Study of reactive oxygen species (ROS) and nitric oxide (NO) as molecular mediators of the sepsis-induced diaphragmatic contractile dysfunction. Protective effect of heme oxygenases

Esther Barreiro Portela
Department of Experimental Sciences and Health
Universitat Pompeu Fabra
Barcelona, Catalonia, Spain

March 2002

Dipòsit legal: B. 36319-2003 ISBN: 84-688-3012-7

UNIVERSITAT POMPEU FABRA

DEPARTMENT DE CIÈNCIES EXPERIMENTALS I DE LA SALUT

AREA DE CONEIXEMENT DE FISIOLOGIA

POMPEU FABRA UNIVERSITY

DEPARTMENT OF EXPERIMENTAL SCIENCES AND HEALTH

PHYSIOLOGY

DOCTORAL THESIS

STUDY OF REACTIVE OXYGEN SPECIES (ROS) AND NITRIC OXIDE (NO) AS MOLECULAR MEDIATORS OF THE SEPSIS-INDUCED DIAPHRAGMATIC CONTRACTILE DYSFUNCTION. PROTECTIVE EFFECT OF HEME OXYGENASES

Thesis presented by: Esther Barreiro Portela
Medical Doctor
Specialized in Respiratory Medicine

Thesis director: Dr. Sabah N.A. Hussain, MD, PhD
Critical Care and Respiratory
Divisions, Royal Victoria Hospital
Associate Professor, McGill University
Montreal, Quebec, Canada

CEXS Supervision: Dr. Joaquim Gea Guiral, MD, PhD
Servei de Pneumologia
Hospital del Mar-IMIM
CEXS, Universitat Pompeu Fabra
Barcelona, Catalonia, Spain

March, 2002

ABSTRACT

Nitric oxide (NO) and reactive oxygen species (ROS) are constitutively synthesized in skeletal muscle, and they are produced in large quantities during active inflammatory processes such as in sepsis. These molecules modulate skeletal muscle contractility in both normal and septic muscles. Modification of tyrosine residues and formation of 3-nitrotyrosine is the most commonly studied covalent modification of proteins attributed to NO. Heme oxygenases (HOs), the rate limiting enzymes in heme catabolism, have been shown to exert protective effects against oxidative stress in several type tissues. We evaluated the involvement of NO synthases (NOS) and HOs in nitrosative and oxidative stresses in sepsis-induced diaphragmatic contractile dysfunction. Sepsis was elicited by injecting rats and transgenic mice deficient in either inducible (iNOS), neuronal (nNOS), or endothelial (eNOS) with 20 mg/Kg *Escherichia Coli* lipopolysaccharide (LPS). Peroxynitrite formation (near diffusion-limited reaction between NO and O_2^-) was monitored by measuring nitrotyrosine immunoreactivity with selective antibodies. Three indirect indices of oxidative stress were also used to detect protein oxidation (carbonyl groups), lipid peroxidation (hydroxynonenal protein adducts), and muscular glutathione concentrations. HO expression was evaluated using anti-HO-1 and –HO-2 antibodies. Rats were also injected with a selective inhibitor of HO activity (Chromium Mesoporphyrin IX) and diaphragmatic contractility studies were conducted to assess the biological significance of these enzymes. We mostly found that protein tyrosine nitration occurs in normal muscle fibers and is mainly localized within the cytosol, whereas the sepsis-mediated increase in protein tyrosine nitration is limited to both the mitochondrial and membrane muscle fractions. Protein tyrosine nitration is limited to protein bands ranging in apparent molecular mass between 66 and 10 KDa. Systemic inhibition of HO activity led to an increase in muscle protein oxidation and lipid peroxidation, and to a substantial decline in diaphragmatic force generation under both normal and septic conditions. The iNOS isoform plays a major role in protein tyrosine nitration in both normal and septic muscles, but it does not modulate HO expression. We conclude from these findings that nitrosative stress occurs in normal skeletal muscle, and sepsis elicits an increase in muscle protein tyrosine nitration. HOs protect muscle contractile machinery from the deleterious effects of ROS in skeletal muscle under both normal and septic conditions.

RESUM

L'òxid nítric (NO) i les espècies oxigenades reactives (ROS) se sintetitzen constitutivament en el múscul esquelètic, i es produeixen abundantment durant processos actius inflamatoris, com ara la sepsi. Aquestes molècules regulen la contracció del múscul normal i del sèptic. D´entre les modificacions moleculars atribuïbles al NO, la més estudiada és la nitrificació de l´aminoàcid tirosina i conseqüent formació de 3-nitrotirosina. Les hemo-oxigenases (HOs), enzims limitants del catabolisme del grup hemo, tenen efectes protectors a nivell cel·lular davant de l'estrès oxidatiu. En aquest estudi es va avaluar el paper de les sintetases del NO (NOS) i de les HOs sobre la formació de radicals nitrificants i oxidants en la disfunció muscular contràctil induïda per la sepsi. La sepsi es va induir mitjançant la injecció de 20 mg/kg del lipolisacàrid (LPS) d´*Escherichia Coli* a rates, i a ratolins deficients en les isoformes de les NOS induïble (iNOS *knockout mice*), neuronal, (nNOS) i endotelial (eNOS). La medició de la immunoreactivitat de la 3-nitrotirosina amb anticossos específics es va utilitzar com a mesura indirecta de la formació de peroxinitrit (reacció entre el NO i l´anió superòxid O_2^-). Es van mesurar l´oxidació proteica (grups carbonil), la peroxidació lipídica (conjugats proteics hidroxinonenals) i les concentracions musculars de glutation com a índexs indirectes d'estrès oxidatiu. Les expressions proteiques de les HOs es van determinar mitjançant anticossos anti-HO-1 i –HO-2. Es va administrar a alguns grups de rates un inhibidor selectiu de l´activitat de les HOs i es van fer estudis de contractilitat per avaluar el significat biològic d´aquests enzims en el múscul normal i sèptic. Els resultats més importants d´aquests estudis són el fet que el procés de nitrificació de proteïnes és abundant en el múscul normal, aquestes proteïnes es localitzen fonamentalment en la fracció citosòlica i tenen uns pesos moleculars aparents entre 10 i 66 KDa. L´increment de proteïnes nitrificades induït per la sepsi està limitat a les fraccions mitocondrial i de membrana. La inhibició sistèmica de l´activitat de les HOs dóna lloc a un increment de l´oxidació proteica, de la peroxidació lipídica i a un descens substancial de la força de contracció diafragmàtica. S´ha demostrat que la isoforma iNOS juga un paper fonamental en la nitrificació de proteïnes en el múscul normal i en la sepsi, sense regular l´expressió de les HOs. Concloem que el fenomen d´estrès "*nitrificatiu*" és present en el múscul normal i que la sepsi indueix un augment de proteïnes nitrificades. Les HOs protegirien l´aparell contràctil muscular dels efectes deleteris de les ROS tant en el múscul normal com en el sèptic.

RESUMEN

El óxido nítrico (NO) y las especies oxigenadas reactivas (ROS) se sintetizan constitutivamente en el músculo esquelético, y son producidas abundantemente en procesos activos inflamatorios, como la sepsis. Esta moléculas regulan el proceso de contracción del músculo normal y séptico. De entre las modificaciones moleculares atribuibles al NO, la más estudiada es la nitrificación del aminoácido tirosina y consecuente formación de 3-nitrotirosina. Las hemo-oxigenasas (HOs), enzimas limitantes del catabolismo del grupo hemo, tienen efectos protectores a nivel celular frente al estrés oxidativo. Evaluamos el papel de las sintetasas del NO (NOS) y de las HOs sobre la formación de radicales nitrificantes y oxidantes en la disfunción muscular contráctil inducida por la sepsis. La sepsis se indujo mediante inyección de 20 mg/kg del lipopolisacárido de *Escherichia Coli* a ratas y a ratones deficientes en las isoformas de las NOS inducible (iNOS *knockout mice*), neuronal (nNOS), y endotelial (eNOS). La medición de la inmunoreactividad de 3-nitrotirosina se utilizó como medida indirecta de la formación de peroxinitritito (reacción entre el NO y el anión superóxido O_2^-). Se midieron la oxidación proteica (grupos carbonilo), la peroxidación lipídica (conjugados proteicos hidroxinonenales) y las concentraciones musculares de glutation, como índices indirectos de estrés oxidativo. Las expresiones proteicas de las HOs se determinaron mediante anticuerpos anti-HO-1 y –HO-2. Se administró un inhibidor selectivo de la actividad de las HOs a algunos grupos de ratas y se hicieron estudios de contractilidad para la evaluación de su significado biológico en el músculo normal y séptico. Los resultados más importantes son que el proceso de nitrificación de proteínas es abundante en el músculo normal, que estas proteínas se localizan en la fracción citosólica, y que tienen unos pesos moleculares aparentes entre 10 y 66 KDa. El incremento de proteínas nitrificadas inducido por la sepsis está limitado a las fracciones mitocondrial y de membrana. La inhibición sistémica de la actividad de las HOs dio lugar a un incremento de la oxidación proteica, de la peroxidación lipídica y a un descenso substancial de la fuerza de contracción diafragmática. Se ha demostrado que la isoforma iNOS juega un papel importante en la nitrificación de proteínas en el músculo normal y en la sepsis, sin regular la expresión de las HOs. Concluimos que el fenómeno de estrés "*nitrificativo*" está presente en el músculo normal, y que la sepsis induce un aumento de proteínas nitrificadas. Las HOs protegerían el aparato contráctil muscular de los efectos deletéreos de las ROS tanto en el músculo normal como en el séptico.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank a number of colleagues and friends who have actively facilitated and contributed to the development of this thesis.

My first largest and sincere debt of gratitude goes to Dr Sabah Hussain for welcoming me into his group, and for his knowledgeable guidance and continuous education throughout these years. I would also like to express my gratitude for his close supervision and guidance of the work presented herein, along with the preparation of the two papers included in this thesis.

My second largest and sincere debt of gratitude goes to Dr. Joaquim Gea Guiral, who has always encouraged me to fulfill the main goals of my research career, helping me to cope, even from the distance, the hardest situations. Dr. Joaquim Gea, with his extremely motivating attitude, has continuously followed very closely every single step of the research work presented herein. Without his generous support, the accomplishment of the objectives implied in this work would have been much harder.

I also want to acknowledge the continuous generous advice provided by Dr. Josep Maria Antó, who has directly followed the evolution of my research career since 1997. Dr. Josep Maria Antó has always expressed his thoughtful ideas regarding the meaning of the word research. I am very grateful for the help he has always offered me to deal with the preparation of this thesis.

I am also very grateful to Dr Sheldon Magder for accepting me as a student and for his wise criticism during my research training in the Critical Care Division of the Royal Victoria Hospital.

I would like to thank the Meakins-Christie Laboratories for accepting me as a research fellow and for the intellectual feed-back provided by all the research directors.

I am particularly grateful for the technical assistance provided by Luigi Franchi.

I would also like to show my gratitude to Dr Alain Comtois for his knowledgeable experience and assistance in the contractility studies.

I am specially grateful to Shawn Mohammed for his assistance in the glutathione measurements.

My warmest thanks also extended to all my work mates and colleagues, Valerie, Anne Claire, Marie Reine, Maria, Jill, David, Selma, Johanne, Roy and Gawiyou for their pleasant company and generous encouragement during these two years at the Meakins-Christie, McGill University, Montreal.

I would like to acknowledge the support provided by the Spanish Institutions who have funded my research training throughout these two years, FUCAP, SEPAR, & SOCAP as well as the European Community Biomed Grant.

I would like to extend my debt of gratitude to Universitat Pompeu Fabra for accepting me as a student, and for giving me the current opportunity of presenting my research work accomplished in Montreal as a Doctoral Thesis.

I am very grateful to all my colleagues from Servei de Pneumologia, Hospital del Mar-IMIM, Barcelona for their patience and inestimable support throughout these years, Joan Maria Broquetas, Víctor Curull, Carles Sanjuás, Mauricio Orozco-Levi, Alba Lucía Ramírez, Miquel Félez, Núria Soler and Angela Roig.

I am also very grateful to my colleagues from Grup de Pneumologia, IMIM (Barcelona) for their support throughout these years, Pepe Palacio, Carme Casadevall, Núria Hernández, and Judith García-Aymerich.

I also want to thank you the strong support and encouragement that I have always received from Batxi Gáldiz from the Basque Country.

I would like to warmly acknowledge the extremely loving support and patience provided by my dearest family and Spanish friends, who, even from the distance, have always strongly approved my decisions and encouraged me to persevere, my father Guillermo, my mother Fina, my brother Guillermo, my beloved best friend Rafael, Pablo, and Mónica Cosío.

Finally, my last and sincere debt of gratitude goes to my beloved friends in Montreal, specially Mario Arcand, and Denis Bédard, who have always expressed their affectionate friendship and support in the hardest days, along with some of our friends Lucie Ricart, Pierre-Yves, Johanne Manseau, Luc, Richard, and Lucie Gauthier, who have made my life in the city of Montreal incredibly enjoyable and unforgettable during these three years.

FUNDS

All the studies carried out for the development of the present thesis have been funded by the **Canadian Institute of Health Research** (CIHR).

Esther Barreiro has been supported by **Sociedad Española de Neumología** (SEPAR), **Fundació Catalana de Pneumologia** (FUCAP), and **Societat Catalana de Pneumologia** (SOCAP) (Spain), and by a **European Community Biomed Grant** (E.U.).

PREFACE

The research work presented herein as a doctoral thesis consists of many different experiments which have entirely been carried out in the Critical Care Division of the Royal Victoria Hospital, at McGill University, Montreal, Quebec, Canada, under the direct supervision of Dr. Sabah N.A. Hussain.

May the reader take note that I have chosen to present this thesis in a manuscript-based format, where it has been included the text of two full papers. The first paper entitled: "**Protein tyrosine nitration in the ventilatory muscles: Role of nitric oxide synthases**" is presented in its galley proof version. It will be shortly published in the *American Journal of Respiratory Cell and Molecular Biology*. The second paper entitled: "**Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress**" has recently been accepted for publication in the *American Journal of Physiology* (*Lung Section*). I have chosen to present the second paper in the corresponding journal format for the better understanding and comprehension of the data. The **letters of acceptance** of these two papers are included at the end of the thesis within the **Addendum** section. The present thesis consists of these two papers along with several chapters which consolidate the final presentation of this work. In this regard, a general **introduction** chapter has been written to provide the necessary information to better understand the papers. **Hypotheses** and **objectives** are as well included within this introduction. It is also provided another chapter summarizing the most general **methods** used within the two papers. Finally, both a general **discussion** of the two papers and a list of the most important **conclusions** has also been prepared for this thesis. Short

abstracts written in English, Catalan, and Spanish appear as the first pages of this section.

May the reader take note that the two research papers are presented as numbered pages independently of the rest of the text. They have been included in a separate section which do not follow the numbering of the remaining thesis chapters.

Several investigators have contributed to the preparation of the papers aforementioned. By order of citation:

Dr A.S. Comtois has actively participated in several animal experiments, including diaphragmatic contractility studies, and has provided scientific guidance in the organization of the manuscripts.

Dr J. Gea has strongly participated in the evaluation of the results and has also provided scientific advice in the preparation of the manuscripts.

Dr. V. Laubach has been directly involved in the prepraration of the knockout mouse experiments.

Dr S.N.A. Hussain has been my supervisor during my stage in Montreal. Throughout these two years he has extended continuous supervision of my experimental work. He has also offered wide scientific contribution to the development of the current thesis, in Barcelona.

Dr L. Lands and S. Mohammed have been entirely responsible for the determination and evaluation of glutathione measurements.

As concerns my contribution to the work presented herein, I am responsible for all the experimental work described except for the glutathione measurements and part of the contractility studies.

The present work presented herein as a doctoral thesis has provided enough data which have contributed to the presentation of the following **abstracts**:

1.- E. Barreiro, J. Gea, S.N.A. Hussain.
Oxidative stress of the ventilatory muscles in septic shock: role of nitric oxide synthases. (NOS).
Am J Respir Crit Care Med 2001; 163 (5): A149.

2.- E. Barreiro, A.S. Comtois, S.N.A. Hussain.
Nitrotyrosine immnoreactivity in the ventilatory muscles: role of nitric oxide synthases.
Am J Respir Crit Care Med 2001; 163 (5): A148.

3.- E. Barreiro, S.N.A. Hussain, J.M. Broquetas, J.Gea.
Estrés oxidativo de los músculos ventilatorios en el shock séptico: papel de las sintetasas del óxido nítrico. Arch Bronconeumol 2001; 37 (suppl. 1): 48.

4.- E. Barreiro, J. Gea, S.N.A. Hussain.
Oxidative stress of the ventilatory muscles in septic shock: role of nitric oxide synthases (NOS).
Eur Respir J 2001; 16 (suppl.31): 421S.

5.- E. Barreiro, J. Gea, A. S. Comtois, S.N.A. Hussain.
Protein nitration in the ventilatory muscles: role of nitric oxide synthases.
Eur Respir J 2001; 16 (suppl.31): 421S.

6.- E Barreiro, AS Comtois, J Gea, SN Hussain.
Role of heme oxygenases in the ventilatory muscles: influence on oxidative stress.
Accepted for presentation at the American Thoracic Society (ATS) meeting, Atlanta, 2002.

TABLE OF CONTENTS

ABBREVIATIONS

Listed below only the abbreviations most frequently used throughout the text.

LPS: lipolysaccharide

E. Coli : *Escherichia Coli*

TNF- α: tumor necrosis factor-α

IL-1: interleukin-1

ROS: reactive oxygen species

NO: nitric oxide

SOD: superoxide dismutase

MnSOD : manganese SOD

CuZnSOD: copper-zinc SOD

NADPH: nicotinamide adenine dinucleotide phosphate hydrogen

BH4: tetrahydrobiopterin

NOS: nitric oxide synthase-s

nNOS: neuronal nitric oxide synthase

iNOS: inducible nitric oxide synthase

eNOS: endothelial nitric oxide synthase

PEG-SOD: Polyethylen glycol-adsorbed-SOD

HOs: heme oxygenase-s

cGMP: cyclic guanylate monophosphate

CrMSPIX : chromium (III) mesoporphyrin IX

SDS: sodium dodecylsulfate

PVDF: polyvinylidene difluoride

HNE: hydroxynonenal

BSA: bovine serum albumin

SSA: sulfosalicylic acid

CO: carbon monoxide

INTRODUCTION

INTRODUCTION

Respiratory muscles are striated muscles that are under both voluntary and involuntary control, and have to overcome elastic and resistive loads. Their resting lenght is established by the balance between the inward recoil forces of the lung and the outward recoil forces of the chest wall[1,2]. The function of the respiratory muscles is essential to life, since they have to generate the required forces for ventilation throughout the existence of the individual.

Sepsis is usually defined as the systemic response to serious infection, including several clinical manifestations such as fever, tachycardia, tachypnea, leukocytosis, and a localized site of infection. When hypotension or multiple organ failure occurs as a consequence of this syndrome the condition is called septic shock[3]. The cardiovascular response observed in septic shock in humans is divided into two hemodynamic phases, which include an initial hyperdynamic phase manifested by hypotension, a significant rise in cardiac output, and a decline in total cardiovascular resistance. Conversely, the late hypodynamic phase is associated with severe hypotension, low cardiac output, and a significant rise in peripheral vascular resistance.

The incidence of both sepsis and septic shock has considerably increased in the last 70 years, and it represents the most common cause of death in the intensive care units[3]. Gram-negative bacteria constitutes by far the most frequent etiology of all the septic shock syndromes. Endotoxin is the distinctive lipolysaccharide (LPS) associated with the cell membrane of gram-negative organisms, and it represents the classic example of an initiator of the septic shock pathogenetic cascade[3]. The

endotoxin molecule consists of an inner oligosaccharide core which shares similarities among common gram-negative bacteria, an outer core with a series of oligosaccharides that are antigenically and structurally diverse, and a core lipid A that is highly conserved across bacterial species[3]. Interestingly, the administration of endotoxin to experimental animals results in a cardiovascular response very comparable to that described in patients with septic shock. A cardiovascular response similar to that of spontaneous sepsis, in addition to fever and mild constitutional symptoms was also observed after the administration of a very small dose of purified endotoxin to normal humans.

Respiratory insufficiency is currently considered to be the most important cause of death in patients with septic shock[4]. Although respiratory failure has traditionally been attributed to lung injury in sepsis, there is now growing evidence that septic shock is also associated with ventilatory pump failure. In this regard, sepsis-induced respiratory muscle failure has been the focus of many recent studies. Burke *et al*[5] already described in 1963 that hypercapnic respiratory failure occurred in patients with fulminating septic shock in the presence of normal PaO_2. Several years later it was shown clinical and electromyographic evidence of diaphragmatic contractile failure in patients with severe sepsis who could not be weaned from mechanical ventilation[6]. This observation was based on the concept previously described by Friman[7] who reported that both maximum force and endurance capacity of various limb muscles significantly declined during acute infections in humans.

Numerous experimental animal studies conducted in the last two decades have also provided great evidence of the existence of an association between septic shock and depressed contractile performance of both limb and ventilatory muscles. Sepsis and/or septic shock in these studies has been induced by the administration of either live microorganisms or bacterial endotoxin to experimental animals, which results in cardiovascular features similar to those observed in the hypodynamic phase of human septic shock. In this regard, Hussain *et al*[8] were the first to report that ventilatory failure of *Escherichia Coli* (*E. Coli*) endotoxic shock in dogs over several hours was due to fatigue of the respiratory muscles, finally leading to hypercapnic respiratory failure. Several years later other studies confirmed these findings as well. Leon *et al*[9] reported that acute *E. Coli* endotoxemia reduced diaphragm force in response only to high frequency stimulation in mechanically ventilated rats. The same group of investigators also demonstrated a decline in diaphragmatic force in response to only high frequency stimulation, in this case, after 3 days of endotoxemia in rats[10]. Shindoh *et al*[11], however, after the same observation time period showed a decrease in diaphragmatic force over a wide range of stimulation frequencies in septic hamsters. Though, both groups of investigators concluded that prolonged endotoxemia induced a decline in diaphragmatic endurance in both experimental animals[10,11]. Most recently, it was shown[12] that peritonitis caused diaphragm weakness in rats, leading to the concept that humans with peritonitis may be as well predisposed to respiratory muscle dysfunction[12].

The factors involved in the respiratory muscle dysfunction can be divided into two categories. On one hand, one group of factors are the consequence of an imbalance between increased ventilatory muscle demands, due to augmentation of

ventilation, plus hypoxemia and increased pulmonary resistances, and poor both muscle oxygen and metabolite extraction. On the other hand, mediators of muscle dysfunction include specific cellular, metabolic, and immune deficiencies that interfere with a number of processes necessary for normal force generation[4]. These defects are mediated by complex interactions between several local and systemic mediators which, together, contribute to the respiratory muscle dysfunction described in sepsis. These mediators are discussed in the following paragraphs.

On one hand, although there is no clear evidence showing their direct impact on muscle function, bacterial endotoxins have been proposed to directly act on skeletal muscles, leading to a sequence of events which ultimately would precipitate muscle failure.

On the other hand, endotoxin has also been proposed to alter arachidonic acid metabolism, leading to release of prostaglandins such as prostacyclin and thromboxane A_2[4]. Pre-injection of chronic endotoxemic rats with indomethacin (cyclooxygenase inhibitor) abrogated both the decline in diaphragmatic force generating capacity and prolongation of twitch relaxation time[10]. In line with this, Murphy *et al*[13] showed similar results during acute bacteremia in piglets. Interestingly, in this study the use of an analogue of thromboxane A_2 elicited comparable changes in diaphragmatic force similar to those described in acute endotoxemia.

Furthermore, it is well known that endotoxin can stimulate monocytes, macrophages, and mast cells to produce tumor necrosis factor alpha (TNF-α),

interleukin-1 (IL-1), and other cytokines. Among them, TNF-α is considered to be a central mediator of immune and inflammatory responses, thus, it has been the focus of several experimental studies. In this regard, Wilcox *et al*[14] reported that 3 hours after systemic infusion of TNF-α was associated with decreased both diaphragmatic pressure and shortening in response to artificial phrenic nerve stimulation, compared to control animals. In line with this, in another study TNF-α messenger RNA was shown to increase in the rat diaphragms after 3 hours of endotoxin administration. Moreover, they also observed that endotoxin-induced diaphragmatic hypocontractility was partially reversed by pre-treating the animals with anti-TNF-α antibodies. These findings led to the idea that TNF-α must play a major role in sepsis-induced muscle contractile dysfunction. Though, the mechanisms whereby TNF-α act on skeletal muscle are not very clear yet. Indeed, it is very likely that this cytokine acts by inducing secondary messenger molecules such as **reactive oxygen species** (**ROS**) and **nitric oxide** (**NO**). To some extent, the study of their action mechanisms in skeletal muscle constitutes the main goals of this research work.

NO is a multifunctional molecule that participates in numerous biological processes in almost all aspects of life, including vasodilatation, bronchodilation, neurotransmission, inhibition of both phagocyte and platelet aggregation, and antimicrobial activity[15-17]. NO is an extremely useful intracellular messenger, since it does not react rapidly with most biological molecules at the dilute concentrations produced *in vivo*. Its biological chemistry can easily be simplified in 3 main reactions[18]. First, NO acts as a signalling molecule through binding and activation of guanylate cyclase[18]. Second, NO may be destroyed by reaction with oxyhemoglobin within a red blood cell to form nitrate[19]. Third, NO can be transformed to

peroxynitrite ($ONOO^-$) by reaction with superoxide anion (O_2^-), which reaction is usually limited by the micromolar concentrations of superoxide dismutases (SOD) in cells. Nevertheless, excessive NO production as occurs during active inflammatory-immune processes, leads to detrimental effects of this molecule on tissues, which have been attributed to its diffusion-limited reaction with superoxide anion to form the powerful and toxic oxidant peroxnitrite[18]. Interestingly, this highly reactive species is considered to be mostly responsible for the majority of damaging effects of excessive NO release[18]. In fact, when the concentration of NO rises to the micromolar range it may outcompete SOD for reaction with superoxide anion, since this reaction is 3-fold faster than that of SOD with superoxide. Therefore, peroxynitrite formation will be the consequence of excessive production of both NO and superoxide, a condition almost invariably occuring at sites of active inflammatory processes[20].

