Accordingly, Mfn-2 genetic ablation was described to reduce the number of contact points between both organelle by about 40% and to alter SR/ER and mitochondrial morphology (Brito and Scorrano 2008). A recent report has demonstrated that Mfn-2 is present in cardiomyocytes, where it plays an essential role in physiological calcium signalling and normal bioenergetic response (Chen et al. 2012). In this study, genetic ablation of Mfn-2 induced a trend towards an increase in the mean distance between junctional SR and the outer mitochondrial membrane in cardiac myocytes. This structural defect was paralleled by a dysfunctional energy demand-supply coupling, impaired mitochondrial calcium uptake and increased mitochondrial ROS production in these cells (Chen et al. 2012).

The Grp75 is a cytosolic chaperone proposed as another candidate to participate in ER-mitochondria interconnection in HeLa cells and rat hepatocytes (Szabadkai et al. 2006). Many evidences support the existence of a physical link between

RyR/Inositol-P_3 receptor (IP_3R) and the outer mitochondrial membrane VDAC through Grp75 (Decuypere et al. 2011). This association has been proved to be critical for the bioenergetics crosstalk and calcium flux between both organelles which function following a "quasisynaptic" organization (Csordás et al. 2006;Szabadkai et al. 2006). High resolution imaging of HeLa cells expressing the sensitive-calcium photoprotein aquaporin targeted to mitochondria indicates that calcium release from the ER is followed by a significant increase of mitochondrial calcium. Calcium concentration reached at the mitochondrial compartment after this intervention is higher than the concentration achieved in the bulk cytosol (Rosario Rizzuto et al. 1998). These results confirm the existence of a privileged calcium exchange pathway between both organelles.

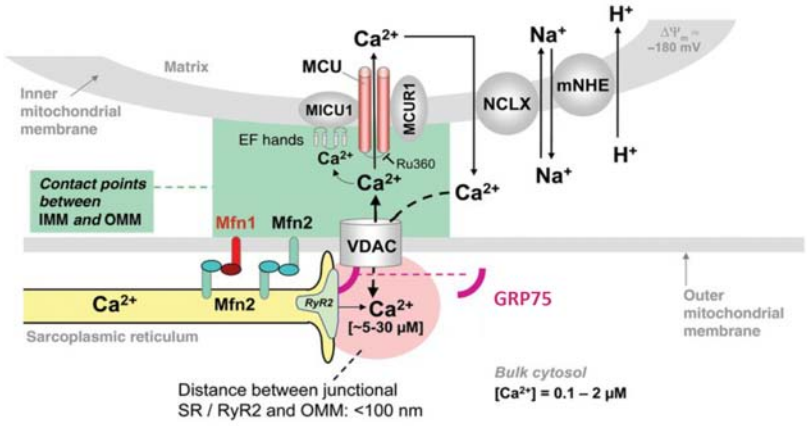


Figure 1.13: Scheme of the anatomy and physiology of the mitochondria-SR microdomains. Due to the proximity between the organelle and the close apposition of the ryanodine receptor (RyR) with the voltage dependent anion channel (VDAC) in the outer mitochondrial membrane the calcium can be taken up by the mitochondrial calcium uniporter to the mitochondrial matrix. Adapted from *Kolhass and Maack 2013.*

In cardiac cells, efficient mitochondrial calcium uptake secondary to SR calcium release is possible because of the existence of locally restricted high calcium subcellular microdomains (Rosario Rizzuto and Pozzan 2006). Indeed, recent data support the view that mitochondrial calcium uptake driven by the MCU, is more dependent on the calcium concentration present around the contact sites between SR and mitochondria than on bulk cytosolic calcium concentration. Computational modelling predicts calcium concentrations of 10–30 µmol/L at a distance of 200 nm from RyRs after SR calcium release (Peskoff and Langer 1998), a concentration that is in the range of MCU $K_{0.5}$. Also, integrative models that considering their algorithm mitochondrial calcium uptake/release during calcium transients and Krebs cycle dehydrogenases ac-

tivity predict rapid mitochondrial calcium transients during each cell contraction (Cortassa et al. 2003), a response that has been empirically confirmed in guinea pigs cadiomyocytes loaded with the mitochondrial calcium probe rhod-2 (Maack et al. 2006).

Mitochondrial and cytosolic calcium transients' kinetics differs from each other. Mitochondrial calcium transients peak slightly earlier than cytosolic ones, supporting the concept of direct tunnelling of calcium from SR to mitochondria. By contrast, the decay of calcium concentration in mitochondrial matrix is slower than in the bulk cytosol (Maack et al. 2006), in agreement with the slow kinetics of the main mitochondrial calcium extrusion mechanism, the mNCX. As a consequence, an increase in calcium transient frequency leads to mitochondrial calcium accumulation in the upper nanomolar range despite normal cytosolic calcium level (Maack et al. 2006). Due to these kinetics differences it can be hypothesized that mitochondria are able to anticipate and respond to changes in a beat-to-beat basis (Maack et al. 2006).

Truncation or modification of the anatomical proximity between SR and mitochondria are increasingly recognized as important players in the pathophysiology of several conditions in the heart, with consequences on ATP availability, sarcomeric calcium and oxidative stress. Failing cardiomyocytes display reduced cytosolic calcium transient amplitude, diminished SR calcium content and dysfunctional SERCA and RyRs activities (Cooper et al. 2013). It has been suggested that these alterations may be the consequence of insufficient energy production and excessive ROS damage (Dorn and Maack 2012). According to recent studies (Nickel, Löffler, and Maack 2013) a common pathophysiological mechanism underlying these deleterious effects could be the impairment of calcium exchange between SR and mitochondria. Low mitochondrial calcium uptake secondary to a defective SR-mitochondria communication could lead to a mismatch on energy demand and supply and to excessive oxidative damage, due to insufficient nicotinamide adenine dinucleotide phosphate (NAD(P)H) regeneration (Chen et al. 2012; Nickel, Kohlhaas, and Maack 2014).

A defective communication between mitochondria and SR can also be the cause of cardiac arrhythmias and sudden death (Brown et al. 2010). Calcium-dependent arrhythmias, and more specifically atrial fibrillation, are generally associated with instability of SR calcium release in a pro-oxidative environment (Mihm et al. 2001). Similarly, sequential dysfunction of SR calcium handling and mitochondrial function has been consistently reported in diabetic cardiomyopathy (Dhalla, Rangi, Babick, et al. 2012).

1.4 Ischemia/reperfusion injury

Ischemic heart disease is the leading cause of deaths worldwide (Pagidipati and Gaziano 2013). The main mechanism by which ischemic heart disease causes its impact on the survival and quality of life of patients is cardiomyocyte death secondary to myocardial ischemia-reperfusion. Massive cardiomyocyte death occurs during acute myocardial infarction and emergency coronary recanalization is usually not able to prevent it. Laboratory research has demonstrated that while a fraction of cardiomyocytes dies during the ischemic episode, a significant part of cell death takes place during the first few minutes of restoration of blood flow (reperfusion). Therefore, there is a hope that beyond effective coronary revascularization, the development of treatments aimed at preventing cell death secondary to ischemia-reperfusion can increase myocardial salvage and improve the prognosis of patients with acute coronary artery disease.

Oxygen deprivation causes the arrest of electron transport chain, leading to mitochondrial depolarization, ATP exhaustion and acidosis derived from anaerobic glycolysis metabolism. It has been observed that after 10-20 minutes of ischemia, a gradual rise in the diastolic tension occurs. This phenomenon, known as ischemic rigor contracture, is due to the formation of rigor complexes between actin and myosin filaments in the absence of bound nucleotides and does not depend on cytosolic calcium concentration (Allshire et al. 1987).

During oxygen deprivation mitochondrial depolarization not only inhibits ATP synthesis, but causes a rapid hydrolysis of the remaining cellular ATP due to a reversion of the FoF1 ATP synthase into FoF1 ATPase (Nicholls and Budd 2000). This reversion contributes to maintain mitochondrial $\Delta\Psi m$ at the expense of ATP hydrolysis by pumping protons out of the matrix, but has deleterious energy consequences for the cell. The persistent operation of FoF1 ATPase in the reverse mode accelerates rigor onset.

Low levels of ATP together with ischemic acidosis cause failure of the sarcolemmal Na^+/K^+ATPase leading to cytosolic sodium overload. The cell tries to correct the sodium overload by the Na^+/Ca^{2+} exchanger (energy independent) working in reverse mode, extruding the sodium at the expense of cytosolic calcium increase (Ruiz-Meana and García-Dorado 2009).

Reperfusion, whether exerted by invasive procedures or by thrombolysis, is the only possible alternative to rescue ischemic cells, however it does not guarantee their survival. Paradoxically, it has been demonstrated that reperfusion itself may precipitate cell death (Ruiz-Meana and García-Dorado 2009). The deleterious effect of reperfusion includes myocardial stunning, reperfusion arrhythmias and necrosis.

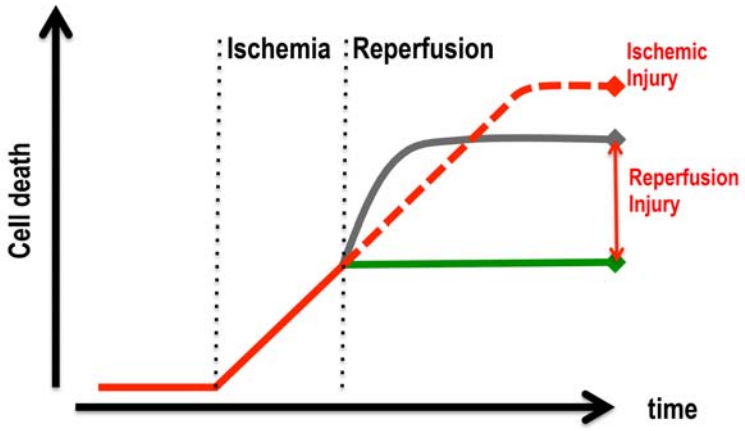


Figure 1.14: Scheme of ischemia-reperfusion injury. Progression in time of the cell death while the ischemia is established. This progression stops at reperfusion. Cell death rate accelerates during the first minutes of reperfusion limiting its therapeutic effect.

Acute reperfusion injury takes place in the very first minutes of restoration of oxygen and has been documented in a huge number of preclinical and clinical models, in which experimental cardioprotective strategies applied at the time of reperfusion have been demonstrated to reduce cell death and infarct size (Garcia-Dorado and Piper 2006) (fig. 1.14).

When reperfusion strategy takes place early enough to safe the ischemic myocardium at risk some cells are able to recover the ionic homeostasis and survive. Those that cannot recover, dye by necrosis. Apoptosis does not play a significant role in reperfusion injury since the expression of the apoptotic program proteins is silenced in adult cardiomyocytes (Sanchis et al. 2008; David Garcia-Dorado et al. 2014). Reperfusion-induced necrosis is associated with hypercontracture, cell membrane rupture and release of the cytosolic content to the extracellular space

pH normalization:

Upon reperfusion, the low pH in the interstitial space is rapidly normalized. A gradient is generated between the intersticium and the cytosol, where the acidosis was inhibiting the contractile apparatus (Blanchard and Solaro 1984). This situation activates the H^+ extruding mechanisms of the cardiomyocytes by the Na^+/H^+ exchanger and the Na^+/HCO_3^- symporter. As consequence, there is a fast normalization of the intracellular pH together with a sodium influx into the cytosol. Normalization of pH reactivates the myofibril contractility and sodium exacerbates the cytosolic calcium overload by activating reverse Na^+/Ca^{2+}exchange. Thus, pH correction during the first

minutes of reperfusion aggravates cytosolic calcium overload in the reoxygenated cardiomyocytes, seriously compromising their survival (fig. 1.15) (Inserte et al. 2011; Piper, García-Dorado, and Ovize 1998).

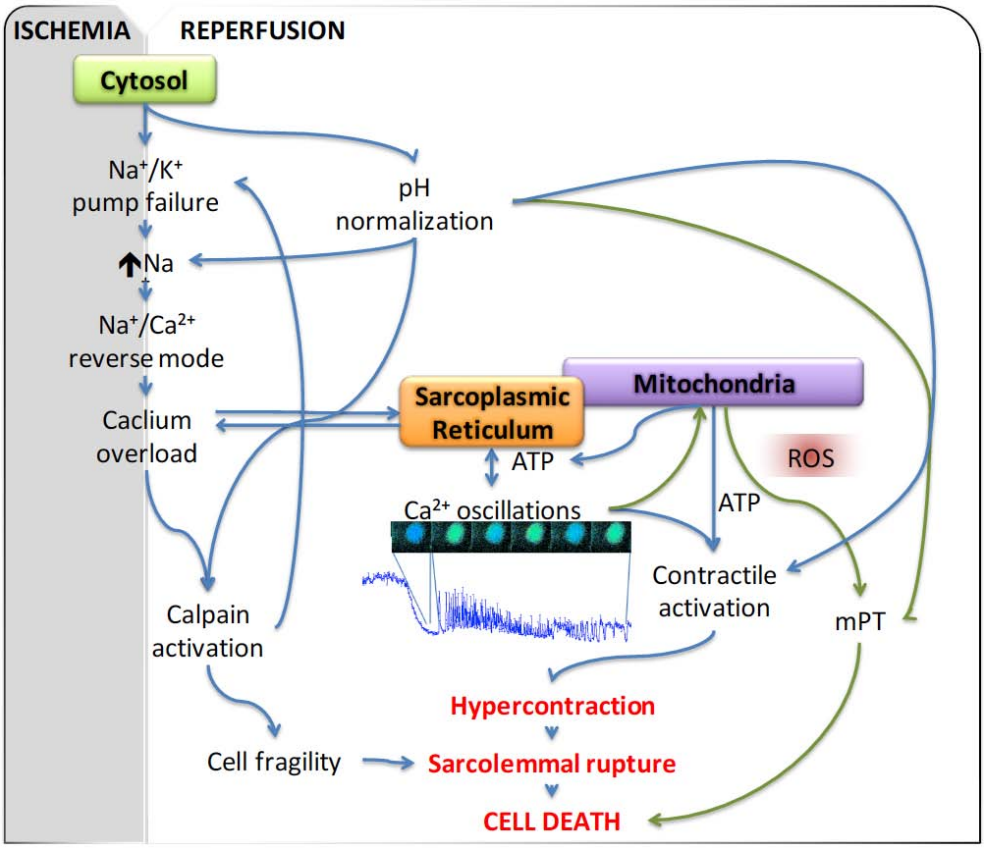


Figure 1.15: Schematic view of the mechanisms inducing cell death during the first minutes of reperfusion.

Reactivation of the ETC and calcium oscillations:

Oxygen restoration reactivates the oxidative phosphorylation and ATP availability in a cytosolic environment of high calcium and sodium concentration. ATP recovery activates SERCA and sarcolemmal Na^+/K^+ATPase. Activation of SERCA results in a temporary calcium sequestration into the SR. If the capacity of the SR is surpassed by the amount of calcium in the cytosol, a cycle of uptake and release in form of calcium oscillations starts (Ruiz-Meana and García-Dorado 2009; Piper, García-

Dorado, and Ovize 1998). These calcium oscillations will come to an end if the calcium extrusion, mediated by the Na^+/Ca^{2+}exchanger in "forward mode", is activated by a sufficient magnitude of transarcolemmal sodium gradient. The restoration of this sodium gradient is dependent of the fast activation of the Na^+/K^+ATPase removing the excess of this ion in the cytosol. Cells, in which this pump has been irreversibly damaged during ischemia, are not able to re-establish the sodium gradient and the Na^+/Ca^{2+}exchanger working in "reverse mode" favours the cytosolic calcium overload (fig. 1.15) (Siegmund, Zude, and Piper 1992; Siegmund, Ladilov, and Piper 1994).

A) **B)**

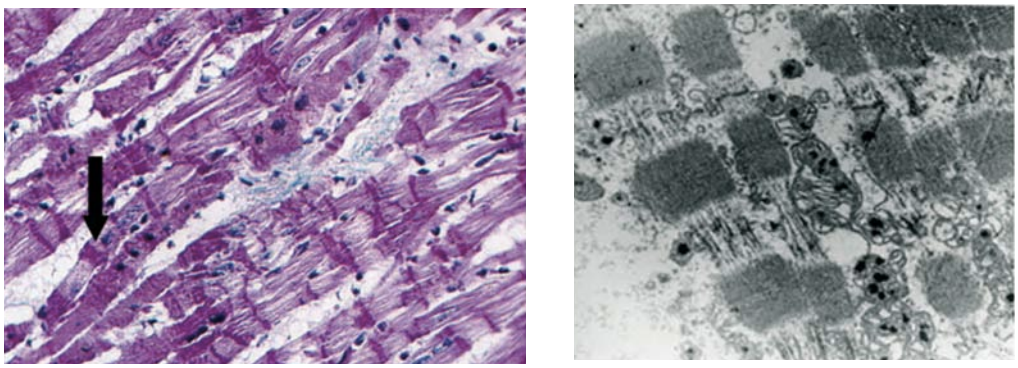


Figure 1.16: A) Histological preparation of porcine myocardium after 50 minutes ischemia where contraction band necrosis (black arrow) can be appreciated. **B)** Electronic microscope image showing the detailed ultrastructure of necrotic bands, edema and mitochondrial disruption. Adapted from *Ruiz-Meana and García -Dorado 2009*.

The re-supply of ATP in the presence of high cytosolic calcium concentration hyperactivates the myofibril contractile apparatus, leading to an uncontrolled sustained and excessive generation of force that finally results in the hypercontraction of the cardiomyocytes characterized by an abrupt cell shortening together with cytoarchitectural disorganization (Piper, García-Dorado, and Ovize 1998; Ruiz-Meana and García-Dorado 2009). These structural changes in the isolated cardiomyocytes are coincident with the characteristics of contraction band necrosis observed in the histological samples (fig. 1.16) (Vander Heide et al. 1986; Ruiz-Meana and García-Dorado 2009). The irreversible injured cells develop membrane disruption accompanied by a sudden release of cytosolic enzymes (lactate dehydrogenase and creatine kinase) to the extracellular space (Ganote 1983; Hearse 1973).

Cell swelling, calpain activation and intercelular juntions:

During reperfusion, the washing out the metabolites accumulated in the extracellular space creates a transarcolemmal osmotic gradient that favours the entry of water into cells. Cell swelling in the presence of a damaged cytoskeleton and a fragile sarcolemmal membrane imposes a mechanical stress that favours the occurrence of of sarcolemmal rupture (Ruiz-Meana et al. 1995). Sarcolemmal rupture can be propagated to adjacent cells by a gap junctions-mediated mechanism (Garcia-Dorado Circulation 1997; Ruiz-Meana Circ Res 1999).

In addition, calcium overload together with pH normalization results in calpain activation. Calpains are calcium dependent proteases that may produce detachment of the Na^+/K^+ATPase from the sarcolemma, impairing ionic normalization during reperfusion and further aggravating cell fragility (Inserte et al. 2011; Garcia-Dorado and Piper 2006) (fig. 1.15).

Role of mitochondria and mitochondrial permeability transition pore in the ischemia-reperfusion injury:

Mitochondria may contribute to ischemia-reperfusion injury by multiple mechanisms, like failure in the recovery of $\Delta\Psi m$ and ATP synthesis, altered calcium handling, burst of ROS production, release of cytochrome c, and others. One of the mechanisms that attracted much attention and has been consistently associated with reperfusion injury is the development of an increased membrane permeability, leading to an energetic collapse secondary to the abrupt uncoupling of oxidative phosphorylation, a phenomenon that is favoured by by calcium overload, ROS, SR calcium oscillations, pH correction and oxidative damage (fig. 1.15) (Griffiths and Halestrap 1995).

This sudden change of mitochondrial permeability was first described in 1976 as a reversibly opening of a proteinaceous pore in the inner mitochondrial membrane (Hunter, Haworth, and Southard 1976). This pore, also known as megachannel or mitochondrial permeability transition pore (mPTP) allows the free circulation of low molecular weight solutes (<1.5 KDa) (Leung and Halestrap 2008). Apart of high mitochondrial calcium concentrations, other stimuli triggers the mPTP opening, like oxidants, Pi and the Pi carrier (PiC) or the adenine nucleotide depletion (Varanyuwatana and Halestrap 2012; Kwong and Molkentin 2015). Its inhibition is mediated by low pH, antioxidants and submicromolar concentrations of cyclosporine-A, which binds to the peptidyl-prolyl cis-trans isomerase cyclophilin D (CyP-D) of the mitochondrial matrix (Brookes et al. 2004; Halestrap and Brenner 2003). Indeed, mitochondria from mice with genetic ablation of CyP-D are highly tolerant to calcium and oxidative stress (Baines et al. 2005).

The molecular nature of mPTP remains elusive. One of the proposed models is that mPTP forms at contact sites between VDAC, located at the mitochondrial outer membrane, and adenine nucleotide translocase (ANT) at the mitochondrial inner membrane. These two proteins would conform the physical configuration of the channel, while other proteins, like matrix CyP-D, would act as external modulators of its permeability (Zoratti and Szabo 1994; Crompton, Virji, and Ward 1998) (fig. 1. 17 A). This theoretical model has been challenged by studies using genetically modified animals showing the dispensable character of most of the proposed components for the opening of mPTP (Baines et al. 2007; Kokoszka et al. 2004).

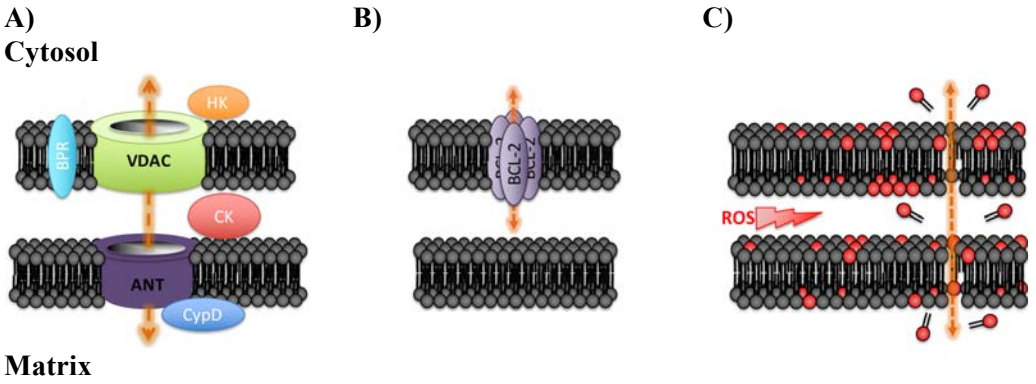


Figure 1.17: Different models of the mPTP structure. **A)** The mPTP is formed at the contact sites by at least the adenosine nucleotide translocator (ANT) and the voltage dependent anion channel VDAC. Other proposed components are the hexokinase (HK), creatine kinase (CK) and the peripheral benzodiazepine receptor. **B)** BCL-2 is postulated to form protein pores in the OMM. **C)** Membrane lipids are highly susceptible to peroxidation and to excise from the membranes forming pores. Modified from *Santos et al. 1998; Hermann, Watson, and Wildering 2014*

Other proposed models suggest that mPTP may be the consequence of massive lipidic peroxidation of mitochondrial membranes secondary to free radical accumulation under pathological conditions (Santos et al. 1998; (X.-J. Wang, Wang, and Xu 2005) (fig. 1.17 C), or the formation of protein-permeable channels from Bcl 2 family at the mitochondrial outer membrane (Kuwana and Newmeyer 2003; Basañez, Soane, and Hardwick 2012) (fig. 1. 17 B). An alternative proposed model states that mPTP is not conformed by a specific protein but rather by an aggregation of misfolded membrane proteins damaged by cellular stresses (He and Lemasters 2002) although this model fails to explain the calcium requirement of mPTP or its regulation by voltage, pH and adenine nucleotides (Bernardi 2013).

Recent findings point to a radically different structure from independent studies based on the concept that FoF1 ATP synthase may conform itself the molecular

entity of mPTP (Giorgio et al. 2013; Alavian et al. 2014). This idea is supported by the observations that CyP-D can bind to the FoF1 ATP synthase at the OSCP subunit located in the lateral stalk, (Giorgio et al. 2009), modulating its conformation and conferring sensitivity to cyclosporin, one of the main regulators of mPTP. In both models CyP-D binding requires Pi and leads to an inhibition of the complex V.

The first proposed model for ATP synthase conforming the mPTP is based on the formation of dimmers of 2 molecules of ATP synthase that give rise to a high conductance channel with permeability properties indistinguishable from that of mPTP in response to calcium (Bernardi et al. 2015) (fig. 1.18 A).

The second model postulates the occurrence of a conformational change at the c-ring of FoF1 ATP synthase in response to calcium that would form a high conductance channel whose biophysical properties are identical to those reported for mPTP (Alavian et al. 2014) (fig. 1.18 B).

A) ATPsynthase dimmers model **B) c-ring ATPsynthase monomer model**

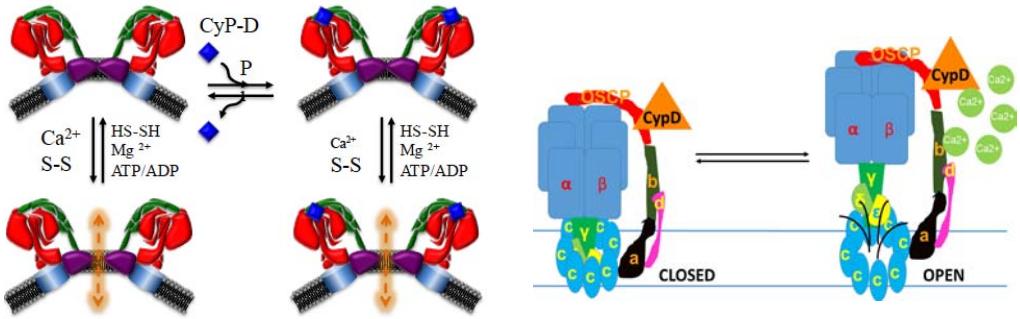


Figure 1.18: Binding of CyP-D, which is favoured by Pi would increase the accessibility of the metal binding sites, allowing PTP formation at lower calcium concentrations **A)** Hypothetical transition of FoF1 ATP synthase dimers to form the PTP. ATP synthase dimers can undergo PTP formation when calcium rather than magnesium is bound, possibly at the catalytic sites, in a reversible process favoured by thiol oxidation. Adenine nucleotides counteract PTP formation in synergy with magnesium. Adapted from *Bernardi 2013*. **B)** Schematic model of pathophysiological mPTP activity. The components of F_1F_O that separate from the c-subunit during this process are not yet completely known. Adapted from *Jonas et al. 2015.*

The mPTP has important physiological roles. Transient openings of the mPTP are involved in ROS (Zorov et al. 2000) and calcium signalling (Bernardi and von Stockum 2012), cardiomyocyte development and mitochondria maturation (Hom et al. 2011). it has been postulated that the pore itself might also have influence over the matrix calcium. Mathematical models suggest that under high intracellular calcium cycling during periods of stress, the calcium efflux pathway mediated by the mNCX

may unable to prevent calcium overload suggesting that the implication of different mechanisms of removing calcium must implicated (Bernardi and von Stockum 2012; Elrod and Molkentin 2013). The observation of the mitochondrial calcium efflux inhibition in rat and mouse cardiomyocytes treated with CsA or lacking CyP-D (Altschuld et al. 1992; Elrod et al. 2010) supported the implication of the mPTP the calcium efflux. Futhermore, an elevated matrix calcium has been found to be related with a shift in substrate utilization from fatty acid oxidation to glycolysis in the working heart what suggests that the mPTP may constitute a control point which links mitochondrial metabolism with myocardial workload through calcium (Elrod and Molkentin 2013).

By contrast, sustained opening of mPTP dissipates $\Delta\Psi m$, uncouples oxidative phosphorylation and leads the cell to a dramatic energetic collapse incompatible with life. The role of mPTP in reperfusion-induced cell death has been extensively documented (Ruiz-Meana et al. 2011; Bernardi and Di Lisa 2015a; Halestrap and Richardson 2015). Indeed, during the first minutes of reperfusion all the conditions previously described capable to induce mPTP opening (i.e. normalization of pH, calcium overload, ROS production) concur in the myocardium and pharmacological inhibitors of mPTP, as well as genetically ablation of Cyp-D, have been proven to effectively reduce reperfusion-induced cell death and infarct size (Crompton 2004; Baines et al. 2005).

However, the concept that mPTP opening induces energy collapse during reperfusion is difficult to reconcile with the fact that hypercontracture is an energy dependent process (Vander Heide et al. 1986). Data from our laboratory suggest that both phenomena, hypercontracture and mPTP opening, may be closely interrelated (Ruiz-Meana et al. 2007). In experiments in which mPTP was induced by intermittent laser illumination in isolated cardiomyocytes, there was a pool of permeabilized mitochondria that coexisted with intact ATP-generating mitochondria within the same cell. This phenomenon explains the mechanism by which some mitochondria release calcium to the cytosol whereas other mitochondria sustain the energy production required for hypercontracture. Mitochondrial calcium release in response to mPTP opening aggravates cytosolic calcium overload of the reperfused cardiomyocytes and promotes hypercontracture and cell death (Ruiz-Meana et al. 2007). These experiments indicate that mPTP opening may induce calcium-dependent hypercontracture during the first minutes of reperfusion. The other way around is also possible, as indicated by studies from our laboratory and others (Ruiz-Meana et al. 2009; Abdallah et al. 2011a) in which SR-driven calcium oscillations were shown to promote mPTP opening in adjacent mitochondria, favouring the occurrence of cell death (fig 1.15).The duration of ischemia is a critical determinant for the specific contribution of mPTP to cell death.

After short ischemic period, genuine energy-dependent hypercontracture is the main mechanism of myocardial cell death and contractile inhibitors have been proven to effectively reduced infarct size (Ruiz-Meana 2011). However, after more prolonged ischemic duration, mPTP opening is much more relevant and addition of CsA is a much more powerful cardioprotective intervention (Ruiz-Meana 2011).

