Nitrosative and oxidative stresses in the ventilatory muscles: Role of nitric oxide synthases and heme oxygenases

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ABSTRACT

Reactive oxygen species (ROS) and nitric oxide (NO) modulate normal and septic skeletal muscle contractility and redox state. We evaluated the involvement of NO synthases (NOS) and heme oxygenases (HO) 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) or neuronal (nNOS) isoforms with 20 mg/kg *E.Coli* lipolysaccharide (LPS). Peroxynitrite formation was monitored by measuring nitrotyrosine immunoreactivity with selective antibodies. Oxidative stress was assessed by measuring protein oxidation (carbonyl groups), lipid peroxidation (hydoxynonenal protein adducts), and muscular glutathione concentrations. HO expression was evaluated using anti-HO-1 and -HO-2 antibodies, and biological significance using HO inhibitor, and contractility studies. We conclude that the iNOS isoform plays a major role in nitrotyrosine formation and protein oxidation in septic ventilatory muscles, and that HO protect muscle contractile machinery from the deleterious effects of ROS in control and septic muscles.

RÉSUMÉ

Dans un modèle de dysfonction diaphragmatique septique, nous avons évalué le rôle des NO synthétases (NOS), hème oxygénases (HO) et des radicaux nitrosylés et oxygénés produits sur la contractilité et l'équilibre redox. Après injection de lipopolysaccharide (*E.Coli*, 20mg/kg), chez des rats et des souris transgéniques déficientes pour la NOS inductible (iNOS) ou neuronale (nNOS), ont été étudiés la contractilité musculaire et la formation de péroxynitrite en mesurant la nitrotyrosine, le stress oxydatif en mesurant l'oxydation des protéines (groupes carbonyles), la péroxydation lipidique (conjugués protéiques hydroxynenonaux), et les concentrations musculaires en glutathion. L'expression des HO a été détectée à l'aide d'anticorps anti-HO-1 et –HO-2. Ces paramètres ont ensuite été étudiés en présence d'inhibiteur des HO. Lors du sepsis, l'isoforme iNOS semble impliquée dans la formation de nitrotyrosine et l'oxydation des protéines des muscles respiratoires. Les HO protégerait l'appareil contractile des effets délétères des RO dans les muscles normaux et septiques.

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PREFACE

During my Master in Science studies in the Critical Care Division of the Royal Victoria Hospital I have contributed to the following manuscripts and abstracts:

A) 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.

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5.- E. Barreiro, J. Gea, A. S. Comtois, S.N.A. Hussain.
Protein nitration in the ventilatory muscles: role of nitric oxide synthases. Submitted to the ERS meeting, Berlin 2001.

B) MANUSCRIPTS

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Protein tyrosine nitration in the ventilatory muscles: role of nitric oxide synthases. Submitted to the Am J Respir Cell Mol Biol (V-2001).

2.- E. Barreiro, L. Lands, S. Mohammed, J. Gea, S.N.A. Hussain.
Oxidative stress of the ventilatory muscles in septic shock: role of nitric oxide synthases. Manuscript in preparation.

3.- E. Barreiro, A.S. Comtois, S. Mohammed, L. Lands, S.N.A. Hussain.
Role of heme oxygenases in the ventilatory muscles: influence on oxidative stress. Manuscript in preparation.

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 one full paper already submitted as well as the text of two more manuscripts which are currently being written for future publication.

Several investigators have contributed to the preparation of both abstracts and manuscripts aforementioned. By order of citation:

Dr AS 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 SNA Hussain is my supervisor. 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, including both abstracts and manuscripts.

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.

I have chosen the option of presenting the data on full unnumbered pages with figure legends included on separate sheets at the end of each study.

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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/ -6: interleukin-1/ -6

ROS: reactive oxygen species

NO: nitric oxide

NOS: nitric oxide synthase-s

nNOS: neuronal nitric oxide synthase

iNOS: inducible nitric oxide synthase

eNOS: endothelial nitric oxide synthase

NADPH: nicotinamide adenine dinucleotide phosphate hydrogen

ATP: adenosin triphosphate

SR: sarcoplasmic reticulum

SNP: nitroprusside

$_L$-NAME : N^G-nitro-$_L$-arginine methyl ester

MVS : Maximal velocity of shortening

RyR : Ryanodine Receptor-s

IFN-γ: interferon γ

TBAR: thiobarbituric acid-reactive substances

HO: heme oxygenase-s

CO: carbon monoxide

SOD: superoxide dismutase

MnSOD : manganese SOD

CuZnSOD: copper-zinc SOD

NO_2Tyr: modified amino acid 3-nitrotyrosine

GSH: reduced glutathione

GSSG: oxidized glutathione

HNE: hydroxynonenal

CrMP : chromium (III) mesoporphyrin IX

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INTRODUCTION AND LITERATURE REVIEW

1.- RESPIRATORY MUSCLES

The ventilatory muscles are striated skeletal muscles that are in control of the rhythmical displacement of the chest wall to move air into and out of the lungs, in order to maintain arterial blood gases within adequate limits[1]. The respiratory muscles are different from the limb skeletal muscles in several aspects, which are directly related to their functional role. Basically, the ventilatory muscles overcome elastic and resistive loads, and they are under both voluntary and involuntary control. Furthermore, the resting length of the respiratory muscles is established by the balance between the inward recoil forces of the lung and the outward recoil forces of the chest wall [2, 1]. Finally, their function is essential to life, since they have to contract rhythmically and generate the required forces for ventilation throughout the whole existence of the individual[3]. The ventilatory pump muscles are roughly classified as inspiratory or expiratory muscles based on their mechanical actions. These actions are redundant in order to ensure several means whereby air can be effectively moved under any physiologic or pathophysiologic condition [4].

2.- SEPSIS AND SEPTIC SHOCK

2.1.- CLINICAL MANIFESTATIONS

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. Furthermore, when hypotension or multiple organ system failure occurs as a consequence of this syndrome the condition is called septic shock [5]. Several organs may be affected in septic shock, such as the central nervous system, the heart, the kidneys, the liver, the gastrointestinal tract, the immune system, and the lungs. Septic shock is characterized by a state of reduced vascular perfusion which leads to extensive impairment of cellular function. 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 vascular 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 is now the most common cause of death in the intensive care units[5].

2.2.- ETIOLOGY AND PATHOGENESIS

Both gram-negative and gram-positive microorganisms as well as fungi can cause sepsis and septic shock. Though, gram-negative bacteria constitutes by far the most frequent etiology of all the septic shock syndromes. Microorganisms lead to septic shock through different mediators, such as polysaccharide structures, toxin A, and the teichoic acid antigens. However, endotoxin, which is the distinctive lipolysaccharide (LPS) associated with the cell membrane of gram-negative organisms, represents the classic example of an initiator of the septic shock pathogenetic cascade[5]. The endotoxin molecule consists of an inner oligosaccharide core that has 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[5]. The lipid A has been the target for many attempts to synthesize either analogues or inhibitors, since it is the structure responsible for many of the toxic properties of endotoxin.

2.3.- EXPERIMENTAL SHOCK

The administration of endotoxin to experimental animals results in a cardiovascular response very comparable to that described in patients with septic shock. Moreover, the administration of a very small dose of purified endotoxin to normal humans produces fever, mild constitutional symptoms, and a cardiovascular response similar to that of spontaneous sepsis. In patients with septic shock and positive blood cultures, detectable plasma endotoxin is associated with increased mortality, leading to the concept that endotoxin is an important mediator in many septic shock patients[5].

2.4.- EVIDENCE OF SEPSIS-INDUCED RESPIRATORY MUSCLE DYSFUNCTION

2.4.1.- PATIENTS

Among the multiple organ system failure, respiratory insufficiency is now considered to be the most important cause of death in patients with septic shock[6]. Respiratory failure has traditionally been attributed to lung injury which causes impaired gas exchange, decreased lung compliance, and increased pulmonary shunting due to bilateral pulmonary infiltration. Although lung injury has been shown to occur in sepsis, there is nowadays growing evidence that septic shock is also associated with ventilatory pump failure. Hence, sepsis-induced ventilatory muscle failure has been the focus of many recent studies. Burke *et al* [7] described that hypercapnic respiratory failure occurred in patients with fulminating septic shock in the presence of normal PaO_2. It was also shown clinical and electromyographic evidence of diaphragmatic contractile failure in patients with severe sepsis who could not be weaned from mechanical ventilation[8]. This observation was based on the concept described by Friman who reported that maximum force and endurance capacity of various limb muscles significantly declined during acute infections in humans [9].

2.4.2.- ANIMAL EXPERIMENTS

There is great evidence as well that experimental septic shock is associated with depressed contractile performance of both limb and ventilatory muscles. Experimental septic shock, which is produced by the systemic administration of live microorganisms or bacterial endotoxin to experimental animals, results in similar cardiovascular features observed in the hypodynamic phase of human septic shock. As a result, Hussain *et al* were the first to report that the ventilatory failure of *Escherichia Coli* (*E. Coli*) endotoxic shock in dogs over several hours was due to fatigue of the respiratory muscles, eventually leading to hyerpcapnic respiratory failure[10]. Other studies also confirmed these findings. For instance, Leon and coworkers[11] reported that acute (less than 60 min) *E. Coli* endotoxemia reduced diaphragm force in response only to high frequency stimulation in mechanically ventilated rats. In another study it was also shown a decline in diaphragmatic force in response to only high frequency stimulation after 3

days of endotoxemia in rats[12]. However, Shindoh and colleagues showed a decrease in diaphragmatic force over a wide range of stimulation frequencies after 3 days of endotoxemia in hamsters[13]. Though, both groups of investigators concluded that diaphragmatic endurance in response to low frequency stimulation was significantly attenuated after prolonged endotoxemia in both experimental animals. More recently, it has also been shown that peritonitis caused diaphragm weakness in rats, leading to the concept that humans with peritonitis might also be predisposed to respiratory muscle dysfunction[14]. Eventually, systemic inoculation of live *Streptococcus pneumoniae* in rats also induced a significant decline in both diaphragmatic force and endurance after 3 days of bacteremia in response to artificial phrenic nerve stimulation[15].

3.- FACTORS INVOLVED IN SEPSIS-INDUCED VENTILATORY MUSCLE FAILURE

3.1.- VENTILATORY, METABOLIC AND HEMODYNAMIC FACTORS

One group of factors are the consequence of the imbalance between the increased ventilatory muscle metabolic demands due to augmentation of ventilation, hypoxemia, and increased pulmonary resistance, and the poor oxygen and metabolite muscle extraction. It has been established that ventilatory demands are increased in septic humans as well as in endotoxemic animals due to a combination of increased minute ventilation and alterations in pulmonary and airway mechanics[6]. It has also been shown that at low blood pressure values, muscle blood flow becomes a function of arterial pressure despite the significant decline in muscle vascular resistance[16]. Therefore, it is predictable that ventilatory muscle blood flow may be insufficient to meet muscle metabolic demands, leading to limitation of muscle aerobic capacity and increased reliance on anaerobic metabolism, and hence, increased lactic acid production[17]. Moreover, maldistribution of blood flow and underperfusion of active muscle fibers would also lead to the same effects[17]. Finally, septic shock and endotoxemia are associated as well with metabolic alterations involving the utilization of carbohydrates, lipids and proteins which are likely to depress skeletal muscle contractile performance[6].

3.2.- MEDIATORS OF MUSCLE DYSFUNCTION

The other group of factors include specific cellular, metabolic, and immune deficiencies which interfere with a number of processes necessary for normal force generation[6]. These defects are mediated by complex interactions between several local and systemic mediators, which together contribute to the respiratory muscle dysfunction described in sepsis.

3.2.1.- ENDOTOXIN

Bacterial endotoxins have been proposed to directly act on skeletal muscles, leading to a sequence of events which ultimately would precipitate muscle failure. Though, there is no clear evidence showing their direct impact on muscle function. Accordingly, Diaz and coworkers demonstrated that *E. Coli* endotoxin had no direct effects on the *in vivo* contractility of isolated rat diaphragm strips after 1h of exposure[18].

3.2.2.- CYTOKINES

It is well known that endotoxin can stimulate monocytes, macrophages and mast cells to produce tumor necrosis factor (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, hence, it has been the focus of several experimental studies. Wilcox and colleagues[19] reported that 3 h after systemic TNF-α infusion in dogs, both diaphragmatic pressure and shortening were depressed compared to control animals in response to artificial phrenic nerve stimulation. Moreover, since peak to peak amplitude of muscle action potential significantly declined after several hours of TNF-α infusion, this decline in contractility appeared to be mediated through neuromuscular transmission failure[19]. In another study it was reported that TNF-α messenger RNA was increased in the rat diaphragms after 3 h of endotoxin infusion[20]. Furthermore, these authors also found that endotoxin-induced diaphragmatic hypocontractility was partially reversed by pre-treating the animals with anti-TNF-α antibodies, leading to the concept that TNF-α plays a major role in sepsis-induced muscle contractile dysfunction. However, it is not clear yet whether TNF-α directly acts on muscle contractile machinery. Diaz and

coworkers [18] showed no direct effect of TNF-α on muscle force contractility in isolated rat diaphragm strips after 1 h of incubation with this cytokine. On the contrary, it is very likely that TNF-α may act by inducing secondary messenger molecules such as **reactive oxygen species (ROS)** and **nitric oxide (NO)**, whose action mechanisms will be discussed below in separate sections, since both have extremely relevant contribution to sepsis-induced ventilatory muscle dysfunction.

3.2.3.- ARACHIDONIC ACID METABOLISM

It has also been shown that endotoxin could alter arachidonic acid metabolism, leading to the release of prostaglandins such as prostacyclin and thromboxane A_2 [6]. Boczkowski *et al* [12] concluded that the decline in diaphragmatic force generating capacity and prolongation of twitch relaxation time observed after 3 days of sublethal endotoxemia was abrogated by pre-injecting the rats with indomethacin, an inhibitor of cyclooxygenase. Indomethacin pre-treatment also led to similar results during acute bacteremia in piglets[21]. However, in this study the effects of indomethacin were attributed to the prevention of thromboxane A_2 release, since the infusion of an analogue of this molecule elicited comparable changes in diaphragmatic force similar to those described in acute endotoxemia[21].

4.- NITRIC OXIDE

4.1.- NITRIC OXIDE BIOCHEMISTRY

NO is a multifunctional molecule which participates in many biological processes in almost all aspects of life, including vasodilatation, bronchodilation, neurotransmission, inhibition of phagocyte and platelet aggregation, and antimicrobial activity[22-24]. Nitric oxide is an uncharged molecule composed of seven electrons from nitrogen and eight electrons from oxygen[25]. The highest occupied orbital in NO contains an unpaired electron, since orbitals can only contain a maximum of 2 electrons. NO only reacts rapidly with a variety of molecules that have orbitals containing unpaired electrons, such as other free radicals and transition metals like heme iron[25]. Therefore,

NO diffuses rapidly through most tissues with little reaction because the majority of biological molecules contain bonds occupied with 2 electrons. In other words, NO does not react rapidly with most biological molecules at the dilute concentrations produced *in vivo*. Hence, this characteristic makes NO an extremely useful intracellular messenger.

Although NO produced in an endothelial cell will diffuse much faster than it will react with most intracellular components, its biological chemistry can easily be simplified in 3 main reactions, using the reaction rates involving NO as a guide [25]. First, NO may act as a signalling molecule through binding and activation of guanylate cyclase[25]. Second, it may be destroyed by reaction with oxyhemoglobin within a red blood cell to form nitrate[26]. Third, it can also be transformed to peroxynitrite ($ONOO^-$) by reaction with superoxide ($O2^-$)[25], whose reaction is usually limited by the micromolar concentrations of superoxide dismutases (SOD) in cells. However, excessive NO production, as it occurs during active inflammatory-immune processes, leads to detrimental effects of NO in tissues, which have been attributed to its diffusion-limited reaction with superoxide to form the powerful and toxic oxidant peroxynitrite. This highly reactive species is considered to be mostly responsible for the majority of the damaging effects of excessive NO release[25]. When the concentration of NO rises to the micromolar range it may outcompete SOD for reaction with superoxide, since this reaction is 3-fold faster than that of SOD. Therefore, peroxynitrite formation will be the consequence of excessive production of both NO and superoxide, a condition almost invariably occurring at sites of active inflammatory-immune processes[27].

4.1.1.- MECHANISM OF ACTION

NO responses are mediated through reactions with thiol or transition metal centers in proteins or both[28-30]. Specifically, NO reactions are mostly mediated by S-nitrosylation of cysteine redox centers or by coordinate interactions with heme or nonheme iron copper[31]. Examples of interaction of NO with heme-containing proteins are on one hand, the binding to cytochrome-*c* oxidase, which reversibly inhibits the enzyme and consequently cell respiration[32], and on the other, the binding to guanylate cyclase, which activates the enzyme, raising cyclic guanosine monophosphate (cGMP) levels [33]. Many actions of cGMP are elicited by cGMP-dependent kinase. However, cGMP effects

are generally moderate in skeletal muscle, and guanylate cyclase activity is relatively low compared with other tissues[31]. Although the mechanisms whereby cGMP acts in skeletal muscles are not well understood, one proposed mechanism is through phosphorylation of the nicotinic acetylcholine receptor subunits[34] by neuronal NOS (nNOS) and its cGMP-dependent protein kinase which are both enriched at the neuromuscular end plate[35]. However, in another study endogenous cGMP-dependent kinase was shown to phosphorylate dystrophin[36].

4.1.2.- PEROXYNITRITE AND TYROSINE NITRATION

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) [27]. For example, reactions with thiol residues to form S-nitrosothiols might be considered as a mechanism of either enzyme regulation or NO transport. Moreover, S-nitrosothiols in proteins or in low-molecular weight thiols, such as glutathione have been detected in the circulation, as well as in respiratory tract lining fluids [23,27]. As well, the mutagenic properties of NO have been attributed to nitrosation of amines by these reactive nitrogen species, most likely through nitrosative deamination of DNA bases[37].

However, more irreversible NO-induced modifications include nitration of aromatic amino acids, lipids, or DNA bases[38,39,37]. The amino acid tyrosine appears to be a primarily susceptible target for nitration. As a matter of fact, 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*[27]. Furthermore, there is growing evidence that nitration of essential tyrosine residues can inactivate many 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 active inflammatory-immune processes.

4.2.- LOCALIZATION AND EXPRESSION OF NITRIC OXIDE SYNTHASES IN SKELETAL MUSCLES

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)[40]. Three isoforms have been identified, two of which are constitutively expressed and were originally purified in the endothelial cells (eNOS, NOS3), and brain cells (nNOS, NOS1). The third, which is an inducible isoform, was initially purified in macrophages (iNOS, NOS2). The requirements for calcium (Ca^{2+}) and calmodulin differ between eNOS and nNOS on one hand, and iNOS on the other. However, the classification of NOS as constitutive or inducible isoforms has been shown to result inconsistent since the inducibility is highly dependent on the stimulus rather than the gene product[31]. Furthermore, the numerical designation is currently being used more frequently as each of the NOS isoform has a wide tissue distribution[31].

4.2.1.- nNOS

This enzyme has been identified in skeletal muscles by immunological, histochemical, and cellular fractionation methods. Recent experiments have shown that skeletal-muscle nNOS is an alternatively spliced form (nNOSμ), which is slightly larger than the brain isoform [41].

Kobzik *et al* [42] found that adult rat skeletal muscle contains a neuronal-type NOS localized in the sarcolemma of some, but not all, muscle fibers. Muscle histochemistry using ATPase staining identified NOS-positive fibers as type II (fast twitch fibers), and NOS-negative fibers as type I (slow twitch fibers). These authors also found prominent staining of cGMP in the area of the sarcolemma, supporting the concept that NOS and guanylate cyclase activities may be coupled. Neuronal-type NOS has also been shown to be enriched at the muscular endplate (9,p.10). In contrast, nNOS do not exhibit a specific fiber type distribution in human skeletal muscles as has been shown to be equally distributed in both type I and type II fibers[43,44].

Developmental regulation in ventilatory muscles varies from that in limb skeletal muscles[45]. Both NOS activity and nNOSμ and eNOS expressions were elevated in fetal and neonatal diaphragm, showing a gradual reduction in the expression levels during late postnatal development. Besides, nNOS was localized at the sarcolemma of both neonatal and adult diaphragm.

The association of nNOS with the sarcolemma is mediated through the interaction of this NOS isoform with the dystrophin complex[46]. Alteration of the dystrophin complex in Duchenne muscular dystrophy and in *mdx* mice (a dystrophic mouse model) causes downregulation of nNOS expression[47] as well as displacement of nNOS from the sarcolemma to the cytoplasm of the skeletal muscle fibers, where it is diffusely distributed at reduced levels [46,48,44].

Chronic muscle activation and mechanical load affected the fiber composition, and increased both nNOS expression and NOS activity in rat skeletal muscles[49,50]. However, Fujii *et al* [51] showed a decrease in NOS activity in the rat ventilatory muscles, after acute inspiratory resistive loading, whereas nNOS expression did not significantly change.

Endotoxemia has also been shown to increase nNOS protein expression mainly in the diaphragm, and to a minor extent in the intercostal and gastrocnemius muscles[52]. A progressive increase in the rat diaphragm nNOS expression was confirmed once more in response to sepsis, reaching its peak after 12 h, and remaining elevated after 24 h of LPS injection [53].

The role of the 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)[54]. It was concluded from these experiments that the nNOS isoform may play a protective role in the prevention of LPS-induced impairment of diaphragmatic force. However, this isoform is not a major factor in the pathogenesis of sarcolemmal injury in septic animals, since similar degrees of sarcolemmal injury were detected in both nNOS knockout and wild type mice.

Muscle nNOS, which represents the majority of NOS activity, is activated by extracellular signals that elevate the cytosolic Ca^{2+} concentration, facilitating the interaction of the enzyme with calmodulin[31]. In contrast, caveolin-3 and a 10-kDa protein

inhibitor of nNOS (PIN) have been reported to inhibit nNOS activity in skeletal muscle[31]. Muscle nNOS interacts with two scaffolding/inhibitory domains of caveolin-3, called caveolae, which is a component of specialized invaginations of the sarcolemma[55]. As has been shown by Guo *et al* [56] embryonic rat diaphragm levels of PIN were much higher than in adult muscle. The authors of this study also reported that levels of PIN protein were similar in rat, mouse, and human diaphragms, showing no relationship with muscle fiber type distribution. Furthermore, PIN was localized in close proximity to the sarcolemma as shown by immunohistochemical analyses.

4.2.2.- eNOS

Kobzik *et al* [57] found in another study that eNOS was localized within the rat skeletal muscle fibers, showing fiber-to-fiber variation in density of antigen expression. Muscle histochemistry using the mitochondrial marker succinate dehydrogenase showed clear correlation with eNOS antigen. Moreover, adenosin triphosphate (ATP)ase fiber typing showed that both type I and type II fibers exhibited eNOS positive staining. However, guinea pig gastrocnemius muscle showed significant eNOS immunoreactivity only in the vascular endothelium[58].

Acute inspiratory resistive loading showed no significant change in eNOS expression in the rat diaphragm[51].

Acute endotoxemia has also been shown to increase eNOS protein expression in both rat diaphragm and gastrocnemius[52]. A progressive increase in the rat diaphragm eNOS expression was observed in response to sepsis, reaching its peak after 24 h of LPS injection[53].

Finally, muscle eNOS may also be regulated by caveolin-3 [31].

4.2.3.- iNOS

iNOS activity varies in skeletal muscles depending on disease state and species investigated . For instance, normal human skeletal muscles express low levels of iNOS[59,60] that are significantly augmented in patients with chronic heart failure (4,164,p.8) or autoimmune inflammatory myopathies [61] as well as in animals and skeletal muscle cell cultures after exposure to LPS or inflammatory cytokines[62] . Muscle iNOS is

systematically expressed in response to LPS injection in experimental animals, reaching its peak at 12 h after the endotoxin administration [62,52,53]. Specifically, Boczkowski *et al*[62] only detected positive iNOS expression in the muscle fibers of the rat diaphragm, whereas iNOS expression was found in rat diaphragm, intercostals, and soleus muscles by Hussain and colleagues [52,53].

Both iNOS mRNA and iNOS protein levels were undetectable or only minimally detectable in various normal skeletal muscles of rats and mice[62,63,52,53]. Nevertheless, Gath and coworkers[58] demonstrated the expression of iNOS 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. Immunohistochemistry analyses revealed a spotty distribution of NOS2 in type I muscle fibers, and these structures were compatible with sarcoplasmic reticulum (SR) and/or the transverse tubular system[58].

Muscle iNOS has been attributed to have a protective role in attenuating the inhibitory effects of LPS on muscle contractility[64]. It was also shown in the same study that the absence of iNOS gene (iNOS knockout mice) upregulated the expression of nNOS in response to sepsis, leading to the notion that iNOS might regulate nNOS transcription, mRNA stability, or protein levels.

iNOS in skeletal muscles may also be regulated by caveolin-3. Specifically, it has recently been reported that caveolin-3 coimmunoprecipitated iNOS from skeletal muscle homogenates exposed to LPS and interferon (IFN)-γ[65].

4.3.- PHYSIOLOGY OF NO IN SKELETAL MUSCLE

4.3.1.- RESTING POTENTIAL

nNOS colocalizes with the N-methyl-D-aspartate receptor subunit 1 (NMDA-1), and both are enriched at neuromuscular junctions[66]. It has been shown that denervated muscle fibers display downregulation of nNOS, and reinnervation restored the enzyme level [67]. The earliest change in muscle postdenervation is depolarization of the membrane potential, which in turn depresses muscle excitability and contractility. Treatment of the diaphragm with sodium nitroprusside (SNP), a NO donor, attenuated the early

postdenervation depolarization[68]. In other words, NOS activity stimulated through NMDA-mediated influx of calcium regulates the resting potential in rat diaphragm[31] .

4.3.2.- CONTRACTILE FUNCTION

4.3.2.1.- DEFINITIONS

a) Excitation-contraction coupling: is the process whereby chemical and/or electrical signals at the cell surface are coupled to the release of calcium from the sarcoplasmic reticulum (SR), which in turn promotes actin-myosin interaction and thereby contracts the muscle fiber [69]. After that, ATP-dependent pumps (Ca^{2+} -ATPases) in the SR resequester the calcium.

b) Twitch contraction: A single action potential which leads to the development of the events described above.

c) Tetanic contraction: Bursts of repetitive action potentials sustain calcium release, producing more vigorous contractions.

d) Maximal velocity of shortening (MVS): is one of most important parameters of the skeletal muscle fiber, which reflects the maximum rate of cross-bridge cycling. It is independent of calcium concentration, maintained constant during titanic contractions, and is relatively resistant to physiological effectors such as pH and temperature[31]. The differences in MVS among muscles are due to myosin ATPase activity [31], which are distributed as follows: type I < type IIa = type IIx < type IIb.

e) Fatigue: is the decline in muscle performance following an intense skeletal muscle activity. Both the extent of skeletal muscle fatigue and recovery from fatigue depend on the intensity and duration of exercise, muscle fiber type composition, fitness, and dietary state[70]. It is currently accepted that excitation-contraction coupling plays a crucial role in the decline of contractile force in fatigued skeletal muscle[31]. Many factors may affect excitation-contraction coupling in exercised and fatigued muscle, such as a fall in intracellular K^+, an increase in intracellular Na^+, accumulation of H^+ and P_i, and a drop in the ATP phosphorylation potential [71].