NO is synthesized from $_L$-arginine by a group of hemoproteins known as NO synthases (NOS) in the presence of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and tetrahydrobiopterin (BH4)[21]. Three isoforms have been identified, two of which are constitutively expressed and were originally purified in the endothelial cells (eNOS, NOS3), and brain cells (neuronal) (nNOS, NOS1). The third one, which is an inducible isoform, was initially purified in macrophages (iNOS, NOS2). The requirements for calcium and calmodulin differ between the constitutive and the inducible isoforms. Since the inducibility of iNOS is highly dependent on the stimulus rather than the gene product[22], this classification of NOS as constitutive and inducible isoforms is nowadays not very consistent.

The isoform nNOS is only localized in the sarcolemma of type II fibers (fast twitch) of rat skeletal muscles[23]. Interestingly, the authors of this study also found that both guanylate cyclase and nNOS activities are coupled (signalling molecule). Conversely, nNOS isoform does not exhibit a specific fiber type distribution in human skeletal muscles as shown to be equally distributed in both type I and type II fibers[24,25]. Interestingly, the role of nNOS isoform in the pathogenesis of LPS-induced diaphragmatic contractile dysfunction and sarcolemmal injury has recently been assessed using mice deficient in the nNOS gene (nNOS knockout mice) by Comtois *et al*[26]. The authors of this study had previously found that sepsis induced sarcolemmal injury, and NO was shown to be a contributing factor as demonstrated by administration of NOS inhibitors to the septic animals[27]. They concluded from the second study that nNOS isoform may play a protective role in the prevention of LPS-induced impairment of diaphragmatic force. Nevertheless, this isoform was not a major factor in the pathogenesis of sarcolemmal injury in septic animals, since similar degrees were detected in both nNOS knockout and wild type mice.

The isoform eNOS is localized in the mitochondria of rat skeletal muscles, without exhibiting a specific fiber type distribution[28].

Both iNOS mRNA and iNOS protein levels were undetectable or only minimally detectable in various normal skeletal muscles of rats and mice[29-32]. Nevertheless, Gath *et al*[33] demonstrated the expression of this isoform in normal skeletal muscles from specific pathogen-free guinea pigs at the mRNA, protein, and activity levels, suggesting a “constitutive” expression of this isoform in skeletal muscle. Muscle iNOS is systemically expressed in response to bacterial

lipolysaccharide injection in experimental animals, reaching its peak at 12 hours after the endotoxin administration[29-31]. Boczkowski *et al*[31] only detected positive iNOS expression in muscle fibers of rat diaphragms, whereas iNOS expression was found in rat diaphragms, intercostals, and soleus muscles by Hussain *et al*[29,30]. The role of iNOS isoform in the pathogenesis of LPS-induced diaphragmatic contractile dysfunction has been recently assessed using mice deficient in the iNOS gene (iNOS knockout mice)[34]. The authors of this study concluded that muscle iNOS has a protective role in attenuating the inhibitory effects of LPS on muscle contractility, as demonstrated by the worsening of diaphragmatic force in septic diaphragms obtained from iNOS knockout mice.

In general, the oxidative modifications induced by peroxynitrite and other reactive nitrogen intermediates include addition or substitution products in which NO is essentially incorporated into the target molecule (nitrosation and nitration reactions)[20]. More irreversible NO-induced modifications, however, include nitration of aromatic amino acids, lipids, and DNA bases[35-37]. In this regard, the amino acid tyrosine appears to be a primarily susceptible target for nitration. Indeed, the formation of free or protein-associated 3-nitrotyrosine is recently being considered as a potential biomarker for the generation of reactive nitrogen species *in vivo*[20]. Moreover, there is increasing evidence that nitration of essential tyrosine residues can either inactivate numerous enzymes or prevent phosphorylation of tyrosine kinase substrates, leading to the concept that tyrosine nitration might not only be considered as a **marker** of nitrosative stress *in vivo*, but as a **direct mediator** of the damaging effects on tissues observed during active inflammatory-immune processes.

A large number of studies have demonstrated the generation of free radicals in contracting skeletal muscle of intact animals using indirect indicators of oxidative stress, such as protein oxidation, lipid peroxidation, and muscle glutathione. Recent lines of evidence have established ROS-mediated oxidative stress as a cause of muscle fatigue[38]. For instance, the antioxidant N-acetylcysteine, which scavenges superoxide anion, hydroxyl radicals, and hydrogen peroxide, and provides cysteine for the *novo* synthesis of glutathione, has been shown to attenuate the development of muscle fatigue[39]. Similarly, other free radical scavengers such as SOD, catalase, and dimethylsulfoxide prevented the rate of development of fatigue of diaphragm strips in response to electrical stimulation of low frequency[40]. In this regard, ROS effects on skeletal muscle function have usually been associated with certain pathophysiological muscle conditions, such as muscle fatigue following strenuous exercise, ischemia-reperfusion injury, inflammatory muscle disease, and various myopathies[41].

Recent evidence, however, has proved that endogenous ROS also regulate contractile function of healthy skeletal muscle[42]. Indeed, they are produced at a relatively low rate in resting muscle fibers where appear to be essential for normal force production, and their levels progressively increase in response to muscle activation[38,40,42]. ROS concentration within the muscle fiber is usually kept at relatively low levels by intracellular antioxidants such as SOD. Interestingly, Reid[38] has recently proposed a model of ROS homeostasis including their biphasic effects on muscle force generation. According to this model, ROS levels in unfatigued muscle fibers are relatively low and are required for normal force production. Both twitch and tetanic muscle forces significantly decline when ROS are scavenged in

these muscles, whereas modest ROS exposure increases force of unfatigued muscle. Finally, excessive ROS production as occurs during exogenous ROS exposure, strong muscle contractions or in sepsis results in oxidative stress, which in turn leads to a decline in muscle force production.

Several studies have demonstrated that sepsis-induced muscle injury is largely mediated by an increase in ROS levels. In this regard, in two studies the group of Supinski[43,44] were the first to report that free radicals contribute to the diaphragmatic dysfunction induced induced by systemic endotoxin injection. These authors concluded that administration of polyethylene glycol-adsorbed-SOD (PEG-SOD) prevented both malondialdehyde formation (index of free radical-mediated lipid peroxidation) and contractile dysfunction in septic hamsters. In line with this, Peralta *et al*[45] demonstrated increased levels of ROS (muscle chemiluminescence) in septic rat hindlimb muscles. Pre-treatment of animals with SOD attenuated the levels of ROS. One year later, the same group of investigators[46] reported in another study that oxidative stress occurs early in rats along with both inhibition of active mitochondrial respiration and inactivation of antioxidant enzymes. Supinski *et al*[47] concluded that endotoxin-induced dysfunction in septic hamsters was not limited to ventilatory muscles, but also occurred in limb skeletal muscle, while cardiac muscle appeared to be resistant to it. The same group of investigators[48] have also shown that free radicals reduce the maximal diaphragmatic mitochondrial oxygen consumption in endotoxin-induced sepsis in rats. Most recently, they have also demonstrated that free radicals play a central role in altering skeletal muscle contractile protein force-generating capacity in septic rat diaphragms[49]. Despite the progress made in the demonstration of the involvement of ROS in sepsis-induced skeletal muscle

dysfunction, the potential sources of ROS production and the mechanisms whereby oxidative stress contributes to this phenomenon are still poorly understood.

The heme molecule is a complex of the transition element iron, linked to the four nitrogen atoms of a tetrapyrrole macrocycle. It is ubiquitously distributed and of vital importance in eukaryotes. It functions as the prosthetic moiety of various heme proteins including: hemoglobin and myoglobin for oxygen transport, various cytochromes involved in electron transport, energy production and chemical metabolism, peroxidases and catalases for hydrogen peroxide activation, and many other enzyme systems[50].

Heme oxygenases (HOs), which were originally identified by Tenhunen *et al* [51] are the rate limiting enzymes of the initial reaction in the degradation of heme to yield equimolar quantities of biliverdin, carbon monoxide (CO) and free iron[52]. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase, and then free iron is rapidly incorporated into ferritin. Three documented isoforms (HO-1, HO-2, and HO-3) catalyze this reaction[53-55]. Although heme still represents the typical inducer, the 32-kDa HO-1 isoform has been shown to be induced by various non-heme products, such as NO[56,57], cytokines[58], shearstress[59], heavy metals[60], endotoxin[61,62], hyperoxia[63], hydrogen peroxide[64], heat shock[65] and many others[64]. The 36-kDa HO-2 protein is mostly constitutively synthesized existing predominantly in the central nervous system and testis[64], though it may also be present in other tissues such as skeletal muscle[66]. Most recently, the 33-kDa HO-3 isoform has been discovered, and it was shown to be present in the spleen, liver, thymus, prostate, heart, kidney, brain, and testis[55]. Both HO-1 and HO-2 hold heme

degrading activity, whereas the catalytic activity exhibited by HO-3 is very poor. The highest HO activity under physiological conditions has been shown to occur in the spleen, where senescent erythrocytes are sequestered and destroyed[52]. Though, this activity may occur in all systemic organs as well.

As afore-mentioned numerous studies have shown that HO-1 enzyme activity may also be stimulated by various non-heme products[56,58,60,61,64], with the common capacity to generate ROS production and/or modify glutathione levels[52]. The demonstration of the induction of HO-1 by agents causing oxidative stress led to the notion that HO-1 might have a cytoprotective role against the excess of oxidants. In this regard, it has been well characterized that several antioxidant enzyme systems are induced in response to oxidative stress, such as manganese (Mn)SOD, copper-zinc (CuZn)SOD, and catalases that are in charge of scavenging ROS in order to maintain cellular homeostasis. Recent evidence, however, shows that transcriptional regulators such as c-FOS, c-Jun, and NF-κB, as well as other stress-response genes, for instance HO-1 are up-regulated in response to oxidative stress-induced injury[52]. Indeed, HO-1 enzyme is now thought to be part of a more general cellular response to oxidative stress.

The cellular protective effects of HO-1 have been shown in various *in vivo* and *in vitro* models of tissue injury[60,67]. Indeed, this protective effect was further proved by observations made using both HO-1 protein deficient mouse and human models. With regard to HO-1 protein deficient mice (HO-1 knockout mice), it was shown that mouse fetuses lacking the HO-1 gene[68,69] did not survive to term, or in case they did, they were abnormal and died within one year of birth. Additionally, these mice showed growth retardation, normochromic and microcytic anemia,

increased blood cellular counts, glomerulonephritis, and evidence of iron deposition in quite a few organs such as liver, kidneys, and signs of chronic inflammation represented by hepatoesplenomegaly. HO-1 knockout mice were also more vulnerable to both death and hepatic necrosis in response to systemic administration of endotoxin[68]. Furthermore, HO-1 deficient embryonic fibroblasts showed higher sensitivity to oxidative stress-induced cytotoxicity, supporting the idea that expression of HO-1 is essential for both cell viability and optimal maintenance of cellular free radical levels[69]. In line with these experimental findings, a patient suffering from deficient levels of HO-1 enzyme was also reported to exhibit growth retardation, anemia, leukocytosis, and increased sensitivity to oxidative stress[70].

It is now thought that HO-1 regulates cellular homeostasis via its three major catabolic by-products: CO, bilirubin, and ferritin. CO has been shown to exert protective effects on cellular homeostasis by activation of guanylate cyclase on the binding of this molecule to the heme moiety of this enzyme, which in turn will lead to the formation of cGMP[71]. This gaseous molecule may also have anti-inflammatory effects through cGMP formation by inhibiting platelet activation or aggregation and neutrophil infiltration[72,73]. Bilirubin was first reported by Stocker *et al*[74] to have beneficial effects in the brain as a physiological antioxidant. This molecule is the major endogenous antioxidant in mammalian tissues, and it accounts for most of the antioxidant activity of human serum[75]. Finally, the incorporation of free iron into the iron storage protein ferritin has also been reported to act as an antioxidant mechanism[64,76].

Little is known about the presence, distribution and functional significance of HOs in skeletal muscle fibers. In this regard, several studies have shown a relationship between muscle contractions and expression of HO-1 enzyme. For instance, HO-1 mRNA levels were shown to increase after repeated muscle contractions in rat limb muscle[77]. The authors concluded that HO-1 induction might underlie an antioxidant pathway in response to increased levels of ROS usually generated during strong muscle contractions. Interestingly, in another study it was shown that hemin-induced HO-1 expression directly correlated with both the content of red fibers and tissue myoglobin in different rat muscles[78]. The authors of this study concluded that HO-1 expression follows a specific fiber-type distribution in skeletal muscle[78]. In addition to HO-1 enzyme increased expression in muscle fiber, it was reported that HO-2 protein is present in satellite cells, vascular endothelial cells, fibroblasts and extrafusal myofibers either associated with the non-junctional sarcolemma or within the subsarcolemmal region[66] .

As previously mentioned, HO-1 gene is also induced by the administration of endotoxin. In line with this, an increase in both HO-1 mRNA levels and activity was detected in the lungs of endotoxemic rats after LPS administration[79]. Interestingly, pre-treatment of animals with hemoglobin led to HO-1 induction that resulted in 100% survival. On the contrary, the administration of a competitive inhibitor of HO activity to the endotoxemic rats completely abrogated the protective effects attributed to HO-1 induction. Two years later, the same group of investigators observed a clear reduction of both lung and systemic inflammatory effects of sepsis, by pretreating animals with hemoglobin[80]. Finally, recent evidence showed that HOs noticeably protect diaphragmatic contractile dysfunction induced by sepsis in rats[81].

In this study both a HO-1 inducer and a competitive inhibitor were used to demonstrate the beneficial effects of this enzyme. The authors of this study concluded that HOs play a protective role on the sepsis-induced respiratory muscle contractile dysfunction by exerting antioxidant effects via their catabolic by-products.

HYPOTHESES AND OBJECTIVES

GENERAL HYPOTHESIS

Septic shock is associated with ventilatory pump failure due to the action of several mediators, including the excessive production of both NO and ROS. At the same time these two types of molecules will induce the up-regulation of HO-1 expression in the context of severe sepsis. The inducible enzyme HO-1 plays a relevant protective role by alleviating sepsis-induced respiratory muscle dysfunction. HO-1 cytoprotective effects are due to the action of its antioxidant by-products.

To test this hypothesis, several experiments were performed to assess on one hand, the presence and localization of protein tyrosine nitration, and on the other to investigate both the expression and localization of HOs and their biological significance in control and septic muscles. These experiments have been divided into two separate papers, and their respective hypotheses are described next.

SPECIFIC HYPOTHESES

1.- Increased levels of both NO and superoxide anion in the ventilatory muscles during sepsis in experimental animals will lead to the formation of the potent ROS peroxynitrite, and the consequent nitration of tyrosine residues. Protein tyrosine nitration in skeletal muscle will be measured as the fingerprint of peroxynitrite formation in this tissue. Nitrotyrosine formation might be a mediator of the sepsis-induced respiratory muscle dysfunction.

2.- HO-1 is induced in the ventilatory muscles during sepsis by several mediators such as NO released by iNOS, peroxynitrite, and endotoxin. These mediators are as well involved in the sepsis-induced diaphragmatic contractile dysfunction. HO-1 increased activity will play a cytoprotective role within the skeletal muscle fiber by attenuating the deleterious effects of ROS on muscle contractile machinery.

GENERAL OBJECTIVES

As afore-mentioned this research work has been divided into two main projects which are presented herein as two separate papers. As to the first paper, our main objective was to assess the levels of nitrosative stress in sepsis-induced ventilatory muscle contractile dysfunction as compared to control muscles. We also investigated the contribution of the different NOS isoforms to the generation of reactive nitrogen species *in vivo* under both normal and septic conditions in rat skeletal muscles.

As to the second paper, our main objectives were to assess both whether HOs are induced in response to sepsis in rat ventilatory muscles, and whether they exert cytoprotective effects in the sepsis-induced diaphragmatic contractile dysfunction.

SPECIFIC OBJECTIVES

A) First paper: "Protein tyrosine nitration in the ventilatory muscles: role of nitric oxide synthases".

1.- To determine the presence, compartmentalization, and localization of nitrotyrosine formation in both normal and septic ventilatory and limb rat skeletal muscles of various fiber type composition.

2.- To investigate, using selective pharmacological inhibitors of NOS isoforms and genetically altered mice which are deficient in a specific NOS isoform, the contribution of the iNOS, eNOS, and nNOS isoforms to protein tyrosine nitration in the ventilatory and limb muscles both under normal conditions and in response to severe sepsis.

B) Second paper: "Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress".

1.- To assess the expression, localization, and functional significance of HO-1 and HO-2 in the regulation of redox status and contractile function of normal ventilatory muscles.

2.- To evaluate the influence of sepsis on the expression of HO-1 and HO-2 in the ventilatory muscles and to assess the contribution of these enzymes to sepsis-induced muscle oxidative stress and contractile dysfunction.

3.- To evaluate the involvement of the iNOS isoform in the regulation of ventilatory muscle HO-1 and HO-2 expression both under normal conditions and in response to severe sepsis.

METHODS

GENERAL METHODS

The methodologies used to assess every specific objective of the current research work are described in detail in the **Methods section** of each separate paper. Nevertheless, the most general methodologies used are briefly summarized within this section.

1.- ANIMAL EXPERIMENTS

Rats: Pathogen-free male Sprague-Dawley rats (250-275 g) were used.

Mice: Three separate groups of adult (8-12 weeks old) mice genetically deficient (knockout) in either iNOS, eNOS, or nNOS isoforms were used to assess the separate contribution of NOS isoforms to nitrotyrosine formation in the diaphragm. For the iNOS isoform, B6/129 hybrid iNOS knockout mice ($iNOS^{-/-}$) were generated. Wild type B6/129 hybrid mice were purchased from Jackson Laboratories (Bar Habor, ME). For the nNOS and eNOS isorforms, C57BL/6 $nNOS^{-/-}$ and $eNOS^{-/-}$ mice were generated. Wild type C57BL/6 mice (the background strains for both $eNOS^{-/-}$ and $nNOS^{-/-}$) mice were purchased from Charles River Inc.

Induction of sepsis: Sepsis was induced by injection with *E. Coli* LPS (serotype 055:B5, 20 mg/kg dissolved in 0.3 ml of saline).

Pharmacological NOS inhibition: Animals were injected with either a selective iNOS inhibitor (1400 W, 20 mg/kg), or with a non-selective NOS inhibitor (L-NAME, 30 mg/kg).

Pharmacological HO activity inhibition: Animals were injected with a selective HO inhibitor [Chromium Mesoporphyrin IX (CrMSPIX), 5μmol/kg)].

Animals were sacrificied at different time points according to each experimental protocol. They were always anesthetized with sodium pentobarbital (30 mg/kg), and the diaphragm, external intercostal, gastrocnemius and soleus muscles were quickly excised, and for immunoblotting frozen in liquid nitrogen, and then stored at –80ºC. However, for immunostaining, the tissues were first flash frozen in cold isopentane for 20 seconds, then immersed in liquid nitrogen, and finally stored at –80ºC.

2.- MUSCLE SAMPLE PREPARATION

Fractionation of muscle samples: We used two distinct methods for separating animal skeletal muscles into two different groups of compartments. On one hand, muscle was fractionated into mitochondria, membrane, and cytosolic fractions. On the other, muscle fibers were separated into myofibrillar, membrane and cytosolic compartments. Separation of mitochondrial, membrane, and cytosolic muscle fractions was achieved using the protocol of Rock *et al* [82]. Fractionation of muscle samples into myofibrillar, membrane, and cytosolic fractions was performed according to the protocol of Fagan *et al* [83].

In various other experiments, only crude homogenates from either rat or mouse skeletal muscles were used.

3.- TECHNIQUES

Immunoblotting: Crude homogenates and various muscle fractions or rat skeletal muscles were mixed with sample buffer, boiled for 5 min, and then loaded onto tris-glycine sodium dodecylsulfate (SDS) polyacrylamide gels, and separated by electrophoresis. Proteins were transferred electrophoretically to polyvinylidene

difluoride (PVDF) membranes. The PVDF membranes were subsequently incubated with primary monoclonal or polyclonal antibodies raised against 3-nitrotyrosine in the experiments corresponding to the first paper. As to the experiments corresponding to the second paper, the PVDF membranes were incubated with both monoclonal anti-HO-1 and anti-HO-2 antibodies. Furthermore, a well characterized polyclonal antibody to detect 4-hydroxy-2-nonenal (HNE) protein adducts was also used to detect lipid peroxidation in both ventilatory and limb muscles of normal and septic animals. Protein oxidation was measured by evaluating the levels of protein carbonyl group formation using the Oxyblot protein oxidation detection kit.

Immunohistochemistry: Frozen tissue sections (5-10 μm thickness) were adsorbed to microscope slides and dried. The sections were fixed with acetone at –20ºC, re-hydrated with PBS, and then blocked with solutions of avidin, biotin, and 3% BSA. The sections were then incubated with primary monoclonal or polyclonal antibodies raised against 3-nitrotyrosine in the first study. As for the second study, sections were incubated with primary monoclonal anti-HO-1 and anti-HO-2 antibodies.

Glutathione measurements: Frozen muscle samples were homogenized in 20 μL 5% 5-Sulfosalicylic acid (SSA) / mg tissue. The homogenate was then centrifuged at 10,000 g for 5 min at 4ºC. Total glutathione concentration was determined by the glutathione reductase recycling method of Tietze[84] adapted for the Cobas Mira S spectrophotometer (Roche Diagnostics, Laval, Quebec).

4.- MUSCLE CONTRACTILITY STUDIES

The biological significance of HO in the sepsis-induced diaphragmatic contractile dysfunction was assessed by evaluating muscle physiological parameters such as force and endurance.

Diaphragmatic strip preparation: Very briefly, diaphragms of control and septic (24 hours post LPS injection) rats both un-treated and treated with CrMSPIX were surgically excised with ribs and central tendon attached, and then placed in an equilibrated Krebs solution chilled at 4ºC. From the central tendon to the rib, a muscle strip (3-4 mm wide) was dissected from the lateral costal portion of the diaphragm. The strip was mounted in a muscle chamber, which was subsequently mounted vertically into a double jacket gut bath (Kent Scientific Instruments). A 4.0 silk thread was used to secure the central tendon to the isometric force transducer (Kent Scientific Instruments).

Force-frequency measurements: Muscle strips were electrically stimulated at constant currents via platinum electrodes mounted in the muscle chamber wich were connected to a square wave pulse stimulator (Grass Instruments, model S48). Muscle lenght was gradually adjusted with a micrometer to the optimal value (L_0) at which maximum isometric muscle force was generated in response to supramaximal stimulation (current of 300-350 mA, 120 Hz frequency). **Muscle force** was evaluated by stimulating the muscle at 10, 20, 30, 50, and 120 Hz while maintaining supramaximal current and stimulation duration (600 ms) constant. **Muscle fatigue** was evaluated by continuously stimulating the muscle at 100 Hz during 3 min.

PUBLICATIONS

FIRST PUBLICATION

Esther Barreiro, Alain S. Comtois, Joaquin Gea, Victor E. Laubach, and Sabah N.A. Hussain, 2002, Protein tyrosine nitration in the ventilatory muscles: roles of nitric oxide synthases, *American Journal of Respiratory and Cell Biology,* 26(4) :438-46. Official Journal of the American Thoracic Society

This paper is presented herein in its Galley version.

SECOND PUBLICATION

Esther Barreiro, Alain S. Comtois, Shawn Mohammed, Larry C. Lands, and Sabah N.A. Hussain. Role of heme oxygenases in sepsis-induced diaphragmatic contractile disfunction and oxidative stress. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 2002 Aug; 283(2) :L476-84

This paper is presented herein in its final resubmitted version.

Protein Tyrosine Nitration in the Ventilatory Muscles

Role of Nitric Oxide Synthases

Esther Barreiro, Alain S. Comtois, Joaquin Gea, Victor E. Laubach, and Sabah N.A. Hussain

Critical Care and Respiratory Divisions, Royal Victoria Hospital and Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada; Respiratory Medicine Department, Hospital del Mar-IMIM, Universitat Pompeu Fabra, Barcelona, Catalonia, Spain; and Department of Surgery, University of Virginia Health System, Charottesville, Virginia

Modification of tyrosine residues and formation of 3-nitrotyrosine is one of the most commonly identified effects of reactive nitrogen species on proteins. In this study we evaluated the presence and localization of tyrosine nitration in various ventilatory and limb muscles. We also assessed the contribution of the neuronal (nNOS), the endothelial (eNOS), and the inducible (iNOS) isoforms of nitric oxide synthase (NOS) to tyrosine nitration in skeletal muscles both under normal conditions and in response to severe sepsis. In normal rats and mice, muscle tyrosine nitration was detected at 52, 48, 40, 30, 18, and 10 kD protein bands. Tyrosine nitration of the majority of these protein bands was significantly reduced within 1 h of *in vivo* NOS inhibition in rats. Diaphragmatic protein tyrosine nitration in mice deficient in the inducible NOS ($iNOS^{-/-}$) averaged $\sim$ 50% of that detected in wild-type ($iNOS^{+/+}$) mice. Injection of bacterial lipopolysaccharides (LPS) in rats produced a significant rise in protein tyrosine nitration in the mitochondrial and membrane fractions but not in the cytosol of ventilatory muscles. Absence of iNOS expression ($iNOS^{-/-}$), but not nNOS ($nNOS^{-/-}$) or eNOS ($eNOS^{-/-}$), in genetically altered mice resulted in a significant reduction in LPS-mediated rise in diaphragmatic nitrotyrosine. We conclude that tyrosine nitration of proteins occurs in normal muscle fibers and is dependent mainly on the activity of the iNOS isoform. Sepsis-mediated increase in protein tyrosine nitration is limited to the mitochondria and cell membrane and is highly dependent on the activity of the iNOS but not the nNOS or eNOS isoforms.