Overall, experimental data consistently indicate that senescent cardiomyocytes show a reduced threshold to tolerate and adapt to a stress, including ischemia-reperfusion injury. Additionally, there are evidences that demonstrate that cardiac cells develop several structural and functional alterations that are associated with changes in calcium handling and bioenergetic capacity as well as increased pro-oxidative status, highly resembling those of failing cardiomyocytes (Strait and Lakatta 2012; North and Sinclair 2012; Fannin et al. 2014). Clinical and epidemiological studies further support the concept that beyond the increased load of comorbidities in the elderly, there is a constitutive impairment of the aging heart to withstand and adequately respond to stressful conditions, increased mortality during coronary heart disease and resistance to cardioprotective strategies (Willems et al. 2005; Roger et al. 2011; Dégano, Elosua, and Marrugat 2013)

Because subcellular microdomains formed by SR and mitochondria are involved in interorganelle calcium exchange, energy demand/supply matching and antioxidant system regeneration, we hypothesized that altered SR-mitochondria communication may play a causative role in cell functional decline during aging and reduced tolerance to ischemia-reperfusion injury. Previous studies have proposed that the increased oxidative damage and reduced ATP production of failing cardiomyocytes develops as a consequence of a defective mitochondrial calcium uptake (Kohlhaas and Maack 2011). Whereas several studies have investigated the impact of age on SR calcium handling on one side and on mitochondrial function on the other side, there are no published studies that have explored the effect of age on interorganelle communication. On the other hand, we have previously demonstrated that SR and mitochondria interplay may have consequences on cell death/survival during the first minutes of reperfusion, favouring mPTP opening and calcium overload, but the potentially harmful effect of aging on calcium handling, recovery of mitochondrial respiratory activity and ROS production after ischemia-reperfusion has not been investigated. The results of this thesis could help to identify new molecular mechanisms of cardiovascular aging involved in the increased vulnerability of elderly patients to ischemia-reperfusion injury that could be useful for the development of more effective and specific cardioprotective strategies in these patients.

CHAPTER 2

Hypothesis and Objectives

It is not known how aging may affect the physical interaction between SR and mitochondria and whether this potential lost of the fine interplay between these organelles can alter the calcium dynamics within the cell and the mitochondrial bioenergetics.

In this thesis, we initially hypothesized that advanced age is determinant for the adequate calcium handling. An impaired SR-mitochondria calcium crosstalk in advanced age may affect mitochondrial function, due to their close anatomical and functional relationship. This effect could result in an increased vulnerability of senescent myocardium to ischemic damage and may underlay the pathophysiology of contractile dysfunction associated with aging.

The main goal of this thesis is to disclose how aging may impair SR-mitochondrial microdomains, calcium dynamics and oxidative damage, focusing on how these possible impairments would affect the tolerance to ischemia and reperfusion of aged hearts.

Different approaches are used to:

1. To characterize mitochondrial bioenergetics in elderly mice.
2. To analyse the calcium dynamics of SR, mitochondria and transfer between both organelle and its relation with the antioxidant capacity of the cardiomyocytes.
3. To characterize the spatial proximity between SR and mitochondria.
4. To gain insight into the relationship between aging, SR calcium handling and mitochondrial function in ischemia-reperfusion.
5. To identify the potential mechanisms responsible for the reduced tolerance to ischemia of senescent myocardium.

CHAPTER 3

Defective sarcoplasmic reticulum-mitochondria calcium exchange in aged mouse myocardium

Abstract - *Mitochondrial alterations are critically involved in the increased vulnerability to disease during aging. We investigated the contribution of mitochondria-sarcoplasmic reticulum (SR) communication in cardiomyocyte functional alterations during aging. Heart function (echocardiography) and ATP/phosphocreatine (NMR spectcopy) were preserved in hearts from old mice (>20months) respect to young mice (5-6months). Mitochondrial membrane potential and resting O_2 consumption were similar in mitochondria from young and old hearts. However, maximal ADP-stimulated O_2 consumption was specifically reduced in interfibrillar mitochondria from aged hearts. Second generation proteomics disclosed an increased mitochondrial protein oxidation in advanced age. Because energy production and oxidative status are regulated by mitochondrial calcium, we investigated the effect of age on mitochondrial calcium uptake. While no age-dependent differences were found in calcium uptake kinetics in isolated mitochondria, mitochondrial calcium uptake secondary to SR calcium release was significantly reduced in cardiomyocytes from old hearts, and this effect was associated with decreased NAD(P)H regeneration and increased mitochondrial ROS upon increased contractile activity. Immunofluorescence and proximity ligation assay identified defective communication between mitochondrial VDAC and SR ryanodine receptor (RyR) in cardiomyocytes from aged hearts associated with altered calcium handling. Age-dependent alterations in SR calcium transfer to mitochondria and in calcium handling could be reproduced in cardiomyocytes from young hearts after interorganelle disruption with colchicine, at concentrations that had no effect in aged cardiomyocytes or isolated mitochondria. Thus, defective SR-mitochondria communication underlies inefficient interorganelle calcium exchange that contributes to energy demand/supply mismatch and oxidative stress in the aged heart.*

Adapted from Fernandez-Sanz C, Ruiz-Meana M, Miro-Casas E, Nuñez E, Castellano J, Barba I. Poncelas M, Rodriguez-Sinovas A, Vazquez J, Garcia-Dorado D. Defective sarcoplasmic reticulum-mitochondria calcium exchange in aged mouse myocardium. 2014 Dec 18;5:e1573

3.1 Introduction

Age is the main independent risk factor for cardiovascular morbidity and mortality (Lloyd-Jones et al. 2010). It increases heart vulnerability to cardiac diseases as well as the severity of their clinical manifestations, and reduces the efficacy of cardioprotective interventions (Boengler, Schulz, and Heusch 2009). At the cellular level, some of the structural and functional age-dependent changes resemble those of failing cardiac myocytes (Strait and Lakatta 2012; North and Sinclair 2012). Specifically, disturbed calcium homeostasis and excitation–contraction coupling (Terentyev et al. 2008), as well as deficient mitochondrial energetics (Rosca et al. 2008) and excessive ROS production (Sawyer et al. 2002), have been consistently reported in senescent cardiomyocytes. These subcellular alterations likely contribute to the reduced adaptive capacity to stress (exercise, beta-adrenergic stimulation) and increased vulnerability to disease of the aged hearts.

In cardiac cells, electrochemical coupling and metabolic adaptations are based upon the coordination between SR and mitochondria tightly interconnected forming an interface to support local ionic exchange and signal transduction in a beat-to-beat basis (G. Csordás, Thomas, and Hajnóczky 2001) (fig. 3.1).

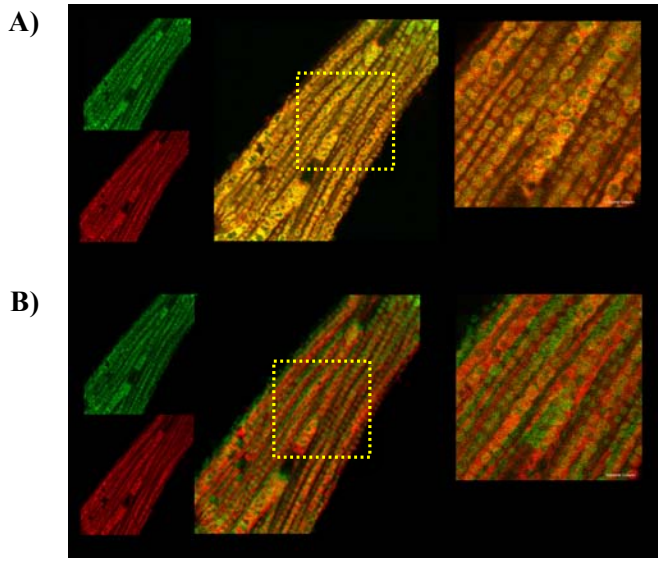


Figure 3.1: Confocal images (100x) of a permeabilized cardiac myocytes simultaneously loaded with Mito-Tracker Green (mitochondria) and ER-Tracker red (sarcoplasmic reticulum), and after merging both fluorochromes, under control conditions (**A**) and 20 min after addition of 1 μmol/l colchicine (**B**). A volume reconstruction of a magnified region (framed by dashed yellow line) is shown at right. Modified from *Ruiz Mena et al. 2009.*

This privileged interorganelle communication facilitates mitochondrial ATP transport for SR calcium cycling and ensures energy replenishment by reciprocal calcium and ADP exchange. Calcium is taken up by mitochondria using a low affinity uniporter whose activity is driven by the elevated calcium concentration in the microenvironment present around RyR (Rizzuto and Pozzan 2006). Indeed, the kinetics of mitochondrial calcium uptake is more dependent on the concentration of calcium at the SR-mitochondria contact points than on bulk cytosolic calcium concentration (Csordás, Thomas, and Hajnóczky 2001). Mitochondrial calcium uptake allows energy supply-demand matching through the activation of Krebs cycle dehydrogenases and electron transport chain activity, and at the same time it regulates the regeneration of Krebs-coupled antioxidative defences (NAD(P)H) (Maack and O'Rourke 2007).

Defective SR-mitochondria crosstalk has been causally linked to the abnormal mitochondrial calcium uptake in failing hearts and may underlie their increased oxidative stress (Kohlhaas and Maack 2010). Also, in diabetic cardiomyopathy, intracellular calcium overload and depletion of energy stores appear to develop as a consequence of sequential SR-mitochondria dysfunction (Dhalla, Rangi, Zieroth, et al. 2012). Atrial fibrillation has been associated with an increased fusion of mitochondria and a subsequent increased colocalization of giant mitochondria with SR, a subcellular remodelling process that contributes to the perpetuation of the arrhythmia (Redpath et al. 2013). Because mitochondria are highly dynamic structures, some molecular links have been proposed to provide a stable physical interorganelle bridge (Cerqua et al. 2010; Y. Chen et al. 2012) while others appear to facilitate direct tunnelling of calcium and other signalling mediators (Szabadkai et al. 2006). In the present study, we hypothesized that aging may negatively impact on mitochondria-SR communication by mechanisms involving defective calcium transmission, and we identified reduced physical interaction between RyR and mitochondrial VDAC as the main responsible of this effect.

3.2 Methods

Young adult (5-6 months) and old (>20 months) C57BL/6 mice were used for in situ functional analysis (echocardiography) and for the obtention of myocardial tissue, isolated cardiomyocytes and mitochondria. Animal handling was approved by Research Commission on Ethics of the Hospital Vall d'Hebron. All procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

3.2.1 Transthoracic Echocardiographic Analysis

Echocardiographic measurements were performed in young and old mice under light anaesthesia (isofluorane 0,5-1%) with a Vivid-Q portable ultrasound system using a ILS 12 MHz transducer (GE Healthcare, USA). Conventional parameters (ejection fraction of the left ventricle [LVEF], left ventricular end-diastolic diameter [LVEDD], left ventricular end-systolic diameter [LVESD], septum wall thickness [SWT] and posterior wall thickness [PWT]) were measured in M-mode recordings at the level of the papillary muscles. Left ventricular fractional shortening was calculated as ([LVEDD-LVESD])/LVEDD) x 100. Left ventricular mass was calculated as 0.8 x (1.04 x (LVEDD+ PWT + SWT)3 – (LVEDD)3) + 0.6.

3.2.2 NMR spectroscopy

Nuclear magnetic resonance (NRM) spectroscopy is based on the study of the different magnetic isotopes of a molecule. In the presence of a magnetic field, the different magnetic isotopes orient their magnetic moments in the same or opposite direction of the magnetic field. These states are separated by an energy ΔE that depends on the strength of the interaction between nucleus and the field. The energy difference of energy between the two states of the magnetic isotopes can be measured by applying an electromagnetic radiation frequency (ν), which makes the nucleus to "flip" from a lower energy state to the next higher one.

Every nucleus has its characteristic magnetic moment and the electromagnetic radiation frequency is directly proportional to the applied magnetic field and the gyromagnetic constant. The exact resonance frequency is characteristic of the chemical environment of the nucleus in a molecule.

$$\nu=\gamma\bullet\beta \text{ effi}$$

ν= electromagnetic radiation frequency

γ= gyromagnetic constant

β effi = magnetic field

To analyse the bioenergetics from old and young mice, hearts from both groups were perfused in a Langendorff system with modified Krebs buffer-KH2PO4

free (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.8 CaCl2, 25 NaHCO3, 1.2 KH2PO4, and 11 glucose, 37 ºC, pH=7.4).

The 31P spectra were acquired in a 9,4T vertical magnet interfaced to a Bruker AVANCE 400 spectrometer tuned to 161.97 MHz; each spectrum consisted in the accumulation of 400 scans and lasted for 14 minutes (min) (Rodríguez-Sinovas et al. 2010). Phosphocreatine (PCr) and γATP peaks were identified in the spectra according to the chemical sift. The areas under the identified peaks were measured by deconvolution (Fig. 3.2). Results were shown and analysed as the ratio PCr/ATP from both groups.

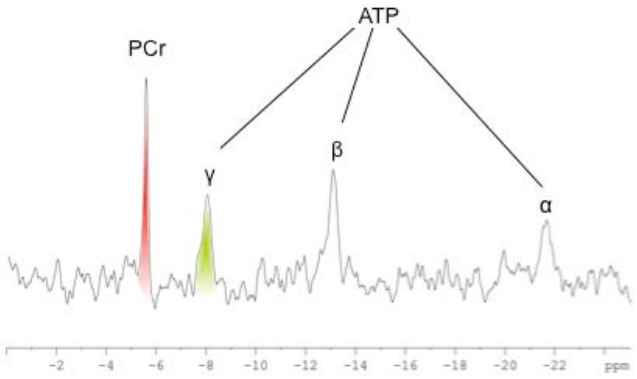


Figure 3.2: Example of a 31P spectra obtained by nuclear magnetic resonance spectroscopy. PCr peak (read) appears just before the γATP peak (green).

3.2.3 Isolation of heart mitochondria

Subsarcolemmal and interfibrillar cardiac mitochondria were isolated by differential centrifugation from mouse hearts as originally described by Palmer *et al.* (Palmer, Tandler, and Hoppel 1977). Ventricular tissue was minced in cold "buffer A", containing (in mmol/L): 290 sucrose, 5 MOPS at pH 7.4, 2 EGTA and 0.2% defatted albumin. Minced tissue was mildly homogenized using a Potter-Elvehjem device. Homogenates were centrifuged at 750xg for 5min. Pellets and supernatants were processed independently to isolate different mitochondrial pools. For subsarcolemmal mitochondria (SSM) fraction, supernatants were centrifuged at 5000xg for 5min. For interfibrillar mitochondria (IFM) fraction, the resulting pellets were resuspended in cold "buffer B", containing (in mmol/L): 100 KCl, 5 MOPS at pH 7.4, 2 EGTA and 0.2% defatted albumin with proteinase K (P2308, Sigma) at 2 mg/g wet weight, and

quickly homogenized. Homogenates were centrifuged at 750xg for 5min. Supernatants were subjected to a subsequent centrifugation at 5000xg for 5min to obtain IFM. Protein was determined by Bradford assay. For Western-blot analysis, SSM and IFM were additionally centrifuged using Percoll® buffer to increase the purity of preparations. Protein was determined by Bradford assay.

3.2.4 Cell fractioning

Different subcellular fractions were obtained by differential centrifugation. Fresh cardiac ventricles were minced and homogenized using a Potter-Elvehjem PTFE pestle-glass tube at 1 400 rpm on ice-cold isolation buffer (in mmol/L): 10 HEPES acid, 225 manitol, 75 sucrose, 0.1 EGTA, pH 7.4 with 10 Tris base).

Nuclei and other cell debris were obtained in the pellet from an initial centrifugation step (750xg for 5 min, 4ºC). Part of the supernatant was treated with 1% DODM - 1% SDS and considered as heart extract fraction. The rest of the supernatant was collected and submitted to a 10 000xg centrifugation at 4ºC for 10 min.

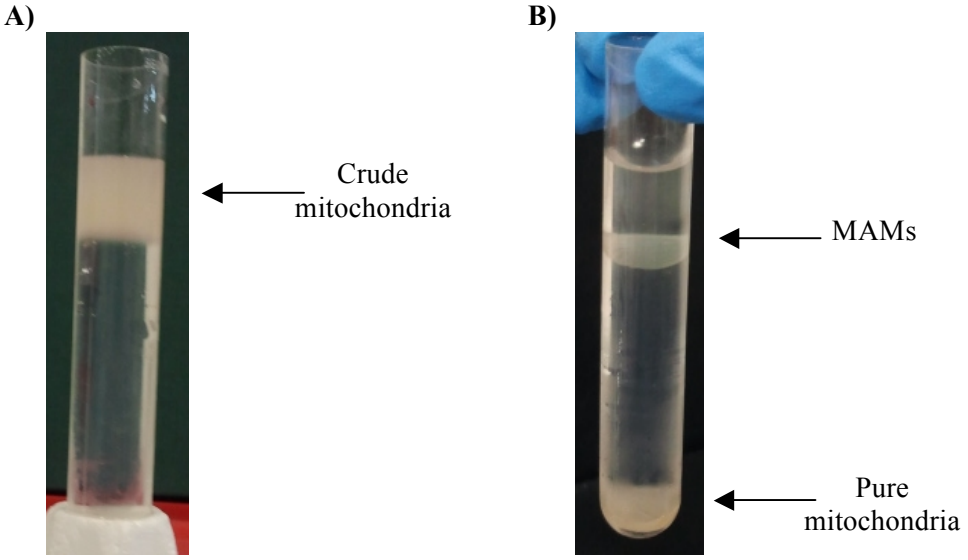


Figure 3.3:A) Crude mitochondria over the 8 ml 30% Percoll® gradient column. **B)** Separated fractions of MAMs and pure mitochondria after ultracentrifuge.

The resulting pellet, containing crude mitochondria, was collected and maintained on ice. The supernatant was ultra centrifuged at 100 000xg, 4ºC for 1 h, to obtain the cytosolic fraction on the supernatant and the heavy microsomal vesicles in the pellet.

One aliquot of the crude mitochondrial fraction was kept on ice for future analysis. The rest of the crude mitochondrial fraction was placed on 8 ml 30% Percoll® gradient column (fig. 3.3 A). The Percoll® gradient column was ultracentrifuged at 90 000xg, 4ºC for 30 min in a swinging rotor for the separation of pure mitochondria (heavy fraction) and mitochondria-associated membranes (MAM, light fraction). Both fractions (fig 3.3 C) were extracted from the column. The pure mitochondria fraction was centrifuged at 7 000xg for 10 min at 4ºC. The pellet contained pure mitochondria. The MAM fraction was centrifuged at 12 000xg for 10 min at 4ºC. The pellet contained free MAM (Wieckowski et al. 2009).

3.2.5 Studies in isolated mitochondria

Respiration assay:

O2 consumption was quantified in SSM and IFM from young and old mice hearts incubated in respiration buffer (in mmol/L: 100 KCl, 5 MOPS pH 7.4, 2 EGTA, 5 KH2PO4, 1 MgCl2, 0.1% defatted albumin) using Clark-type oxygen electrodes (Hansatech, UK) after the addition of specific substrates for each of the respiratory complexes (complex 1: 2mmol/L malate + 5mmol/L glutamate; complex 2: 6mmol/L succinate with 0.5µmol/L rotenone to inhibit complex 1; complex 3: reduced form of 2,3 dimethoxy-5-methyl-1,4benzoquinone; complex 4; ascorbate + NNN'N' tetramethyl-p-phenylene diamine (TMPD)). Maximal O2 consumption (state 3) was obtained with 0.25mmol/L ADP. Respiratory control rate was calculated as ADP stimulated O2 consumption (state 3)/non-stimulated O2 consumption (state 2). Citrate synthase activity was determined by colorimetry and was used to normalize data on mitochondrial respiration (Matsuoka and Srere 1973).

Mitochondrial calcium uptake:

Mitochondrial calcium uptake was fluorometrically quantified in 150µg of SSM or IFM incubated in 235µL of respiration buffer with 0.5µmol/L Calcium Green-5N (CG5N hexapotassium salt, Invitrogen). Mitochondria were exposed to an external calcium pulse of 30µmol/L in the presence of 1µmol/L cyclosporine A (CsA) to avoid membrane permeabilization. Changes in 530nm fluorescence were recorded every 10s. Fluorescence decay after calcium addition reflected the kinetics of mitochondrial calci-

um uniporter and could be effectively inhibited by 10µmol/L of the specific blocker Ruthenium 360 (Ru360).

3.2.6 Isolation of mouse cardiac myocytes

For the obtention of cardiomyocytes, hearts from young (<6 months) or old (>20 months) mice were perfused in a Langendorff system for 15min with a modified Krebs buffer (in mmol/L: 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose and 10 butanedione monoxime, pH7.4) with 0.03% type II collagenase (Serva). Perfused tissue was subjected to differential centrifugation.

Calcium tolerant rod-shaped cardiomyocytes (fig. 3.4) were selected by sedimentation in 4% BSA gradient, and plated on laminin-coated cover slips. Only when the initial yield of rod-shaped cardiac myocytes was >50%, preparations were considered suitable for experiments.

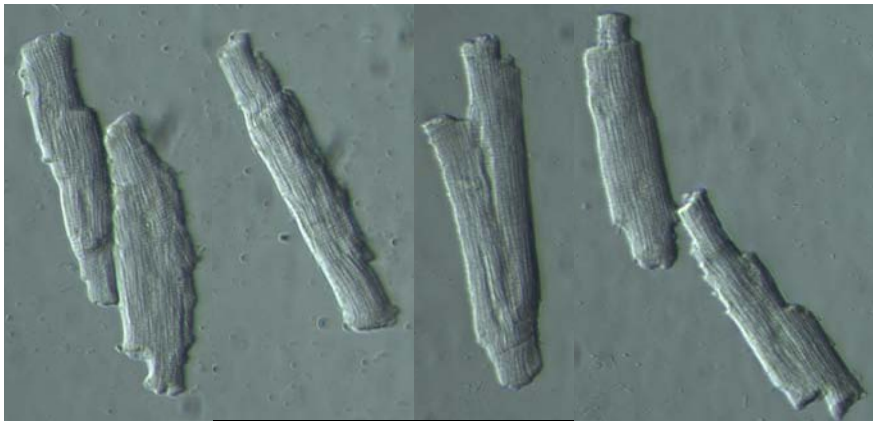


Figure 3.4: Freshly isolated rod-shaped cardiomyocytes from mouse heart. Non-published image from our laboratory.

3.2.7 Lysosome, mitochondria and lipofuscin staining

Lysosomes were visualized in cardiomyocytes loaded with 50nmol/L lysotracker green (30min, 37ºC) and excited at 560nm using an Ar/Kr laser confocal system (Yokogawa CSU10, Nipkow spinning disk), set on an Olympus IX70 (VoxCell Scan, Visitech, UK) at 60X oil immersion objective lens. Mitochondrial pool was estimated in confocal images of intact cells after labelling mitochondria with 1 µmol/L MitoTracker® Red CMXRos (30min, 37ºC). Lipofuscin pigment was observed as auto-

fluorescence after 488nm laser excitation. In all cases, cell fluorescence was analysed in background-subtracted images using commercially available software (VoxCell Scan, Visitech, UK).

3.2.8 Mitochondrial membrane potential (ΔΨm) in intact cardiomyocytes

Resting ΔΨm was analysed in isolated cardiomyocytes loaded with 10µmol/L JC-1 (6min, 37ºC) and excited at 488nm (Ar/Kr laser confocal system). Green (520nm) and red (590nm) emission lights were simultaneously recorded at 60X (CCD cameras, Hamamatsu, Japan) and 590/520nm fluorescence ratio was calculated as an index of ΔΨm and expressed relative to maximal mitochondrial membrane depolarization achieved with 200µmol/L dinitrophenol (DNP).

3.2.9 Induction of SR-mitochondria calcium transfer in permeabilized cardiomyocytes

To analyse the fraction of mitochondrial calcium uptake that is dependent on SR calcium transfer without the contribution of sarcolemma/cytosol, cardiomyocytes were loaded with 5µmol/L rhod-2 using the cold-warm protocol (60min at 4ºC followed by 30min at 37ºC) and the sarcolemma of the cells was subsequently permeabilized with 10 µmol/L digitonin (2 min, 37ºC) in intracellular-like buffer (in mmol/L: 5 MgCl2, 10 HEPES, 250 sucrose, 25 Tris, 5 succinate, 2 ATP, pH 7.2) under calcium free conditions (2mmol/L EGTA). SR calcium release was induced with a pulse of caffeine (10mmol/L) and mitochondrial Calcium uptake was monitored as changes in rhod-2 fluorescence throughout time with respect to the initial value (F/F0, Ex: 561nm/Em: 605nm) using an Ar/Kr laser confocal system (Yokogawa CSU10, Nipkow spinning disk), set on an Olympus IX70 (VoxCell Scan, Visitech, UK) at 60X.

3.2.10 Quantification of NAD(P)H regeneration

NAD(P)H regeneration from NAD(P) was determined in intact cardiomyocytes as autofluorescence after excitation at 340nm (Em:450nm) with the aid of a xenon lamp (Visitech monochromator, UK) on the stage of an inverted microscope (Olympus IX70, Japan), at X40. For calibration, maximal reduction state of NAD(P)H was achieved after the addition of 2mmol/L sodium cyanide, and minimal reduction state was achieved after the addition of 200µmol/L DNP. Results were expressed as the ratio

of NADH(P)H/NAD(P)+ and as % of the reduced form respect to total NAD(P)H pool according to calibrated data(Y. Chen et al. 2012). To test the effect of increasing contractile activity on NAD(P)H consumption/regeneration, electrical pacing of the cells was increased from 1Hz to 5Hz.

3.2.11 Cytosolic and mitochondrial ROS production

ROS production in cytosolic and mitochondrial compartments was analysed in freshly isolated cardiomyocytes simultaneously labelled with 10µmol/L of the cytosolic ROS-sensitive fluorochrome 2',7'-dichlorodihydrofluorescein diacetate (DCF, 30 min, 37ºC) and 5µlmol/L of the mitochondrial ROS-sensitive fluorochrome MitosSOX red (5µmol/L, 10 min, 37ºC). Cells were electrically stimulated at low (1Hz) and high frequencies (5Hz). At the end of the protocol, 250µmol/L of the mitochondrial respiratory complex I inhibitor rotenone was added to induce a peak of ROS production. Fluorescence was recorded using an Ar/Kr laser confocal system (Visitech, UK, Ex: 488nm/Em: 520-580nm) on the stage of an inverted microscope (Olympus IX70). Results were expressed as relative changes respect to peak ROS induced by rotenone. Image acquisition was set at 1 image/10s to avoid phototoxicity.

3.2.12 SR calcium handling

SR Calcium transients in field-stimulated cardiomyocytes:

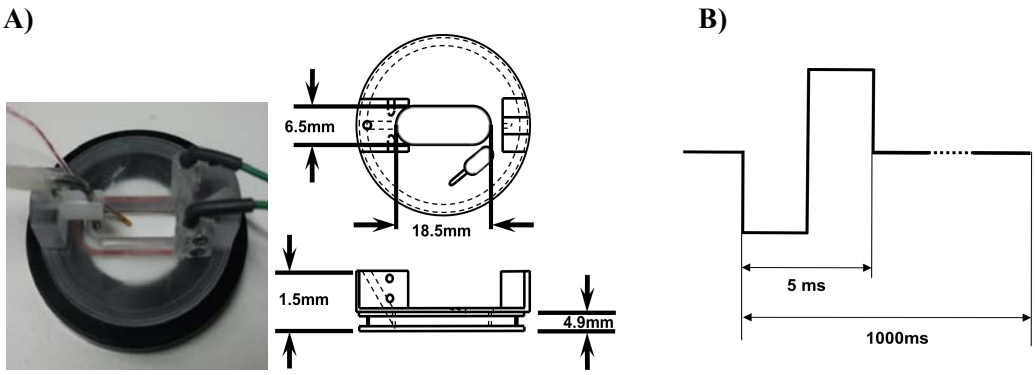


Figure 3.5: A) Field stimulation microperfusion chamber and schematic view. **B)** Final obtained biphasic pulse of 5 ms duration. Amplitude and frequency was set up according to the experiments.

Intact cardiomyocytes loaded with the cytosolic Calcium indicador fluo-4 (5µmol/L, 30 min at 37ºC) were superfused with a control HEPES buffer (in mmol/L:

150 NaCl, 5.4 KCl, 10 HEPES acid, 2 $CaCl_2$, 1 glucose, 2.5 piruvate, 5 creatine, 5 taurine, pH=7.4) and electrically stimulated at 1Hz (biphasic pulse, SIU-102, Warner Instruments, USA) (fig. 3.5). Fluorescence changes throughout time were recorded using an Ar/Kr laser confocal system (Yokogawa CSU10, Nipkow spinning disk), set on an Olympus IX70 (VoxCell Scan, Visitech, UK) at 60X, and expressed as changes throughout time with respect to the initial value (F/F0) (Ex: 488nm/Em: 520nm). Image acquisition was set at 40 images/s. Data were analysed with VoxCell Scan software (Visitech, UK).

Spontaneous SR Calcium sparks:

A) **B)**

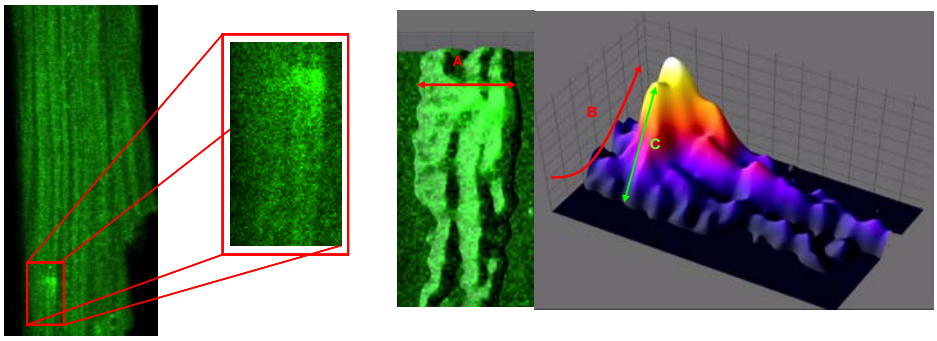


Figure 3.6: A) Line scan image from a Fluo 4-AM loaded cardiomyocyte. Spontaneous Sparks (red square) were recognized and analysed by the SparkMaster ImageJ plug-in. **B)** Tridimensional reconstructions of a Spark. A is the width at half maximum, B is the time to peak and C is the amplitude. These parameters where measured from every Spark recognised by the program. Non-published image from our laboratory

Spontaneous Calcium sparks were analysed in intact fluo-4 loaded cardiomyocytes incubated in control HEPES buffer using a spectral confocal microscope (Ex: 488nm, Olympus Spectral Confocal Microscopy FV1000, Olympus). Spark frequency (sparks x ($100\mu m^{-1}$) x (s^{-1})), amplitude ($\Delta F/F_0$), rate (($\Delta F/F_0$ x (s^{-1})) and morphology (full width at half-maximal amplitude, µm) were quantified using Spark Master plugin of Image J software (NIH, USA) (fig. 3.6).