4.3.2.2.- STUDIES

The force-frequency relationship that defines excitation-contraction coupling can be modified by effects on the sarcolemma, SR or myofilaments. NO has been shown to modify excitation-contraction coupling at each of these levels[72]. Though, the consequences of such modulation seems to be dependent on the muscle preparation, protocol, and both concentration and source of NO.

a) Intact animal preparation: Murrant *et al*[73] showed that gastrocnemius force production was markedly attenuated by the infusion of S-nitro-N-acetylcysteine (SNAC) at the higher frequency twitch and tetanic contractions. In another study it was shown that infusion of the NOS inhibitor, N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME), in the gastrocnemius increased force production during slow-twitch contractions[74], suggesting that either exogenous or endogenous NO can attenuate force production of limb muscles *in situ*.

Infusion of $_L$-NAME did not affect *ex vivo* diaphragmatic contractility of rats after inspiratory resistive loading. Conversely, both transdiaphragmatic pressure-frequency curves and muscle endurance in pigs were slightly decreased by both $_L$-NAME and SNP[75] . A compromise in diaphragmatic blood flow might explain $_L$-NAME effects on the one hand, whereas direct effects on muscle function might explain nitroprusside results, on the other. In conclusion, systemic administration of either NO donors or inhibitors have only moderate effects on diaphragm function in healthy animals[31].

b) Isolated muscle (bundles) preparations: nNOS activity contributes to the intrinsic differences in force generation by type I and type II fibers as shown by Kobzik *et al* [42]. These authors observed that force produced by soleus, diaphragm, and extensor digitorum longus (EDL) was inversely correlated with their NOS activities. Furthermore, the administration of NOS inhibitors significantly reduced the differences in the force-frequency relationship among muscle groups. More recently it has been established using both NOS inhibitors (7-nitroindazole, aminoguanidine, and $_L$-NAME) or the NO scavenger hemoglobin, and the NO substrate, L-arginine, or the NO donors that NO and/or related molecules attenuated both twitch and tetanic contractions of rat diaphragm without affecting maximal force production [31].

nNOs appears to be the isoform with force-inhibitory activity. This is indicated by the demonstration that contractile function remains unchanged in mice deficient in eNOS[76], and by both the relative selectivity of 7-nitroindazole for nNOS and the fact that most of NOS activity is associated with nNOS localized within the sarcolemma[42]. Furthermore, the upregulation of nNOS in iNOS deficient mice in response to LPS also confirmed that nNOS can decrease force generation in mouse diaphragm *in vitro*[64].

It has also been shown that NO is essential for optimal myofilament function during active shortening, leading to increased MVS in mice and rats[77,78]. These investigators observed that NOS inhibition reduced MVS, while the administration of NO donors prevented this decrease.

c) Isolated muscle fibers: It has been shown that 100 μM S-nitrosyl-N-acetylpenicillamine (SNAP) or 1mM SNP reduced both maximal calcium-activated contraction and MVS of skinned muscle fibers from both slow- and fast-twitch rat limb muscles [31]. Perkins *et al* [79] described similar findings using rabbit soleus single fiber preparations. These findings were attributed to inhibition of myosin ATPase, which in turn regulates the rate of actin-myosin cross-bridge cycling. Despite these conclusions, these studies using NO donors added to single muscle fibers in very high concentrations are not likely to have a physiological translation[31].

4.3.2.3.- MECHANISMS

a) cGMP: It has been reported that intracellular cGMP account for most of the NO effects on skeletal muscle[42,80]. Though, cGMP effects on the mechanical parameters are usually moderate, with the exception of MVS, which is increased by cGMP analogs (8%) and reduced by guanylate cyclase inhibitors (20%)[31].

Guanylate cyclase activity in skeletal muscle is lower than in most other tissues. Only one study has shown that cGMP significantly increased in contracting EDL muscles, and NOS inhibitors blocked the increase due to electrical stimulation[81]. However, these changes in cGMP are difficult to put within physiological context since they are very small.

b) Ryanodine Receptors (RyR). b.1) Definition: RyR are channels that control the levels of intracellular calcium in skeletal muscle by releasing Ca^{2+} from the SR. They are regulated by voltage-sensing surface membrane L-type Ca^{2+} channels, known as dihydropyridine receptors (DHPRs), via a direct physical interaction [69]. The channel activity of the skeletal muscle RyR isoform (RyR1) is affected by several endogenous modulators such as Mg^{2+}, ATP, or calmodulin[82]. Both RyR and DHPRs are known to be redox responsive. Furthermore, skeletal muscle RyR activity has also been shown to be modified by nitrogen reactive species via a c-GMP pathway[83] .

RyR are potential targets for NO and related molecules since they contain the greatest number of sulfhydryls of any identified protein[31]. Specifically, the tetrameric mammalian skeletal RyR has a total of 404 cysteine residues.

b.2) Studies: It has been reported that NO or NO-related species attenuated calcium release from isolated skeletal muscle SR, and this effect was blocked by NOS inhibitors[84]. Kobzik *et al* [42] had already proposed a cGMP-independent inhibition of contractile function induced by NO. In contrast, in other studies it has been shown that NO donors activate calcium release from SR[85] . These two opposite findings suggest that NO or NO donors interact with the skeletal muscle RyR in a especially complex manner.

It has recently been described both nitrosative and oxidative modifications of the cardiac Ca^{2+} release channel, involving up to 12 thiols per subunit and leading to either reversible or irreversible alteration of RyR ion channel activity, respectively[86]. This activation of the RyR by multiple covalent modifications is possibly disclosing how RyR are affected in situations where great quantities of both reactive nitrogen and oxygen species are produced, leading to nitrosative and oxidative stress, respectively[31]. It was also shown that the NO effect on RyR activity is critically dependent on redox state and calmodulin[87]. The discrepancies found among these studies might be easily explained by the generation of different modifications (nitrosation vs. oxidation) and/or interactions with various types of thiol[31]. Besides, the redox state (oxygen tension, receptor thiol content, buffer, and pH) was not strictly controlled in any of the studies.

Although the redox state of skeletal muscle *in vivo* has not yet been adequately summarized, it has already been well documented that reactive nitrogen and oxygen species are increased in exercising and fatiguing muscle[72]. Moreover, both nitrosative and

oxidative stresses directly affect RyR activity, though the extent of S-nytrosilation and oxidation in these channels has not yet been identified [31].

c) Ca^{2+} ATPase: It has been shown that nNOS activity correlated with inhibition of the SR Ca^{2+} -ATPase [88]. In another study it was concluded that this loss of activity might probably result in part from S-nitrosylation or oxidation of critical thiols[89].

d) Interactions with ROS: Reid *et al* [90]discovered that superoxide generated by diaphragm muscle bundles increased both twitch and tetanic forces developed during submaximal stimulation. Moreover, the addition of superoxide dismutase was shown to decrease isometric force in rat diaphragm. Consequently, superoxide is required for optimal force production *in vitro*, and promotes excitation-contraction coupling[90.31]. Since NO scavenges superoxide three times faster than superoxide dismustase, it might be raised the concept that NO may serve as a scavenging mechanism. However, it is not clear yet whether the inhibitory effects of NO on skeletal muscle contractility are the consequence of a direct NO-mediated effect or an indirect effect by its scavenging of superoxide.

4.3.2.4.- CYTOKINES

It has been shown that exposure of C_2C_{12} skeletal muscle myocytes to endotoxin, IL-1α, TNF-α, and IFN-γ was able to induce the iNOS gene and produce NOS activity[91]. This activity was independent of the state of myocyte differentiation. It was speculated in this study that cytokine-induced NO production might supply further oxygen during vigorous exercise through vasodilation as had been observed in normal volunteers after prolonged exercise who showed increased plasma levels of cytokines. This conclusion is in line with the increased diaphragmatic oxygen uptake and the reduction in phrenic vascular resistance during endotoxemia in mechanically ventilated dogs described by Hussain *et al* [92].

Although, it is believed that cytokine induction of NOS may have detrimental effects, it is not clear yet whether some beneficial effects might also be derived.

4.3.3.- EXERCISE

It has been shown that both eNOS and nNOS expression increase in response to chronic exercise regimen in rats[31]. In another study, where electrical stimulation of rat tibialis anterior and EDL muscles was applied that both nNOS expression and NOS activity were increased [50]. However, Fuji *et al* [51] reported a decline in NOS activity by 50% in rat ventilatory muscles (diaphragm and intercostals), but not in abdominal or limb muscles in response to 3h of inspiratory resistive loading, with no significant change in both eNOS and nNOS expressions in any type of muscle with respect to control animals. Clearly, this was a post-translational effect. Eventually, the effects of chronic exercise on eNOS expression remain still speculative.

4.3.4.- GLUCOSE UPTAKE

Both eNOS activity in endothelial cells and iNOS expression in rat and human myoblasts are increased via a phosphatidylinositol (PI) 3-kinase-dependent pathway in response to insulin or insulin-like factors[93]. Furthermore, both NOS activity and the expression of eNOS and nNOS are also increased in response to contractile activity[31]. It has also been shown that NOS activity increases glucose transport in intact skeletal muscles in both rats and human individuals [31]. However, current evidence points to the fact that insulin-mediated NO effects are originally microvascular, not myocyte.

4.3.4.1.- STUDIES

a) In vivo: Muscle blood flow in rats and humans is increased by insulin[94], and this effect is considered to increase the delivery of glucose to the muscle cell. Actually, it has been proposed that increases in muscle perfusion can account for almost 30% of the effects of insulin on glucose uptake[31]. Furthermore, there is good evidence that insulin-mediated vasodilation is partly NO dependent. Therefore, increases in muscle perfusion contribute to increased skeletal muscle glucose transport, independent of the insulin-mediated rises of the GLUT-4 transporter, which is not NO-dependent[31].

b) Isolated muscles:

Constitutive NOS: Several investigators have studied NOS effects on basal glucose uptake in isolated muscle preparations, reaching different conclusions, though there is an agreement among them that constitutive NOS inhibitors do not modify the sensitivity of isolated muscle preparations to insulin nor the effects on glucose uptake in insulin-stimulated muscles[31] .

iNOS and NO donors: Insulin-stimulated glucose transport in both isolated soleus and EDL muscles and cultured L6 muscle cells was inhibited by both iNOS and NO donors[95]. Moreover, GLUT-4 expression in L6 cells was decreased by iNOS [95]. As a result, both iNOS and exogenous NO reduce insulin-mediated glucose uptake by downregulating its transporter [31].

4.3.4.2.- MECHANISMS OF ACTION

a) cGMP: The effect of cGMP on skeletal muscle glucose metabolism is controversial. Kapur *et al* [96] found no effect on either basal or insulin-stimulated glucose transport in isolated soleus muscles treated for 1 h with 8-bromo-cGMP (a cell-permeable analog). Conversely, other investigators found that the same compound significantly increased glucose transport activity[97]. Moreover, both groups found that high concentrations of SNP mimicked the effects induced by 8-bromo-cGMP. Concisely, it can be assumed that the cGMP pathway may increase glucose utilization, though the physiological role as well as the link to NOS activity has not yet been established.

b) Glucose transporters: GLUT-4 expression on the cell surface of isolated rat epitrochlearis muscle was increased by SNP[97]. On the contrary, while iNOS induction by cytokines and LPS caused a 50% increase in the levels of expression of the GLUT-1 transporter, those of the GLUT-4 transporter decreased by 50% in L6 myocytes, with no alteration in GLUT-3 expression[95]. Moreover, the modifications of GLUT-1 protein were prevented by $_{L}$-NAME, but not those of GLUT-4. These controversial effects might be attributed to secondary metabolic consequences, such as inhibition of mitochondrial respiration, induced by the use of high doses of SNP and iNOS [31].

4.3.5.- BLOOD FLOW

NO is associated with the microvascular response to muscle contraction, hypoxia , vascular occlusion, and changes in sympathetic drive both in resting and exercising skeletal muscles [31] . It has recently been shown that nNOS mediates the sympathetic vasoconstriction intrinsic to resting skeletal muscle[98]. In effect, it has been reported that only α_2-adrenergic vasonconstriction is inhibited during contraction of the fast-twitch fiber-type muscles, which express nNOS[42,96]. What' s more, both $_L$-NAME and 7-nitroindazole reversed this inhibition of the vasoconstrictor response to lumbar sympathetic nerve stimulation[98] in rats. Studies using nNOS mutant mice and *mdx* mice[99], which are deficient in functional nNOS[46], demonstrated that this isoform is responsible for the attenuation of α-adrenergic vasoconstriction during hindlimb contractions. However, it is not clearly stated in this study whether NO originates within the skeletal muscle fiber, or in other contiguous structures such as the femoral artery, the lumbar sympathetic cell bodies, or the postganglionic fibers[31].

4.3.6.- TISSUE RESPIRATION

4.3.6.1.- DEFINITIONS

Four different steps will determine the oxygen utilization in a given tissue: the oxygen carrying capacity of **hemoglobin**, the **blood flow**, the **oxygen extraction** by the cell, and its **consumption** by the mitochondria. NO covers these functions in a fashion that is released from hemoglobin and eNOS in response to hypoxia, improving blood flow, and acting as a break at the level of the mitochondria[31]. Mitochondrial oxygen consumption in skeletal muscle appears to be regulated by NO synthesized by eNOS localized within the microvascular endothelial cells, and by both eNOS and nNOS located within the skeletal muscle fibers.

4.3.6.2.- STUDIES

King *et* al [100] reported, in contrast to expectation, that the infusion of $_L$-NAME increased hindlimb skeletal muscle oxygen uptake regardless of decreases in blood flow. While systemic oxygen consumption did not change, cardiac output severely dropped. In

another study it was shown that endothelial agonists of decreased tissue oxygen consumption in slices of dog triceps brachii[101]. The same response was obtained using a NO donor, and the opposite effect using nitro-$_L$-arginine. Furthermore, 8-bromo-cGMP declined oxygen consumption, and the major site of NO action was proposed to be the mitochondria. More recent studies have shown that NOS inhibitors increase oxygen consumption in healthy human volunteers [31].

NO synthesized within a vascular endothelial cell would have to escape the hemes of myoglobin and be capable of targeting the hemes of the respiratory chain cytochromes in order to inhibit respiration in skeletal muscle fibers. The difficulty understanding this complex situation led to the concept that there might be a skeletal muscle source of NO. In fact, it was shown that eNOS is present within the mitochondria of skeletal muscle [57,96] as described in former section. Moreover, it was also demonstrated that mitochondrial respiration through reversible interactions with cytochrome-*c* oxidase is regulated by NO[102].

Other targets that have been shown to inhibit mitochondrial respiration in skeletal muscle include creatine kinase[103], and the SR Ca^{2+}-ATPase isoform in fast-twitch (SERCA1a) and slow-twitch (SERCA2a) skeletal muscle[104].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aconitase, and complex 1 of the respiratory chain are additional NO targets, whose inhibition might influence cell respiration or calcium homeostasis in muscle fibers exposed to nitrosative stress [31].

Finally, NO effects on oxygen consumption depend on blood flow. Accordingly, Hussain *et al* [105] already demonstrated that infusion of a NOS inhibitor into the phrenic artery had no effect on oxygen extraction by contracting diaphragm under poor oxygen delivery conditions.

4.3.7.- INJURY

There is evidence that NO and its related oxidation products are involved in the pathophysiology of many inflammatory conditions and degenerative diseases. However, their implications in different forms of muscle injury and fatigue is not yet well understood. Nevertheless, apoptosis has been shown to be induced in rat skeletal muscle

myoblasts by NO donors[106]. Another study demonstrated that iNOS was remarkably induced in crushed limb muscle, where it was also shown that delayed induction of iNOS may destroy muscle tissue and contribute to rhabdomyolysis[107]. They also reported an induction of eNOS associated with capillary perfusion in response to vasodilatation occurring after injury. An increase in nNOS expression was also observed in a ischemia-reperfusion model[108]. In conclusion, all three isoforms may contribute to various forms of skeletal muscle injury.

4.3.8.- MYOBLAST DIFFERENTIATION

Differentiation of embryonic muscle cells implicates fusion of mononucleated myoblasts into multinuclear myotubes. Cultured myoblasts have NOS activity, which is critically dependent on time of differentiation [31]. In another study, NOS activity increased between gestational days 10 and 12 in cells from chick embryos, and intracellular levels of cGMP correlated well with NOS activity [31].

5.- REACTIVE OXYGEN SPECIES

5.1.- SOURCES OF ROS FORMATION IN SKELETAL MUSCLE

5.1.1.- MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

Oxygen free radicals are normal mediators in mitochondrial electron transport chain reactions[109]. The first step in oxygen reduction includes the reaction of molecular oxygen with a semiquinone radical to form ubiquinone and superoxide[110] . The electron transport chain usually reduces most oxygen (95-99%) by successively supplying three additional electrons to these superoxide anions, and these molecules remain bound to the electron transport chain until fully reduced[110]. The remaining 1-5% of oxygen molecules leave the oxygen transport chain as superoxide. The rate of superoxide anion production is proportional to the partial pressure of oxygen in the mitochondria [110].

The fact that strenuous exercise produce a several fold increase in oxygen utilization has led to the proposal that this superoxide release may increase as well in

contracting muscles[110]. Furthermore, it has also been suggested that large exercise-induced superoxide production may overcome mitochondrial antioxidant defences, leading to superoxide leakage to the cytosol and other muscle sites, which may comprise initiating damaging reactions in tissues[111,112]. Consequently, it could be proposed that the mitochondrial electron transport plays a central role in the free radical production in contracting muscles. Based on this assumption, Ambrosio *et al*[113] found that administration of an inhibitor of the nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase (Amytal), a component of the electron transport chain, reduced oxygen radical formation following ischemia/reperfusion of intact rabbit hearts. In addition, Amytal also prevented membrane lipid peroxidation and improved functional recovery following ischemia/reperfusion in this organ.

5.1.2.- XANTHINE OXIDASE

Superoxide may also be produced in muscle as a by-product of the xanthine oxidase catalyzed conversion of hypoxanthine to xanthine and uric acid, which varies between tissues and species. This pathway has been shown to play a role in mediating the free radical generation and tissue damage observed after periods of transient tissue ischemia, and following limb skeletal muscle reperfusion[114]. Allopurinol reduced muscle fatigue rate of dog hindlimb, which supports the xanthine oxidase-mediated free radical production in contributing to muscle fatigue[115]. Most recently, it has bee demonstrated that the xanthine oxidase pathway (oxypurinol) contributes to the generation of superoxide by the contracting diaphragm[116]. Nevertheless, ischemia-related muscle injury does not include this pathway in humans [117].

5.1.3.- NADPH OXIDASE

NADPH oxidase has been identified as a new source of ROS in non-phagocytes during the past few years. NADPH oxidase is complex membrane-associated enzyme system found in a variety of cells of mesodermal origin and leukocytes. This enzyme catalyzes the production of superoxide by one electron reduction of molecular oxygen using NADPH or NADH as the electron donor[118]. The activity of this enzyme is also increased by cytokines, phospholipase A_2 agonists, and protein kinase C, among

others. The superoxide generated constitutes the initial material for the production of other ROS. They contribute to eliminate foreign organisms in phagocytes, though they may also induce tissue damage by acting on both cellular proteins and lipids.

The core component of NADPH oxidase in neutrophils includes 2 membrane-bound subunits, and 3 cytosolic subunits which associate with the former subunits upon activation. Similar NADPH oxidase, but not identical to neutrophil NADPH oxidase, has recently been identified in vascular, mesangial, and microglial cells[119]. NADPH oxidase in these cells is constitutively active, uses NADH as the electron donor, and its activity does not require the whole five subunits. Furthermore, our group has currently described[120] the existence of a NADPH oxidase system within the skeletal muscle fiber, as well as its contribution to ROS production. The characteristics of this enzyme are similar to those of non-phagocyte NADPH oxidases described in other tissues. This NADPH oxidase enzyme complex is constitutively active, and significantly contributes to ROS production within the skeletal muscle fibers in both normal conditions and in response to sepsis.

5.1.4.- MICROSOMAL P-450 SYSTEM

This system generates superoxide via a NADPH dependent reaction. It has been shown to play an important role in the generation of free radicals and tissue damage in response to oxygen administration [110], as well as in mediating the damage produced by several cellular toxins[110].

5.1.5.- BY-PRODUCT OF ARACHIDONIC ACID METABOLISM

Free radicals may also be produced as a product of the conversion of PgG_2 to PgH_2 within the arachidonic acid metabolism. This conversion reaction is an important source of free radicals in the brain following periods of ischemia [121].

5.2.- EVIDENCE OF ROS PRODUCTION IN SKELETAL MUSCLE

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.

5.2.1.- PROTEIN OXIDATION

Reaction of free radicals with proteins results in the formation of characteristic sidegroup modifications, such as sulfhydryl group oxidation, nitrosylation of tyrosine residues by peroxynitrite, and formation of carbonyl groups[110] . Both acute and chronic limb muscle exercise have been shown to increase limb muscle carbonyl sidegroup concentrations[1212,123]. Furthermore, increased diaphragmatic concentrations of protein carbonyl sidegroups were also reported following chronic respiratory loading by Supinski *et al* [124].

5.2.2.- LIPID PEROXIDATION

Different forms of muscle activity, either inspiratory resistive loading or limb skeletal muscle exercise, have shown to be associated with lipid peroxidation. For instance, limb muscle exercise led to an increase in the levels of the widely used muscle lipid peroxidation by-products, the thiobarbituric acid-reactive substances (TBAR)[110]. Diaphragmatic TBAR levels were also increased following resistive loading breathing. Moreover, increases in 8-isoprostane, another lipid peroxidation by-product, were also shown in response to both acute and chronic respiratory loading [124].

5.2.3.- TISSUE GLUTATHIONE

Several experiments have demonstrated increased oxidized glutathione (GSSG), diminished levels of reduced glutathione (GSH), and a rise in GSSG/GSH ratio in exercising muscles[125,126] as well as in peripheral blood and liver[124]. Other investigators have also reported that a wide range of acute inspiratory loads of sufficient magnitude to result in the generation of respiratory muscle failure can lead to alterations in diaphragm glutathione concentrations along with a decline in diaphragm force generating capacity[126,127,110].

5.4.- ROS-MEDIATED MUSCLE CONTRACTILITY DYSFUNCTION

Recent lines of evidence have established ROS-mediated oxidative stress as a cause of muscle fatigue[128]. The antioxidant N-acetylcysteine, which scavenges superoxide, hydroxyl radicals, and hydrogen peroxide, and provides cysteine for the synthesis of glutathione, was shown to attenuate the development of muscle fatigue[129]. Moreover, it appeared to be especially effective in preventing reductions in the diaphragm force in response to low frequency electrical stimulations [129.130]. Similarly, SOD, catalase, and dimethylsulfoxide (free radical scavengers) prevented the rate of development of fatigue of diaphragm strips in response to 20 Hz electrical stimulation frequencies[131]. However, no protective effect of these scavengers was observed in response to high stimulation frequencies (100 Hz) in the same experiments. Consequently, it could be interpreted from these findings that the pattern of muscle activation determines the contribution of free radicals to the development of muscle fatigue.

A xanthine oxidase pathway (allopurinol used as inhibitor) was shown to contribute to the development of muscle dysfunction as a result of rhythmic contractions[115]. High intensity contractions was proposed as a model of relative ischemia in the muscle, which may induce free radical generation in a fashion similar to that described in ischemia/reperfusion models, at least in dogs[115]. However, this pathway (oxypurinol used as inhibitor) did not intervene in the radical-mediated lipid peroxidation of the decerebrated rat diaphragms during inspiratory resistive loading[132].

Supinski *et al*[133] reported that polyethylene-glycol adsorbed-superoxide dismutase (PEG-SOD) and dimethylsulfoxide (DMSO) were capable of attenuating the rate of fatigue development of *in situ*, blood perfused canine diaphragm strips during a 2h long period of repetitive contractions elicited by 20 Hz electrophrenic stimulation. These two free radical scavengers, also prevented diaphragm lipid peroxidation, detected by TBAR levels as compared to only saline treated animals[133]. Nevertheless, the scavenger administration did not increase the rate of recovery of force over the first hour following the cessation of contractions in the same study. The authors concluded that pre-treatment with free radical scavengers prevents both muscle lipid peroxidation and attenuates the rate of development of muscle fatigue during periods of muscle contraction in response to low frequency stimulation[133]. The same authors in another study[134] concluded that N-

acetylcysteine administration preserved the pressure generating capacity of decerebrate unanesthetized rat respiratory muscles and slowed the development of respiratory failure during respiratory loading. The same group of investigators also described that chronic respiratory loading-induced alterations in the diaphragmatic force generation may be attributed to free radical-mediated protein oxidation, but not to free radical-induced lipid peroxidation [123].

Interventions reducing muscle concentrations of natural antioxidants designed to increase the severity of muscle fatigue are also described in the literature. Accordingly, Anzueto *et al* [135] found that in nonloaded experiments, the diaphragms of vitamin E deficient rats developed lower *in vitro* forces than normal diaphragms. Moreover, in loaded experiments, the diaphragms of vitamin E deficient animals showed lower forces than either normal loaded rats or vitamin E deficient nonloaded animals. The authors concluded that vitamin E deficiency predisposes to the development of diaphragmatic contractile dysfunction during respiratory loading. Finally, the administration of compounds that decrease muscle glutathione stores accelerated the rate of development of rat diaphragm fatigue in response to a given regimen of exercise [110].

5.5.- EFFECTS OF ROS ON SKELETAL MUSCLE CONTRACTION

As seen so far, 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[136]. However, recent evidence has proved that endogenous ROS also regulate contractile function of healthy skeletal muscle[137]. They are produced at a relatively low rate in resting muscle fibers, appear to be essential for normal force production[137], and their levels progressively increase in response to muscle activation[128,131,137]. ROS concentration within the muscle fibers is usually kept at relatively low levels by intracellular antioxidants such as SOD.

It has been shown that selective reduction of ROS by catalase or SOD treatment results in a decline in muscle force which is reversible by enzyme washout[90,130]. In contrast, exposure to low levels of exogenous ROS increases muscle force[90,138]. Furthermore, Reid[128] has recently proposed a model of ROS homeostasis including their

biphasic effects on muscle force generation (Figure 1, right-side panel). ROS levels in unfatigued muscle fibers are relatively low and are required for normal force production (point A). Both twitch and tetanic muscle forces significantly decline when ROS are scavenged in these muscles (point A to B) [90,139]. Conversely, modest ROS exposure increases force of unfatigued muscle (point A to C). Finally, excessive ROS production, as occurs during exogenous ROS exposure, strong muscle contractions or in sepsis, results in a state of oxidative stress leading to a decline in muscle force production (point D).