The formation of 3-nitrotyrosine (NO_2Tyr) is the most commonly studied covalent modification of proteins attributed to nitric oxide (NO). Elevated NO_2Tyr formation has been documented in acute lung injury, sepsis, rheumatoid arthritis, amytrophic lateral sclerosis, Alzheimer, and liver transplantation (1, 2). Several mechanisms are involved in tyrosine nitration, including peroxynitrite (formed from the near diffusion-limited reaction between NO and O_2^- anions), the reaction of NO with protein tyrosyl radicals (3), the reaction of nitrite and peroxidases (4), and finally nitrous acid (formed in acidic environment such as the stomach) (5).

Little information is available regarding protein tyrosine nitration in skeletal muscles. Recent studies have documented significant NO_2Tyr formation in the limb and ventilatory muscles of animals with severe sepsis (6–8). Despite this recent progress, many aspects of NO_2Tyr formation in skeletal muscles remain unexplored. For instance, it is unclear whether NO_2Tyr formation occurs in normal skeletal muscle fibers and whether this formation is dependent on the fiber-type composition of various muscles. Constitutive NO synthesis and nitric oxide synthase (NOS) expression inside skeletal muscles is highly dependent on fiber-type composition especially in rats and mice (9, 10). Another important question that remained unanswered is the contribution of the neuronal (nNOS), endothelial (eNOS), and the inducible (iNOS) isoforms of NOSs to NO_2Tyr formation in muscle fibers. We should emphasize that all of these isoforms contribute to elevated NO synthesis in the muscles of septic animals (6). Finally, the nature of tyrosine-nitrated proteins inside skeletal muscle fibers remains unexplored. Numerous proteins have been shown to be tyrosine nitrated in nonmuscle cells and organs including cytosolic and contractile proteins such as actin, catalase, glutathione-S-transferase, tubulin, carbonic anhydrase, and several mitochondrial enzymes including Mn-superoxide dismutase (Mn-SOD), aconitase, ATP synthase, and glutamate dehydrogenase (11–13). Whether these proteins, particularly Mn-SOD, actin, and tubulin, are also tyrosine nitrated inside skeletal muscle fibers remain unclear.

We hypothesized in this study that protein tyrosine nitration occurs inside normal skeletal muscle fibers and is dependent on NO synthesis. This hypothesis is based on the fact that skeletal muscles are among few organs in which the two components required for peroxynitrite formation (O_2^- and NO) are constitutively synthesized (9, 14). We also hypothesized, on the basis of an excellent correlation between the time course of NO_2Tyr formation and iNOS expression in the ventilatory muscles of septic rats (6), that protein tyrosine nitration in these muscles is dependent mainly on the activity of iNOS. To test these hypotheses, we assessed the presence and localization of protein tyrosine nitration in normal ventilatory and limb muscles of various fiber-type compositions. We also evaluated, using selective pharmacologic inhibitors of NOS isoforms and genetically altered mice, which are deficient in a specific NOS isoforms, the contribution of the iNOS, eNOS, and nNOS isoforms to protein tyrosine nitration in the ventilatory and limb muscles both under normal conditions and in response to severe sepsis.

Materials and Methods

Reagents for protein measurement were purchased from Bio-Rad Inc. (Hercules, CA). Gels and loading buffers for immunoblotting were obtained from Novex Inc. (San Diego, CA). Aprotinin, leu-

(*Received in original form* ■■■ *and in revised form* ■■■)

Address correspondence to: Dr. S. Hussain, Room L3.05, 687 Pine Avenue West, Montreal, PQ, H3A 1A1 Canada. E-mail: sabah.hussain@muhc.mcgill.ca

Abbreviations: bovine serum albumin, BSA; endothelial isoforms of nitric oxide synthase, eNOS; inducible isoforms of nitric oxide synthase, iNOS; lipopolysaccharides, LPS; myeloperoxidase, MPO; nitric oxide, NO; nitric oxide synthase, NOS; neuronal isoforms of nitric oxide synthase, nNOS; 3-nitrotyrosine, NO_2Tyr; optical densities, OD; phosphate-buffered saline, PBS; phenylmethylsulphonyfluoride, PMSF; polyvinylidene difluoride, PVDF; sodium dodecylsulfate, SDS.

Am. J. Respir. Cell Mol. Biol. Vol. 26, pp. 1–9, 2002
Internet address: www.atsjournals.org

peptin, trypsin inhibitor, pepstatin A, and phenylmethylsulphonyfluoride (PMSF) were purchased from Sigma Chemicals (St. Louis, MO). Monoclonal, polyclonal, and horseradish peroxidase–conjugated polyclonal antibodies for NO_2Tyr were obtained from Cayman Chemical Inc. (Ann Arbor, MI), Upstate Biotechnology (Lake Placid, NY), and Academy Biomedical Co. (Houston, TX), respectively. Secondary antibodies for immunoblotting and immunohistochemistry were obtained from Transduction Laboratories Inc. (Lexington, KY) and Jackson ImmunoResearch Inc. (West Grove, PA), respectively. Reagents for enhanced chemiluminescence detection were obtained from Chemicon Inc. (Temecula, CA).

Animal Preparation

Rat Experiments. The Animal Research Committee of McGill University and University of Virginia approved all procedures. Pathogen-free male Sprague-Dawley rats (250–275 g) were used. The animals were housed in the animal facility of the hospital, fed food and water *ad libitum*, and studied 1 wk after arrival.

Nitrotyrosine formation in normal rat muscles. Male rats were anesthetized with sodium pentobarbital (30 mg/kg), and the diaphragm, intercostals, gastrocnemius, and soleus muscles were quickly excised and frozen in liquid nitrogen. For immunostaining, the tissues were first flash frozen in cold isopentane (20 s), then immersed in liquid nitrogen and stored at −80°C.

Acute NOS inhibition. To evaluate the acute influence of NOS inhibition on muscle nitrotyrosine formation, three groups of male rats were studied. Group 1 served as control, whereas Groups 2 and 3 were injected intraperitoneally with either a selective iNOS inhibitor (1400W, 20 mg/kg) or a nonselective NOS inhibitor (L-NAME, 30 mg/kg). Both inhibitors were dissolved in 0.3 ml of phosphate-buffered saline (PBS). All animals were killed 1 h later and the diaphragm (mixed fiber composition) and soleus (rich in type I fibers) muscles were quickly excised and frozen in liquid nitrogen.

Sepsis. Five groups of pathogen-free male Sprague-Dawley rats (250–300 g, $n = 5$ in each group) were studied 1 wk after arrival. Group 1 was injected with normal saline (control group). Groups 2, 3, and 4 were injected intraperitoneally with *Escherichia coli* lipopolysaccharides (LPS) (serotype 055:B5; Sigma Inc., 20 mg/kg dissolved in 0.3 ml of PBS) and killed by an overdose of sodium pentobarbital 6, 12, and 24 h after the injection, respectively. Group 5 animals were injected with a selective iNOS inhibitor (1400W, 20 mg/kg) 30 min before LPS administration and every 8 h thereafter and were killed 24 h after LPS administration. The diaphragm was quickly dissected, frozen in liquid nitrogen, and stored at −80°C.

Mice Experiments. To evaluate the separate effects of NOS isoforms on nitrotyrosine formation in the diaphragm, we studied three separate groups of adult (8- to 12-week-old) mice genetically deficient (knockout) in iNOS, eNOS, or nNOS isoforms. For the iNOS isoforms, B6/129 hybrid iNOS knockout mice ($iNOS^{-/-}$) were generated as mentioned previously (15) and a full colony of these mice was maintained at McGill University. Wild-type B6/129 hybrid mice ($iNOS^{+/+}$) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred to serve as experimental controls. NO_2Tyr formation in normal mice muscles was studied in two groups ($n = 6$ in each group) of wild-type (control–$iNOS^{+/+}$) and iNOS knockout (control–$iNOS^{-/-}$) mice. Animals were anesthetized with sodium pentobarbital (30 mg/kg), the chest was then opened, and the diaphragm was quickly excised and frozen in liquid nitrogen. To evaluate the role of iNOS in sepsis-induced NO_2Tyr formation, two groups ($n = 6$ in each group) of $iNOS^{+/+}$ (LPS–$iNOS^{+/+}$) and $iNOS^{-/-}$ (LPS–$iNOS^{-/-}$) mice were injected with intraperitoneal *E. coli* LPS (20 mg/kg) and killed 24 h later. The diaphragm was excised and frozen in liquid nitrogen as mentioned above. For the nNOS and eNOS isoforms, C57BL/6 $nNOS^{-/-}$ and $eNOS^{-/-}$ mice were generated as mentioned previously (16, 17). The $nNOS^{-/-}$ mouse colony was maintained at McGill University, whereas the $eNOS^{-/-}$ mouse colony was placed at the University of Virginia. Wild-type (WT) C57BL/6 mice (the background strain for both $eNOS^{-/-}$ and $nNOS^{-/-}$ mice) were purchased from Charles River Inc. The roles of eNOS and nNOS isoforms in NO_2Tyr formation in normal muscles were assessed by comparing diaphragm NO_2Tyr levels among control $nNOS^{-/-}$ and $eNOS^{-/-}$ mice and their corresponding wild-type animals. These groups were designated as control–$nNOS^{-/-}$, control–$nNOS^{-/-}$, control–$eNOS^{+/+}$ and control–$eNOS^{-/-}$ mice. Sampling of the diaphragms from these animals was performed as mentioned above. The involvement of the nNOS isoform in sepsis-induced NO_2Tyr formation was evaluated by injecting two groups ($n = 6$ in each group) of $nNOS^{+/+}$ (LPS–$nNOS^{+/+}$) and $nNOS^{-/-}$ (LPS–$nNOS^{-/-}$) mice with *E. coli* LPS (20 mg/kg i.p.). Using an identical approach, we compared diaphragmatic NO_2Tyr formation after 24 h of *E. coli* LPS (20 mg/kg) injection in $eNOS^{+/+}$ (LPS–$eNOS^{+/+}$) and $eNOS^{-/-}$ (LPS–$eNOS^{-/-}$) mice. All animals were killed after 24 h of LPS injection and the diaphragm was excised and frozen as mentioned above.

Muscle Sample Preparation

Fractionation of muscle samples. Separation of mitochondrial, membrane, and cytosolic muscle fractions was achieved using the protocol of Rock and colleagues (18). The entire procedure was done at 4°C. In brief, frozen muscle samples were homogenized in 6 vol/wt ice-cooled homogenization buffer A (tris-maleate 10 mM, EGTA 3 mM, sucrose 275 mM, DTT 0.1 mM; leupeptin 2 μg/ml; PMSF 100 μg/ml; aprotinin 2 μg/ml, and pepstatin A 1 mg/100 ml, pH 7.2). Samples were then centrifuged at 1000 × *g* for 10 min. The pellet (P1) was discarded, whereas the supernatant (S1) was designated as *crude homogenates*. These homogenates were then centrifuged at 12,000 × *g* for 20 min to yield supernatant (S2) and pellet (P2). Pellet (P2) was then resuspended in buffer B (tris-maleate 10 mM, EDTA 0.1 mM, and KCl 135 mM) and then centrifuged at 12,000 × *g* for 20 min to yield S3 and P3. The resulting pellet (P3) was resuspended in buffer A and designated as the *mitochondrial fraction*. Both S2 and S3 fractions were pooled and were used to separate the membrane and cytosolic fractions by centrifugation for 1 h at 100,000 × *g*. The resulting supernatant (S4) was designated as the *cytosolic fraction*, whereas the pellet (P4) was resuspended in buffer C (HEPES 10 mM and sucrose 300 mM, pH 7.2), treated for 1 h with 600 mM KCl, and then centrifuged again at 100,000 × *g* for 1 h. Pellet was resuspended in buffer A and designated as the *membrane fraction*.

Fractionation of muscle samples into myofibrillar, membrane, and cytosolic fractions was performed according to the protocol of Fagan and colleagues (19). In brief, muscle samples were homogenized in ice-cooled pyrophosphate buffer (tris-maleate 0.01M, 0.1M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 2 mM $Na_4P_2O_7$, 0.1M Na_2PO_3, and 1 mM DTT, pH 6.8). Samples were then centrifuged at 1000 × *g* for 10 min. The pellet (P1) was then washed four times with 10-vol low-salt buffer followed by one wash with low-salt buffer containing Triton X-100 (0.02%) and one wash with sodium deoxycholate (0.02%). The pellet was then washed two additional times in low-salt buffer and was finally suspended in pyrophosphate buffer and designated as the myofibrillar fraction. The supernatant (S1) was then centrifuged at 100,000 × *g* for 1 h to yield supernatant (S2, cytosolic fraction). The resulting pellet (P2) was resuspended in buffer A (*see above*) and designated as membrane fraction. Protein concentrations of various muscle fractions were measured according to the Bradford technique (BioRad Inc.).

Immunoblotting. Crude homogenates and various muscle fractions of rat diaphragmatic samples (80 μg per sample) were

mixed with sample buffer, boiled for 5 min at 95°C, and were then loaded onto 8 or 10% tris-glycine sodium dodecylsulfate (SDS) polyacrylamide gels and separated by electrophoresis (150 V, 30 mA for 1.5 h). Lysates of human and rat neutrophils were used as positive controls (including control for species differences). Proteins were transferred electrophoretically (25 V, 375 mA for 2 h) to methanol presoaked polyvinylidene difluoride (PVDF) membranes, and then blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. The PVDF membranes were subsequently incubated with primary monoclonal or polyclonal antibodies raised against NO_2Tyr and dissolved in 1% BSA. In few samples, we also probed membrane with monoclonal antibodies specific to iNOS and nNOS proteins (Transduction Laboratories Inc.). In addition, the efficiency of separating muscle samples into different fractions was verified by probing various muscle fractions with selective antibodies to cytochrome oxidase (mitochondrial marker), caveolin-3 (marker of sarcolemma), and troponin I (myofibril protein marker). After three 10-min washes with wash buffer on rotating shaker, the PVDF membranes were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies. Specific proteins were detected with a chemiluminescence kit (Chemicon Inc.) The blots were scanned with an imaging densitometer and optical densities (OD) of positive nitrotyrosine protein bands were quantified with SigmaGel software (Jandel Scientific Inc.) Total NO_2Tyr OD was calculated for each sample by adding OD of individual positive protein band. Specificity of anti-NO_2Tyr antibodies was evaluated by preincubation of each primary antibody with either 10-mM nitrotyrosine or 10-fold excess peroxynitrite-tyrosine–nitrated bovine serum albumin (generously provided by Dr. Ischiropoulos, University of Pennsylvania).

Immunohistochemistry. Frozen tissues sections (5–10 μm thickness) were adsorbed to microscope slides and dried. The sections were fixed with acetone at −20°C, rehydrated with PBS containing 1% BSA (pH 7.4), and were then blocked with solutions of avidin, biotin (15 min each at room temperature), and then 3% BSA for 30 min. The sections were incubated for 1 h at room temperature with primary monoclonal or polyclonal antibodies raised against NO_2Tyr. For negative control, the primary antibody was replaced with nonspecific mouse or rabbit IgG. After three rinses with PBS, sections were incubated with biotin-conjugated anti-mouse or anti-rabbit secondary antibodies at room temperature for 1 h followed by exposure to Cy3-labeled strepavidin for 1 h. Sections were then washed, mounted with coverslips, and examined with a Nikon fluorescence microscope and photographed with a 35 mm camera (Nikon Inc.).

Statistical analysis. Values are presented as means ± SEM. Differences in OD of individual NO_2Tyr protein band or total sample NO_2Tyr OD between various conditions were compared with one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons. *P* values of less than 5% were considered significant.

Results

Protein Tyrosine Nitration in Normal Muscles

Figure 1 illustrates NO_2Tyr immunoreactivity in normal rat diaphragms detected with three different antibodies. Monoclonal anti-NO_2Tyr antibody (Cayman Chemical Inc.) detected five nitrated protein bands of 52, 48, 40, 30, and 10 kD. (Figure 1A). The ODs of each of these protein bands expressed as a percentage of total muscle NO_2Tyr OD are listed in Table 1. Polyclonal anti-NO_2Tyr antibody (Upstate Biotechnology) also detected the above-mentioned nitrated proteins in addition to an 18-kD protein band (Figure 1 B). The intensity of nitrated protein bands detected with the polyclonal antibody differed from those delineated by the monoclonal antibody (Table 1). Similarly, HRP-conjugated anti-NO_2Tyr polyclonal antibody (Academy Biomedical) detected positively nitrated protein bands of 52, 40, 30, 18, and 10 kD; however, an additional positive band of 66 kD was also detected and that of 48 kD was not detected in normal rat diaphragms (Figure 1C). Unlike the monoclonal antibody, which preferentially detects 30- and 52-kD protein bands, the intensity of the 10-kD protein band was the highest proportion of total diaphragmatic NO_2Tyr signal detected with the HRP-conjugated anti-NO_2Tyr antibody (Figure 1C and Table 1). When various rat muscles were compared with respect to nitrotyrosine immunoreactivity, intercostals muscles showed similar pattern of nitrotyrosine proteins to that of the diaphragm, whereas gastrocnemius and soleus muscles have weaker protein nitration at 10 kD compared with the diaphragm and intercostals muscles (Figure 1D). Figure 1E illustrates the specificity of monoclonal anti-NO_2Tyr in detecting NO_2Tyr formation in rat diaphragm. Preincubation of monoclonal anti-NO_2Tyr antibody with 10-fold excess of peroxynitrite-tyrosine–nitrated bovine serum albumin completely eliminated NO_2Tyr immunoreactivity (*two right lanes*, *underlined*). Similar results were obtained when this antibody or polyclonal antibodies were preincubated with 10 mM nitrotyrosine (data not shown).

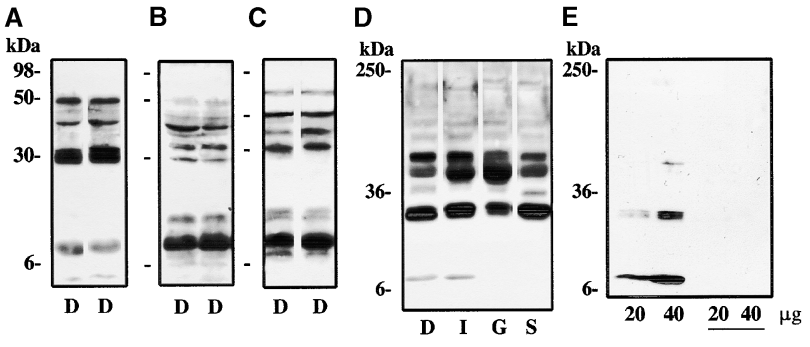


Figure 1. Representative immunoblots of two different rat diaphragmatic (*D*) homogenates probed with monoclonal (Cayman Chemical Inc. [*A*]), polyclonal (Upstate Biotechnology Inc. [*B*]) and HRP-conjugated polyclonal (Academy Biomedical Co. [*C*]) anti-NO_2Tyr antibodies. Note the differences in the intensity of individual protein bands among the three immunoblots. (*D*) A representative immunoblot of rat diaphragm (*D*), intercostals (*I*), gastrocnemius (*G*), and soleus (*S*) muscles probed with monoclonal anti-NO_2Tyr antibody. (*E*) Selectivity of monoclonal anti-NO_2Tyr antibody. Rat diaphragmatic samples (20 and 40 μg total protein per lane) were probed with monoclonal anti-NO_2Tyr antibody (*two left lanes*). The *two right lanes* (*underlined*) show diaphragmatic muscle samples probed with the same antibody, which was preincubated with 10-fold excess of nitrated bovine serum albumin. Note that this preincubation resulted in disappearance of positive nitrotyrosine protein bands shown in the *left two lanes*.

TABLE 1

ODs of various nitrotyrosine protein bands as percentage of total nitrotyrosine OD detected in the diaphragm of normal rats using various anti-nitrotyrosine antibodies

OD (% of Total)	66 kD	52 kD	48 kD	40 kD	30 kD	18 kD	10 kD
Monoclonal antibody	—	21.3 ± 0.8	13.6 ± 0.6	17.6 ± 0.4	30.6 ± 1.5	—	16.8 ± 2.0
Polyclonal antibody	—	19.2 ± 0.2	12.5 ± 0.8	17.7 ± 1.7	12.6 ± 0.5	12.6 ± 0.8	24.8 ± 1.8
HRP-polyclonal antibody	6.5 ± 0.5	20.7 ± 3.7		14.0 ± 5.0	19.7 ± 1.0	10.3 ± 1.7	27.7 ± 1.1

Definition of abbreviations: optical densities, OD.
Values are mean ± SE (n = 5 samples detected with each antibody).

Figure 2 illustrates the presence of NO_2Tyr immunoreactivity in various muscle fractions. Monoclonal anti-NO_2Tyr antibody detected positive NO_2Tyr protein bands mainly in the cytosolic fraction, whereas only weak bands were detected in the myofibrillar, membrane, and mitochondrial fractions (Figures 2A and 2B).

Localization of nitrotyrosine immunoreactivity in normal rat muscles is shown in Figure 3. Monoclonal anti-NO_2Tyr antibody detected positive staining in gastrocnemius (Figures 3A and 3B) and soleus (Figure 3C) in close proximity to the sarcolemma. Positive NO_2Tyr staining was also detected in nerve fibers (*arrows* in Figure 3D), but not in blood vessels traversing skeletal muscles (Figure 3D). Polyclonal anti-NO_2Tyr also detected positive NO_2Tyr in the diaphragm of normal rats in close proximity to the sarcolemma (Figure 3E). We should emphasize that we detected in this muscle punctate cytosolic-positive NO_2Tyr staining suggesting that a proportion of tyrosine-nitrated proteins is not close to the sarcolemma. Replacement of primary antibodies with nonspecific mouse IgG or rabbit IgG completely eliminated positive NO_2Tyr staining (Figure 3F).

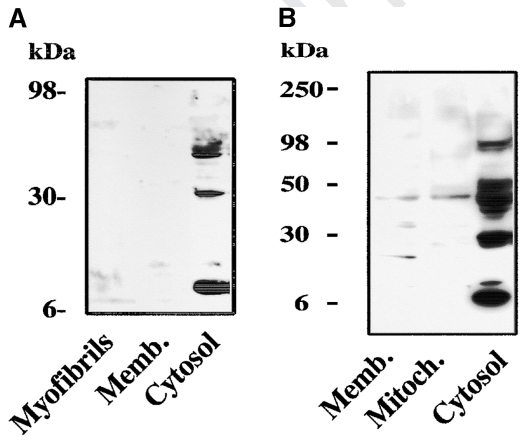


Figure 2. (*A*) Representative immunoblot of myofibrillar, membrane, and cytosolic fractions of normal rat diaphragm samples probed with monoclonal anti-NO_2Tyr antibody. (*B*) Membrane, mitochondrial and cytosolic fractions of a rat diaphragm probed with monoclonal anti-NO_2Tyr antibody. Note that the majority of tyrosine nitrated protein bands are localized in the cytosolic fraction.

Role of NOS in Protein Tyrosine Nitration in Normal Muscles

Examination of crude homogenates (Figure 4A) and various muscle fractions (Figure 4B) after 1 h of *in vivo* administration of either a selective iNOS inhibitor (1400W) or nonselective NOS inhibitor (L-NAME) in normal rats revealed significant reductions in total diaphragmatic NO_2Tyr levels (Figure 4C). Administration of 1400W reduced NO_2Tyr OD at 52 and 48 kD to 72 and 64% of control diaphragms, respectively ($P < 0.05$). Those of 30 and 10 kD remained similar to control muscles. The inhibitory effect of 1400W on tyrosine nitration of 52- and 48-kD proteins was much more pronounced when muscle homogenates were fractioned into mitochondrial, cytosolic, and membrane fractions (Figure 4B). L-NAME administration produced relatively larger reduction in NO_2Tyr levels of crude diaphragm homogenates compared with that elicited by 1400W, and lowered NO_2Tyr ODs of 52-, 48-, and 30-kD protein bands to 20, 48, and 81% of control muscles, respectively ($P < 0.01$).

In addition to pharmacologic inhibition of NOS activity in rats, we compared diaphragmatic NO_2Tyr OD among wild-type mice and mice deficient in either iNOS, eNOS, or nNOS (knockout) isoforms (Figure 5). Anti-NO_2Tyr antibodies detected in the diaphragms of control–$iNOS^{+/+}$ (B129/C57Bl6 wild-type), control–$nNOS^{+/+}$ and control–$eNOS^{+/+}$ (Bl6 wild-type) mice several nitrated protein bands, which are similar to those detected in the diaphragms of normal rats (Figures 5A–5C). In control–$iNOS^{-/-}$ mice, the intensities of these bands, particularly those of 52-, 48-, 40-, and 30-kD protein bands, were significantly lower than in $iNOS^{+/+}$ mice (Figure 5A). By comparison, absence of nNOS or eNOS proteins has no effects on the intensity of NO_2Tyr protein bands compared with wild-type mice (Figures 5B and 5C). Figure 5D shows total diaphragmatic NO_2Tyr ODs in the six groups of control animals. Total NO_2Tyr OD was significantly lower in $iNOS^{-/-}$ mice compared with that of $iNOS^{+/+}$ mice ($P < 0.01$), whereas no significant differences were observed in total diaphragmatic NO_2Tyr levels between $eNOS^{-/-}$ and $nNOS^{-/-}$ animals and their corresponding wild-type animals.