SR Calcium load:

Total SR Calcium load was analysed in intact cardiomyocytes loaded with the cytosolic calcium indicator fluo-4 (5µmol/L, 30 min at 37ºC). Cells were submitted to a brief field-stimulation at 1Hz for 30 s to allow calcium transient stabilization fol-

lowed by a single pulse of 10mmol/L caffeine. Maximal amplitude of caffeine-induced peak fluorescence was normalized by the initial fluorescence value (F/F0) and considered as an index of total SR calcium load.

3.2.13 Spatial proximity between SR and mitochondria

Immunolabeling and colocalization of RyR and VDAC:

Isolated cardiomyocytes placed on laminin-coated coverslips were fixed (99.6% acetone cooled at -20ºC, 5 min), permeabilized (0.025 % Titon X-100 in PBS) and incubated at 4ºC overnight with mouse monoclonal RyR antibody (34C Abcam, 1:50) and rabbit polyclonal VDAC antibody (15895Abcam, 1:50) in PBS-BSA 1%. This was followed by 1h incubation at room temperature with secondary Alexa anti-mouse-561 and Alexa antirabbit-488. Nuclei were stained with 10µg/ml Hoeschst-33342. Mounted samples were observed with a spectral confocal microscope (Olympus Spectral Confocal Microscopy FV1000, Olympus). Degree of RyR-VDAC overlap was quantified with Mander's coefficient analysis (JaCop, ImageJ software).

Proximity ligation assay (PLA):

Mouse cardiomyocytes were isolated and labelled with RyR and VDAC primary antibodies, raised in two different species, under the conditions previously described. The secondary antibodies were conjugated to PLA oligonucleotide probes PLUS and MINUS, able to hybridize with their complementary connector oligos (PLA probe rabbit PLUS and PLA probe rat MINUS; Olink Bioscience, Uppsala, Sweden) (fig. 2.7-A).

The hybridization reaction was done at 37°C for 1 h (fig. 3.7 B). If both proteins are in close proximity (≤ 40nm) these hybridization and ligation reactions take place. After a positive hybridization-ligation reaction, the resulting double PLUS strand will prime a rolling-circle amplification. It will go around the circle for approximately 1000 turns, creating a concatemeric DNA (fig. 3.7 C).

This repetitive DNA is complementary to detection probes, which are connected to a fluorophore. Each couple of proteins will yield a concatemeric DNA able to bind 500-1000 fluorescent probes (fig. 3.7 D). Ligation and amplification reactions were performed according to the Duolink® InSitu kit user manual (Olink Bioscience, Uppsala, Sweden).

Preparations were mounted and cell images were registered with a spectral confocal microscope (FluoView-1000, Olympus). Positive fluorescent cross-reactivity was quantified in background-substracted images using BlobFinder software (Centre for Image Analysis, Uppsala University, Sweden).

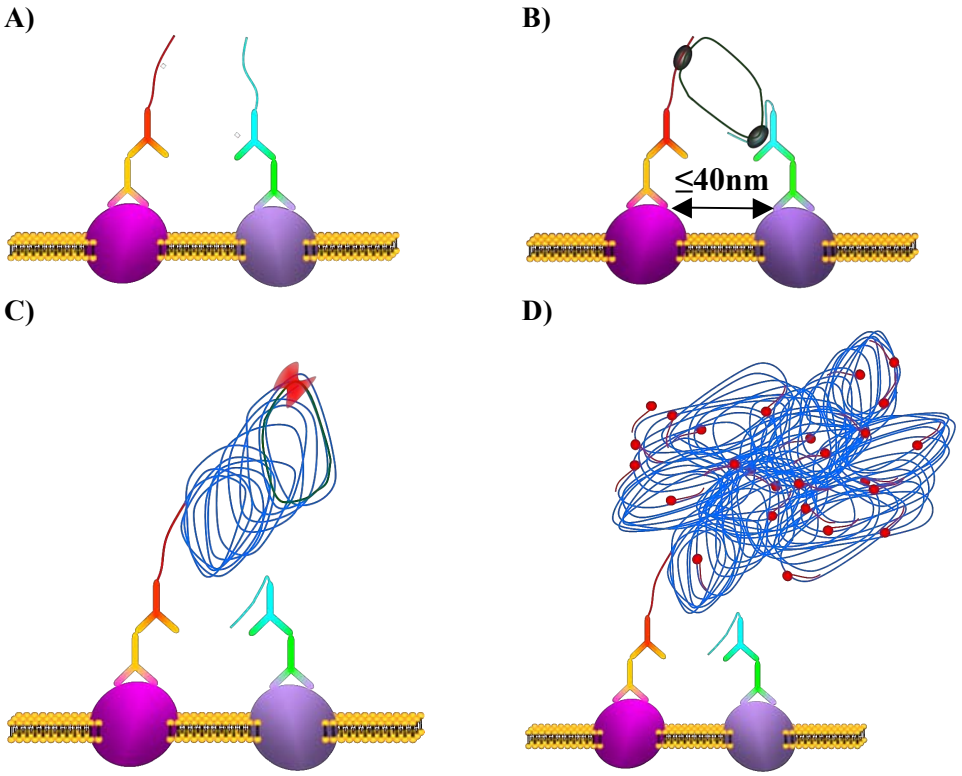


Figure 3.7: A) Schematic representation of primary and secondary conjugated antibodies. **B)** Minimum distance required for an efficient hybridization of PLA oligonucleotide probes. **C)** After ligation takes place generating a double PLUS strand is created, suitable to be used by the polymerase and creating a large concatemeric DNA. **D)** Final product after the hybridization of the fluorescent detection probes with the concatemeric polymerized DNA.

3.2.14 Western blot

Mouse hearts homogenates, microsomal, MAM and mitochondria extracts were obtained as described above. Equal amounts of protein (70µg) supplemented with 1% SDS (Bio-Rad, 161-0301) were subjected to polyacrylamide gel electrophoresis. Samples were preheated (95ºC, 5min) in SDS reducing buffer, separated on 6%-12% acrylamide/bis (Bio-Rad) gels and transferred to Amersham Hybond-ECL nitrocellulose membranes (GE Healthcare). The membranes were blocked in TBS-T solution (Tris-buffered saline, 0.1% Tween-20) with 5% non-fat milk powder. Proteins were detected with RyR (Abcam, ab2868), VDAC1 (Abcam, ab15895), GRP75 (Santa Cruz Biotechnology Inc, sc-13967), GAPDH (Gene Tex, GT239), Mfn-2 (Abcam, ab56889) and ANT1/2 (Santa Cruz Biotechnology Inc, sc-9299) antibodies in TBS-T with 3%

BSA (Sigma). Horseradish peroxidase-conjugated IgGs were used as secondary antibodies: anti-mouse (Sigma, A4416), anti-rabbit (Pierce, 31460), and anti-goat (Thermo Scientific, 31402). Peroxidase reactions were carried out and visualized using Supersignal West Dura Extended Duration Substrate (Thermo Scientific) and the chemiluminescence imaging system LAS-3000 (Fujifilm). Band intensities were quantified with Science Lab-2001 Image Gauge (Fujifilm).

3.2.15 Beta-galactosidase quantification in mouse hearts

Beta-galactosidase (β-gal) was determined in heart extracts from young and old mice using the soluble β-galactosidase method(Lee et al. 2006). Frozen tissue (0.1-0.2g) was homogenized in 0.1mol/L citrate (pH 4.5) and centrifuged at 12000g for 7 min. The supernatants were diluted in citrate assay buffer containing 2-nitrophenyl-β-D-galactopyranoside (2mg/mL) and 1mmol/L $MgCl_2$. After overnight incubation at 37ºC, one volume of 1mol/L potassium carbonate was added at each sample, and absorbance of O-nitrophenol, the product resulting from the enzyme activity, was read at 420nm ($E_{mM\ pH10}$ 21.3). -gal was expressed as µmols of O-nitrophenol/g tissue.

3.2.16 Measurement of glutathione levels in mouse hearts.

Total glutathione concentration (GSHtot) and oxidized glutathione fraction (GSSG) were determined in heart extracts from young and old animals using the glutathione reductase enzymatic method (Tietze 1969), based on the reaction between Ellman's reagent (DTNB) with glutathione to form a spectrophotometric detectable product at 412 nm.

A standard curve of reduced glutathione (0-50mmol/L) was used to calculate glutathione amounts in mice extracts. Briefly, frozen heart extracts from old and young animals were thawed (50 µL) and subsequently assessed in phosphate_EDTA buffer containing glutathione reductase (17 U/mL), NAD(P)H (0.016mg/mL) and DTNB (0.042mg/mL). To determine GSSG, a duplicate of each sample was previously incubated with 3mmol/L of 1-methyl-2-vinylpyridinium triflate, a thiol-scanvenging reagent, which rapidly scavenges reduced form of glutathione without interfering with the enzyme activity. Results were expressed in nmol GSH/mg protein.

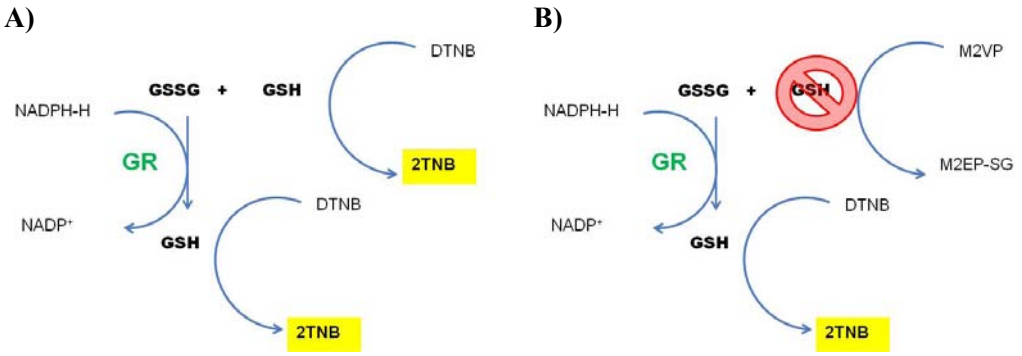


Figure 3.8: A) Ellman's reagent is able to react with the reduced glutathione (GSH). Additionally, the oxidised glutathione (GSSG) is reduced by the glutathione reductase (GR) making it also able to react with the Ellman's regent. In this procedure total glutathione (GSHtot) in the sample is detected. **B)** Blockage of the GSH with the alkylating 1-methyl-2-vinylpyridinium triflate (M2VP). GSSG is reduced by the GR and only the GSH originated from the GSSG reacts with Ellman's reagent. With this strategy only GSSG from the sample is detected.

3.2.17 Differential quantitative mitochondrial proteomic analysis

Mitochondrial and microsomal fractions were isolated by differential centrifugation (as described above). Peptides and proteins were identified and quantified by differential high-throughput proteomic analysis performed by stable isotopic labelling using a previously described protocol (Bonzon-Kulichenko et al. 2011; Navarro and Vázquez 2009; Navarro et al. 2014). Changes in the abundance and composition of cysteine-thiol redox status of mitochondria and microsomal proteins were quantified using de GELSILOX methodology (Martinez-Acedo et al. 2012). Analyses of samples by LC-MS/MS were performed as previously described (López-Ferrer et al. 2006). Functional protein classification was done using the Gene Ontology database. Proteomics results were analysed as previously described (Jorge et al. 2009).

3.2.18 Statistical Analysis

Data are expressed as mean ± SEM. For comparisons between 2 groups 2-tailed Student's t-test for independent samples was used. For comparison of groups with more than one factor a factorial ANOVA analysis was performed followed by post-hoc comparisons when necessary. Differences of $p<0.05$ were considered statistically significant. When samples did not follow a normal distribution, the non-parametric test of median was used. All statistical analyses were performed with SPSS v.15 software.

3.3 Results

3.3.1 Aging phenotype

Echocardiographic analysis showed thinner ventricular wall and a trend towards increased LV end-diastolic volume in old mice, as well as a non-significant trend towards reduced ejection fraction (table 3.1).

Table 3.1: Echocardiographic study of young and old mice.

	Young (n=12)		Old (n=14)		
	Mean	**SEM**	**Mean**	**SEM**	**p**
Body weight (g)	38.30	± 4.00	34.59	± 1.90	0.41
SWT (mm)	0.87	± 0.05	0.79	± 0.021	0.19
PWT (mm)	0.88	± 0.04	0.72	± 0.029	0.004*
LVEDD (mm)	4.09	± 0.17	4.30	± 0.174	0.39
LVEDDIN (mm/g)	0.12	± 0.01	0.13	± 0.009	0.17
PWT/LVEDD	0.22	± 0.01	0.17	± 0.01	0.016*
SWT/LVEDD	3.13	± 0.2	3.75	± 0.25	0.07
LVm (mg)	115.42	± 11.89	100.98	± 6.25	0.25
EDLvol (mL)	46.98	± 4.1	55.12	± 4.29	0.37
LVm/EDLvol (mg/mL)	3.32	± 0.15	1.88	± 0.08	0.014*
LVEF (%)	71.01	± 2.73	66.85	± 2.06	0.22
HR (bpm)	455.45	± 9.75	436.36	± 3.24	0.32

SWT: septum wall thickness; **PWT**: posterior wall thickness; **LVEDD**: left ventricular end-diastolic diameter; **LVEDDIN**: LVEDD indexed by weight; **LVm**: left ventricular mass; **EDLVvol:** end-diastolic left ventricular volume**; LVEF**: left ventricular ejection fraction; **HR**: heart rate; **bpm**; beats per minute.

Isolated cardiomyocytes from aged hearts displayed an increase in lysosome vesicles, lipofuscin pigment and β-galactosidase activity, as hallmarks of cell senescence (fig. 3.9A-D, F).

There were no age-dependent differences in mitochondrial pool, as quantified by Mitotracker red staining, quantification of citrate synthase activity and total cardiac mitochondrial yield (fig. 3.9 E-F and fig. 3.10).

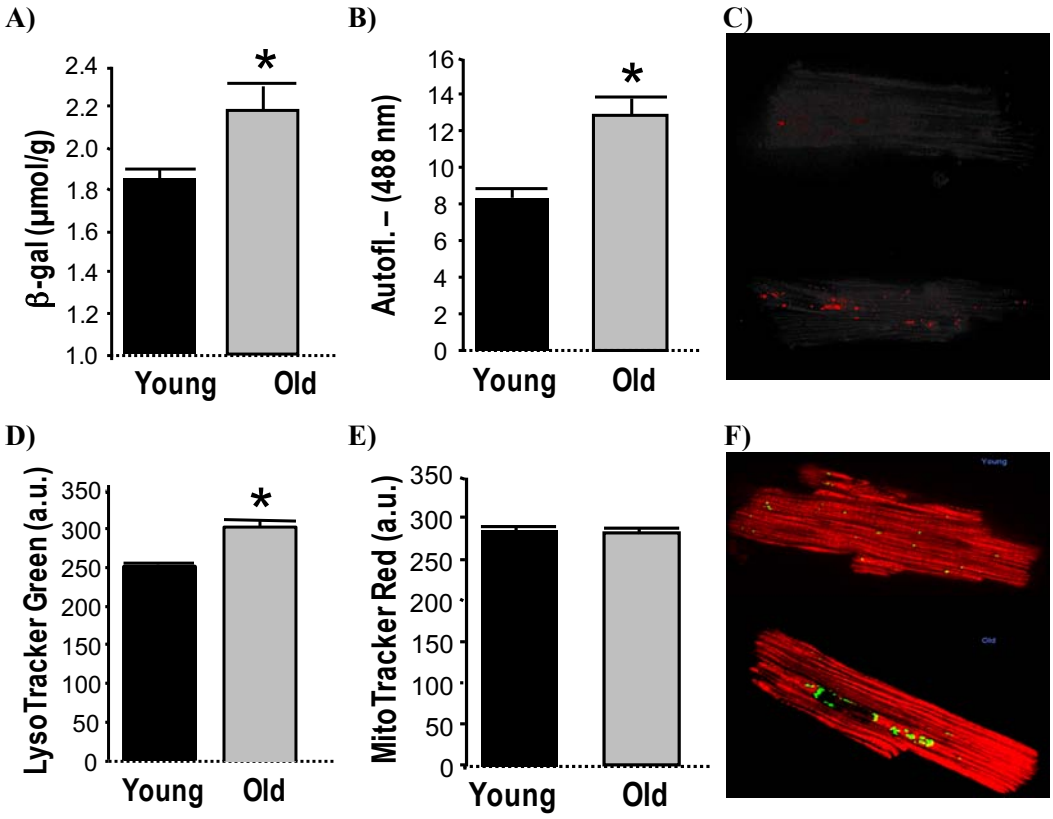


Figure 3.9: A) Effect of advanced age on β-galactosidase activity and lipofuscin autofluorescence in intact mouse cardiomyocytes. **A)** Lysosomal β -galatosidase activity is enhanced in aged cardiomyocytes (8.34±0.57 in young vs. 12.94±0.94 in old; $p<0.01$). **B)** Liposfuscine accumulation is increased with aging (1.9±0.05 in young and 2.2±0.14 in old; $p<0.05$). **C)** Representative image of lipofuscin autofluorescence from young and old isolated cardiomyocytes. Mean±SE; from 4 replicates per group (2 hearts). **D)** LysoTracker® Green fluorescence was significantly increased in aged cardiomyocytes (252.50±2.15 in young vs. 303.75±8.96 in old; $p<0.01$.). **E)** Mitochondrial pool (MitoTracker® Red labeling) remain constant despite of age (281±7.73 in young vs. 290±1.84 in old; p=n.s.). **F)** Representative image of co-labelled intact mouse cardiomyocytes from young and old mice with MitoTracker® Red and LysoTracker Green were an obvious increased of lysosomic vesicles appear with aging. Mean±SE from n=33-28 cells, 4-3 hearts per group.

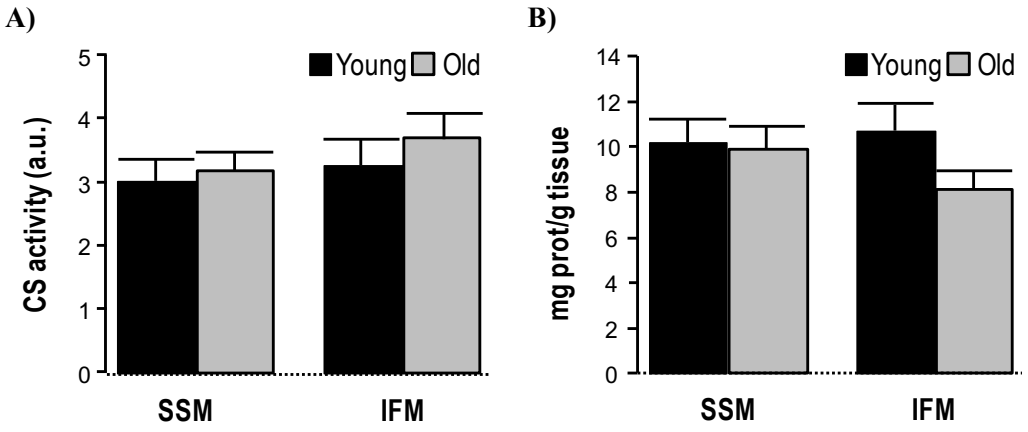


Figure 3.10: Changes observed in the citrate synthase activity (**A**) and mitochondrial yield determined as mitochondrial protein respect to total cardiac protein (**B**) in isolated SSM and IFM from young and old mice (n=14-25 replicates, 5-10 hearts).

3.3.2 Effect of aging on energy metabolism and mitochondrial respiration

Myocardial ATP/phosphocreatine ratio was similar in hearts from both groups of ages. Advanced age did not induce mitochondrial membrane depolarization under resting conditions as quantified in JC-1 loaded isolated cardiomyocytes (fig. 3.11 A-B).

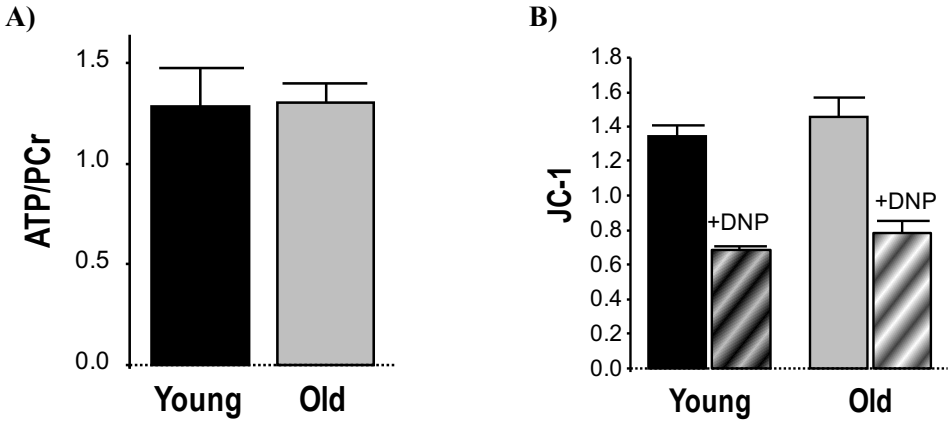


Figure 3.11: A) ATP/PCr in intact mouse hearts, quantified by NMR spectroscopy (1.29±0.1 in young vs. 1.31±0.02 in old; n.s.) Mean±SE from n=3 hearts. **B)** Mitochondrial membrane potential (JC-1 ratiofluorescence) in intact cardiac myocytes from young and old mouse hearts under resting conditions and after induction of maximal mitochondrial depolarization with DNP (basal: 1.342±0.065 in young vs. 1.454±0.115 in old; n.s. DNP: 0.682±0.027 in young vs. 0.782±0.068 in old; n.s.). Mean±SE from n=14-26 cardiomyocytes per group, 5 hearts).

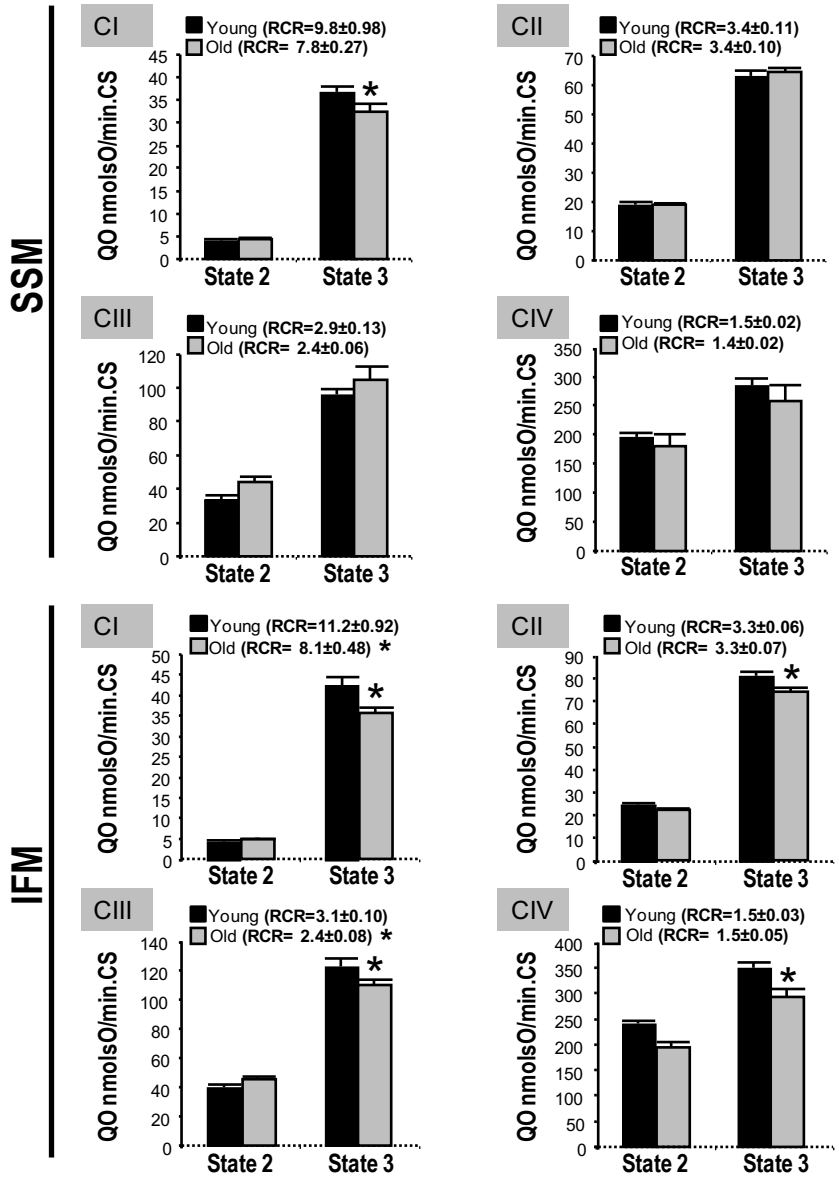


Figure 3.12: Resting O_2 consumption State-2) and ADP-stimulated O_2 consumption (State-3) was measured in freshly isolated Subsarcolemmal (SSM) and interfibrillar (IFL) mitochondria from young and old mouse hearts, mediated by substrates of complexes 1 to 4, normalized by citrate synthase (CS) activity. RCR (respiratory control rate, State-3/State-2). In SSM population no changes related with aging were observed at any complex activity except at State-3 from complex I. Nevertheless, this decreased activity was compensated in the RCR. In IFM population all complexes were significantly affected with aging at State-3, especially complexes I and II were these decreased function was also evident in their RCR. Mean±SEM from n=14-28 replicates (5-10 hearts).

Both O_2 consumption at rest (state-2) and after ADP-stimulation (state-3) were largely preserved in subsarcolemmal mitochondria from aged mouse hearts, independently of the substrates used to feed the respiratory complexes, except a slight decrease in complex I-mediated state-3 respiration, without changes in respiratory control rate (fig. 3.12). By contrast, interfibrillar mitochondria from aged hearts displayed a depression of ADP-stimulated O_2 consumption for any substrate used to feed respiratory complexes, with reduced respiratory control rate dependent on complexes 1 and 3 (fig. 3.12). These data indicate an altered capacity of interfibrillar mitochondria from aged hearts to respond to maximal stimulation, despite preserved respiration under resting conditions.

3.3.3 Aging is associated with altered SR RyR gating properties

Field stimulation of cardiomyocytes disclosed a reduction in the amplitude of SR calcium transients and a decreased rate of calcium rise in cardiomyocytes from old hearts, without differences in the time to peak or time to 50% decay. SR calcium content, quantified as maximal caffeine-induced calcium release, was similar in cardiomyocytes from both groups of ages (fig. 3.13).

In non-stimulated cardiomyocytes, aging was associated with a significant increase in the frequency of spontaneous SR calciumsparks with decreased calciumdiffusion, without changes in their rate or amplitude (fig. 3.14). These data indicate altered RyR gating properties in old cardiomyocytes.

3.3.4 Advanced age depresses SR-mitochondria calcium transfer and NAD(P)H regeneration

In digitonin-permeabilized cardiomyocytes from young hearts, induction of SR calcium release with caffeine was followed by a rapid increase in mitochondrial calcium uptake that was severely depressed in cardiomyocytes from old hearts (fig. 3.15 A). Calcium uptake by mitochondria was dependent on mitochondrial calcium uniporter and could be prevented by the specific inhibitor Ru360 (fig. 3.15 B). However, this depression in mitochondrial calcium uptake kinetics observed in intact cardiomyocytes of aged hearts could not be reproduced in isolated mitochondria exposed to an external calcium pulse *in vitro* (fig. 3.15 C). Field-stimulation at 1Hz did not result in net consumption of NAD(P)H in cardiomyocytes from any group of age, but rather induced a slight increase in young cells. However, acceleration of electrical pacing from 1Hz to 5Hz was associated with a decay in NAD(P)H/NAD(P)+ in cardiomyocytes from old

hearts but not from young hearts, indicating an inefficient NAD(P)H regeneration (fig. 3.15 D).

All together, these data suggest a defective calcium transfer from SR to mitochondria, with concomitant reduced bioenergetic feedback response (NAD(P)H regeneration), in cardiomyocytes of aged hearts, despite normal mitochondrial calcium uniporter activity.