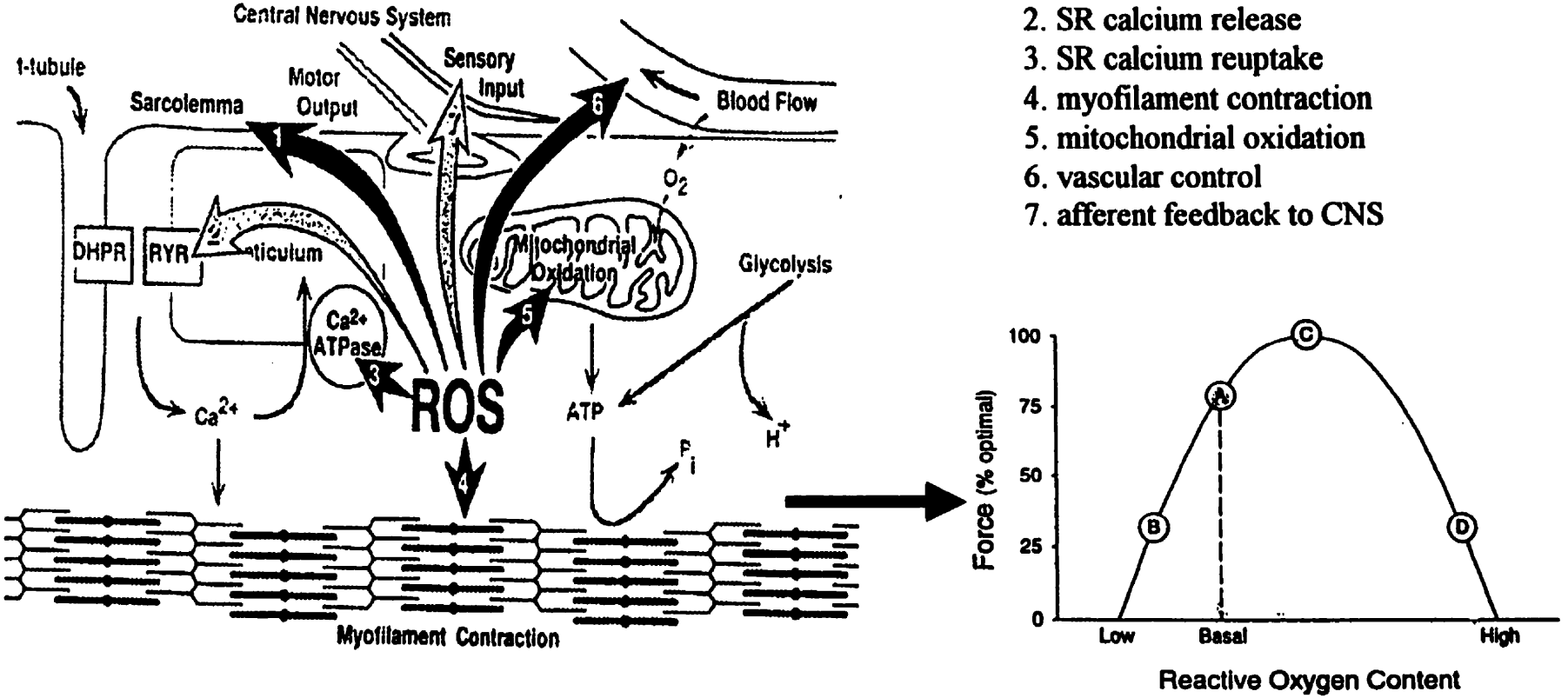
5.6.- ROS-SENSITIVE SITES IN NORMAL SKELETAL MUSCLE

The current literature proposes several mechanisms whereby ROS may increase muscle force. As illustrated in (Figure 1, left-side panel) ROS may enhance the open probability of SR calcium release channels[140], and inhibit the calcium-dependent ATPase[141] (pathways 2 and 3). Both actions predispose to higher temporary calcium levels in contracting muscle, which in turn will lead to increased muscle force. ROS may also increase calcium sensitivity of the myofilaments[140] (pathway 4). In summary, there are multiple sites in unfatigued muscle fibers that may be sensitive to ROS, as has also been described with NO.

5.7.- ROS-SENSITIVE SITES IN FATIGUED MUSCLE

The intracellular mechanisms whereby ROS contribute to fatigue still remain poorly understood. Several intracellular sites have been proposed to be influenced by ROS, but sarcolemma-related processes such as excitation-contraction coupling appear to be particular targets for ROS actions. What's more, excitation-contraction coupling is influenced by ROS at several levels. For instance, ROS oxidizes regulatory proteins involved in Na^+-K^+ pump and Na^+-K^+-Cl^- transporter[142]. Inhibition of t-tubule voltage sensor, which in turn affects depolarization-induced contraction, has also been shown to be influenced by ROS [143]. Another interesting target of ROS action is the SR. Several studies have demonstrated that different forms of oxidative stress cause an initial release of calcium from SR, which has been shown to be mediated by oxidation of critical thiol groups located in close proximity to the calcium release channel[144]. Specifically, Favero

Effects of ROS on skeletal muscle force production. Mechanisms



Reid M.B. Muscle fatigue: mechanisms and regulation. In: Exercise and Oxygen Toxicity, 2nd edition, C.K. Sen, L. Parker, and O. Hanninen, eds; Elsevier Science B.V. Amsterdam.

Figure 1. Biphasic effects of ROS on skeletal muscle force production

et al [144] found that hydrogen peroxide stimulated both calcium release and ryanodine binding to the calcium release channel. However, it was reported in another study that an oxidant stress-induced inhibition of SR calcium release might account for the development of low frequency fatigue during sustained muscle exercise[110,143]. Another target for ROS actions is the SR Ca^{2+}-dependent ATPase pump as a result of protein thiol modification and nitrotyrosine formation[141,145]. It has also been shown that oxygen radicals may result in inhibition of mitochondrial respiration, reduction in trans-mitochondrial calcium gradients, and depletion of mitochondrial antioxidants[146]. Finally, it has also been reported that contractile proteins constitute another cellular target for ROS action by oxidizing critical sulfhydryl groups[147].

In summary, although several cellular targets have been shown to be influenced by ROS actions, the relative contribution to muscle fatigue *in vivo* remains still unknown. Furthermore, the relative sensitivity of these molecular targets within the muscle fiber appears to be dependent on different experimental conditions in the different studies.

5.8.- OXIDATIVE STRESS IN SEPTIC SKELETAL MUSCLES

Several studies have demonstrated that sepsis-induced muscle injury is largely mediated by an increase in ROS levels. Shindoh *et al* [13,148] were the first to report that free radicals also contribute to the diaphragmatic dysfunction induced by systemic endotoxin injection. The authors concluded that administration of PEG-SOD prevented malondialdehyde (MDA) formation (index of free radical-mediated lipid peroxidation) and contractile dysfunction in septic hamsters. Peralta and coworkers[149]demonstrated increased levels of ROS (muscle chemiluminescence) in septic rat hindlimb muscles which was attenuated by SOD pre-treatment. The same group of investigators[150] reported in another study that oxidative stress occurs early in rats along with inhibition of active mitochondrial respiration, and inactivation of antioxidant enzymes. Supinski *et al* [151] assessed lipid peroxidation using 8-isprostane (index of lipid peroxidation) and muscle contractile dysfunction in septic hamsters. They concluded that endotoxin-induced dysfunction was not limited to the ventilatory muscles, but also occurred in limb skeletal muscle, while cardiac muscle appeared to be resistant to it. Most recently, it has been

shown that free radicals reduce the maximal diaphragmatic mitochondrial oxygen consumption in endotoxin-induced sepsis in rats[152]. Finally, it has also been reported that free radicals play a central role in altering skeletal muscle contractile protein force-generating capacity in the septic rat diaphrams [153].

Despite this progress, the potential sources of ROS production in septic skeletal muscles as well as the mechanisms whereby oxidative stress contributes to the sepsis-induced skeletal muscle dysfunction are still poorly understood.

6.- HEME OXYGENASES

6.1.- DESCRIPTION

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[154].

Heme oxygenase (HO), which was originally identified by Tenhunen and colleagues[155], is the rate limiting enzyme of the initial reaction in the degradation of heme. HO oxidatively cleaves the α-meso carbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa, carbon monoxide (CO), and free iron[156]. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase, and then free iron is rapidly sequestered into ferritin. Three documented isoforms (HO-1, HO-2, and HO-3) catalyze this reaction[157,158,159]. 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[160, 161], cytokines [162], shearstress[163], heavymetals[164], endotoxin[165,166], hyperoxia[167], hydrogen peroxide[168], heat shock[169] and many others[168]. The 36-kDa HO-2 protein is mostly constitutively synthesized existing predominantly in the central nervous system and testis[168], though it may also be present in other tissues such as skeletal muscle[170]. 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[159]. 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 [156]. Though, this activity may occur in all systemic organs as well.

6.2.- HO-1 INDUCTION AND ITS CYTOPROTECTIVE ROLE

As mentioned previously, several studies have shown that HO-1 enzyme activity may also be stimulated by various non-heme products[160,162,164,165,168], with a common capacity to generate ROS production and/or modify glutathione levels[156]. 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 oxidative stress. 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, which scavenge ROS in order to maintain cellular homeostasis. However, recent evidence shows that transcriptional regulators such as c-FOS, c-Jun, and NF-κB, as well as the expression of other stress-response genes, such as HO-1, are up-regulated in response to oxidative stress-induced injury[156]. As has been shown that HO-1 induction plays a protective role in oxidative stress, this enzyme is now thought to be part of a more general response to such conditions.

Several studies have reported that HO-1 provides protective effects in various *in vivo* and *in vitro* models of tissue injury[164,171]. Moreover, this protective effect was further proved by the observations made in the HO-1 deficient mouse and human models. Specifically, it was shown that mouse fetuses lacking the HO-1 gene[172,173] (HO-1 knockout mice) did not survive to term, or in the case they did, they were abnormal and died within one year of birth. Additionally, these mice showed growth retardation, normochromic and microcytic anemia, evidence of iron deposition in several organs such as liver, kidneys, signs of chronic inflammation represented by hepatosplenomegaly, increased blood cellular counts, and glomerulonephritis[172]. Furthermore, HO-1 deficient embryonic fibroblasts showed higher sensitivity to oxidative stress-induced

cytotoxicity[172], supporting that the expression of HO-1 is essential for cell viability and optimal maintenance of cellular free radical levels. Finally, HO-1 knockout mice were more vulnerable to both death and hepatic necrosis in response to systemic endotoxin administration[172]. Similarly, it has recently been reported a clinical case of a HO-1 deficient patient who exhibited growth retardation, anemia, leukocytosis, and increased sensitivity to oxidant stress, in line with the phenotypic alterations described in the HO-1 knockout mice[174]. In fact, various diseases have recently been shown to associate with increased expression of HO-1. For instance, acute renal failure, myocardial ischemia, hypertension, cerebrovascular accident, Alzheimer's disease, asthma, and chronic obstructive lung disease, constitute some of the well characterized disorders[168]. Consequently, these findings might lead to the concept that HO-1 levels can be used as a biological marker to diagnose and monitor patients suffering from these diseases[168].

Interestingly, it has been demonstrated that HO expression is well preserved evolutionarily, since it has been identified in prokaryotic bacteria, plants and fungi [168]. This preservation has led to the concept that HO may play a protective role as a regulator of key cellular processes, aside from its ability to degrade heme.

6.3.- MECHANISMS OF CYTOPROTECTION INDUCED BY HO-1

It is currently believed that HO-1 regulates cellular homeostasis via its three major catabolic by-products: carbon monoxide (CO), bilirubin, and ferritin, though its cytoprotective functions have not yet been clearly identified.

6.3.1.- CO

Because of its strong affinity for hemoglobin, CO is one of the most commonly and powerful tissue toxic agent[168] which may lead to anoxia and death of to living organisms. However, accumulating evidence suggests that low concentrations of CO may exert protective effects on cellular homeostasis[168]. First evidence and confirmation that this molecule may have signaling functions similar to NO was provided by a couple of studies[175,176], where it was respectively shown that CO generated from HO can regulate vasomotor tone and neurotransmission. These effects were shown to be mediated through the activation of guanylate cyclase on the binding of CO to the heme

moiety of this enzyme, which in turn, will lead to cGMP formation[175]. Though, CO is thought to be 50-100 times less potent that NO with respect to activation of guanylate cyclase, which is the common mechanism whereby these two gaseous molecules modulate smooth muscle cell functions[175,176]. Nevertheless, both CO chemical stability and its exclusive reaction with heme may lead to the accumulation of higher levels of CO within the cell, which in turn will counteract its relative inadequacy of binding guanylate cyclase with regard to NO[168]. Furthermore, CO may have anti-inflammatory effects through guanylate cyclase activation and consequent formation of cGMP, by inhibiting platelet activation or aggregation and neutrophil infiltration[178,179] Moreover, in the first study[178] it was shown that CO attenuated total lung apoptotic index within the airways in response to hyperoxia compared to the group of animals exposed to hyperoxia in the absence of CO.

More recently, it has also been shown that changes in CO levels in exhaled breath characterized both HO-1 activity and cellular stress, thereby correlating with the severity of the disease[177,180].

6.3.2- BILIRUBIN

Bilirubin was first reported to have beneficial effects as a physiological antioxidant in the brain by Stocker *et al* [181]. This molecule is the major endogenous antioxidant in mammalian tissues, and it accounts for most of the antioxidant activity of human serum[182]. However, bilirubin may also act as a toxic agent that accumulates in the serum of neonates, causing jaundice, and it may also induce neurotoxicity by depositing in the brain[168].

6.3.3.- FERRITIN

The sequestration of free iron into the iron storage protein ferritin has also been reported to act as an antioxidant mechanism by several investigators[183,168].

6.4.- HO-1 INDUCTION IN SKELETAL MUSCLE

Little is known about the presence, distribution and functional significance of HO in skeletal muscle fibers. HO-1 mRNA levels were shown to increase after repeated muscle contractions in rat limb muscle[184]. HO-1 induction (in this study) might underlie an antioxidant pathway in response to increased ROS levels generated during strong muscle contractions. Furthermore, it was also reported that NO donors such as SNP, and hemin are potent inducers of HO-1 mRNA, protein, and activity in skeletal myoblast cells[185]. Interestingly, in another recent study it has been shown that hemin-induced HO-1 expression directly correlated with both, the content of red fibers and tissue myoglobin in different rat muscles[186]. As HO-1 expression was significantly higher in type I fiber muscles such as soleus, the authors concluded that HO-1 expression follows a fiber-type specific distribution in the skeletal muscle [186].

Finally, it has recently been reported that HO-2 is present in satellite cells, vascular endothelial cells, fibroblasts and extrafusal myofibers either associated with the non-junctional sarcolemma or within the subsarcolemma region[170].

6.5.- ROLE OF HO-1 IN SEPSIS

As previously mentioned, mice deficient in the HO-1 gene were more susceptible to death in response to endotoxin administration[173]. In another study, it was shown that LPS administration induced increased levels of HO-1 mRNA as well as a rise in HO-1 activity in the lungs of endotoxemic rats[187]. Interestingly, pre-treatment of animals with hemoglobin led to HO-1 induction, which resulted in 100% survival. On the contrary, tin protoporphyrin, a competitive inhibitor of HO, completely abrogated the protective effects attributed to HO-1 induction in the endotoxemic rats[187]. These findings led to the hypothesis that HO-1 plays a protective role in lungs of septic rats, which might have future therapeutical implications. This hypothesis was supported by a more recent study from the same laboratory indicating that pre-treatment of rats with hemoglobin reduced both lung and systemic inflammatory effects of sepsis, through a ferritin-independent pathway[188]. In another study[165] intratracheal LPS instillation in rats induced ferritin protein expression in the lung. In addition, HO-1 expression both at the protein and mRNA levels was increased as well as its activity in this organ[165].

Finally, recent evidence has shown that HO has a protective role in the diaphragmatic contractile dysfunction of endotoxemic rats by using both hemin as a HO-1 inducer and zinc protoporphyrin IX (ZnPP-IX) as a competitive inhibitor[189].

6.6.- HO-1 REGULATION AND INTERACTIONS

Several cytokines, such as interleukin (IL)-6, activate HO-1 gene transcription both *in vivo* and *in vitro* [190,191]. Most recently, it has been reported that TNF-α and IL-1 induced HO-1 expression via protein kinase C, Ca^{2+}, phospholipase A_2, and oxidant production in human vascular endothelial cells[192]. An increase in HO-1 expression is mediated by NO donors in both vascular smooth muscle cells and skeletal myocytes[185,193]. In line with this finding, Foresti *et al* [194] reported that HO-1 induction in response to peroxynitrite formation in vascular endothelial cells might have a protective effect against the toxicity caused by peroxynitrite.

Finally, Baum and co-workers[170] described the colocalization of both HO-2 and nNOS in rat skeletal limb muscles. This colocalization took place only in the non-junctional sarcolemma region of extrafusal type II myofibers outside costameres. Moreover, HO-2 expression did not change in *mdx* mice, and it was not present in patients suffering from Duchenne's muscular dystrophy [170].

7.- GENERAL HYPOTHESIS

Septic shock is associated with ventilatory pump failure due the action of different mediators including NO release and ROS production. HO-1 expression in the diaphragm is significantly up-regulated during the course of septic shock. Up-regulation of HO-1 in septic animals is mediated in part through enhanced NO release. HO-1 plays an important protective role in improving muscle function in septic animals. The protective role of HO-1 is due to its anti-oxidant activities.

To test our hypothesis we decided to divide our research work in three main projects which are subsequently organized and presented in three corresponding chapters: 1) role of nitric oxide synthases in protein tyrosine nitration in the diaphragm muscle; 2) role of nitric oxide synthases in oxidative stress in the diaphragm muscle; and 3) role of HO-1 in the diaphragm: influence on oxidative stress. These three projects share a common link, namely HO-1 would have a protective effect in attenuating the deleterious effects of both excessive NO and ROS release by the contracting septic diaphragm. A general summary of results is presented in a separate section, and the conclusions derived from the three studies as well as the common links established among them will also be described in the same section.

8.- GENERAL OBJECTIVES

8.1.- NITROSATIVE STRESS

The aim of this study is to assess the nitrosative stress in sepsis-induced ventilatory muscle contractile dysfunction, as well as the contribution of the different NOS to nitrosative stress. This will be achieved in chapter 1.

8.2.- OXIDATIVE STRESS

The main objective of this project is to investigate the state of oxidative stress in the respiratory muscles of endotoxemic rats, as well as to assess the role of NO in oxidative stress-mediated ventilatory muscle dysfunction in sepsis. This will be accomplished in chapter 2.

8.3.- HEME OXYGENASES

The aim of this study is to assess whether HO are induced in response to septic shock in rat ventilatory muscles to exert their cytoprotective effects on these septic muscles. The biological significance of HO-1 induction constitutes the main objective. This will be accomplished in chapter 3.

PROTEIN TYROSINE NITRATION IN THE VENTILATORY MUSCLES: ROLE OF NITRIC OXIDE SYNTHASES

CHAPTER # 1

9.- INTRODUCTION

Many of the cytotoxic and pathological effects of excessive NO formation have been attributed to the capacity of NO to produce covalent modifications of numerous biological molecules. The most commonly studied metabolite of *in-vivo* production of reactive nitrogen intermediates is modified amino acid 3-nitrotyrosine (NO_2Tyr). The significant interest in the biological relevance of NO2Tyr arose from several observations documenting a marked increase in protein-linked NO_2Tyr in many pathologies including acute lung injury, sepsis, rheumatoid arthritis, amyotrophic lateral sclerosis, Alzheimer and liver transplantation [25,195]. Although the mechanisms responsible for protein nitration in normal skeletal muscles remain unclear, the majority of proposed pathways responsible for the *in-vivo* formation of NO_2Tyr require the presence of NO[195]. These mechanisms include peroxynitrite (formed from the near diffusion-limited reaction between NO and O_2^- anions), the reaction of NO with protein tyrosyl radicals[196], the reaction of nitrite and peroxidases[27], and finally nitrous acid (formed in acidic environment such as the stomach)[197].

Little information is available regarding mechanisms, sites and functional significance of protein tyrosine nitration in skeletal muscles. Skeletal muscle fibers are among few organs in which the two components required for peroxynitrite formation (O_2^- and NO) are constitutively synthesized[198,42]. Whether sufficient concentrations of these components are formed in normal muscle fibers to cause peroxynitrite formation and tyrosine nitration has not yet been determined. There is evidence, however, that NO_2Tyr formation in the ventilatory and other skeletal muscles develops in pathological conditions where the production of both superoxide and NO is augmented as in sepsis[53,199-201]. Despite these recent observations regarding protein tyrosine nitration, 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. Muscle constitutive NO synthesis is highly dependent on fiber type composition since the nNOS is abundant in type II muscle fibers, whereas the (eNOS) is present mainly in type I fibers [42,57]. Also unclear are the targets of tyrosine nitration inside skeletal muscle fibers. Recent studies have documented that Mn-SOD[202], neurofilament L[203], actin and other

cytoskeletal proteins[204], α-tubulin[205], tyrosine hydroxylase[204], lung surfactant protein A and α1-antitrypsin[206] are tyrosine nitrated in various cells and that in most cases, tyrosine nitration leads to inhibition of protein activity. Whether these proteins particularly Mn-SOD, actin and tubulin are also tyrosine nitrated inside skeletal muscle fibers remain unclear. The dependence of NO_2Tyr formation both in normal muscles and muscles of septic animals on different NOS isoforms has not been assessed. Our group has recently established that the expression of both the nNOS and iNOS isoforms are induced in response to LPS injection in animals and that both isoforms contribute to enhanced NO production in septic muscles[53]. Nevertheless, it is not yet known whether both the nNOS and iNOS isoforms contribute equally or differentially to protein tyrosine nitration in septic muscles.

10.- SPECIFIC HYPOTHESES

Increased levels of NO and superoxide radical in the ventilatory muscles during sepsis in experimental animals will lead to the formation of the potent reactive species peroxynitrite, and the consequent nitration of tyrosine residues. NO_2Tyr formation might be a mediator of the sepsis-induced respiratory muscle dysfunction.

11.- SPECIFIC OBJECTIVES

11.1.- To determine the presence, compartmentalization, and localization of NO_2Tyr formation in normal ventilatory and limb rat skeletal muscles of various fiber type compositions.

11.2.- To assess the dependence of NO_2Tyr formation in normal rat skeletal muscles on NO synthesis by the different NOS isoforms.

11.3.- To establish the presence, compartmentalization, and localization of NO_2Tyr formation in septic rat skeletal muscles.

11.4.- To investigate the regulation of NO_2Tyr formation in skeletal muscles during sepsis by determining the contribution of the different NOS isoforms to this formation.

12.- MATERIALS AND METHODS

12.1.- REAGENTS

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). LPS (serotype 055:B5), L-NAME, aprotinin, leupeptin, trypsin inhibitor, pepstatin A, ethylene glycol-bis (β-amino ethyl ether) tetraacetic acid [EGTA], ethylenediaminetetraacetic acid [EDTA], tris-maleate, dithiothreitol [DTT] and phenylmethylsulphonyl fluoride [PMSF] were purchased from Sigma Chemicals (St. Louis, MO). 1400 W was obtained from Cayman Chemical Company (Ann Arbor, MI). Monoclonal, polyclonal and horseradish peroxidase-conjugated polyclonal antibodies for 3-nitrotyrosine were obtained from Cayman Chemical Inc. (Ann Arbor, MI), Upstate Biotechnology (Lake Placid, NY), and Academy Biomedical Company (Houston, TX), respectively. Antibodies for both iNOS and nNOS were obtained from Transduction Laboratories Inc. (Lexington, KY). Secondary antibodies for immunoblotting and immunohistochemistry were obtained as well from Transduction Laboratories Inc. and Jackson ImmunoResearch Inc. (West Grove, PA), respectively. The biotin blocking system was obtained from Dako (Carpinteria, CA). AquaPerm mounting medium was purchased from Shandon (Pittsburg, PA) Reagents for enhanced chemiluminescence detection were obtained from Chemicon Inc. (Temecula, CA).

12.2.- ANIMAL PREPARATION

12.2.1.- COMMON PROCEDURES

The Animal Research Committee of McGill University approved all procedures. The interventions summarized within this section were common for the different animal experiments. Pathogen-free male Sprague-Dawley rats (250-275 g) were used for several experiments. Two separate groups of mice genetically deficient (knockout) in either iNOS or nNOS isoforms were also used to evaluate the separate effects of the nNOS and iNOS isoforms on nitrotyrosine formation. For the iNOS isoform, adult (8-12 weeks old) B6/129 hybrid iNOS knockout mice ($iNOS^{-/-}$) were generated as mentioned previously[207]. Wild type B6/129 hybrid mice ($iNOS^{+/+}$) were purchased from Jackson Laboratories (Bar Harbor, Maine) and bred to serve as experimental controls. For the nNOS isoform, adult (8-12 weeks old) C57BL/6 $nNOS^{-/-}$ mice were generated as mentioned previously[208]. Wild type (WT) C57BL/6 mice were purchased from Charles River Inc. All animals were always injected intraperitoneally. All animals, rats and mice, were always injected a sedation dose of sodium pentobarbital (30 mg/kg) prior to sacrifice. The animals were always alive and properly perfused at the moment of the sacrifice. The diaphragm, intercostals, and for comparison gastrocnemius, and soleus muscles were quickly excised and frozen in liquid nitrogen and stored at -80°C, for western-blot analyses. For immunostaining, the tissues were first flash frozen in cold isopentane (20 s), then immersed in liquid nitrogen to be immediately stored at –80°C. The animals died as soon as the diaphragm was removed.

12.2.2.- RAT EXPERIMENTS

12.2.2.1.- ANIMAL GROUPS

a) 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 non-selective NOS inhibitor ($_L$-NAME, 30 mg/kg). All animals were sacrificed 1 hr later, and the diaphragm (mixed fiber composition) and soleus (rich in type I fibers) muscles were quickly dissected.

b) Sepsis: Seven groups of rats (n=5 in each group) were studied. Group 1 was injected with normal saline (control group). Groups 2, 3, 4, 5, 6, and 7 were injected intraperitoneally with *E. coli* LPS (20 mg/kg) and killed by an overdose of sodium pentobarbital 1, 3, 6, 12 and 24 hrs after the injection, respectively. Group 7 animals were injected with a selective iNOS inhibitor (1400W, 20 mg/kg) 30 min before the LPS administration and every 8 hrs thereafter, and were sacrificed 24 hrs after the LPS injection.

12.2.3.- MOUSE EXPERIMENTS

12.2.3.1.- iNOS KNOCKOUT MICE

a) normal mouse muscles : two groups (n=6 in each group) of animals were established, wild type (control-iNOS$^{+/+}$) and iNOS knockout (control-iNOS$^{-/-}$) mice.

b) septic mouse muscles: two groups (n=6 in each group) of animals were established, iNOS$^{+/+}$ (LPS- iNOS$^{+/+}$) and iNOS$^{-/-}$ (LPS-iNOS$^{-/-}$). Mice were injected intraperitoneally *E. coli* LPS (20 mg/kg) and sacrificed 24 hrs later.

In both cases, the 4 muscles were excised and frozen in liquid nitrogen as mentioned above.

12.2.3.2.- nNOS KNOCKOUT MICE

a) normal mouse muscles: two groups (n=6 in each group) of mice were established, wild type (control-nNOS$^{+/+}$) and nNOS knockout (control-nNOS$^{-/-}$).

b) septic mouse muscles: two groups (n=6 in each group) of mice were established, nNOS$^{+/+}$ (LPS-nNOS$^{+/+}$) and nNOS$^{-/-}$ (LPS-nNOS$^{-/-}$). Mice were injected *E. coli* LPS (20 mg/kg i.p.), and sacrificed 24 hrs later .