Protein Tyrosine Nitration in Septic Muscles

The influence of sepsis on NO_2Tyr formation in the ventilatory and limb muscles was assessed both in rats and

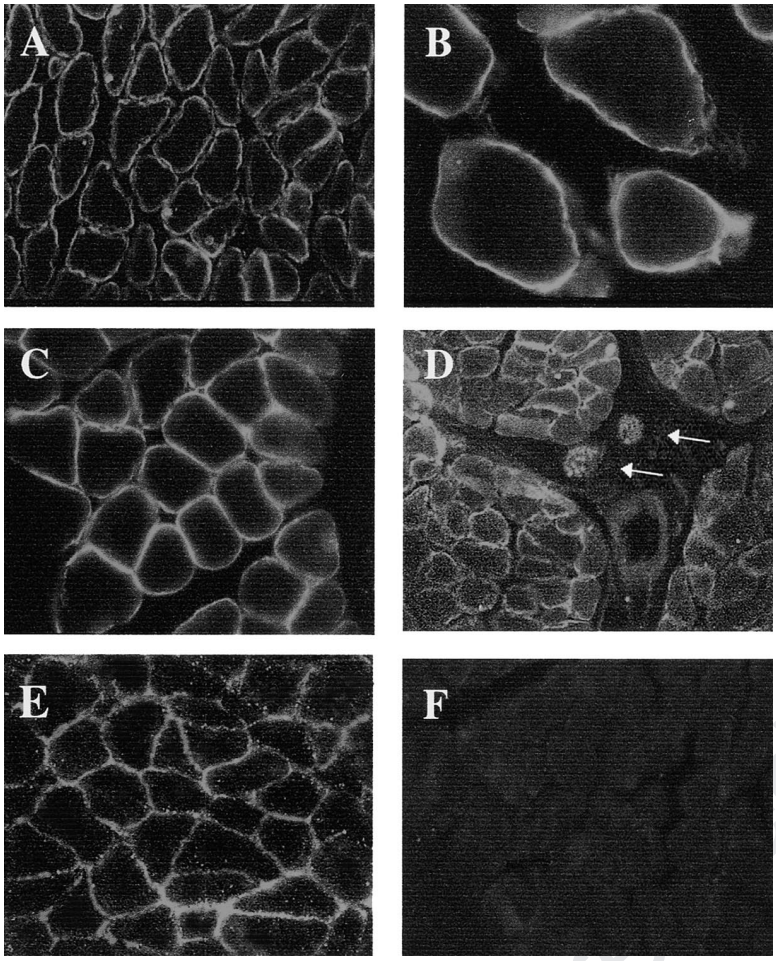


Figure 3. Immunohistochemical localization of tyrosine-nitrated proteins in normal rat muscles. Monoclonal anti-NO_2Tyr antibody detected positive staining in gastrocnemius (*A* and *B*) and soleus (*C*) muscle sections in close proximity to the sarcolemma. Positive NO_2Tyr staining was also detected in nerve fibers (*arrows* in *D*) but not in blood vessels traversing gastrocnemius muscle. Detection of protein tyrosine nitration in the diaphragm of rats with polyclonal anti-NO_2Tyr antibody is shown in (*E*), whereas (*F*) shows negative control staining in which the primary anti-NO_2Tyr antibody was replaced by nonspecific mouse IgG. Magnification of all panels except (*B*) is ×200. (*B*) is magnified at ×400.

mice. Injection of *E. coli* LPS in rats elicited a significant induction of the iNOS isoform in the diaphragm and intercostals muscles, which peaked after 12 h of LPS administration (Figure 6). In addition to iNOS induction, LPS injection produced a significant rise in nNOS protein expression, which peaked after 24 h of LPS administration (Figure 6). Injection of LPS in rats produced a significant rise in NO_2Tyr OD of crude diaphragmatic homogenates with total NO_2Tyr OD after 6, 12, and 24 h of LPS injection reaching 120 ± 3, 134 ± 7, and 175 ± 6% of control samples, respectively ($P < 0.05$, Figure 7A). Fractionation of muscle homogenates revealed that the increase in NO_2Tyr formation in response to LPS injection was localized mainly in the mitochondrial and membrane fractions rather than in the cytosolic fraction (Figures 7B and 7C). The rise in mitochondrial and membrane NO_2Tyr levels in response to LPS injection was evident mainly at 52-, 48-, and 30 kD-protein bands rather than at the 10-kD band.

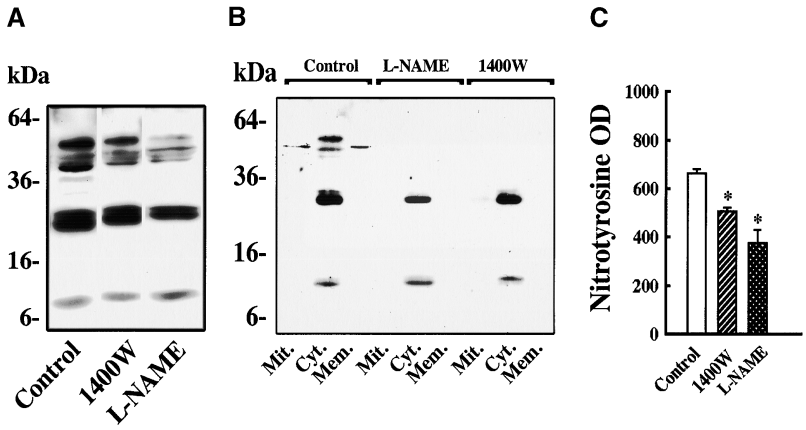


Figure 4. Effects of acute (within 1 h) inhibition of NO synthesis on the intensity of protein tyrosine nitration in the crude lysates (*A*) and in the mitochondrial, cytosolic, and membrane fractions (*B*) of the diaphragm. Rats were injected with either 1400W (selective iNOS inhibitor) or L-NAME (nonselective NOS inhibitor). (*C*) Total muscle NO_2Tyr OD of control, 1400W-, and L-NAME–treated animals. *$P < 0.05$ compared with control.

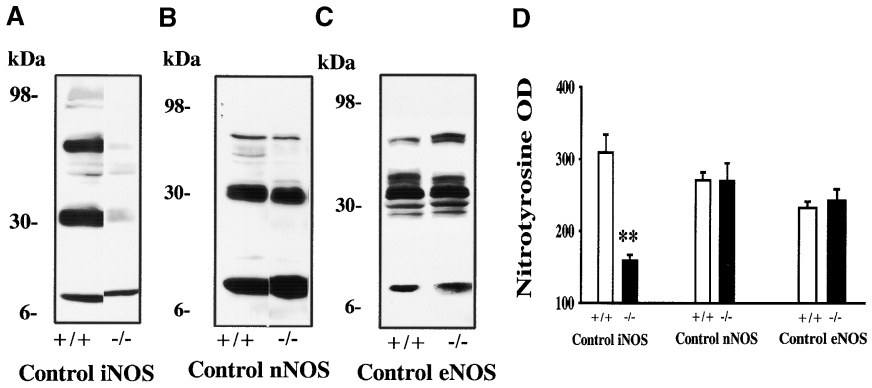


Figure 5. (*A–C*) Representative NO_2Tyr immunoblots of diaphragmatic samples obtained from control $iNOS^{-/-}$, $nNOS^{-/-}$, and $eNOS^{-/-}$ mice and their corresponding wild-type ($^{+/+}$) animals. Note that the absence of iNOS but eNOS or nNOS had a major effect on tyrosine nitration of diaphragmatic proteins. (*D*) Mean values of total diaphragmatic NO_2Tyr OD in control wild-type and NOS knockout mice. $^{**}P <$ 0.01 compared with wild-type mice. Note that the total diaphragmatic NO_2Tyr OD is significantly lower in $iNOS^{-/-}$ mice compared with $iNOS^{+/+}$ mice.

Selective inhibition of iNOS activity in septic rats by the administration of 1400W before and after LPS injection resulted in significant attenuation of LPS-induced rise in diaphragmatic NO_2Tyr formation. Indeed, NO_2Tyr OD of 52-, 48-, and 30-kD protein bands in septic rats that received 1400W reached 63, 76, and 77% of corresponding values in rats receiving only LPS, respectively ($P < 0.05$).

The involvement of NOS isoforms in LPS-induced rise in muscle NO_2Tyr formation was also assessed in wild-type mice, $iNOS^{-/-}$, $nNOS^{-/-}$, and $eNOS^{-/-}$ mice. LPS injection in $iNOS^{+/+}$ mice increased total diaphragmatic NO_2Tyr OD by a mean value of 56% of control–$iNOS^{+/+}$ mice ($P < 0.05$, Figure 8A). Only the 10-kD protein band did not show any rise in tyrosine nitration in response to LPS injection in $iNOS^{+/+}$ mice (Figure 8A). By comparison, LPS injection in $iNOS^{-/-}$ mice did not elevate diaphragmatic tyrosine nitration (mean values of 91% of control–$iNOS^{-/-}$ mice, Figure 8A). Injection of LPS in $nNOS^{+/+}$ and $nNOS^{-/-}$ mice produced a similar degree of increased tyrosine nitration in diaphragmatic lysates (mean values of 161 and 168% of corresponding control animals, respectively). Moreover, similar augmentation in diaphragmatic tyrosine nitration was noticed among $eNOS^{+/+}$ and $eNOS^{-/-}$ mice in response to LPS injection (154 and 149% of corresponding control animals, respectively). Figure 8B summarizes total diaphragm NO_2Tyr OD measured in response to LPS injection in $nNOS^{-/-}$, $iNOS^{-/-}$, and $eNOS^{-/-}$ mice (results are expressed as % of their corresponding septic wild-type animals). Whereas total tyrosine nitration in LPS–$nNOS^{-/-}$ and LPS–$eNOS^{-/-}$ mice was similar to their corresponding wild-type animals, total tyrosine nitration in LPS–$iNOS^{-/-}$ mice was significantly lower than LPS–$iNOS^{+/+}$ mice. These results suggest that the absence of iNOS, but not nNOS or eNOS, significantly alters the response of muscle tyrosine nitration to LPS injection.

Discussion

The main findings of this study are that (*i*) abundant NO_2Tyr formation is detected in the cytosolic fraction of normal rat and mouse ventilatory and limb muscles; (*ii*) NO_2Tyr immunoreactivity in the ventilatory and limb muscles is limited to specific protein bands ranging in apparent molecular mass between 66 and 10 kD; (*iii*) pharmacologic inhibitors and knockout mice experiments revealed that in normal ventilatory muscles, the iNOS isoform is a major contributor to NO_2Tyr formation; and finally, (*iv*) injection of LPS resulted in augmentation of muscle NO_2Tyr formation, particularly in the mitochondrial and membrane fractions, and was dependent in large part on muscle iNOS activity.

Little information is available regarding NO_2Tyr formation in the ventilatory and limb muscles. Supinski and colleagues (20) used dot-blotting technique to demonstrate a significant increase in NO_2Tyr formation in the diaphragm of septic rats. Our group reported recently that two protein bands (50 and 42 kD) are tyrosine nitrated in

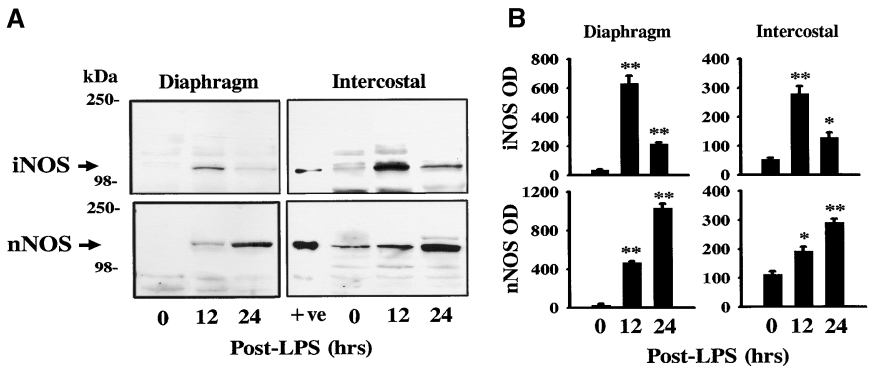


Figure 6. (*A*) Expression of iNOS (*top*) and nNOS (*bottom*) proteins in the diaphragm and intercostals muscles of rats in response to LPS injection. 0 refers to control animals, whereas +ve refers to positive controls. Note the transient nature of iNOS expression and the upregulation of nNOS protein after 24 h of LPS administration. (*B*) Optical densities of nNOS and iNOS proteins (mean values of five animals) in the diaphragm and intercostals muscles of control (0 h) and LPS-injected animals. *, ** $P <$ 0.05 and 0.01, respectively, compared with control values (0 h).

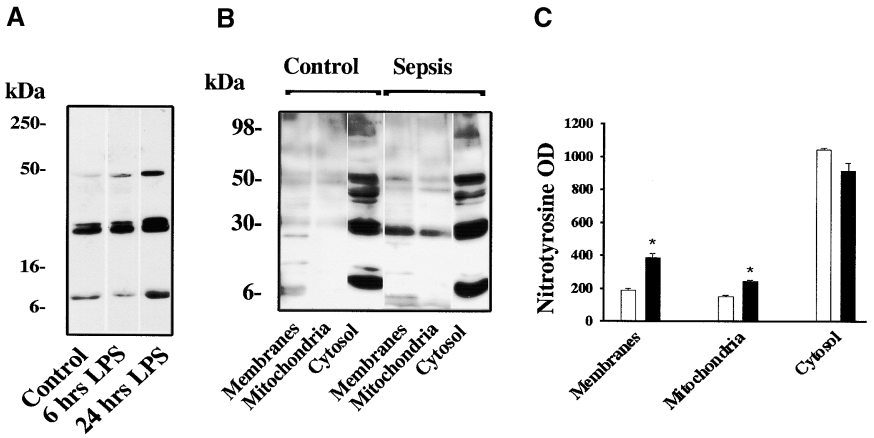


Figure 7. (*A*) Influence of LPS injection on diaphragmatic NO_2Tyr formation. Notice that NO_2Tyr levels rose substantially 24 h after LPS compared with control samples. (*B*) Localization of NO_2Tyr formation in control and septic diaphragmatic muscle samples. Notice the appearance of tyrosine-nitrated proteins in the mitochondrial and membrane fractions but not the cytocolic fractions of rat diaphragms in response to LPS injection. (*C*) Mean values of total NO_2Tyr OD in the three fractions of diaphragmatic samples obtained from control (*open bars*) and septic (24 h after LPS injection; *filled bars*) rats. $*P < 0.05$ compared with control animals.

normal rat diaphragms, and two additional protein bands (196 and 86) are heavily nitrated in the diaphragm of septic rats (6). In a more recent study, Boczkowski and colleagues (8) described tyrosine nitration of a single 105-kD protein band in diaphragmatic mitochondria of septic rats and in response to exposure of isolated muscle mitochondria to SIN-1 (peroxynitrite donor). We detected in the current study abundant protein tyrosine nitration in the ventilatory and limb muscles of both normal rats and mice. This tyrosine nitration was noticeable with three different antibodies, though various intensities of specific proteins bands were apparent depending on the type of the antibody used. The fact that more tyrosine nitrated protein bands were detected with polyclonal antibodies is not surprising since these antibodies are capable of detecting more diverse epitopes than monoclonal antibodies. We attribute the failure to detect abundant protein nitration in normal skeletal muscles in previous studies to lack of protein separation (as in Supinski and colleagues [20]), incomplete separation of various proteins (as in our previous study [6]), or measurement of NO_2Tyr formation in one muscle fraction (mitochondria, in Boczkowski and colleagues [8]).

Our finding of a significant reduction in intensity of tyrosine-nitrated proteins in the diaphragm of rats injected with NOS inhibitors and in the diaphragms of $iNOS^{-/-}$ mice confirms that NO_2Tyr formation in skeletal muscle fibers is dependent on NO production. Moreover, we found that the level of specific tyrosine nitrated proteins (52 and 48 kD) declined significantly within 1 h of NOS inhibition, whereas other nitrated proteins (30 and 10 kD) were less sensitive to rapid NOS inhibition (Figure 4). We speculate that increased turnover rate of tyrosine-nitrated proteins can explain this finding. Our speculation in this respect is supported by the observation of Souza and colleagues (21), namely, that protein nitration enhances susceptibility to degradation by the proteasome. Another possible mechanism for rapid decline in tyrosine nitration after 1 h of NOS inhibition is that an enzymatic mechanism exists to denitrate nitrated tyrosine residues. The existence of such mechanism (nitrotyrosine denitrase) has been proposed by Kamisaki and colleagues (22), who found that homogenates of rat lung and spleen are capable of modifying tyrosine-containing proteins in a time and protein concentration-dependent fashion. The exact nature and molecular structure of "nitrotyrosine denitrase" remain to be elucidated.

Although the exact contribution of each of the NOS isoforms expressed in skeletal muscle fibers to protein tyrosine nitration in normal muscle remains speculative, our experiments in which NOS isoforms were pharmacologically inhibited or genetically altered in mice clearly suggest that the iNOS isoform is the main contributor to NO_2Tyr formation in normal muscles. These results are rather surprising since relatively low levels of iNOS protein have been detected in normal skeletal muscles of various species compared with the other two isoforms (nNOS and eNOS) (6, 23, 24). It is possible that even low levels of iNOS protein are sufficient to elicit protein tyrosine nitration in skeletal muscles because of the much higher rate of NO synthesis by the iNOS isoform compared with the nNOS and eNOS isoforms. Although previous studies in neurons suggest that the nNOS isoform is involved in protein tyrosine nitration (25), our results using $nNOS^{-/-}$ and

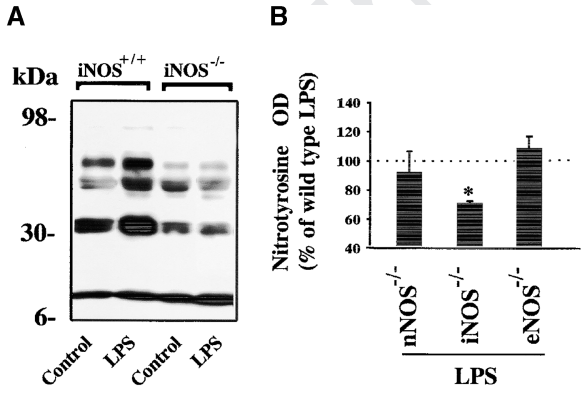


Figure 8. (*A*) Comparison of diaphragmatic protein tyrosine nitration in wild-type ($iNOS^{+/+}$) and iNOS knockout ($iNOS^{-/-}$) mice under control condition and after 24 h of LPS injection. (*B*) Mean values of total NO_2Tyr OD of diaphragmatic muscle samples obtained after 24 h of LPS injection in $iNOS^{-/-}$, $nNOS^{-/-}$, and $eNOS^{-/-}$ mice. Values are expressed as percentage of corresponding septic wild-type animals. $*P < 0.05$ compared with wild-type animals.

$eNOS^{-/-}$ mice suggest that both of these isoforms do not play a major role in protein tyrosine nitration in normal skeletal muscles.

Previous studies have documented an increase in protein tyrosine nitration in the ventilatory muscles of septic humans and rats (6–8). It has also been shown that systemic inhibition of NOS in septic animals reduces mitochondrial tyrosine nitration (8). The contribution, however, of various NOS isoforms to the rise in protein tyrosine nitration in septic animals or the presence of tyrosine nitration in various muscle compartments were not assessed in the above-mentioned studies. We found that muscle NO_2Tyr levels increase significantly in septic animals and that tyrosine nitration was mainly localized in the mitochondrial and membrane fractions but not in the cytosolic fraction. Our results also indicate that LPS-induced protein nitration is mediated primarily by the iNOS isoform but not by the nNOS and eNOS isoforms despite the fact that the levels of these isoforms in the ventilatory muscles are elevated in response to LPS injection (Figure 6) (6). The mechanisms responsible for *in vivo* nitration of tyrosine residues remain the focus of intense investigation and debate over the past several years. The most widely accepted mechanism of *in vivo* tyrosine nitration is peroxynitrite, which is formed from the near diffusion-limited reaction between NO and superoxide anions (1). The identity of peroxynitrite as the reaction product of NO and superoxide anions, as well as its ability of peroxynitrite to nitrate tyrosine residues at physiologic pH has recently been confirmed by Reiter and colleagues (26). We propose that protein tyrosine nitration in skeletal muscles is mediated primarily by peroxynitrite and that iNOS is the primary source of NO required for peroxynitrite formation. Other proposed pathways mediating protein tyrosine nitration include the reaction of NO with tyrosyl radical generated by prostaglandin H synthase-2 (3), and oxidation of NO_2^- by H_2O_2 at physiologic pH, which can result in the formation of peroxynitrous acid and consequently lead to nitration of tyrosine residues (2). We speculate that these two pathways are not likely to contribute to tyrosine nitration in skeletal muscles simply because the first pathway necessitates the presence of abundant prostaglandin H synthase-2 expression, which is not usually present in skeletal muscle fibers. The second pathway requires relatively high concentrations of H_2O_2 (> 1 mM), although normal skeletal muscle H_2O_2 levels are quite small (27). Finally, it has been proposed recently that myeloperoxidase (MPO), utilizing both NO_2^- and H_2O_2, is capable of nitrating tyrosine residues (28). We believe that this pathway does not play a major role in tyrosine nitration in normal skeletal muscle because MPO is localized mainly in polymorphonuclear leukocytes, whereas protein tyrosine nitration is detected inside muscle fibers. Moreover, the MPO pathway requires relatively high levels of NO_2^- and H_2O_2 and more than 1 h to produce tyrosine nitration (29).

A major finding in our study is that muscle NO_2Tyr is limited to specific protein bands ranging in apparent mass between 66 and 10 kD, though the intensity of these bands in normal muscles and the changes in their intensity in response to inhibition of NOS differed significantly depending on the antibody used. Tyrosine nitration is a selective process, which is dependent on several factors such as the nature of the nitrating agent, the exposure of the aromatic ring to the surface of protein, the location of tyrosine on a loop structure, and the presence of glutamate in the local environment of the tyrosine residue (2). Interestingly, tyrosine nitration is not influenced by protein abundance or the abundance of tyrosine residues in a given protein (2). Major progress has recently been made in identifying tyrosine-nitrated proteins. The majority of these proteins are cytosolic, including catalase, glutathione-S-transferase, carbonic anhydrase III, tyrosine hydroxylase, cAMP-dependent protein kinase, lactate dehydrogenase, glycogen synthase, and transketolase (12, 13, 30). In addition, cytoskeletal proteins such as actin, neurofilament L, tubulin, and myofibrillar creatine kinase have recently been shown to be tyrosine nitrated (11, 12, 32, 33). Tyrosine nitration of several mitochondrial proteins such as aconitase, Mn-SOD, ATP synthase, glutamate dehydrogenase, and glutamate oxaloacetate transaminase-2 has also been described (12, 33). Finally, recent studies suggest that nuclear proteins such as histones II-S and VIII-S can be tyrosine nitrated (13). The influence of tyrosine nitration on protein function remains in most cases unclear. There is, however, evidence that tyrosine nitration may result in loss of function. For instance, nitration of specific tyrosine residues of Mn-SOD or α1-antitrypsin causes a significant inhibition of activity of these proteins (33, 34). Nitration of C-terminal tyrosine residue in α-tubulin compromises microtubule organization and binding of microtubule-associated proteins (32).

Our findings that the majority of tyrosine-nitrated proteins in normal skeletal muscles are residing in the cytosol is in agreement with the observations Aulak and colleagues (12) that described NO_2Tyr formation in cultured A459 cells and in the liver and lungs of LPS-injected rats. We did observe, however, that protein tyrosine nitration rose significantly in the mitochondrial and membrane fractions in response to LPS injection. Although our study does not provide a comprehensive list of tyrosine-nitrated proteins in skeletal muscles, we can exclude several proteins from the list of tyrosine-nitrated proteins (12, 13, 32, 33). The fact that we did not observe abundant tyrosine nitration in the myofibrillar fraction excludes the possibility of sarcomeric actin and myofibrillar creatine kinase. Moreover, we can exclude Mn-SOD from the list of possible tyrosine-nitrated proteins in our study because the apparent molecular masses of tyrosine-nitrated proteins in various muscle fractions do not correspond to that of Mn-SOD and that this protein is localized in the mitochondria where little tyrosine nitration was detected in normal muscles. Other previously described tyrosine-nitrated proteins such as neurofilament L and lung surfactant protein A can also be excluded because they are not usually abundantly expressed inside normal skeletal muscle fibers. One possible candidate for tyrosine nitration in the cytosol of normal muscles is tubulin, which has a similar molecular mass (49 kD) to protein bands detected by various anti-NO_2Tyr antibodies in normal muscle samples (Figure 1). Both the α and β isoforms of tubulin are known to undergo tyrosine nitration under specific conditions (12, 32). We have conducted several experiments in which tyrosine-nitrated pro-

teins in normal diaphragm muscle lysates were immunoprecipitated with monoclonal and polyclonal anti-NO_2Tyr antibodies. The immunocomplexes were then separated on SDS-PAGE, transferred to PVDF membranes and probed with anti-tubulin antibody (Sigma Chemical Inc.). We were unable to detect positive tubulin protein bands in the NO_2Tyr immunocomplexes suggesting that tubulin is not tyrosine nitrated in normal rat diaphragm. Clearly, more research is needed to elucidate the identity of tyrosine-nitrated proteins in skeletal muscle.

In summary, our study indicates that there is abundant protein tyrosine nitration in the cytosol of both ventilatory and limb muscles and that tyrosine nitration is limited to specific proteins ranging in molecular mass between 66 and 10 kD. We also found that sepsis elicits a significant augmentation of NO_2Tyr formation in the ventilatory muscles that is localized in the mitochondrial and membrane fractions. Finally, NO_2Tyr formation both in normal and septic muscles appears to be dependent on the activity of the inducible NOS.

