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ROLE OF NITRIC OXIDE AND ITS INTERACTION WITH SUPEROXIDE IN PORCINE MODEL OF SEPTIC SHOCK

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Abstract

Although nitric oxide (NO) from inducible nitric oxide synthase (NOSII) is proposed to be the major factor in the vascular abnormalities of sepsis in rats, its role in higher order species is not well established. This thesis thus, addresses the role of NO in septic pigs. I also studied the interaction of NO with superoxide (O_2^-) because of the important modulating effect that O_2^- has on NO. I chose pigs because their sensitivity to lipopolysaccharide (LPS) and the pattern of changes in blood pressure, cardiac output, and systemic vascular resistance, are closer to the human response than what is observed in rats and mice.

I first hypothesized that induction of NOSII in pigs is the cause of sepsis-induced hypotension as occurs in rodents. To test this, I treated pigs with LPS to simulate sepsis. In contrast to what is observed in rats and mice, plasma nitrite did not change during the 4 hours of the LPS infusion and there was no increase in calcium (Ca)-independent nitric oxide synthase (NOS) activity in lung tissue from endotoxemic pigs compared to control animals. Furthermore, there was only minimal induction of NOSII mRNA. However, there was increased nitrotyrosine formation suggesting formation of peroxynitrite as observed in endotoxic rats. Thus, NOSII does not play a major role in the vascular dysfunction of the early phase of hyperdynamic sepsis in pigs, but NO from other sources may still have a role.

Although the production of NO from endothelial NOS (NOSIII) in response to LPS is decreased in septic rats, there are inconsistent results in other experimental models. My second hypothesis was that NOSIII is not down regulated in septic pigs, and NO from NOSIII, could contribute to peroxynitrite formation. In support of this, western

blot analysis of samples from aorta and vena cava showed no down regulation of NOSIII. There also was an increase in Ca^{++}-dependent NOS activity of aorta and vena cava after 2-hour of endotoxemia, which indicates increased constitutive NOS activity. Morover, there was an increase in NOSI in the vena cava. LPS produced a leftward shift in the dose- response curves of SVR and RVR in response to the NOS inhibitor, L-NAME (N^G-nitro-L-arginine-methyl-ester). This increased sensitivity of endotoxemic pigs to NOS inhibition supports the evidence of increased NOS activity in the vessels of endotoxemic pigs.

Peroxynitrite is formed from NO and superoxide (O_2^-). Since NO was not increased in endotoxemic pigs, I next hypothesized that O_2^- is increased in endotoxemic pigs and this reacts with NO from constitutive NOS to form peroxynitrite. To test this hypothesis, I first studied rats to confirm that O_2^- is increased in arterial vessels of endotoxic rats. There was a small increase in basal O_2^- in endotoxic rats. Stimulation of NAD(P)H oxidase by giving the substrate, NADH, did not result in different O_2^- production in control and endotoxic rats. However, inhibition of NOS increased O_2^- in endotoxic rats. This indicates an interaction between NO production and O_2^- in endotoxic rats. In septic pigs, the basal level of O_2^- also was increased. However, in contrast to rats, NADH increased O_2^- production significantly more in arterial rings of endotoxic pigs compared to control pigs. Moreover, L-NAME decreased O_2^- production in NADH-treated arterial rings of endotoxemic pigs. These results suggest increased NADPH oxidase activity in arterial vessels of endotoxemic pigs is NOS-dependent. Furthermore, we observed an increase in luminol chemiluninesence in arterial rings of endotoxemic pigs, which also supports the increased production of peroxynitrite.

Skeletal muscle force is decreased in sepsis, and superoxide dismutase alleviates this decrease, indicating a role for $O_2^{\cdot}$. However, the source of $O_2^{\cdot}$ generation is not known in skeletal muscles. I hypothesized that NAD(P)H oxidase in skeletal muscle could be responsible. I demonstrated by immunohistochemistry and western blot analysis, that the componenets of NAD(P)H oxidase, gp91 ^{phox}, p22 ^{phox}, and p47 ^{phox} are present in skeletal muscle and it has similar behavior to other non-phagocytic NADPH oxidases. The addition of L-NAME to NADH-treated diaphragm strips of endotoxic rats, increased $O_2^{\cdot}$ production in endotoxic rats indicating that $O_2^{\cdot}$ production counters the increased NO formation as we observed in rat aorta. These observations indicate that $O_2^{\cdot}$ generation from NADPH oxidase in skeletal muscles can play a role in the pathophysiology of sepsis.

Résumé

Bien que la production de monoxyde d'azote (NO) par la NO synthase inductible (NOSII) soit proposée comme un facteur majeur intervenant dans les anomalies vasculaires chez le rat septique, son rôle chez les espèces supérieures n'est pas bien défini. Ainsi, cette thèse étudie le rôle du NO chez les cochons septiques, ainsi que les interactions entre la voie du NO et l'anion superoxide O_2^-. J'ai choisi d'étudier le cochon vu sa sensibilité au lipopolysaccharide (LPS). De plus, chez le cochon, les changements survenant dans la pression artérielle, le débit cardiaque et les résistances vasculaires systémiques, reflètent mieux le sepsis humain que les modèles murins.

Premièrement, j'ai émis l'hypothèse que l'induction de la NOSII chez le cochon est la cause de l'hypotension induite par le sepsis, comme chez le rongeur. Pour vérifier cette hypothèse, j'ai traité des cochons avec du LPS pour simuler le choc septique. Contrairement à ce qui observé chez le rat et la souris, la concentration de nitrite dans le plasma n'a pas changé durant les 4 heures d'infusion de LPS. De plus, il n'y a pas eu d'augmentation de l'activité de la NOS calcium (Ca^{++})-indépendante (NOS) dans les tissus pulmonaires des cochons endotoxémiques par rapport aux contrôles. Il n'y avait par ailleurs qu'une induction minimale de l'ARN messager de la NOSII. Par contre, il y avait une augmentation de synthèse de nitrotyrosine, suggérant la formation de peroxynitrite, comme chez les rats endotoxémiques. Ainsi, NOSII ne joue pas un rôle majeur dans l'insuffisance vasculaire lors de la première phase du sepsis hyperdynamique chez le cochon, mais le NO provenant d'autres sources pourrait néanmoins jouer un rôle.

Alors que la production de NO par la NOSIII endothéliale diminue chez le rat septique, d'autres modèles expérimentaux montrent des résultats contradictoires. Ma seconde hypothèse était que la NOSIII n'est pas inhibée chez le cochon septique, et que le NO produit par la NOSIII pourrait contribuer à la formation de peroxynitrite. Conformément à cette théorie, des "western blot" effectués sur des échantillons d'aortes et de veines caves n'ont pas démontré d'inhibition de la NOSIII. De plus, l'activité de la NOS Ca^{++}-dépendante a augmenté dans l'aorte et la veine cave après 2 heures d'endotoxémie, indiquant une augmentation de l'activité de la NOS constitutive. Par ailleurs, il y avait aussi une augmentation de la NOSI dans la veine cave. Le LPS a induit une déviation vers la gauche des courbes dose-réponse de SVR et de RVR au L-NAME, un inhibiteur des NOS. Cette plus grande sensibilité à l'inhibition du NO chez le cochon endotoxémique confirme l'augmentation d'activité de la NOS dans les vaisseaux de cochons endotoxémiques.

Le peroxynitrite est produit par la réaction entre NO et O_2^-. Vu que le NO n'augmente pas chez le cochon endotoxémique, j'ai émis l'hypothèse que O_2^- augmente chez le cochon endotoxémique et réagit avec le NO provenant de la NOS constitutive, pour former le peroxynitrite. Pour vérifier cette hypothèse, j'ai d'abord étudié des rats, afin de confirmer l'augmentation de O_2^- dans les vaisseaux artériels des rats endotoxémiques. Il y a eu une légère augmentation du niveau basal d'O_2^- chez le rat endotoxémique. La stimulation de la NADPH oxydase par son substrat NADH n'a pas modifié la production d'O_2^-, pas plus que chez les rats contrôles. Par contre, l'inhibition de la NOS a augmenté l'O_2^- chez les rats endotoxémiques. Ceci démontre une interaction entre O_2^- et la production de NO chez les rats endotoxémiques. Chez les cochons septiques, le niveau

basal de O_2^- a aussi augmenté. Contrairement aux rats, NADH a augmenté la production d'O_2^- de manière significative dans les parois artérielles des cochons endotoxémiques, comparativement aux contrôles. De plus, chez les cochons endotoxémiques, L-NAME a réduit la production d'O_2^- dans les parois artérielles traitées avec NADH. Ces résultats suggèrent que l'augmentation de l'activité de la NADPH oxydase dans les vaisseaux artériels des cochons endotoxémiques dépend du NO. Nous avons aussi observé une augmentation de chémiluminescence dans les parois artérielles des cochons endotoxémiques, ce qui confirme l'augmentation de la production de peroxynitrite.

La force développée par le muscle squelettique est diminuée lors du sepsis, et la superoxyde dismutase réduit cette diminution, indiquant un rôle pour O_2^-. Par contre, la source d'O_2^- dans les muscles squelettiques n'est pas connue. Mon hypothèse est que la NADPH oxydase dans les muscles squelettiques pourrait en etre responsable. J'ai démontré par immunohistochimie et par "western blot" la présence de composants de la NADPH oxydase, $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ dans les muscles squelettiques, similaires aux autres NADPH oxydases non phagocytaires. L'addition de L-NAME aux échantillons de diaphragmes traités avec du NADH chez les rats endotoxiques a augmenté la production d'O_2^- chez ces derniers, indiquant que la production d' O_2^- a inhibé la formation de NO, comme dans l'aorte de rat. Ces observations démontrent que la synthèse d'O_2^- à partir de la NADPH oxydase dans les muscles squelettiques peut jouer un rôle dans la physiopathologie du sepsis.

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ABBREVIATION

3'UTR	3' untranslated region
Akt	protein kinase B
ANOVA	analysis of variance
AP-1	activating protein-1
ATP	adenosine 5'-triphosphate
BAEC	bovine aortic endothelial cell
BH4	tetrahydrobiopterine
BSA	bovine serum albumine
CAPON	nNOS adaptor protein
CD14	LPS receptor, receptor for endotoxin
cDNA	complementary DNA
CGMP	cyclic guanosine monophosphate
CO	cardiac output
Co	cobalt
COS-7cells	monkey kidney fibroblast
C-rel	transcription factor nuclear factor kappa-B (a member from Rel family proteins)
Cu	copper
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)
eNOS	endothelial nitric oxide synthae
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
Fe	iron
FMN	flavin adenine mononucleotide
GC	guanylate cyclase
GFP	green fluorescent protein
$Gp91^{phox}$	91 KDa glycoprotein of phagocytic NAD(P)H oxidase
GPIIb/IIIa	glycoprotein IIb/IIIa
GRE	glucocorticoid responsive elements
H_2O_2	hydrogen peroxide
Hb	hemoglobin
HEPES	N-2-hydroxyethylpip[erazine-N'-2 Ethane sulfonic acid
HSP90	heat shock protein
HUVEC	human umbilical vein endothelial cells
I-κB	inhibitory protein for transcription factor NF-κB
IFN-γ	interferon - gamma
IL-10	interlukin-10

IL-2	interlukin-2
IL-4	interlukin -4
IL-8	interlukin-8
iNOS	inducible nitric oxide synthase
IP_3	inositol-triphosphate
IV	intravenous
K_{ca}	calcium-dependent potassium channel
KDa	kilodalton
KI	potassium iodide
LDL	low density lipoprotein
L-NAME	N^G-nitro-L-arginine-methyl-ester
L-NMMA	N^G-methyl-L-arginine
L-NNA	N^G-nitro-L-arginine
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaI	sodium iodide
NANC	non-adrenergic non-cholinergic
NE	norepinephrine
NEM	N-ethylmaleimide
NF-κB	nuclear factor kappa-B
NMDA receptor	N-methyl-D-asparatic acid receptor
nNOS	neuronal NOS
NO	nitric oxide
NO^-	nitrosyl
NO^+	nitrosonium
NO2	nitrogen dioxide
$NO3^+$	nitronium
NOS	nitric oxide synthase
NOSI	neuronal nitric oxide synthase
NOSII	inducible nitric oxide synthase
NOSIII	endothelial nitric oxide synthase
O_2^-	superoxide
ONOOH	peroxynitrous acid
$OONO^-$	peroxynitrite
$P22^{phox}$	22 KDa protein subunit of phagocytic NAD(P)H oxidase
$P40^{phox}$	40 KDa protein subunit of phagocytic NAD(P)H oxidase
$P47^{phox}$	47 KDa protein subunit of phagocytic NAD(P)H oxidase
$P67^{phox}$	67 KDa protein subunit of phagocytic NAD(P)H oxidase
P50, P52, P65, P100	components of NF-κB
P_{art}	arterial pressure
PCR	polymerase chain reaction
P_{cw}	pulmonary occlusion pressure
PDZ	*post*-synaptic density protein-95,*d*iscs-large,*Z*O-1
PGE2	prostaglandin E2