In both cases, the 4 muscles were excised and frozen in liquid nitrogen as previously mentioned.

12.3.- MUSCLE FRACTIONATION

12.3.1.- MITOCHONDRIA, CYTOSOL, AND MEMBRANE FRACTIONS

Separation of mitochondrial, membrane and cytosolic muscle fractions was

achieved using the protocol of Rock *et al* [209]. The entire procedure was done at 4°C. In brief, 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 (P1) was discarded, whereas the supernatant (S1) was designated as **crude homogenates**. These homogenates were then centrifuged at 12,000g for 20 min to yield supernatant (S2) and pellet (P2). Pellet (P2) was then re-suspended in buffer B (tris-maleate 10 mM, EDTA 0.1 mM and KCl 135 mM) and then centrifuged at 12,000g for 20 min to yield S3 and P3. The resulting pellet (P3) was re-suspended 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 hr at 100,000g. The resulting supernatant (S4) was designated as the cytosolic fraction, whereas the pellet (P4) was re-suspended in buffer C (HEPES 10 mM, sucrose 300 mM, pH 7.2), treated for 1 hr with 600 mM KCl and then centrifuged again at 100,000g for 1 hr. Pellet was re-suspended in buffer A and designated as the membrane fraction.

12.3.2.- MYOFIBRILLAR, CYTOSOL, AND MEMBRANE FRACTIONS

Fractionation of muscle samples into myofibrillar, membrane and cytosolic fractions was performed according to the protocol of Fagan *et al* [210]. In brief, muscle samples were homogenized in ice-cooled pyrophosphate buffer (tris-maleate 0.01M, 0.1M KCl, 2 mM $MgCl_2$, 2mM EGTA, 2mM $Na_4P_2O_7$, 0.1M Na_2PO_3, 1 mM DTT, pH6.8). Samples were then centrifuged at 1000g for 10 min. The pellet (P1) was then washed 4 times with 10 volumes of low-salt buffer followed by 1 wash with low-salt buffer containing Triton X-100 (0.02%) and 1 wash with sodium deoxycholate (0.02%). The pellet was then washed 2 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 100,000g for 1 hr to yield supernatant (S2, cytosolic fraction). The resulting pellet (P2) was re-suspended 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.).

12.4.- WESTERN BLOT ANALYSIS

Crude homogenates are the supernatants obtained from samples homogenized in 6 volumes (wt/vol) of homogenization buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1mM DTT, 1mg/ml PMSF, 0.32 mM sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A), centrifuged at 4°C for 30 min at 5,000 rpm. 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 12% tris-glycine sodium dodecylsulfate (SDS) polyacrylamide gels and separated by electrophoresis. Lysates of human and rat neutrophils were used as positive controls (including control for species differences). Proteins were transferred electrophoretically 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 anti-3-nitrotyrosine monoclonal or polyclonal antibodies, dissolved in 1% BSA. In few samples, we also probed membrane with monoclonal antibodies specific to iNOS and nNOS proteins. Additionally, the efficiency of separating muscle samples into different fractions was verified by probing various muscle fractions with selective primary antibodies to cytochrome oxidase (mitochondrial marker), caveolin-3 (marker of sarcolemma) and troponin I (myofibril protein marker). After the incubation with the respective primary antibodies, several 10-min washes with wash buffer (PBST or TBST) on rotating shaker were done. The PVDF membranes were then further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies. Specific proteins were detected with a chemiluminescence kit. 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 3-nitrotyrosine OD was calculated for each sample by adding OD of individual positive protein band. Specificity of anti-3 nitrotyrosine antibodies was evaluated by pre-incubation of each primary antibody with either 10 mM of nitrotyrosine or 10-fold excess of peroxynitrite-tyrosine nitrated bovine serum albumin (generously provided by Dr. Ischiropoulos, University of Pennsylvania).

12.5.- 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 or polyclonal 3-nitrotyrosine antibodies. For negative control, the primary antibody was replaced with non-specific 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 hr followed by exposure to Cy3-labelled streptavidin for 1 hr. Sections were then washed, mounted with coverslips and examined with a Nikon fluorescence microscope and photographed with a 35 mm camera (Nikon Inc.).

12.6.- 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 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).

13.- EXPERIMENTAL RESULTS

13.1.- PROTEIN TYROSINE NITRATION IN NORMAL MUSCLES

Figure 2 illustrates NO_2Tyr immunoreactivity in normal rat diaphragms detected with three different antibodies. Monoclonal anti-NO_2Tyr antibody detected five nitrated protein bands of 52, 48, 40, 30 and 10 kDa. (Figure 2A). Polyclonal anti-NO_2Tyr antibody also detected the above-mentioned nitrated proteins in addition to an 18-kDa-protein band (Figure 2 B). Similarly, HRP-conjugated anti-NO_2Tyr polyclonal antibody detected positively nitrated protein bands of 52, 40, 30, 18 and 10 kDa, and an additional

positive band of 66 kDa, whereas that of 48 kDa was not detected (Figure 2C). The intensity of 10-kDa protein band was the highest proportion of total diaphragmatic NO_2Tyr signal detected with the HRP-conjugated anti-NO_2Tyr antibody (Figure 2 C). 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 kDa, compared to the ventilatory muscles (Figure 2D). Figure 2E illustrates the specificity of monoclonal anti-NO_2Tyr in detecting NO_2Tyr formation in rat diaphragm. Pre-incubation 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 pre-incubated with 10 mM nitrotyrosine (results not shown).

Figure 3 illustrates the presence of NO_2Tyr immunoreactivity in various muscle fractions. Anti-NO_2Tyr antibodies detected positive NO_2Tyr protein bands mainly in the cytosolic fraction, whereas only weak bands were detected in the myofibrillar, membrane and mitochondrial fractions (Figure 3A and B).

Localization of nitrotyrosine immunoreactivity in normal rat muscles is shown in figure 4. Monoclonal anti- NO_2Tyr antibody detected positive staining in gastrocnemius (panels A and B) and soleus (panel C) in close proximity to the sarcolemma. Positive NO_2Tyr staining was also detected in nerve fibers (arrows in panel D), but not in blood vessels traversing skeletal muscles (panel D). Polyclonal anti-NO_2Tyr also detected positive NO_2Tyr in the diaphragm of normal rats in close proximity to the sarcolemma (panel E). Replacement of primary antibodies with non-specific mouse IgG or rabbit IgG completely eliminated positive NO_2Tyr staining (panel F).

13.2.- ROLE OF NOS IN PROTEIN TYROSINE NITRATION IN NORMAL MUSCLES

Examination of muscle samples after 1 hr of *in-vivo* administration of either a selective iNOS inhibitor (1400W) or non-selective NOS inhibitor ($_L$-NAME) in normal rats revealed significant reduction in total diaphragmatic and soleus muscle NO_2Tyr levels (Figure 5C). In the diaphragm (Figure 5A), administration of 1400W significantly reduced NO_2Tyr OD at 52 and 48 kDa ($p<0.05$). Those of 30 and 10 kDa remained similar to control muscles. $_L$-NAME administration produced relatively larger reduction in diaphragmatic NO_2Tyr levels compared with that elicited by 1400W and significantly lowered NO_2Tyr ODs of 52, 48 and 30 kDa protein bands ($p<0.01$). Similarly, administration of 1400W or $_L$-NAME significantly reduced soleus muscle NO_2Tyr levels compared with control values (panel B). As in the diaphragm, the reduction in total NO_2Tyr OD elicited by L-NAME was relatively greater than that produced by 1400W injection (Figure 5B).

In addition to pharmacological inhibition of NOS activity in rats, we compared diaphragmatic NO_2Tyr OD among wild type mice and mice deficient in either iNOS or nNOS (knockout) isoforms (Figure 6). Monoclonal anti- NO_2Tyr antibody detected in the diaphragms of control-$iNOS^{+/+}$ (B129/C57Bl6 wild type) and control-$nNOS^{+/+}$ (Bl6 wild type) mice several nitrated protein bands, which are similar to those detected in the diaphragms of normal rats (panels A and B). In control-$iNOS^{-/-}$ mice, the intensities of these bands particularly those of 52, 48, 40 and 30 kDa-protein bands were significantly lower than in $iNOS^{+/+}$ mice (Figure 6A). By comparison, absence of nNOS in control-$nNOS^{-/-}$ mice had no effect on the intensity of NO_2Tyr protein bands compared with wild type ($nNOS^{+/+}$) mice (Figure 6B). Figure 6C shows total diaphragmatic NO_2Tyr ODs in the four 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 NO_2Tyr levels between $nNOS^{+/+}$ and $nNOS^{-/-}$ mice.

13.3.- 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 mice. Injection of *E. coli* LPS in rats elicited a significant induction of the iNOS isoform in the diaphragm, which peaked after 12 hrs of LPS administration (Figure 7). In addition to iNOS induction, LPS injection produced a significant increase in nNOS protein expression, which peaked after 24 hrs of LPS administration (Figure 7). Similar results regarding iNOS and nNOS expressions were found in the intercostals and gastrocnemius muscles (results not shown).

Injection of LPS in rats produced a significant rise in NO_2Tyr OD of crude diaphragmatic homogenates after 6, 12 and 24 hrs of LPS injection ($p<0.05$, Figure 8A). Fractionation of muscle homogenates revealed that the increase in NO_2Tyr formation in response to sepsis was localized mainly in the mitochondrial and membrane fractions rather than in the cytosolic fraction (Figure 8B and C). The rise in mitochondrial and membrane NO_2Tyr levels in response to LPS injection was evident mainly at 52, 48 and 30 kDa protein bands rather than at the 10 kDa band. Continuous selective inhibition of iNOS activity in septic rats achieved by 1400W resulted in significant attenuation of LPS-induced rise in diaphragmatic NO_2Tyr formation, especially those of 52, 48 and 30 kDa protein bands ($p<0.05$) (data not shown) .

After 24 hrs of LPS injection, diaphragmatic NO_2Tyr OD was significantly lower in $iNOS^{-/-}$ mice compared with $iNOS^{+/+}$ mice (Figure 9A). This was due mainly to lower level of protein nitration of 50, 48 and 30 kDa protein bands in LPS-$iNOS^{-/-}$ compared with LPS-$iNOS^{+/+}$ mice (Figure 9 A and B). The absence of the nNOS isoform in $nNOS^{-/-}$ mice had no significant effect on LPS-induced NO_2Tyr formation in the diaphragm with total and individual protein band NO_2Tyr OD being similar to those measured in LPS-$nNOS^{+/+}$ mice (Figure 9B).

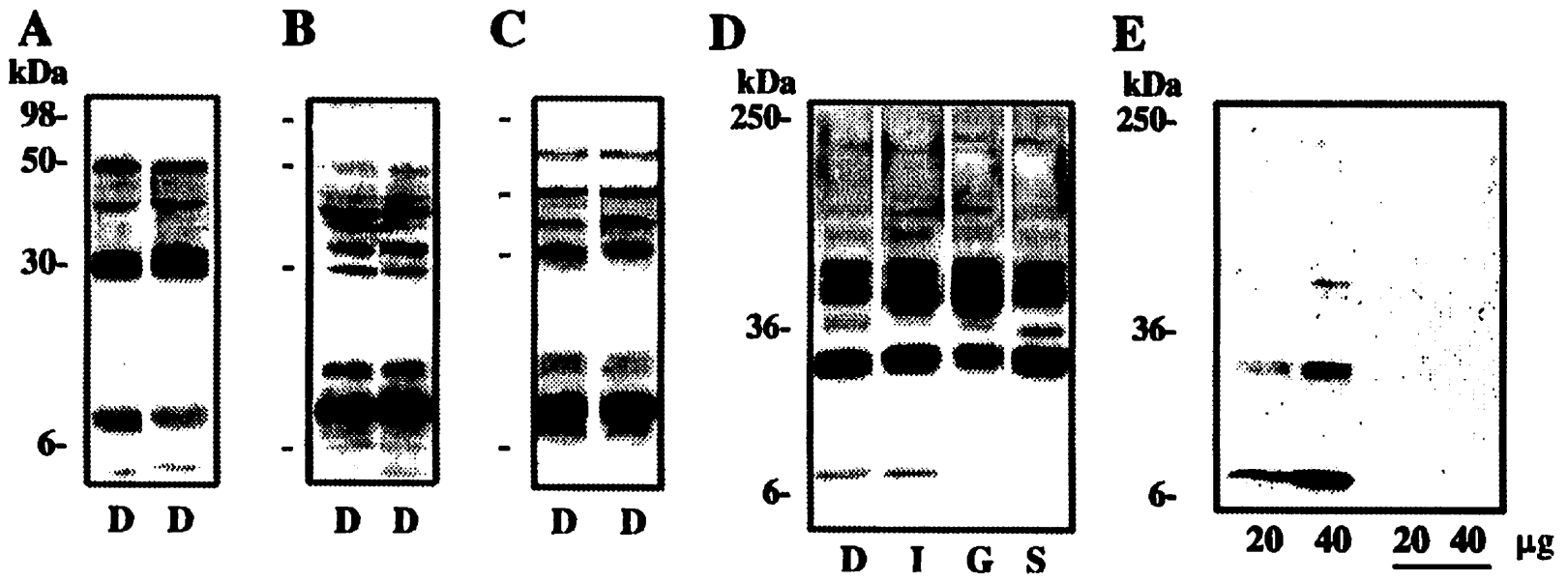


Figure 2. Nitrotyrosine immunoreactivity in normal rat diaphragms

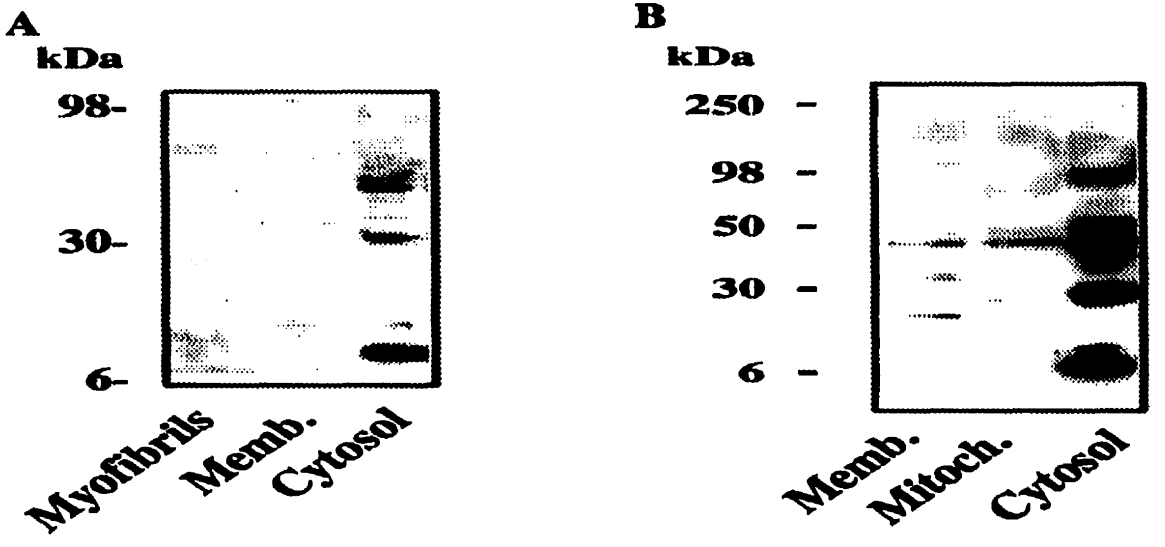


Figure 3. Nitrotyrosine immunoreactivity in different muscle compartments

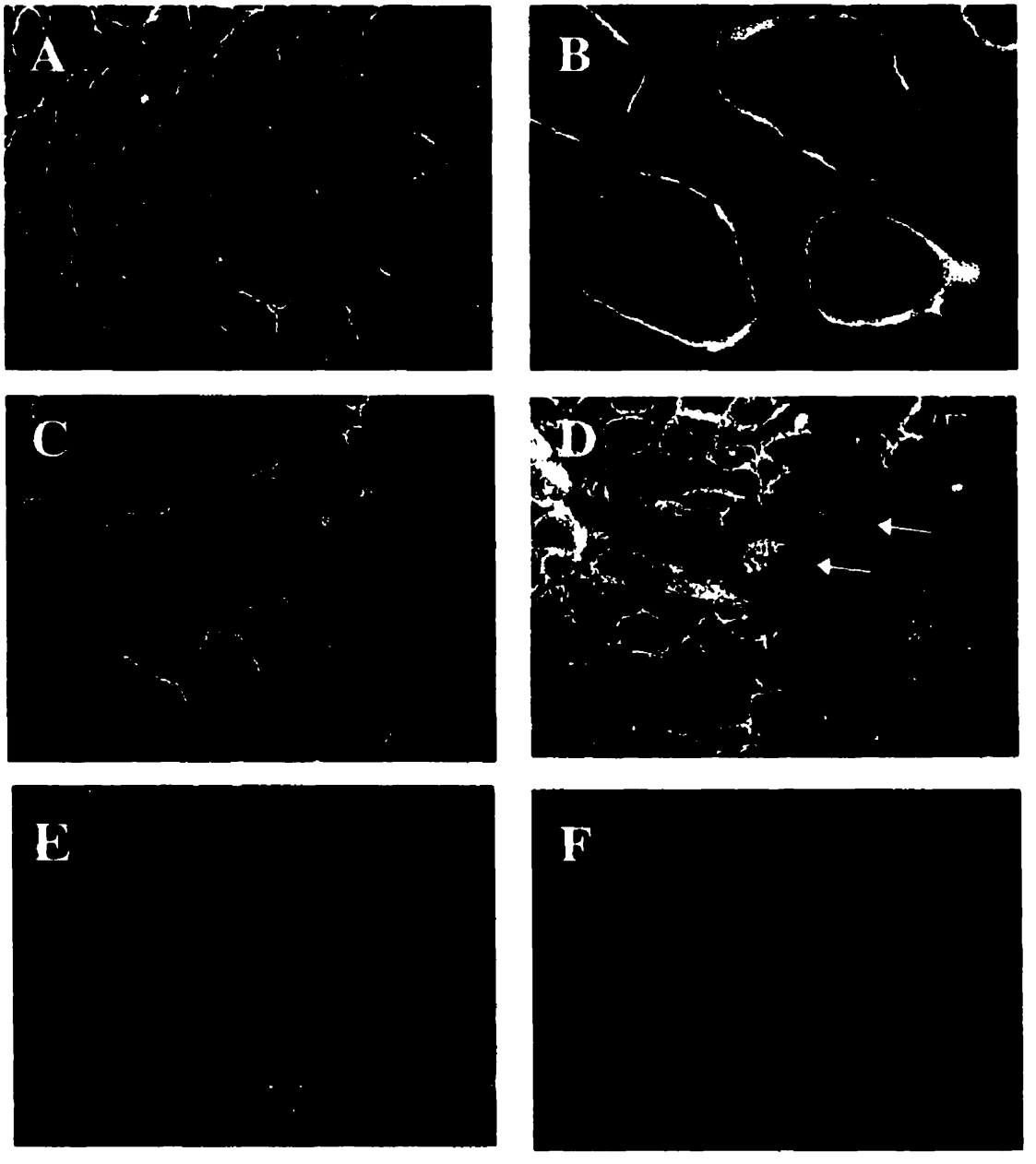


Figure 4. Localization of Nitrotyrosine immunoreactivity in normal rat muscles

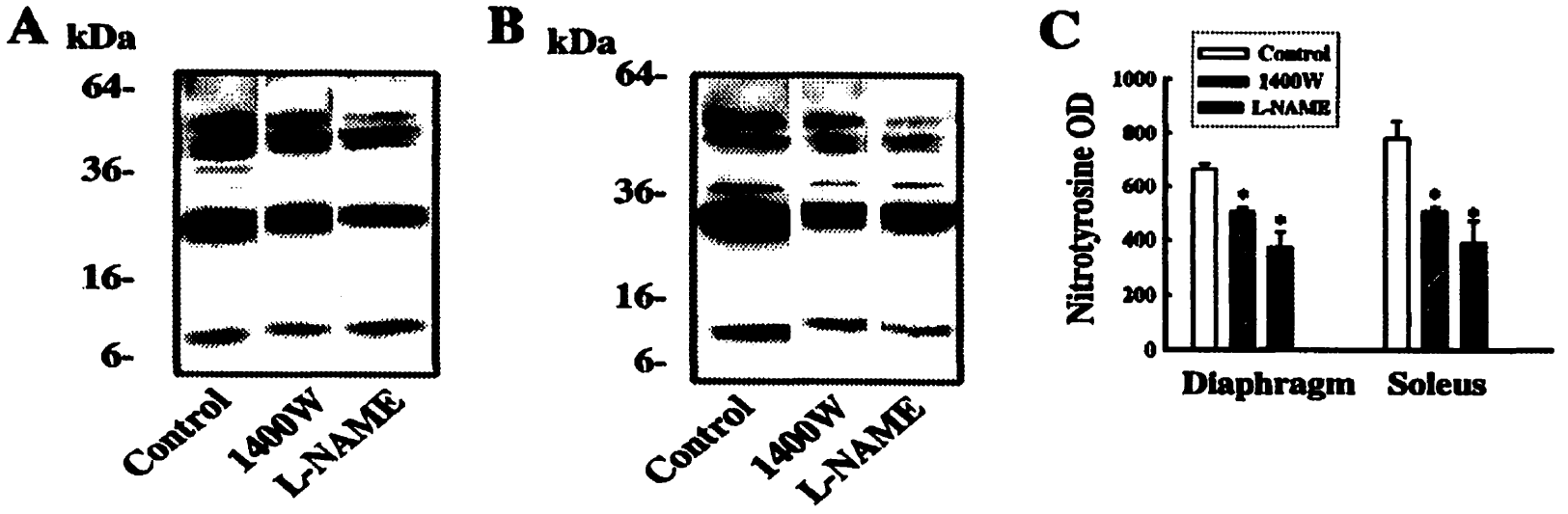


Figure 5. Effect of NOS on protein nitration in normal muscles

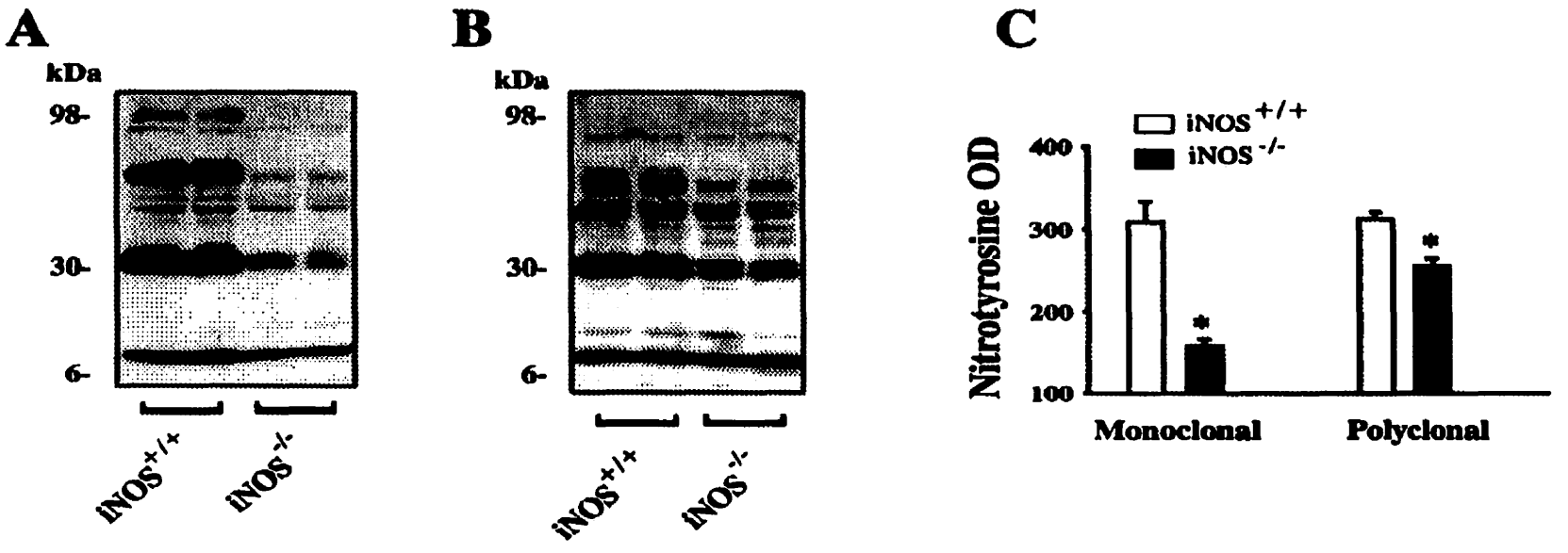


Figure 6. Effect of NOS (iNOS knockout mice) on protein nitration in normal muscles