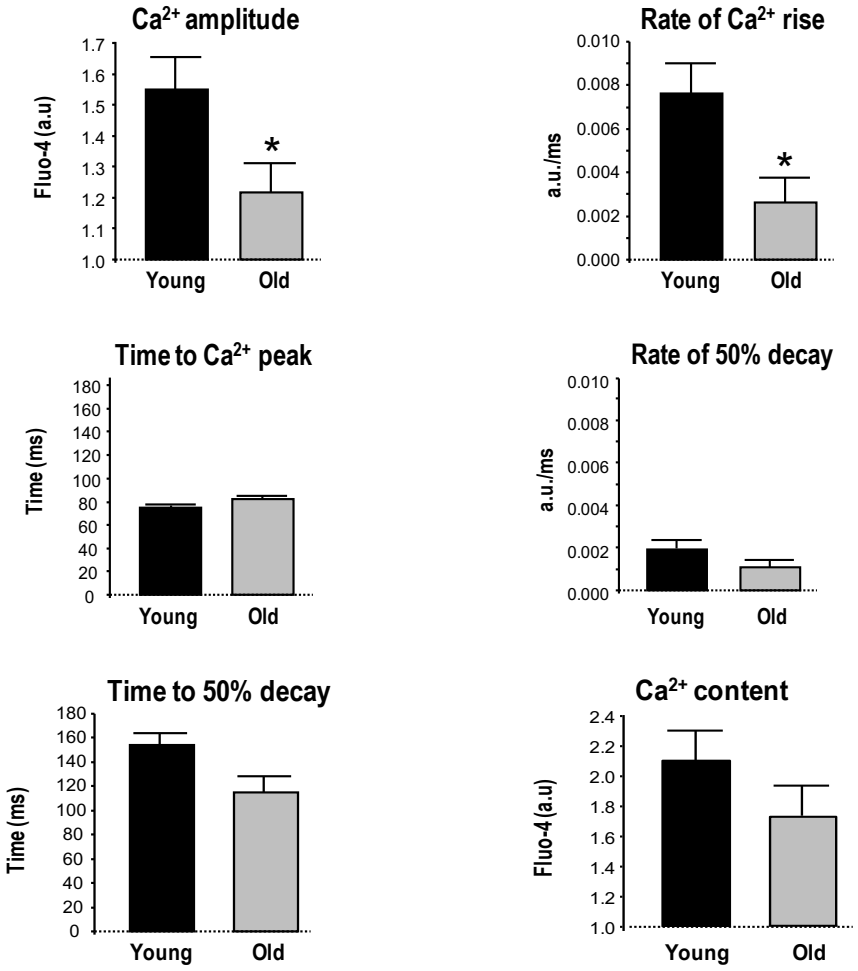


Figure 3.13: Parameters of SR calcium release/uptake kinetics in field-stimulated (0,5Hz) Fluo 4-AM loaded cardiac myocytes from young and old mouse hearts. Aging was associated with a decreased transient amplitude (0.355±0.095 in young vs. 0.218±0.093 in old; $p<0.05$) and rate of calcium rise (0.0077±0.0013 in young vs. 0.0026±0.0011 in old; $p<0.05$) while no effects were observed in the time to peak (74±2.1 in young vs. 82±3.2 in old; p=n.s.), rate of 50% of decay (0.0019± 0.0004 in young vs. 0.0011±0.0004 in old; p=n.s.), time to 50 % decay (160.1±11.0 in young vs. 115.2±13.3 in old; p=n.s.) SR Ca^2 content. Mean±SEM from n=9-13 cardiomyocytes per group (6 hearts).

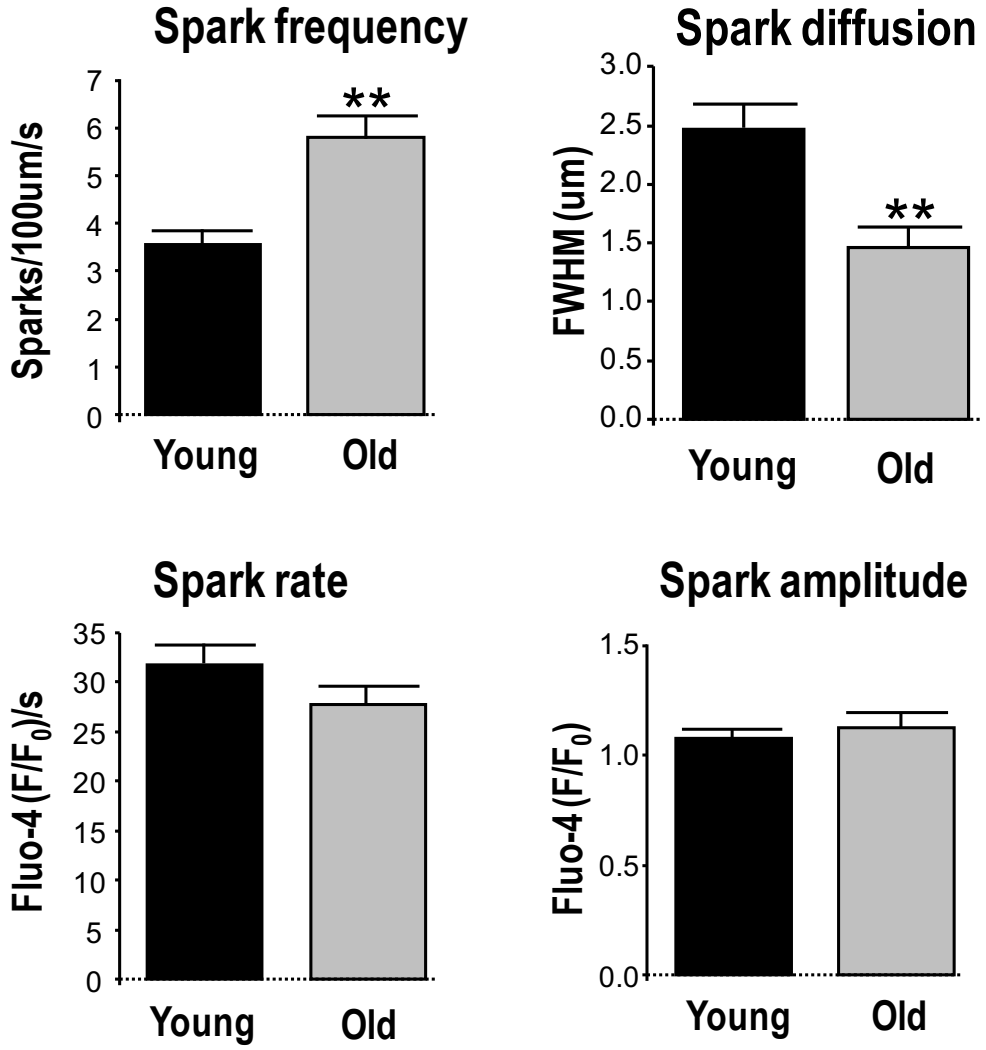


Figure 3.14: Spontaneous spark in quiescent isolated cardiomyocytes loaded with Fluo-4. Aging was related with a significant increase of spark frequency (3.38±0.41 in young vs. 5.81±0.60 in old; $p<0.01$) and a decreased diffusion (2.50±0.16 in young vs. 1.52±0.13 in old; $p<0.01$). The rate (31.35± 2.75 in young vs. 15.07±2.92 in old; p=n.s.) and the amplitude (1.06± 2.75 in young vs. 1.12±0.03 in old; p=n.s.) were not affected by aging. FWHM= full width at half maximum (µm). Mean±SEM from n=9-13 cardiomyocytes per group, 6 hearts. Mean±SEM from n=1601 sparks, 6-6 cells/40 z-stacks per cell (4 hearts).

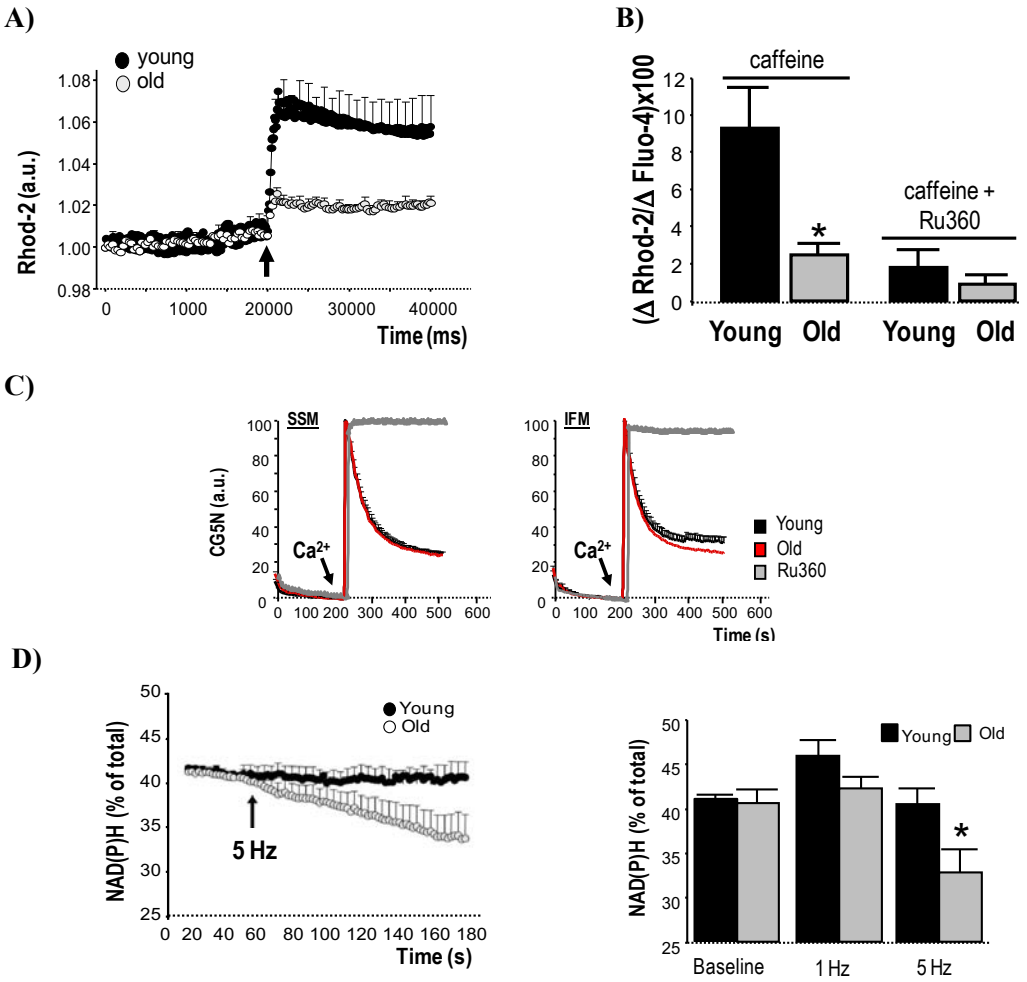


Figure 3.15: **A)** Mitochondrial calcium uptake throughout time in response to SR calcium release (10mmol/L caffeine, arrow) in digitonin-permeabilized Rhod 2-AM loaded cardiac myocytes from old and young mouse hearts, 8-11 cardiomyocytes per group (n=5 hearts). **B)** Maximal mitochondrial calcium uptake (Rhod 2-AM) normalized by maximal SR calcium release (fluo-4) in young and old permeabilized mouse cardiomyocytes (9.32±2.24 in young vs. 2.52±0.59 in old; $p<0.05$). Addition of 10µmol/L Ru360 (a specific blocker of the mitochondrial calcium uniporter) prevented caffeine-induced mitochondrial calcium uptake in both groups of ages (Young: 9.32±2.24 controls vs.1.84±0.93 Ru360; $p<0.05$. Old: 2.52±0.59 vs. 1.07±0.38 Ru360; p=n.s.) Mean±SEM from n=7-11 cardiomyocytes per group (5 hearts). **C)** Absence of age-dependent differences in the in vitro mitochondrial calcium uptake kinetics (CG5N fluorescence), when exposing isolated subsarcolemmal (SSM) and interfibrillar (IFM) cardiac mitochondria to an external calcium pulse of 30µmol/L (arrow). Addition of 10µmol/L Ru-360 prevented mitochondrial calcium uptake. Mean±SEM from 4 replicates per group (6 hearts). **C)** Kinetics of the NAD(P)H consumption in its reduced form, expressed respect to total cell NAD(P)H, during 5Hz stimulation (left panel). Mean±SEM from n=10-19 cardiomyocytes per group (9 hearts). NAD(P)H regeneration 2 min after electrical stimulation of intact cardiomyocytes from young and old mouse hearts at 1Hz (45.96±1.63 in young vs. 42.33±1.28; p=n.s.) and 5Hz (40.75±1.64 in young vs. 33.15±2.42; $p<0.05$) to induce high contractile activity (right panel).

3.3.5 Aged cardiomyocytes have less glutathione and increased mitochondrial ROS production

Total glutathione levels (GSHtot) quantified in myocardial tissue were significantly decreased in aging hearts (fig. 3.16 A). The oxidised fraction of glutathione (glutathione disulfide, GSSG) showed a trend towards an increase in the aging myocardium (fig. 3.16 A insert).

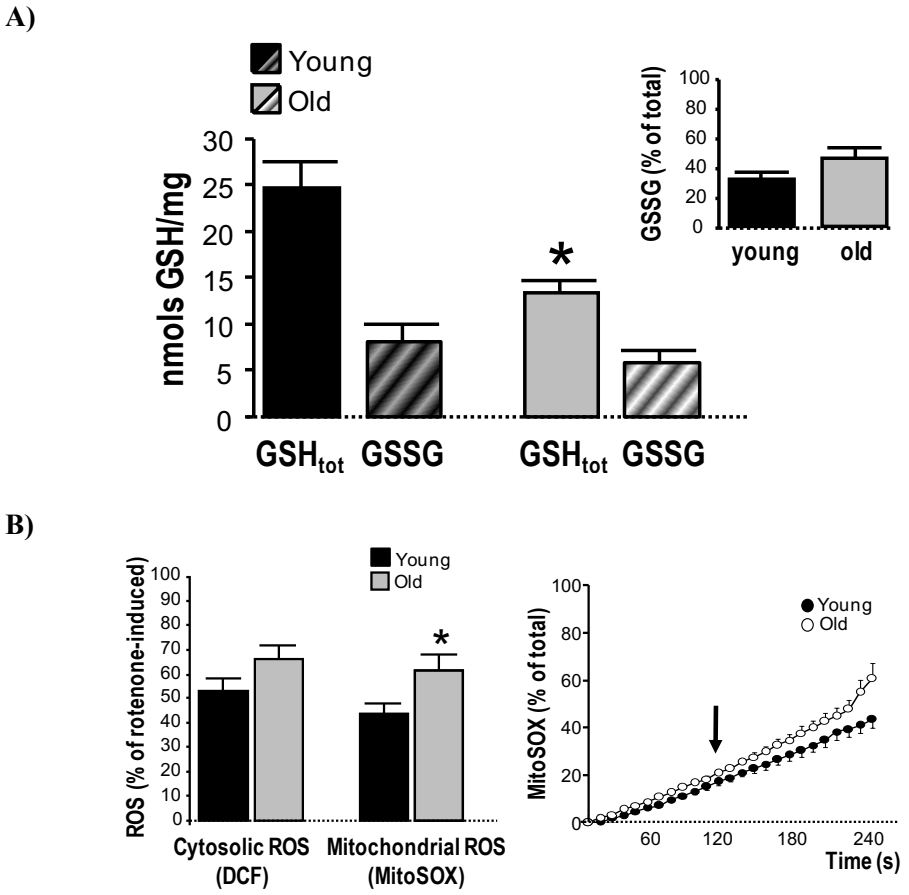


Figure 3.16: **A)** Total glutathione (GSHtot) and oxidised glutathione levels (GSSG) in myocardial tissue of young and old mice (GSHtot: 25±2.8 in young vs. 13±1.2 in old; $p<0.05$. GSSG: 8±1.8 in young vs. 6±1.4 in old; p=n.s.). The inset shows the fraction of oxidised glutathione respect to total one (32±4.7 in young vs. 42±8.7 in old; p=n.s.). **B)** Cytosolic and mitochondrial ROS production of isolated cardiomyocytes from young and old hearts submitted to increasing frequencies of field stimulation (0Hz-1Hz-5Hz), as quantified by CARBOXY-H2DCFDA (43.9±3.82 in young vs. 61.23±6.42; $p<0.05$) and MitoSOX (53.4±488 in young vs. 66.15±5.94 in old; p=n.s.) fluorescence (left). Kinetics of short-term mitochondrial ROS production during pacing (right). Mean±SEM from n=9-12 cardiomyocytes per group (4 hearts).

In isolated cardiomyocytes submitted to electrical stimulation, aging was associated with a significant increase in the short-term mitochondrial ROS production during high frequency pacing (5Hz). There was a non-significant trend towards an increased cytosolic ROS levels in aging cardiomyocytes after high frequency pacing, but we cannot rule out that this is due to diffusion of mitochondrial ROS towards the cytosolic (fig. 3. 16 B)

3.3.6 Spatial proximity between mitochondria and SR decreases with aging

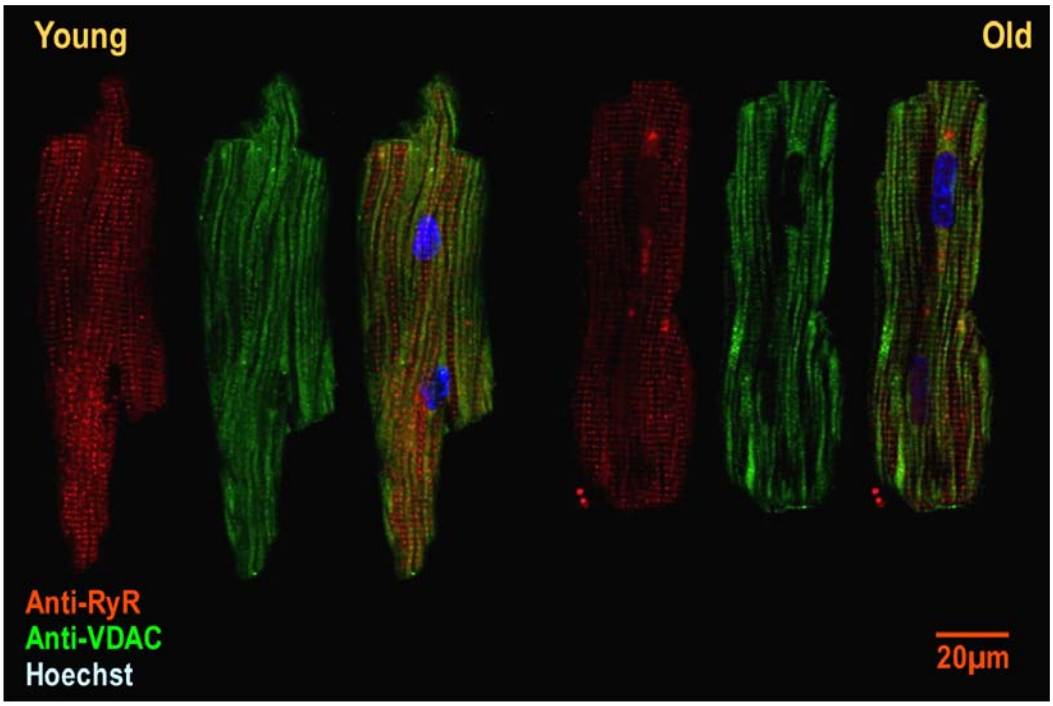
Simultaneous immunelabelling of permeabilized cardiomyocytes with anti-RyR and anti-VDAC disclosed a pattern of close proximity and similar spatial organization between SR and mitochondria (fig. 3. 17 A). Blind quantification of immunocolocalization degree by Mander's coefficient analysis revealed an age-dependent reduction of the fraction of RyR overlapping with VDAC (m1) without significant differences in either RyR or VDAC total fluorescence (fig. 3. 17 B).

Proximity ligation assay, specifically addressed to quantify SR-mitochondria clusters of <40nm distance, demonstrated an age-dependent reduction in the positive amplification fluorescent spots (fig. 3. 18 A, B). These data indicate a reduction in the fraction of SR RyR closely juxtaposed with mitochondrial VDAC.

The effect of age on SR-mitochondria dissociation was not explained by a reduced expression of the main proteins postulated to tether both organelles. Western blot quantification of Mfn-2, VDAC-1, RyR-2 and Grp75 in total cardiac homogenates, microsomal fractions and mitochondria showed no differences between both groups of age (fig. 3. 19).

These results were consistent with those of quantitative proteomics analysis (not shown). However, individual peptide quantification by GELSILOX of the same data revealed an increase in the proportion of peptides containing oxidized Cys detected in mitochondrial VDAC and a concomitant reduction in the same peptides containing the reduced Cys form in both SSM and IFM from old mouse hearts, suggesting that aging induces oxidative damage in this protein (fig 3.20 A). Consistently, proteins from MAM fraction displayed a trend towards and increased oxidative state (fig. 3.20 B). These data are compatible with the existence of an oxidised microenvironment around SR-mitochondria contact points, despite normal protein expression.

A)



B)

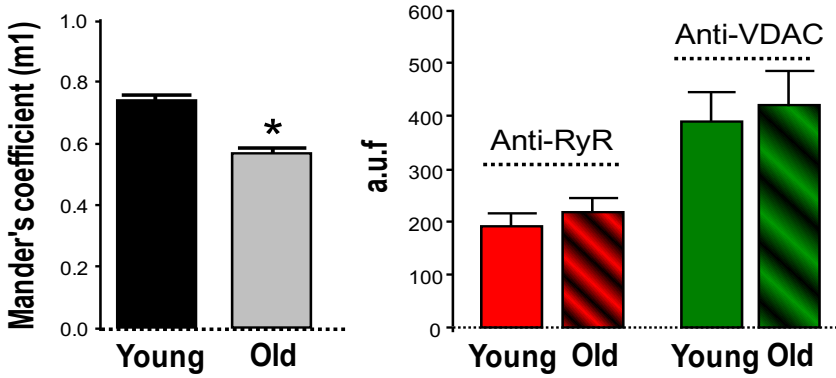
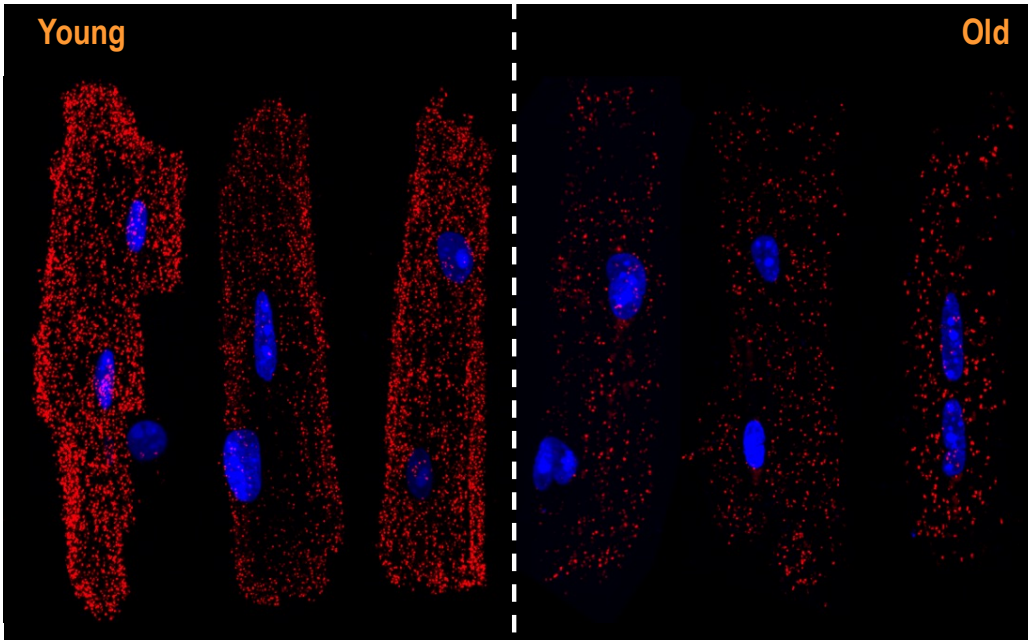


Figure 3.17: A) Confocal fluorescent images of a young and old mouse cardiomyocyte simultaneously labelled with anti-RyR (red), anti-VDAC (green) and Hoechst (blue) for visualization of SR, mitochondria and nuclei, respectively. **B)** Effect of aging on RyR-VDAC spatial interaction, as quantified by Mander's coefficient (m1) analysis (0.742±0.019 in young vs. 0.506±0.029 in old: $p<0.05$), expressed as the percentage of RyR -respect to total RyR fluorescence- that overlaps with VDAC (left panel); in the right panel, total RyR and VDAC fluorescence (RyR: 191±25.38 in young vs. 218±27.49 in old; p=n.s. VDAC: 388±58.09 in young vs. 419±61.64 in old; p=n.s.). Mean±SEM from, 4-6 cardiomyocytes per group, 4 ROIS per cardiomyocytes, 50 z-frames per ROI (4 hearts).

A)



B)

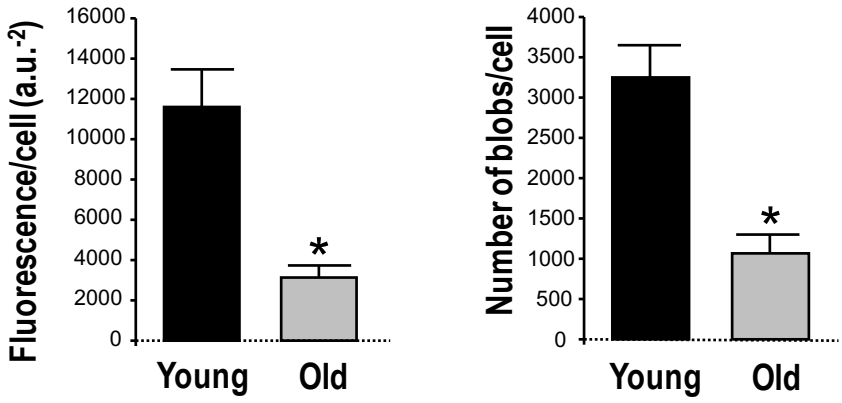


Figure 3.18: A) Confocal fluorescent images of the RyR-VDAC interaction in different individual cardiomyocytes isolated from young and old mouse hearts, detected by proximity ligation assay (PLA). Positive cross-reactivity -reflecting an intermolecular distance of <40nm- is shown in red, nuclei are depicted in blue (Hoechst). **B)** Aging was associated with a significant reduction in cell fluorescence resulting from RyR-VDAC cross-reactivity (left panel, 11625086±185878.7 in young vs. 306548.6±64505.6 in old; $p<0.05$) and in the number of amplification spots (right panel; 3249.4±397 in young vs. 1071.2±293 in old; $p<0.05$), as quantified by PLA assay. Mean±SEM from 1071-3250 blobs per group (15 cardiomyocytes, 50 z planes per cell, 2 hearts).

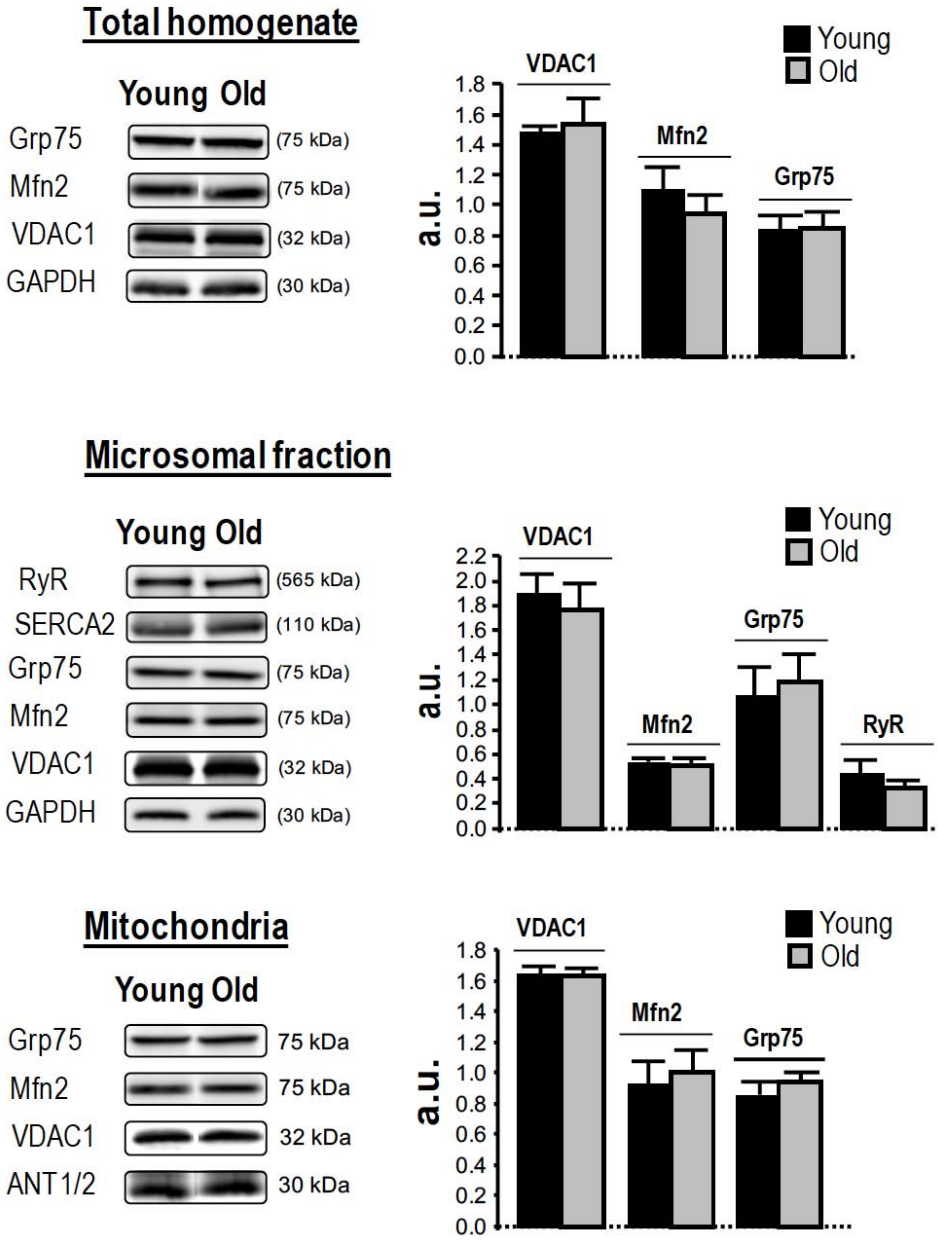


Figure 3.19: Western blot representative bands and quantification of the expression of proteins involved in SR and mitochondria calcium transport and interorganelle communication in whole heart homogenate, microsomal and mitochondrial fractions from young and old mice. Each protein of interest was normalized by the corresponding protein of reference as follows: in total homogenates, VDAC1/Grp75, Mfn2/GAPDH and Grp75/GAPDH; in microsomal fraction: Mfn2/VDAC1, VDAC1/Grp75, Grp75/GADPH and RyR/Grp75; in mitochondria: VDAC1/Grp75, Grp75/ANT1/2 and Mfn2/ANT1/2. Mean±SEM from n=8 replicates from 4 hearts/group.