PGI_2	prostaglandin I_2
PI_3K	phosphatidyl inositol-3-OH-kinase
PIN	protein inhibitor of neuronal nitric oxide synthase
PKC	protein kinase C
PKG	protein kinase G
PMN	polymorphonuclear
Po_2	partial pressure of oxygen
P_{pa}	pulmonary artery pressure
PPM	part per million
P_{ra}	right atrial pressure
PSD-93	postsynaptic density protein
PVDF	polyvinyl difluoride
PVR	pulmonary vascular resistance
RAW264.7	mouse peritoneal macrophage cell line
RE	response element
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
R-SH	thiols
RTPCR	reverse transcription polymerase chain reaction
RVR	resistance to venous return
SDS	sodium dodecyl sulfate
SNC	S-nitrosocystein
SOD	superoxide dismutase
SVR	systemic vascular resistance
TGF-β	transforming growth factor-beta
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminoethane
TRIzol	monophasic solutions of phenol and guanidine isothiocyanate suitable for isolating total RNA, DNA, and proteins.
ZnSO4	zinc sulfate

PREFACE

The McGill Faculty of Graduate studies and Research permits the choice of two options in the submission of a thesis. Option A is the conventional format known to all universities whereas option B is in the form of published or publishable papers. This work is submitted in the format of option B according to the thesis guideline:

Candidates have the option, subject to the approval of their department, of including as part of their thesis, the text of one or more papers submitted, or to be submitted, or the clearly duplicated text (not the reprint) of one or more published papers. If this option is chosen, connecting text, providing logical bridges between the different papers are mandatory. The text must conform to the Thesis Preparation Guidelines with respect to font size, line spacing and margin sizes and must be bound together as an integral part of thesis. The thesis must include as separate chapters or section: 1) a Table of Contents, 2) a general abstract in English and French, 3) an Introduction which clearly states the rationale and objectives of the research, 4) a comprehensive review of the literature (in addition to that covered in introduction to each paper, 5) a final conclusion and a summary, and 6) a comprehensive bibliography or reference list, at the end of the thesis, after the final conclusion and summary. Additional material must be provided, where appropriate (e.g. in appendices) in sufficient details to allow a clear and precise judgment to be made of the importance of the research reported in the thesis. In the case of a manuscript co-authored by the candidate and others, the candidate must have made a substantial contribution to all the papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work

and to what extent. The supervisor must attest to the accuracy of this statement at the Ph.D. oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidates' interest to clearly specify the responsibilities of the authors of the co-authored papers.

Four papers are presented in this thesis. These are the following:

1- Javeshghani,D. ; Magder,S. Presence of nitrotyrosine with minimal iNOS induction in endotoxic pigs (submitted to American Journal of Physiology)

2- Javeshghani,D. ; Magder,S. Regional changes in constitutive NOS and the hemodynamic consequences of its inhibition in porcine endotoxemia. (submitted to American Journal of Physiology)

3- Javeshghani,D. ; Quinn,M. ; Magder,S. Vascular production of superoxide anion by NADPH oxidase and its interaction with nitric oxide synthase in endotoxemia.

4- Javeshghani,D. ; Quinn,M. ; Magder,S; Hussain,S.N.A. Production of superoxide radicals in the ventilatory muscles by NADPH oxidase.

I am the first author of all papers. .

Dr Hussain's laboratory assisted in carrying out the western blotting for the paper 4, and Dr Hattori,R. a researcher in Japan and Dr Quinn, M. generously provided the monoclonal antibody for NOSII, and NADPH oxidase respectively.

CHAPTER ONE

GENERAL INTRODUCTION

1-General Introduction:

Nitric oxide (NO), a noxious unstable gas which is produced as a byproduct of automobile exhaust, electric power stations and lightning was discovered in body fluids about 13 years ago. This inorganic gas is produced by many species such as the barnacle, fruit flies, horseshoe crabs, chickens, trout and humans, and participates in variuos functions (64)

1.1-Chemistry:

NO is a poorly water-soluble small molecule and has a high partition coefficient (solubility in H_2O at 0, 20, and 60° C is 7.34, 4.6, 2.37 ml/100ml respectively), so it tends to exist as gas. It is composed of one atom of nitrogen and one atom of oxygen and in contrast to nitrous oxide, N_2O, an inhalational anesthetic commonly referred to as laughing gas, NO is an uncharged molecule with an unpaired electron so it is a radical molecule. This makes it highly reactive with other substances but it does not self-react, because of the length of its bond, which is intermediate between double and triple bond length. It has a half-life of 2-30 seconds, and because it is uncharged, it can diffuse easily across membrane (160) (64) (4).

1.2-History:

It was not until 19980's that production of NO by mammals was appreciated. There had been clues that this occurred. For example, the level of nitrate in urine is higher than the level in the diet of animals such as rat, pig, human (64), but it was not considered that mammalian cells produced NO. Instead, it was believed that the source of nitrate production was the bacteria in the gut. In 1981, Steven R. Tannenbaum and coworkers demonstrated that a significant amount of nitrogen oxides are produced by mammals, and the amount is

increased by factors, that result in inflammation (94). Further support for the biosynthesis of nitrogen oxide in human was obtained in 1981 by Dennis Stuehr and Michael Marletta who demonstrated that macrophages of mice, which were injected with inflammatory mediators or infectious agents, produced significant levels of nitrite, and nitrate (244). This led to the development of the technique of induction of nitrogen monoxide biosynthesis by adding cytokines or bacterial products to pure cultures of mouse macrophages or macrophage-like cell line (121) (111).

There was other evidence which indicated NO is a biological mediator. Since the 18^{th} century nitroglycerin has been prescribed by physicians to treat patients with angina. This occures by the dilation of blood vessel by this compound. However, it was not known how this dilation occured. In 1977 Fried Murad and colleagues showed that agents which release NO activate soluble guanylyl cyclase which produces guanosine monophosphate cGMP (128) (127). Louise J. Ignarro and coworker observed that in order to make vascular smooth muscle relax, trinitroglycerine and sodium nitrite require the presence of thiols in muscle tissue to form S-nitrosothiols, which serve as precursors of NO. This evidence gave a molecular mechanism for the action of nitroglycerine and other NO-releasing substances (117).

Around that time Furchgott and Zawadzki found that relaxation of arterial strips depends upon the presence of endothelium, since removal of endothelium causes acetylcholine induced contraction of strip (82). They proposed that acetylcholine releases a factor from the endothelial layer of the blood vessel, which diffuses into the neighboring smooth muscle layer and causes relaxation. Furchgott named this endothelium-derived relaxing factor (EDRF) (82). Later on, other substances such as histamine, serotonin,

bradykinin, substance P and thrombin were also found to release of EDRF from endothelial cells and induce smooth muscle cell relaxation (81).

The chemical nature of EDRF was unknown until 1987, when it was found that the EDRF effect on the biological system is due to NO (116) (199) (197). Richard M. J. Palmer, Louise J. Ignarro, Salvador Moncada and coworkers found that the biological effect of EDRF is dependent on the production of NO from L-arginine (196) (199) (116). They also measured the release of NO from endothelial cells by chemiluminescence technique and argued that it was sufficient to explain the relaxation in vascular smooth muscle by EDRF.

Once it was known that macrophages could generate NO, and NO requires L-arginine, in 1980s, David S. Bredt and Solomon H. Snyder, predicted that L-arginine would be converted to L-citrulline. They thus used the conversion of tritium labeled L-arginine to L-citruline to assay nitric oxide synthase activity and monitor the purification of the enzyme from rat cerebellum. They were unable to find pure enzyme, but they could measure the substrate conversion in crude homogenates. The reason was that the enzyme activity was dependent upon Ca/calmodulin complex. (24). This dependency of the enzyme made a physiological link between the N-methyl-D-asparatic acid (NMDA) receptor and the enzyme. During neurotransmission, the release of glutamate from presynaptic neuron activates NMDA receptors of the postsynaptic neuron. This receptor activation causes channels in the same postsynaptic neuron to open, which allows Ca^{++} to enter due to its electrochemical gradient. Calcium ions bind to calmodulin and make a complex, which associates with the enzyme NOS. Thereafter, in the presence of oxygen and NADPH, the enzyme produces L-citrulline and NO from L-arginine. (24) (25).

Since that time the importance of NO as biological signal has been appreciated. A great deal of research has been focused on NO, and the various functions of NO in biological systems (176) have been identified including smooth muscle relaxation (217), platelet activation (212), neurotransmission (25;54), tumor cell lysis (162), bacterial killing (170), and stimulation of hormonal release (54).

1.3-Measurement:

1.3-i- Reason for NO measurement:

With the discovery of NO, interest in its measurement increased exponentially. Moreover there were problems with the old techniques to study NO. For example synthesis of NO is not the only means by which endothelium alters vascular tone (70). Endothelium also can promote vasodilation by production of prostaglandins such as prostacyclin, EDRF, or endothelium-derived hyperpolarizing factor (EDHF) (161) (80) (106). An observed hemodynamic phenomenon may be the summed effect of these endothelial-derived factors. For example, acetylcholine can change vascular tone through the production of prostaglandins (76), release of EDRF, or endothelium hyperpolarizing factor (EDHF) (142). The mechanism of vasodilation in different vascular beds can vary. For example, bradykinin causes pulmonary vasodilation by releasing NO (44) but in the brain bradykinin alters cerebral vascular tone via superoxide production (144). Therefore determination of NO as a factor made researchers develop techniques for its measurement. (4). In the following sections only common techniques will be described briefly.

1.3-ii- Indirect assay of NO:

Several assays indirectly determine the presence of NO, but none are specific. NO can increase guanylate cyclase (GC) activity resulting in elevation of cyclic guanosine

monophosphate (c-GMP). Thus, as discussed above, NO activates soluble guanylate cyclase to increase c-GMP. However, c-GMP can increase for many reasons independent of NO synthesis. For example, atrial natriuretic factor stimulates the particulate form of GC to increases c-GMP, independent of NO (214). On the other hand, NO signaling transduction can be independent of the production of cGMP by GC. For example, it can activate calcium-dependent potassium channels (K_{ca}) in vascular smooth muscles by NO (21). There can be dissociation between c-GMP concentration and vascular responses. For example, although the vasodilatory effect of acetylcholine correlates well with elevated c-GMP in intact bovine pulmonary artery endothelium, in damaged endothelium, acetylcholine increases c-GMP but the rings constrict (287) (1).

Another method to measure NOS activity is the measurement of L-citrulline, a co-product of the action of NOS on L-arginine. This assay determines the Ca-dependent and Ca-independent NOS activity.

None of these assays determine NO directly but provides information at different levels. Therefore one needs to collect information from two or three techniques for NO measurement to overcome the nonspecificity of each assay (4).

1.3-iii- Direct assays of NO:

A good sampling method must have rapid sample acquisition and avoidance of oxygen (O_2). In O_2-free solutions NO is stable for several days, but in the presence of O_2, NO reacts with it. Hence, contamination with O_2 decreases the recovery of NO. It is impossible to get rid off contamination by oxygen, but contamination can be minimized. Another means of recovering NO is to reduce nitrite by acidification and transforming it into NO. Acidification transforms all nitroso compounds, alkyl or inorganic nitrites, and

nitrosoamines in vitro back to NO. This gives the exact amount of NO if all nitroxides were originally derived from NO before reacting with oxygen. However if they were not, this result overestimates the amount of NO. If the sample is not acidified the assay measure only the NO present at the time of sampling (4). Sodium or potassium iodides (NaI, KI) have been used as reducing agents and the effect of varying the KI concentration on the observed NO production from nitrite has been studied (3). It was found that chemiluminescence NO readings were directly proportional to the KI dose untill a concentration of 1 M, at which concentration the reaction plateaus. It is thus recommended that 1 M KI be used to decrease the variability in transformation to NO (3).