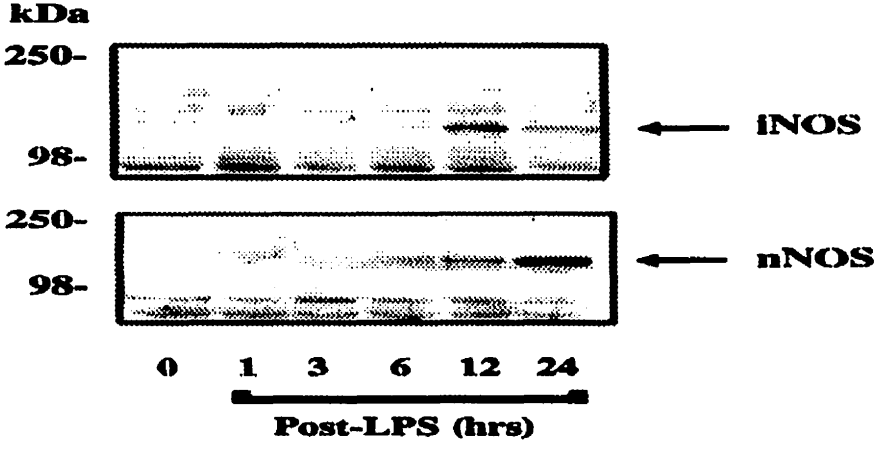


Figure 7. Time course of expression of iNOS and nNOS in sepsis

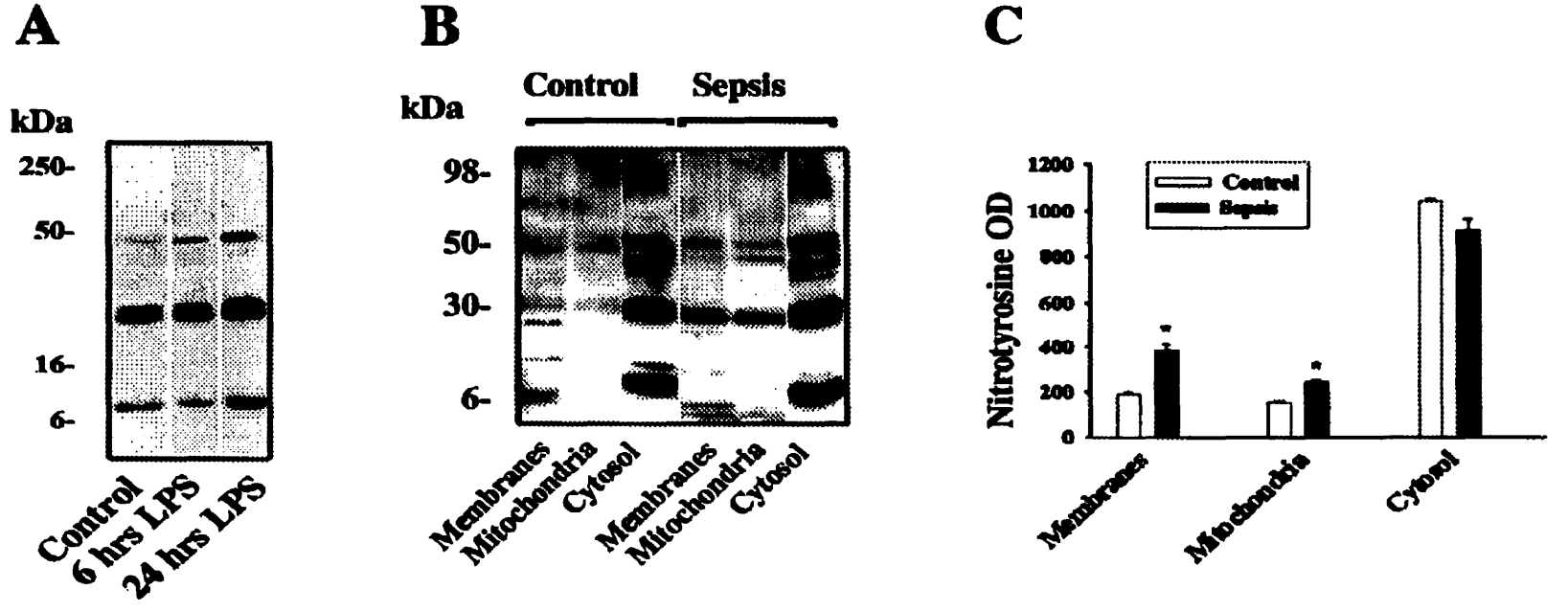


Figure 8. Nitrotyrosine immunoreactivity in septic rat diaphragms

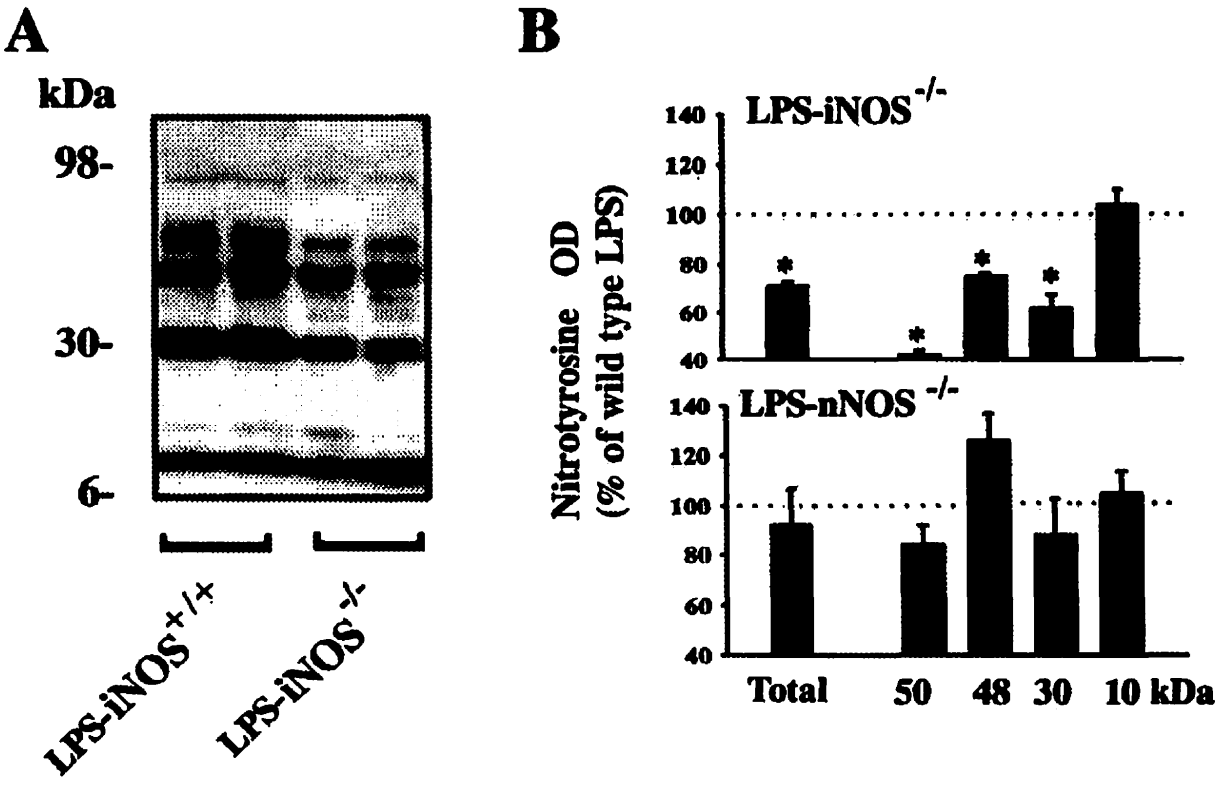


Figure 9. Effect of NOS inhibition on nitrotyrosine formation in septic muscles

14.- MAIN FINDINGS

14.1 PROTEIN TYROSINE NITRATION IN NORMAL MUSCLES

Abundant NO_2Tyr formation was detected in the cytosolic fraction of different rat skeletal muscles using three different anti-NO_2Tyr antibodies. These nitrated proteins were localized within the skeletal muscle fiber in close proximity to the sarcolemma. Finally, six distinct proteins appear to be nitrated in normal skeletal muscles with apparent molecular weights ranging from 10 kDa to 66 kDa.

14.2.- ROLE OF NOS IN TYROSINE NITRATION IN NORMAL MUSCLES

Either iNOS or both constitutive isoforms (nNOS and eNOS) appear to be involved in tyrosine nitration in normal rat ventilatory and limb muscles as demonstrated by the reduction in protein nitration elicited by both a selective iNOS inhibitor and a constitutive NOS inhibitor in both the rat diaphragm and the soleus muscles. Interestingly, a great reduction in tyrosine nitration was detected in the control diaphragms of mice deficient in the iNOS isoform compared with control wild type mice. Although the administration of the constitutive NOS inhibitor, L-NAME elicited a significant reduction in total levels of nitrated proteins, diaphragms from mice deficient in the nNOS isoform did not show any significant decline in protein nitration with respect to the control wild type group.

14.3.- PROTEIN TYROSINE NITRATION IN SEPTIC MUSCLES

E. Coli LPS injection was associated with a significant induction of the iNOS isoform, which reached its peak at 12 h of the LPS administration. Additionally, nNOS protein expression progressively increased in the course of sepsis to reach its peak at 24 h of endotoxemia.

Sepsis was associated with a significant increase in muscle NO_2Tyr formation particularly in the mitochondrial and membrane fractions, but not in the cytosolic compartment. Finally, the increase in protein nitration induced by sepsis is largely dependent on muscle iNOS activity as demonstrated by using both pharmacological and genetic iNOS inhibition.

15.- FIGURE LEGENDS

Figure 2: Representative immunoblots of two different rat diaphragmatic (D) homogenates probed with **A**) monoclonal, **B**) polyclonal, **C**) and HRP-conjugated polyclonal anti-NO_2Tyr antibodies. Note the differences in the intensity of individual protein bands among the three immunoblots. **D**) A representative immunoblot of normal 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 pre-incubated with 10-fold excess of nitrated bovine serum albumin. Note that this pre-incubation resulted in disappearance of positive nitrotyrosine protein bands shown in the left two lanes.

Figure 3: **A**) Representative immunoblot of myofibrillar, membrane and cytosolic fractions of normal rat diaphragm samples probed with polyclonal 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.

Figure 4: 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 panel **E**, whereas panel **F** shows negative control staining in which the primary anti-NO_2Tyr antibody was replaced by non-specific mouse IgG.

Figure 5: Effects of acute (within 1 hr) inhibition of NO synthesis on the intensity of protein tyrosine nitration in the diaphragm (**A**) and soleus (**B**) muscles. Rats were injected with either 1400W (selective iNOS inhibitor) or L-NAME (non-selective NOS inhibitor). Panel **C** shows total muscle NO_2Tyr OD of control, 1400W and L-NAME-treated animals. *$P<0.05$ compared with control. Note that diaphragmatic and

soleus muscle NO_2Tyr intensities declined significantly in response to NOS inhibition by either 1400W or L-NAME.

Figure 6: **A)** Intensity of protein tyrosine nitration in diaphragmatic samples obtained from control wild type ($iNOS^{+/+}$) and iNOS knockout ($iNOS^{-/-}$) mice. Two separate diaphragmatic samples are shown for each animal group. **B)** Comparison of protein tyrosine nitration in diaphragmatic samples obtained from control wild type ($nNOS^{+/+}$) and nNOS knockout ($nNOS^{-/-}$) mice. Two separate diaphragmatic samples are shown for each animal group. **C)** 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.

Figure 7: Expression of iNOS (top) and nNOS (bottom) proteins in the diaphragms of rats in response to LPS injection. 0 refers to control animals. Note the transient nature of iNOS expression and the up-regulation of nNOS protein after 24 hrs of LPS administration.

Figure 8: **A)** Influence of LPS injection on diaphragmatic NO_2Tyr formation. Notice that NO_2Tyr levels rose substantially 24 hrs 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 and septic (24 hrs after LPS injection) rats. *$P<0.05$ compared with control animals.

Figure 9: **A)** Comparison of diaphragmatic protein tyrosine nitration in wild type ($iNOS^{+/+}$) and iNOS knockout ($iNOS^{-/-}$) mice injected 24 hrs earlier with LPS. Two separate diaphragmatic samples are shown in each animal group. **B)** Mean values of total muscle and individual protein NO_2Tyr OD of diaphragmatic muscle samples obtained after 24 hrs of LPS injection in $iNOS^{-/-}$ and $nNOS^{-/-}$ mice. Values are expressed as percentage of corresponding septic wild type animals. *$P<0.05$ compared with wild type animals.

OXIDATIVE STRESS IN THE VENTILATORY MUSCLES: ROLE OF NITRIC OXIDE SYNTHASES

CHAPTER # 2

In the foregoing study, the occurrence of nitrosative stress in response to sepsis has been demonstrated as assessed by the increased levels of nitrotyrosine formation in the ventilatory muscles. These increased levels of nitrotyrosine formation appear to be dependent on NO synthesized by the iNOS isoform. This finding along with the fact that skeletal muscle fibers normally synthesize the constituents required for peroxynitrite formation, NO and superoxide, led us propose the concept that protein tyrosine nitration in skeletal muscles is primarly mediated by peroxynitrite in both control and septic muscles. However, this powerful ROS can also oxidize proteins at different residues other than tyrosine in skeletal muscle in response to inflammatory-immune processes or in strenuous exercise, which may lead to the development of oxidative stress. Oxidative stress is defined as the final deleterious effects of certain oxidant agents on cells and tissues which have not been counteracted by the normal antioxidant systems responsible for keeping cellular homeostasis. The next and second study of this master thesis will deal with this topic. It will focus on the study of the redox status of the ventilatory muscles of septic animals by using three well established indices of oxidative stress in skeletal muscle as well as in other tissues.

16.- INTRODUCTION

There is accumulating evidence that sepsis-induced skeletal muscle injury is largely mediated by an increase in ROS levels[149,150], leading to the development of various phenomena such as protein oxidation[210], lipid peroxidation[13,148,211,212] and alterations in muscle glutathione metabolism[213] . Moreover, it has been well established that peroxynitrite (the reaction product of superoxide and NO) is the principal molecule responsible for most oxygen radical-mediated tissue damage effects in pathophysiological conditions[110]. Additionally, cellular *in vivo* conditions such as sepsis, which involve excessive production of both superoxide and NO, favor the formation of peroxynitrite[214]. Peroxynitrite, which has a relatively long half-life, and directly crosses cell membranes can react either with cellular constituents or decompose to form reactive by-products. One of the most important consequences of peroxynitrite effects on cells, is its capability to oxidize proteins and non-protein thiols, as well as other molecules such as deoxyribose,

methionine, membrane phospholipds, and DNA. Alternatively, peroxynitrite nitrates tyrosine residues to form nitrotyrosine, phenomenon which has been extensively addressed in the preceding chapter.

Among different molecules, proteins constitute one of the major targets of ROS, including peroxynitrite. Protein oxidation is known to modify the side chains of methionine, histidine, and tyrosine as well as to form cysteine disulfide bonds[215]. Metal catalyzed oxidation of proteins, which occurs in the presence of molecular oxygen, iron, copper, and an appropriate electron donor, introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues in a site-specific manner[216] . The introduction of these carbonyl groups constitutes a hallmark of the oxidation status of proteins. Protein oxidation is a very important phenomenon, which can modify the biochemical characteristics of proteins such as enzymatic activity, DNA binding of transcription factors, and the susceptibility to proteolytic degradation[217,218].

Another consequence of ROS interactions is their reaction with polyunsaturated fatty acids, resulting in the formation of cytotoxic aldehydes such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde, which are the end products of lipid peroxidation[219]. Accordingly, Supinski *et al* [220] showed that peroxynitrite induces contractile dysfunction and lipid peroxidation in the rat diaphragms. Additionally, the modifications induced by these two lipid peroxidation products have been implicated in several degenerative diseases, in aging[221,222] and in sepsis[13,148]. Particularly, HNE cross-links and influences many enzymes such as Na^+-K^+-ATPase, and glucose-6-phosphate dehydrogenase[223] upon reaction with the amino groups of the proteins leading to the formation of what is known as Michael adducts[223]. HNE-bound proteins can be immunodetected by using specifically designed antibody anti-Michael adduct, which has resulted in a convenient index of lipid peroxidation.

The tripeptide glutathione (γ-glycyl-glutamyl-cysteine) is a major source of non-protein thiols and one of the most important antioxidants in mammals. Furthermore, glutathione has been proposed to have other roles, such as the regulation of both protein synthesis and degradation, detoxification and metabolism of various substances in the liver, and transmembrane transport of amino acids[224]. The oxidative capacity of a given tissue determines its glutathione concentration. Certain metabolic properties of skeletal

muscle, such as its oxidative capability, its patterns of amino acid utilization as well as its antioxidant enzyme activities have conferred an exceptional competence in relation to glutahione metabolism on this tissue[224]. The rate-limiting γ-glutamyl-cysteine synthase catalyzes the initial reaction in the synthesis of glutathione to form γ-glutamyl-cysteine from cysteine and glutamate [224]. The final addition of glycine to this dipeptide is catalyzed by glutathione synthase[224]. Additionally, the molecule undergoes changes between the reduced (GSH) and the oxidized state (GSSG). The oxidation of GSH to GSSG is catalyzed by glutathione peroxidase, while the reduction of GSSG to GSH is catalyzed by glutathione reductase[224] . The GSH represents more than 90% of the total glutathione levels. Finally, it was reported that the effectiveness of glutathione as an antioxidant within a tissue depends on glutathione concentrations in this tissue as well as on the capacity of the tissue to import GSH and export GSSG [224].

In summary, as indicated both protein oxidation[210] and lipid peroxidation[13,148,200] along with changes in glutathione concentrations occur in skeletal muscles either following strong contractions or in sepsis[213,225,226]. Our current and second study aimed to investigate the oxidative stress of the ventilatory muscles in response to sepsis.

17.- SPECIFIC HYPOTHESES

Sepsis will lead to an increased production of ROS in the skeletal muscles of septic animals. The excessive ROS production will target both proteins and lipids, leading to increased levels of protein oxidation and lipid peroxidation, respectively, within the skeletal muscle fibers. Glutahione levels in skeletal muscle, a major antioxidant, will be diminished by the effect of excessive ROS production in rat septic skeletal muscles. Excessive NO production by iNOS during sepsis will lead to the formation of peroxynitrite, which in turn will oxidize proteins and lipids, and reduce glutathione levels in rat ventilatory muscles.

18.- SPECIFIC OBJECTIVES

The main objective of this study is to investigate the role of NO in the sepsis-induced oxidative stress of the rat ventilatory muscles. The assessment of the redox state of these muscles has been approached using three different indices of oxidative stress along with their corresponding experiments designed to determine the participation of NO in each phenomenon. Consequently, the project comprises three more specific objectives:

18.1.- To determine the levels of protein oxidation in the septic rat skeletal muscles and the involvement of NO in this process.

18.2.- To determine the levels of lipid peroxidation in response to sepsis in the rat ventilatory muscles, and to assess the role of NO in this phenomenon.

18.3.- To assess the levels of glutathione in the rat respiratory muscles during sepsis and the participation of NO in this process.

19.- MATERIALS AND METHODS

19.1.- REAGENTS

Oxyblot protein oxidation kit was purchased from Intergen Company (NY). Aminoguanidine was obtained from Sigma Chemicals (St Louis, MO). Polyclonal anti-HNE Michael adducts antibody was purchased from Calbiochem (San Diego, CA). The rest of reagents employed in this study have already been described in section 12.1.

19.2.- ANIMAL PREPARATION

19.2.1.- COMMON PROCEDURES

Pathogen-free male Sprague-Dawley rats (250-275 g) were used for some experiments. In this study both iNOS and nNOS knockout mice[207,208] and the corresponding wild type mice were also used to evaluate the contribution of both iNOS and nNOS isoforms to oxidative stress. More details are described in section 12.2.1.

19.2.2.- RAT EXPERIMENTS

19.2.2.1.- ANIMAL GROUPS

a) Sepsis: Six groups of rats (n=5 in each group) were studied. Group 1 was injected with normal saline (control group). Groups 2, 3, 4, 5, and 6 were injected with *E.Coli* LPS (20 mg/Kg) and killed by an overdose of pentobarbital 1, 3, 6, 12, and 24 hrs after the LPS injection, respectively.

b) NOS inhibition: A selective iNOS inhibitor, aminoguanidine (30mg/kg), with similar inhibitory properties to 1400W, was used to evaluate the contribution of the inducible NOS isoform to oxidative stress. 3 groups were established. Group 1 animals were injected with aminoguanidine 30 min prior to sacrifice. Group 2 rats were injected with aminoguanidine 30 min prior to the LPS injection, and the animals were sacrificed 3h later. Group 3 animals were again injected with aminoguanidine 30 min before the LPS injection, and 6 h after this injection, and they were sacrificied at 12 h of endotoxemia.

19.2.3.- MOUSE EXPERIMENTS

19.2.3.1.- iNOS KNOCKOUT MICE

Experiments were organized according to section 12.2.3.1.

19.2.3.2.- nNOS KNOCKOUT MICE

Experiments were organized according to section 12.2.3.2.

19.3.- MYOFIBRILLAR, CYTOSOL, AND MEMBRANE FRACTIONS

This method of muscle fractionation has been described in section 12.3.2.

19.4.- PROTEIN OXIDATION - CARBONYL GROUP CONTENT

The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The oxyblot protein oxidation detection kit contains all the 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 was added as well as 2-mercaptoethanol to the sample mixture.

19.5.- WESTERN BLOT ANALYSIS

Crude homogenates, muscle fractions, and derivatized proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described in section 12.4. Specifically, the DNP-derivatized protein samples were electrophoretically separated followed by Western blotting. The PVDF membranes were then incubated with primary antibody specific to the DNP moiety of the proteins and with specific HRP-conjugated secondary antibody. Crude homogenates and muscle fractions were also incubated with anti-HNE protein adducts antibody.

19.6.- GLUTATHIONE MEASUREMENTS

19.6.1.- TISSUE PROCESSING

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, or following derivatization, for oxidized glutathione (GSSG) measurement.

19.6.2.- DERIVATIZATION FOR GSSG MEASUREMENTS

Based on the method of Coutelle[227], samples for GSSG measurement were derivatized by vortexing 400 μL of diluted sample with 50 μL of a mixture of 250 μL TEA (Triethanolamine), 250 μL 0.9% SSA and 50 μL 2-VP (2-Vinylpyridine), for 10 min at RT.

19.6.3.- GLUTATHIONE ASSAY

Glutathione concentration was determined by the glutathione reductase (GR) recycling method of Tietze[228], adapted for the Cobas Mira S spectrophotometer (Roche Diagnostics, Laval, Quebec)[229]. Briefly, the Cobas Mira S pipettes 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 at 37° C, 15 µL GR (2 U/100 µL) is added and the reaction monitored every 24 s for 12 min. Under these conditions, the method is linear for GSH concentrations between 0.1 and 6 µM.

19.7.- STATISTICAL ANALYSIS

See section 12.6 for more details.

20.- EXPERIMENTAL RESULTS

20.1.- INDICES OF OXIDATIVE STRESS IN SEPTIC MUSCLES

Figure 10 illustrates different oxidized proteins in the septic rat diaphragms. Significant increase of the levels of carbonyl groups were detected in response to sepsis compared with the control group (Figure 10A). Muscle protein oxidation was concentrated to specific protein bands ranging in apparent mass between 50 and 10 KDa. Figure 10B illustrates the presence of several oxidized proteins in three muscle fractions. Oxidized protein bands were mainly localized in the myofibrilar and membrane compartments of the septic rat diaphragms. The time course of the expression of lipid peroxidation in response to sepsis using anti-HNE protein adducts antibody is shown in figure 11A. No significant differences were found between the levels of HNE-protein adducts in response to sepsis and the control diaphragms. Muscle lipid peroxidation was concentrated to specific protein bands ranging in apparent molecular weight between 64 and 10 KDa. Figure 11B illustrates the presence of several HNE-protein adducts in three muscle compartments. These HNE-protein adducts were mainly localized in the cystosolic fraction in both the control and septic diaphragms. Figure 12 shows both total carbonyl group OD and total HNE-protein adducts OD in the course of sepsis.

Diaphragmatic carbonyl groups OD were significantly higher after 6,12, and 24 h of endotoxemia with respect to the control diaphragms. Conversely, no significant differences were found in HNE-protein adducts OD between groups.

20.2.- ROLE OF NOS IN OXIDATIVE STRESS IN CONTROL MUSCLES

20.2.1.- iNOS INHIBITION

20.2.1.1.- PHARMACOLOGICAL INHIBITION

Selective inhibition of the inducible NOS with aminoguanidine injected 30 min prior to the sacrifice elicited a significant reduction in carbonyl group formation with respect to the control diaphragm (saline) (Figure 13A). Conversely, aminoguanidine did not elicit any modification in the intensity of the HNE-protein adduct bands (Figure 13B).

20.2.1.2.- GENETIC DELETION OF iNOS: iNOS KNOCKOUT MICE

The control diaphragms of mice deficient in the iNOS isoform did not show any reduction in the levels of expression of both carbonyl groups and HNE-protein adducts compared to control diaphragms of wild type mice as illustrated in Figure 14A and 14 B, respectively. These findings are more clearly shown in figure 14 C where total diaphragmatic carbonyl groups and HNE-protein adducts OD of both iNOS knockout and wild type mice are represented.

20.2.2.- nNOS INHIBITION

The control diaphragms of mice deficient in the nNOS isoform showed an increase in carbonyl group formation with respect to control diaphragms of wild type mice as illustrated in figure 15A. Conversely, no modifications were detected between control diaphragms of both nNOS knockout and wild type mice with regard to the levels of expression of HNE-bound proteins (Figure 15B). Figure 15C shows total diaphragmatic carbonyl groups and HNE-protein adducts OD of both nNOS knockout and wild type mice.

20.3.- ROLE OF NOS INHIBITION IN SEPTIC MUSCLES

20.3.1.- iNOS INHIBITION

20.3.1.1.- PHARMACOLOGICAL INHIBITION

Selective continuous iNOS inhibition induced by aminoguanidine significantly reduced the levels of carbonyl groups in septic rat diaphragms after 12 h of endotoxemia with respect to the12 h-septic diaphragms which were not treated with the iNOS inhibitor (Figure 13A). In contrast, no modifications were detected between these two groups of animals with regard to the levels of expression of HNE-protein adducts (Figure 13B).

20.3.1.2.- GENETIC DELETION OF iNOS: iNOS KNOCKOUT MICE

Interestingly, septic diaphragms of mice deficient in the iNOS isoform showed a significant decrease in carbonyl group formation with respect to septic diaphragms of wild type mice as illustrated in figure 14A. In contrast, no modifications were detected between septic diaphragms of both iNOS knockout and wild type mice with regard to the formation of HNE-protein adducts (Figure 14B). These findings are more clearly shown in figure 14 C where total diaphragmatic carbonyl groups and HNE-protein adduct OD of both iNOS knockout and wild type mice are represented.

20.3.2.- nNOS INHIBITION

Interestingly, sepsis elicited a significant increase in the diaphragmatic content of carbonyl groups in both wild type and nNOS knockout mice with respect to their corresponding control diaphragms (Figure 15A). In contrast, no modifications were observed between the septic diaphragms of both wild type and nNOS knockout mice regarding the levels of HNE-protein adduct formation (Figure 15B). These findings are more clearly illustrated in figure 15C, where total OD of both carbonyl groups and HNE-protein adducts of septic diaphragms of both wild type and nNOS knockout mice are depicted.

20.4.- MUSCLE GLUTATHIONE

20.4.1.- SEPTIC RAT DIAPHRAGMS

Sepsis elicited a significant decline in the total rat diaphragm glutathione levels with respect to the control diaphragms (upper panel, Figure 16). Furthermore, a significant increase in the oxidized fraction of diaphragmatic glutathione was observed in response to endotoxemia, leading to an augmentation in the muscular GSSG/total ratio (lower panel, Figure 16).

20.4.2.- PHARMACOLOGICAL iNOS INHIBITION IN SEPTIC RAT DIAPHRAGMS

Continuous iNOS inhibition with aminoguanidine did not elicite any significant modification in the total levels nor in the oxidized fraction of diaphragmatic glutathione (upper and lower panels, Figure 16).