A)

	Ox		Red	
VDAC1	SSM	IFM	SSM	IFM
YQVDPDACFSAC	-3.3	-5.4	4.19	2.68
VDAC2				
WNTDNTLGTEIAIEDQICQGLK	-2.5	2.63	4.95	
WCEYGLTFTEK	0.1	7.8	4.75	

B)

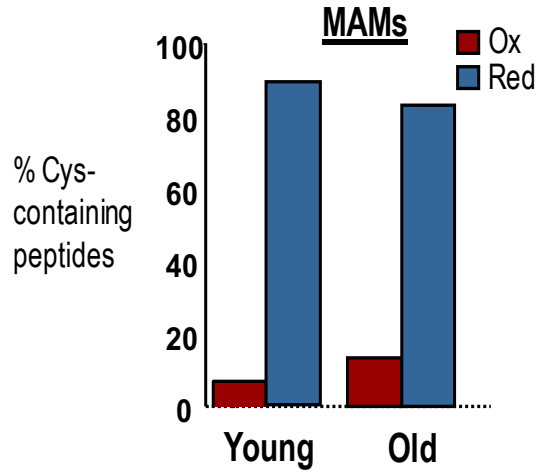


Figure 3.20: A) The table represent the standardized variable at the peptide level as in negative values indicate an increase (in green) and positive values a decrease (in red) in the peptides containing Cys in oxidized form (Ox) or in reduced form (Red) in mitochondrial VDAC proteins in hearts from old mice. **B)** Quantitative red-ox proteomics using GELSILOX of proteins from mitochondria-associated membranes (MAMs). The graph represents the percentage of Cys-containing peptides identified in oxidized (red) or reduced (blue) forms.

3.3.7 Increased oxidation of mitochondrial respiratory proteins in aged hearts

High-throughput quantitative proteomics followed by systems biology analysis indicated that there were no age-dependent changes in relative abundance of proteins of any of the five oxidative phosphorylation complexes in SSM or IFM (fig. 3.21 A).

However, GELSILOX analysis revealed that aging was associated with an increased proportion of mitochondrial peptides containing oxidized Cys and a decrease in those containing reduced Cys in proteins of the oxidative phosphorylation complexes (fig. 3.21 B).

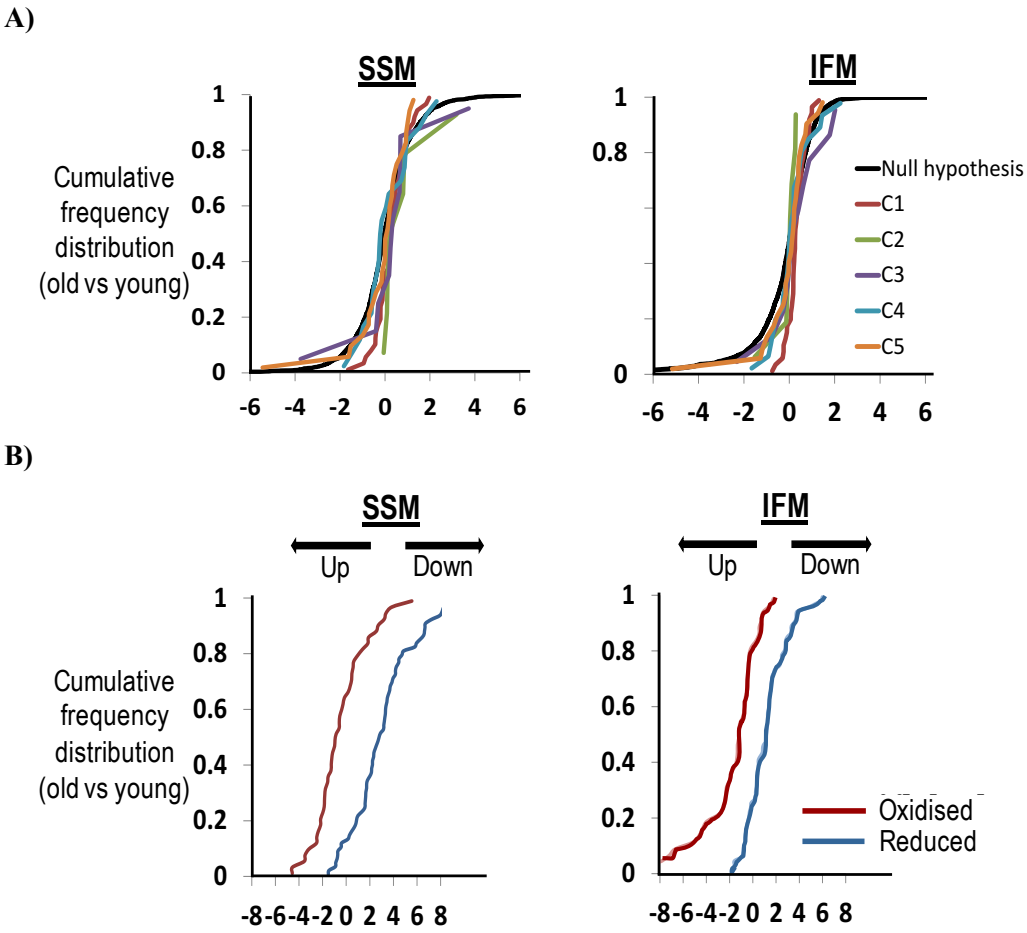


Figure 3.21: A) The abundance of mitochondrial respiratory complexes (1 to 5) in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria. Data are shown as cumulative distributions of the standardized variable at the protein level (i.e. corrected log2-ratios of proteins expressed in units of standard deviation) for all oxidative phosphorylation proteins. The black sigmoid is the theoretical null hypothesis distribution; a displacement towards the left indicates an increase in protein concentration. All the categories follow very closely the null hypothesis distribution, indicating that aging does not affect the abundance of mitochondrial respiratory proteins. **B)** Alterations in the abundance of oxidized (red) and reduced (blue) cysteine (Cys)-containing peptides in SSM and IFM from young and old mouse hearts. Peptides containing Cys residues in different oxidation states were quantified using the GELSILOX method. The sigmoid curves represent the cumulative distribution of the standardized variable at the peptide level (i.e. corrected log2-ratios of peptides expressed in units of standard deviation), for all peptides containing either oxidized or reduced Cys sites that belong to proteins from oxidative phosphorylation complexes.

3.3.8 Disruption of SR-mitochondria connection in young cardiomyocytes mimicks age-induced alterations in calcium handling

Addition of 0.75µmol/L colchicine to intact cardiomyocytes induced a partial disruption of SR-mitochondria interaction, confirmed by both decreased RyR-VDAC immunocolocalization (fig. 3. 22 A) and reduced number of amplification clusters in PLA assay (fig. 3. 22 B).

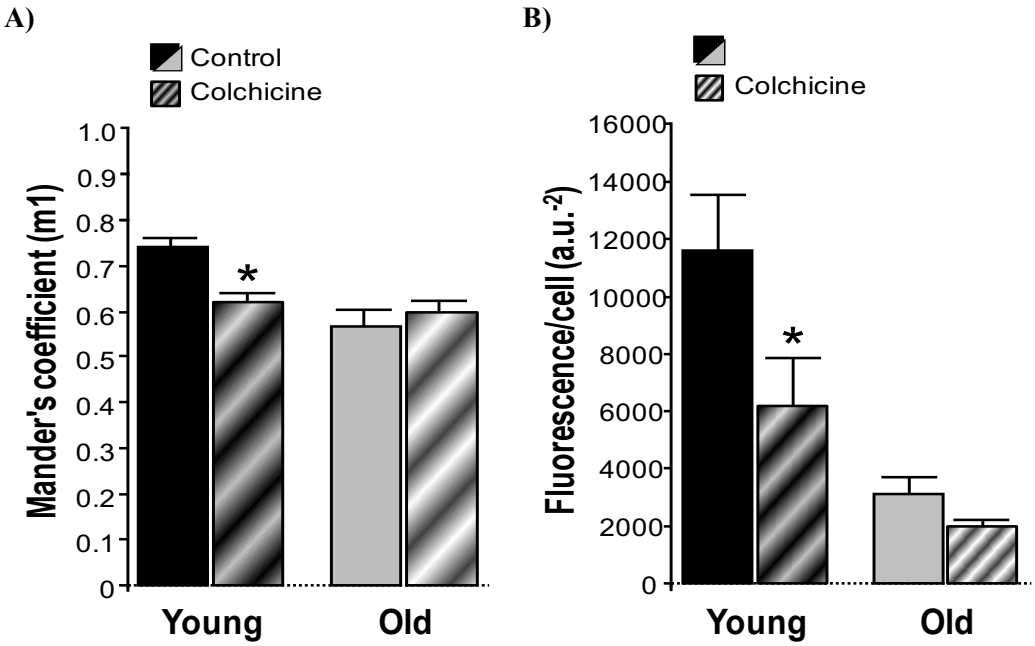


Figure 3.22: A) Effect of 0.75 µmol/l colchicine on the Mander's coefficient from isolated cardiomyocytes immonolabelled with anti-RyR and anti-VDAC antibodies (young: 0.742±0.019 control vs. 0.622±0.019 colchicine; $p<0.05$ - old: 0.506±0.029 control vs. 0.597±0.026 colchicine; p=n.s.). **B)** PLA fluorescence of isolated cardiomyocytes from young and old mouse hearts with and without colchicine treatment. (young: 11625086±185878.7 in control vs. 6166713±167610.6 colchicine; $p<0.05$ – old: 306548.6±64505.6 control vs. 201975.8±14775.8 colchicine; p=n.s.).

The effect of this intervention was significantly less pronounced in cardiomyocytes of aged hearts. In cardiomyocytes from young mice, colchicine induced a parallel depression in the amplitude of SR calciumtransients in intact cells (fig. 3.23 A) and decreased mitochondrial calciumuptake secondary to SR calcium transfer (caffeine) in permeabilized cells, but it had only little non-significant effect in cardiomyocytes from old mice (fig. 3. 23-B).

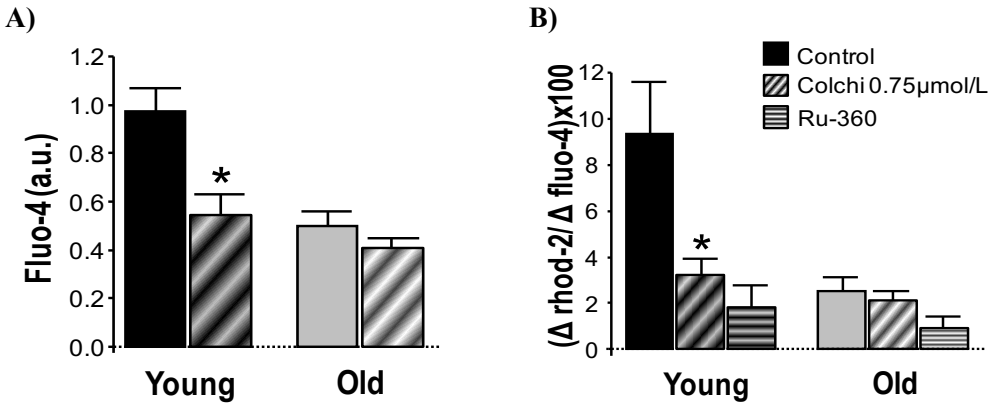


Figure 3.23: A) Changes in the amplitude of calcium transients on field stimulated cardiomyocytes fluo-4 loaded from young and old mouse hearts due to the addition of 0.75 µmol/l of colchicine(young: 0.973±0.10 in control vs. 0.550±0.08 colchicine; $p<0.05$ – old: 0.504±0.058 control vs. 0.410±0.004 colchicine; p=n.s.). **B)** Effect of colchicine treatment in the up taken capacity (Rhod 2-AM fluorescence) of mitochondria in response of SR-Ca^{2+} release in digitonin-permeabilized cardiomyocytes from young and old mice (young: 9.32±2.24 in control vs. 3.23±0.69 colchicine; $p<0.05$ – old: 2.52±0.59 control vs. 2.51±0.39 colchicine; p=n.s.). RU360 10 µmol/L specifically inhibited the mitochondrial calcium uptake. Mean±SEM from n=6-11).

These data indicate that partial pharmacological disruption of SR-mitochondrial communication induces calcium handling alterations that resemble those that are constitutively present in old cardiomyocytes. Nevertheless, colchicine did not have any effect on calcium uptake kinetics or respiration efficiency in isolated mitochondria of any group of age (fig. 3. 24 A-B).

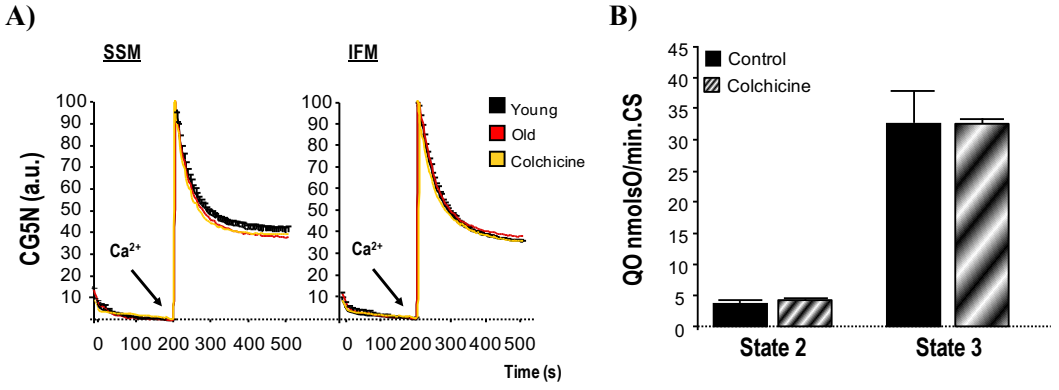


Figure 3.24: A) In vitro mitochondrial calcium uptake (CG5N fluorescence) kinetics in isolated SSM and IFM from young and old mouse hearts in control conditions (black and red) and in presence of 0.75 µmol/L colchine (yellow). **B)** Complex-1 mediated O_2 consumption (State-2) and ADP-stimulated O_2 consumption (State-3) in SSM from young mouse hearts, normalized by citrate synthase activity, in the absence (control) or in the presence of colchicine (bottom panel). Mean±SEM of 4 replicates per group (6 hearts).

3.4 Discussion

The results of the present study indicate that SR-mitochondria communication is altered in aged cardiomyocytes, as manifested by reduced physical interaction among RyR and VDAC and depressed local calcium transfer from SR to mitochondria, despite normal mitochondrial calcium uniporter activity and SR calcium content. As a consequence of reduced calcium exchange, mitochondrial regeneration of the electron donor NADH and the antioxidant NAD(P)H is depressed in old cardiomyocytes when exposed to increased frequency of pacing, reflecting an uncoupled bioenergetic feedback response, and mitochondrial ROS production is concomitantly increase under these conditions. Interfibrillar mitochondria -the population of mitochondria closely juxtaposed with SR- displayed reduced maximal O_2 consumption and altered respiratory control rate. Glutathione levels are reduced in aging myocardium and differential redox proteomics using GELSILOX identified an age-dependent increase in Cys oxidation of mitochondrial VDAC and a trend towards an overall increase in the oxidative state of MAM proteins. This microenvironmental oxidation at the mitochondria-SR contact points was associated with abnormal RyR gating properties in SR from aged cardiomyocytes. These data identify a novel subcellular mechanism of functional decline during aging that could help to the development of cardioprotective strategies specifically addressed to the aged heart.

3.4.1 Disruption of SR-mitochondria spatial relationship during aging

In cardiac myocytes, SR is surrounded by a dynamic network of mitochondria extending along myofibril length (Yoshikane, Nihei, and Moriyama 1986). The positioning of mitochondria is supported by mitochondrial anchorage to cytoskeleton and SR through proteolysis-sensitive electron-dense tethering structures (György Csordás et al. 2006). Several molecular entities have been proposed to bridge the terminal cisternae of the SR or ER with mitochondria, as Mfn-1 and 2 (Chen et al. 2012), the complex formed by RyR/IP3 and the outer mitochondrial membrane VDAC connected through the cytosolic chaperone Grp75 (Szabadkai et al. 2006), and other proteins related with apoptotic pathway (Cerqua et al. 2010), but their exact contribution to physical or chemical coupling or whether they may be redundant remains controversial. In cardiac cells, mitochondrial calcium uptake secondary to SR calcium release, essential for short and long-term metabolic adaptations, gives rise to locally restricted high calcium cell domains(Rosario Rizzuto and Pozzan 2006). To accomplish this, up to 90%

of the calcium release units of ventricular SR are close to mitochondria, with an estimated interorganelle surface distance of 37nm (Sharma et al. 2000).

Our results demonstrate a partially disrupted VDAC and RyR interaction in ventricular cardiomyocytes from aging hearts, determined by two independent techniques, immunocolocalization and PLA. Mander's coefficient analysis of SR and mitochondria immunolabelling indicates that while most RyR fraction (>75%) overlaps with mitochondrial VDAC in young cardiomyocytes, this interaction is reduced to <60% in old cardiomyocytes. Proximity ligation assay, specifically addressed to detect those SR-mitochondria native clusters in which the spatial distance between RyR and VDAC is ≤40nm, confirmed the dissociating effect of age at this subcellular level.

Altered RyR-VDAC interaction in old hearts cannot be explained by a decrease of the expression of these proteins, because the amount of RyR and VDAC, as quantified by immunofluorescence labelling, Western blot and proteomics, was similar in both groups of ages. The expression of other proteins potentially involved in SR-mitochondria bridging, like Mfn-2 and the cytosolic chaperone Grp75, also remained preserved in old hearts at the different subcellular fractions.

Because mitochondria are the major producers of superoxide, it is reasonable to expect that sustained mitochondrial ROS production derived from respiratory activity locally impacts on redox-sensitive proteins at the subcellular microdomains. Indeed, our experiments show increased mitochondrial ROS production in aging cardiomyocytes submitted to increased contractile activity and redox microdomains have been described at the interface between SR and mitochondria, in which NADPH oxidases (NOXs) contribute to ROS generation (Eisner, Csordás, and Hajnóczky 2013). Our proteomics data points out to an increased overall oxidation of the MAM fraction, the subcellular proteinaceous SR-mitochondria tethering structure involved in intracellular calcium signalling and bioenergetics regulation (van Vliet, Verfaillie, and Agostinis 2014), as well as an increased oxidation of mitochondrial VDAC. However, the efficiency of our proteomics approach to specifically obtain Cys-containing peptides within RyR molecules is too low to get a reliable quantification of their redox state, probably due to the deep localization of the thiol sites in the receptor. RyR channels contain multiple, potentially redox-sensitive cysteine residues, and cysteine thiol oxidation appears to increase RyR channel activity (Zima and Blatter 2006).

Our functional data on SR calcium handling are fully consistent with an oxidation-induced alteration of the RyR gating properties, manifested by a substantial increase in spontaneous calcium spark frequency and altered morphology (reduced calcium diffusion pattern) and decreased calcium transient amplitude in the aged cardiomyocytes, despite no significant alteration in total SR calcium content. These SR calcium abnormalities have been attributed to increased frequency of RyR opening events of

less unitary duration and have been described in oxidative environments like those present in failing and aging hearts (Terentyev et al. 2008; Cooper et al. 2013; Zhou et al. 2011). It remains to be elucidated what type of conformational changes may occur in RyR proteins exposed to oxidative stress and how these changes may potentially modify their interaction with other proteins.

3.4.2 Defective SR-mitochondria communication impacts on local calcium handling

Our experiments demonstrate that old cardiomyocytes exhibit a depressed mitochondrial calcium uptake that is secondary to reduced calcium transfer from SR. This concept is supported by several observations: 1) it is specifically manifested in response to RyR stimulation with caffeine; 2) it takes place in digitonin-permeabilized cardiomyocytes, in which the contribution of sarcolemma and cytosol to mitochondrial calcium handling is absent; 3) mitochondrial calcium uptake is strictly normal when exposing isolated mitochondria to external calcium *in vitro*. Our data also indicate that reduced SR calcium transfer in aged cardiomyocytes results in defective NAD(P)H regeneration when cells are submitted to increased contractile activity (high-rate pacing), indicating an altered bioenergetic feedback response. Moreover, impaired mitochondrial calcium uptake in aged cardiomyocytes can be reproduced in young cells after pharmacological disruption of SR-mitochondrial physical interaction with colchicine. Of note, this disrupting manoeuvre does not have any effect on mitochondrial calcium uniporter activity or O_2 consumption, which could reduce mitochondrial calcium uptake by mechanisms independent of interorganelle communication.

An increasing number of evidences support the view that the coordination between mitochondrial ATP supply and mechanical activity demand is highly dependent on an efficient calcium transfer from SR to mitochondria (Y. Chen et al. 2012; Konstantinidis et al. 2012), and that altered SR-mitochondria calcium exchange may underlie the pathophysiology of several cardiac diseases(Ruiz-Meana, Fernandez-Sanz, and Garcia-Dorado 2010). In an acute setting, like the first phase of myocardial reperfusion, rapid and cyclic SR-induced calcium oscillations -in a hyperoxidative cellular milieu- trigger mitochondrial permeabilization, hypercontracture and cell death (Ruiz-Meana et al. 2009; Abdallah et al. 2011b). In this context, genetic or pharmacological inhibition of SR-mitochondria calcium transfer have been demonstrated to have beneficial effects on cell survival (Paillard et al. 2013).

By contrast, chronically insufficient mitochondrial calcium uptake due to defective SR-mitochondrial spatial organization contributes to oxidative stress and ener-

gy deficiency (Chen et al. 2012). This is because calcium released by RyR is partially transferred to adjacent mitochondria, where it activates calcium-sensitive Krebs dehydrogenases and other downstream enzymes involved in energy production, like F1-F0 ATPase and adenine nucleotide translocase, as well as antioxidant pool regeneration (Glancy and Balaban 2012; Kohlhaas et al. 2010; Brandes and Bers 2002). Therefore, ablation of Mfn-2 not only disrupts SR-mitochondrial cytoarquitecture but has been demonstrated to alter calcium transients and mitochondrial calcium uptake and to induce bioenergetic-redox mismatch in cardiac myocytes submitted to beta-adrenergic stimulation (Chen et al. 2012). In a recent 3D integrated cardiomyocyte computational model (Hatano et al. 2013), increasing SR-mitochondria distance from 50nm to 200nm depressed mitochondrial calcium uptake by 17% and NADH generation by 11% when pacing rates were switched from 0.5 to 2 Hz (Hatano et al. 2013). In our experiments, depressed NAD(P)H regeneration in response to pacing stress occurred only in cardiomyocytes from aged hearts, indicating impaired bioenergetic feedback response secondary to inadequate mitochondrial calcium uptake, and was associated with increased ROS production at the mitochondrial compartment. This mechanism may aggravate the age-associated excess of toxic ROS in a vicious cycle. Importantly, the impact of aging on mitochondrial maximal O_2 consumption capacity and respiratory control rate was specifically manifested in IFM. This finding is consistent with previous observations suggesting a greater susceptibility of IFM to aging (S. W. Fannin et al. 1999; Judge et al. 2005) and should be put in the context that this mitochondrial population represents the most important fraction of total cell mitochondria closely juxtaposed with SR, although other mitochondria may contact with RyR2 (Min et al. 2012). Remarkably, resting O_2 consumption, ATP/PCr and $\Delta\Psi m$ are preserved in old hearts, indicating that age-dependent mitochondrial energy deficiency develops only under stressful conditions (ADP-induced maximal O_2 consumption, increased contractile activity) and involves the mitochondrial bioenergetics feedback response (NADPH regeneration, respiratory control rate). This bioenergetic inefficiency cannot be attributed to reduced mitochondrial mass, which seems to remain preserved in aging mouse cardiomyocytes as quantified by specific staining with Mitotracker red, total cardiac mitochondrial yield quantification and citrate synthase activity measurement.

Aging is characterized by augmented entropy, in which random and stochastic episodes not genetically controlled result in biologically defective molecules. Some covalent modifications –i.e. oxidation, glycosilation, phosphorylation, conformational changes and genomic mutations- eventually exceed cell repair capability (Decuypere et al. 2011). An insidious increase in the oxidation status has been consistently observed during senescence (Boengler, Schulz, and Heusch 2009; North and Sinclair 2012). Our data confirm that mitochondrial respiratory complexes are over-oxidized in the aged

cardiomyocytes. This post-translational damage can reflect excessive ROS generation, decreased antioxidant capacity or reduced degradation of oxidized proteins. Recent evidences have suggested the defective mitochondrial calcium uptake is one of the mechanisms involved in impaired antioxidant regeneration in failing cardiomyocytes (Kohlhaas and Maack 2010; Liu and O'Rourke 2009).

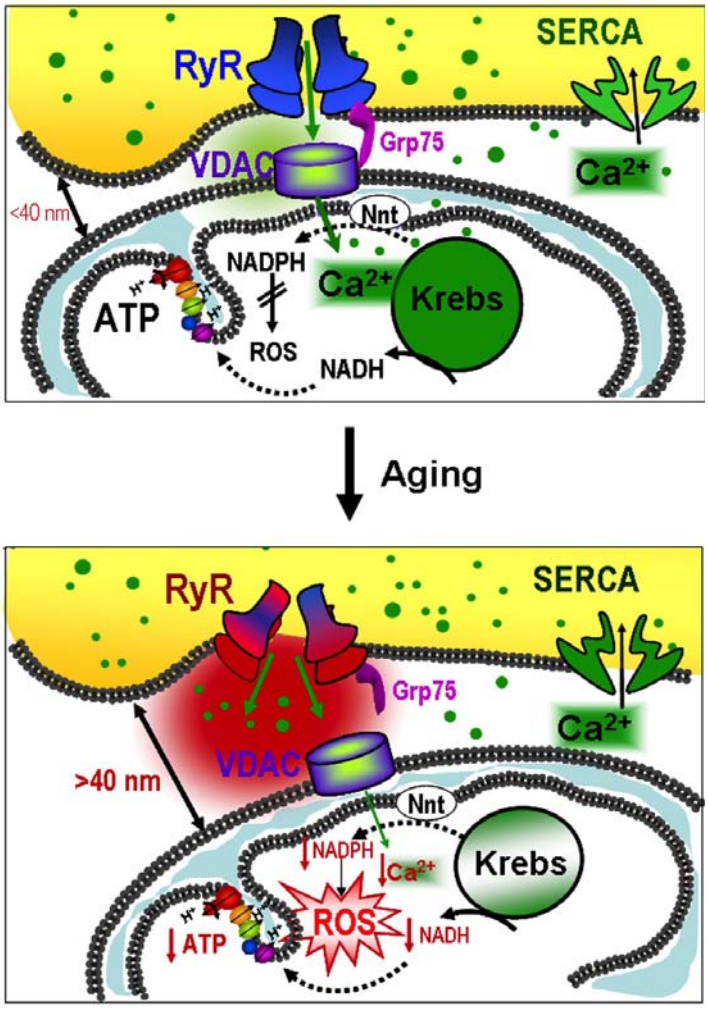


Figure 3.25: Schematic representation of the proposed mechanism by which aging induces SR-mitochondria disruption in cardiomyocytes.

In conclusion, we propose a pathophysiological mechanism summarized in figure 3.25, in which mitochondrial ROS derived from oxidative phosphorylation locally impacts on different protein targets, including electron transport chain and bridging proteins of the interorganelle space. Oxidative damage does not have significant consequences on mitochondrial energy production in old hearts under resting conditions but eventually disrupts the intimate connexion between mitochondria and SR. The resulting alteration in RyR gating properties and SR calcium handling may, however, amplify cell damage in a positive feedback, as deficient calcium transfer from RyR to adjacent mitochondria further increases oxidative damage (impairing NAD(P)H regeneration) and ultimately leads to inefficient energy production.

CHAPTER 4

Altered FoF1 ATP synthase and susceptibility to mitochondrial permeability transition pore during ischemia and reperfusion in aging cardiomyocytes

Abstract - *Aging is a major determinant of the incidence and severity of ischemic heart disease. Preclinical information suggests the existence of intrinsic cellular alterations that contribute to ischemic susceptibility in senescent myocardium, by mechanisms not well established. We investigated the role of altered mitochondrial function in the adverse effect of aging. Isolated perfused hearts from old mice (>20mo) displayed increased ischemia-reperfusion injury as compared to hearts from adult mice (6mo) despite delayed onset of ischemic rigor contracture. In cardiomyocytes from aging hearts there was a more rapid decline of mitochondrial membrane potential (ΔΨm) as compared to young ones, but ischemic rigor shortening was also delayed. Transient recovery of ΔΨm observed during ischemia, secondary to the reversal of mitochondrial FoF1 ATP synthase to ATPase mode, was markedly reduced in aging cardiomyocytes. Proteomic analysis demonstrated increased oxidation of different subunits of ATP synthase. Altered bionergetics in aging cells was associated with reduced mitochondrial calcium uptake and more severe cytosolic calcium overload during ischemia-reperfusion. Despite attenuated ROS burst and mitochondrial calcium overload, mitochondrial permeability transition pore (mPTP) opening and cell death was increased in reperfused aged cells. In vitro studies demonstrated a significantly reduced calcium retention capacity in interfibrillar mitochondria from aging hearts. Our results identify altered FoF1 ATP synthase and increased sensitivity of mitochondria to undergo mPTP opening as important determinants of the reduced tolerance to ischemia-reperfusion in aging hearts. Because ATP synthase has been proposed to conform mPTP, it is tempting to hypothesize that oxidation of ATP synthase underlie both phenomena.*

Adapted from Fernandez-Sanz C, Ruiz-Meana M, Castellano J, Miro-Casas E, Nuñez E, Inserte I, Vázquez J, Garcia-Dorado D. Altered FoF1 ATP synthase and susceptibility to mitochondrial permeability transition pore during ischemia and reperfusion in aging cardiomyocytes. Thromb. Haemost. 2015 Mar;113(3):441-51.