1.3-iii- a- Chemiluminescence assay:

The principle of this technique is based on light that is generated from the reaction of NO and ozone. The product of ozone and NO is an excited molecule of nitrogen dioxide ($NO2^*$) which emits a photon. The total number of photons produced is proportional to the NO concentration. The sample is injected into a tight chamber under vacuum condition (about 3 mmHg to remove O_2). If the sample is a liquid, NO is driven into the gas phase by bubbling the injected solution through an inert gas such as helium (12ml/min X 60 sec). This process is called stripping which takes advantage of the low solubility of NO in aqueous solutions. Once stripping is complete, the gaseous specimen is drawn through a needle valve into the reaction chamber or photomultiplier tube (PMT) and reacts with ozone, which is generated with an ozone generator. Because the chamber has a vacuum generated by a pump, any leakage through the rubber piece of the needle valve allows room air to be sucked into the chamber and results in an increase in background signal. The needle valve is adjusted to make the NO signal appear as a brief spike, and avoid entrapment of

foam from the sample. To use chemiluminescence one needs to calibrate the assay. Gaseous NO or aqueous solutions containing various amounts of NO (20-1000 pmole) are used for calibration. For example, with a special tight syringe, 5 to 50 µl of 8PPM NO can be injected, and a calibration curve is produced by plotting the signal from PMT (mV) vs the known concentration of NO. Linearity of the chemiluminescence with NO has been reported to be from 20-200 pmole (130) and 300-3000 pmole (4). One can optimize the assay by changing the integration time, the period of time during which photons are counted before that value is presented and counting resumes. The integration time should be short enough so the counts do not saturate the photomultiplier tube but long enough to detect small signals. It has been suggested that integration time of .06 sec is adequate for most experiments, but if one uses .25 sec for integration time it results in a more linear and sensitive NO/chemiluminescence curve. The specificity of chemiluminescence depends on whether one uses, acid or reducing agents such as potassium iodide (KI) or sodium iodide (NaI). In the absence of these agents the signal is almost exclusively due to NO and the signal is small (about one log) compared to when these reducing agents are present. Therefore acidification may extract NO from non-NO sources as was mentioned before. which is a disadvantage. Dimethyl sufloxide (DMSO) is one of the solvents that can cause chemiluminescence at high doses. This problem has been solved by using both a low concentration of DMSO, (less than .1 ml/10ml) as well as interposing a red filter, to absorb light at wavelengths emitted by reaction of ozone with DMSO, in front of photomultiplier tube. One can use also 1M solution of a Fe^{++} compound to determine if chemiluminescence is due to NO. This solution reacts with NO, and removes it, and thus decreases the signal. It

is also required to keep the PMT cool, have a good vacuum to decrease O2 contamination and finally to dry the gaseous sample to decrease the humidity, which lowers the signal. (4)

1.3-iii- b- Diazotization assay:

Spectrophotometeric methods can also be used to determine NO with the Griess reagent. This is a mixture of sulfanilic acid and N-(1-naphtyl)ethyl)ethylenediamine that reacts with nitric oxides in the sample and generates a colored substance. Then its absorbance is measured at 548 nm by a spectrophotometer (4). This method can be used to measure plasma or serum nitrites. The nitrate in plasma or serum can also be included by its conversion to nitrite by reduction. This reduction can be done by bacterial nitrite reductase, or by metals such as cadmium, or by vanadium at high temperature (90°C). Cadmium is inexpensive but toxic. Plasma or serum samples after reduction are diluted with nitrite free water, and then mixed with 30% (w/v) zinc sulfate ($ZnSO_4$) to precipitate protein and the supernatant is used for the assay. This technique measures values between 1-100 μmole of Na NO3 (268).

1.3-iii- c- Electron paramagnetic resonance (EPR) assay:

NO has an unpaired electron in its π orbital and is paramagnetic. This electron can be excited by EPR spectroscopy, and when electron returns to its previous level of energy, its produces a characteristic spectrum of NO. However since this return process is rapid, spin traps are needed to measure the radical. Spin traps are compounds that interact with radicals and make a more stable product that can be detected by EPR (78) (296) (38). The identity of radical, defined by EPR, is characterized by the magnitude and multiplicity of hyperfine splitting of the spin adduct spectra, and the amount of radical is proportional to signal. This technique has a detection threshold of one nmole and has an advantage over the

chemiluminescence in that it can be used to measure NO in blood and diazotization is not necessary. However, the equipment required performing EPR is expensive and requires specialist expertise (4).

1.3-iii- d- Microelectrode NO assay:

Local release of NO from a single cell, or tissue can be measured by a microelectrode designed for NO. Based on the observation that metaloporphyrins catalyze the oxidation of NO ($NO\bullet - e^- \rightarrow NO^+$) which generates electrical current, Malinski and Taha used carbon fiber coated with thin polymeric porphyrin layer. When they coated the carbon fiber with a layer of Nafion, a negatively charged and anion impermeable material, a 20 fold excess of $NO2^-$ could not produce any current, but they could detect NO from cultured cells very well. The detection threshold of this technique is 10^{-20}M and has a short response time of 10 msec. (4) (30)

1.4- Reaction with other targets:

NO readily reacts with, superoxide radical (O_2^-), to yield peroxynitrite ($ONOO^-$) (4) (107). Because of its small size, NO can pass easily through cell membrane, and once within the cell, it avidly binds to transition metals such as Fe, Cu, Co, and Mn (4). Nearly all compounds are possible targets for NO, including proteins, carbohydrate, lipids and nucleic acids (108), (238). Because of the many possible interactions (122) (171) that NO can have, its availability can be limited. An important target that will be discussed further elsewhere in this thesis is superoxide (O_2^-). NO availability can be limited by its interaction with transition metals in proteins such as iron centers in hemoproteins, and proteins containing none heme iron-sulfur centers (218) (238). Additionally metal and thiol-containing proteins

serve as other target sites for NO which include enzymes, receptors, transcription factors, ion channels and signalling proteins (32) (46).

1.4-i- Reaction of NO with heme-containing proteins:

NO reacts readily with transition metals. For example NO reacts with heme-iron of guanylate cyclase (GC) which activates GC by a conformational change, and elevates cGMP. This in turn reduces the intracellular calcium concentration, and produces physiological responses such as smooth muscle relaxation, and inhibition of platelet aggregation. Another example is the reaction of NO with the heme-iron of hemoglobin. NO has a 10^6 -fold greater affinity for Hb than oxygen and stability is greater than HbO_2. Exposure of animals to NO results in binding of NO to hemoglobin and the production of nitrosylated Hb (HbNO) and methemoglobin Hb (Fe^{III}) in red blood cells. The proportion of these two depends on the state of oxygenation of hemoglobin. In arterial blood at a normal O_2 saturation (94-99%), almost all NO is converted to methemoglobin and nitrate and there is little production of HbNO, whereas in venous blood there is more HbNO and less nitrite (107). The interaction of NO with heme can inhibit enzyme responses such as activity of cytosolic enzymes, indoleamine 2,3-dioxygenase, cytochrome p450, and even, NOS itself. In addition NO can interact with other heme-containing proteins including, catalase, myoglobin, cytochrome C, cyclooxygenase, and peroxidase (129) (256) (107).

1.4-ii- Reaction of NO with none heme-iron:

NO can react with the non-heme iron sulfur cluster of several enzymes and inhibit them by altering the configuration of catalytic sites (107). These include members of the mitochondrial electron transport chain including NADH-ubiquinone oxidoreductase (complex I), NADH succinate oxidoreductase (complex II), the tricarboxylic acid enzyme

cis-aconitase and ribonucleotide reductase which is involved in cell division (20) (107). NO binds to iron storage proteins and iron-sulfur containing enzymes such as succinate oxidoreductase, NADH-ubiquinone oxidoreductase and cis-aconitase, and liberates iron which can cause lipid preoccupation (218) (291).

1.4-iii- Reaction of NO with superoxide anion:

An important target of NO is O_2^-, which produces the product, peroxynitrite (204) (119). Peroxynitrite has a pKa of 6.8 and half-life of about 1 second under physiological condition. This means that peroxynitrite is sufficiently stable to diffuse away from the site of formation (293). When protonated, the resulting peroxynitrous acid (ONOOH) decomposes to form potent toxic oxidants with reactivities equal to hydroxy radical and nitrogen dioxide. The rate of peroxynitrite formation depends upon the product of concentration of NO and O_2^-. Thus relatively small increases in O_2^- and NO production may greatly enhance the rate of peroxynitrite formation to potential toxic level (16). The reaction of NO with O_2^- is three times faster than the reaction of O_2^- with SOD (18). The reaction of NO with O_2^- is even 30 times faster than the reaction of NO with oxyhemoglobin (137).

Peroxynitrite has been implicated as a toxic metabolite, which leads to vascular (267) and myocardial dysfunction (230) (118). Peroxynitrite reacts with other molecules such as nucleic acids, proteins, and lipids (16) (249) (59) (35). The results of these studies (16) (249) (59) (35) indicate that peroxynitrite is a toxic molecule. This compound is known to initiate lipid peroxidation (210), sulfhydryl oxidation (211), and nitration of aromatic amino acids such as tyrosine (263) (17). The toxicity of peroxynitrite is because it can deplete energy stores of cells (295). Peroxynitrite inactivates mitochondrial actonitase (40). It also damages DNA, which results in activation of poly-adenosine diphosphate

ribosyl synthase. Massive activation of this enzyme depletes the cell of NAD and ATP by poly-ADP-ribosylation (295). However, low concentrations (nmolar) of peroxynitrite decrease leukocyte rolling and adhesion (150). Moreover it has been demonstrated that neutrophil-induced myocardial dysfunction is reduced by low concentration of peroxynitrite (150). Additionally, peroxynitrite increases platelet aggregation, but only in the absence of thiols, whereas it inhibits platelet aggregation in the presence of thiols (166).

There are in vitro observations that the effects of peroxynitrite are reversible. For example incubation of coronary arteries from dogs with peroxynitrite resulted in vasorelaxation which was reversed by washing (154) and incubation of aortic strips from rats with peroxynitrite resulted in their relaxation. This was inhibited by addition of oxyhemoglobin as NO scavenger (177).

In sepsis where the formation of either or both NO and O_2^- are increased high levels of peroxynitrite could be formed and be toxic. We used luminol chemiluminesence to measure perxynitrite and showed in this study (chapter two) that peroxynitrite formation is increased in sepsis. We found that low levels of peroxynitrite are formed even under normal physiological condition, suggesting that peroxynitrite may have some physiological role. However at high concentration peroxynitrite may induce a generalized permanent irreversible change in the reactivity of vasculature. Therefore, whether peroxynitrite is cytotoxic or beneficial, appear to be dependent on its concentration and also its environmental condition.

1.4-iv- Reaction of NO with thiols:

NO can react with SH group of amino acids, organic acids, and sugars to yield S-nitrosothiols. However, NO does not directly nitrosate thiols (R-SH) (204) (18). This

reaction is thought to occur through generation of intermediate nitrosonium (NO^+) and nitrosyl (NO^-) species (86) that have strong affinity for R-SH (86). There is evidence that thiols, such as S-nitrosothiols (SNOs) and NO-adducts exert bioactivity without releasing NO (86).

Interaction of sulfhydryl groups with NO appears to serve a regulatory function (86). For example, it can inhibit the post-translational traffiking of $P47^{phox}$ of the NADPH-oxidase complex to the neutrophil cell membrane (86). S-nitrosylation may activate pathways such as hexose monophosphate shunt (86) by depleting glutathione (109). These modifications are thought to occur by producing conformational changes similar to phosphorylation of a protein (86). S-nitrosylation which is the reaction of NO^+ with sulfhydryl group of cysteine results in regulation that is different from tyrosine nitration by nitronium ($NO3^+$) which is not regulatory (86). Nitrothiols in serum are not inactivated by hemoglobin (18). Therefore it appears that nitrothiols are a pool of nitric oxide, which prolong the half-life of nitric oxide.

1.5- Biology of NO:

NO is produced from L-arginine in two steps: L-arginine is converted to N-hydroxy-L-arginine and then to NO and L-citrulline by NOS (243) (26) (237).

Three NOS isoforms have been purified. All isoforms require nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), and tetrahydrobiopterin (BH4) as cofactors. NOSs were initially classified into two groups: constitutive and inducible forms. The first group are Ca/calmodulin dependent and can be activated by mediators such as bradykinin, acetylcholine, calcium ionophore, histamine, leukotriene, and platelet activating factor (24)

(148) (183) (197). Another isoform was classified as an inducible form, and is induced by bacterial lipopolysaccharide (LPS), or cytokines (110) (241) (62). The initial NOS nomenclature reflected the observation that the NO production only occurred in the inflammatory cells when they were activated, hence the term inducible or iNOS. In contrast constitutive, refers to isoforms, which are expressed all the time. However, it is now known that constitutive isoforms can be induced under different physiological conditions, such as increased shear stress (84), or nerve injury (73). Conversely the iNOS can be expressed constitutively in some cells such as bronchial epithelial cells (98).