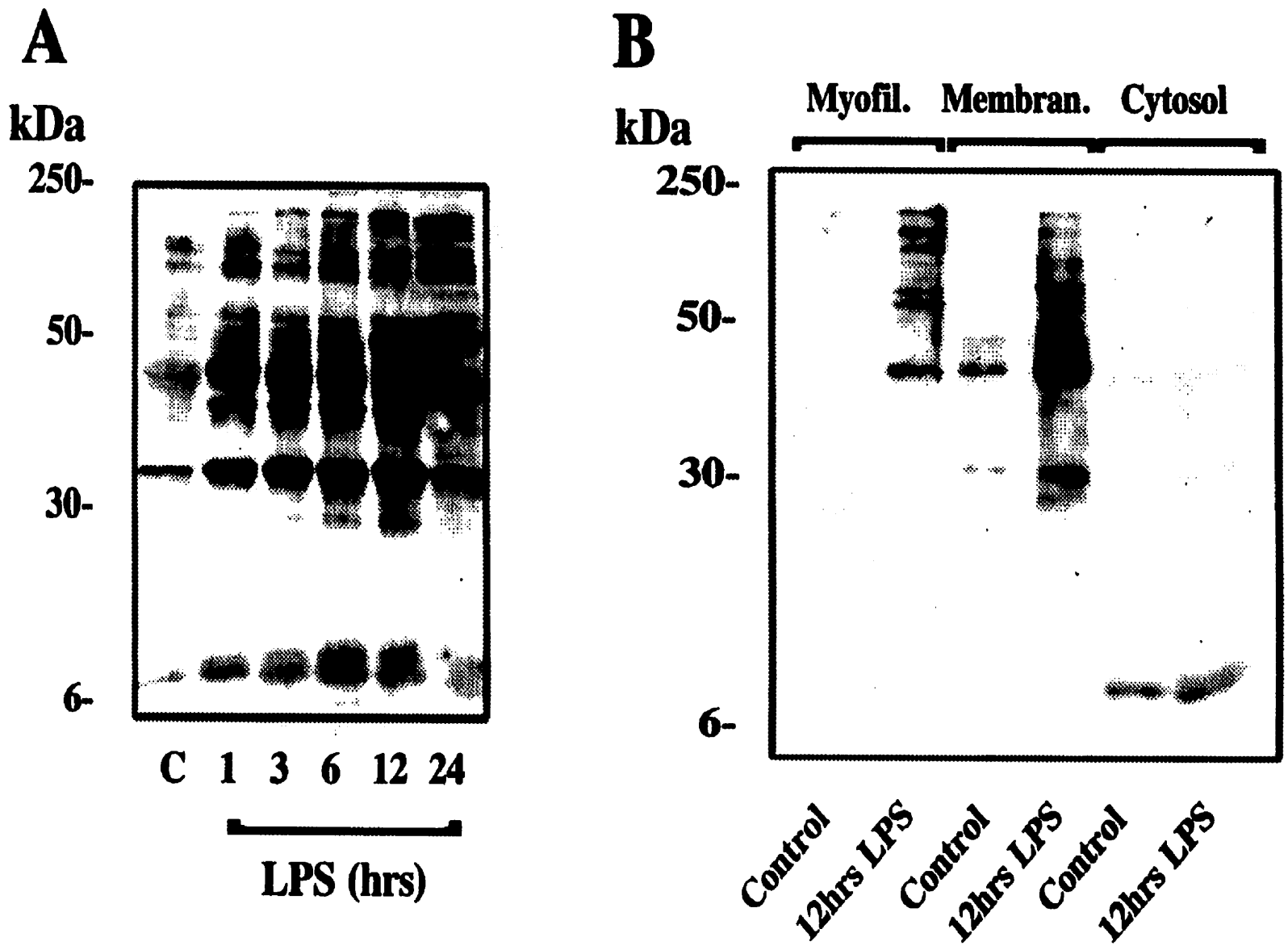


Figure 10. Time course of expression of oxidized proteins in septic diaphragms

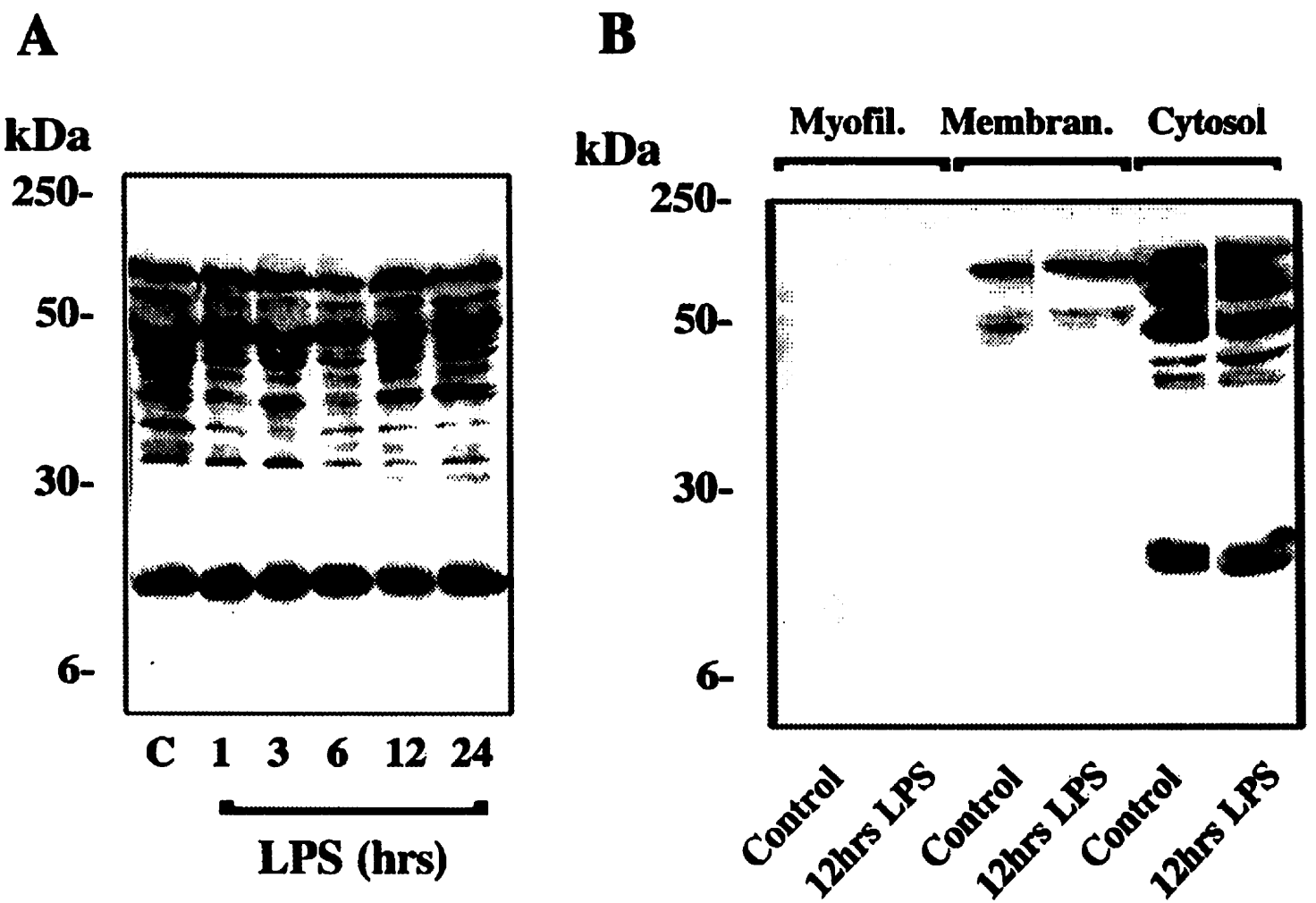


Figure 11. Time course of expression of HNE-bound proteins in septic diaphragms

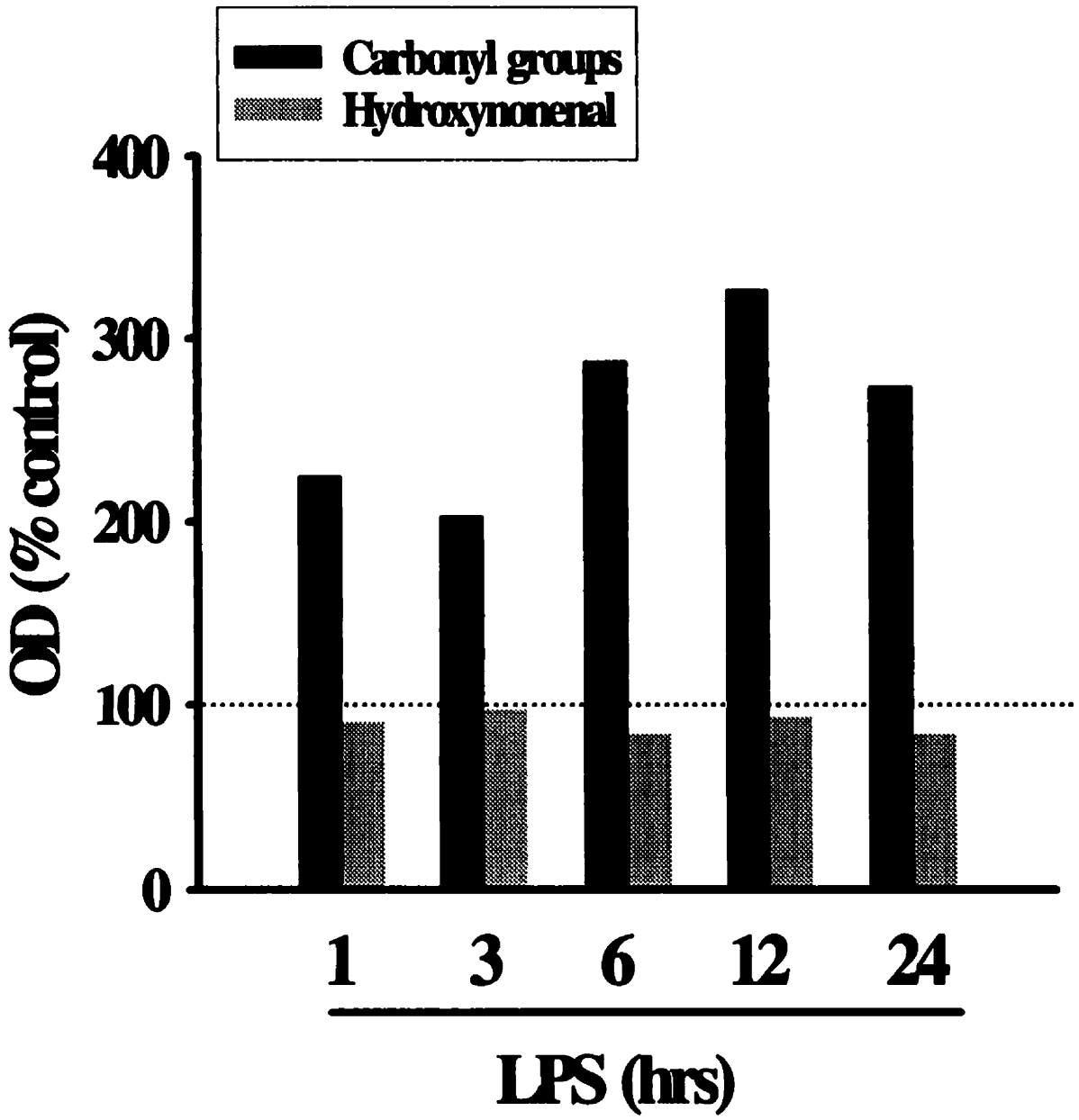


Figure 12. Total OD of HNE-bound proteins and carbonyl group formation in sepsis

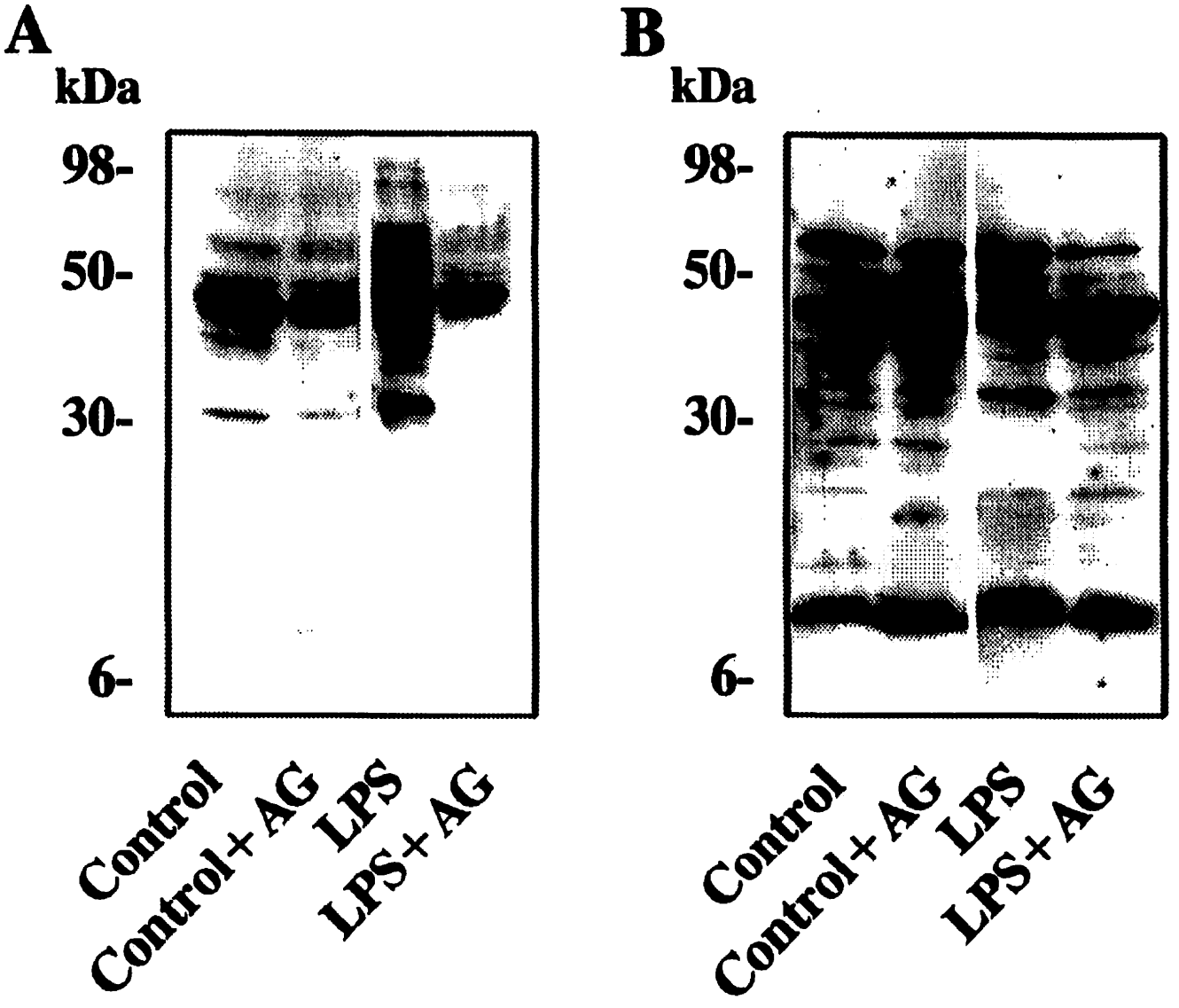


Figure 13. Effect of iNOS inhibition on the levels of carbonyl groups and HNE-bound proteins

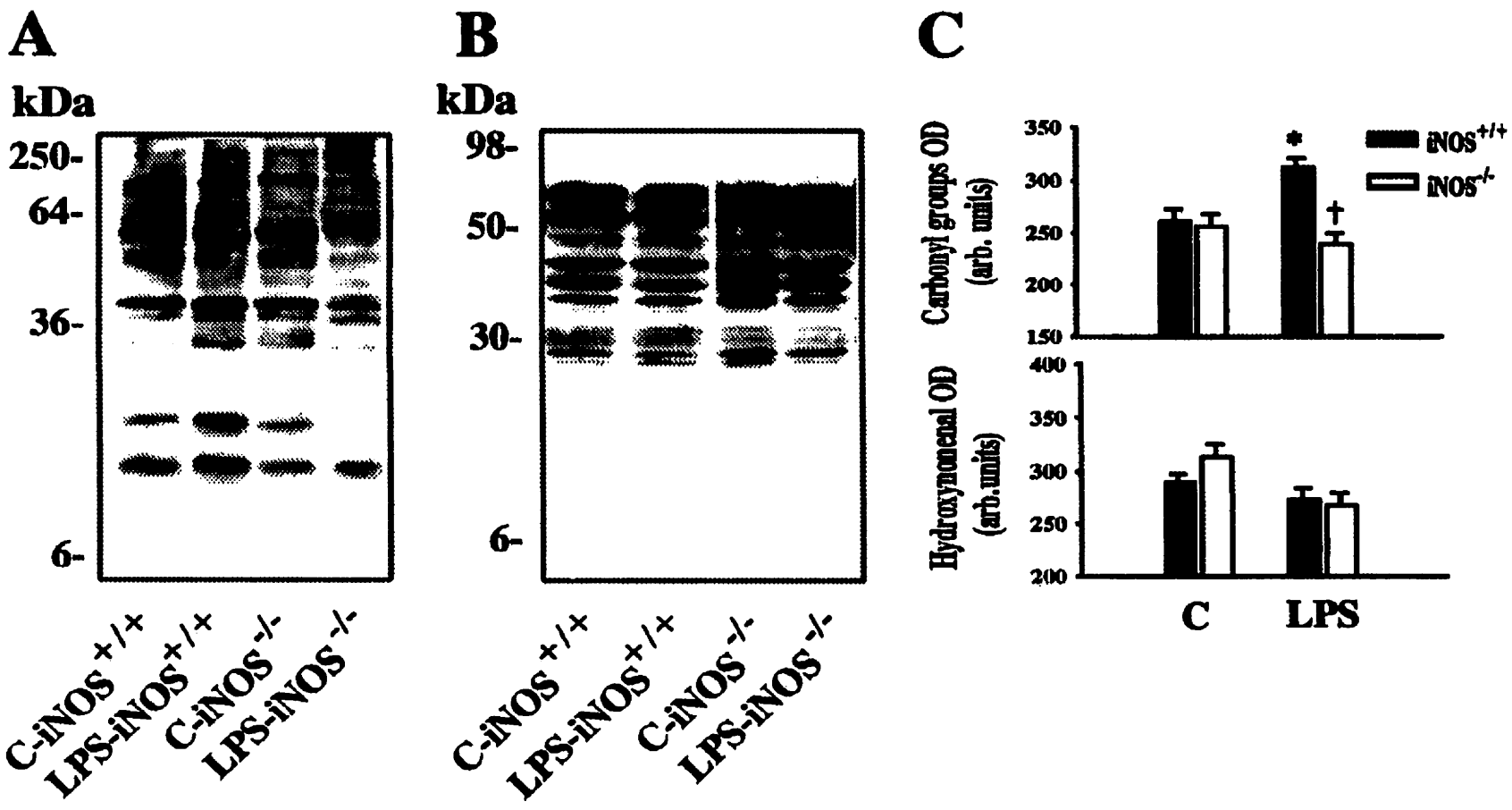


Figure 14. Effect of iNOS inhibition (iNOS knockout mice) on the levels of carbonyl groups and HNE-bound proteins in normal and septic muscles

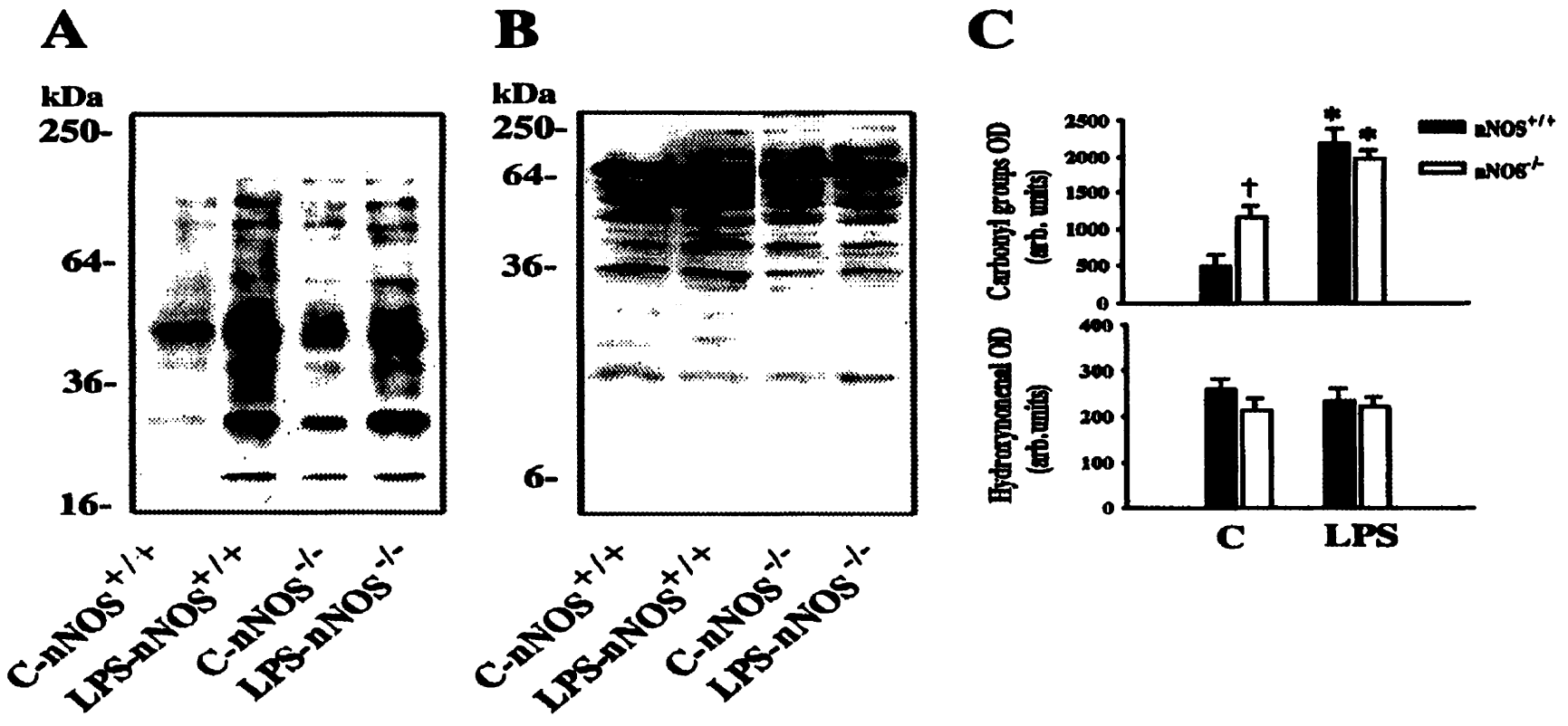


Figure 15. Effect of nNOS inhibition (nNOS knockout mice) on the levels of carbonyl groups and HNE-bound proteins in normal and septic muscles

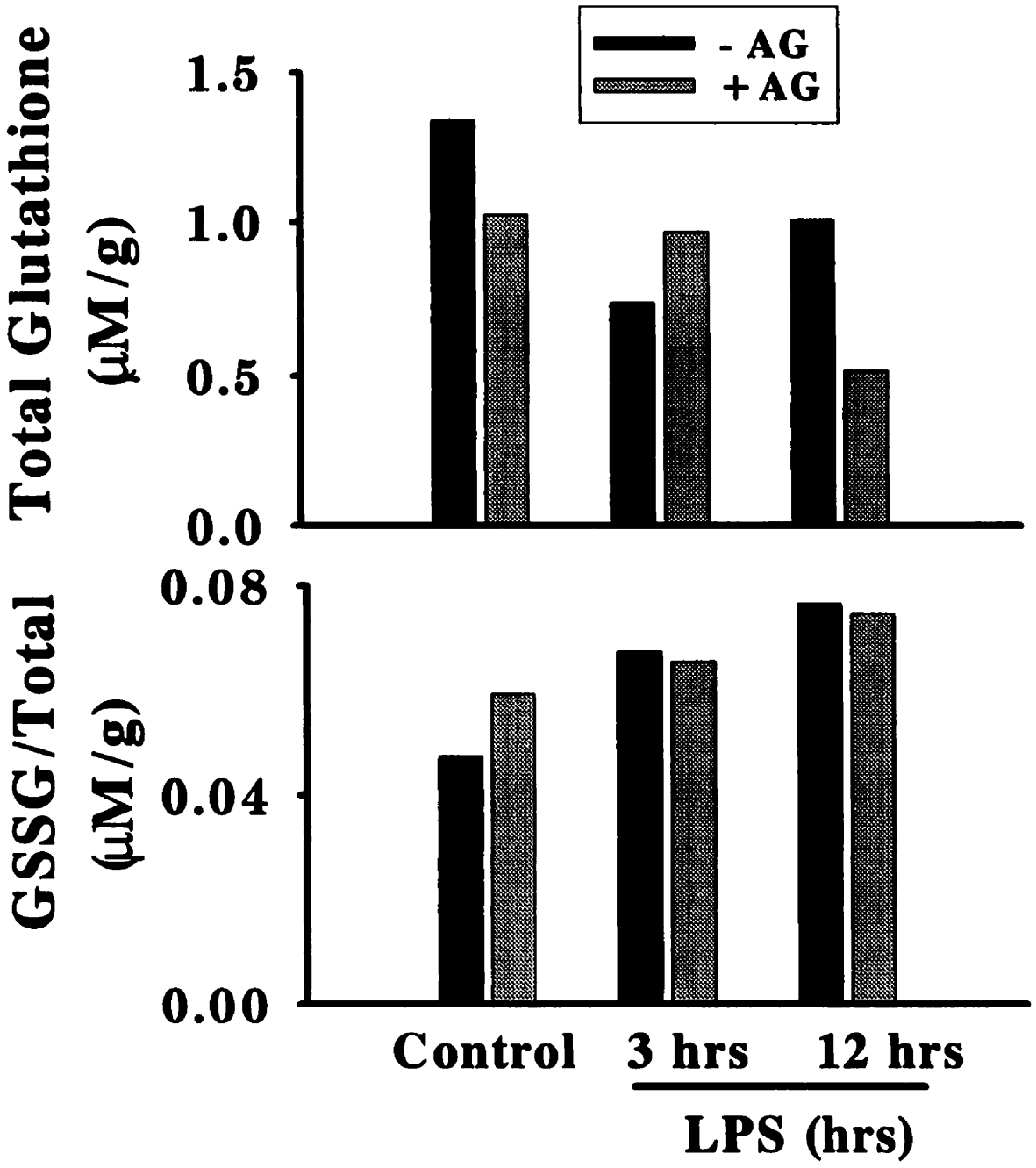


Figure 16. Glutathione concentrations in rat diaphragms

21.- MAIN FINDINGS

21.1- INDICES OF OXIDATIVE STRESS IN RAT DIAPHRAGMS

Sepsis produced a significant rise in total protein oxidation of rat diaphragms, and these oxidized proteins are mainly localized in the mitochondrial and myofibrilar fractions, but not in the cytosolic compartment. Lipid peroxidation occurs in both control and septic rat diaphragms and these HNE-bound proteins are mainly localized in the cytosol. No significant change of this index of oxidative stress was observed in response to sepsis in our experiments. Interestingly, a significant decline in the total diaphragmatic glutathione levels and an increase in the oxidized glutathione fraction were detected during sepsis.

21.2.- ROLE OF NO IN THESE INDICES OF OXIDATIVE STRESS

21.2.1.- ROLE OF iNOS

Sepsis-induced protein oxidation is clearly dependent on the activity of iNOS as demonstrated by using both a pharmacological inhibitor of iNOS and an iNOS knockout mouse model. Particularly, the absence of iNOS greatly reduced the levels of protein oxidation in the septic rat diaphragms, and had little effects on control the rat diaphragms (Aminoguanidine). In contrast, the lack of iNOS during the same type of experiments did not induce any significant modification in the levels of diaphragmatic lipid peroxidation nor in the levels of both total and oxidized glutathione in control and septic rat diaphragms. In summary, the iNOS isoform modulates the levels of protein oxidation in response to sepsis, and has no effects on peroxidation and diaphragmatic glutathione.