4.1 Introduction

Aging of human population has substantially increased the global burden of ischemic heart disease in the last years (Moran et al. 2014). Advanced age has been described as a major and independent risk factor of coronary syndromes (Lloyd-Jones et al. 2010; Moran et al. 2014) having profound effects on their epidemiology, as the proportion of elderly persons is unprecedentedly expanding worldwide (Burch et al. 2014). Not only is there a progressive increase in the incidence of myocardial infarction with aging, but also an exacerbation of the clinical manifestations and higher mortality rates (Lloyd-Jones et al. 2010; Shih et al. 2010). Although this can be partially explained by a compromised functional reserve in older patients, increased burden of comorbidities or more frequent extra-cardiac complications (Ekerstad et al. 2011), preclinical studies in different models and current clinical data are consistent with the notion that susceptibility to ischemia-reperfusion is increased in the old heart because of a constitutive impairment of cellular capacity to tolerate and adapt to ischemic stress (Willems et al. 2005; Strait and Lakatta 2012).

A considerable number of evidences implicate mitochondria in functional and structural damage secondary to both ischemic injury and senescence (Balaban, Nemoto, and Finkel 2005; Lesnefsky and Hoppel 2006; Q. Chen et al. 2008). One of the postulated mechanisms linking both phenomena is the excess of mitochondrial-derived ROS production and concomitant oxidative damage of some key molecules, specifically those involved in mitochondrial bioenergetic activity (Sadek et al. 2003; Lesnefsky et al. 2006). Aging is associated with electron transport chain defects in myocardial tissue, mainly in interfibrillar mitochondria (Fannin et al. 1999; Lesnefsky et al. 2001), and it is well known that mitochondria become a source of pathological ROS production after an ischemic insult (Sadek et al. 2003; Paradies et al. 2004). To date, it is not clear whether these changes are cause or consequence of other cellular functional perturbations.

Age-dependent oxidative changes may have adverse consequences on calcium handling and bioenergetics (Balaban, Nemoto, and Finkel 2005; Cooper et al. 2013). In the present study we analyzed cytosolic calcium handling and mitochondrial function in cardiomyocytes from aged mouse hearts in which tolerance to ischemia-reperfusion is reduced. The information on the mechanisms responsible for the reduced tolerance to ischemia of senescent myocardium could be essential for devising interventions to limit infarct size in older patients and the deleterious consequences it has on their prognosis.

4.2 Methods

Mitochondria, cardiomyocytes and hearts were obtained from young (4-6 months) and old (>20 months) C57BL/6 mice. Animal handling was approved by the Ethical Committee of the Vall d'Hebron Research Institute and experiments were performed in accordance with the European Union legislation (EU directive 2010/63EU) and Recommendation 2007/526/EC, regarding the protection of animals used for scientific purposes.

4.2.1 Ischemia-reperfusion in perfused hearts

After sodium pentobarbital overdose (150mg/Kg i.p.), mouse hearts were quickly excised and perfused through the aorta in a Langendorff apparatus with a modified Krebs–Henseleit bicarbonate buffer (in mmol/L: 140NaCl, 24$NaHCO_3$, 2.7KCl, 0.4KH_2PO_4, 1$MgSO_4$, 1.8$CaCl_2$and 11glucose, 95% O_2–5% CO_2 at 37ºC) at constant flow perfusion pressure of 80mmHg. Left ventricular (LV) pressure was monitored with a water-filled latex balloon inserted into the LV and inflated to obtain an end-diastolic pressure (LVEDP) of 6-8mmHg. LV developed pressure (LVdevP) was calculated as the difference between LV systolic pressure and LVEDP. After 30 min of normoxic perfusion, mouse hearts were subjected to 60 min of normothermic global ischemia followed by 60 min of reperfusion.

Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in samples collected from the coronary effluent at different times throughout the perfusion period and was considered an indirect measure of necrosis. After 60 min of reperfusion, heart slices were incubated in 1% triphenyltetrazolium chloride to outline the area of necrosis. Hearts submitted to normoxic perfusion during 120 min served as time control series.

4.2.2 Freshly isolated cardiomyocytes

Ischemia-reperfusion in isolated cardiomyocytes

Freshly isolated cardiomyocytes were obtained from mouse hearts by retrograde collagenase perfusion (Ruiz-Meana et al. 2006). Rhod-shaped calcium tolerant cardiomyocytes were selected by differential centrifugation and albumin gradient and were plated on laminin-coated glass bottom coverslips (for confocal studies) or on 4% fetal calf serum pretreated multiwell dishes. To simulate ischemia-reperfusion in cardiomyocytes, two independent approaches were used: anoxic workstation under con-

trolled atmosphere of 0% O_2-5% H_2 at 37ºC (Invivo$_2$ Workstation Ruskin, UK) and microscope-adapted microperfusion closed chamber (RC-43C/BS4 64-0371 Harvard Apparatus). For the anoxic workstation, cardiomyocytes were plated in 96-wells dishes, placed within the anoxic atmosphere and incubated for 15 min with glucose-free acidic ischemic buffer (previously deoxygenated in an autoclave and bubbled with N_2 for 20 min) containing (in mmol/L): 140 NaCl, 3.6 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 20 HEPES, pH 6.4, and supplemented with 4µmol/L resazurin, 100µmol/L ascorbic acid, 0.5mmol/L dithionite and 100U/ml superoxide dismutase.
For reperfusion, ischemic buffer was washed out and oxygenated glucose-containing control buffer (pH 7.4) was added for 10min.

To simulate ischemia in the microperfusion closed chamber (fig. 4.1), laminin-attached cardiomyocytes placed on the stage of an inverted microscope were superfused, with the aid of a peristaltic pump, within a closed microperfusion closed chamber with ischemic buffer (see above) at pH 6.4, continuously bubbled with N_2 for 15min. Reoxygenation was induced by switching to oxygenated, glucose-containing control superfusion, at pH 7.4.

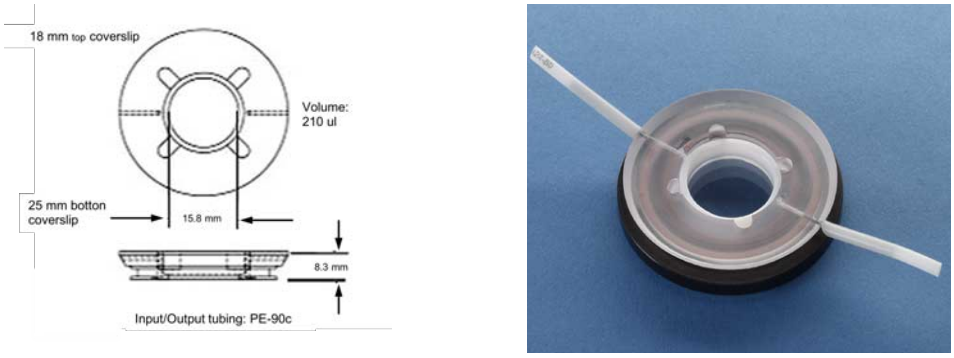


Figure 4.1: Schematic representation and image of the microperfusion closed chamber RC-43C, BS4 64-0371 (Harvard Apparatus).

Rigor development, hypercontracture and cell death during ischemia-reperfusion

To analyse the effect of ischemia-reperfusion injury on cell morphology (fig 4.2), the rate of ischemic rigor shortening development (defined as 25-40% reduction of cell length with preserved squared-shape morphology) (fig. 4.2 B) and of reperfusion-induced hypercontracture (defined as >70% reduction of cell length with concomitant disruption of cytoarchitecture) (fig, 4.2 C) was quantified.

Reperfusion-induced sarcolemmal disruption was analysed by two independent methods: a) visual quantification of 0.04% trypan blue positive cells (BX41 Olympus)

and b) spectrophotometrical analysis of LDH release (photometer Multiskan FC, Thermo Scientific™) expressed respect to total LDH induced by massive osmotic shock.

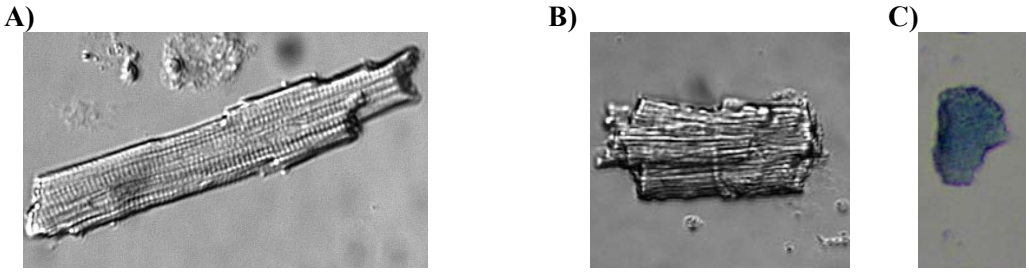


Figure 4.2: A) Freshly isolated Rhod-sahped cardiomyocytes under normoxic conditions. **B)** Example of rigor contraction (25-40% reduction of cell length with preserved squared-shape morphology) developed during simulated ischemia (0% O_2-5% H_2 at 37ºC. Invivo$_2$ Workstation Ruskin, UK). **C)** Example of hypercontraction (>70% reduction of cell length with concomitant disruption of cytoarchitecture) and sarcolemmal rupture (trypan blue positive)developed during reperfusion.

Cytosolic and mitochondrial calcium changes in ischemia-reperfusion

The effect of aging on cytosolic calcium handling during ischemia and reperfusion was investigated in isolated cardiomyocyte sincubated with 5µmol/L of the acetoxymethyl ester of the calcium-sensitive fluorochrome fluo-4 (Molecular Probes) for 30 min at 37ºC, washed and subsequently submitted to transient ischemia-reperfusion on the stage of the microscope. During ischemia-reperfusion, cells were excited at 488nm with an Ar/Kr laser confocal system (Yokogawa CSU10, Nipkow spinning disk) set on an Olympus IX70 (VoxCell Scan, Visitech, UK) at 60X oil immersion objective lens, and changes in 505nm emission were captured with a CCD digital camera (ORCA Hamamatsu, Japan) and monitored throughout time in cytosolic regions of interests previously defined within the cells. Cell fluorescence was analysed in background-subtracted images using commercially available software (VoxCell Scan, Visitech, UK). To analyze the changes in mitochondrial calcium, a cold-warm protocol was used to reduce cytosolic compartmentalization of the dye. Briefly, isolated cardiomyocytes were incubated with 5µmol/L of the acetoxymethyl ester of the mitochondrial calcium-sensitive fluorochrome rhod-2 (Molecular Probes) for 60 min at 4ºC (this step is required to inhibit AM cleavage from cytosolic unspecific esterases and to improve mitochondrial uptake and retention of the fluorochrome), washed and post-incubated for additional 30 min at 37º (Trollinger, Cascio, and Lemasters 1997). Loaded cells were submitted to transient 15 min ischemia-10 min reperfusion while being excited at 560nm. Changes in 580nm emission were monitored throughout time in

mitochondrial regions previously defined within the cells. In all experiments, perfusion was initiated with 2 min normoxic buffer to obtain baseline fluorescence. Changes in cytosolic and mitochondrial calcium were expressed as arbitrary units of fluorescence (a.u.) respect to the initial normoxic value.

Mitochondrial membrane potential (ΔΨm) during ischemia-reperfusion:

For the analysis of ΔΨm, isolated cardiomyocytes from young and old mouse hearts were incubated with 10 μmol/L of the membrane-permeant form of JC-1 (Molecular Probes) for 8 min at 37ºC, washed and subsequently submitted to transient ischemia-reperfusion on the stage of the microscope. During ischemia-reperfusion period, cells were excited at 488nm using an Ar/Kr laser confocal system, and simultaneous changes in 525nm and 590nm emission wavelengths were monitored throughout time. Capture speed was set at 1 image/30s to avoid phototoxicity. The fluorescence ratio obtained from both emissions was normalized respect to the baseline normoxic value.

Mitochondrial permeability transition pore and ROS production during reperfusion:

To investigate the effect of age on the occurrence of mitochondrial permeability transition pore (mPTP) opening and ROS production during ischemia-reperfusion, isolated cardiomyocytes were simultaneously incubated with 5 μmol/L of the acetoxymethyl ester of calcein (Molecular Probes), 15 min at 37ºC, and MitoSoxTM (Molecular Probes), 10 min at 37ºC, washed and post-incubated with control buffer supplemented with 1mmol/L $CoCl_2$ for additional 10min, to quench cytosolic fluorescence (18). Cells were subsequently submitted to ischemia-reperfusion in a microchamber placed on the stage of the microscope and were alternatively excited at 488nm (calcein) and 561nm (MitoSox) with the Ar/Kr laser confocal system. In all perfusion buffers, 0.5 mmol/L $CoCl_2$ was present to prevent cytosolic fluorescence contribution to mitochondrial fluorescence quantification. Decline in calcein fluorescence was indicative of mPTP opening, whereas increase in MitoSox fluorescence corresponded to mitochondrial ROS production.

4.2.3 Isolation of subsarcolemmal and interfibrillar heart mitochondria.

To obtain SSM and IFM mitochondria, fresh cardiac ventricles were minced and homogenized in iced cold buffer A (in mmol/L: 290 sucrose, 5 MOPS at pH 7.4, 2

EGTA and 0.2% defatted albumin) using a Potter-Elvehjem device. Nuclei and other cell debris were pelleted in an initial centrifugation step at 750xg (5 min, 4ºC). The supernatant was centrifuged at 5000xg (5 min, 4ºC) and SSM were precipitated in the resulting pellet. IFM were obtained by treating the initial 750g pellet with 1ml isolation buffer B (in mmol/L: 100 KCl, 5 MOPS at pH 7.4, 2 EGTA and 0.2% defatted albumin) and 3mg/ml proteinase K for 1min. Interfibrillar homogenate was centrifuged at 750xg (5min, 4ºC). After discarding the pellet, supernatant was centrifuged at 5000xg (5min, 4ºC) to precipitate the IFM. Mitochondrial protein concentration and citrate synthase activity were measured by colorimetric assays.

Mitochondrial calcium retention capacity:

To investigate the effect of aging on mitochondrial calcium tolerance, a concentration of 0.2-0.4mg/ml of SSM or IFM from young and old mouse hearts was suspended in the assay buffer (in mmol/L: 200 sucrose, 6 MOPS, 5 KH_2PO_4, 1 $MgCl_2$, 10 succinate, 0.001 rotenone) and plated in a microplate fluorometer (Max GeminiXS, Molecular Devices) in the presence of 2µmol/L of the hexapotassium salt impermeant calcium sensitive dye calcium-Green™ 5N. Consecutive pulses of 4µmol/L calcium were added to the mitochondrial suspension, and mitochondrial calcium uptake was detected as decay of fluorescence emission at 532nm (Ex: 506nm), recorded every 15s during 2min for each calcium pulse. Calcium was added repeatedly until total mitochondrial calcium retention capacity was exceeded, a phenomenon that could be detected by the increase in buffer fluorescence reflecting the occurrence of mPTP. In parallel experiments, mitochondrial calcium retention capacity was assessed in the presence of either 0.2mmol/L cyclosporin (CsA, yo inhibit mPTP) or 10 µmol/L Ru360 (to inhibit mitochondrial calcium uniporter).

Differential quantitative mitochondrial proteomics:

Peptides of mitochondrial FoF1 ATP synthase complex were identified and quantified by differential high-throughput proteomic analysis performed by stable isotopic labelling in SSM and IFM isolated from young and old mouse hearts, using a previously described protocol (Bonzon-Kulichenko et al. 2011).

Changes in the abundance and composition of cysteine-thiol redox status of FoF1 ATP synthase peptides were quantified using de GELSILOX methodology (Martinez-Acedo et al. 2012) (fig. 4.3). Analyses of samples by LC-MS/MS were performed as previously described (Bonzon-Kulichenko et al. 2011). Functional protein classification was done using the Gene Ontology database. Proteomics results were analyzed as previously described (Jorge et al. 2009).

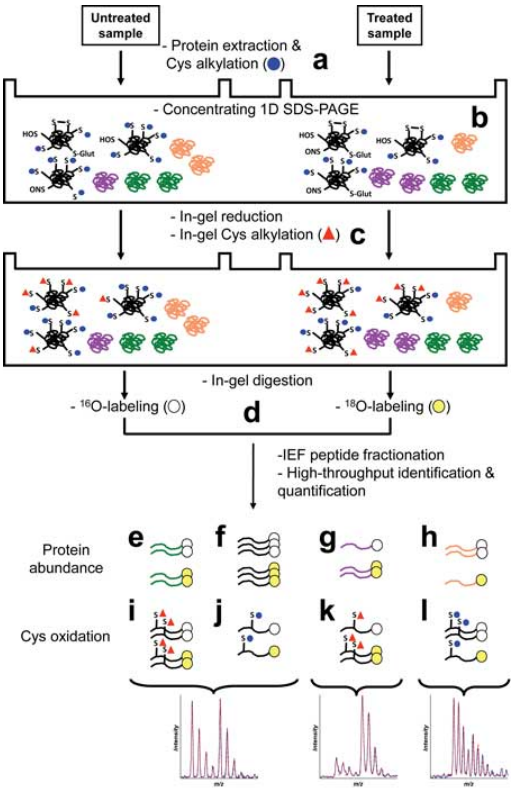


Figure 4.3: Schematic representation of the Gelsilox technique. The sample is treated with an alkilalating regeant (**a**). Afterwards the sample in run on a concentratrting 1D gel (**b**) followed by a reducing and diferentialy alkilating second electrophoresis (**c**). Finally, the gell is digested and samples from each group are differentially labelled with ^{16}O or ^{18}O (**d**).

4.2.4 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). For comparisons between 2 groups with normal distributions, 2-tailed Student's t-test for independent or paired samples was used. For comparisons between more than two groups with normal distribution, one-way ANOVA and planned contrasts was used. Differences in temporal evolution were assessed by repeated-measures factorial ANOVA. When samples did not follow a normal distribution, the non-parametric Mann-Whitney U or Kruskal-Wallis tests were used for each design as needed. Differences of $p<0.05$ were considered statistically significant. In experiments involving isolated cardiomyocytes, cells were derived from $n\geq 3$ independent isolation procedures. All statistical analyses were performed with SPSS v.15 software.

4.3 Results

4.3.1 Ischemia and reperfusion injury in aging hearts

In isolated Langendorff-perfused mouse hearts, there were no differences in LV function between groups at baseline. Previous morphometric analysis of the hearts of these mice by echocardiography showed no age-related hypertrophy, but a trend towards increased LV end-diastolic volume in old mice (Fernandez-Sanz et al. 2014).

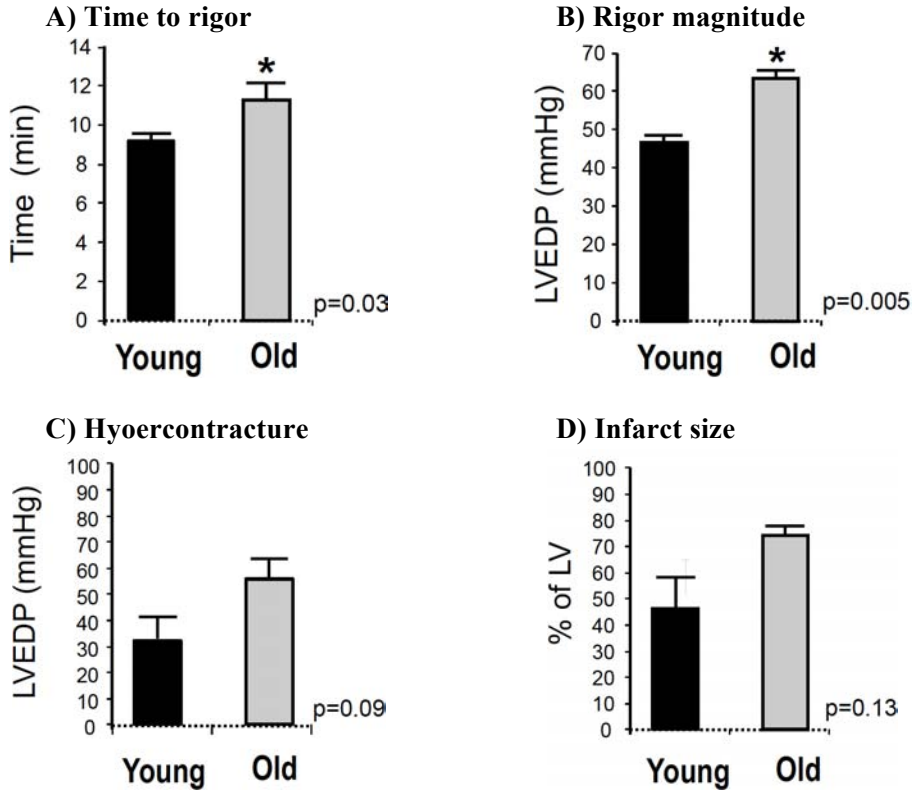


Figure 4.4: Ischemia and reperfusion in isolated perfused hearts. A) Time to the onset of ischemic rigor contracture and magnitude of rigor contracture, as manifested by peak left ventricular end-diastolic pressure (LVEDP) during ischemia in Langendorff-perfused hearts from young and old mice. **B)** Reperfusion-induced hypercontracture, as manifested by peak LVEDP during reperfusion, and infarct size, expressed as % of the left ventricle, in Langendorff-perfused hearts from young and old mice. Data correspond to mean ± SEM of 3-5 hearts per group.

Ischemia resulted in a complete cessation of contraction and a progressive increase in LVEDP reflecting development of ischemic rigor. Hearts from old mice

showed a significant delay in the time to ischemic rigor (fig. 4.4 A) and an increase in its magnitude (fig. 4.4 B). During reperfusion, there was a trend towards age-related increased hypercontracture, defined as maximal rise in LVEDP and infarct size (fig. 4.4 B-C).

4.3.2 Ischemia and reperfusion injury in isolated aging cardiomyocytes

After 15min of ischemia, rigor cell shortening was more pronounced with aging (fig. 4.5 A). While young cardiomyocytes shortened around 25% respect to their initial length, cardiomyocytes from aging hearts shortened more than 40% (fig. 4.5 A insert). Paradoxically, the time at which rigor contracture developed –an accepted marker of severe ATP depletion- was significantly delayed with aging (fig. 4.5 B). During reperfusion, cardiomyocytes experienced an additional cell shortening coincident with energy restoration, usually accompanied by massive morphological distortion (hypercontracture). The mean length of reperfused cardiomyocytes was significantly shorter in the aging group (fig. 4.5 A).

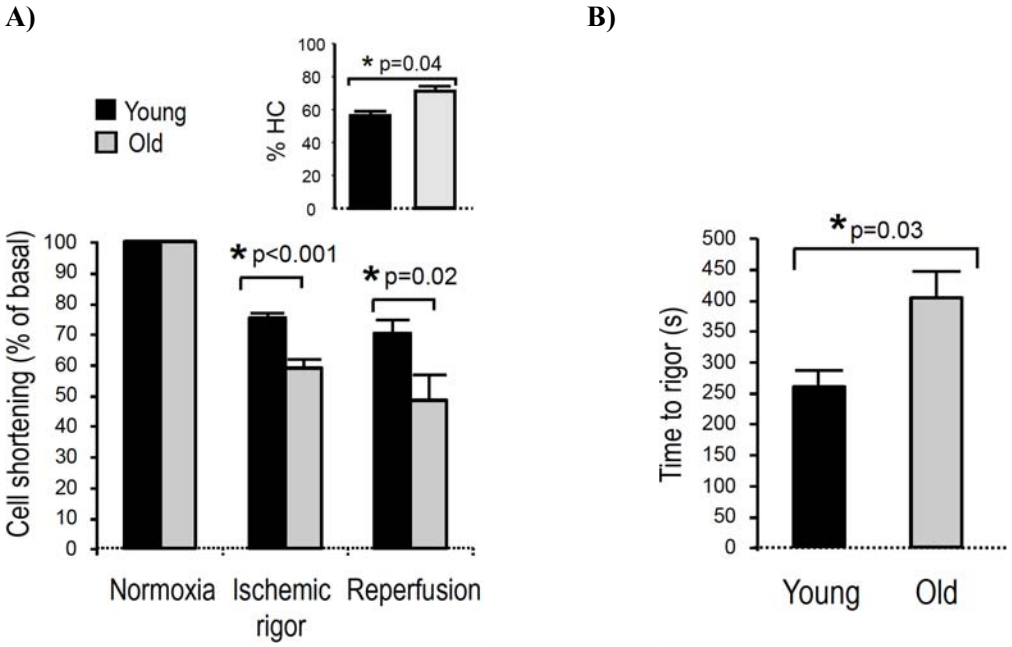


Figure 4.5: Effect of aging on ischemia-reperfusion injury in isolated cardiomyocytes. A) Morphological changes associated with ischemia and reperfusion. B) Time to rigor contracture in young and old cardiomyocytes. Mean ± SEM of n>10 cells per group.

4.3.3 Reperfusion-induced sarcolemmal disruption

During the first minutes of reperfusion, a proportion of isolated cardiomyocytes developed sarcolemmal rupture and death, quantified by the release of LDH to the extracellular medium. Reperfusion-induced cell death was significantly increased in cardiomyocytes from aging hearts (fig 4.6 A). The rate of sarcolemmal rupture associated to hypercontracture, as detected by trypan blue positive staining, was significantly increased in cardiomyocytes from aging hearts (fig.4.6 B).

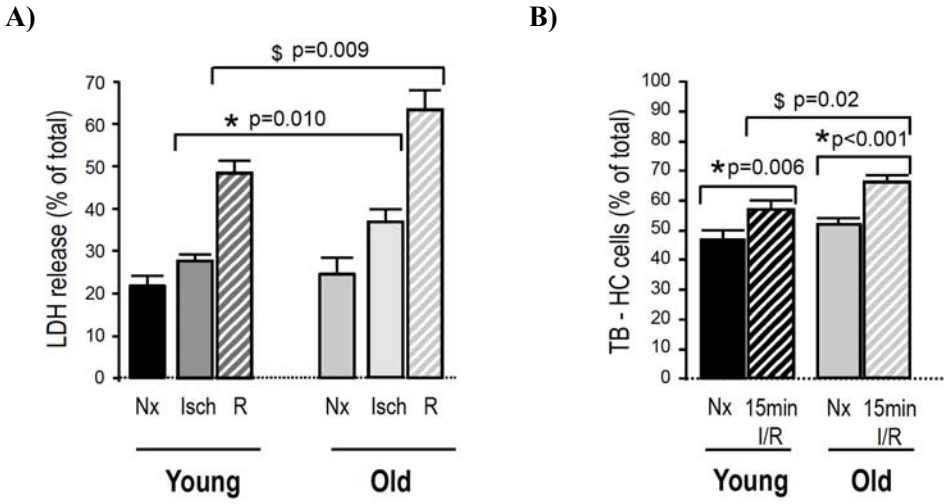


Figure 4.6: Effect of aging on ischemia-reperfusion injury in isolated cardiomyocytes. A) Cell death during ischemia and reperfusion, determined by LDH release respect to total cell LDH content. **B)** Percentage of hypercontracted cells during reperfusion that develop membrane disruption, as determined by trypan blue (TB) positive staining in young and old cardiomyocytes. Mean ± SEM of 3 replicates per group. Isch: ischemia; R: reperfusion; I/R: ischemia and reperfusion.

4.3.4 Cytosolic and mitochondrial calcium handling is impaired in aging cardiomyocytes during ischemia-reperfusion

Fluo-4 loaded isolated cardiomyocytes submitted to transient ischemia experienced an increase in cytosolic calcium concentration (fig. 4.7 A). During the first minutes of reperfusion, cytosolic calcium overload was partially corrected in surviving cells from both groups of ages. The number of cells presenting sarcolemmal rupture at reperfusion was higher in the aging group (fig. 4.7 B) and cytosolic calcium immediately before reperfusion-induced membrane rupture was significantly higher in aging cells (fig. 4.7 B).