Acknowledgments: The authors are grateful to Mr. Luigi Franchi for his technical assistance and to Ms. C. Mutter and Ms. R. Carin for their assistance in editing the manuscript. This study is funded by a grant from the Canadian Institute of Health Research. Dr. S. Hussain is a scholar of F.R.S.Q. Dr. E. Barreiro is supported by FUCAP, SOCAP, (Spain) and Biomeed (EU).

References

1. Beckman, J. S. and W. H. Koppenol. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol. (CellPphysiol.)* 271:c1424–c1437.
2. Ischiropoulos, H. 1998. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Bioche. Bioph.* 356:1–11.
3. Gunther, M. R., L. C. Hsi, J. F. Curtis, J. K. Gierse, L. J. Marnett, T. E. Eling, and R. P. Mason. 1997. Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical nitrotyrosine formation. *J. Biol. Chem.* 272:17086–17090.
4. van der Vliet, A., J. P. Eserich, M. K. Shigenaga, and C. E. Ross. 1999. Reactive nitrogen species and tyrosine nitratin in the respiratory tract: epiphenomena or pathologic mechanism of disease? *Am. J. Respir. Crit. Care Med.* 160:1–9.
5. Knowles, M. E., D. J. McWeeny, L. Couchman, and M. Thorogood. 1974. Interaction of nitrite with proteins at gastric pH. *Nature* 247:288–289.
6. El-Dwairi, Q., A. Comtois, Y. Guo, and S. N. A. Hussain. 1998. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. *Am. J. Physiol.* 274:C770–C779.
7. Lanone, S., A. Mebazaa, C. Heymes, D. Henin, J. J. Poderoso, Y. Panis, C. Zedda, T. Billiar, D. Payen, M. Aubier, and J. Boczkowski. 2000. Muscular contractile failure in septic patients: role of the inducible nitric oxide synthase pathway. *Am. J. Respir. Crit. Care Med.* 162:2308–2315.
8. Boczkowski, J., C. L. Lisdero, S. Lanone, A. Samb, M. C. Carreras, A. Boveris, M. Aubier, and J. Poderoso. 1999. Endogenous peroxynitrite mediates mitochondrial dysfunction in rat diaphragm during endotoxemia. *FASEB J.* 13:1637–1647.
9. Kobzik, L., M. B. Reid, D. S. Bredt, and J. S. Stamler. 1994. Nitric oxide in skeletal muscle. *Nature* 372:546–548.
10. Kobzik, L., B. Stringer, J. L. Balligand, M. B. Reid, and J. S. Stamler. 1995. Endothelial type nitric oxide synthase in skeletal muscle fibers:mitochondrial relationship. *Biochem. Biophys. Res. Commun.* 211:375–381.
11. Mihm, M. J., C. M. Coyle, B. L. Schanbacher, D. M. Weinstein, and J. A. Bauer. 2001. Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. *Cardiovasc. Res.* 49:798–807.
12. Aulak, K. S., M. Miyagi, L. Yan, K. A. West, D. Massillon, J. W. Crabb, and D. J. Stuehr. 2001. Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc. Natl. Acad. Sci. USA* 98:12056–12061.
13. Kuo, W. N., J. M. Kreahling, V. P. Shanbhag, P. P. Shanbhag, and M. Mewar. 2000. Protein nitration. *Mol. Cell. Biochem.* 214:121–129.
14. Reid, M. B. 1996. Reactive oxygen and nitric oxide in skeletal muscle. *News Physiol. Sci.* 11:114–119.
15. Laubach, V. E., E. G. Shesely, O. Smithies, and P. A. Sherman. 1995. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc. Natl. Acad. Sci. USA* 92:10688–10692.
16. Huang, P. L., T. M. Dawson, D. S. Bredt, S. H. Snyder, and M. C. Fishman. 1993. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75:1273–1286.
17. Shesely, E. G., N. Maeda, H.-S. Kim, K. M. Desai, J. H. Krege, V. E. Laubach, P. A. Sherman, W. C. Sessa, and O. Smithies. 2001. Elevated blood pressure in mice lacking endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* 93:13176–13181.
18. Rock, E., C. Napias, C. Sarger, and J. Chevallier. 1985. Simultaneous preparation of membrane fractions from small amounts of skeletal muscle: a study on mitochondrial fractions from Med^J mice. *Biochem. Biophys. Res. Commun.* 128:113–119.
19. Fagan, J. M., M. Ganguly, G. Tiao, J. E. Fischer, and P. O. Hasselgren. 1996. Sepsis increases oxidatively damaged proteins in skeletal muscle. *Arch. Surg.* 131:1326–1332.
20. Supinski, G., D. Stofan, D. Nethery, and A. DiMarco. 1999. Apocynin improves diaphragamtic function after endotoxin administration. *J. Appl. Physiol.* 87:776–782.
21. Souza, J. M., I. Choi, Q. Chen, M. Weisse, E. Daikhin, M. Yudkoff, M. Obin, J. Ara, J. Horwitz, and H. Ischiropoulos. 2000. Proteolytic degradation of tyrosine nitrated proteins. *Arch. Biochem. Bioph.* 380:360–366.
22. Kamisaki, Y., K. Wada, K. Bian, B. Balabanli, K. Davis, E. Martin, F. Behbod, Y.-C. Lee, and F. Murad. 1998. An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc. Natl. Acad. Sci. USA* 95:11584–11589.
23. Gath, I., E. I. Closs, U. Godtel-Armbrust, S. Schmitt, M. Nakane, I. Wessler, and U. Forstermann. 1996. Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications of contractile function. *FASEB J.* 10:1614–1620.
24. Boczkowski, J., S. Lanone, D. Ungureanu-Longrois, G. Danialou, T. Fournier, and M. Aubier. 1996. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats: role of diaphragmatic contractile dysfunction. *J. Clin. Invest.* 98:1550–1559.
25. Ayata, C., G. Ayata, H. Hara, R. T. Mathews, M. F. Beal, R. J. Ferrante, M. Endres, A. Kim, R. H. Christie, C. Waeber, P. L. Huang, B. T. Hyman, and M. A. Moskowitz. 1997. Mechanisms of reduced striated NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. *J. Neurosci.* 17: 6908–6917.
26. Reiter, C. D., R. J. Teng, and J. S. Beckman. 2000. Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J. Biol. Chem.* 275:32460–32466.
27. Nethery, D., L. A. Callahan, D. Stofan, R. Mattera, A. DiMarco, and G. Supinski. 2000. PLA_2 dependence of diaphragm mitochondrial formation of reactive oxygen species. *J. Appl. Physiol.* 89:72–80.
28. van der Vliet, A., J. P. Eiserich, B. Halliwell, and C. E. Cross. 1997. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite: a potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* 272:7617–7625.
29. Sampson, J. B., Y. Ye, H. Rosen, and J. S. Beckman. 1998. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Bioph.* 356:207–213.
30. Ara, J., S. Przedborski, A. B. Naini, V. Jackson-Lewis, R. R. Trifiletti, J. Horwitz, and H. Ischiropoulos. 1998. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc. Natl. Acad. Sci. USA* 95:7659–7663.
31. Crow, J. P., Y. Z. Ye, M. Kirk, S. Barnes, and J. S. Beckman. 1997. Superoxide dismutase catalyzes nitration of tyrosines by peroxynitrite in the rod and head domains of neurofilament-L. *J. Neurochem.* 69:1945–1953.
32. Eiserich, J. P., A. G. Estevez, T. Bamberg, Y. Z. Ye, P. H. Chumley, J. S. Beckman, and B. A. Freeman. 1999. Microtubule dysfunction by post-translational nitrotyrosination of α-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. USA* 96:6365–6370.
33. MacMillan-Crow, L. A., J. P. Crow, J. D. Kerby, and J. S. Beckman. 1996. Nitration and inactivation of manganese superoxide dismutase in chronic jection of human renal allografts. *Proc. Natl. Acad. Sci. USA* 93:11853–11858.
34. Moreno, J. J., and W. A. Pryor. 1991. Inactivation of α1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.* 5:425–431.

Annotations from 4634.pdf

Page 1

Annotation 1; Label: Comp2; Date: 2/14/2002 3:20:25 PM
Ed:
Please provide received/revised dates.

Annotation 2; Label: Comp2; Date: 2/14/2002 3:20:02 PM
in

Page 2

Annotation 1; Label: Comp2; Date: 2/14/2002 3:21:06 PM
Au:
wt/vol instead?

Page 6

Annotation 1; Label: Comp2; Date: 2/14/2002 3:21:55 PM
Au:
Information missing here? "not"?

Page 9

Annotation 1; Label: Comp2; Date: 2/14/2002 3:22:42 PM
Au:
The reference 31 "Crow, Ye, Kirk, Barnes, Beckman, 1997" is not cited in the text. Please add an in-text citation or delete the reference.

Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress

ESTHER BARREIRO, ALAIN S. COMTOIS, SHAWN MOHAMMED, LARRY C. LANDS, AND SABAH N.A. HUSSAIN

Critical Care and Respiratory Divisions, Royal Victoria Hospital and Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada. Respiratory Medicine Department, Montreal Children's Hospital, McGill University, Montreal, Quebec, Canada. Department of Respiratory Medicine, Hospital del Mar-IMIM, Universitat Pompeu Fabra, Barcelona, Catalonia, Spain.

Barreiro, Esther, Alain S. Comtois, Shawn Mohammed, Larry C. Lands, and Sabah N.A. Hussain. Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress. *Am. J. Physiol. (Lung Cell Mol Physiol).* Heme oxygenases (HOs), essential enzymes for heme metabolism, play an important role in the defence against oxidative stress. In this study we evaluated the expression and functional significance of HO-1 and HO-2 in the ventilatory muscles of normal rats and rats injected with bacterial lipopolysaccharide (LPS). Both HO-1 and HO-2 proteins were detected inside ventilatory and limb muscle fibers of normal rats. Diaphragmatic HO-1 and HO-2 expressions rose significantly within 1 hr and 12 hrs of LPS injection, respectively. Inhibition of the activity of the inducible nitric oxide synthase (iNOS) in rats and absence of this isoform in $iNOS^{-/-}$ mice did alter sepsis-induced regulation of muscle HOs. Systemic inhibition of HO activity with chromium mesoporphyrin IX enhanced muscle protein oxidation and hydroxynonenal formation in both normal and septic rats. Moreover, *in-vitro* diaphragmatic force generation declined substantially in response to HO inhibition both in normal and septic rats. We conclude that both HO-1 and HO-2 proteins play an important role in the regulation of muscle contractility and in the defence against sepsis-induced oxidative stress.

heme oxygenases, nitric oxide, nitric oxide synthase, sepsis, diaphragm

---

HEME OXYGENASES (HOs) ARE the rate-limiting enzymes of the initial reaction in the degradation of heme, which yields equimolar quantities of biliverdin IXa, carbon monoxide (CO), and free iron (8). Biliverdin is subsequently converted to bilirubin, and free iron is rapidly incorportated into ferritin. So far, three HO isoforms have been identified, HO-2 and HO-3 are constitutively expressed in various cells, whereas HO-1 is transcriptionally activated in response to a variety of stimuli including bacterial lipopolysaccharide (LPS), heat stress, hypoxia and exposure to nitric oxide (NO) (1,7,9). There is increasing evidence that HOs play important roles in the cellular defence against oxidative stress and the deleterious effects of pro-inflammatory cytokines and LPS. Indeed, pharmacological inhibition of HO activity renders rats more sensitive to LPS-induced mortality (27). Similarly, mice which are deficient in HO-1 are very susceptible to the effects of reactive oxygen species and develop a high mortality in response to LPS administration (28). In endothelial cells, gene transfer of HO-1 protects against oxidant-induced lung injury (36). The anti-oxidant properties of HOs are believed to be mediated by products of HO activity including bilirubin, CO and ferritin (26,30).

Little is known about the existence and functional significance of HOs in skeletal muscles. Baum *et al*. (4) described the presence of HO-2 protein inside normal mammalian skeletal muscles and reported that this protein co-localizes with the neuronal nitric oxide synthase (nNOS) in close proximity to the non-junctional sarcolemma. HO-2 has also been identified at the neuromuscular junctions of normal skeletal muscles (20). More recent studies have indicated that HO-1 is expressed at relatively low levels in normal *in-vivo* skeletal muscles and *in-vitro* cultured myoblasts and that HO-1 expression is elevated in response to increased muscle activation or exposure to exogenous hemin and nitric oxide (NO)(13,35). The functional significance of HOs in skeletal muscle was not addressed in these studies. Very recently, Taille *et al*. (32) described for the first time the involvement of HOs in LPS-induced oxidative stress and contractile dysfunction of the ventilatory muscles. These authors observed that injection of LPS in rats evokes HO-1 induction in the ventilatory muscles of rats with no change in HO-2 expression. It was also reported that inhibition of HO activity augments LPS-induced muscle oxidative stress and worsens LPS-induced muscle contractile dysfunction (32). Despite this recent progress, many aspects of the biological roles of HOs in the ventilatory or limb muscles remain unclear. For instance, it is unclear whether HOs play any role in regulating redox status or contractile function of normal skeletal muscles. The observations that both enzymes, particularly HO-2, are expressed at various sites within normal muscle fibers and inside blood vessels supplying muscles (16), suggests a functional role for these enzymes in normal muscle fibers. Moreover, our preliminary

experiments in rats suggested that LPS evokes significant up-regulation of muscle HOs with HO-1 expression being elevated early in sepsis, whereas HO-2 is elevated within 12 hrs of LPS injection. These results suggest that there are differences in the response of HO-1 and HO-2 to LPS injection. Finally, there is increasing evidence that exogenous NO donors exert a significant stimulatory effect on HO expression in a variety of cells, including cultured myocytes (17). Whether or not endogenous muscle NO production, particularly in septic animals where the inducible (iNOS) nitric oxide synthase is highly expressed, participates in the regulation of muscle HO expression remains unclear.

The main objectives of this study are: 1) to assess the expression, localization and functional significance of HO-1 and HO-2 in the regulation of redox status and contractile function of normal ventilatory muscles; 2) to evaluate the influence of sepsis (induced by LPS injection) on the expression of HO-1 and HO-2 in the ventilatory muscles and to assess the contribution of these enzymes to sepsis-induced muscle oxidative stress and contractile dysfunction, and finally 3) to evaluate the involvement of the iNOS isoform in the regulation of ventilatory muscle HO-1 and HO-2 expression both under normal conditions and in response to severe sepsis.

MATERIALS AND METHODS:

Reagents. Gels and loading buffers for immunoblotting were obtained from Novex Inc. (San Diego, CA). *E. Coli* LPS (serotype 055:B5), bovine serum albumin (BSA), aprotinin, leupeptin, trypsin inhibitor, pepstatin A, tris-maleate, dithiothreitol (DTT) and phenylmethyl-sulphonyl fluoride (PMSF) were purchased from Sigma Chemicals (St. Louis, MO). 1400 W was obtained from Cayman Chemical Company (Ann Arbor, MI). Monoclonal antibodies for HO-1 and HO-2 were obtained from Transduction Laboratories (Lexington, KY). Pure HO-1 and HO-2 proteins were purchased from StressGen Biotechnologies (Victoria, BC). Secondary antibodies for immunohistochemistry were purchased from Jackson ImmunoResearch Inc. (West Grove, PA). Reagents for enhanced chemiluminescence detection were obtained from Chemicon Inc. (Temecula, CA). Chromium mesoporphyrin IX (CrMSPIX) was purchased from Porphyrin Products, Inc. (Logan, UT). Oxyblot protein oxidation kit was purchased from Intergen Company (NY).

Preparation of CrMSPIX: CrMSPIX was dissolved in 500 µl of 10% (wt/vol) ethanolamine. After addition of 7 ml of distilled water and adjusting the pH to 7.4 with HCl, the volume was adjusted to 10 ml in order to obtain a final solution of 650 µM concentration. For the inhibition of HO activity, we injected animals with a single dose of CrMSPIX (5 µmol per kg body weight) in one single injection.

Animal preparations:

Rat Experiments: The Animal Research Committee of McGill University approved all procedures. Pathogen-free male Sprague-Dawley rats (250-275 g) were used in all protocols. The animals were housed in the animal facility of the hospital, were fed food and water *ad libitum* and were studied 1 week after arrival. At the end of all experimental protocols, the animals were anesthetized with sodium pentobarbital (30 mg/kg) and the diaphragm (and intercostals, gastrocnemius and soleus in a few animals) was quickly excised and frozen in liquid nitrogen. For immunostaining, the tissues were first flash frozen in cold isopentane (20 s), then immersed in liquid nitrogen and stored at -80ºC.

a) HO-1 and HO-2 expressions in normal and septic muscles: We studied 8 groups (n=3 in each group) of male rats. Group 1 animals served as control and were injected with 0.3 ml normal saline and were sacrificed 1 hr later. All the remaining groups received intraperitoneal injection of *E. coli* LPS (serotype 055:B5, Sigma Inc., 20 mg/kg) and were sacrificed 1, 3, 6, 12, 24, 48 and 72 hrs later.

b) iNOS inhibition and HO-1 and HO-2 expressions: We evaluated the involvement of the iNOS isoform in the regulation of diaphragmatic HO-1 and HO-2 expression by studying two groups (n=5 in each group) of male rats. Group 1 received an injection of *E. coli* LPS as mentioned above and was sacrificed 24 hrs later. Group 2 animals received an injection of 1400W (20 mg/kg, a selective iNOS inhibitor) 30 min before LPS administration and every 8 hrs thereafter and were sacrificed 24 hrs after LPS administration.

c) Effects of HO inhibition*:* We evaluated the functional significance of HOs in regulating redox status and muscle contractility by inhibiting HO activity with CrMSPIX. Four groups of male rats (n=6 in each group) were studied. Group 1 animals served as controls and were injected with 0.3 ml of normal saline and sacrificed 1 hr after the injection. Group 2 animals were injected with CrMSPIX (5 µmol/kg body weight i.p) followed an hour later by i.p. injection of 0.3 ml of normal saline. These animals were sacrificed 1 hr after saline injection. Groups 3 animals were injected with *E. coli* LPS (20 mg/kg) and were sacrificed 24 hrs later, whereas CrMSPIX (5 µmol/kg body weight i.p) was injected 1 hr prior to LPS injection in group 4 animals. Animals in that group were sacrificed 24 hrs after LPS injection.

Mice Experiments: To further evaluate the role of iNOS in the regulation of muscle HO-1 and HO-2 expressions, we studied adult (8-12 weeks old male) mice genetically deficient (knockout) in iNOS. B6/129 hybrid iNOS knockout mice ($iNOS^{-/-}$) were generated as previously described (21). We also studied wild type B6/129 hybrid mice ($iNOS^{+/+}$) (purchased from Jackson laboratories, Bar Harbor, Maine) and bred to serve as experimental controls. In each genotype ($iNOS^{+/+}$ and $iNOS^{-/-}$), HO-1 and HO-2 protein expressions were studied in four groups of mice (n=5 in each group). Group 1 animals were injected with saline and served as control, whereas group 2, 3 and 4 animals received i.p. injection of *E. coli* LPS (20 mg/kg) and were sacrificed 6, 12 and 24 hrs later. The diaphragm was excised and frozen in liquid nitrogen as described above.

Immunoblotting: Frozen muscle samples were homogenized in 6 v/w ice-cooled homogenization buffer A (tris-maleate 10 mM, EGTA 3 mM, sucrose 275 mM, DTT 0.1 mM; leupeptin 2µg/ml; PMSF 100µg/ml; aprotinin 2µg/ml, pepstatin A 1 mg/100ml, pH 7.2). Samples were then centrifuged at 1000g for 10 min. The pellet was discarded, whereas the supernatant was designated as crude homogenate. Total muscle protein level in each sample was determined with the Bradford technique (Biorad Inc.). Crude homogenate samples (80 µg per sample) were mixed

with SDS sample buffer, boiled for 5 min at 95° C and were then loaded onto 8 or 10% tris-glycine sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and separated by electrophoresis (150 V, 30 mA for 1.5 hrs). Pure HO-1 and HO-2 proteins were used as positive controls. Proteins were transferred electrophoretically (25 V, 375 mA for 2 hrs) to methanol pre-soaked polyvinylidene difluoride (PVDF) membranes, and then blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature. The PVDF membranes were subsequently incubated overnight at 4°C with primary monoclonal anti-HO-1 and anti-HO-2 antibodies. After three 10 min washes with wash buffer on a rotating shaker the PVDF membranes were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody. Specific proteins were detected with an enhanced chemiluminescence (ECL) kit (Chemicon Inc.) The blots were scanned with an imaging densitometer and optical densities (OD) of positive HO-1 and HO-2 protein bands were quantified with SigmaGel software (Jandel Scientific Inc.).

Protein Oxidation: We measured protein oxidation in muscle samples by evaluating the levels of protein carbonyl using immunoblotting. Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury (22). The conventional assay for protein carbonyls is a colorimetric procedure that measure the binding of dinitrophrenylhydrazine (DNP), however, western blotting using anti-DNP antibody provides a much more sensitive method than colorimetric procedures. We used an Oxyblot kit, which contains all solutions required for the derivatization of the samples as well as the antibodies required for the immunodetection. Briefly, 15 µg of protein were used per derivatization reaction. Proteins were then denatured by addition of 12% SDS. The samples were subsequently derivatized by adding 10 µl of 1X DNPH solution and incubated for 15 min. Finally, 7.5 µl of neutralization solution and 2-mercaptoethanol were added to the sample mixture. Both derivatized and un-derivatized (negative control) muscle samples were then separated on SDS-PAGE and transferred onto PVDF membranes, as described above. Membranes were probed with polyclonal anti-DNP moiety antibody. Positive proteins were detected with HRP-conjugated anti-rabbit secondary antibody and an ECL kit as described. Total levels of carbonyls in each muscle sample were calculated by adding OD of individual positive protein band.

Measurements of 4-hydroxy-2-nonenal (HNE): Peroxidation of membrane lipids results in free radical-mediated fragmentation of polyunsaturated fatty acids and the formation of various aldehydes, alkenals and hydroxyalkenals. 4-Hydroxy-2-nonenal (HNE) is an α,β-unsaturated aldehyde and represents the most cytotoxic product of lipid peroxidation. We used a well-characterized polyclonal antibody to detect HNE protein adducts in the ventilatory and limb muscles of normal and septic rats (31). Crude muscle homogenates were separated on SDS-PAGE and transferred to PVDF membranes as described above. Membranes were probed with polyclonal anti-HNE antibody (Calbiochem, San Diego CA) and specific proteins were detected with HRP-conjugated antibody and an ECL kit. Adding OD of individual positive protein band calculated total level of HNE in each muscle sample.

Immunohistochemistry: Frozen tissues sections (5-10 µm thickness) were adsorbed to microscope slides and dried. The sections were fixed with acetone at -20°C, re-hydrated with PBS (pH 7.4), and were then blocked with solutions of avidin, biotin (15 min each at room temperature) and then 3% BSA for 30 min. The sections were incubated for 1 hr at room temperature with primary monoclonal anti-HO-1 and anti-HO-2 antibodies. To evaluate the negative control staining, the primary antibody was replaced with non-specific mouse IgG. After three rinses with PBS, sections were incubated with biotin-conjugated anti-mouse or anti-rabbit secondary antibodies at room temperature for 1 hr followed by exposure to Cy3-labelled streptavidin for 1 hr. Sections were then washed, mounted with cover-slips and examined with a fluorescence microscope and photographed with a 35 mm camera (Olympus Inc.).

Glutathione Measurements: Frozen muscle samples were homogenized in 20 µL 5% SSA (5-Sulfosalicylic acid)/mg tissue. The homogenate was then centrifuged at 10,000 g for 5 min, at 4° C. The supernatant was diluted 1:5.5 in ddH_2O, aliquoted, and immediately stored at -70° C, for total glutathione (GSH) measurement. Total glutathione concentration was determined by the glutathione reductase (GR) recycling method of Tietze (34) adapted for the Cobas Mira S spectrophotometer (Roche Diagnostics, Laval, Quebec). Briefly, we placed 250 µL NADPH (0.3 mM), 30 µL DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid)) (6.0 mM) and 95 µL of sample, standard or 0.9% SSA into cuvettes. After a 4-min incubation period at 37° C, 15 µL GR (2 U/100 µL) was added and the reaction was monitored every 24 s for 12 min. Under these conditions, the method is linear for GSH concentrations between 0.1 and 6 µM.

Diaphragmatic muscle strip preparation: Diaphragms of control and septic (24 hrs post LPS) rats un-treated and treated with CrMSPIX were surgically excised with ribs and central tendon attached and were placed in an equilibrated (95% 0_2 - 5% CO_2; pH 7.38) Krebs solution chilled at 4°C that had the following composition (mM): 118.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1 KH_2PO_4, 25 $NaHCO_3$, and 11.0 glucose. From the central tendon to the rib, a muscle strip (3-4 mm wide) was dissected from the lateral costal portion of the diaphragm. The rib was left attached to the strip and was used to secure the diaphragm strip in the custom built Plexiglas muscle chamber. The strip was mounted in a muscle chamber and the muscle chamber was mounted vertically into a double jacket gut bath (Kent Scientific Instruments). A 4.0 silk thread was used to secure the central tendon to the isometric force transducer (Kent Scientific Instruments). Muscle strips were electrically stimulated at constant currents via platinum electrodes mounted in the muscle chamber which were connected to a square wave pulse stimulator (Grass Instruments, model S48). After an equilibration period of 30 min (temperature of 22-25°C), the organ bath temperature was increased to 35 °C and the maximum current necessary to elicit maximum force during 120 Hz stimulation-frequency (600 ms duration) was then identified. Muscle length was then gradually adjusted with a micrometer to the optimal value (Lo) at which maximum isometric muscle force was generated in response to supra-maximum stimulation (current of 300-350 mA, 120 Hz frequency). Force-frequency relationships of diaphragmatic strips were then constructed by varying the stimulation frequency (between 10 and 120 Hz) while maintaining supra-maximal current and stimulation duration (600 ms) constant. Tetanic contractions were digitized at a frequency of 1 KHz with a personal computer and stored on the hard disk for later analysis. At the end of the experiment, the strips were blotted dry and weighed. Muscle length (cm) and weight (g) were measured and used to calculate the cross sectional area. Isometric forces were normalized for muscle cross sectional area estimated by using the value of 1.056 g/cm^3 for muscle density. The peak force in N/cm^2

was measured for each contraction within the force frequency curve.