21.2.2.- ROLE OF nNOS

The absence of nNOS as demonstrated by using a nNOS knockout mouse model, increased the levels of protein oxidation only in the control rat diaphragms, suggesting that nNOS might have a protective role against protein oxidation in normal skeletal muscles. In contrast, this effect was not detected in the septic rat diaphragms. Finally, the absence of nNOS did not induce any modification in the levels of lipid peroxidation in both control and septic rat diaphragms.

22.- FIGURE LEGENDS

Figure 10: Representative immunoblots showing the time course of carbonyl group formation in rat diaphragms. **A)** homogenates containing carbonyl groups, including control (C) and septic (LPS) animals were derivatized, and the DNP moieties were detected with specific antibody. **B)** Myofibrillar, membrane, and cytosolic fractions of control and septic (LPS) rat diaphragms probed with specific anti-DNP moiety antibody. Note that the majority of oxidized proteins were localized in the myofibrillar and membrane fractions.

Figure 11: Representative immunoblots of HNE-protein adducts of rat diaphragms at different time points of endotoxemia. A) homogenates probed with polyclonal anti- HNE antibody. B) Myofibrillar, membrane, and cytosolic fractions of control and septic rat diaphragms probed with specific anti-HNE-protein adducts antibody. Note that the majority of these HNE-bound protein bands were localized in the cytosolic compartment.

Figure 12: Mean values of total diaphragmatic carbonyl content and HNE-bound proteins of septic rats. Note that total diaphragmatic carbonyl group content was significantly higher after 12 and 24 hrs of endotoxemia. The values from control diaphragms are represented by the 100% line.

Figure 13: Effects of continuous iNOS inhibition on the intensity of protein oxidation (A) and lipid peroxidation (B) in rat diaphragms. Animals were injected with aminoguanidine (AG) 30 min before the sacrifice in the control groups. In the septic groups, animals were injected with aminoguanidine both 30 min before and 6 hrs after the LPS injection.

Figure 14: **A)** Comparison of carbonyl group formation in diaphragmatic samples obtained from both control (C) and septic (LPS) wild type ($iNOS^{+/+}$) mice, and both control and septic iNOS knockout ($iNOS^{-/-}$) mice. **B)** Intensity of HNE-bound proteins in diaphragms obtained from both control an septic wild type and iNOS knockout mice, respectively. **C)** Mean values of total diaphragmatic protein oxidation and lipid peroxidation OD in both control and septic wild type and iNOS knockout mice. *$P<0.05$ septic diaphragms from wild type mice compared with the corresponding control.

Figure 15: Comparison of carbonyl group formation in diaphragmatic samples obtained from both control (C) and septic (LPS) wild type ($nNOS^{+/+}$) mice, and both control and septic nNOS knockout ($nNOS^{-/-}$) mice. **B**) Intensity of HNE-bound proteins in diaphragms obtained from both control an septic wild type and nNOS knockout mice, respectively. **C**) Mean values of total diaphragmatic protein oxidation and lipid peroxidation OD in both control and septic wild type and nNOS knockout mice. *$P<0.05$ both septic groups of mice compared with the corresponding control groups.

Figure 16: Mean values of total diaphragmatic glutathione levels (top panel) and diaphragmatic oxidized fraction (bottom panel). Black bars (- AG) represent both control and septic animals (underlined), whereas iNOS inhibition elicited by aminoguanidine (+ AG) is depicted in grey bars.

ROLE OF HEME OXYGENASES IN THE VENTILATORY MUSCLES : INFLUENCE ON OXIDATIVE STRESS

CHAPTER # 3

So far, we have successfully demonstrated that both nitrosative and oxidative stresses occur in the rat ventilatory muscles in response to sepsis. Our findings are consistent with current published data (For review see General Introduction). We suggest that these two forms of cellular stress might account for most of the sepsis-induced diaphragmatic contractile failure already shown by our group[53] . Finally, our last hypothesis was designed beyond this scope with the intention of assessing whether the expression of various antioxidant enzyme systems, such as the HO pathway, could be upregulated to counteract the deleterious effects of nitrosative and oxidative stresses in the ventilatory muscles. The generation of this hypothesis led us to the organization and design of the next and last study included in this master thesis, which is presented below.

23.- INTRODUCTION

Sepsis-induced respiratory muscle contractile dysfunction appears to be mediated by both nitrosative and oxidative stresses, through the formation of peroxynitrite. The highly ROS is the consequence of the excessive production of NO and superoxide, condition invariably occurring in inflammatory-immune processes. We have demonstrated in the preceding studies that these two phenomena occur in the respiratory muscles of septic animals. However, there is still little evidence about what are the mechanisms utilized by the diaphragm to counteract the deleterious effects of these forms of stress.

HO-1, the rate-limiting enzyme in heme catabolism, has also been shown to be induced in response to non-heme products such as endotoxin[156,166] , nitric oxide[160,161], cytokines[162], and many other conditions which have in common their capacity to produce ROS. Among others, strong skeletal muscle contractions led to an increase in HO-1 mRNA levels, suggesting that this enzyme could have antioxidant effects[184], since strenuous exercise is known to cause muscular oxidative stress. Furthermore, it was also reported that NO donors such as SNP, and hemin are potent inducers of HO-1 mRNA, protein, and activity in skeletal myoblast cells[185]. In another study, it was also shown[194] that peroxynitrite induces the expression of HO-1 in bovine aortic endothelial cells, suggesting that this pathway may provide cytoprotection against the cytotoxic effects of

peroxynitrite[194]. Several recent studies have also shown that endotoxemia leads to the induction of both HO-1 expression and activity. For instance, it was reported that *E. Coli* LPS administration increased levels of HO-1 mRNA and in HO-1 activity in the lungs of endotoxemic rats[187]. On the contrary, tin protoporphyrin, a competitive inhibitor of HO, completely abrogated the protective effects attributed to HO-1 activity. Most recently, Boczkowski *et al* [189] have shown the presence of HO-1 and HO-2 proteins in the septic rat diaphragms, and demonstrated protective effects of the HO on the endotoxin-mediated diaphragmatic dysfunction. In fact, HO-1 is now widely considered to be part of a general response to oxidative stress, which might have significant therapeutical implications[156].

The current study aimed at assessing whether HO-1 is induced in skeletal muscles, including the diaphragm, of experimental animals in response to sepsis. Finally, the biological significance of HO activity in the sepsis-induced diaphragmatic contractile dysfuntion was another of our main goals in this study. To confirm this hypothesis, contractility studies were conducted as part of the experiments shown in this research work. The physiological role of HO was assessed by using a selective inhibitor of HO activity, chromium (III) mesoporphyrin IX (CrMP), which does not inhibit the activity of either NOS or soluble guanylate cyclase at low concentrations as recently shown by Appleton *et al* [230].

24.- SPECIFIC HYPOTHESES

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 also responsible for the sepsis-induced diaphragmatic contractile dysfunction. The induction of HO-1 expression would have a protective role by attenuating the decline in diaphragmatic contractility observed in sepsis.

25.- SPECIFIC OBJECTIVES

25.1.- To determine the levels of expression and localization of both HO-1 and HO-2 in both control and septic rat skeletal muscles.

25.2- To evaluate the role of NO in the regulation of HO-1 expression.

25.3.- To investigate the influence of HO activity on oxidative stress, assessed by the three indices used in the preceding study (carbonyl groups, HNE-bound proteins, and the diaphragmatic glutathione levels) in both control and septic rat diaphragms.

25.4.- To assess the biological significance of HO activity in both control and septic rat diaphragms.

26.- MATERIALS AND METHODS

26.1.- REAGENTS

Both polyclonal anti-HO-1 antibody and pure HO-1 protein were purchased from StressGen Biotechnologies Corp. (Victoria, BC). CrMP was purchased from Porphyrin Products, Inc. (Logan, UT). Monoclonal antibodies for HO-1 was obtained from Transduction Laboratories (Lexington, KY). The rest of reagents used employed in this study have already been described in both sections 12.1 and 19.1.

26.2.- PREPARATION OF THE METALLOPORPHYRIN

Stock solutions of CrMP were prepared according to the protocol described by Vreman *et al* [231]. Briefly, the metalloporphyrin 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 volumen was adjusted to 10 mL in order to obtain a final solution of 650 µM concentration. All animals treated with CrMP received a total dose of 5 µmol CrMP/Kg body weight in one single injection.

26.3- ANIMAL EXPERIMENTS

26.3.1.- COMMON PROCEDURES

Pathogen free Sprague-Dawley rats (250-275 g) were used for some experiments. Besides, in this study both iNOS knockout[207] and the corresponding wild type mice were used to evaluate the contribution of the iNOS isoform to HO-1 induction. More details are explained in section 12.2.1.

26.3.2.- RAT EXPERIMENTS

26.3.2.1.- ANIMAL GROUPS

a) Sepsis: Six groups of rats (n=5 in each group) were studied. Group 1 was injected with normal saline (control group). Groups 2, 3, 4, 5, and 6 were injected with *E.Coli* LPS (20 mg/Kg) and sacrificed after the removal of the diaphragm (previous injection of 30mg/Kg pentobarbital) 1, 3, 6, 12, and 24 hrs after the LPS injection, respectively.

b) NOS inhibiton: A powerful selective iNOS inhibitor, 1400W, was used for this group of experiments. Animals were injected with 1400W (20 mg/kg) 30 min before the E.Coli LPS administration and every 8 hrs thereafter, and were sacrificed 24 hrs after LPS administration.

c) HO-1 inhibition: Six groups of rats (n=5) were established. Group 1 was injected with saline (control group). Group 2 was injected with CrMP (5 μmol/Kg body weight) 1h prior to sacrifice (control group). Groups 3, 4, 5, and 6 were injected with *E.Coli* LPS (20 mg/Kg), and sacrificed 6h (groups 3 and 4) and 24h (groups 5 and 6) after the *E.Coli* LPS injection. Groups 4 and 6 were injected with CrMP 1h before the *E.Coli* LPS injection.

d) Contractility studies: Four groups of rats (n=6) were established. Group 1 was injected with saline (control group) and the animals were sacrificed 24h later. Group 2 animals were injected with CrMP 1h prior to the injection of saline and sacrificed 24h later (control group). Groups 3 and 4 were injected with *E.Coli* LPS (20 mg/Kg) and sacrificied 24h after this injection, and group 4 received an additional injection of CrMP 1h prior to the *E. Coli* LPS injection.

26.3.3.- MOUSE EXPERIMENTS

26.3.3.1.- iNOS KNOCKOUT MICE

A) normal mouse muscles : 2 groups (n=4 in each group) of animals were established, wild type (control-iNOS$^{+/+}$) and iNOS knockout (control-iNOS$^{-/-}$) mice.

B) septic mouse muscles: 6 groups (n=4 in each group) of animals were established. In the group of iNOS$^{+/+}$ (LPS- iNOS$^{+/+}$) animals were injected intraperitoneally with *E.Coli* LPS (20 mg/Kg i.p.) and sacrificed 6h, 12h, and 24h after the LPS injection. The same applies to the group of iNOS$^{-/-}$ (LPS-iNOS$^{-/-}$), animals were injected with *E. Coli* LPS (20 mg/Kg i.p.) and sacrificed at the same time periods afterwards.

26.4.- PROTEIN OXIDATION – CARBONYL GROUP CONTENT

The procedure was described in section 19.4.

26.5.- WESTERN BLOT ANALYSIS

Crude homogenates and derivatized proteins were separated by SDS-polyacrylamide gel electrophoresis as previously shown in sections 12.4 and 19.5. Briefly, in this study different concentrations of pure HO-1 protein were prepared and detected with polyclonal anti-HO-1 antibody. Crude homogenates were probed for both monoclonal anti-HO-1 and anti-HO-2 antibodies, as well as for anti-HNE protein adducts antibody. The DNP-derivatized protein samples were incubated with the corresponding anti-DNP moiety antibody. Different concentrations of pure HO-1 protein were also prepared and detected by immunoblotting with polyclonal anti-HO-1 antibody.

26.6.- IMMUNOHISTOCHEMISTRY

The protocol employed in this study is identical to the one described in section 12.5. Specifically, in this study the tissue sections were incubated with both monoclonal antibodies against HO-1 and HO-2.

26.7.- GLUTATHIONE DETERMINATION

The methodology followed for the determination of diaphragmatic glutathione has already been described in detail in sections: 19.6.1, 19.6.2, and 19.6.3.

26.8.- CONTRACTILITY STUDIES

The protocols employed in these studies have previously been described by our group[53,232].

26.8.1.- DIAPHRAGMATIC STRIP PREPARATION

The diaphragms from the four groups of rats were surgically excised with ribs and central tendon attached and placed in an equilibrated (95%O_2 - 5%CO_2; pH 7.38) Krebs solution chilled at 4°C that had the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1 KH_2PO4, 25 $NaHCO_3$, and 11.0 glucose. From the central tendon to the rib, a muscle strip (2 mm wide) was dissected free 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 the muscle chamber, which was placed vertically in 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).

26.8.2.- FORCE-FREQUENCY MEASUREMENTS

Muscle strips were stimulated electrically at constant currents via platinum electrodes mounted in the muscle chamber and 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 (standard temperature at which the muscle is still alive) and the maximum current necessary to elicit maximum force during 120-Hz stimulation frequency (600 ms duration) was identified. Muscle length was then gradually adjusted with a micrometer to the optimal value at which maximum isometric muscle force was generated in response to supramaximal stimulation (current of 300-350 mA, 120-Hz frequency). This new resting

length was defined as L_0. Muscle force was evaluated by stimulating the muscle at 10, 20, 30, 50, 100, and 120 Hz while maintaining constant supramaximal current and stimulation duration (600 ms). Muscle fatigue was evaluated by continuously stimulating the muscle at 100-Hz during 3 min. Both the force and the fatigue signals were registered by means of a paper recorder (Linseis, L6514B, Princeton, NJ). At the end of the experiment, the strip was blotted dry and weighed. Force was expressed in Newtons/cm^2, and the muscle cross-sectional area was calculated as the ratio of trimmed muscle mass (g) to strip length (cm) X 1.056 g/cm^3 (muscle density)[233] .

26.9.- STATISTICAL ANALYSIS

Differences between various groups were compared by 2-way analysis of variance for repeated measures. Any differences detected were evaluated *post hoc* by the Student Neuman-Keuls test. See section 12.6 for more details.

27.- EXPERIMENTAL RESULTS

27.1.- HO-1 EXPRESSION IN SKELETAL MUSCLES

Different dilutions of pure HO-1 protein were detected at 32kDa (Figure 17A). This result confirmed that HO-1 is a 32 kDa protein and was used as positive control in our experiments. Basal HO-1 expression was detected in both normal ventilatory (diaphragm) and limb (soleus) muscles (Figure 17B). Interestingly, HO-1 expression progressively increased in response to sepsis in both muscles, reaching its peak within 3 to 6h of endotoxemia. At 24 h the expression was again similar to that of control muscles. These results can be more clearly observed in figure 17C, where total HO-1 OD in both muscles from septic animals are illustrated.

Localization of HO-1 in normal and septic muscles is shown in figure 18. Monoclonal anti-HO-1 antibody detected positive staining in gastrocnemius muscles (panels A and B) within the skeletal muscle fiber. Positive HO-1 staining was also detected within the endothelial cells of a blood vessel traversing the muscle (panel C).

Replacement of primary antibody with non-specific mouse IgG completely eliminated positive HO-1 staining (panel D).

27.2.- ROLE OF NOS IN HO-1 EXPRESSION

27.2.1.- PHARMACOLOGICAL iNOS INHIBITION

The administration of a selective iNOS inhibitor (1400W) did not induce any significant change in HO-1 expression in response to sepsis in the diaphragms of septic rats injected only with *E.Coli* LPS (Figure 19A).

27.2.2.- GENETIC INHIBITION – iNOS KNOCKOUT MICE

Both control and septic diaphragms from mice deficient in the iNOS isoform did not show any significant difference in HO-1 expression compared with the wild type group as illustrated in figure 19B.

27.3.- ROLE OF HO ACTIVITY IN DIAPHRAGMATIC OXIDATIVE STRESS

27.3.1.- LEVELS OF PROTEIN OXIDATION

Interestingly, the blockade of HO activity achieved by the administration of CrMP significantly increased the levels of carbonyl groups in both control and septic rat diaphragms (Figure 20A). These findings are more clearly shown in figure 20C (top panel) where total diaphragmatic carbonyl group OD in both control and septic animals are expressed.

27.3.2.- LEVELS OF LIPID PEROXIDATION

The inhibition of HO activity did not elicit any modification in the levels of HNE-bound proteins in both control and septic diaphragms as shown in figure 20B. Figure 20C (bottom panel) illustrates total HNE OD in both control and septic animals.

27.3.3.- MUSCLE GLUTATHIONE LEVELS

Figure 21 shows total levels of glutathione of both control and septic rat diaphragms. The blockade of HO activity achieved by CrMP induced a tendency to increase the levels of total diaphragmatic glutathione only in septic animals.

27.4.- BIOLOGICAL SIGNIFICANCE OF HO ACTIVITY

Figure 22 shows the changes in diaphragmatic force-frequency relationship in the four groups of animals. Injection of LPS elicited a significant decline in diaphragmatic force compared with the control group. The inhibition of HO activity was also associated with a significant reduction in the diaphragmatic force in normal muscles. Interestingly, the blockade of HO activity in septic animals elicited a dramatic reduction in the diaphragmatic force compared with both control and septic diaphragms with intact HO activities. The decline in diaphragmatic force production over time (endurance) during repetitive stimulations (100 Hz) in the four groups of animals was also observed in the 4 groups of animals. Particularly, the blockade of HO activity in septic rats was associated with a greater decline in diaphragmatic force generation over time with respect to both control (saline treated group) and septic rat diaphragms (data not shown).

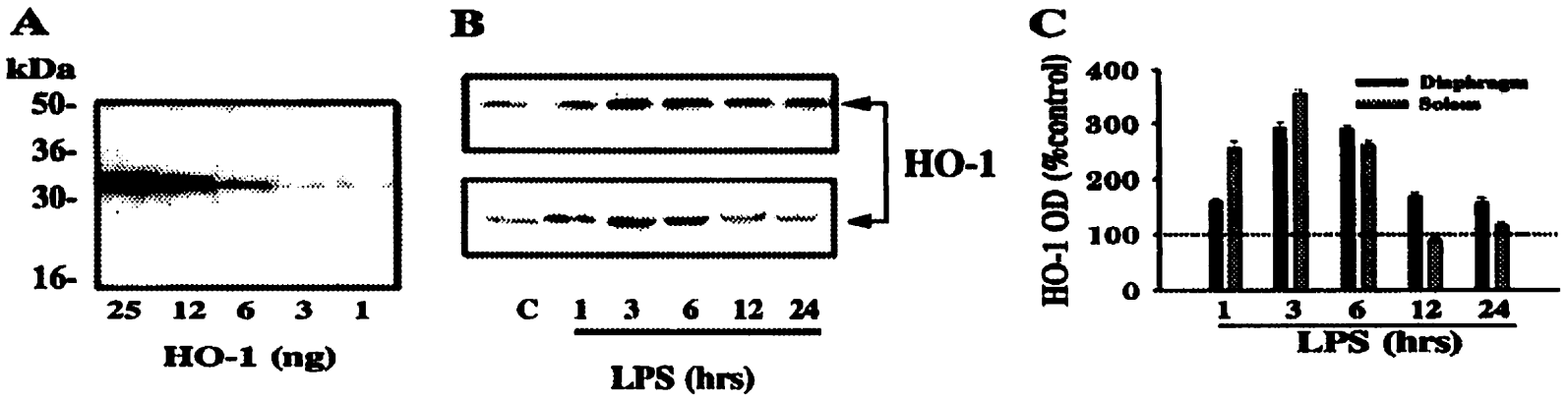


Figure 17. Expression of HO-1 protein in normal and septic rat muscles

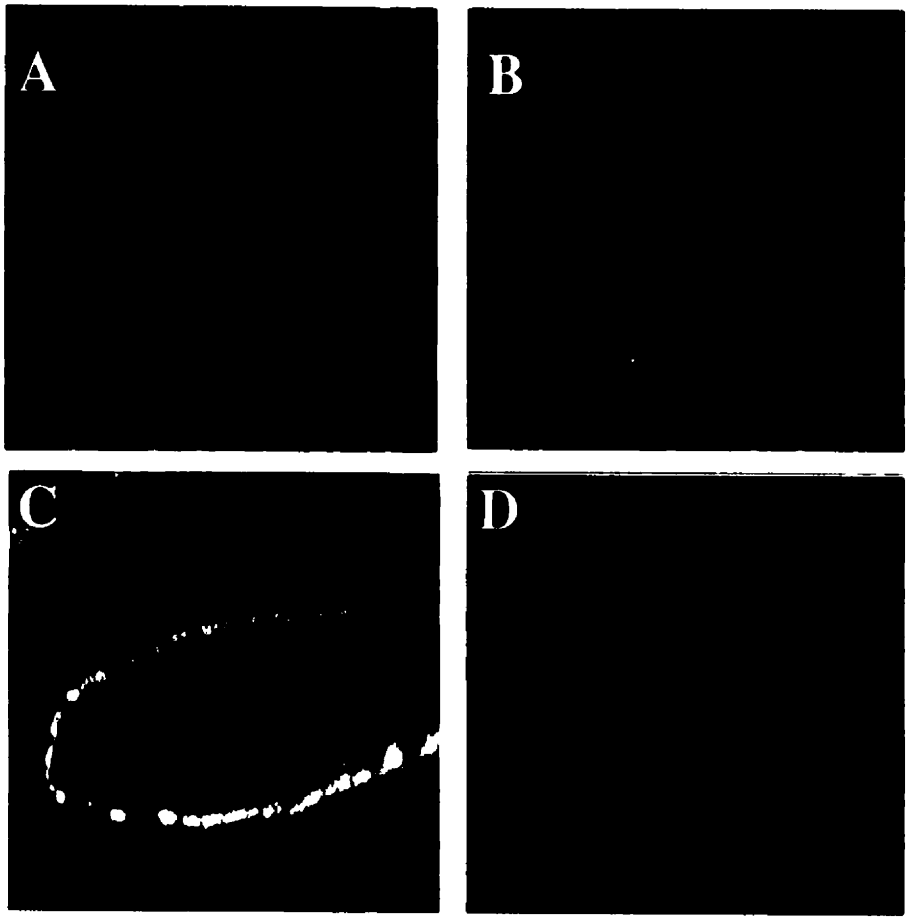


Figure 18. Localization of HO-1 protein in gastrocnemius muscles

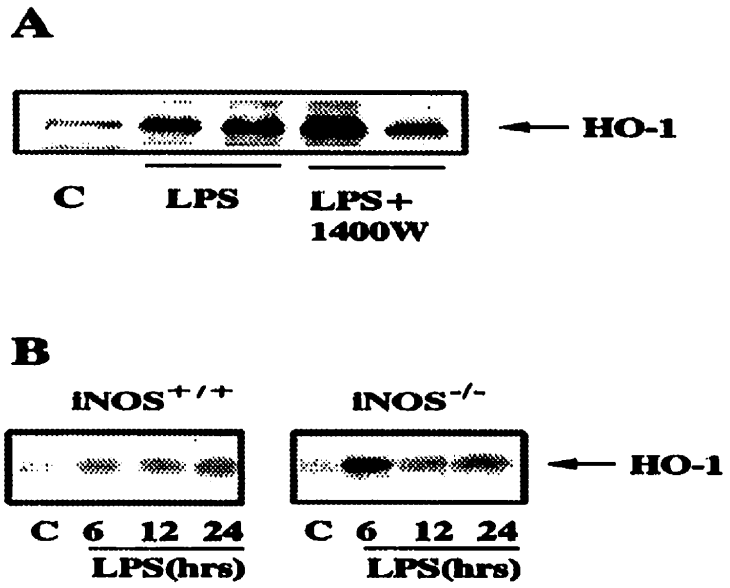


Figure 19. Effect of NOS on HO-1 expression

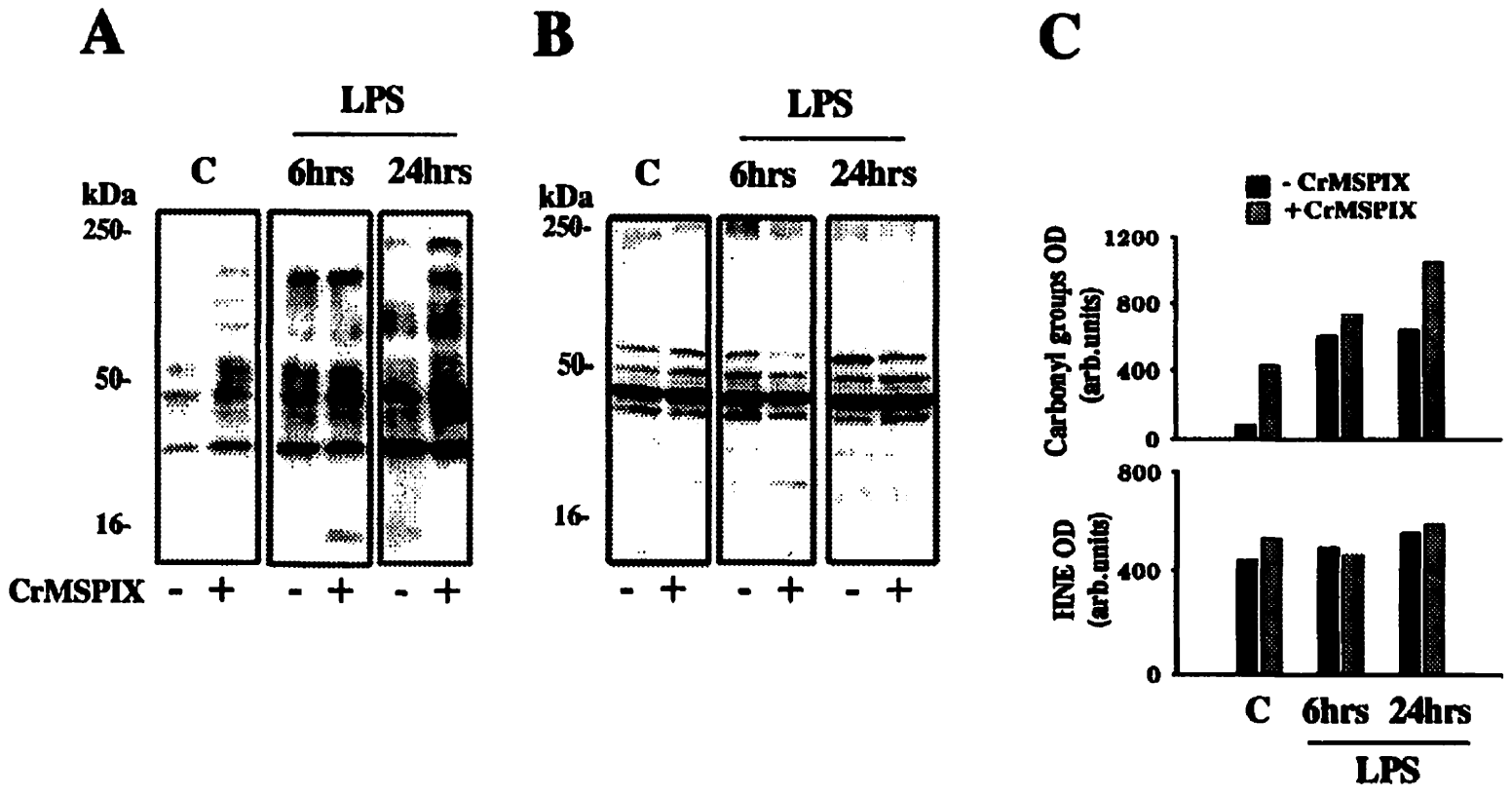


Figure 20. Effect of HO-1 inhibition on the levels of carbonyl groups and HNE-bound proteins in normal and septic rat muscles