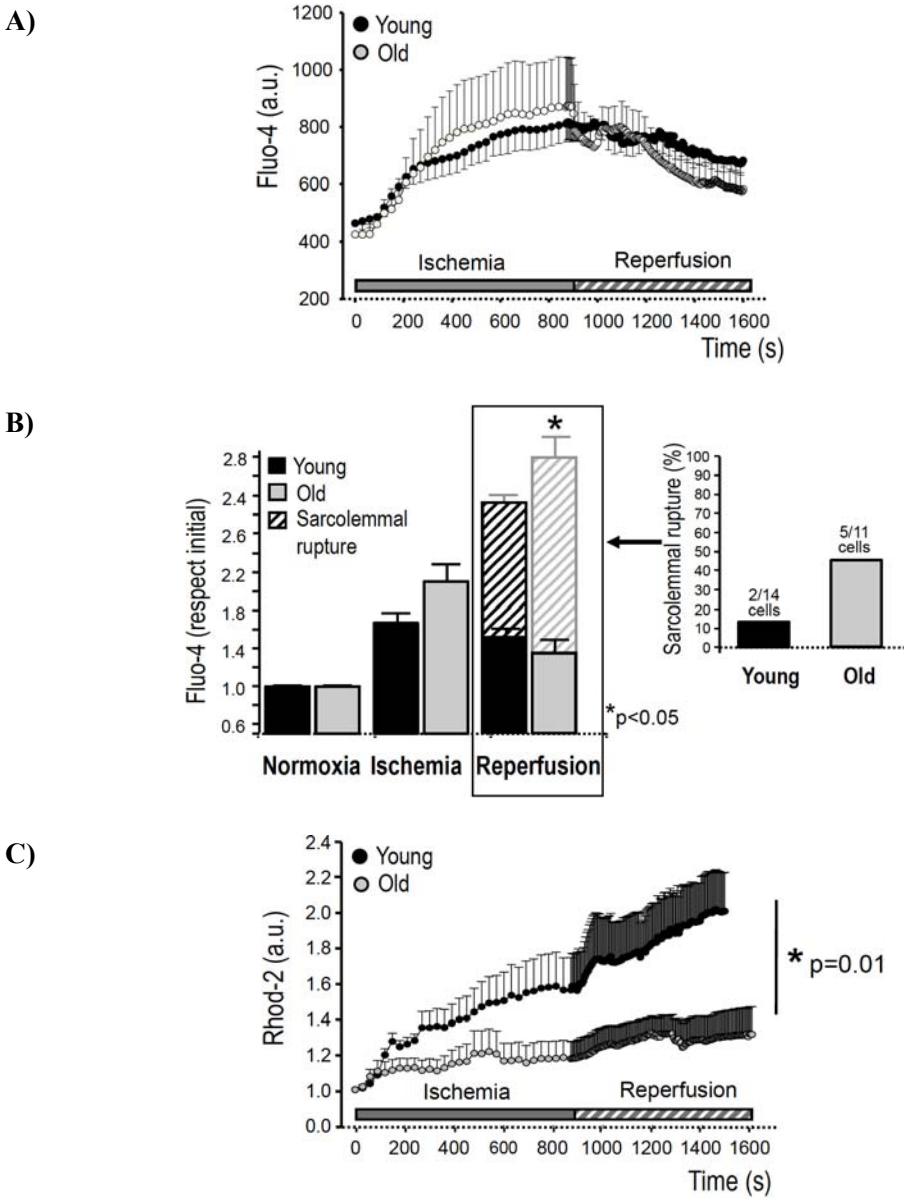
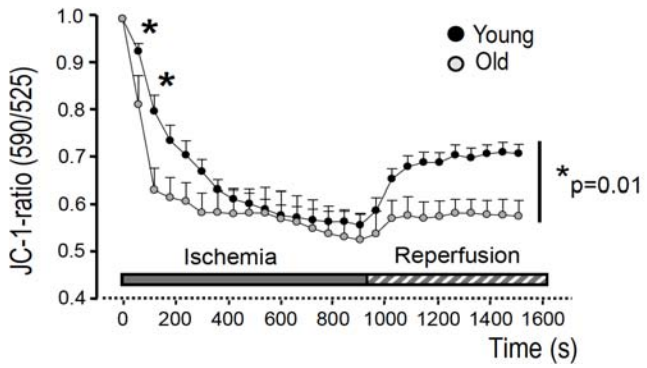


Figure 4.7: Cytosolic and mitochondrial calcium handling during ischemia-reperfusion in isolated cardiomyocytes. A) Cytosolic calcium kinetics throughout ischemia and reperfusion in cardiomyocytes from young and old mouse hearts. Data are expressed as mean ± SEM of n=6-12 cells per group. **B)** Final degree of cytosolic calcium overload, respect to the initial normoxic value, obtained the end of ischemic period and after 10min of reperfusion. Faded bars correspond to cytosolic calcium level immediately before sarcolemmal rupture. Inset shows the percentage of cells experiencing reperfusion-induced sarcolemmal rupture in this series. Data are expressed as mean ± SEM of n=11-14 cells per group. **C)** Mitochondrial calcium kinetics throughout ischemia and reperfusion in cardiomyocytes from young and old mouse hearts. Data are expressed as mean ± SEM of n= 6-11 cells per group. a.u.: arbitrary units.

The kinetics of mitochondrial calcium uptake in rhod-2 loaded cardiomyocytes subjected to ischemia-reperfusion disclosed an impaired ability of mitochondria from old hearts to uptake and accumulate calcium both during ischemia and reperfusion (fig. 4.7 C). The fact that mitochondrial calcium is reduced in the presence of higher cytosolic calcium concentration in aging cells denotes that there is no cross-interference between the two markers.

A)



B)

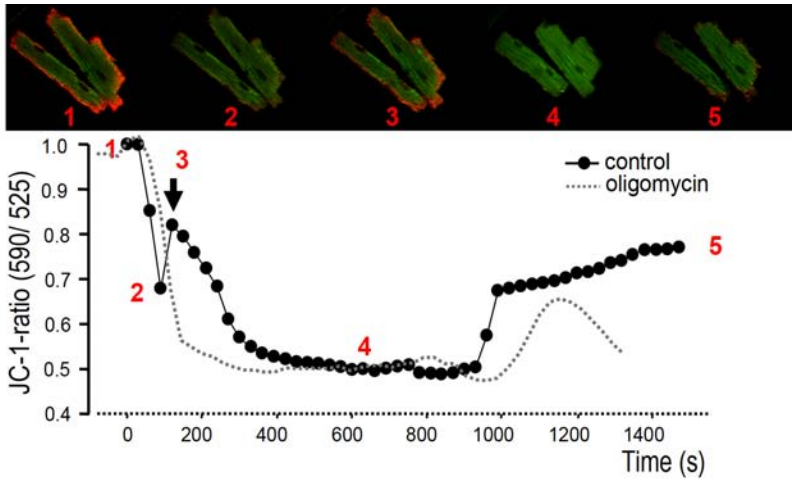


Figure 4.8: ΔΨm, FoF1 ATP synthase reversal and cysteine oxidation. A) Changes in ΔΨm during ischemia and reperfusion respect to the initial normoxic value, quantified by JC-1 ratiofluorescence, in isolated cardiomyocytes from young and old mouse hearts. Mean ± SEM of n=6-8 cells per group. **B)** Confocal imaging sequence of a JC-1 loaded pair of young cardiomyocytes and the corresponding fluorescence monitorization throughout ischemia and reperfusion, to illustrate the initial decline of ΔΨm during the first minutes of ischemia (images 1 and 2), the transient repolarization wave (image 3, arrow), the end-ischemic degree of mitochondrial depolarization (image 4) and the partial recovery of ΔΨm during the first minutes of reperfusion (image 5). Grey dotted line corresponds to ΔΨm in an oligomycin-treated cell, in which transient ΔΨm repolarization during ischemia is prevented.

4.3.5 Aging is associated with altered ΔΨm during ischemia and reperfusion

During ischemia, isolated cardiomyocytes developed a progressive decline of ΔΨm, quantified by JC-1 ratiofluorescence. Mitochondrial membrane depolarization was accelerated in old cardiomyocytes (fig. 4.8 A). Upon reperfusion, there was a rapid although incomplete recovery of ΔΨm in young cardiomyocytes, which was significantly depressed in the aged cells (fig. 4.8 A). Immediately before maximal ischemic depolarization, a rapid and transient recovery of ΔΨm took place in 80% of the control young cells (fig. 4.8 B). This repolarization wave corresponded to the reversal of FoF1 ATP synthase because it could be prevented by the addition of 10µmol/L of the specific ATP synthase inhibitor oligomycin (fig. 4.9 A). By contrast, and despite faster mitochondrial depolarization, only 30% of aging cardiomyocytes experienced a reversal of ATP synthase (fig. 4.9 A, insert). The delay in rigor contracture development observed in aging cells could be reproduced with oligomycin, suggesting that it is secondary to the inability of the ATP synthase to revert to its ATPase mode. Second generation proteomics combined with GELSILOX analysis demonstrated an increase in the number of oxidised Cys residues in the mitochondrial ATP synthase of the aging hearts (fig. 4.9 B), without changes in its expression levels (data not shown).

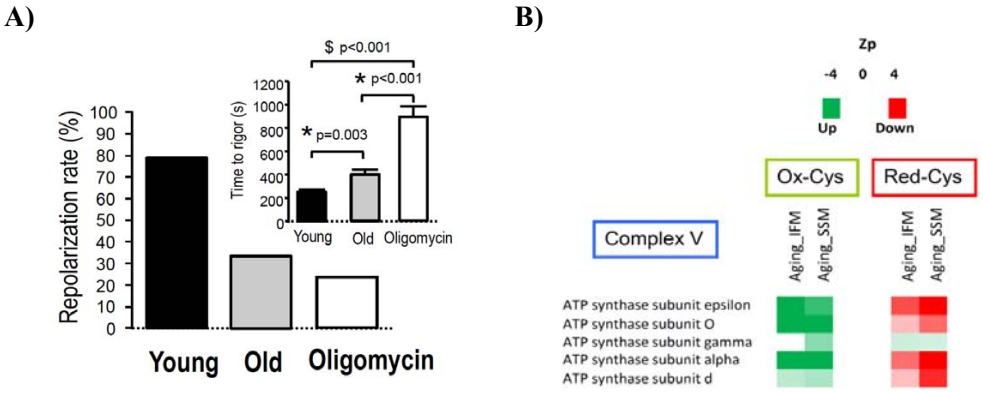


Figure 4.9: FoF1 ATP synthase repolarization and cysteine oxidation. A) Percentage of cells experiencing a transient repolarization wave during ischemia among cardiomyocytes from young and old mouse hearts, and cardiomyocytes from young mouse hearts treated with 10µmol/L of oligomycin to inhibit mitochondrial ATPase. The inset shows the time at which rigor contracture developed in the same groups of cells**. B)** Quantitative redox proteomics using GELSILOX of the cysteine containing peptides detected in mitochondrial FoF1 ATP synthase (complex V) in subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) from aging hearts. Increase in the peptide-containing oxidised cysteine is shown in green and correlates with decrease in the peptide-containing reduced cysteine (in red) in different subunits of FoF1 ATP synthase.

4.3.6 Effect of age on mPTP and ROS production during reperfusion

Reperfusion triggered mitochondrial calcein release, reflecting the occurrence of mPTP opening, in cardiomyocytes from both groups of ages (fig. 4.10 A). The extent of mitochondrial permeabilization was greater in aged cardiomyocytes (fig. 4.10 A). Concomitantly, there was a narrow and high peak of mitochondrial ROS production, detected by MitoSox fluorescence, during the first minutes of reperfusion (fig. 4.10 B).

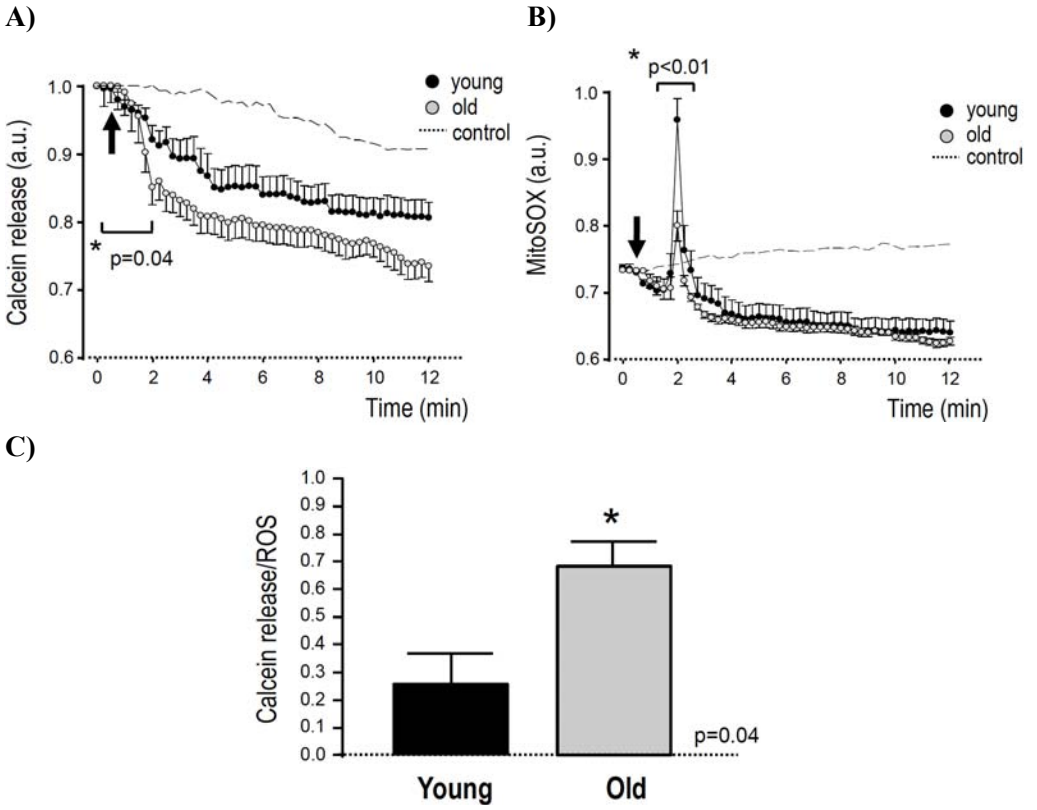


Figure 4.10: mPTP opening and mitochondrial ROS production during reperfusion. A) Mitochondrial calcein fluorescence decay, reflecting mPTP opening, during reperfusion in isolated cardiomyocytes from young and old mouse hearts. Arrow points the onset of reperfusion. Control cells were not submitted to previous ischemia. **B)** Reperfusion-induced mitochondrial ROS production in isolated cardiomyocytes from both groups of age. Arrow points the onset of reperfusion. Control cells were not submitted to previous ischemia. **C)** Ratio between mitochondrial calcein release and mitochondrial ROS production in the first minutes of reperfusion in isolated cardiomyocytes from both groups of age, as an index of the susceptibility of mitochondria to undergo permeabilization in response to ROS. Data correspond to mean ± SEM of n=7-12 cells per group.

This peak of ROS appeared to be associated with reactivation of mitochondrial respiratory activity, as it overlapped with restoration of ΔΨm and was significantly attenuated in aged cells (fig. 4.10 B). The proportion of mitochondrial calcein released in relation to maximal ROS production during the first minutes of reperfusion, an index of the mPTP sensitivity to ROS, was significantly increased in cardiomyocytes from aged hearts (fig. 4.10 C). These data indicate a reduced threshold of mitochondria from aged cells to undergo membrane permeabilization.

4.3.7 Aging reduces mitochondrial tolerance to calcium overload

Isolated mitochondria exposed *in vitro* to consecutive external calcium pulses eventually developed CsA-sensitive mPTP when the threshold of their calcium retention capacity was exceeded, as determined by extramitochondrial calcium by CG5N fluorescence (fig. 4.11 A). Mitochondrial calcium uptake was prevented by Ru-360, indicating that it occurred through the mitochondrial calcium uniporter (fig. 4.11 A). Subsarcolemmal mitochondria (SSM) showed a less calcium retention capacity than interfibrillar mitochondria (IFM) regardless of age (fig. 4.11 B). However, aging importantly impaired calcium tolerance in IFM population (fig. 4.11 B).

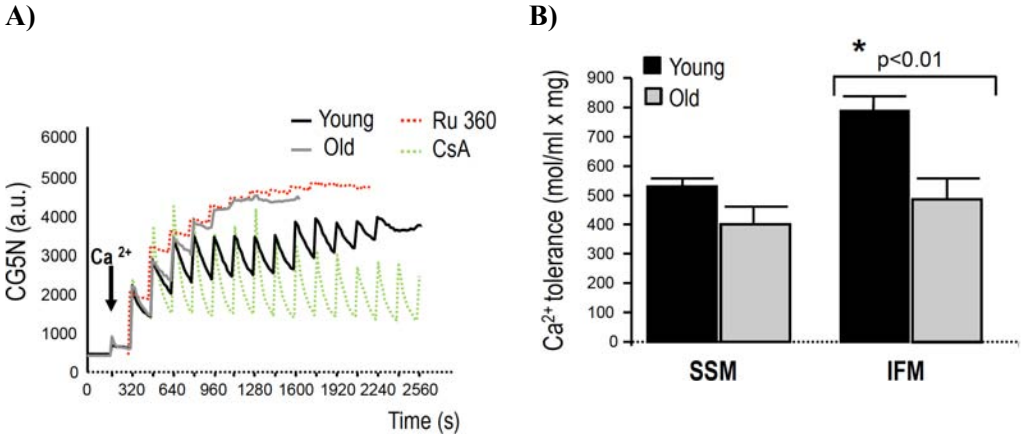


Figure 4.11. Effect of aging on calcium retention capacity in cardiac mitochondria: A) Calcium uptake and accumulation in mitochondria isolated from young and old mouse hearts and submitted to consecutive external calcium pulses of 4µmol/L, quantified by changes in extramitochondrial CG5N fluorescence. In a subset of experiments either 10 µmol/L Ru360 (inhibitor of the mitochondrial calcium uniporter) or 1µmol/L CsA (inhibitor of mPTP) was added during calcium exposure. **B)** Quantification of total calcium tolerance in subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) from both groups of age before membrane permeabilization takes place. Data correspond to mean ± SEM of 5 replicates per group.

4.4 Discussion

The present study provides evidence that altered FoF1 ATP synthase function contributes to the reduced tolerance to ischemia in aging hearts. Altered FoF1 ATP synthase activity is associated with oxidative damage of this protein complex and causes more rapid dissipation of ΔΨm, reduced mitochondrial calcium uptake and delayed ATP exhaustion marked by the delayed occurrence of ischemic rigor contracture. Mitochondria from old cardiomyocytes are more prone to undergo mPTP during reperfusion or during external calcium exposure *in vitro*. Because recent studies suggest that dimmers of ATP synthase form the mPTP, it is tempting to speculate that impaired ATP synthase activity observed in aging hearts contributes to both altered energy metabolism and increased susceptibility to mPTP.

4.4.1 Ischemia and reperfusion in the aging heart: energy exhaustion

During ischemia, cytosolic ATP levels progressively decline because ATP expenditure is not adequately compensated by its regeneration through mitochondrial oxidative phosphorylation. Arrest of oxidative phosphorylation results in mitochondrial depolarization by a dual mechanism: 1) interruption of H^+ extrusion to the intermembrane space, and 2) dissipation of ΔΨm mainly through proton-driven ATP synthase, although other uncoupling proteins may play a role (Sack 2006) . Importantly, when ΔΨm declines beyond certain level, ATP synthase reverses to proton-pumping ATPase, in a futile attempt to maintain mitochondrial intermembrane H^+ gradient (Di Lisa et al. 1995). Reversal of ATP synthase during ischemia converts mitochondria into major cellular ATP consumers (Di Lisa et al. 1995) and accelerates ATP fall to the critical threshold of 50-100 μmol/L, in which rigor-type calcium-independent actin-myosin interaction develops (Allshire et al. 1987). Up to 50% of cellular ATP is consumed by reverse mode of the mitochondrial FoF1 ATPase (Grover et al. 2004). Rigor-type contracture, manifested as an abrupt cell shortening in unrestrained isolated cardiomyocytes and increased resting tension in intact myocardium, is an ATP-consuming response by itself, and may propagate from cell-to-cell through gap junctions(M. Ruiz-Meana et al. 2001).

Aging cardiomyocytes have preserved mitochondrial membrane potential and respiratory activity under resting conditions (Fernandez-Sanz et al. 2014). However, during oxygen deprivation they develop a paradoxical response, consisting of an acceleration of mitochondrial membrane depolarization and a concomitant delay in the development of rigor contracture. This response was consistently observed in isolated

cardiomyocytes and intact hearts submitted to global ischemia, and could be explained by failure of FoF1 ATP synthase to revert to the ATP consuming mode that precipitates rigor shortening during ischemia. The observation that oligomycin –an FoF1 ATPase inhibitor- when present during ischemia prevents the occurrence of the transient repolarization wave in JC-1 loaded cardiomyocytes and delays rigor development, mimicking the effect of aging, supports that this is indeed the case. The failure of ATP synthase to revert its activity may also explain the increased rate of mitochondrial depolarization in aging cardiomyocytes. Although the exact mechanism of this failure cannot be established with the present data, quantitative proteomics revealed increased cysteine oxidation at different subunits of ATP synthase complex in aging hearts, including subunit rotation γ and oligomycin-senstive subunit Fo, without changes in their abundance. Interestingly, the extent of cysteine cross-linking as a result of disulphide bond formation and S-glutathionylation has been shown to have important functional consequences, and to be negatively correlated with the hydrolytic activity of ATP synthase (S.-B. Wang et al. 2013).

4.4.2 Mitochondrial calcium handling during ischemia-reperfusion

The acceleration of mitochondrial membrane depolarization observed in aging cardiomyocytes during ischemia may have important consequences on cellular calcium handling. Mitochondria exhibit a striking ability to accumulate enormous amounts of calcium (Rizzuto, Bernardi, and Pozzan 2000), crucial for buffering cytosolic calcium in conditions in which SR calcium uptake through SERCA ATPase is not thermodynamically favoured, like during ischemia (Tran et al. 2009). Moreover, the pro-oxidative status present during ischemia and reperfusion has been described to promote calcium leak from sarcoplasmic reticulum by ryanodine receptors (Fauconnier et al. 2011; Mazurek, Bovo, and Zima 2014), increasing even more the concentration of calcium at the SR-mitochondria microenvironment. We have previously demonstrated that aged cardiomyocytes have abnormal spontaneous spark behaviour, consistent with altered RyR gating properties, although resting cytosolic calcium, sarcoplasmic reticulum content or mitochondrial calcium uniporter activity is similar to those observed in young cardiomyocytes (Fernandez-Sanz et al. 2014). On the other hand, there is solid evidence indicating that calcium can be significantly overloaded in restricted subcellular areas before significant changes can be detected in the cytosol (Rizzuto and Pozzan 2006) and that mitochondrial calcium uptake is indeed more dependent on the calcium levels reached within these microdomains than on bulk cytosolic calcium concentration (Rizzuto et al. 1998; Rizzuto and Pozzan 2006; Ruiz-Meana, Fernandez-Sanz, and

Garcia-Dorado 2010). Calcium uptake and accumulation by mitochondria requires ΔΨm, a condition that is fully met during the initial phase of ischemia, prior to maximal ΔΨm dissipation (Saotome et al. 2005; Ruiz-Meana et al. 2006). Importantly, the driving ΔΨm force for mitochondrial calcium uptake is not depressed in aging cells under normoxic conditions (Fernandez-Sanz et al. 2014). Mitochondrial calcium uptake has been demonstrated to actively participate in cytosolic calcium control both under normoxia and ischemia, alleviating calcium overload and improving cell survival upon reperfusion (Ruiz-Meana et al. 2006). This may explain why pharmacological o genetic inhibition of mitochondrial calcium uptake confers no protection against mPTP opening or necrosis in reperfusion (Ruiz-Meana et al. 2006; Herzig, Maundrell, and Martinou 2013). It is therefore plausible that depressed mitochondrial calcium uptake secondary to accelerated mitochondrial depolarization during ischemia, and impaired ΔΨm recovery during reperfusion, significantly aggravates cytosolic calcium overload and hypercontracture in aging cells. Defective communication between SR and mitochondria during aging may further contribute to this effect by further impairing mitochondrial calcium uptake (Fernandez-Sanz et al. 2014).

Previous observations indicated an age-related increase in diastolic calcium during ischemia and early reperfusion that may account for the increased sensitivity to injury (Ataka et al. 1992; O'Brien, Ferguson, and Howlett 2008) The mechanisms responsible for impaired calcium handling in aging myocardium are far from being elucidated. It has been proposed that a decline in SR proteins that participate in calcium removal plays a causative role (Cain et al. 1998), although other studies found no changes in the decay of calcium transient in aging, suggesting normal SERCA activity (O'Brien, Ferguson, and Howlett 2008). Some studies describe changes in relaxation times compatible with reduced SERCA participation and compensatory increase in sarcolemmal Na^+/Ca^{2+} exchanger activity(Fowler et al. 2007), while others advocate post-translational modifications, like increased oxidation of SERCA and phospholamban (Babušíková et al. 2012) or changes in the phosphorylation status of SR cycling proteins secondary to reduced CaMKII expression (A. Xu and Narayanan 1998). To our knowledge, this is the first evidence indicating that depressed mitochondrial calcium uptake during ischemia and reperfusion can be causally involved in the aggravation of cytosolic calcium overload of senescent cardiomyocytes.

4.4.3 Mitochondrial ATP synthase oxidation and mPTP opening

Our results demonstrate an enhanced susceptibility to mPTP opening in mitochondria from aging hearts. This observation was obtained both in intact cardiomyo-

cytes submitted to ischemia/reperfusion and in isolated mitochondria exposed *in vitro* to calcium overload. The results in intact cardiomyocytes indicate that mPTP opening occurs more easily during reperfusion in aging cells but do not rule out the possibility that this depends on more intense triggering conditions. However, the fact that isolated mitochondria, in which the contribution of cytosolic environment and SR interaction is excluded, exhibit less calcium tolerance suggests that sensitivity to mPTP opening is intrinsically increased with aging. The increased susceptibility to mPTP opening associated with aging is specifically manifested in interfibrillar mitochondria, in full agreement with previous observations (Hofer et al. 2009). Other studies have demonstrated an enhanced susceptibility of senescent mitochondria to undergo mPTP and proposed several mechanisms, including excess of membrane cardiolipin oxidation (Petrosillo et al. 2010), increased mitochondrial calcium content (Pepe 2000) or impairment of cytosolic cardioprotective signaling pathways, mainly Akt/GSK-3 beta (Zhu et al. 2010), or several posttranslational modifications such as acetylation (Hafner et al. 2010). Our experiments indicate that the increased rate of mPTP opening in aging may be independent of cytosolic signaling pathways, as it occurs in normoxic isolated mitochondria, but also that it takes place in cells with lower mitochondrial calcium uptake, and is not associated with increased ROS production during the initial phase of reperfusion. All these observations suggest an intrinsic alteration of mPTP in older hearts.

The long-standing concept that mPTP forms at the contact sites of VDAC and ANT has been challenged by evidences indicating that both molecules are dispensable for pore formation in genetically modified mitochondria (Kokoszka et al. 2004; Baines et al. 2007). Recent studies propose dimerization of FoF1 ATP synthase as the molecular entity conforming pore structure (Antoniel et al. 2014). In our experiments, mitochondria from aging hearts exhibited an increased cysteine oxidation in several subunits of FoF1 ATP synthase, and this structural modification was correlated with a functional failure of the molecule during ischemia, resulting in increased rate of H^+ dissipation and higher rates of mPTP opening, despite lower driving force for mitochondrial calcium uptake. These findings further are consistent with the view that FoF1 ATP synthase may directly participate in mPTP opening, and suggest a new link between bioenergetic failure, calcium handling and membrane permeabilization in mitochondria from aging hearts.

Recent proteomics surveys have revealed that lysine acetylation is a widespread cellular modification that is particularly abundant in mitochondrial enzymes and proteins, with known implications in aging and longevity. MS-based proteomics approaches led to the identification of thousands of novel acetylation sites (Choudhary et al. 2009; Rardin et al. 2013). The reversible and nutrient-sensitive nature of these ace-

tyl modifications has strongly implicated mitochondrial NAD+-dependent deacetylases (sirtuins) in energy homeostasis. Thus, mice lacking the predominant mitochondrial deacetylase SIRT3 exhibit depleted cardiac ATP levels (Ahn et al. 2008). Since mitochondria and metabolism are sensitive targets for damage during ischemia-reperfusion injury, the role of SIRTs in cardioprotection is of particular interest. Recently, it has been demonstrated that decreased SIRT3 may enhance the susceptibility of cardiac-derived cells and adult hearts to ischemia-reperfusion injury and may contribute to ischemia-reperfusion injury in the aged heart (Porter et al. 2014). The downstream cardioprotective mechanism of SIRT3 remains unclear but it has been related to the acetylation of CypD lysine 166, a residue that lies adjacent to the cyclosporin-A binding pocket of this protein, suggesting that its acetylation might regulate the mPTP (Hafner et al. 2010). SIRT3 may also enhance antioxidant defense via deacetylation and activation of MnSOD (Qiu et al. 2010) and regulate oxidative phosphorylation by deacetylating specific OxPhos subunits (Ahn et al. 2008). Overall, acetylation of cardiac mitochondria could be associated with an inability of the heart to respond adequately to cardiac stress. Our proteomics analysis found a negligible number of acetylated peptides in mitochondria from aging hearts (0.26% of the total number of identified peptides vs 3% of oxidized Cys-containing peptides), in agreement with previous reports (Choudhary et al. 2014).

4.4.4 Conclusions and implications

Our data demonstrate that aged cardiomyocytes exhibit more pronounced bioenergetic failure, more severely altered cytosolic and mitochondrial calcium, and reduced tolerance to the stress imposed by ischemia-reperfusion, by mechanisms that converge into higher mPTP opening and cell death. These findings could be related to the observed increased oxidation of some critical subunits of the FoF1 ATP synthase molecule.

Figure 4.12 summarizes the proposed pathophysiological mechanism. Importantly, advanced age is associated with partial loss in the efficiency of some cardioprotective interventions, like ischemic pre- and postconditioning or drug-induced cardioprotection. It has been proposed that abrogation of cardioprotection may be related with deficient activity of some signal transduction pathways, like depressed mitochondrial Cx43 or PKC translocation (Tani et al. 2001; Boengler, Schulz, and Heusch 2009) or reduced expression/phosphorylation of STAT3 in aged myocardium (Boengler et al. 2008), among other potential mechanisms. Characterization of these mechanisms should help to identify pharmacologic targets in the elderly and to refine therapeutic strategies specifically addressed to this group of patients.

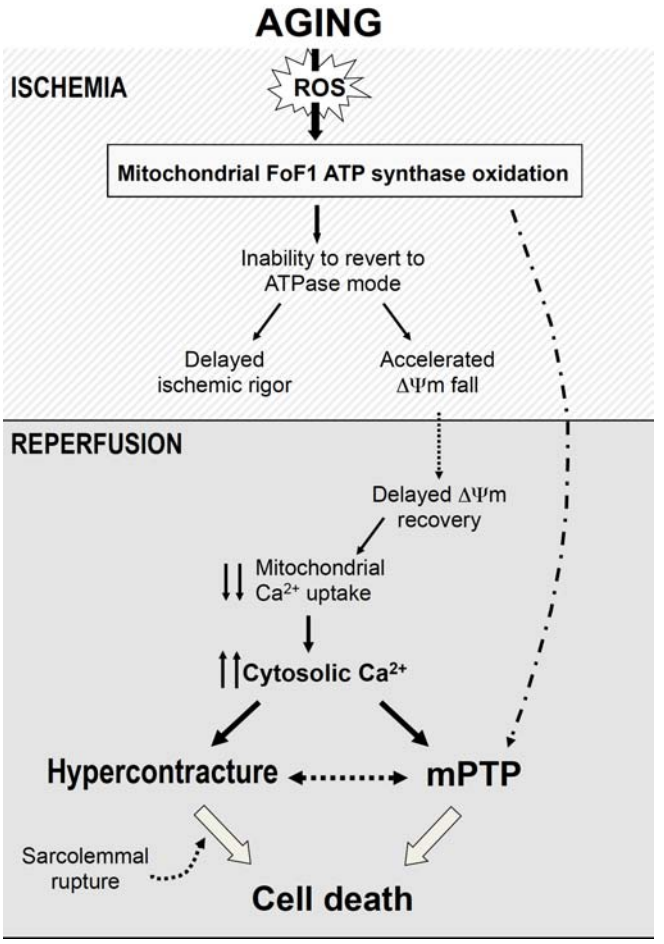


Figure 4.12. Diagram with a proposed pathophysiological mechanism.