Statistical Analysis: Values are presented as means ± SEM. Differences in muscle force and OD of HO-1 and HO-2 proteins, total HNE and total carbonyls among various conditions were compared with one-way ANOVA followed by Tukey test for multiple comparisons. P values of less than 5% were considered significant. Statistical analyses were performed with SigmaStat software (Jandel Scientific, Chicago, IL).

RESULTS

Expression and localization of HO-1 and HO-2 in normal rat skeletal muscles: Pure HO-1 and HO-2 proteins were detected in crude diaphragmatic homogenates as clear bands with apparent molecular masses of 32 and 36 kDa, respectively (figure 1A). Similarly, HO-1 and HO-2 proteins were also detected in soleus, intercostals and gastrocnemius muscle samples obtained from normal rats (results are not shown). Figure 2 illustrates the localization of HO-1 and HO-2 proteins in the diaphragm and leg muscles of normal rats. The anti-HO-2 antibody detected positive HO-2 protein staining at the sub-sarcolemmal region of normal gastrocnemius muscle sections (panel A). Similar positive HO-2 staining was found in close proximity to the non-junctional sarcolemma of rat diaphragms (panel B). HO-2 protein was also present in endothelial cells of normal muscle sections. Positive HO-1 protein staining was detected with a monoclonal anti-HO-1 antibody in close proximity to the sarcolemma of gastrocnemius muscle sections (panels C and D) and in the endothelial cells of a blood vessel traversing this muscle (panel E). Replacement of primary antibodies with non-specific mouse IgG completely eliminated positive HO protein staining (panel F).

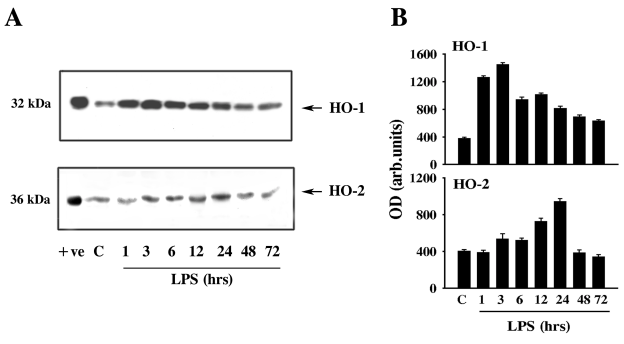


Fig. 1. A) Representative immunoblots indicating the time course of HO-1 and HO-2 protein expression in normal rat diaphragms (C) and in the diaphragms of rats sacrificed after 1, 3, 6, 12, 24, 48 and 72 hrs of LPS injection. +ve refers to positive controls (pure HO-1 and HO-2 proteins). B) Mean values (±SEM) of HO-1 and HO-2 optical densities in the diaphragms of normal and LPS-injected rats. Note the differences in the time course of HO-1 and HO-2 induction in response to LPS injection.

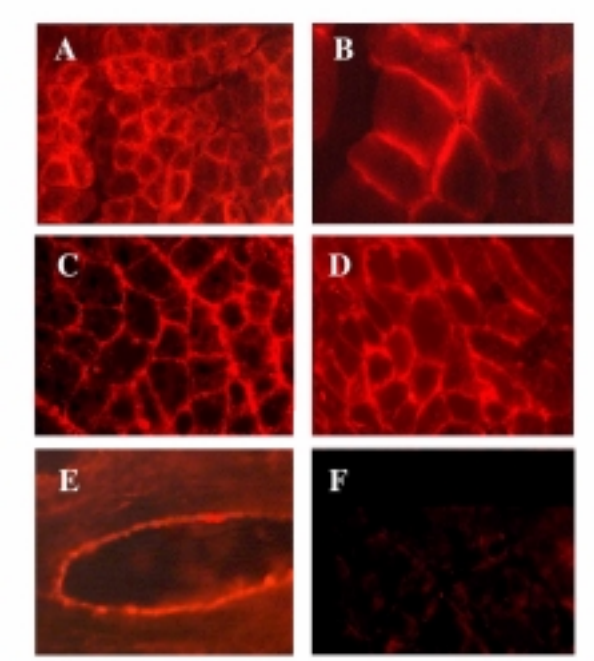


Fig. 2. Localization of HO-2 protein in the gastrocnemius and the diaphragm of normal rats is shown in panels A and B, respectively. Note that HO-2 protein is localized in close proximity to the sarcolemma. Panels C and D show positive HO-1 protein staining in the gastrocnemius of normal rats, whereas panel E indicates the presence of HO-1 protein in blood vessels supplying leg muscles of normal rats. Panel F represents negative control staining in which the primary anti-HO-1 or HO-2 antibodies were replaced with non-specific rabbit IgG.

Muscle HO expression in septic rats and mice: Figure 1 illustrates the effect of LPS injection on diaphragmatic HO-1 and HO-2 protein expression in rats. HO-1 protein levels rose rapidly by more than 3-fold within 1 and 3 hrs of LPS injection with gradual decline thereafter. After 72 hrs of LPS injection, HO-1 expression was slightly higher than control values (figure 1A and B). Unlike HO-1 expression, the intensity of HO-2 protein rose substantially after 12 and 24 hrs of LPS injection with a return to values similar to control values after 48 hrs of LPS injection (figure 1). The changes in HO-1 and HO-2 protein expression in response to LPS injection in wild type mice are shown in figure 3. Diaphragmatic HO-1 expression rose substantially within 6 hrs of LPS injection and remained elevated even after 24 hrs of LPS injection (figure 3 A and B). We were unable to detect HO-2 protein in immunoblots of diaphragms obtained from control and septic wild type mice.

Role of iNOS in HO expression: We evaluated the role of iNOS in LPS-induced HO-1 and HO-2 expressions in muscles by examining mice deficient in iNOS and by injecting rats with a selective iNOS inhibitor (1400W). Figure 3 shows that diaphragmatic HO-1 expression rose substantially after 6, 12 and 24 hrs of LPS injection in iNOS$^{-/-}$ mice. The degree of

HO-1 induction after LPS injection in iNOS$^{-/-}$ mice was similar to that observed in iNOS$^{+/+}$ mice (figure 3). Figure 4 shows that treatment with 1400W (selective iNOS inhibitor) did not alter the induction of diaphragmatic HO-1 and HO-2 expressions observed 24 hrs after LPS injection.

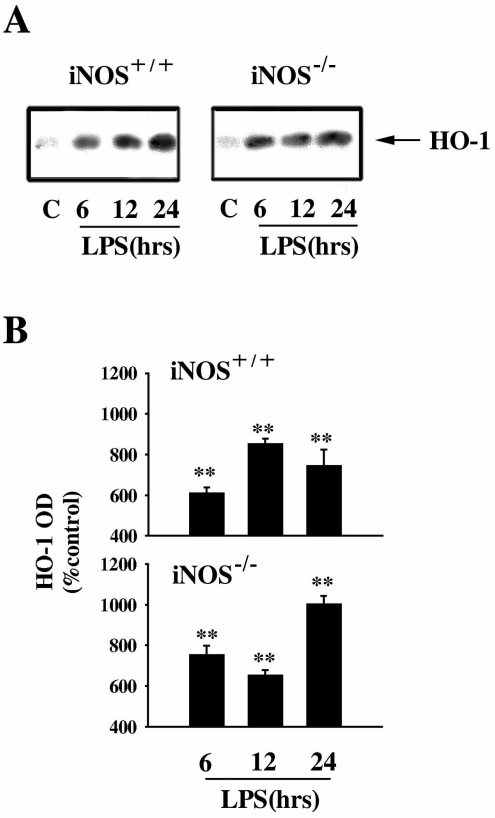


Fig. 3. A) Representative immunoblots of HO-1 protein in the diaphragm of wild type (iNOS$^{+/+}$) and iNOS knockout (iNOS$^{-/-}$) mice both under control conditions (C) and 6, 12, and 24 hrs after LPS injection. B) Mean (±SEM) values of HO-1 protein optical densities (expressed as percentage of control values) of diaphragmatic samples obtained from iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. ** P<0.01 compared with control values. Note that the induction of HO-1 protein in response to LPS injection was observed in both genotypes.

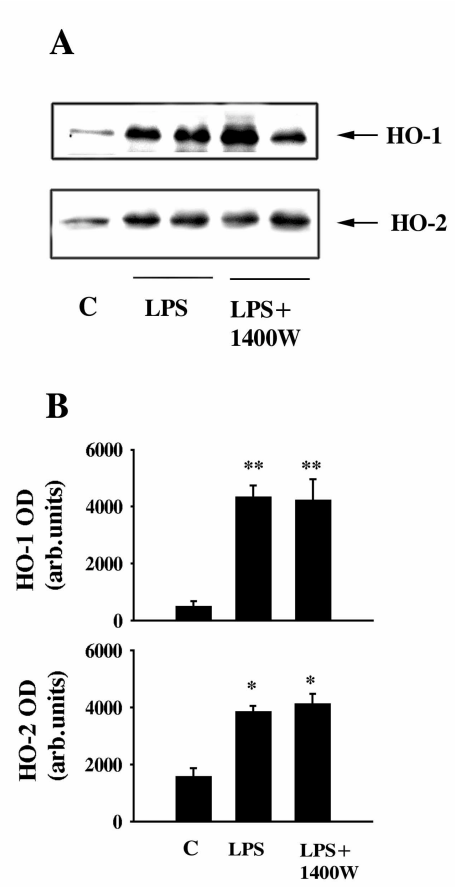


Fig. 4. A) Representative immunoblots of HO-1 and HO-2 proteins in the diaphragms of normal rats (C) and rats examined after 24 hrs of LPS injection. Also shown are diaphragm samples obtained from LPS-injected rats pre-treated with a selective iNOS inhibitor (1400W). B) Mean values of optical densities of HO-1 and HO-2 proteins in the diaphragms of normal rats and rats injected 24hrs earlier with LPS (with or without pre-treatment with 1400W). **, * P<0.01 and 0.05 compared with control rat diaphragms.

HOs and muscle oxidative stress: Measurement of carbonyl groups in diaphragmatic lysates using immunoblotting indicates the presence of relatively few oxidized proteins in control diaphragms (apparent molecular masses of 65, 46 and 34 kDA, figure 5A). Injection of LPS resulted in a significant increase in the intensity of these pre-existing oxidized proteins as well as the appearance of new oxidized proteins with molecular masses of 236, 150, 137 and 44 kDa (figure 5A). Administration of CrMSPIX elicited substantial reduction in the OD of the majority of oxidized proteins both under control conditions and after LPS injection (figure 5A and C). The anti-HNE antibody detected four main protein bands in control diaphragms with molecular masses of 74, 49, 44 and 40 kDa (figure 5B). Injection of LPS resulted in a small increase in intensity of the 74-kDa protein band. Administration of CrMSPIX in control and septic animals elicited a significant rise in total HNE intensity primarily due to the appearance of a strong positive protein band at 24 kDa (figure 5B and C). Figure 6 illustrates the changes in total diaphragmatic glutathione levels both in control and septic rats.

Injection of LPS resulted in a significant decline in total glutathione to values, which are about one third of those found in control diaphragms (figure 6). Injection of CrMSPIX did not alter the level of total glutathione in the diaphragm of control and septic rats.

Diaphragmatic contractility: Figure 7 shows that LPS injection had elicited by 24 hrs a substantial decline in diaphragmatic force generation with mean

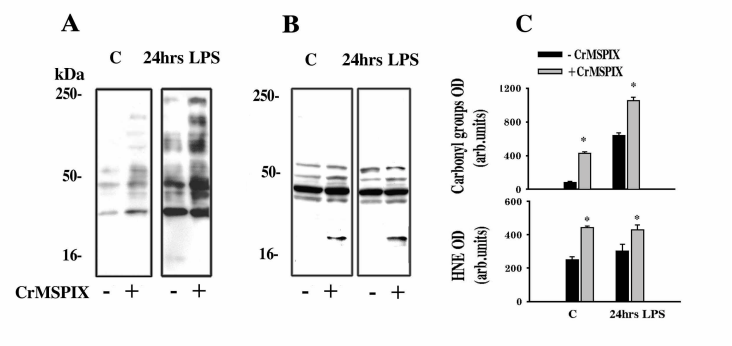


Fig. 5. A) Representative immunoblots of protein carbonyl groups in the diaphragms of control and LPS-injected rats (with or without pre-treatment with HO inhibitor, CrMSPIX). Note that administration of CrMSPIX augmented carbonyl group intensity both in control and septic diaphragms. B) Representative immunoblots of HNE (index of lipid peroxidation) in control and LPS-injected rats (with or without pre-treatment with CrMSPIX). Note the appearance of a 22-kDa-protein band in response to HO inhibition. C) Mean values of total optical densities of carbonyl groups and HNE in control and septic diaphragms (with and without pre-treatment with CrMSPIX). * P<0.05 compared with animals without CrMSPIX injection.

force averaging 38, 45, 52, 45, 48, 51 and 52% of those generated in response to 1, 10, 20, 30, 50, 100 and 120 Hz stimulation in control diaphragms. Administration of CrMSPIX elicited a significant decline in diaphragmatic force both under control conditions and in response to LPS injection (figure 7).

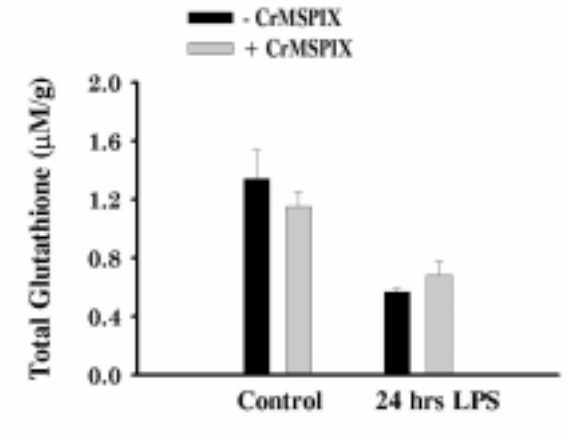


Fig. 6. Total diaphragmatic glutathione levels in the diaphragms of control and LPS-injected rat with or without CrMSPIX injection. Note the decline n total glutathione level in septic diaphragms. Treatment with CrMSPIX had no effect on LPS-induced decline in total glutathione.

HOs and iNOS expression: We assessed the influence of HO activity on muscle iNOS expression by measuring diaphragmatic iNOS protein expression after 6 and 24 hrs of LPS injection (with or without CrMSPIX administration). LPS injection elicited in the diaphragm a transient expression of iNOS protein which disappeared after 24 hrs of LPS

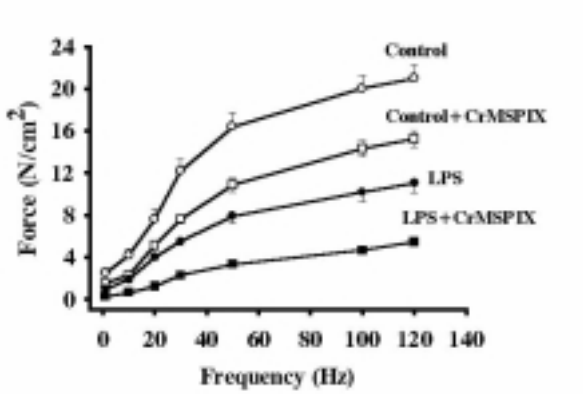


Fig. 7. Force-frequency relationships of in-vitro isolated strips of diaphragms excised from control (open circles) and after 24 hrs of LPS injection (filled circles). Also shown is the effect of CrMSPIX treatment in control (open squares) and LPS-injected (filled squares) animals. Note the substantial decline in diaphragmatic force in response to CrMSPIX treatment.

injection (figure 8). Administration of CrMSPIX had no effect on the time course of diaphragmatic iNOS expression suggesting that muscle HO activity does not modulate iNOS expression.

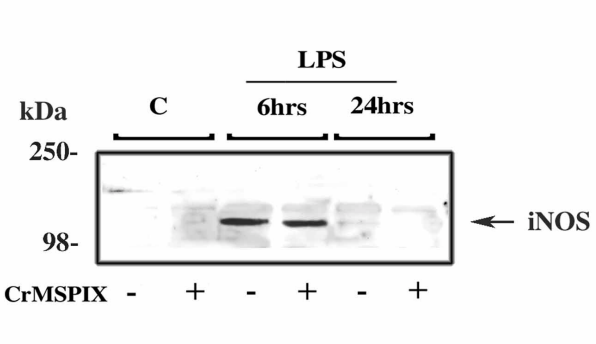


Fig. 8. Representative immunoblots of iNOS protein in the diaphragms of control animals and animals sacrificed after 6 and 24 hrs of LPS injection. Note that administration of HO inhibitor (CrMSPIX) did not alter the transient LPS-induced iNOS expression.

DISCUSSION

The main findings of this study are that: 1) both HO-1 and HO-2 enzymes are constitutively expressed inside skeletal muscle fibres of rats and mice; 2) the expression of both enzymes increases significantly in response to LPS injection but at different time courses; 3) systemic inhibition of HO activity augmented muscle protein oxidation, lipid peroxidation and significantly reduced the ability of muscle fibers to generate force both in normal rats and in rats injected with LPS; 4) the iNOS isoform is not involved in the regulation of muscle HO expression.

Expression of HO in normal and septic muscles: Our study indicates that both HO-1 and HO-2 proteins are expressed, albeit at low levels, in normal limb and ventilatory muscles. This finding is in agreement with previous studies documenting the existence of these proteins in *in-vivo* mammalian muscles and cultured myocytes (4,20,32,35). In accordance with Baum *et al*. (4), we report here the expression of HO-1 and HO-2 in blood vessels traversing skeletal muscles.

Little is known about the factors that regulate HO expression in skeletal muscle fibers. In non-muscle cells, many conditions such as heat shock, ischemia, hypoxia, endotoxin, pro-inflammatory cytokines and hemin induce HO expression, particularly that of HO-1 (for review, see (8)). In skeletal muscles, Essig *et al.* reported that exhaustive running or artificial stimulation induce muscle HO-1 mRNA expression. Hemin is another inducer of HO-1 expression in cultured myocytes (35). We report here that diaphragmatic HO-1 and HO-2 expression in rats and HO-1 expression in mice was significantly elevated in response to LPS injection. Taille *et al.* (32) reported that LPS injection in rats elicits a significant induction of diaphragmatic HO-1 expression with no change in HO-2 expression. The contradictory results regarding the influence of LPS on HO-2 between our study and that of Taille *et al*. (32) is likely to be due to the differences in dosage of LPS used in the two studies (4 mg/kg in Taille's study compared with 20 mg/kg in our study). We should emphasize that Taille *et al*. did not investigate the mechanisms responsible for induction of HO-1 and HO-2 expression in LPS injected animals.

We speculate that the following mechanisms are responsible for up-regulation of muscle HO expression in septic animals. *First*, we propose that the induction of HOs inside muscle fibers late in sepsis might be due in part to increased reactive oxygen species (ROS) production (23,29) which are known to enhance HO expression (8). However, it is unlikely that ROS were involved in early (after 1 hour of LPS injection) up-regulation of muscle HO-1 expression since (figure 1). *Second*, HO-1 induction in septic muscles might have been mediated by stress activated (c-fos and c-jun) and pro-inflammatory (NFκB) transcription factors. Bindings sites for these transcription factors have been clearly identified in the promoter of HO-1 (8). In addition, TNF-α and IL-1α activate HO-1 expression in endothelial cells, an action which is mediated via protein kinase C, Ca^{++} and phospholipase A2 (33). On the basis of these observations, it is likely that LPS and pro-inflammatory cytokines, particularly TNF-α are directly responsible for elevated muscle HO-1 expression in septic animals. *Third*, exposure of mesangial, endothelial, smooth muscle cells and cultured myocytes to exogenous NO donors has recently been shown to elicit a significant induction of HO-1 mRNA and activity (9,11,17, 35). Under normal conditions, NO is produced inside skeletal muscle fibers by the neuronal and endothelial NOS isoforms (19). However, muscle NO production rises significantly in septic animals as a result of iNOS induction (5,18). We have excluded an involvement of iNOS in regulating muscle HO expression in the current study on the basis that inhibition of iNOS activity in rats and absence of iNOS expression in $iNOS^{-/-}$ mice did not alter the effects of LPS on HO-1 and HO-2 expressions. *Fourth*, it has been shown that peroxynitrite (formed from the near-diffusion reaction between NO and $O_2^{\cdot -}$ radicals) regulates HO-1 mRNA expression and activity in endothelial cells (15). In septic rats, tyrosine nitration (footprint of peroxynitrite formation) increases significantly in the ventilatory and limb muscles in response to LPS injection (12). On the basis of these findings, it is plausible that induction of diaphragmatic HO expression in septic animals might have been caused by increased peroxynitrite formation. We believe that this was not likely since inhibition of iNOS activity by 1400W, a procedure known to reduce peroxynitrite formation, had no effect on LPS-induced muscle HO expression.

Role of HO in oxidative stress: One of the major findings in our study is that administration of the HO inhibitor, CrMSPIX, increased diaphragmatic protein oxidation and lipid peroxidation (as measured with HNE antibody) both under normal conditions and in response to LPS injection. Taille *et al*. (32) described a similar rise in protein oxidation and malondialdehyde contents in response to HO inhibition in septic muscles but not in control diaphragms. We speculate that difference between our findings with respect to normal rat diaphragms and that of Taille *et al*. are due to the use of two different inhibitors of HO activity (chromium (III) mesoporphyrin IX in our study vs zinc protoporphyrin IX in Taille's study). Appleton *et al*. (3) reported that chromium (III) mesoporphyrin IX is a more potent and more selective inhibitor of HO activity than zinc protoporphyrin IX. Despite this difference, both our study and that of Taille *et al.* suggest that muscle HO activity plays an important and protective role in attenuating muscle oxidative stress particularly in septic animals. This suggestion is in accordance with previous findings in non-muscle cells (15,27). Moreover, mice deficient in HO-1 have been shown

to be vulnerable to mortality and hepatic cirrhosis induced by LPS (28). The protective effects of HOs have been attributed to the anti-oxidant properties of the products of HO activity (bilirubin, CO, and ferritin)(14,25,26).

Effects of HO inhibition on muscle contractility: A major finding in our study is that inhibition of HO activity evoked a significant impairment of diaphragmatic contractility (figure 7). Although the exact mechanisms through which HO activity influence muscle contractility remain speculative, we propose that HOs, through the anti-oxidant properties of their products, promote excitation-contraction coupling by inhibiting the negative effects of ROS on sarcoplasmic (SR) Ca^{++} release-channels and SR Ca^{++} uptake (2,6). In addition, reduction of maximum muscle force by HO inhibition suggests that HOs may also exert their effects at the level of contractile proteins. These effects may include attenuation of the deleterious effects of ROS on myofibrillar Ca^{++} sensitivity and myofibrillar creatine kinase activity (24). Finally, it is possible HOs influence muscle function by promoting vascular dilation though an NO-independent activation of guanylate cyclase and a rise in cGMP (10).

In summary, our results indicate the presence of HO-1 and HO-2 proteins inside ventilatory muscle fibers and that the expression of both enzymes is up-regulated albeit with different time courses, in response to LPS injection in rats. Our results also suggest that HO activity plays very important and protective roles in attenuating oxidative stress and promotion of sub-maximum and maximum force generation both under normal conditions and in response to severe sepsis.

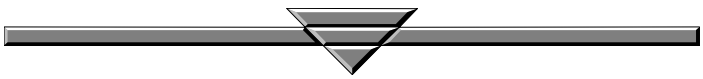
The authors are grateful to Mr. Luigi Franchi for his technical assistance and to Mrs. C. Mutter and Mrs. R. Carin for their assistance in editing the manuscript. This study is funded by a grant from the Canadian Institute of Health Research. Dr S. Hussain is a scholar of the FRSQ. Dr. E. Barreiro is supported by FUCAP, SOCAP (Spain), and BIOMED (E.U.). Dr. L.C. Lands is a clinician-scientist of the FRSQ.

Address for reprint requests: Dr. S. Hussain. Room L3.05, 687 Pine Ave. West, Montreal, Quebec H3A 1A1,Canada

REFERENCES:

1. Abraham NG, Drummond GS, Lutton JD, and Kappas A. The biological signifcance and physiological role of heme oxygenase. *Cell.Physiol.Biochem.* 6: 129-168, 1996.
2. Andrade FH, Reid MB, Allen DG, and Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J.Physiol.* 509: 565-575, 1998.
3. Appleton SD, Chretien ML, McLaughlin BE, Vreman HJ, Stevenson DK, Brien JF, Nakatsu K, Maurice DH, and Marks GS. Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations. *Drug Metab Dispos.* 27: 1214-1219, 1999.
4. Baum O, Feussner M, Richter H, and Gossrau R. Heme oxygenase-2 is present in the sarcolemma region of skeletal muscle fibers and is non-continuously co-localized with nitric oxide synthase- 1. *Acta Histochem.* 102: 281-298, 2000.
5. Boczkowski J, Lanone S, Ungureanu-Longrois D, Danialou G, Fournier T, and Aubier M. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats. Role of diaphragmatic contractile dysfunction. *J.Clin.Invest.* 98: 1550-1559, 1996.
6. Brotto MAP and Nosek TM. Hydrogen peroxide disrupts calcium release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J.Appl.Physiol.* 81: 731-737, 1996.
7. Camhi SL, Alam J, Otterbein LE, Sylvester SL, and Choi AMK. Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am.J.Respir.Cell Mol.Biol.* 13: 387-398, 1995.
8. Choi AMK and Alam J. Heme oxygenase-1: function, regulation and implication for a novel stress-inducible protein in oxidant-induced lung injury. *Am.J.Respir.Cell Mol.Biol.* 15: 9-19, 1996.
9. Datta PK and Lianos EA. Nitric oxide induces heme oxygenase-1 gene expression in mesangial cells. *Kidney International* 55: 1734-1739, 1999.
10. Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton WR, Lee ME, Nabel GJ, and Nabel EG. Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat.Med* 7: 693-698, 2001.
11. Durante W, Kroll MH, Christodoulides N, Peyton KJ, and Schafer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ.Res.* 80: 557-564, 1997.
12. El-Dwairi Q, Comtois A, Guo Y, and Hussain SNA. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. *Am.J.Physiol.(Cell Physiol)* 274: C770-C779, 1998.
13. Essig DA, Borger DR, and Jackson DA. Induction of heme oxygenase-1 (HSP32) mRNA in skeletal muscle following contractions. *Am.J.Physiol.* 272: C59-C67, 1997.
14. Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, and Snyder SH. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat.Cell Biol* 1: 152-157, 1999.
15. Foresti R, Sarathchandra P, Clark JE, Green CJ, and Motterlini R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem.J.* 339: 729-736, 1999.
16. Grozdanovic Z and Gossrau R. Expression of heme oxygenase-2 (HO-2)-like immunoreactivity in rat tissues. *Acta Histochemica* 98: 203-214, 1996.
17. Hartsfield CL, Alam J, Cook JL, and Choi AMK. Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. *Am.J.Physiol.(Lung Cell.Mol.Physiol.)* 273: L980-L988, 1997.