Based on physical and biochemical properties of purified enzymes, NOS is now classified into three isoforms (228) (242). NOSI (nNOS; bNOS) is Ca/calmodulin dependent and found in the soluble fraction in brain tissue. NOSII (iNOS) is Ca-independent, and found in macrophages in cytosolic fraction. NOSIII (eNOS) is Ca/calmodulin dependent and is localized to the particulate fraction of endothelial cells. These isoforms come from distinct genes (174).

1.6- NOSI:

1.6-i-Gene:

NOS I gene has been mapped to chromosome 12, in human, chromosome 5 in mouse, and 12 in rat. The three species have 86-88% identity in their coding sequence. The full gene structure is known in humans and has a complex structural organization (101). Its length is 200kb. The major NOS I RNA is made of 29 exons. Its open reading frame codes for a protein of 1434 amino acids with a predicted molecular mass of 160.8 KDa. It has been suggested that the three isoforms of NOS were derived from a common ancestor, since there is a high homology in the size of exons and location of splicing junctions. (73). NOS I gene

has multiple alleles that vary in size. Cloning studies of cDNAs from human or rodent tissues have revealed different structures of mRNA. The reasons given for the variability in NOSI transcripts include alternative promoters in a transcriptional unit, cassett exon deletion or insertion, and use of alternate polyadenylation site. A characteristic of NOSI gene is possession of alternative promoter activity. Therefore, assembly of alternative exons and different promoters can lead to NOSI proteins with different cellular localization and perhaps different function (73).

1.6-ii- NOSI Regulation:

NOSI was originally thought to be a constitutively expressed enzyme, but recent observations suggest that it may be regulated in physiological and pathological condition (73). NOSI is upregulated by stress-inducing stimuli such as spinal cord and nerve injuries, immobilization stress, electrical stimulation, formalin and phenobarbital administration (73) (200).

Hypoxia has been shown in most studies to increase the mRNA, protein and activity of NOSI (99) (234) (206) (125). This upregulation has been suggested to be due to two mechanisms (73). One mechanism may be that hypoxic exposure of animal activates a stress pathway that has been shown to increase mRNA, protein, and activity in the hypothalamic pituitary-adrenal system (135). The second mechanism is binding of hypoxia-inducible factor to the NOSI genomic sequence and consequent upregulation of NOSI. However, there is also evidence that show oxygen deprivation decreases NOSI (55). Similarly asphyxia of rats in prenatal period decreased a NOSI protein that had the size of 150 KDa, but this was not associated with changes in NOSI mRNA or NOSI activity.

Body fluid volume and plasma osmolality can upregulate NOSI expression. It has been shown that hypovolemia increases expression of NOSI in paraventricular and supraoptic nuclei (261). Chronic salt loading or water deprivation increases plasma osmolality, which has been shown to upregulate NOSI in hypothalamohypophysal region (189). This suggests a potential role for this isoenzyme in osmoreceptor signal transduction. However, a low salt diet upregulated NOSI in kidney (73). The pathway and molecular mechanism of these different responses are not known.

Lipopolysaccharide alone or combined with IFN-γ has been shown to decrease both mRNA and protein of NOSI (89) (13) in guinea pig skeletal muscle and rat brain, spleen, stomach, and rectum, although in one study it was upregulated in brain (102). Interestingly it has been shown that NOSI is required for production of fever in septic animals. Pre-treatment with 7-nitroindazole, an inhibitor of NOSI in LPS-treated animals lowered body temperature 1.5°C compared to other LPS-treated rat group. The mechanism of this regulation is not known.

Sex hormones can also regulate NOSI expression. Estrogen has been demonstrated to upregulate this isoform in several tissues in rats (73). NOSI protein was increased in neutrophils of women with high levels of circulating estrogen compared to periods of low estrogen (83). Treatment of neutrophils, obtained from men, with 17-β estradiol also increased the level of NOSI (83). Furthermore, incubation of neutrophils with estrogen-receptor antagonists, tomoxifen and ICI 182780 inhibited the upregulation of NOSI in neutrophils treated with 17-β estradiol. The inhibitory effect of ICI 182780 was greater than tomoxifen. Together these observations indicate that estrogen receptors regulate the

expression of NOSI in neutrophils, but the mechanism is unknown. In particular 5'-flanking region of NOSI does not contain estrogen response elements.

Testosterone also has been shown to have a positive effect on NOSI regulation (219) (227). Reilly et al (227) castrated two groups of rat and they implanted testosterone pellets in one group. After several days they compared the ratio of intracavernosal pressure to mean arterial pressure in response to electrical stimulation in both groups and showed that the testosterone treated group had a larger ratio than the untreated group. By reverse transcription polymerase chain reaction (Rt-PCR) on penile tissue they showed that the densities of NOSI was higher in testosterone- treated rats which further supports, upregulation of NOSI by testosterone (219). Again the molecular mechanism of this upregulation by testosterone is not known.

In situ hybridization with a NOSI riboprobe of brain sections, of rats that were injected with cortisone showed a lower density than non-cortisone treated group, indicating that cortisone down-regulates NOSI (215).

NOSI interaction with other proteins is a mechanism for its regulation. Calmodulin is one these proteins. At resting intracellular concentration NOSI is inactive, but it is activated when intracellular Ca^{++} levels increase sufficiently to maintain calmodulin binding. When intracellular Ca^{++} decreases to basal level, calmodulin is released (143).

NOSI contains a PDZ domain at its N-terminus that allows this protein to interact with other proteins, which regulate NOSI activity. With this domain, NOSI interacts with PDZ domain of other proteins such as post-synaptic density proteins (PSD-93) in brain, (PSD-95), α_1-syntrophin, and NOSI-adaptor protein (CAPON) (123) in skeletal muscle (143). However the significance of this interaction is not known. NOSI can also interact

with other proteins. For example, caveolin-3 (266) and a protein inhibitor of NOSI, which is called PIN, both inhibit NOSI (124) (126), whereas heat shock protein 90 (Hsp90) activates NOSI (143).

Availability of L-arginine, the substrate of NOSI, and tetrahydrobiopterin (BH_4), the cofactor for NOS activity, are also important for the regulation of NOSI activity (264). In vitro results indicate that deficiency of BH_4 or L-arginine increases O_2^- production by NOSI (264) (281). However, the mechanism of regulation is not known.

1.6-iii- Function:

This isoform plays a role in physiological neuronal functions such as neurotransmitter release, penile erection, neuronal development, regeneration, synaptic plasticity, and regulation of gene expression. In various neurological diseases, this enzyme produces excessive NO, which leads to neuronal injury (73) (200). In knockout mice, the stomach is grossly distended and urethral sphinncter is dysfunctional These observation indicate the importance of NOSI in gastric motility, and bladder function (200).

The role of NOSI in the regulation of blood pressure is not clear. It has been reported that this isoform does not change blood pressure acutely (147), but chronic administration of 7-nitroindazole increases the blood pressure (194). Recent findings from homozygote NOSIII knockout mice show that acute and chronic inhibition of NOSI decreases the blood pressure. The results of this study indicate that NOSI either acts through the central nervous system or possibly affects on other mediators which, affect vascular tone. On the other hand NOSI transcript has been detected in vascular endothelial cells which indicate it may have a role in the vasculature (231). Therefore, the role of NOSI in regulation of vascular tone remains unclear.

1.6-iv-distribution:

NOSI was originally characterized in rat and porcine cerebellum (168) (229) (24), but was later found to be widely expressed in different parts of central nervous system in humans (23), rats (246) (100), and mice (91). NOSI is also found in non neuronal tissues including myocytes of skeletal muscle in rat (236), guinea pig (88), and human (181), epithelial cells of lung in rat (187), endothelial cells of brain vessels in rat (231), and human (7), macula densa cells of kidney in rat (258), gonadotrophs and folliculostellate cells of pituitary gland in rat (41), adrenal glands in rat (120), and neutrophils in human (271).

NOSI is found in particulate fraction, indicating that it is membrane association. It has been shown in skeletal muscle that NOSI is anchored to both caveolin3 (191), which itself is located in plasma membrane (266), and to α-syntrophin, which is a member of dystrophin-glycoprotein complex (28).

1.7- NOSIII:

1.7-i- Gene:

The human NOSIII mRNA is encoded by 26 exons and spans 21-22Kb of genomic DNA and is located on chromosome 7. Full-length cDNAs have been isolated from human, murine, bovine, and porcine endothelial cells. The deduced amino acid sequence predicts a protein of 133 KDa. The coding cDNA sequence of human and other species is around 90%. Amino acid homology between NOSIII and NOSI is about 52% and between NOSIII and NOSII is 58% (73) (74) (148).

1.7-ii- Distribution:

After identification of NOSIII in endothelial cells, (77) the purified protein had a molecular mass of 135 KDa (208), The purified protein was used to produce antibodies for

immunohistochemical studies. Beside endothelial cells NOSIII was shown to be present in cells relevant to cardiovascular system such as in neutrophils (87) platelets (272), cardiac myocytes (12), and smooth muscle cells (48).

1.7-iii- Regulation:

NOS III was originally characterized and localized in both cytoplasmic and particulate fraction of aortic endothelial cells (77) (208), however, the major EDRF/NO activity was associated with particulate fraction of endothelial cells indicating that NOSIII is localized to the membrane. In these experiments (77) bovine aortic endothelial cells (BAEC) was cultured and stimulated with chemicals such as bradykinin, or inhibited with methylene blue, or L-NAME. The particulate or cytosolic fraction was added to the rat fetal lung fibroblasts (RFL) and the cGMP content of these cells was measured as an indicator of NOS activity. Stimulation of BAEC with bradykinin greatly increased the EDRF/NO activity, whereas mehtylene blue and L-NAME decreased activity. Both cytosolic and particulate fractions had EDRF/NO activity, but most of this activity was associated with particulate fraction of BAEC. Addition of calmodulin to KCl-washed particulate fraction restored activity, and incubation with calcium-chelating agents reduced it. The authors (77) concluded that this EDRF/NO activity is dependent on calcium and calmodulin. However, the activity of NOSIII is dependent on its surrounding microenviroment. NOSIII can be active independent of changes in calcium concentration (57) as will be explained in a later section of NOSIII regulation.

NOSIII, like other proteins, is synthesized in ribosome, and then transported to Golgi apparatus and plasma membrane. Association of NOSIII with Golgi membrane occurs by acylation (233) (232) (153). Myristoylation is one example of acylation that

has been shown to be important for localizing NOSIII and regulation of its function (233) (232) (153). Sessa et al (233) transfected human embryonic kidney cells with either mutant or wild type NOSIII cDNA. Because alanine is an amino acid that can be acylated by myristic acid to associate with membrane of the organelle, they constructed a mutant by changing glycine-2 to alanine. Therefore, the synthesized protein is not myristoylated and does not associate with membrane (233). Cells transfected with wild type NOSIII produced more NO than the mutant-transfected. By using mannosidase, a Golgi marker, they showed with light microscopy that NOSIII in wild type NOSIII-transfected cells is localized to Golgi apparatus but in mutant-infected it is not (233). Thus, they showed that inhibition of acylation by myristoylation inhibits compartmentalization of NOSIII. Moreover, this localization into membrane is necessary for the enzyme to use the substrate efficiently to produce NO (233).

Fatty acylation reactions are essential for membrane association and targeting of NOSIII (153) (152). This process needs proper molecular signals to translocate NOSIII. Liu et al (152) ligated the carboxy terminus of wild type or truncated NOSIII cDNA, to a green fluorescent protein (GFP) to generate chimeras, and transfected NIH3T3 cells to study localization of NOSIII. They found that the sequence that determines Golgi targeting of NOSIII, is the first 35 amino acids (of N-terminus) of NOSIII, which has both myristoylation and palmitoylation sites. They also demonstrated that mutation of N-myristoylation site inhibits palmitoylation, and association of NOSIII to Golgi. Instead, this mutation randomly distributes NOSIII into cytosol. In comparison, mutation of palmitoylation sites of NOSIII that was by replacing a serine with leucine resulted in

association of only small fraction of the protein with the Golgi, but the rest of the mutants were mislocalized to other organells other than Golgi.

Considering the great diversity of tissues in which NO is synthesized, it is likely that there are important cell specific regulatory pathways. One example is the interaction of caveolins with NOSIII, which represents a protein-protein interaction.

Once NOSIII associates with the Golgi apparatus, it has the capacity to move to the plasma membrane and interact with caveolae (65). Caveolae are small invaginations in plasma membrane and have the chief structural proteins, caveolins (191). Caveolin-1 in endothelial cells and caveolin-3 in cardiac myocytes have been shown to co-immunoprecipitate with NOSIII (66), which indicates that the targeting of NOSIII to caveolae in plasma membrane, is tissue-specific.