CHAPTER 5

General discussion

Cardiovascular diseases are the leading cause of death and between them ischemic heart disease is the most fatal among men and women (Lloyd 2009). Moreover, the number of people aged over 60 is increasing dramatically all around the world in both developed and developing countries (United Nations Population Fund 2012). Aged population is more susceptible to suffer a myocardial infarction accompanied by aggravation of its clinical outcome which in part is due to the age associated comorbidities and other extra cardiac complications. However, recent studies and preclinical assays have proven that the senescent heart is constitutively less tolerant to ischemic diseases due to intrinsic changes of the cardiac cells independently of vascular factors and other comorbidities(Willems et al. 2005).

5.1 Aging impairs the local calcium handling as a result of SR-mitochondria spatial relationship disruption

The first part of this thesis investigates the effect of aging on mitochondrial function and calcium handling in cardiac myocytes. The obtained results indicate an age-dependent disruption of the communication between SR and mitochondria in cardiac cells from aged mice. Close contact points exist between SR and mitochondria that involve direct molecular links connecting RyR and VDAC (Decuypere et al. 2011). The disruption of these links is related with alterations in calcium exchange between both organelles in aged cardiomyocytes. This defective calcium transfer from SR to mitochondria has important consequences on mitochondrial function. First, it uncouples regeneration of the antioxidant NADPH, an effect that results in an increased production of mitochondrial ROS and concomitant oxidative damage. Second, it results in a general decline of respiratory activity, as mitochondrial calcium is a main regulator of Krebs cycle. Specifically, aging is associated with decreased respiratory control ratio RCR at complexes I and III, the main producers of mitochondrial ROS. Importantly, these mitochondrial functional consequences observed in aging are more

pronounced in the interfibrillar mitochondria population (the one that is in close contact with SR), while subsarcolemmal mitochondria remain preserved. Impaired regeneration of reduced species and altered complex I and III-mediated respiration further aggravates the oxidative microenvironment at the SR-mitochondria microdomains following a vicious cycle (Ungvari et al. 2007, Dai, Rabinovitch, and Ungvari 2012).

5.2 Aged-related FoF1 ATP synthase alteration and its potential role in mPTP opening and reperfusion injury.

The second part of this thesis investigates the mechanisms by which senescence is associated with a lower tolerance of cardiomyocytes to ischemia-reperfusion injury. The obtained results indicate an age-dependent structural and functional alteration of FoF1 ATP synthase likely resulting from the pro-oxidative microenvironment described in the first part of our work. In particular, OSCP and d FoF1 ATP synthase subunits, which might be potentially involved mPTP formation, showed an increased Cys oxidation in mitochondria from aging mouse hearts, as disclosed by differential quantitative proteomic analysis. It has been previously shown that Cys crosslinking from the mitochondrial FoF1 ATP synthase has important functional consequences on its hydrolytic activity (Wang et al. 2013). In our study, the oxidative damage of FoF1 ATP synthase in aging cardiomyocytes is related with detrimental functional consequences in cells challenged by an ischemic insult. Specifically, it resulted in the inability of the molecule to revert to the ATPase mode and faster $\Delta\Psi m$ decay during ischemia. As a consequence, mitochondrial calcium uptake was severely depressed, an effect that was mirrored by a higher degree of cytosolic calcium overload.

Reperfusion of aging cardiomyocytes was accompanied by a partial failure to recover $\Delta\Psi m$ upon reperfusion and more severe cytosolic calcium overload. These effects resulted in higher hypercontracture, mPTP opening and cell death in reperfused aged cells respect to young ones. The ability of mitochondria to accumulate huge amounts of calcium has long been recognized as a buffering mechanism, especially under conditions of calcium overload as those present in ischemia-reperfusion (Rizzuto, Bernardi, and Pozzan 2000; Tran et al. 2009). The results of the present work show that during aging, mitochondrial calcium uptake is depressed by two independent mechanisms. On one hand, the more pronounced decay and failure to recover of $\Delta\Psi m$ during ischemia/reperfusion may depress the activity of mitochondrial calcium uniporter, whose activity is driven by the proton motive force. On the other hand, the constitutive disruption of SR-mitochondria reduces mitochondrial calcium uptake coming from SR. Increased cytosolic calcium overload present in aging cells may explain the

higher rate of hypercontracture. In addition to that, we observed an increased mPTP opening that could be the consequence of the oxidative alteration of FoF1 ATP synthase, in agreement with the concept recently proposed by two independent groups according to which FoF1 ATP synthase may be the molecular entity conforming the e mPTP (Giorgio et al. 2013; Bernardi 2013; Antoniel et al. 2014;Alavian et al. 2014; Bernardi et al. 2015; Jonas et al. 2015). These studies suggested that modification of FoF1 ATP synthase by ROS-dependent thiol oxidation modifies the accessibility of divalent metals and promotes the replacement of magnesium by calcium, favouring the opening of the channel Bernardi et al. 2015 (fig. 5.1).The oxidative damage of the FoF1 ATP synthase in the subunits forming the ring described in the present thesis may be thus a causative mechanism of the increased mPTP susceptibility during the first minutes of reperfusion in the aged cardiomyocytes.

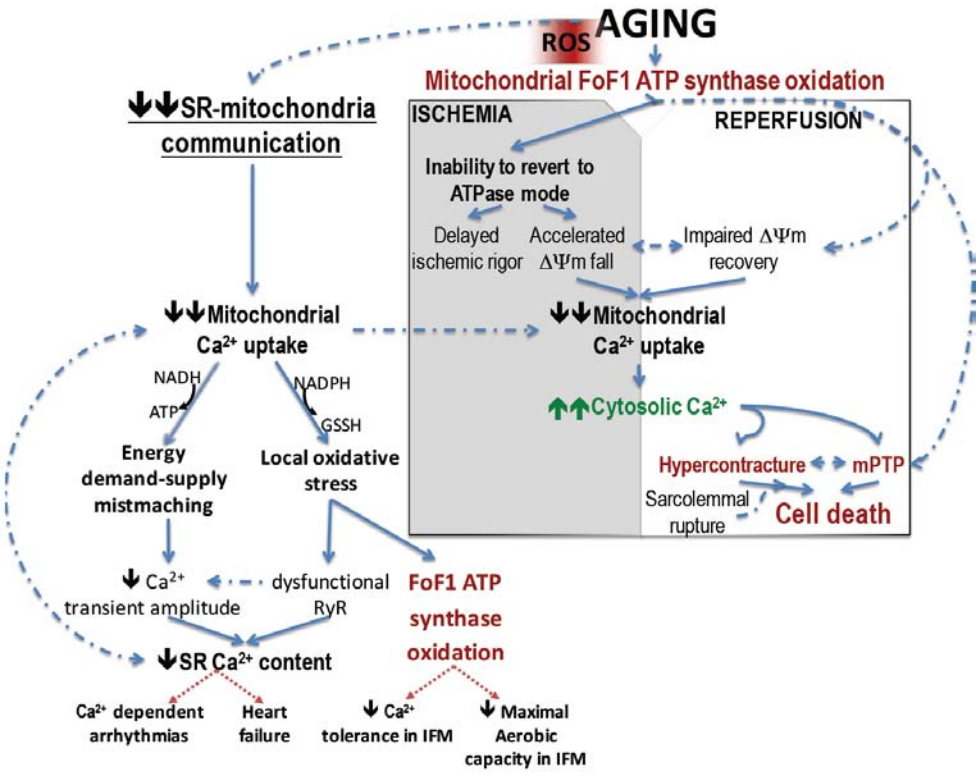


Figure 5.1: Proposed mechanism of the effect aging on the SR-mitochondria crosstalk and its consequences on the ischemia-reperfusion injury

Overall, the present thesis proposes a general pathophysiological mechanism in which the mitochondrial ROS generated as a consequence of respiratory activity may have a local oxidative impact at the SR-mitochondria microdomains, affecting several proteins, including the bridging structure between both organelles (RyR and VDAC molecular link) and FoF1 ATP synthase at the mitochondrial side, among others. The functional impact of this post-translational modifications have detrimental consequences on calcium handling, bioenergetics and antioxidative defence regeneration and make senescent cells more susceptible to mPTP opening and cell death during ischemia-reperfusion injury, as summarized in Figure 5.1.

5.3 Novelty and new insights

This thesis presents innovative data on the importance of SR-mitochondria microdomains in the functional decline of cardiomyocytes during aging. Moreover, a new pathophysiological mechanism is proposed to explain the increased vulnerability of senescent myocardium to ischemia-reperfusion damage

5.3.1 Microdomains disruption: advantage or disadvantage for ischemia-reperfusion injury

It has been previously described that SR-mitochondria microdomains disruption can be protective against ischemia-reperfusion injury by reducing the mitochondrial calcium overload and concomitant mPTP opening secondary to SR calcium oscillations (Abdallah et al. 2011b; Paillard et al. 2013) (fig.1.15). This mechanism does not contradict the findings of the present thesis, since they refer to an acute setting. After a long-term deficient SR-mitochondria communication, like that present during aging, microdomains disruption is associated to other functional consequences – i.e. deficient mitochondrial calcium uptake, altered respiratory function, inefficient ROS scavenging, accumulation of oxidative damage – that eventually lead to an increased reperfusion injury and cell death (fig. 5.1).

5.3.2 Localized oxidative damage

Ultrastructural studies have demonstrated the existence of anatomical and functional microdomains formed by the close apposition between SR and mitochondria (Chen et al. 2012). Not only calcium is exchanged in a priviledged manner, but also ROS generation and oxidative modifications appear to be confined following a

spatio-temporal pattern (Davidson and Duchen 2006; Ruiz-Meana, Fernandez-Sanz, and Garcia-Dorado 2010; Kaludercic, Deshwal, and Di Lisa 2014).

IFM, which are the mitochondria located in close proximity with the SR, appear to be specifically altered by advanced age. These findings are consistent with previous observations showing a reduced mitochondrial respiration in aged IFM (Lesnefsky et al. 2001; Hoppel, Moghaddas, and Lesnefsky 2002; Moghaddas, Hoppel, and Lesnefsky 2003) that may be secondary to their SR-dependent regulation by calcium. Thus, age-dependent defective SR-mitochondria communication induces both altered mitochondria respiration and defective antioxidant regeneration, and may aggravate the increased ROS production observed in the senescent cells (fig. 5.2).

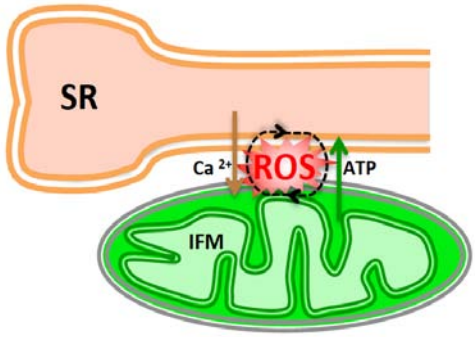


Figure 5.2: Aging specifically alters IFM function.

Increased ROS production at the microdomains level is expected to impact on proteins from both SR and mitochondria. Unfortunately, the proteomics approach did not succeed in the identification of RyR peptides with enough efficiency and therefore in the evaluation redox state of the protein. Nevertheless, the obtained functional results of the RyR calcium handling from aged cells – i.e. decreased calcium transient amplitude, increased spark frequency are coincident with those observed in oxidative environments like failing and aged hearts (Di Lisa et al. 1995; Ekerstad et al. 2011).

5.3.3 FoF1 ATP synthase: target and effector

During ischemia, proton motive force declines and FoF1 ATP synthase reverses to a proton-pumping ATPase in a futile attempt to maintain the mitochondrial proton gradient (Bernardi et al. 2015). When the enzyme works in the hydrolysis mode, the counter clockwise rotation of the γ subunit and of the c ring cause proton translocation into the intermembrane space, maintaining the $\Delta\Psi m$ at the expense of ATP consump-

tion (Nam et al, Noji et al). As a consequence, the cell experience a fast consumption of the ATP stores precipitating the development of rigor contraction (Grover et al. 2004). The present work suggests that aged cells have an oxidative-dependent failure of FoF1 ATP synthase to revert to its hydrolysing mode, a phenomenon that could explain the delay in rigor onset and the acceleration of mitochondrial depolarization during ischemia. This is supported by previous studies where cysteine cross-linking, as a result of cysteine oxidation, has major consequences on the hydrolytic capacity of the FoF1 ATP synthase (Wang et al. 2013).

Under these circumstances the aging cardiomyocyte confront the ischemia-reperfusion under more adverse circumstances that promote the development of hypercontracture, mPTP opening and cell death. Moreover, mPTP opening is also favoured by a reduction in calcium tolerance displayed by IFM of the aging heart. All these functional alterations are possibly linked with the intrinsic damage of the mPTP structure in which the role of the FoF1 ATP synthase appears to be critical (Giorgio et al. 2013; Antoniel et al. 2014; Alavian et al. 2014; Bernardi et al. 2015; Jonas et al. 2015) (fig 5.1).This thesis identifies a new mechanism of FoF1 ATP synthase molecular modification with adverse consequences on the cell tolerance to ischemia-reperfusion damage.

5.3.4 Aging cardiomyocytes are functionally competent but have a reduced threshold to to stress

The results of this thesis show that under resting conditions aging does not have significant functional consequences on the heart. Indeed, echocardiography of aged mice showed a well preserved cardiac function with only minor changes at rest - thinner ventricular wall and a trend towards increased LV end-diastolic volume and reduced ejection fraction in old mice without significant differences-. In mitochondria, maximal stimulation of aerobic capacity induced by saturating concentrations of ADP disclosed a defective response in IFM, but respiratory function was similar at rest in mitochondria from young and old mice. Also, mitochondrial calcium uptake was preserved in isolated mitochondria from aging animals, and only stimulation with high calcium concentrations disclosed their reduced capacity to tolerate calcium. In cardiac myocytes, uncoupled antioxidant regeneration was manifested under high frequency pacing.

All these findings indicate that myocardial tissue is capable to age without a significant functional deficit, although it becomes more vulnerable to stressful conditions (i.e. energy demanding stimuli, ischemia-reperfusion injury). In view of our results, it is tempting to speculate that under stressful conditions aged hearts can not cope

the oxidative damage due to a mismatch in energy-demand system secondary to a defect at the microdomains level.

5.4 Methodological strategies

Isolated mitochondria. The ability to assess mitochondrial function under conditions where circulatory and regulatory systems are intact is an advantage of the *in vivo* techniques. Nevertheless, an accurate assessment of mitochondrial function *in vitro* is crucial to understand the aging process at the subcellular level and the potential underlying pathophysiological mechanisms (Lanza and Nair 2009). Experiments performed in isolated mitochondria allow a reductionist approach to probe mitochondrial function at specific and controlled environment. Moreover, the ability to quantify expression of various mitochondrial proteins, mitochondrial enzyme activities, and post-translational protein modifications make possible the study of a great amount of complementary data (Lanza and Nair 2009).

Isolated cardiomyocytes. The isolation of functionally and morphologically intact adult ventricular cardiomyocytes is essential for the analysis of their size, contractile activity, biochemical and molecular properties. Cardiomyocytes are not proliferative cells and although some atrial cardiac cell lines are commercially available, they do not fully represent adult ventricular cardiomyocytes. The primary isolation and culture of adult cardiomyocytes provide a powerful and well established model for heart research at cellular and molecular levels. Isolated cardiomyocytes allow biochemical, physiological and pharmacological research in conditions free from interaction with other organs and systemic circulation, such as endogenous neurohormonal and hormone like factors (Li et al. 2014).

A microperfusion chamber set on the stage of an inverted microscope was used to allow real-time fluorescence monitorization of living cardiomyocytes loaded with specific fluorochromes and submitted to ischemia-reperfusion conditions. Specifically, RC-43C/BS4 64-0371 from Harvard Apparatus was utilized. Once the chamber is fully assembled, a complete closed and isolated environment is created inside. Three different reservoirs were set up. The exchange between the solutions was controlled by individual one way Luer-Lock stopcocks for each reservoir followed by a multi port manifold in line heater (SHA-6/BS4 64-0104 Harvard Apparatus). With this mechanism, it was possible to control the perfusion solution temperature and to avoid the mixture in between different perfusion solutions.

Permeabilized cardiomyocytes. permeabilization of cardiomyocytes' sarcolemma under controlled conditions makes possible to maintain all intracellular struc-

tures and their interactions fully preserved. The main advantage of this model is that mitochondria or SR can be studied in their cytosolic environment without disrupting them from other cellular structures, as it happens in traditional isolation procedures (Saks et al. 1998). In the present work, this strategy was used to investigate mitochondrial uptake of calcium released by SR without the contribution of other calcium transport/buffering systems.

When permeabilized cardiomyocytes are fixed, immunofluorescent and PLA studies can be performed to study the microdomains structure, characteristics and protein profiles. Compared to other techniques, like immunoprecipitation, PLA allows a very specific visualisation of protein-protein interactions (≤40 nm) in native cells through high resolution confocal images, without the need of overexpressing proteins.

Langendorff perfused hearts. This model allows a proper physiological study of the heart, reducing the variability of *in vivo* models. The cardiac preparation is not influenced by hormones or blood cells, which are washed out by the perfused solution.

Each of these models have strengths but also weaknesses. The combination of these models provides an accurate and complementary biological information at different degrees of complexity increasing the validity of our findings.

5.5 Unsolved issues and future work

To ensure the effectiveness of ROS as local messengers, targets and sources are brought to close distance at focal contacts of the organelles. Examining the cross-talk and interaction between the organelles that arrange these focal signalling systems is technically challenging, hence nowadays only a few studies have provided direct evidences in this regard (Csordás and Hajnóczky 2009).

Alterations of interorganelle-contacts directly affect cellular physiology and, as expected, are associated with a broad spectrum of pathologies (Bravo-Sagua et al. 2014). The ER–mitochondria contacts are of great importance in the pathogenesis of pulmonary arterial hypertension (Sutendra et al. 2011) and Alzheimer's disease (Schon and Area-Gomez 2013). As mentioned before, loss of Mfn2 in cardiomyocytes diminishes the contact area between SR and mitochondria, causing the impairment of protein association at MAM and subsequently the imbalance of calcium signalling and bioenergetics (Chen et al. 2012). Likewise, in Charcot–Marie–Tooth type 2A neuropathy (CMT2A), caused by Mfn2 mutation (Züchner et al. 2004), contacts between ER/SR and mitochondria would be lost. Interestingly, Mfn2 down-regulation has been observed in rats with cardiac hypertrophy (Fang et al. 2007) and in skeletal muscle cells

of patients with type 2 diabetes or obesity (Bach et al. 2005). All these evidences suggest an initial common mechanism, despite different cell types and organs, for diseases like arrhythmia, heart failure, type 2 diabetes and CMT2A.

Overall, despite the evident importance of SR/ER-mitochondria contacts in the context of aging or diseases, further research is needed to elucidate whether their alteration is a cause or a consequence of such pathologies (fig. 5.3).

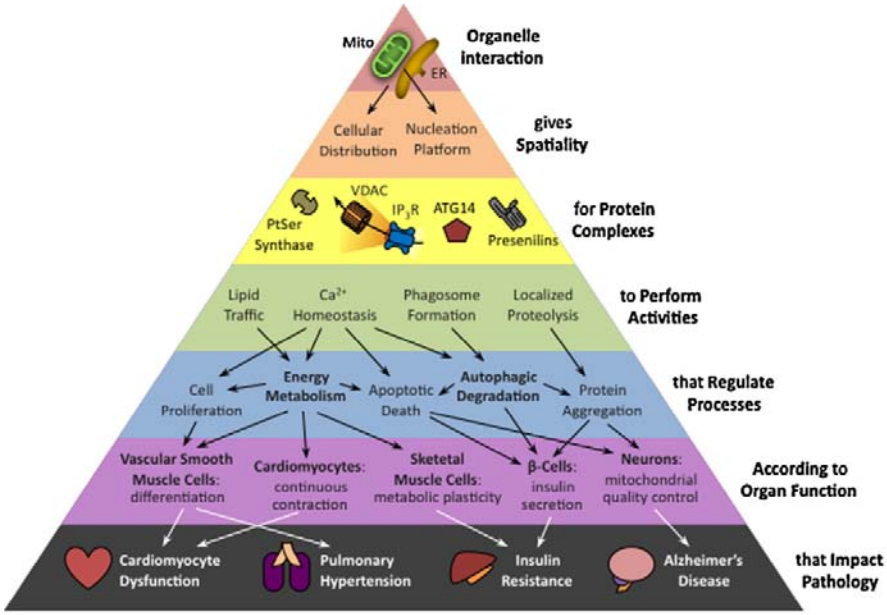


Figure 5.3: Pathophysiological implications of organelle interaction. The ER–mitochondria interface harbours many cellular processes, giving them location within the cellular space, and providing a nucleation platform for complex formation. Lipid biosynthetic enzymes, calcium handling machinery, autophagosome-seeding proteins and protease complexes among others, regulate key functions whose interaction determines both homeostasis and disease. Modified from Bravo-*Sagua et al. 2014*.

The development of new mass spectrometry-based methods has brought to light new information about oxidative modifications of the different FoF1 ATP synthase subunits (Bernardi et al. 2015) that demonstrates its susceptibility to oxidation/nitration stress associated with heart failure (Kane and Van Eyk 2009), central nervous system disorders (Sultana et al. 2006; Poon, Calabrese, et al. 2006), caloric restriction (Poon, Shepherd, et al. 2006) and aging (Groebe et al. 2007; Haynes et al. 2010).

Specifically, a significant increase of Tyr nitration in the FoF1 ATP synthase α-subunit is observed after ischemia-reperfusion of mouse heart (Liu et al. 2009). Con-

sistently, in canine dysynchronous heart failure the formation of an intersubunit disulphide bridge between αC251 and γC78 has been observed, which reverted after cardiac resynchronization therapy (Wang et al. 2011). This α-γ intersubunit disulphide formation has been proposed to be involved in mPTP activation (Petronilli et al. 1994). The fact that these residues are distant in the FoF1 ATP synthase assembled complex suggests that the α-γ intersubunit disulphide may only form in a misfolded/aggregated enzyme. Moreover, it has been demonstrated that myocardial infarction in dogs causes several nitric oxide–related chemical modifications of the d-subunit (Sawicki and Jugdutt 2007).

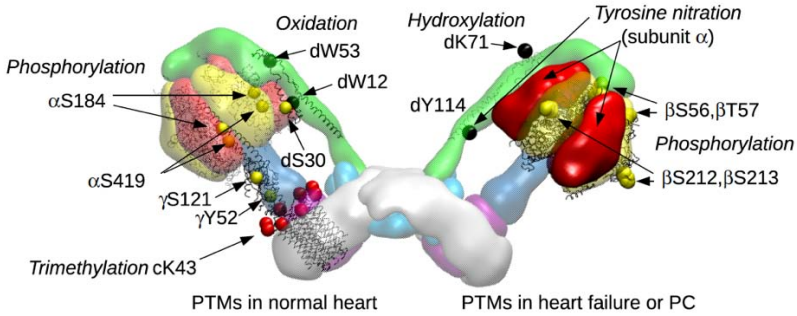


Figure 5.4: Posttranslational modifications of F0F1 ATP synthase in the normal and failing heart. Left monomer, The residues involved in post-translational modifications in normal heart Right monomer, Residues involved in PTMs associated with preconditioning or heart failure *Modified from Bernardi et al. 2015*.

According to our results, in isolated mitochondria from aging cardiomyocytes quantitative proteomics revealed an increased Cys oxidation at several FoF1 ATP synthase subunits which might potentially be involved in mPTP formation. The detected Cys oxidation of the OSCP subunit may affect CyP-D binding to the peripheral stalk and alter its "desensitization" role. FoF1 ATP synthase d-subunit post-translational modifications may also affect the protein kinase C (δPKC) regulatory capacity during ischemia-reperfusion since δPKC plays key roles in ischemia-reperfusion as a modulator of the FoF1 ATP synthase in cardiomyocytes through direct binding to subunit d (Nguyen, Ogbi, and Johnson 2008).

These data suggest that different FoF1 ATP synthase subunits are actively involved in several oxidative modifications, whose implication in mPTP formation and regulation remains to be elucidated (Bernardi et al. 2015). The discovery that mPTP is formed by the FoF1 ATP synthase opens new questions about the mechanisms that switches on this important complex from an energy-conserving to an energy-dissipating device. The answer of these questions will improve our understanding of

the pathophysiological events that trigger the transition in heart diseases, and to set a logical frame for therapeutic strategies (Bernardi and Di Lisa 2015b).

Advanced glycation end products (AGEs) are sugar-derived posttranslational modifications of proteins that have been involved in the pathogenesis of many age-related disorders and have been related with the increased oxidative stress of aging (Brownlee 1995). AGEs irreversibly accumulate on long-lived human proteins as a function of time and have deleterious functional consequences in several organs, including the heart (Simm 2013). The accumulation of AGEs during aging (Ott et al. 2014) may decrease the tolerance to ischemia reperfusion injury in the senescent myocardium by mechanisms related with glycation of intracellular proteins involved in the mitochondrial function and calcium homeostasis in cardiomyocytes, and/or specific membrane receptor activation (RAGEs) which bind to AGEs and activate harmful signalling pathways. Therefore, they can be a potential trigger of the oxidative stress described in the present thesis at the microdomain level. Further investigation is needed to elucidate how AGEs contribute to the functional mismatch in cardiomyocytes during aging and its increased vulnerability to ischemia- reperfusion injury.

5.6 Summary of results

1. Despite of preserved respiration under resting conditions, aging is related with an altered respiratory capacity of IFM under maximal stimulation (ADP-dependent O_2 consumption), defective SR-mitochondria calcium crosstalk and inefficient bioenergetic feedback response (NAD(P)H regeneration).

2. Aging is associated with altered RyR gating properties as disclosed by reduced calcium transient amplitude, decreased rate of calcium rise and increased calcium sparks frequency in cardiomyocytes from aging hearts.

3. Spatial communication between SR and mitochondria is decreased with aging. Immunocolocalization analysis and PLA disclosed an altered structure of the SR-mitochondria microdomains demonstrated by a reduction in the fraction of RyR juxtaposed with the mitochondrial VDAC. Pharmacological disruption of SR-mitochondria connection with colchicine in young cardiomyocytes is able to mimic the anatomical change present in aging cells at the SR-mitochondria microdomains and results in similar alterations in calcium handling.

4. Aged cardiomyocytes have lower levels of total glutathione and a trend towards an increase of the oxidized fraction of glutathione. Proteomic analysis revealed that aging is related with an increased proportion of mitochondrial protein containing oxidized Cys. Additionally, advanced age is related with an increase in the mitochondrial ROS production in isolated cardiomyocytes submitted to high frequency pacing.

5. In both Langendorff-perfused hearts and isolated cardiomyocytes from old mice the development of ischemic rigor is significantly delayed. Paradoxically, reperfusion-induced cell death and sarcolemmal rupture are significantly increased in isolated cardiomyocytes form aging heart.

6. Mitochondrial calcium uptake is severely depressed in aging cardiomyocytes submitted to ischemia-reperfusion. This effect is mirrored by a significantly higher cytosolic calcium overload. Upon reperfusion, extremely elevated cytosolic calcium is associated with higher hypercontracture rate in aging cells.

7. Altered $\Delta\Psi m$ is observed during ischemia and reperfusion in isolated cardiomyocytes from old mice. Mitochondrial depolarization is accelerated in ischemia and the degree of $\Delta\Psi m$ recovery is lower during reperfusion. These effects are secondary to the failure of the FoF1 ATP synthase to activate its reverse mode under energy deprivation conditions in aged cardiomyocytes (absence or transient repolarization wave). Failure of FoF1 ATP synthase to revert to the hydrolase mode is associated with increased Cys oxidation at several subunits of the molecule, including the delta rotor, and correlates with a reduced threshold to undergo membrane permeabilization of aged cells during reperfusion.

8. The increased rate of hypercontracture, mitochondrial failure and calcium overload observed in cardiomyocytes from aging mice resulted in a significantly higher cell death during the first minutes of reperfusion.

5.7 Conclusion

Advanced age is associated with a disruption of SR-mitochondria communication in ventricular cardiomyocytes that results in a mismatch of energy demand-supply and a defective regeneration of antioxidative enzymes. This effect exacerbates the oxidative stress at the microdomains, affecting several protein targets, as FoF1 ATP synthase, and underlying the increased susceptibility of aging cells to undergo hypercontracture, mPTP opening and cell death under ischemia-reperfusion. Overall, this data identifies new targets with potential therapeutic value specifically addressed to elderly people with ischemic heart disease.

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Publications

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1. Urresti J, Ruiz-Meana M, Coccia E, Arévalo JC, Castellano J, **Fernandez-Sanz C,** Galenkamp K, Planells Ferrer L, Moubarak R, Llecha-Cano N, Reix S, Garcia-Dorado D, Barneda-Zahonero B, Comella JX. LFG/FAIM2/TMBIM2 inhibits Fas ligand-mediated endoplasmatic reticulum-calcium release mandatory for apoptosis in type II cells. J Biol Chem. 2015 Accepted with minor revisions.

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2. **Fernandez-Sanz C**, Ruiz-Meana M, Miro-Casas E, Ignasi Barba, Nuñez E, Vazquez Defective SR-mitochondria calcium transfer and increased mitochondrial oxidative state in aged myocardium. J. American Heart Association's Scientific Sessions 2013. Dallas, Texas, 2013. Oral presentation. *Circulation.* 2013; 128: A17508

3. **Fernandez-Sanz C**, Ruiz-Meana M, García-Dorado D. Effect of age on mitochondrial - sarcoplasmic reticulum communication. PROMISE Cardiovascular Network Retreat. Giessen, Germany, 2013. Oral presentation.

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