18. Hussain SNA, Giaid A, El-Dwairi Q, Sakkal D, Hattori R, and Guo Y. Expression of nitric oxide synthase isoforms and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. *Am.J.Respir.Cell Mol.Biol.* 17: 173-180, 1997.
19. Kobzik L, Reid MB, Bredt DS, and Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372: 546-548, 1994.
20. Kusner LL, Kim E, and Kaminski HJ. Heme oxygenase-2 expression at rat neuromuscular junctions. *Neurosci.Lett.* 273: 143-146, 1999.
21. Laubach VE, Shesely EG, Smithies O, and Sherman PA. Mice Lacking Inducible Nitric Oxide Synthase are Not Resistant to Lipopolysaccharide-Induced Death. *Proc.Natl.Acad.Sci.USA* 92: 10688-10692, 1995.
22. Levine RL, Williams JA, Stadtman ER, and Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Meth.Enzym.* 233: 346-357, 1994.
23. Llesuy S, Evelson P, Gonzalez-Flecha B, Peralta J, Carreras MC, Poderoso JJ, and Boverism A. Oxidative stress in muscle and liver of rats with septic syndrome. *Free Rad Biol Med* 16: 445-451, 1994.
24. Mekhfi H, Veksler V, Mateo P, Maupoil V, Rochette L, and Ventura-Clapier R. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. *Circ.Res.* 78: 1016-1027, 1996.
25. Minetti M, Mallozzi C, Di Stasi AM, and Pietraforte D. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. *Arch.Biochem.Biophys.* 352: 165-174, 1998.
26. Otterbein LE, Mantell LL, and Choi AMK. Carbon monoxide provides protection against hyperoxic lung injury. *Am.J.Physiol.(Lung Cell.Mol.Physiol.)* 276: L688-L694, 1999.
27. Otterbein LE, Sylvester SL, and Choi AMK. Hemoglobin provides protection against lethal endotoxemia in rats: the role of heme oxygenase-1. *Am.J.Respir.Cell Mol.Biol.* 13: 595-601, 1995.
28. Poss KD and Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc.Natl.Acad.Sci.U.S.A.* 94: 10925-10930, 1997.
29. Shindoh C, DiMarco A, Nethery D, and Supinski G. Effect of PEG-superoxide dismutase on the diaphragmatic response to endotoxin. *American Review of Respiratory Disease* 145: 1350-1354. 1992.
30. Stokcer R, Yamamoto Y, McDonagh AF, Glazer AN, and Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043-1046, 1987.
31. Szweda LI, Szweda PA, and Holian A. Detection of 4-hydroxy-2-nonenol adducts following lipid peroxidation from ozone exposure. *Methods Enzymol.* 319: 562-570, 2000.
32. Taille C, Foresti R, Lanone S, Zedda C, Green C, Aubier M, Motterlini R, and Boczkowski J. Protective role of heme oxygenases against endotoxin-induced diaphragmatic dysfunction in rats. *Am.J.Respir.Crit Care Med* 163: 753-761, 2001.
33. Terry CM, Clikeman JA, Hoidal JR, and Callahan KS. TNF−α and IL-1α induce heme oxygenase-1 via protein kinase C, Ca^{2+}, and phospholipase A_2 in endothelial cells. *Am.J.Physiol.(Cell Physiol)* 276: H1493-H1501, 1999.
34. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal.Biochem.* 27: 502-522, 1969.
35. Vesely MJJ, Exon DJ, Clark JE, Foresti R, Green CJ, and Motterlini R. Heme oxygenase-1 induction in skeletal muscle cells: hemin and sodium nitroprusside are regulators in vitro. *Am.J.Physiol.(Cell Physiol)* 275: C1087-C1094, 1998.
36. Yang L, Quan S, and Abraham NG. Retrovirus-mediated HO gene transfer into endothelial cells protects against oxidant-induced injury. *Am.J.Physiol* 277: L127-L133, 1999.



SUMMARY OF RESULTS

SUMMARY OF RESULTS

1) Muscle tyrosine nitration was detected at 52, 48, 40, 30, 18, and 10 KDa protein bands which were localized in the cytosol of normal skeletal muscles of both rats and mice.

2) Within 1 hour of *in vivo* pharmacological NOS inhibition protein tyrosine nitration was considerably reduced.

3) Diaphragms from mice deficient in the iNOS protein ($iNOS^{-/-}$) showed a 50% reduction in protein tyrosine nitration compared to diaphragms obtained from wild type mice ($iNOS^{+/+}$).

4) Sepsis elicited by LPS injection induced a rise in protein tyrosine nitration in the mitochondrial and membrane fractions of the ventilatory muscles, but not in the cytosolic compartment.

5) Absence of iNOS expression either by pharmacological inhibition or by using iNOS knockout mice resulted in a significant reduction in LPS-mediated diaphragmatic nitrotyrosine formation. Conversely, protein tyrosine nitration was not modified in diaphragms from mice deficient in either the eNOS isoform ($eNOS^{-/-}$ mice) or the nNOS isoform ($nNOS^{-/-}$ mice).

6) Both HO-1 and HO-2 proteins were detected within both ventilatory and limb muscle fibers of normal rats.

7) Interestingly, diaphragmatic both HO-1 and HO-2 expressions significantly rose within 1 hour and 12 hours of endotoxemia, respectively.

8) Inhibition of the activity of the iNOS isoform in rats, and absence of this protein in mice genetically altered ($iNOS^{-/-}$ mice) did not modify the expression of muscle HOs.

9) Systemic inhibition of HO activity using the selective inhibitor CrMSPIX significantly increased both muscle protein oxidation (carbonyl group formation) and lipid peroxidation (hydroxynonenal protein adducts) in normal and septic rats.

10) Contractility studies showed that *in vitro* diaphragmatic force generation substantially declined in response to HO activity inhibition both in normal and septic rats.

DISCUSSION

DISCUSSION

The formation of 3-nitrotyrosine is the most frequently studied covalent modification of proteins attributed to NO. Increased nitrotyrosine formation has been reported in acute lung injury, sepsis, rheumatoid arthritis, amyotrophic lateral sclerosis, Alzheimer, and liver transplantation[18,85]. Several mechanisms are involved in tyrosine nitration, including the reaction of NO with protein tyrosil radicals[86], the reaction of nitrite and peroxidases[20], nitrous acid formed in acidic environment such as the stomach[87], and the reactive species peroxynitrite, which is formed from the near-diffusion limited reaction between NO and superoxide anions[18].

Since both NO and ROS are constitutively synthesized in skeletal muscle, it could be argued that nitrotyrosine formation may be an important occurring phenomenon within the skeletal muscle fiber. Little information, however, is still available regarding protein nitration in skeletal muscles. In this regard, various recent studies have established significant nitrotyrosine formation in both ventilatory and limb muscles of septic animals[30,88,89]. For instance, our group[30] previously reported that two protein bands (50 and 42 KDa) are tyrosine nitrated in normal rat diaphragms, whereas two additional protein bands (196 and 42 KDa) are strongly nitrated in septic rat diaphragms. Most recently, Boczkowski *et al*[89] described tyrosine nitration of a single 105 KDa protein band within diaphragmatic mitochondria of septic rats. In the same study, the authors also showed the presence of the same nitrated protein band in response to exposure of isolated muscle mitochondria to SIN-1 (peroxynitrite donor). Moreover, Supinski *et al*[90] also demonstrated a significant increase in nitrotyrosine formation in septic rat diaphragms using dot blotting technique. Interestingly, in the present study we

detected abundant protein tyrosine nitration in the ventilatory and limb muscles of both normal rats and mice. This tyrosine nitration was noticeable with three different antibodies, though various intensities of specific protein bands were apparent depending on the type of antibody used. More tyrosine nitrated protein bands were detected with polyclonal antibodies. This is due to the fact that these antibodies are capable of detecting more epitopes than monoclonal antibodies. It is worth mentioning that either the lack of protein separation, incomplete separation of various proteins, or measurement of nitrotyrosine formation only in one muscle fraction are conditions which may account for the failure to detect abundant protein nitration in normal skeletal muscles in previous studies.

We have demonstrated using both NOS pharmacological inhibitors and mice genetically modified that nitrotyrosine formation in skeletal muscle fibers is dependent on NO synthesis. Although the precise contribution of each of the NOS isoforms expressed in skeletal muscle fibers to protein nitration in normal muscle remains speculative, our experiments based on the use of either pharmacological NOS inhibition or genetically deficient mice suggest that the iNOS isoform is the main contributor to nitrotyrosine formation in normal muscles. These results are very surprising since skeletal muscles of various species have been shown to express very low levels of the iNOS protein compared with the constitutive isoforms nNOS and eNOS[30,31,33]. Though, it is possible that even very low levels of iNOS protein are sufficient to produce NO, which in turn will elicit protein tyrosine nitration in normal skeletal muscles, since the rate of NO synthesized by iNOS isoform is much higher than that of either nNOS or eNOS.

As above-mentioned several studies have documented an increase in protein tyrosine nitration in the ventilatory muscles of both septic humans and rats[30,88,89]. Moreover, Boczkowski *et al*[89] showed that systemic inhibition of NOS reduced mitochondrial tyrosine nitration in septic animals. Nevertheless, the regulation of protein tyrosine nitration in septic animals by the different NOS isoforms as well as the identification of nitrotyrosine formation in various muscle compartments have not been assessed in those studies. In our current study we found in septic animals that both muscle nitrotyrosine levels significantly increase, and that tyrosine nitration is mainly localized within the mitochondrial and membrane fractions, but not in the cytosolic fraction. Interestingly, our results also indicate that LPS-induced protein nitration is primarily mediated by the iNOS isoform, but not by the nNOS nor the eNOS isoforms, despite the fact that the levels of both enzymes are increased in response to LPS injection[30].

The mechanisms involved in the *in vivo* nitration of tyrosine residues have been the focus of intense research work over the last few years. In this regard, the most widely accepted mechanism is the formation of peroxynitrite, whose identity as the reaction product of NO and superoxide anions, as well as its ability to nitrate tyrosine residues at physiological pH has recently been confirmed by Reiter *et al*[91]. Based on that, we propose that both protein tyrosine nitration in skeletal muscles is primarily mediated by peroxynitrite and that iNOS protein is the primary source of NO required for peroxynitrite formation. Therefore, protein tyrosine nitration is now considered to be the fingerprint of peroxynitrite formation. The other mechanisms briefly mentioned in the first paragraph of this section are not likely to be involved in *the in vivo* protein tyrosine nitration in skeletal muscle. In fact, the pathway

characterized by the reaction of NO with tyrosil radicals generated by prostaglandin H synthase-2[86] requires the presence of this substance in large quantities, which is not usually the case in skeletal muscle. As to the oxidation reaction of NO_2^- by H_2O_2 at physiological pH which can result in the formation of peroxynitrous acid and consequent nitration of protein tyrosine residues[85], this reaction is not likely to occur in skeletal muscle since the concentrations of H_2O_2 are very low in this tissue[92]. Finally, myeloperoxidases utilizing both NO_2^- and H_2O_2 have been shown to nitrate tyrosine residues[93]. We do not consider this pathway to be very likely to nitrate proteins within the skeletal muscle fiber, since myeloperoxidases are mainly localized inside polymorphonuclear leukocytes. Furthermore, these enzymes require relatively high levels of both NO_2^- and H_2O_2 and more than 1 hour to nitrate protein tyrosine residues[94].

We conclude from our observations that muscle nitrotyrosine is limited to specific protein bands ranging in apparent molecular mass between 66 and 10 KDa, representing one of the major findings in our study. Tyrosine nitration is a selective process which depends on several factors such as the nature of the nitrating agent, the exposure of the aromatic ring to the surface of protein, the location of tyrosine on a loop structure, and the presence of the amino acid glutamate in the local environment of the tyrosine residue[85]. Interestingly, protein tyrosine nitration is not influenced by protein abundance nor by the amount of tyrosine residues in a given protein[85]. Furthermore, the identification of tyrosine nitrated proteins constitutes a step further in the last few years. So far, the majority of the proteins identified are localized within the cytosol. Examples of these proteins are catalase, glutathione-S-transferase, carbonic anhydrase III, tyrosine hydroxylase, cAMP-dependent protein kinase,

lactate dehydrogenase, glycogen synthase, and transketolase[95-97]. Additionally, cytoskeletal proteins such as actin, neurofilament L, tubulin, and myofibrillar creatine kinase have recently been shown to be tyrosine nitrated[95,98,99,100]. Moreover, several mitochondrial proteins such as aconitase, Mn-SOD, ATP synthase, glutamate dehydrogenase, and glutamate oxaloacetate transaminase-2 have also been shown to be tyrosine nitrated[95,100]. Finally, recent evidence has shown that several nuclear proteins such as histones II-S and VIII-S are as well tyrosine nitrated[96]. Whether protein tyrosine nitration affects protein function still remains unclear. Though, it has already been shown that nitration of specific tyrosine residues of Mn-SOD and α-1 antitrypsin is associated with a significant inhibition of the activity of these enzymes[100,101]. Another example is the nitration of the c-terminal tyrosine residue in α-tubulin which compromises both microtubule organization and binding of microtubule-associated proteins[99].

Interestingly, in our study whilst protein tyrosine nitration significantly rose in both the mitochondrial and membrane fractions in response to LPS injection, most of the nitrated proteins in normal skeletal muscles were localized within the cytosolic compartment. The latter is consistent with the finding reported by Aulak *et al*[95], who described nitrotyrosine formation in cultured A459 cells, and in both liver and lungs of LPS-injected rats.

Despite the fact that our study does not provide a comprehensive list of tyrosine nitrated proteins in skeletal muscles, several proteins can be excluded from the above-mentioned list of tyrosine nitrated proteins[95,96,99,100]. In this regard, both sarcomeric actin and myofibrillar creatine kinase can be excluded, since abundant

tyrosine nitration in the myofibrillar fraction was not observed. The enzyme Mn-SOD should also be excluded because the apparent molecular masses of the different tyrosine nitrated proteins detected in our study do not correspond to that of Mn-SOD. Furthermore, this enzyme is localized within the mitochondria, and in normal muscles very little nitrated protein bands were found in this compartment. Othe proteins which have been described to be nitrated such as neurofilament L and lung surfactant protein A can also be excluded since they are not usually abundantly expressed within normal skeletal muscle fibers. Finally, based on the fact that tubulin, which is a cytosolic protein and has an apparent molecular mass of 49 KDa, we conducted a series of experiments to assess whether one of our nitrated protein bands might be tubulin. After immunoprecipitation of normal diaphragm muscle lysates with both monoclonal and polyclonal anti-nitrotyrosine antibodies, which were subsequently separated by electrophoresis and probed with anti-tubulin antibody, we were unable to detect positive tubulin bands in the nitrotyrosine immunocomplexes. We conclude from this finding that tubulin is not tyrosine nitrated in normal rat diaphragm. More studies appear required to clearly elucidate the identity of these nitrated proteins and to establish to what extent this phenomenon is involved in the sepsis-induced skeletal muscle dysfunction.

The development of oxidative stress in the ventilatory muscles in response to sepsis, most probably mediated by peroxynitrite formation, has also been demonstrated in our study by using three well established indices of oxidation. ROS generated *in vivo* are involved in the pathogenesis of several disorders, including cancer, aging, stroke, and cardiovascular diseases[102]. Recent evidence has shown that cytokines, such as TNF-α which is a mediator of the sepsis-induced ventilatory

muscle failure, also stimulates the production of ROS during *E.Coli* endotoxemia[103]. Skeletal muscle may be a major site of oxidation-induced tissue damage in sepsis, since it constitutes the major protein pool within the body. According to this hypothesis, several lines of evidence have confirmed this hypothesis by showing increased levels of ROS in skeletal muscles of septic animals[44-46]. In line with this, in the present study, we have also demonstrated an increase in both the levels of diaphragmatic protein oxidation and lipid peroxidation, and a decrease in total diaphragmatic glutathione levels. Our results are consistent with data reported by Fagan *et al*[83] who found that limb skeletal muscle proteins from septic rats contained significantly more carbonyl groups than muscle proteins from control animals. We have conducted a more comprehensive study on sepsis-induced ventilatory muscle oxidative stress, but data corresponding to this work is not reported herein.

After demonstration of the occurrence of both nitrosative and oxidative stresses in septic ventilatory muscles, we hypothesized whether these muscles might develop protective mechanisms against the damaging effects of sepsis. We also assessed to what extent these two forms of stress mediate the sepsis-induced respiratory muscle dysfunction. In line with this, our study indicates that both HO-1 and HO-2 proteins are expressed at low levels in normal limb and ventilatory muscles. Previous studies have also documented the existence of these two proteins in *in vivo* mammalian tissues and cultured myocytes[66,81,104,105]. Baum *et al*[66] also reported the expression of both HO-1 and HO-2 in normal skeletal muscle and in blood vessels traversing the muscles.

Little information is still available regarding the factors involved in the regulation of HO expression in skeletal muscle. Numerous conditions in non-muscle

cells have been proposed to induce both HO-1 expression and activity, which is the inducible isoform. Among others, heat shock, ischemia, hypoxia, endotoxin, pro-inflammatory cytokines, and hemin are known to induce HO-1 expression[52]. In skeletal muscles, Essig *et al*[77] reported that either exhaustive running or artificial stimulation resulted in marked HO-1 mRNA expression. In cultured myocytes hemin was also shown to increase HO-1 expression[105]. In our study, LPS injection induced a marked increase in both diaphragmatic HO-1 and HO-2 expression in rats, and only in diaphragmatic HO-1 expression in mice. In the study published by Taillé *et al*[81] it was shown that both HO-1 and HO-2 enzymes are constitutively expressed in normal skeletal muscle. These authors also reported an increase in HO-1 expression in rat diaphragms in response to LPS injection, with no change in HO-2 expression. We claim that the experimental conditions (differences in dosage of LPS used in the two studies) may account for the discrepancies found between the two studies regarding HO-2 expression in response to LPS.

We propose several mechanisms whereby HO expression may be regulated in septic animals. On one hand, it can be speculated that the induction of HOs inside muscle fibers late in sepsis might be due in part to increased ROS production which are known to enhance HO expression[43,46,52]. It is unlikely, however, that ROS were involved in the up-regulation of muscle HO-1 expression as early as after 1 hour of LPS injection, since previous reports have documented that enhanced muscle ROS production occurs after several hours of LPS injection[83]. On the other hand, HO-1 induction in septic muscles might have been mediated by stress activated (c-fos and c-jun) and pro-inflammatory (NFκB) transcription factors, since binding sites for these transcription factors have been clearly identified within the promoter region of

HO-1[52]. Additionally, both TNF-α and IL-1α have been shown to activate HO-1 expression in endothelial cells, an action which is mediated via protein kinase C, Ca^{2+}, and phospholipase A_2[106]. Based on these observations, both LPS and pro-inflammatory cytokines, particularly TNF-α are very likely to be directly responsible for increased muscle HO-1 expression in septic animals. Another likely mechanism is based on the observations that exposure of mesangial, endothelial, smooth muscle cells, and cultured myocytes to exogenous NO donors elicits a significant induction of both HO-1 mRNA and activity[56,105,107,108]. It is worth mentioning that under normal conditions NO is synthesized by the constitutive NOS isoforms (nNOS and eNOS)[23]. However, the NOS isoform responsible for muscle NO increased production in response to sepsis is the iNOS[29,31]. Based on our findings, we have excluded the involvement of this isoform in the regulation of muscle HO expression in sepsis, since both inhibition of iNOS activity in rats and absence of iNOS expression in $iNOS^{-/-}$ mice did not modify the effects of LPS on both HO-1 and HO-2 expressions. Finally, the reactive species peroxynitrite has been shown to regulate both HO-1 mRNA expression and activity in endothelial cells[109]. As shown in the first study herein, protein tyrosine nitration (the fingerprint of peroxynitrite formation) is significantly increased in both the ventilatory and limb muscles in response to LPS injection. Therefore, it could be argued that induction of diaphragmatic HO expression in septic animals might be due to peroxynitrite formation in those muscles. This pathway, however, is not very likely, since inhibition of iNOS activity by injecting the animals with 1400W, which is known to clearly reduce peroxynitrite formation, had no effect on LPS-induced muscle HO expression.

One of the major findings in the present study is the fact that inhibition of HO activity by injecting the animals with CrMSPIX clearly increased both protein oxidation and lipid peroxidation, both under normal conditions and in response to LPS injection. Similarly, Taillé *et al*[81] also showed a rise in protein oxidation and malondialdehyde contents in response to HO inhibition in septic muscles, but not in control diaphragms. We speculate that the discrepancies found between the two studies are due to the use of two different inhibitors of HO activity (Chromium Mesoporphyrin IX in our study vs zinc protoporphyrin IX in the study published by Taillé *et al*[81]). We decided to use CrMSPIX, since Appleton *et al*[110] reported that this competitive inhibitor of HO activity is both more powerful and more selective than zinc protoporphyrin IX. Despite this difference, we can conclude from both studies that muscle HO activity clearly plays a relevant and protective role in alleviating the deleterious effects of oxidants on muscle, particularly in septic animals. Previous findings have also shown similar results in non-muscle cells[109,79]. Furthermore, mice deficient in HO-1 protein (HO-$1^{-/-}$ mice) have been shown to be extremely vulnerable to mortality and hepatic cirrhosis induced by sepsis[69]. The protective effects of HO activity have been attributed to the antioxidant properties of its by-products by scavenging muscle oxidants (biliverdin, CO, and ferritin)[72,111,112]. In fact, bilirubin is one of the most abundant endogenous antioxidant in mammalian tissue, and has been shown to efficiently scavenge peroxyl radicals *in vitro* and *in vivo*[112,113]. Ferritin, another by-product of HO catabolism of heme, has recently been shown to induce protection against cellular damage[114], though there is no evidence yet in the literature about its possible protective effects in skeletal muscle. Finally, the CO pathway might also contribute to the protective effects exerted by HO activity in skeletal

muscle, as it does in other tissues[72]. Though, the precise sites of CO effects in skeletal muscle still remain unexplored.

The most remarkable finding in our second study is that inhibition of HO activity elicited a significant impairment of diaphragmatic contractility. As previously mentioned, despite the fact that the exact mechanisms whereby HO activity promotes muscle contractility still remain unknown, we propose several possible pathways. In this regard, HO activity may promote excitation-contraction coupling by inhibiting the deleterious effects of ROS on both sarcoplasmic Ca^{2+} release-channels and sarcoplasmic Ca^{2+} uptake[115,116]. Contractile proteins may also be a target for the action of HO activity. Indeed, attenuation of the deleterious effects of ROS on both myofibrillar Ca^{2+} sensitivity and myofibrillar creatine kinase activity have been shown in cardiac myofibrils[117]. Finally, HOs are likely to influence muscle function by promoting vascular dilation by increasing cGMP via activation of guanylate cyclase[118].

All the experiments described in this study were designed with the aim of better understanding the mechanisms whereby diaphragmatic contractile dysfunction occurs in sepsis. As to the first study, we have documented for the first time that the phenomenon of protein tyrosine nitration is abundantly present in normal muscles and that several proteins of various apparent molecular weights are nitrated in both ventilatory and limb muscles of control animals. We have also first demonstrated in our current study that these nitrated proteins are mainy localized in the cytosol of normal skeletal muscle fibers in close proximity to the sarcolemma. Interestingly, in this study we have also shown for the first time that the inducible isoform is the

major modulator of protein nitration under both normal and septic conditions. Another original finding demonstrated in this study is that sepsis leads to an increase in tyrosine nitration of proteins localized within the mitochondrial and myofibrilar fractions of rat diaphragms, but not of those localized in the cystosol.

In relation to the second study presented herein we have to acknowledge that the recent work published by Taillé *et al*[81] shows various similar findings to those described in this thesis. Both studies demonstrate that HOs exert major antioxidant effects and protect muscle contractile machinery from the inhibitory effects of ROS in both normal and septic skeletal muscles. Nevertheless, we are the first to report that the inducible NOS isoform does not modulate HO expression as assessed by using both pharmacological inhibition and a transgenic mouse model deficient in the iNOS protein. Additionally, in the last study of the current thesis, a collection of experiments were originally designed to investigate the role of HO activity in muscle glutathione concentrations in endotoxemia. As far as we are concerned, this is the first time that the role of HO in relation to muscle glutathione concentrations has been addressed.