Calmodulin has been demonstrated to interact with caveolin (173) and reciprocally regulate NOSIII (173). Incubation of BAEC lysates with calcium and calmodulin, followed by immunoprecipitation with caveolin antibody, revealed NOSIII in the supernatants, but not in the bound fraction. This indicates that calmodulin prevents the interaction of NOSIII to caveolin. In the absence of calcium and calmodulin NOSIII is bound to caveolin. In the same study (173) the association of NOSIII to caveolin, attenuated NOSIII activity, whereas addition of increasing concentration of calmodulin dissociated NOSIII from caveolin and increased activity (173) (67).

Another example of protein-protein interaction that regulates NOSIII activity occurs with heat shock protein, Hsp90 (143) (84). NOSIII co-precipitates with Hsp90, when expressed in heterologous expression system, COS-7 cells. Stimulation with shear stress, or agonist stimulants such as histamine, or vascular endothelial growth factor

(VEGF) resulted in association of Hsp90 with NOSIII and increased NOS activity. The antibiotic geldanamycin, a specific inhibitor of Hsp90-mediated process, attenuated the cGMP production from these human or bovine vascular endothelial cells. This inhibitor also blocked acetylcholine-induced relaxation of aorta indicating that Hsp90 can modulate NOSIII by inducing a conformational change in enzyme or by stabilizing its dimeric form (84).

Recent studies have shown that there are subcellular mechanisms, which regulate NOSIII (79) (57). In these studies the direct effect on NOSIII of protein kinase-B (Akt), a downstream effector of phosphatidylinositol-3-OH-kinase (PI(3)K) were studied. Mutants of NOSIII were constructed for these studies by changing serine 1177 in human or 1179 in bovine NOS to aspartic acid or alanine. Akt phosphorylated wild type-NOSIII, and increased NO production by stimulation of NOS activity in cells co-transfected with Akt and wild type NOSIII cDNA. In contrast, NOSIII mutants were resistant to phosphorylation (57). Moreover mutation of acylation sites on NOSIII prevented phosphorylation and activation of NOSIII by Akt. Because acylation of NOSIII is necessary for its membrane attachment, these results clearly indicate, only NOSIII anchored to membrane can be phosphorylated by Akt (57). Furthermore, stimulation of cells by shear-stress results in the phosphorylation of the serine on NOSIII by Akt, that was unaffected by complete removal of extracellular (57) or intracellular (79) calcium ion or inclusion of calmidazolium, an inhibitor of calmodulin. This indicates that the signalling pathway of activation of NOSIII by shear-stress is not regulated by Ca and calmodulin. Additionally, pretreatment of cells with Wartmannin and LP294002, inhibitors of PI(3)K, decreased NO production in response to the same stimuli that

activated Akt, further supporting that the stimulation of PI(3)K signalling pathway activates Akt, which, thus, activates NOSIII, independent of calcium and calmodulin (57) (79).

1.7-iii-a- Effect of hypoxia:

NOSIII is regulated by hypoxia but this depends on the tissue and time. In cultured human pulmonary artery endothelial cells (294) and bovine pulmonary endothelial cells (151) hypoxia reduced mRNA and/ or protein level of NOSIII, however, another study (220) demonstrated the opposite. Exposure of bovine aortic endothelial cell to low oxygen pressure increased NOSIII mRNA, and protein as well as NOS activity (6). Others have reported upregulation of NOSIII protein in ischemic cerebral vessels (292). Additionally, prolonged hypoxic exposure of conscious rats has been reported to upregulate mRNA of NOSIII (99) (209) and downregulate protein level of NOSIII (99). The reason for these different findings is not clear.

The molecular mechanisms responsible for regulating NOSIII by hypoxia are not known. However, it is known that hypoxia induces a number of factors, including hypoxia-inducible factor-1α (HIF-1α) (288) which could upregulate NOSIII (289). It is also possible that hypoxia indirectly induces NOSIII by stimulating the secretion of erythropoietin, which in turn causes polycythemia which leads to increased fluid shear stress, which is known to upregulate NOSIII.

1.7-iii-b- Effect of LPS and cytokines:

Regulation of NOSIII by cytokines and lipopolysaccharide (LPS) is different. Exposure of cultured bovine aortic endothelial cell (BAEC) (184) and human umbilical vein endothelial cell (HUVEC) (285) to tumor necrosis factor -α (TNF-α) decreased the

level of mRNA. This was thought to be due to decreased stability of NOSIII mRNA. BAEC treated with TNF-α also had markedly increased in NOS activity as evidenced by increased cGMP and a slight decrease in the level of mRNA (148) whereas rats treated with lipoloysaccharide (LPS) (155) had a decreased NOSIII RNA in lung, heart, and aorta compared to untreated animals. These observations indicate that cytokine regulatory effect on NOSIII is dependent on cell type or experimental model.

Tissue or cytokine-dependency of NOSIII regulation has been observed in other studies as well. Intraperitoneal treatment of rats with LPS increased NOSIII mRNA in the liver 8 fold at 12hr but it then decreased (33). The increase did not occur in the kidney. Administration of interleukin-2 (IL-2), a potent activator of interferon-γ (γ-INF) and TNF-α production has also been shown to increase NOSIII immunoreactivity in astrocyte cells of the brain (15). Taken together, it appears that cytokines and LPS may regulate NOSIII expression differently in different tissues.

The mechanism of downregulation of NOSIII by TNF-α in BAEC is thought to be due to destabilization of NOSIII transcript (99) (285), through a cytosolic protein, which interacts, with the 3'untranslated (UTR) region of NOSIIImRNA. Using band shift assay and cross-linking assay Alonso and colleagues (2) (224) identified a 129-nucleotide uridine-cytidine rich region in the middle of the NOSIII mRNA 3'-UTR that binds a 60 KDa cytosolic protein in endothelial cells. They showed that incubation of TNF-α with BAEC, increased complex formation of this cytosolic protein with the NOSIII mRNA specific sequence, and decreased NOSIII mRNA expression. However it is not yet known if other cytokines affect NOSIII mRNA expression through the same protein. Moreover the sequence of amino acids of this is not known yet.

Various other factors such as estrogen, TGF-β, oxidized LDL and also proliferative status of cell are important in the regulation of NOSIII as well (73) (19).

1.8- NOSII:

This enzyme was originally purified from murine macrophage (283) and later was cloned from various cell types including rat vascular smooth muscle (90), cardiac myocytes (104) and hepatocytes (188).

1.8-i- Gene:

NOSII is encoded by a single-copy gene comprised of 26 exons and 25 introns and is located on human chromosome 17 (179). The promoter regulatory region of the murine gene has several DNA-regulatory sequences for binding of transcription factors. IFN-γ response element (IFN-γ-RE), nuclear factor kappa B (NF-κB) binding motifs, nuclear factor IL-6 (NF-IL6) binding sites, IFN-α response element (IFN-α-RE), activating protein 1 (AP-1) sites, and tumor necrosing factor response elements (TNFα-RE) (200) (75) (42).

1.8-ii- Regulation:

This isoform of NOS is regulated at transcriptional, posttranscriptional, and translational level (200).

Expression of NOSI can be regulated transcriptionally by cytokines such as TNF-α, IL1-β, IL-2, IL-6 or IFN-γ or bacterial lipopolysaccharide (LPS) (200). NF-κB is the essential factor for mediation of NOSII transcription. Although transcription binding sequences exist on the NOSII gene for several transcription factors, it appears that the presence of NF-κB site is also essential for their effect on transcription (200). NF-κB is

composed of a group of nuclear proteins including C-rel, P50/P105 (NF-κB_1), P65 (Rel A), P52/P100 (NF-κB_2) and Rel B. In resting cells, NF-κB is in the cytoplasm as a complex with the inhibitory protein, IκB, which belongs to the I-κB family. Following activation of cells by a number of agents (eg. LPS and TNF-α), I-κB is first phosphorylated and then degraded. This allows NF-κB to translocate to nucleus and bind to the regulatory region on the NOSII and promote gene activatation (254) (11) (274). IκB can be upregulated by a number of factors for example, glucocorticoids and NO (9) (96). To induce NOSII investigators combine cytokines to potentiate the induction of NOSII. However, at transcriptional level the interaction of these factors with each other and with NF-κB is not known.

Postranscriptional regulation of NOSII expression occurs by a number of mechanisms. For example, LPS increases the half-life of NOSII RNA. On the other hand, TGF-β inhibits NOSII expression by increasing degradation of NOSII protein, and decreasing the stability of NOSII mRNA (75).

Expression of NOSII is downregulated by a number of factors including TGF-β1, 2, and 3 (277) (276) (253), IL-4, IL-8, IL-10 (75) (134), glucocorticoids, (136) (226) (250), prostaglandin E2 (PGE2)(51) and NO itself (96)

To date it is not known whether the activity of NOSII protein can be regulated by interaction with other proteins. It has serine and tyrosine residues, which can be phosphorylated, but the significance of the phosphorylation sites is not yet known.

Recently it has been shown that phosphatidylinositol 3-kinase (PI3-kinase) inhibits NOSII induction (52). Treatment of macrophage cell line RAW 264.7 with

wortmannin, an inhibitor of PI3, increased the expression of NOSII and nitrite production by LPS. In addition, cotransfection of the cells with both the active subunit of PI3 and NOSII, decreased NOSII promotor activity in response to LPS. In the same study, the authors (52) found that PI3 inhibition by wortmannin occurs in response to LPS, IFN-γ, but not to IL-1β or TNF-α. The authors (52) showed that NF-κB activity was increased with inhibition of IP3, indicating that IP3 decreases iNOS induction by NF-κB in these cells. In the same study (52) LPS increased phosphorylation activity as measured by phosphorylation of phosphoinositol. However, it is not known if IP3 can phosphorylates iNOS itself.

Activation of NOSII by the LPS component of Gram-negative bacteria occurs by the interaction of LPS with LPS binding protein, and this complex interacts with cell surface molecules CD14 or may be transduced across the membrane via the toll-like receptor-2 (257). In addition, products of Gram-positive bacteria can induce NOSII. These include cell wall components such as lipoteichoic acid, as well as secreted extracellular toxins such as staphylococcal protein toxic shock syndrome toxin-1 (157) (290).

Availability of cofactors and substrate for NOSII is another way of regulating the activity of NOSII. To produce NO, this protein must be dimer. Tetrahydobiopterin, a cofactor of NOSII promotes the assembly of the enzyme subunit (45;260). With, reduced availability of L-arginine, the substrate of iNOS, the enzyme produces O_2^- (282). Thus these are other postranslational mechanisms, which, can regulate NOSII expression.

1.8-iii- Distribution:

NOSII has been reported to be induced in different cells, such as macrophages (159), lymphocytes (223), polymorphonuclear leukocytes (PMN) (68), platelets (43), hepatocytes (188), chondrocytes (198), cardiac myocytes (265), keratinocytes (105), adipocytes (186), neurons (178), pancreatic ilets (61), glial cells (286), vascular smooth muscle cells (182), retinal epithelial cells (202), mesangial cells (225) airway epithelium (98), renal tubular epithelium (274), colorectal carcinoma (140), and pulmonary artery (39). NOSII is a cytosolic protein and on SDS page the purified NOSII from macrophages migrates with a molecular mass of 125 to 135 KDa. In general, cells derived from rodents are more easily stimulated to produce NOSII than in higher order species.

Most studies on the expression of NOSII are on rats. In humans there are reports of NOSII in conditions other than sepsis, such as heart failure, as well as constitutive expression in human bronchial epithelium (104) (265). Yet, there are few reports of direct evidence of NOSII expression in other tissues in human sepsis. Probably in higher species expression of this protein is under tighter control. Thus the role of NOSII in sepsis in species higher than rodents is not known.

1.8-iv-Function:

Whereas the constitutive NOSs produce small amounts of NO, production of NO from NOSII is sustained for hours. This is believed to cause the vasodilation in septic shock in rodents (244). High levels of NO are cytotoxic and inhibit a number of enzymes including complex I and complex II of electron transporting system, glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme, and ribonucleotide reductase, a key enzyme in the synthesis of DNA (179).

Whether the expression of NOSII is helpful or not, depends on the condition. For example in inflammation it is beneficial. NO has antibacterial effect through the formation of nitrotyrosine (63) or possibly through other mechanisms such as inhibition of respiratory chain enzymes, iron-sulfur proteins and other metalloproteins. On the other hand in autoimmune disease it is detrimental (93) (269) (221). In heart failure, NOSII is expressed in endothelial and vascular smooth muscle cells and the heart myocytes but the functional significance of it is not known (104) (265).