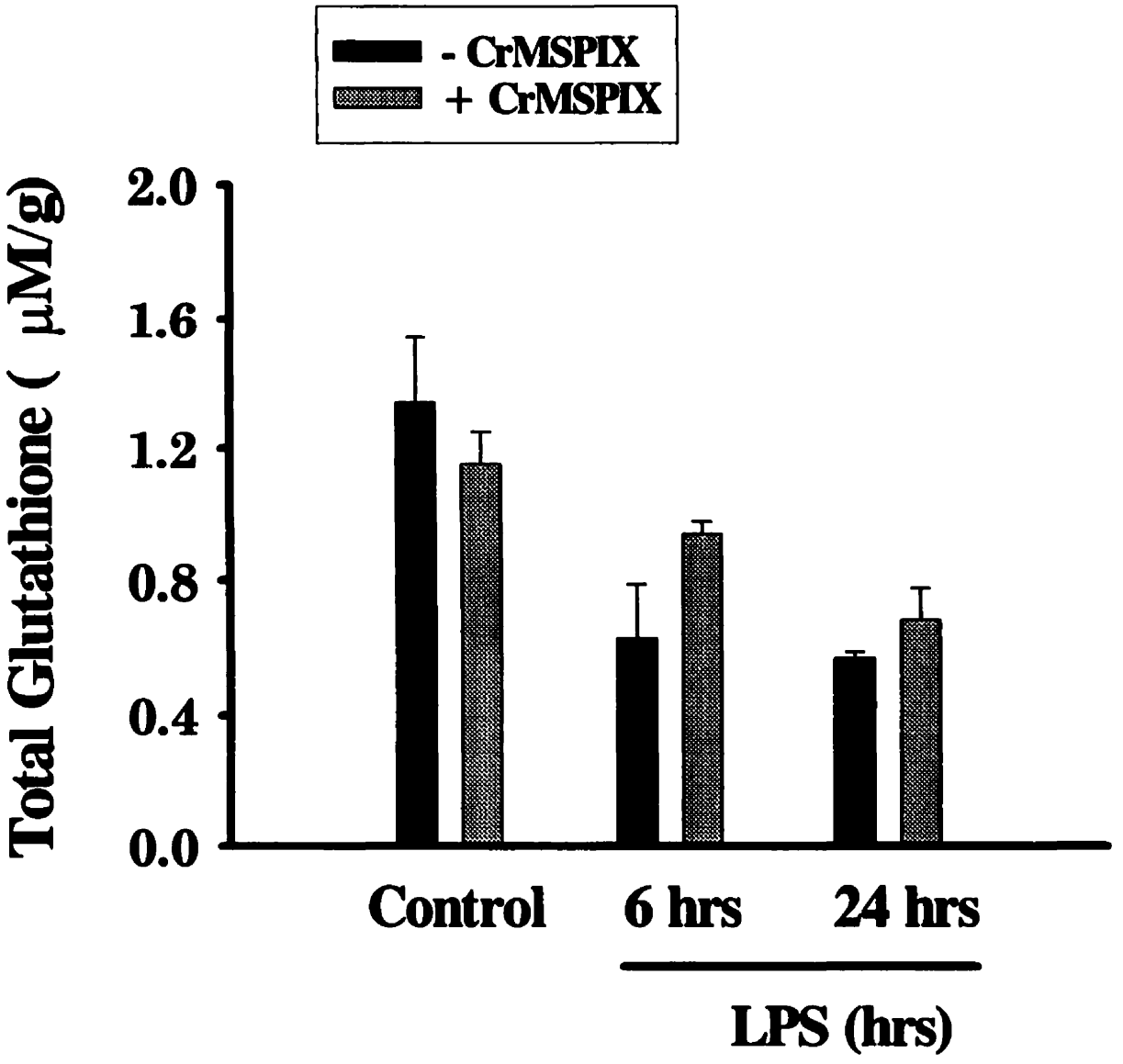


Figure 21. Effect of HO-1 inhibition on diaphragmatic glutathione concentrations

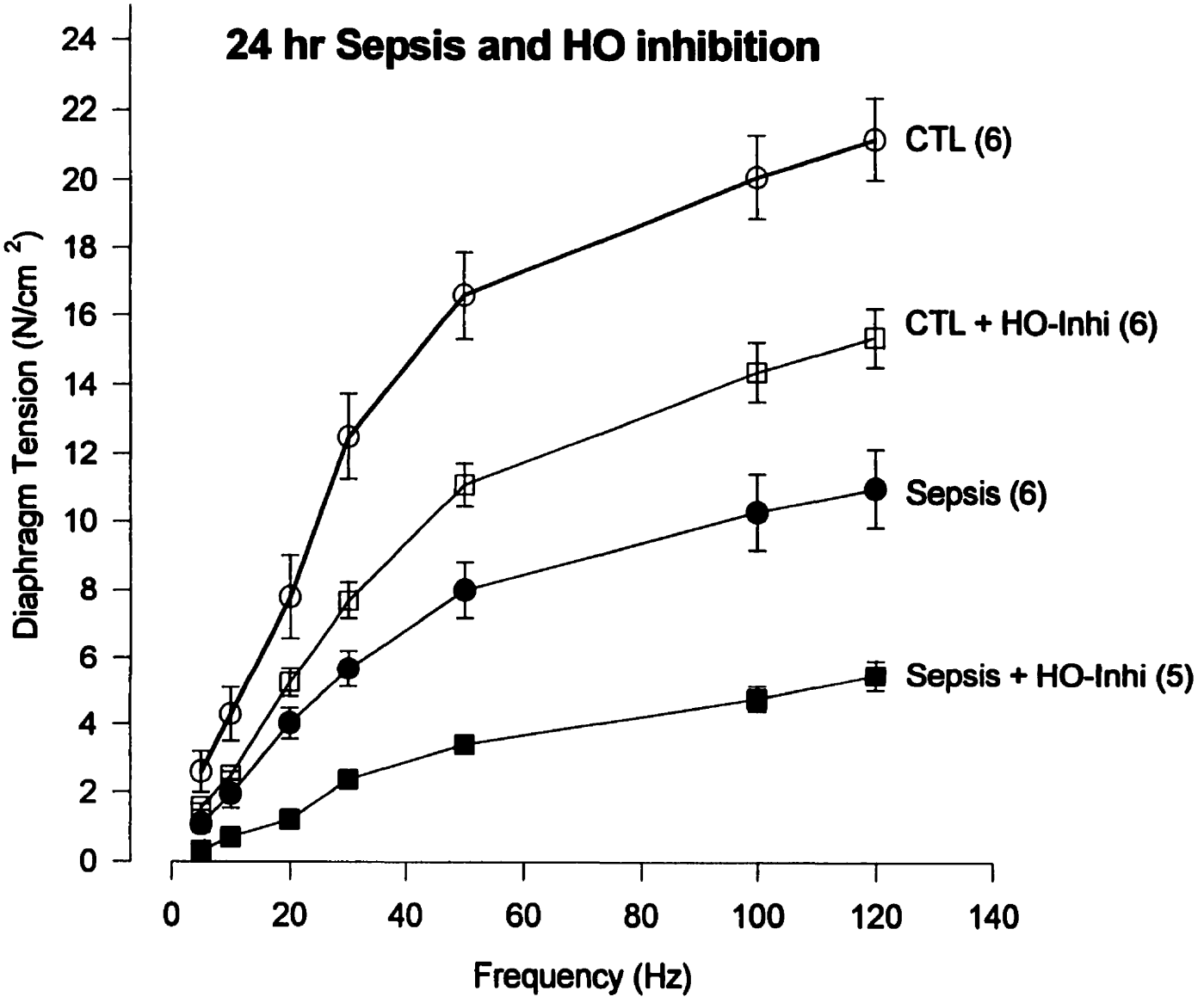


Figure 22. Force-frequency curve of rat diaphragm strips

28.- MAIN FINDINGS

28.1.- HO-1 EXPRESSION IN SKELETAL MUSCLES

HO-1 enzymes was constitutively expressed in normal skeletal muscles of rats. These two isoforms are localized within the muscle fibers as well as in the endothelial cells of the blood vessels traversing the muscle.

Sepsis elicited an increase in HO-1 expression in both the diaphragm and a limb muscle of rats during the first 6 hours after LPS injection. The increase was followed by a decline of the baseline values at 24 h after the injection.

28.2.- ROLE OF NOS IN HO-1 EXPRESSION

HO-1 expression was not dependent on the activity of iNOS as demonstrated by both pharmacological inhibition of iNOS in rats and mice deficient in the iNOS isoform in both control and septic conditions.

28.3.- ROLE OF HO ACTIVITY IN DIAPHRAGMATIC OXIDATIVE STRESS

Our data demonstrated that HO may exert a protective effect on the sepsis-mediated diaphragmatic oxidative stress, as demonstrated by the increase in total levels of protein oxidation in the presence of the HO inhibitor. In contrast, the blockade of HO activities had a tendency to increase the total diaphragmatic glutathione levels in response to sepsis. However, these results are not conclusive, and more experiments are required to confirm these findings.

28.4- BIOLOGICAL SIGNIFICANCE OF HO ACTIVITY

Our results suggest that HO activity has a protective role in the sepsis-mediated diaphragmatic contractile dysfunction, since the blockade of these enzymes elicited a greater loss of force of these muscles in response to sepsis. Interestingly, in this study HO activity has also been shown to exert a protective role in the contractility of the control rat diaphragms.

29.- FIGURE LEGENDS

Figure 17: **A)** Immunoblot of different dilutions (ng) of HO-1 pure protein probed with polyclonal anti-HO-1 antibody. Note that HO-1 expression corresponds to a protein band of 32 kDa. **B)** Time course of HO-1 protein expression in homogenates of two different rat muscles, diaphragm (top panel) and soleus (bottom panel) from control (C) and septic animals (LPS). **C)** Mean values of total OD HO-1 OD in septic rat diaphragms (black bars) and septic rat soleus (grey bars).

Figure 18: Immunohistochemical localization of HO-1 in normal-septic rat muscles. Monoclonal anti-HO-1 antibody detected positive staining in gastrocnemius (panels **A** and **B**) and in endothelial cells of blood vessels (panel **C**). Panel **D** shows negative control staining in which the primary was replaced by non-specific mouse IgG.

Figure 19: **A)** Effects of pharmacological inhibition of iNOS synthesis (1400W) on the intensity of HO-1 expression in control (C) and septic (LPS) rat diaphragms. **B)** Intensitiy of HO-1 expression in diaphragmatic samples obtained from control (C) and septic (LPS) wild type mice ($iNOS^{+/+}$), and iNOS knockout ($iNOS^{-/-}$) mice.

Figure 20: Effects of inhibition of HO activity (+ CrMSPIX) on two indices of oxidative stress, protein oxidation (panel **A**) and lipid peroxidation (panel **B**), in control (C) and septic rat diaphragms (LPS). **C)** Total diaphragmatic OD of carbonyl groups (top panel) and HNE-bound proteins (bottom panel) in the absence and in the presence of HO activity (+CrMSPIX) and (-CrMSPIX), respectively, from both control (C) and septic (LPS) rats.

Figure 21: Total diaphragmatic glutathione levels in the absence and in the presence of HO activity (+CrMSPIX) and (-CrMSPIX), respectively, from both control (C) and septic (LPS) rats.

Figure 22: Effects of HO inhibitor (HO-Inhi) in control-saline injected (CTL +) and LPS combined with HO inhibitor (sepsis + HO-Inhi). Note the significant decline in diaphragmatic force in both control and septic groups of animals pre-treated with the HO inhibitor compared with the respective control (CTL) and septic (Sepsis) groups.

SUMMARY AND FINAL CONCLUSIONS

30.- DISCUSSION AND CONCLUSIONS

30.1.- DISCUSSION

Little information is available regarding NO_2Tyr formation in the ventilatory and limb muscles. Supinski *et al* [200] used dot blotting technique to demonstrate a significant increase in NO_2Tyr formation in the diaphragm of septic rats. Our group recently reported that two protein bands (50 and 42 kDa) are tyrosine nitrated in the normal rat diaphragms and two additional protein bands (196 and 86) are heavily nitrated in the diaphragm of septic rats[53]. In a more recent study, Boczkowski *et al* [201] described tyrosine nitration of a single 105-kDa 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 *et al*[200]), incomplete separation of various proteins (as in our previous study[53]) or measurement of NO_2Tyr formation in only mitochondria (in Boczkowski *et al* [201]) and not in the membrane and cytosol.

The abundant NO_2Tyr formation detected in control rat skeletal muscles was dependent on NO synthesis. Specifically, the iNOS isoform was the major contributor to the development of protein nitration in these muscles as confirmed by using both pharmacological inhibition in rats and mice deficient in the iNOS isoform, whose diaphragms showed lower levels of tyrosine nitration compared with the corresponding wild type mice. Surprisingly, the administration of a selective iNOS inhibitor elicited a reduction of protein nitration in the control rat muscles, which "constitutively" express very low levels of the iNOS isoform[53,58,62]. One likely explanation to account for this finding is that even very low levels of this isoform are sufficient to produce NO, which in turn will elicit protein tyrosine nitration in normal skeletal muscles, since the rate of NO

synthesis by the iNOS isoform is much higher than that of either nNOS or eNOS. However, the observation that $_L$-NAME, a constitutive NOS inhibitor, produced a relatively greater reduction on muscle NO_2Tyr formation levels than 1400W suggests that the constitutive NOS isoforms may also contribute to protein tyrosine nitration in normal muscles. In fact, the administration of $_L$-NAME elicited a significant reduction in protein tyrosine nitration in rat soleus muscle, which is essentially composed by type I (slow-twitch) fibers. Among the constitutive NOS, nNOS is the major contributor to NO synthesis in normal skeletal muscles, and in rats its expression is restricted to the sarcolemma of type II (fast-twitch) skeletal muscle fibers. In contrast, soleus muscles contain abundant mitochondria within the muscle fibers which may express high levels of eNOS. Therefore, we conclude that the reduction in protein tyrosine nitration elicited by the administration of a constitutive NOS inhibitor may be due to the specific inhibition of the eNOS activity in soleus muscles.

A major finding of our study is that muscle NO_2Tyr is limited to specific protein bands ranging in apparent mass between 10 and 66 kDa, though the intensity of these bands in normal muscles and the changes in their intensity in response to inhibition of NOS significantly differed depending on the antibody used. Tyrosine nitration is a selective process. It 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 glutamate in the local environment of the tyrosine residue[195]. Interestingly, tyrosine nitration is not influenced by protein abundance or the abundance of tyrosine residues in a given protein[195].

Several proteins have been identified to be targets for tyrosine nitration including Mn-SOD[202], neurofilament L [203], actin and other cytoskeletal proteins [204], α-tubulin[205], tyrosine hydroxylase[204], lung surfactant protein A and α1-antitrypsin[206] . In most of these proteins, tyrosine nitration leads to changes in both protein structure and function. For instance, nitration of specific tyrosine residues of Mn-SOD or α1-antitrypsin causes a significant inhibition of activity of these proteins[202,206] .

Nitration of c-terminal tyrosine residue in α-tubulin compromises microtubule organization and binding of microtubule-associated proteins[205] . Whether the above-mentioned proteins contribute to the protein nitration detected in normal skeletal muscle

samples remains to be established. However, we can exclude certain proteins such as Mn-SOD, and α-actin because these proteins are present in the mitochondrial and myofiber fractions, respectively, whereas tyrosine nitration in normal muscle samples is limited to the cytosolic fraction. Other 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. By comparison, it is possible that a few nitrated protein bands in this study are actually α-tubulin, F-actin and other cytoskeletal proteins.

Another major finding in this study is that the injection of *E. Coli* LPS resulted in augmentation of muscle NO_2Tyr formation particularly in the mitochondrial and membrane fractions and was largely dependent on muscle iNOS activity.

Our results also indicate that LPS-induced protein nitration is mediated primarily by the iNOS isoform, but not by the nNOS isoform despite the fact that nNOS protein expression significantly rose in response to *E. Coli* LPS injection (Figure 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[25]. The identity of peroxynitrite as the reaction product of NO and superoxide anions, as well as the ability of peroxynitrite to nitrate tyrosine residues at physiological pH has recently been confirmed by Reiter *et al* [234]. 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[196], and oxidation of NO_2^- by H_2O_2 at physiological pH which can result in the formation of peroxynitrous acid and consequently leads to nitration of tyrosine residues[195]. 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 (> 1mM), and normal skeletal muscle H_2O_2 levels are quite small[235]. Finally, it has recently been

proposed that myeloperoxidase (MPO), utilizing both NO_2^- and H_2O_2, is capable of nitrating tyrosine residues [236]. 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 hr to produce tyrosine nitration [237].

The development of oxidative stress in the ventilatory muscles in response to sepsis, most probably mediated by peroxynitrite formation, has been confirmed in this 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. cardiovascular diseases and others[238] . Recent evidence has demonstrated that cytokines, such as TNF-α which is a mediator of the ventilatory muscle failure described in sepsis, also stimulates the production of ROS during *E. Coli* endotoxemia[239]. Skeletal muscle may be a major site of oxidation-induced tissue damage in sepsis, since it constitutes the major protein pool within the body. Several lines of evidence have confirmed this hypothesis by showing increased levels of ROS in skeletal muscles of septic animals[148-150].

In our study, total levels of protein oxidation in the rat ventilatory muscles were increased in response to sepsis, and these oxidized proteins were mainly localized in both the mitochondrial and myofibrilar muscle fractions. Our results are similar to those published by Fagan *et al* [210] who found that limb skeletal muscle proteins from septic rats contained significantly more carbonyl groups than muscle proteins from control animals. These authors also reported that the oxidation of sarcoplasmic and myofibrilar proteins occurred as early as 8 h after the induction of sepsis by cecal ligation puncture[210]. This last finding is also consistent with the increase in diaphragmatic protein oxidation levels detected in our study after 6 to 12 h of the *E. Coli* LPS injection.

It has been shown using *in vitro* systems that oxidatively damaged proteins are rapidly degraded by purified proteases[240]. Fagan *et al* [210] also confirmed in their study that limb skeletal muscle proteins from septic animals were rapidly degraded in the absence of energy, leading to the notion that an ATP-independent proteolytic pathway might play a role in the degradation and muscle wasting observed in septic patients[210]. Though, it

could also be argued from these findings that oxidatively damaged proteins, which are mainly localized in the myofibrilar and membrane (SR) compartments of the rat diaphragms, may cause alterations in the normal muscle excitation-contraction coupling, which in turn would contribute to the sepsis-induced skeletal muscle contractile dysfunction.

In our study, sepsis-induced protein oxidation depended on the activity of iNOS, as supported by the results obtained after the treatment of rats with a selective iNOS inhibitor and in an iNOS knockout mouse model. We conclude on the basis of these results that excessive production of NO, mainly by the iNOS isoform along with the generation of superoxide, within the skeletal muscle fibers in response to sepsis may contribute to the formation of the powerful oxidant species peroxynitrite, which in turn will lead to skeletal muscle protein oxidation. Therefore, the nNOS isoform appeared to have a protective role in protein oxidation of the control rat diaphragms. We hypothesized that this isoform might have a cytoprotective effect in normal muscles by scavenging superoxide anions. Though, more experiments are required to further investigate the role of nNOS in counteracting protein oxidation in normal skeletal muscles.

Despite the existing evidence that lipid peroxidation significantly increases in septic skeletal muscles compared with control animals[13,148,200,211,241] , we did not detect any significant change in the levels of HNE-bound proteins in response to endotoxemia. Particularly, lipid peroxidation was shown to occur in both normal and septic rat diaphragms, and sepsis did not induce any modification in the expression of this phenomenon in our rat diaphragmatic samples. Furthermore, these HNE-bound proteins were mainly localized in the cytosolic muscle fraction. Neither iNOS nor nNOS activities were implicated in the development of lipid peroxidation in the rat diaphragms. We think that the various conditions employed to induce sepsis as well as the methods used to detect the levels of lipid peroxidation may account for the discrepancies found among the different studies.

It has been well established that a decline in reduced glutathione (GSH) and an increase in oxidized glutathione (GSSG) levels are good markers of oxidative stress in the ventilatory muscles during loaded breathing[126,127,225,226]. Based on this assumption we assessed the levels of both total and oxidized glutathione in the diaphragms of control and

septic animals. Interestingly, our findings confirmed the published data from resistive breathing animal models. Total glutathione was significantly reduced in the rat diaphragms within the course of sepsis, whereas the oxidized fraction was increased in these muscles. The modifications in muscle glutathione levels were not dependent on iNOS activity. Furthermore, HO activity did not appear to have a biological effect on diaphragm glutathione metabolism. Though, more experiments should be conducted in order to confirm these findings.

It has recently been shown that total glutathione concentrations (GSH and GSSG) were significantly higher in gastrocnemius muscles of rats injected with live *E.Coli* bacteria[242]. The authors concluded that the enhanced muscle glutathione concentrations might be explained by an increase in the requirements for antioxidants to counterbalance the oxidative stress induced by sepsis. However, in our study, animals were observed for a shorter period of time (12 and 24 h) compared with the observation time-period of endotoxemia (48 h) selected by Malmezat and colleagues[242]. Therefore, the discrepancies found between these two studies might be explained by the differences in the observation time-periods used in each experimental design.

Most recently, the same group of investigators[213] has shown that glutathione metabolism increased during the acute phase of sepsis in rats, and that glutathione synthesis accounts for 40% of the increased cysteine utilization during infection. These authors concluded that the increased requirements for cysteine identified in the course of infection might be due to the augmentation of glutathione metabolism to counterbalance the deleterious effects of ROS on intracellular structures. On the basis of these and our finding, we hypothesize that the utilization of muscle glutathione reservoirs to rapidly counteract the harmful effects of oxidants within the muscle fibers may account for the reduction in total muscle glutathione levels detected in the early phases of sepsis. In contrast, new synthesis of glutathione by either the liver or other tissues may lead to an increase in the levels of total muscle glutathione in the late phases of septic shock.

Finally, in the last study we have demonstrated that both HO-1 and HO-2 are constitutively expressed in normal rat skeletal muscles as well as in the endothelial cells traversing those muscles. Specifically, the expression of HO-1 also increased in response to sepsis. In line with our findings, Boczkowski and colleagues[189] have also shown an

increase in HO-1 protein expression in response to sepsis, but in their study this increase was detected after 24 h of LPS injection. They observed as well that HO-1 protein returned to basal levels 6 days after endotoxin administration. Furthermore, the late increase in HO-1 expression observed in this study coincided with the increase in HO-1 activity. We claim that both experimental conditions (different doses of LPS) and the different observation time periods selected in each study might account for the discrepancies found between the two studies.

Our data suggest that HO-1 protein expression was not associated with the activity of iNOS, as demonstrated by using both a powerful pharmacological inhibitor and an iNOS knockout mouse model. This finding led us to the notion that in certain conditions the upregulation of the genes of both inducible enzymes, which has been shown to share common stimulators, may occur independently from each other[243].

Another interesting finding of this study is that sepsis-induced protein oxidation is dependent on the activity of HO. One likely explanation to account for this discovery is that HO activity through its by-products (biliverdin, CO, and ferritin) would scavenge peroxynitrite, which in turn would lead to a decline in the levels of protein oxidation. Therefore we suggest that this mechanism may protect skeletal muscle against sepsis-induced oxidative stress by reducing the levels of protein oxidation, and this reduction could be mediated by the by-products of HO activity. 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* [244,245] . Ferritin, another by-product of HO catabolism of heme, has recently been shown to induce protection against cellular damage[246], 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[178]. Though, the precise sites of CO effects in skeletal muscle still remain unexplored. The most remarkable finding of this study is the demonstration that HO activity has an extremely important biological significance on the sepsis-induced diaphragmatic dysfunction. Moreover, the blockade of HO activity further reduced both diaphragmatic force and endurance in response to sepsis. This finding is consistent with what recently was reported by Boczkowski *et al*[189]. The systemic inhibition of HO activity elicited a

significant reduction in these diaphragmatic contractile parameters in the control rat diaphragms. In line with what stated in the foregoing paragraph, we conclude from these two findings that heme oxygenases exert major antioxidant effects and protect the skeletal muscle machinery from the deleterious effects of excessive ROS production in both control and septic animals. We claim that the antioxidant effects exerted by the by-products of HO-1 on skeletal muscle may take place at different ROS-sensitive sites as shown in figure 1. In this regard, HO-1 may reduce the sepsis-induced muscle contractility dysfunction by either facilitating the SR calcium release, improving the sarcolemma function, or enhancing myofilament contraction.

30.2.- FINAL CONCLUSIONS AND FUTURE CONSIDERATIONS

In summary, we have demonstrated and extensively documented that both nitrosative and oxidative stresses occur in the ventilatory muscles of septic animals. The iNOS isoform appears to be the major regulator of these two forms of skeletal muscle stresses, most likely through the formation of the highly ROS, peroxynitrite. Total muscle glutathione levels are decreased while the oxidized fraction is increased in septic rat diaphragms. Finally, HO exert major antioxidant effects and protection from the sepsis-induced diaphragmatic contractile dysfunction by reducing the levels of protein oxidation in the skeletal muscles of septic animals.

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. The identification of the oxidized proteins localized in the membrane (SR) and myofibrilar fractions, which might be implicated in the skeletal muscle contractile machinery, also needs to be further elucidated. More experiments are required to further investigate the role of nNOS in counteracting protein oxidation in normal skeletal muscles. Finally, the development of therapeutical approaches specifically designed to upregulate HO-1 expression might be hopeful strategies for precluding sepsis-induced respiratory failure.

30.3.- CLAIMS TO ORIGINAL RESEARCH

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 localized in the cytosol of the skeletal muscle fibers in close proximity to the sarcolemma. Interestingly, in this study it is also shown for the first time that nitrotyrosine formation is dependent on both the constitutive and the inducible isoforms in normal muscles, whereas the inducible isoform is the major modulator of protein nitration in sepsis. Another original finding demonstrated in this study is that sepsis leads to an increase in tyrosine nitration of proteins localized in the mitochondrial and myofibrilar fractions of rat diaphragms, but not of those localized in the cystosol.

With regard to the second study, we have first demonstrated in our experiments that sepsis-induced skeletal muscle protein oxidation, which mainly occurs in the myofibrilar and membrane compartments of the rat diaphragms, is modulated by the activity of the iNOS isoform. We also provide first report of both a decrease in total diaphragmatic glutathione concentrations and an increase in the oxidized fraction within acute and subacute endotoxemia in rats.

In relation to the third and last study presented herein we have to acknowledge that the recent work published by Boczkowski and colleagues[189] shows various similar findings to those described in this thesis. Both studies demonstrate that HO 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.

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