Future studies appear required to further investigate the biological significance of protein tyrosine nitration, as well as to identify the nature of these nitrated proteins. Finally, the development of therapeutical approaches specifically designed to upregulate HO-1 expression might be hopeful strategies for precluding sepsis-induced respiratory muscle failure.

FINAL CONCLUSIONS

FINAL CONCLUSIONS

1) Abundant protein tyrosine nitration occurs in the cytosolic fraction of normal skeletal muscles.

2) Sepsis induces an increase in protein tyrosine nitration which mainly occurs in both the mitochondrial and membrane fractions of both ventilatory and limb muscles.

3) Protein tyrosine nitration is limited to specific protein bands ranging in apparent molecular weight between 66 and 10 KDa.

4) The inducible NOS isoform appears to be the main regulator of protein tyrosine nitration in both normal and septic skeletal muscles.

5) Both HO-1 and HO-2 appear to be constitutively expressed at low levels in normal skeletal muscle.

6) The expressions of both HO-1 and HO-2 are up-regulated in response to sepsis.

7) The inducible NOS isoform is not involved in the modulation of HO expression as assessed by using both a selective pharmacological iNOS inhibitor and a transgenic mouse model deficient in the iNOS protein.

8) HOs exert major antioxidant effects and protect muscle contractile machinery from the inhibitory effects of ROS in both normal and septic muscles. Consequently, these enzymes promote sub-maximum and maximum force generation both under normal conditions and in response to severe sepsis.

LIST OF REFERENCES

REFERENCES

1.- Farkas G, Cerny F, Rochester DF. Contractility of the ventilatory pump muscles. *Med Sci Sports Exerc* 1996; 28: 1106-1114.

2.- Fenn O. A comparison of respiratory and skeletal muscles. In: *Perspectives in Biology*. Houssay Memorial Papers, C.F. Cori, V.G. Foglia, L.Leloir, and S. Ochoa (eds.). Elsevier Science BV, Amsterdam 1963.

3.- Parrillo JE. Shock syndromes related to sepsis. In: *Cecil Textbook of Medicine*. Goldman Bennet (eds). W.B. Saunders Company. Philadelphia 2000. pp: 507-512.

4.- Hussain SNA. Respiratory muscle dysfunction in sepsis. *Mol Cell Biochem* 1998; 179: 125-134.

5.- Burke JF, Pontoppidan H, Welch CE. High output respiratory failure. *Ann Surg* 1963; 158: 581-594.

6.- Cohen CA, Zagelbaum G, Gross D, Roussos C, Macklem PT. Clinical manifestations of inspiratory muscle fatigue. *Am J Med* 1982; 73: 308-316.

7.- Friman G. Effects of acute infectious disease on isometric strength. *Scand J Clin Lab Invest* 1977; 37: 303-308.

8.- Hussain SNA, Simkus G, Roussos C. Respiratory muscle fatigue: A cause of ventilatory failure in septic shock. *J Appl Physiol* 1985; 58: 2033-2040.

9.- Leon A, Boczkowski J, Dureuil B, Desmonts JM, Aubier M. Effects of endotoxic shock on diaphragmatic function in mechanically ventilated rats. J *Appl Physiol* 1992; 72: 1466-1472.

10.- Boczkowski J, Dureuil B, Pariente R, Aubier M. Preventive effects of indomethacin on diaphragmatic contractile alterations in endotoxemic rats. *Am Rev Respir Dis* 1990; 142: 193-198.

11.- Shindoh C, DiMarco A, Nethery D, Supinski G. Effect of PEG-superoxide dismutase on the diaphragmatic response to endotoxin. *Am Rev Respir Dis* 1992; 145: 1350-1354.

12.- Krause KM, Moody MR, Andrade FH, Addison AT, Miller CC III, Kobzik L, Reid MB. Peritonitis causes diaphragm weakness in rats. *Am J Respir Crit Care Med* 1998; 157: 1277-1282.

13.- Murphy TD, Gibson R, Standaert TA, Woodrum DE. Diaphragmatic failure during group streptococcal sepsis in piglets: the role of thromboxane A_2. *J Appl Physiol* 1995; 78(2): 491-498.

14.- Wilcox PG, Wakai Y, Walley KR, Cooper DJ, Road J. Tumor necrosis factor α decreases in vivo diaphragm contractility in dogs. *Am J Respir Crit Care Med* 1994; 150: 1368-1373.

15.- Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; 43: 109-142.

16.- Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994; 149: 538-551.

17.- Fang FC. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 1997; 100: S43-S50.

18.- Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* 1996; 271 (40): C1424-C1437.

19.- Goretski J, Hollocher TC. Trapping of nitric oxide produced during denitrification by extracellular hemoglobin . *J Biol Chem* 1988; 263: 2316-2323.

20.- van der Vliet A, Eiserich JP, Shigenaga MK, Cross CE. Reactive nitrogen species and tyrosine nitration in the respiratory tract: Epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999; 160: 1-9.

21.- Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994; 298: 249-258.

22.- Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev* 2001; 81: 209-237.

23.- Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 1994; 372: 546-548.

24.- Gossrau R. Caveolin-3 and nitric oxide synthase I in healthy and diseased skeletal muscle. *Acta Histochem* 1998; 100: 99-112.

25.- Grozdanovic Z, Gossrau R. Co-localization of nitric oxide synthase I (NOS-I) is deficient in the sarcolemma of striated muscle fibers in patients with Duchenne muscular dystrophy, suggesting an association with dystrophin. *Acta Histochem* 1996; 98: 61-69.

26.- AS Comtois, E. Barreiro, Huang PL, Marette A, Perrault M, Hussain SNA. Lipopolysaccharide-induced diaphragmatic contractile dysfunction and sarcolemmal injury in mice lacking the neuronal nitric oxide synthase. *Am J Respir Crit Care* Med 2001; 163: 977-982.

27.- Lin MC, Ebihara S, El-Dwari Q, Hussain SNA, Yang L, Gottfried SB, Comtois A, Petrof BJ. Diaphragm sarcolemmal injury is induced by sepsis and alleviated by nitric oxide synthase inhibition. *Am J Respir Crit Care Med* 1998; 158: 1656-1663.

28.- Kobzik L, Stringer B, Balligand JL, Reid MB, Stamler JS. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. *Biochem Biophys Res Commun* 1995; 211(2): 375-381.

29.- Hussain SNA, Giaid A, El Dwari Q, Sakkal D, Hattori R, Guo Y. Expression of nitric oxide synthases and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. *Am J Respir Cell Mol Biol* 1997; 17: 173-180.

30.- El-Dwari Q, Comtois A, Guo Y, Hussain SNA. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. *Am J Physiol* 1998; 274: C770-C779.

31.- Boczkowski J, Lanone S, Ungureanu-Longrois D, Danialou G, Fournier T, Aubier M. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats. *J Clin Invest* 1996; 98: 1550-1559.

32.- Thompson M, Becker L, Bryant D, Williams G, Levin D, Margraf L, Giroir BP. Expression of the inducible nitric oxide synthase gene in diaphragm and skeletal muscle. *J Appl Physiol* 1996; 81: 2415-2420.

33.- Gath I, Closs EI, Godtel-Armbrust U, Schmitt S, Nakane M, Wessler I, Forstermann U. Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications for contractile function. *FASEB J* 1996; 10: 1614-1620.

34.- Comtois AS, El-Dwari Q, Laubach V, Hussain SNA. Lipopolysaccharide-induced diaphragmatic contractile dysfunction in mice lacking the inducible nitric oxide synthase. *Am J Respir Crit Care Med* 1999; 159: 1975-1980.

35.- Tamir S, Burney S, Tannenbaum SR.DNA damage by nitric oxide. Chem Res Toxicol 1996; 9: 821-827.

36.- Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 1996; 9: 836-844.

37.- Rubbo H, Darley-Usmar V, Freeman BA. Nitric oxide regulation of tissue free radical injury. *Chem Res Toxicol* 1996; 9: 809-820.

38.- Reid MB. Muscle fatigue: mechanisms and regulation. In: Exercise and Oxygen Toxicity. Seck CK, Packer L, Hannine O (eds). Elsevier Science BV, Amsterdam 1998.

39.- Shindoh C, DiMarco A, Thomas A, Manubay P, Supinski G. Effect of N-acetylcysteine on diaphragm fatigue. *J Appl Physiol* 1990; 68: 2107-2113.

40.- Reid MB, Shoji T, Moody MR, Entman ML. Reactive oxygen in skeletal muscle: II. Extracellular release of free radicals. *J Appl Physiol* 1992; 73: 1805-1809.

41.- Jackson MJ, Edwards RH. Free radicals and trials of antioxidant therapy in muscle diseases. *Adv Exp Med Biol* 1990; 264: 485-491.

42.- Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, West MS. Reactive oxygen in skeletal muscle: I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol* 1992; 73: 1797-1804.

43.- Shindoh C, DiMarco A, Nethery D, Supinski G. Effect of PEG-superoxide dismutase on the diaphragmatic response to endotoxin. *Am Rev Respir Dis* 1992; 145: 1350-1354.

44.- Supinski G, Nethery D, DiMarco A. Effect of free radical scavengers on endotoxin-induced respiratory muscle dysfunction. *Am Rev Respir Dis* 1993; 148: 1318-1324.

45.- Peralta JG, Llesuy S, Evelson P, Carreras MC, Gonzalez B, Poderoso JJ. Oxidative stress in skeletal muscle during sepsis in rats. *Cir Shock* 1993; 39: 153-159.

46.- Llesuy S, Evelson P, Gonzalez B, Peralta JG, Carreras MC, Poderoso JJ, Boveris A. Oxidative stress in muscle and liver of rats with septic syndrome. *Free Radic Biol Med* 1994; 16: 445-451.

47.- Supinski G, Nethery D, Stofan D, DiMarco A. Comparison of the effects of endotoxin on limb, respiratory, and cardiac muscles. *J Appl Physiol* 1996; 81: 1370-1378.

48.- Callahan LA, Stofan D, Szweda LI, Nethery D, Supinski G. Free radicals alter maximal diaphragmatic mitochondrial oxygen consumption in endotoxin-induced sepsis. *Free Radic Biol Med* 2001; 30: 129-138.

49.- Callahan LA, Nethery D, Stofan D, DiMarco A, Supinski G. Free radical-induced contractile protein dysfunction in endotoxin-induced sepsis. *Am J Respir Cell Mol Biol* 2001; 24: 210-217.

50.- Abraham NG, Drummond GS, Lutton JD, Kappas A. The biological significance and physiological role of heme oxygenase. *Cell Physiol Biochem* 1996; 6: 129-168.

51.- Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 1968; 61: 748-755.

52.- Choi AMK, Alam J. Heme oxygenase-1 : Function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am J Respir Cell Mol Biol* 1996; 15: 9-19.

53.- Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms and clinical implications. *FASEB J* 1988; 2: 2557-2568.

54.- McCoubrey WK, Ewing JF, Maines MD. Human heme oxygenase-2: characterization and expression of a full-length cDNA and evidence suggesting that the two HO-2 transcripts may differ by choice of polyadenylation signal. *Arch Biochem Biophys* 1992; 295: 13-20.

55.- McCoubrey WK, Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 1997; 247: 725-732.

56.- Datta PK, Lianos EA. Nitric oxide induces heme oxygenase-1 gene expression in mesangial cells. *Kidney Int* 1999; 55: 1734-1739.

57.- Polte T, Abate A, Dennery PA, Schroder H. Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. *Arterioscler Thromb Vasc Biol* 2000; 20: 1209-1215.

58.- Terry CM, Clikeman JA, Hoidal JR, Callahan KS. Effect of tumor necrosis factor-α and interleukin-1α on heme oxygenase-1 expression in human endothelial cells. *Am J Physiol Heart Circ Physiol* 1998; 274: H883-H891.

59.- Wagner CT, Durante W, Christodoulides N, Hellums JD, Schafer AI. Hemodynamic forces induce the expression of heme oxygenase in cultured vascular smooth muscle cells. *J Clin Invest* 1997; 100: 589-596.

60.- Eyssen-Hernandez R, Ladoux A, Frelin C. Differential regulation of cardiac heme oxygenase and vacular endothelial growth factor mRNA expressions by hemin, heavy metals, heat shock, and anoxia. *FEBS Lett* 1996; 382: 229-233.

61.- Carraway MS, Ghio AJ, Taylor JL, Piantadosi CA. Induction of ferritin and heme-oxygenase-1 by endotoxin in the lung. *Am J Physiol* 1998; 275: L583-L592.

62.- Camhi SL, Alam J, Wiegand GW, Chin BY, Choi AMK. Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5' distal enhancers: role of reactive oxygen intermediates and AP-1. *Am J Respir Cell Mol Biol* 1998;18: 226-234.

63.- Lee PJ, Alam J, Sylvester SL, Inamdar N, Otterbein L, Choi AMK. Regulation of heme oxygenase-1 expression *in vivo* and *in vitro* in hyperoxic lung injury. *Am J Respir Cell Mol Biol* 1996; 14: 556-568.

64.- Otterbein LE, Choi AMK. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol* 2000; 279: L1029-L1037.

65.- Stulhemeier KM. Activation and regulation of Hsp32 and Hsp70. *Eur J Biochem* 2000; 267: 1161-1167.

66.- Baum O, Feussner M, Richter H, Gossrau R. Heme oxygenase-2 is present in the sarcolemma region of skeletal muscle fibers and is non-continuously co-localized with nitric oxide synthase-1. *Acta Histochem* 2000; 102: 281-298.

67.- Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME. Induction of heme oxygenase is a rapid protective response in rhabomyolysis in the rat. *J Clin Invest* 1992; 90: 267-270.

68.- Poss KD, Tonegava S. Heme oxygenase-1 is required of mammalian iron reutilization. *Proc Natl Acad Sci USA* 1997; 94: 10919-10924.

69.- Poss KD, Tonegava S. Reduced stress defense in heme oxygenase-1 deficient cells. *Proc Natl Acad Sci USA* 1997; 94: 10925-10930.

70.- Yachie A, Moore AR, Frederick R, Willoughby DA. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 1999; 103: 129-135.

71.- Morita T, Perrella MA, Lee, ME, Kourembanas S. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc Natl Acad Sci USA* 1995; 92: 1475-1479.

72.- Otterbein LE, Mantell LL, Choi AMK. Carbon monoxide provides protection against hyperoxic lung injury. *Am J Physiol* 1999 ; 276 : L688-L694.

73.- Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell R, Choi AMK. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000; 6: 422-428.

74.- Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987; 235: 1043-1045.

75.- Gopinathan V, Miller NJ, Milner AD, Rice-Evans CA. Bilirubin and ascorbate antioxidant activity in neonatal plasma. *FEBS Lett* 1994; 349: 197-200.

76.- Balla G, Jacob SH, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 1992; 267: 18148-18153.

77.- Essig DA, Borger DR, Jackson DA. Induction of heme oxygenase-1 (HSP32) mRNA in skeletal muscle following contractions. *Am J Physiol* 1997; 272: C59-C67.

78.- Vesely MJJ, Sanders R, Green CJ, Motterlini R. Fibre type specificity of haem oxygenase-1 induction in rat skeletal muscle. *FEBS Lett* 1999; 458: 257-260.

79.- Otterbein L, Sylvester SL, Choi AMK. Hemoglobin provides protection against lethal endotoxemia in rats: the role of heme oxygenase-1. *Am J Respir Cell Mol Biol* 1995; 13: 595-601.

80.- Otterbein L, Chin BY, Otterbein SL, Lowe VC, Fessler HE, Choi AMK. Mechanims of hemoglobin-induced protection against endotoxemia in rats: a ferritin-independent pathway. *Am J Physiol* 1997; 272: L268-L275.

81.- Taillé C, Foresti R, Lanone S, Zedda C, Green C, Aubier M, Motterlini R, Boczkowski J. Protective role of heme oxygenases against endotoxin-induced diaphragmatic dysfunction in rats. *Am J Respir Crit Care Med* 2001; 163: 753-761.

82.- Rock E, Napias C, Sarger C, Chevallier J. Simultaneous preparation of membrane fractions from small amounts of skeletal muscle: a study on mitochondrial fractions from Med^{J} mice. *Biochem Biophys Res Commun* 1985; 128: 113-119.

83.- Fagan JM, Ganguly M, Tiao G, Fischer JE, Hasselgren PO. Sepsis increases oxidatively damaged proteins in skeletal muscle. *Arch Surg* 1996; 131:1326-1332.

84.- Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969; 27: 502-522.

85.- Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Bioph* 1998; 356:1-11.

86.- Gunther M, Hsi LC, Curtis JF, Gierse JK, Marnett LJ, Eling TE, Mason RP. Nitric oxide trapping of the tyrosil radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical nitrotyrosine formation. *J Biol Chem* 1997; 272: 17086-17090.

87.- Knowles ME, McWeeny DJ, Couchman L, Thorogood M. Interaction of nitrite with proteins at gastric pH. Nature 1974; 247: 288-289.

88.- Lanone S, Mebazaa A, Heymes C, Henin D, Poderoso JJ, Panis Y, Zedda C, Billiar T, Payen D, Aubier M, Boczkowski J. Muscular contractile failure in septic patients: role of the inducible nitric oxide synthase pathway. *Am J Resp Crit Care Med* 2000;162:2308-2315.

89.- Boczkowski J, Lisdero CL, Lanone S, Samb A, Carreras MC, Boveris A, Aubier M, Poderoso J. Endogenous peroxynitrite mediates mitochondrial dysfunction in rat diaphragm during endotoxemia. *FASEB J* 1999;13:1637-1647.

90.- Supinski G, Stofan D, Nethery D, DiMarco A. Apocynin improves diaphragamtic function after endotoxin administration. *J Appl Physiol* 1999; 87:776-782.

91.- Reiter CD, Teng RJ, Beckman JS. Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 2000; 275:32460-32466.

92.- Nethery D, Callahan LA, Stofan D, Mattera R, DiMarco A, Supinski G. PLA_2 dependence of diaphragm mitochondrial formation of reactive oxygen species. *J Appl Physiol* 2000; 89:72-80.

93.- van der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem 1997;* 272:7617-7625.

94.- Sampson J B, Ye Y, Rosen H, Beckman JS. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch Biochem Bioph* 1998; 356:207-213.

95.- Aulak K S, Miyagi M, Yan L, West KA, Massillon D, Crabb JW, Stuehr DJ. Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 2001; 98:12056-12061.

96.- Kuo N, Kreahling JM, Shanbhag VP, Shanbhag PP, Mewar M. Protein nitration. *Mol Cell Biochem* 2000; 214:121-129.

97.- Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, Ischiropoulos H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* 1998; 95:7659-7663.

98.- Mihm M J, Coyle CM, Schanbacher BL, Weinstein DM, Bauer JA. Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. *Cardiovasc Res* 2001; 49:798-807.

99.- Eiserich J P, Estevez AG, Bamberg T, Ye YZ, Chumley PH, Beckman JS, Freeman BA. Microtubule dysfunction by post-translational nitrotyrosination of α-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc Natl Acad Sci USA* 1999; 96:6365-6370.

100.- MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS. Nitration and inactivation of manganese superoxide dismutase in chronic jection of human renal allografts. *Proc Natl Acad Sci USA* 1996; 93:11853-11858.

101.- Moreno J J, Pryor WA. Inactivation of α1-proteinase inhibitor by peroxynitrite. *Chem Res Toxicol* 1991; 5:425-431.

102.- Stadtman ER. Protein oxidation and aging. *Science* 1992; 257: 12220-1224.

103.- Lloyd SS, Chang AK, Taylor FB, Janzen EG, McCay PB. Free radicals and septic shock in primates: the role of tumor necrosis factor. *Free Radic Biol Med* 1993; 14: 233-242.

104.- Kusner LL, Kim E, and Kaminski HJ. Heme oxygenase-2 expression at rat neuromuscular junctions. *Neurosci Lett* 1999*;* 273: 143-146.

105.- Vesely MJJ, Exon DJ, Clark JE, Foresti R, Green CJ, and Motterlini R. Heme oxygenase-1 induction in skeletal muscle cells: hemin and sodium nitroprusside are regulators in vitro. *Am J Physiol (Cell Physiol)* 1998; 275: C1087-C1094.

106.- Terry CM, Clikeman JA, Hoidal JR, and Callahan KS. TNF–α and IL-1α induce heme oxygenase-1 via protein kinase C, Ca^{2+}, and phospholipase A_2 in endothelial cells. *Am J Physiol (Cell Physiol)* 1999; 276: H1493-H1501.

107.- Durante W, Kroll MH, Christodoulides N, Peyton KJ, and Schafer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res* 1997; 80: 557-564.

108.- Hartsfield CL, Alam J, Cook JL, and Choi AMK. Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. *Am J Physiol (Lung Cell.Mol.Physiol.)* 1997; 273: L980-L988.

109.- Foresti R, Sarathchandra P, Clark JE, Green CJ, and Motterlini R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem J* 1999; 339: 729-736.

110.- Appleton SD, Chretien ML, McLaughlin BE, Vreman HJ, Stevenson DK, Brien JF, Nakatsu K, Maurice DH, and Marks GS. Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations. *Drug Metab Dispos* 1999; 27: 1214-1219.

111.- Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, and Snyder SH. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol* 1999; 1: 152-157.

112.- Minetti M, Mallozzi C, Di Stasi AM, and Pietraforte D. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. *Arch Biochem Biophys* 1998; 352: 165-174.

113.- Clark, Foresti R, Sarathchandra P, Kaur H, Green C, Morttelini R. Heme oxygenase-1 derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol* 1999; 278: H643-H451.

114.- Ferris C, Jaffrey S, Sawa A, Takahaashi M, Brady S, Barrow R, Tysoe S, Wolosker H, Baranano D, Dore S, Poss K, Snyder S. Heme oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol* 1999; 1: 152-157.

115.- Andrade FH, Reid MB, Allen DG, and Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol* 1998; 509: 565-575.

116.- Brotto MAP, Nosek TM. Hydrogen peroxide disrupts calcium release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J Appl Physiol* 1996; 81: 731-737.

117.- Mekhfi H, Veksler V, Mateo P, Maupoil V, Rochette L, and Ventura-Clapier R. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. *Circ Res* 1996; 78: 1016-1027.

118.- Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton WR, Lee ME, Nabel GJ, and Nabel EG. Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat Med* 2001; 7: 693-698.

ADDENDUM

AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY®
AN OFFICIAL JOURNAL OF THE AMERICAN THORACIC SOCIETY

Dr. Sabah N.A. Hussain
McGill University
Royal Victoria Hospital/Meakins-Christie Labs
687 Pine Avenue West, Room I.3.05
Montreal, Quebec H3A 1A1, Canada

FAX: 514-843-1686

RE: MS # 4634

Dear Dr. Hussain:

I am pleased to inform you that your revised manuscript entitled "Protein tyrosine nitration in the ventilatory muscles: Role of nitric oxide synthase" has been accepted for publication in the *American Journal of Respiratory Cell and Molecular Biology.*

Further changes in the text are not possible in the galley proofs, except for corrections of errors in editing and typesetting. Thus, please contanct the editorial office within seven days if there are any changes you wish to make to your manuscript before it is typeset. You are encouraged to submit a final version on disk, accompanied by a hard-copy printout (including tables) as well with any changes highlighted. In addition, artwork on disk is acceptable, but must be on a separate disk from the text. On the disk's label, please specify the file name, MS-DOS or Macintosh, and the word-processing software used. WordPerfect or Microsoft Word files are preferred, but all files are acceptable.

Please also note that you will be billed at the rate of $40 per printed page.

Thank you for submitting your paper to the *Journal.* I look forward to seeing it in a future issue.

Sincerely,

Joe G. N. Garcia, MD
Associate Editor

Subject: AJP: Lung Cellular and Molecular Physiology - LCMP-00495-2001.R1

Dear Dr. Hussain:

I am pleased to inform you that your manuscript

Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress, (LCMP-00495-2001.R1), has been accepted and is eligible for immediate online publication in Articles in Press. If you no longer wish to participate, please e-mail tbynum@the-aps.org indicating this decision.

In case there are changes in authorship (addition, deletion, change of order), a Change of Authorship Form must be obtained from http://www.the-aps.org/publications/journals/pub_author_form.PDF, and all the authors must sign it. The signed form should then be faxed to 301-571-1814.

There are two steps to the final publication of your work.

1. FOR ARTICLES IN PRESS:

If you still wish to participate, please log on to www.apscentral.org where you will find your manuscript listed under Manuscripts Accepted for Articles in Press. Please follow

the instructions given directly under the heading on how to proceed. You are to submit a single, clean PDF (no changes tracked in red) containing all the elements of the manuscript. When you have completed the process you will be issued with an electronic acknowledgment. Please note that this submission will

be designated as a revision. This, of course, is not the case.

2. FOR PRINT PRODUCTION:

PLEASE SEND THIS MATERIAL WITHIN 48 HOURS OF RECEIPT OF THIS MESSAGE so that the production schedule can proceed as quickly as possible. As the PDF version cannot be copyedited, you are still required

to mail one hard copy of the final, accepted version, an electronic file of the text, preferably in MS Word, and figures,

either saved as .TIFF or .EPS files, and TWO sets of camera-ready artwork to APS Central, 9650 Rockville Pike, Bethesda, MD

20814. Please clearly mark your manuscript "FINAL ACCEPTED VERSION" and be sure that the MANUSCRIPT NUMBER

(LCMP-00495-2001.R1) is on
the upper right-hand corner on each page. These files may be
saved on two 3.5 inch high density, PC-formatted disks, a zip
disk, or a CD-ROM. Full instructions on how to prepare accepted

manuscripts for print publication are available at
http://www.the- aps.org/publications/journals/pub_ajp_info.htm

under Submission of Accepted Manuscripts. As we do not have the

technology in place yet to allow you to upload your native files

for copyediting, we appreciate that this may seem like a
duplication of effort, but we are working toward this goal.

Thank you for your continued interest in the journal and
congratulations to you and your colleagues on such an excellent study.

Sincerely,
Dr. Paul Schumacker
Associate Editor