1.9- Role of NO in blood coagulation:

Nitric oxide is involved in regulation of platelet activation. Platelets are blood elements that ensure the integrity of vessel wall. The contact of platelets to altered biological or nonbiological surfaces, is called adhesion and when platelets interact with each other, they form a hemostatic plug. The process of adhesion involves expression of specific receptors on platelet surface or change in the affinity of receptors such as GPIIb/IIIa. This occurs when platelets are exposed to subendothelial surface or collagen. In the process of aggregation, platelets can bind to fibrinogen, which traps other blood elements and forms a blood clot. In vessels, the formation of this clot is finely controlled by stimulatory factors from activated platelets and inhibitory factors such as prostacyclin PGI2 from endothelial cells adjacent to the area of altered endothelial surface. In vivo, platelets are kept in an aggregation -disaggregation equilibrium in order to ensure a rapid response to activating signal. Elevation of cGMP negatively modulates the aggregatory process of platelets (31). Activation of guanylate cyclase by nitric oxide produces cGMP. This results in activation of protein kinase G, which by an unknown mechanism leads to inhibition of phospholipase C, inositol 1,4,5-triphosphate and Ca mobilization in platelet

(31) (273). Because NO can inhibit platelet aggregation, platelet aggregation has been used as a method of assessing NO generation (213).

Although generation of NO has been shown in many in vitro studies to inhibit platelet aggregation (31) (284), Marietta et al (165) suggest that elevation of NO is not necessarily associated with inhibition of aggregation. They infused L-arginine into 10 healthy volunteers for 30 minutes, collected platelets, and measured L-citrulline generation and platelet aggregation. The citrulline level was increased indicating that nitric oxide was increased, but this was not associated with platelet aggregation, which they believe was due to decreased platelet sensitivity to NO. This unexpected response of platelets could have been due to the experimental conditions, but other studies have demonstrated similar results. For example, Picunio et al (207) administered LPS to conscious rats and observed a rapid and transient decrease in circulating platelets, which they concluded was because of platelet activation and aggregation. The disappearance of single platelets, which was used, as an index of platelet aggregation, was associated with significant increase in nitrite indicating nitric oxide has increased. Similarly Bar et al (14) isolated platelets from pregnant and non-pregnant volunteers and assessed aggregation and P-selectin expression as index of platelet activation. They observed that the cytokines, which were supposed to increase nitric oxide, increased platelet aggregation, in non-pregnant woman but inhibited aggregation from pregnant woman. These results contrast with another study where LPS, which is supposed to release cytokines, decreased platelet aggregation (235). This antiaggregatory effect was also associated with increased nitrite production, increased cGMP, and decreased in calcium mobilization. Thus these results present a contradictory picture of NO effect on platelet

activity. Taken together under physiological condition nitric oxide inhibits platelet adhesion and aggregation.

1.9-i- NO effect on leukocytes:

In addition to its effect on platelets, nitric oxide has been shown to affect the interaction of leukocytes with vascular endothelium. Leukocytes move freely in blood stream. When leukocyte are required to reach the site of injury in the extravascular space they first have to marginate. During this process they slow down by a process called rolling, which is followed by adhesion to blood vessel lumen or endothelial cells. They then extravasate to extravascular space. Interaction of leukocyte with endothelial cells involves expression of receptors such as P-selectin which has been used as an index of leukocyte adhesion. Nitric oxide has been proposed to be a regulator of leukocyte-endothelial interaction, however, observations (193) (192) (145) (149) (95) are not consistent. For example Lefer et al (149) used three isoforms of NOS-knockout mice to study rolling and adherence of leukocytes to endothelial cells in postcapillary venules under video-microscopy. P-selectin expression was higher in NOSIII and NOSI knockouts than NOSII knockout or wild type mice. NOSIII knockouts had a higher rolling and adherence than the NOSI that was significantly attenuated by the administration of P-selectin ligand or monoclonal antibody against P-selectin for its neutralization. This group (149) further observed that administration of thrombin into mesenteric vessels, resulted in a higher and faster expression of P-selectin in NOSIII knockout mice. Therefore, they demonstrated that NOSIII isoform has greater effect in the leukocyte-endothelial interaction than the two other NOS isoforms. Similarly, Kosonen et al (145) studied this interaction in co-cultures of endothelial cells and

polymorphonuclear leukocyte (PMN). They added human PMN leukocyte to cultured rabbit endothelial cells that were supplemented with nitric oxide-releasing compounds. They further induced adhesion by treatment with TNF-α and measured the number of adhered leukocytes to endothelial cells by staining with fluorescein isothiocyanate labelled CD18 monoclonal antibody. They demonstrated that NO-donors decreased leukocyte adhesion in a dose dependent manner after 30 minutes incubation. Nitric oxide resulted in an increase of cGMP in leukocyte, and cGMP-analogues decreased the leukocyte adherence as well. Thus these observations (149) (145) demonstrate that nitric oxide decreases rolling and adherence of leukocyte to endothelial cells.

In contrast, there are other studies (193) (192), which indicate the opposite effect of nitric oxide on leukocyte. For example, Okayama et al (193), who also used endothelial cell culture, and assessed P-selectin expression as an index of leukocyte adherence. They treated human umbilical vein endothelial cells (HUVEC) with different inhibitors to analyze the mechanism of adherence and observed that treatment with an NO-donor increased leukocyte adhesion and P-selectin expression. The induction of adherence by NO-donor was inhibited by an inhibitor of protein kinase G (KT5823), but not by a protein kinase C inhibitor (Go6976), a trypsin kinase inhibitor (genistein) or a protein kinase inhibitor (H-89). They also (192) observed that the same NO-releasing compound does not effect leukocyte adhesion if used at .5mM instead of 1mM. Additionally, another study demonstrated no relationship between NO and leukocyte extravasation (47). In this study, they administered oyster glycogen intraperitoneally and collected leukocytes that also had NOSII. Using an NOS inhibitor, L-iminomethyl-lysine (L-NIL), they reported reduction of the NO concentrationin lavage fluid, as was

measured by a decrease in nitrite and nitrate, and there was no change in leukocyte / endothelial cell interaction. The reason for the discrepant responses of leukocyte in the above mentioned experiment could be differences in the microenvironment of leukocytes. Because NO can react with other molecules it possibly produced molecules which could have had the opposite effect on leukocytes. In general, it appears that nitric oxide inhibits leukocyte adhesion and aggregation.

1.10- Role of NO in Vasculature:

It was originally thought that NO dilates vessels by decreasing the intracellular calcium concentration of vascular smooth muscle (115). However, the available data in the literature do not conclusively show this. Bolotina et al (21) used patch clamping technique to test the direct effect of freshly dissolved NO on membrane patches of rabbit aortic smooth muscle and observed activation of K^{+}_{ca} channel which would decrease intracellular Ca^{++}. They used N-ethylmaleimide (NEM) an inhibitor of nitrosylation, and demonstrated that the activation of K^{+}_{ca} channel by NO was inhibited. Additionally, they applied myristic acid, a fatty acid, which activates of K^{+}_{ca} and showed that this occurs in NEM pretreated membrane patches indicating that NO modulation of channels differ from that caused by the fatty acid. To support further the direct effect of NO they used methylene blue, a guanylate cyclase inhibitor. This did not change the NO-induced relaxation of isolated aortic ring, although the cGMP content of these rings was significantly reduced. They concluded that nitric oxide directly activates K^{+}_{ca} channels, which could cause hyperpolarization and relaxation of vascular smooth muscle by outward diffusion of potassium ions through K^{+}_{ca} channels. In contrast, Davison et al (53) believe that the vasodilatory effect of NO donors is not due to decomposition and release

of NO from these compounds. They injected both L and D isomers of S-nitrosocysteine (SNC) into a closed chamber that had either rat blood or porcine aortic smooth muscle cell. The NO released by L- and D-SNC, upon addition to the cells in chamber, was carried in a stream of nitrogen gas under vacuum to a chemiluminescence NO analyzer. They found that the amount of NO produced from decomposition of these two isomers of S-nitrosocystein were equal, however they observed that L-SNC is a more potent vasodilator agent than D-SNC suggesting that the vasodilatory effect of S-nitrosocystein is not simply due to its decomposition to NO. They argued that the nitrothiol moiety itself is biologically active.

There are studies which indicate that NO relaxation of smooth muscle involves guanylate cyclase-catalyzed production of 3', 5'-cyclic monophosphate (cGMP) with subsequent activation of protein kinase G (PKG) (5) (49). For example, Archer et al (5) demonstrated that exposure of vascular rings to saturated NO solutions results in relaxation associated with increased cGMP level. This relaxation effect was mimicked by the addition of inhibitors of cGMP-phosphodiestrase. Addition of potassium chloride (KCl) which decreases the concentration gradient of K and blockers of K channel nearly eliminated the relaxation in response to NO and inhibitors of cGMP-phosphodiestrase. Inhibitors of K channel dephosphorylation, okadeic acid, enhanced whole cell K-current and pretreatment of vascular smooth muscle cell with methylene blue inhibited the effect of NO. Thus, this study (5) shows that NO increases cGMP which results in phosphorylation of a K channel and its activation. This activated K channel hyperpolarizes the cell and relaxes the vessel. However, in this study (5) they did not use NEM, an inhibitor of nitrosylation to make sure that NO and not its compound induce

relaxation. Whether NO or its compounds such as nitrothiols have different effect on potassium channel in smooth muscle is not known, however Campbell et al (37) demonstrated that nitric oxide and S-nitrosothiols exert discrete and opposite effect on Ca-channels in ventricular myocyte. Also, it is not clear whether that the concentration of nitrothiols in vivo are high enough to allow NO to function as a vasodilator.

Cyclic GMP is a second messenger. It can mediate NO signalling by I) activation of cGMP-dependent protein kinase (60) (156) (180) II) regulation of phosphodiestrase activity (56) (114) and III) regulation of ion channels (5) (50). In smooth muscle cells elevation of cGMP activates cGMP dependent protein kinase G (PKG) (180). PKG can phosphorylate the inositol 1,4,5-triphosphate receptor (IP3)(141) and leads to a decrease in calcium concentration and finally muscle relaxation. However the significance of IP3 phosphorylation in the process of relaxation is not known. In summary relaxation of vascular smooth by NO could occur directly or indirectly by the elevation of cGMP which leads to activation of a K channel and relaxation of smooth muscle.

1.11- NO as a neurotransmitter:

Synaptic functions for NO were first established in peripheral nervous system. Researchers observed that certain actions of autonomic nervous system were not blocked by using cholinergic or adrenergic nerve fibers, which indicates that these non-adrenergic non-cholinergic (NANC) are mediated by other neurotransmitters. After EDRF was identified as a neurotransmitter, similarities between NANC and EDRF suggested that they are the same substances (27) (34). For example, bovine retractor penis muscle has inhibitory nerves whose neurotransmitter was unknown. Electrical stimulation relaxed this muscle and this was inhibited by oxyhemoglobin, which binds to nitric oxide (22). Definitive evidence in

these experiments was obtained when specific inhibitors of NOS were used such as monomethyl arginine (34). Thereafter, neurotransmission by nitric oxide was demonstrated in different tissues supplied by autonomic nerves including myentric plexus of gastrointestinal tract, cavernosal nerve and adventitia of deep cavernosal arteries, and cerebral blood vessels (54) (167)

Nitric oxide is an unusual neurotransmitter (195) because it is not released as packaged vesicles and is not released by exocytosis. In the central nervous system it is produced by activation of N-methyl-D-aspartic acid (NMDA) receptor in postsynaptic membrane. NMDA receptor activation allows Ca entry across postsynaptic membrane and results in increasing intracellular Ca. This in turn activates NOS, which produces NO. Nitric oxide can diffuse to presynaptic activating soluble guanylate cyclase to catalyze production of cGMP (146). Elevation of cGMP can affect the function of ion channeles, phosphodiestrase activity, or activate cGMP-dependent protein kinases that in turn affect subcellular function (146).

In addition to the brain, peripheral nerves use NO as neurotransmitter. For example, inhibition of NOS results in increased adrenergic vasoconstrictor response and regulation of bronchial vascular tone,. It is believed that ATP released from the sympathetic neurons causes the release of nitric oxide, which can act on the pulmonary vasculature to relax. Furthermore neurons of the gastrointestinal system, urinary tract, and vascular bed of bronchi stain positively for NOS. (237)

1.12- Septic Shock:

Septic shock is defined as a state of inadequate tissue perfusion for the metabolic demands of the body as a result of overwhelming bacterial infection (8) (201). It is the

cause of large number of death in North America (10) (203). Septic shock is characterized by hypotension, tachycardia, high cardiac output, and low systemic vascular resistance (92). The main cardiovascular manifestation of septic shock is a marked decrease in blood pressure despite adequate restoration of circulating blood volume and vasoconstrictor therapy (92).

Increased production of nitric oxide by the inducible form of nitric oxide synthase (NOSII) has been proposed to be a major factor in the hypotension and loss of vascular tone that occur in sepsis (175) (240) (247). For example incubation of vascular smooth muscle cells with tumor necrosis factor and interlukin-1 have been shown to induce NOSII and increase NO production. This induction is associated with increased cGMP and decreased contractile response to noradrenaline. Pre-treatment with L-NAME and cycloheximide abolishes the increase in cGMP and NOS activity and is independent of presence of Ca in these cells (36). Injection of LPS into rats, increases NOS activity 10-15 fold by 10 hours post injection. (139). LPS injection results in widespread expression of NOSII mRNA and its protein (113) (278). The induction of NOSII is associated with increased nitric oxide in exhaled gas (239) (172), increased concentration of plasma nitrite /nitrate in rats and mice(259) and a marked increase in Ca-independent NOS activity which is consistent with NOSII activation (278). Increased formation of nitric oxide by this isoform has been further supported by using selective inhibitors of this isoform (222). Inhibition of NOS restores blood pressure in endotoxic dogs (131) (133) rats (255) pigs (164) (172) and septic human (205). In LPS-treated mice, aminoguanidine, a selective inhibitor of NOSII increases the survival rate (278). Finally the role of NOSII has been tested in NOSII-gene knockout mice (163) (280). Survival

was significantly increased in knockouts. Furthermore mutant mice were resistant to LPS and were protected from cardiovascular collapse induced by LPS, whereas wild type mice died when challenged with LPS.

Nitric oxide itself or through the elevation of cGMP, leads to decreased intracellular Ca^{++}. This results in vasorelaxtion. Nitric oxide can also react with superoxide to produce peroxynitrite which participates in irreversible changes in tissues (35) (252) (59) (119) These changes probably cause passive relaxation of vessel to circulating volume and hypotension. On the other hand, there is a lot of evidence, which is inconsistent with the hypothesis that NO from the NOSII is responsible for the hemodynamic abnormalities of sepsis. Szabo et al (251) measured blood pressure and Ca-independent NOS activity as an indicator of NOSII induction. They found that hypotension occurred 2-3 hrs before the elevation of Ca-independent NOS activity. Expression of NOSII is not correlated with the level of NO (248) (262). INOS knockout mice had a reduced resistance to Listeria monocytogenase suggesting non-NOSII-dependent mechanism(s) are functioning (163). Cardiac output is decreased in rodents (222) and hypotension is biphasic. There is an immediate and transient decrease in pressure which recovers partially within 15 minutes followed by, a delayed and prolonged hypotension (113) (278) (239). However, in humans, cardiac output is not decreased by sepsis and hypotension is monophasic in response to LPS (245). The increase in the plasma concentration of nitrite /nitrate in endotoxemic rodents is 5-6 times greater than in control rats (259) (72), but the increase in septic patients is much less (190) (97). Moreover the sensitivity of rodents to LPS is much less than in humans. Rodents require doses of endotoxin (163) (270) (139) which are orders of magnitude

greater than those used in human studies (245). Although the presence of NOSII mRNA in human tissue is documented, there are only a few such reports. The role of NOSII in species other than mice and rodents is thus not clear.

1.13- Rational for our study:

The pathophysiological role of NOSII in hyperdynamic sepsis is not clear. Moreover it is not clear whether there is only increased NO production or increased production of radicals such as superoxide as well, which interact with NO to produce peroxynitrite. Therefore an objective of this study was to determine if NOSII plays a role in animals larger than rodents and second to investigate if O^{-}_{2} is increased and interacts with NO to produce peroxynitrite.

1.14- Animal models in Sepsis:

Many different animals and approaches have been used to study sepsis. LPS is one of the commonly used agents to induce sepsis. LPS, is a stable and relatively pure compound. LPS is convenient to use, for it can be stored in lyophilized form until it is used. LPS is a macromolecular glycolipid within the Gram-negative bacterial cell wall and is composed of a polysaccaride chain and a core. The polysaccharide chain accounts for antigenic variability among species and strains of Gram-negative bacteria, and the core has the toxic properties. In contrast to LPS, bacteria are typically stored frozen, grown in culture for 18-24 hours prior to use, washed several times to remove culture medium and solubilized bacterial products. Using fecal suspensions or bowel devascularization and perforation are other means of inducing sepsis by bacteria. Whereas doses of LPS can be accurately and readily measured, doses of live bacteria are not quantified accurately and it is more difficult to

ensure reproducibility of the septic challenge. In fact in septic models of bowel devascularization or perforation, the dose of infecting organism is not known.

The largest amount of experimental work is in rats. Small mammals (rats, mice, guinea pigs) are inexpensive to purchase and maintain. It is economically feasible to use a large sample size. Moreover they are suitable for studies in which organ function is examined such as Langendorf preparation to examine cardiac function. In addition rats are ideal for studies of microvascular function with the use of intravital microscopy to study blood flow in organs such as mesentry, kidney, or liver.

Although it is possible to measure cardiac output and pulmonary artery pressure in rats, catheterization of larger species (sheep, dogs, pigs, and primates) for the measurement of cardiovascular parameters such as ventricular pressure is easier. Because of their small circulating volume, blood sampling in rodents and mice is limited.

In rabbit models of sepsis, depending on the dosage of endotoxin that has been used, both low (279) and high (69) cardiac outputs with hypotension have been demonstrated. The dose of LPS is lower than in the rat. It has been observed that doses of 50-500 μg result in hypotension, decreased systemic vascular resistance and increased cardiac output (69).

Other animals, which have been used, are sheep (216) (158) (169). Adminitration of low doses of LPS over several days produces a hyperdynamic response in unanesthetized sheep. In this animal, catheters are positioned in the atria and arterial vessels. A low dose of .6-1.5 μg/kg of LPS is infused every 12-hour for 5 days and after 4-5 days of recovery the studies are performed. A high cardiac output does not develop as early as in other models such as pigs or humans, which may be because of the low dose or species difference (245). It has been also observed that the hyperdynamic pattern develops after 7-9 hours of 9-

24ng/kg.hr for 24 hours administration. Live bacteria (1.5x 10^9 microorganism) also have been used. The hyperdynamic phase is preceded by a initial drop in cardiac output (216). The results obtained from this model show that once the hyperdynamic pattern develops, it stays for a long time (169), suggesting that, this model is good for longer studies of sepsis. It is also easy to handle this model.

In pigs large doses of LPS 100-200μg/kg produce no change in cardiac output by 40 minutes, whereas the mean arterial pressure and systemic vascular resistance drop and they die (29) (185). Similar results have been obtained with.5μg/kg/hr, 5μg/kg/hr, and 15μg/kg/hr for 2 hours (138) (58) (275). We found that in this study (chapter two) by infusing an appropriate amount of fluid, to keep the right atrial pressure from falling, This hyperdynamic model is produced with a small increase in cardiac output and fall in blood pressure and a fall in systemic vascular resistance. This hyperdynamic model is closer to the human septic pattern than other septic models in the early phase of sepsis.

1.15- Role of NO in septic shock:

Increased nitric oxide production has been proposed to be a major factor in pathophysiology of sepsis and in particular, a cause of hypotension (240) (175). There are a number of pieces of evidence that support the concept of NO induced hypotension.

Thimermann and Vane (255) observed that injection of E.coli lipopolysaccharide (LPS) (15 mg/kg) into rats resulted in a long lasting low level of systemic arterial blood pressure. This hypotension was attenuated by L-NMMA, an inhibitor of NOS, but restored by injection of L-arginine, the substrate for NOS.

Excess production of NO from NOS, in particular NOSII, is believed to be responsible for the hypotension and loss of vascular tone. For example, Hom et al (113)

administered LPS intravenously into rats and observed hypotension in a biphasic pattern. The first phase of hypotension was immediate and transient and the second phase was prolonged and delayed. Pre-treatment with dexamethasone attenuated the prolonged second phase of hypotension. This indicates that it is likely that NOSII is induced during sepsis. Dexamethasone, a glucocorticoid, binds to glucocorticoid-responsive elements (GREs), and negatively regulates induction of cytokines and NOSII (233). It has been observed that pretreatment of rats with LNMMA significantly attenuated the hyporesponsiveness to NE which occurs with an infusion of LPS. Northern blot images of different tissues such as brain, heart, kidney, liver, lung, and spleen revealed a high level of induction of NOSII mRNA compared to non-LPS treated animals. Western blot analysis of the tissues in these animals shows a high level of induction of NOSII protein in septic rats but not control animals.

The presence of NOSII is associated with a large increase in plasma concentration of nitrate/nitrite (85) (103) (112), and Ca-independent NOS activity (139) (85). For example Gardiner et al injected LPS in rats and produced hypotension. The hypotension was associated with increased plasma nitrite NOII activity in heart, liver, lung and aorta. This was prevented by pretreatment of animals with L-NMMA. Knowles et al (139) used a spectrophotometric method to measure the oxidation of oxyhemoglobin to methemoglobin by NO as an index of NOS activity, as well as. L-citrulline generation when extracts of tissues were incubated with radiolabelled L-arginine to measure NOS activity. They injected different doses of LPS into rats and followed the experiment for different periods and collected lung, liver and brain tissues. They found an increase in NOS activity in the liver and lung, which was LPS dose-dependent, but it was not abolished by addition of EDTA, a

calcium chelator. This indicates that the increased NOS activity resulted from the Ca-independent isoform of NOS. They concluded that this isoform of NOS is likely the cause of increased nitrite level in endotoxin-treated rats.

Increase expired NO is also seen with endotoxin. Stewart et al (239) bled one group slightly to make them hypotensive and administered LPS (20 mg/kg) to another, to make them hypotensive. They also measured mean arterial pressure and collected expiratory gases for NO measurement by chemiluminescence. They showed that in normal hypotensive rats, NO production was not increased, but in LPS-treated rats it was increased after a few hours. Exsanguination of LPS-treated rats increased NO production further. Since this dose of LPS has been shown to upregulate NOSII in septic rats (113), and a few hours were required for the increase in it, it is likely that NOSII was responsible for this high level of expired NO. This group (113) believes that the lung produced the NO, and the NO was not from other tissues and transported by circulation. However, it can be argued that it is not only the lung but also pulmonary vessels that produced the NO, which was not bound to hemoglobin because of the exanguination (239).

Kilbourn and Belloni (132) tested the potential of cytokines to stimulate cultured murine endothelial cells to produce nitrite. INF-γ, in combination with tumor necrosis factor, interlukin-1 or endotoxin increased nitrite accumulation in the culture medium. Depletion of L-arginine from culture medium or addition of N^G-monomethyl-L-argininee (L-NMMA), an inhibitor of nitric oxide synthase blocked the accumulation of nitrite and addition of L-arginine resulted in production of nitrite by these endothelial cells. Thus stimulation of cultured endothelial cells with cytokines produced arginine-derived nitrogen oxide.

Cytokines increase the Ca-independent NOS activity. Busse and Mulsch (36) isolated smooth muscle cells from rabbit aorta and cultured them. They further prepared the cytosolic fractions and measured the cGMP produced by soluble guanylate cyclase as an index of NOS activity. They found that the cytosol obtained from TNFα/INFγ-treated segments induced a marked increase in guanylate cyclase (GC) activity which is consistent with the release of NO by increased NOS activity. In further support for a role of NOS, when the cytokine treated cytosol of cultured smooth muscle cells was treated with L-NNA, GC activity was decreased, but increased several fold when L-arginine was added. They also found that the GC activity of cytokine treated smooth muscle cells was Ca-independent indicating that it was likely NOSII.

Endotoxin also activates NOS activity in the vasculature. Fleming and her colleagues (71) incubated rat aortic rings in culture dishes that contained LPS for 5 hrs and measured contractile force followed by cGMP content of the rings as an index of NO release. Incubation of vessels with LPS, resulted in decreased tension and hyporeactivity to noradrenaline. They also found that the addition of LNMMA restored the contractile response in LPS-treated rings. The cGMP level of LPS-treated aortic rings was approximately 10 times higher than the cGMP level in un-treated aortic rings. Thus they demonstrated that endotoxin-induced vasodilation is cGMP dependent.

Patients who receive TNF as part of chemotherapy for cancer have a marked hypotension. Kilbourn and his colleagues (131) hypothesized that this could be due to TNF release of NO. Thus they administered TNF to anesthetized dogs and observed a fall in systemic blood pressure, which was reversed with an inhibitor of NO. Administration of L-arginine, the substrate of NOS, produced hypotension, and this was reversed by inhibitors of

NOS. However, when they administered nitroglycerine, which forms NO without NOS, followed by the administration of inhibitors of NOS, blood pressure was not restored. This indicates that TNF induces hypotension by activation of NOS, which releases NO.

The hypothesis of NO-induced hypotension also has been tested in humans. Petros et al (205) injected L-NMMA and N^G-nitro-L-argininee methyl ester (L-NAME) which inhibit NOS to two patients with sepsis to inhibit the production of NO. These two patients had a progressive fall of both systemic vascular resistance and blood pressure despite the use of vasoconstrictor and volume replacement. After treatment with NOS inhibitors, the hypotension was reversed and systemic vascular resistance increased, and in one of them the cardiac output increased, but in the other it decreased and the patient died because of recurrent intra-abdominal sepsis.

REFERENCE

1. Alonso-Galicia, M., A.G. Hudetz, H. Shen, D.R. Harder, and R.J. Roman. Contribution of 20-HETE to vasodilation action of nitric oxide in cerebral microcirculation. Stroke 30(12): 2727-2734, 1999.

2. Alonso, J., L. Sanchez de Minguel, M. Monton, S. Casado, and A. Lopez-Farre. Endothelial cytosolic proteins bind to the 3' untranslated region of endothelial nitric oxide synthase mRNA: regulation by tumor necrosis factor alpha. Mol.Cell Biol. 17: 5719-5726, 1997.

3. Aoki, T. Continuous flow determination of nitrite with membrane separation/chemiluminesence detection. Biomed.Chromatogr 4: 128-130, 1990.

4. Archer, S. Measurement of nitric oxide in biological models. FASEB J. 7: 349-360, 1993.

5. Archer, S.L., J.M. Huang, V. Hampl, D.P. Nelson, P.J. shultz, and E.K. Weir. Nitric oxide and cGMP cause vasodilation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc.Natl.Acad.Sci.USA 91: 7583-7587, 1994.

6. Arnet, U.A., A. McMillan, J.L. Dinerman, B. Ballerman, and C.J. Lowestein. Regulation of endothelial nitric oxide synthase during hypoxia. J.Biol.Chem. 271: 15069-15073, 1996.

7. Asano, K., C.B. Chee, B. Gaston, C.M. Lilly, C. Gerard, J.M. Drazen, and J.S. Stamler. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc.Natl.Acad.Sci.USA 91: 10089-10093, 1994.

8. Astiz, M.E., E.C. Rachow, J.L. Falk, B.S. Kaufman, and M.H. Weil. Oxygen delivery and consumption in patients with hyperdynamic septic shock. Crit.Care Med. 15: 26-28, 1987.

9. Auphan, N., J.A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 270: 286-290, 1995.

10. Avontuur, J.A., H.A. Bruining, and C. Ince. Sepsis and nitric oxide. Adv.Exp.Med.Biol. 388: 551-561, 1996.

11. Baeuerle, P.A. and T. Henkel. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12: 141-179, 1994.

12. Balligand, J.L., L. Kobzike, X. Han, D.M. Kaye, L. Belhassen, D.S. Ohara, R.A. Kelly, T.W. Smith, and T. Michel. Nitric oxide-dependent parasympathetic signalling is due to activation of constitutive endothelial

(type III) nitric oxide synthase in cardiac myocytes. J.Biol.Chem. 270: 14582-14586, 1995.

13. Bandypadhyay, A., S. Chakder, and S. Ratton. Regulation of inducible and neuronal nitric oxide synthase gene expression by interferone-gamma and VIP. Am.J.Physiol. 272: C1790-C17971997.

14. Bar, J., A. Zosmer, M. Hod, M.G. Elder, and M.H.F. Sullivan. The regulation of platelet aggregation in vitro by interlukin-1β and tumor necrosis factor-α changes in pregnancy and pre-eclampsia. Thromb.Haemost. 78: 1255-1261, 1997.

15. Barna, M., T. Komatsu, and C.S. Reiss. Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. Virology 223: 331-343, 1996.

16. Beckman, J.S., T.W. Beckman, J. chen, P.A. Marshall, and B.A. Freeman. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc.Natl.Acad.Sci.USA 87: 1620-1624, 1990.

17. Beckman, J.S. and J.P. Crow. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. Biochem.Soc.Trans. 21: 330-334, 1993.

18. Beckman, J.S. and W.H. Koppenol. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am.J.Physiol. 271: C1424-C14371996.

19. Block, K.D. Regulation of endothelial NO synthase mRNA stability. RNA-binding proteins crowd on 3'-untranslated region. Circ.Res. 85: 653-655, 1999.

20. Bolanos, I.P., S.J.R. Heales, J.M. Land, and J.B. Clark. Effect of peroxynitrite on mitochondrial respiratory chain: differential suseptibility of neurons and astrocytes in primary culture. J.Neurochem 64: 1965-1972, 1995.

21. Bolotina, V.M., S. Najibi, J.J. Palacino, P.J. Pagano, and R.A. Cohen. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 368: 850-853, 1994.

22. Bowman, A., J.S. Gillespie, and D. Pollock. Oxyhemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. Eur.J.Pharmacol. 85: 221-224, 1982.

23. Bredt, D.S., C.E. Gatt, P.M. Hwang, M. Fotuchi, T.M. Dawson, and S.H. Snyder. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7: 615-624, 1991.

24. Bredt, D.S. and S.H. Snyder. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc.Natl.Acad.Sci.USA 87: 682-685, 1990.

25. Bredt, D.S. and S.H. Snyder. Nitric oxide, a novel neuronal messenger. Neuron 8: 3-11, 1992.

26. Bredt, D.S. and S.H. Snyder. Nitric Oxide: a Physiologic Messenger Molecule. Ann.Rev.Biochem. 63: 175-195, 1994.

27. Brenman, J.E. and D.S. Bredt. Nitric oxide signalling in nervous system. Methods Enzymol. 269: 119-129, 1996.

28. Brenman, J.E., D.S. Chao, H.H.A.K. Xia, and D.S. Bredt. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular. Cell (82)8: 743-752, 1995.

29. Breslow, M.J., C.F. Miller, S.D. Parker, A.T. Walman, and R.J. Traystman. Effect of vasopressors on organ blood flow during endotoxin shock in pigs. Am.J.Physiol. 252(2pt2): H291-H3001987.

30. Brovkovych, V., S. Patton, F. Brovkovych, L. Kiechle, I. Huk, and T. malinski. In situ measurement of nitric oxide, superoxide and peroxynitrite during endotoxemia. J.Physiol.Pharmacol. 48(4): 633-644, 1997.

31. Brune, B. and K. Hanstein. Rapid reversibility of nitric oxide induced platelet inhibition. Thromb.Res. 90: 83-91, 1998.

32. Brune, B. and E.G. Lapentina. Phosphorylation of nitric oxide synthase by protein kinase A. Biochem.Biophys.Res.Commun. 181: 921-926, 1991.

33. Bucher, M., K.P. Ittner, M. Zimmermann, K. Wolf, J. Hobbhahn, and A. Kurtz. Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteic acid. FEBS Lett 412: 511-514, 1997.

34. Bult, H., G.E. Boeckxstaens, P.A. Pelckmans, F.H. Jordaens, Y.M. Van Maercke, and A.G. Herman. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345: 346-377, 1990.

35. Burney, S., J.L. Caulfield, J.C. Niles, J.S. Wishnok, and S.R. Tannenbaum. The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat.Res. 424: 37-49, 1999.

36. Busse, R. and A. Mulsch. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett 275(1-2): 87-90, 1990.

37. Campbell D.L., J.S. Stamler, and H.C. Strauss. Redox modulation of L-type calcium channel in ferret ventricular myocytes. J.Gen.Physiol. 108: 277-293, 1996.

38. Carmichael, A.J. Reactions of active oxygen and nitrogen species studied by EPR and spin trapping. Free Radic.Res.Commun 19 Suppl 1: S1-S161993.

39. Carville, C., S. Adnot, S. Eddahibi, E. Teiger, D. Rideau, and B. Raffestin. Induction of nitric oxide synthase activity in pulmonary arteries from normoxic and chronically hypoxic rats. Eur.Respir.J. 10: 437-445, 1997.

40. Castro, L., R. Radi, and Radi. Acontitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J.Biol.Chem. 269: 29409-29415, 1994.

41. Ceccatelli, S., A.L. Hulting, X. Zhang, L. Gustafsson, M. Villar, and T. Hokfelt. Nitric oxide synthase in the rat anterior pituitary gland and the role of the nitric oxide synthase in regulation of luteinizing hormone secretion. Proc.Natl.Acad.Sci.USA 90: 11292-11296, 1993.

42. Chartrain, N.A., D.a. Geller, P.P. Koty, N.F. Sitrin, A.k. Nussler, E.P. Hoffman, T.R. Billiar, N.I. Hutchinson, and J.S. Mudgett. Molecular cloning, structure, and chromosomal localization of human inducible nitric oxide synthase gene. J.Biol.Chem. 269: 6765-6774, 1994.

43. Chen, L.Y. and J.L. Mehta. Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets. J.Cardiovas.Pharmacol. 27: 154-158, 1996.

44. Cherry, P.D., R.F. Furchgott, J.V. Zawadzki, and D. Jothianandan. Role of endothelial cells in relaxation of isolated arteries by bradykinin. Proc.Natl.Acad.Sci.USA 79: 2106-2110, 1982.

45. Cho, H.J., E. Martin, Q.W. Xie, S. Sassa, and C. Nathan. Inducible nitric oxide synthase: identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin. Proc.Natl.Acad.Sci.USA 92: 11514-11518, 1995.

46. Clementi, E., C. Sciorati, M. Riccio, M. Miloso, J. Meldolesi, and G. Nistico. Nitric oxide action on growth factor elicited signals. J.Biol.Chem. 270: 22277-22282, 1995.

47. Cockrell, A., S. Laroux, D. Jourdheuil, s. Kawachi, L. Gray, H. Van der Heyde, and M.B. Grisham. Role of inducible nitric oxide synthase in leukocyte extravasation in vivo. Biochem.Biophys.Res.Commun. 257: 684-686, 1999.

48. Comin, L., T. Bachetti, G. Gaia, E. Pasini, L. Agnoletti, P. Pepi, C. Ceconi, s. Curello, and R. Ferrari. Aorta and skeletal muscle NO synthase expression in experimental heart failure. J.Mol.Cell Cardiol. 28: 2241-2248, 1996.

49. Cornwell, T.L. and T.M. Lincoln. Regulation of intracellular ca^{++} level in cultured vascular smooth muscle cells. J.Biol.Chem. 264(2): 1146-1155, 1989.

50. Corrier, G.O., L.C. Fuchs, A.P. Winecoff, A.D. Ginlumian, and R.E. White. Nitrovasodilators relax mesentric microvessels by cGMP-induced stimulation of ca-activated K channels. Am.J.Physiol. 273(42): H76-H841997.

51. D'Acquisto, F., L. Sautebin, T. Iuvone, M.D. Rosa, and R. Carnuccio. Prostaglandins prevent inducible nitric oxide synthase protein expression by inhibiting nuclear factor-κB activation in J774 macropgages. FEBS Lett 440: 76-80, 1998.

52. D'iaz-Guerra, M.J.M., A. Castrillo, P. Martin-Sanz, and L. Bosca. Negative regulation by phosphatidylinositole 3-kinase of inducible nitric oxide synthase expression in macrophages. J.Immunol. 162: 6184-6190, 1999.

53. Davisson, R.L., M.D. Travis, J.N. Bates, and S.J. Lewis. Hemodynamic effects of L-S-nitrosocysteine in the rat. Circ.Res. 79: 256-262, 1996.

54. Dawson, T.M. and V.L. Dawson. Nitric oxide synthase: role as a transmitter/ mediator in brain and endocrine system. Ann.Rev.Med. 47: 219-227, 1996.

55. DeAlba, J., A. Cardenas, M.A. Moro, J.C. Leza, P. Lorenzo, and Lizasoain.I. Down-regulation of neuronal nitric oxide synthase by nitric oxide after oxygen-glucose deprivation in rat forebrain slices. J.Neurochem 72: 248-254, 1999.

56. Degerman, E., P. Belfrage, and V.C. Manganiello. Structure, localization, and regulation of cGMP-inhibited phosphodiestrase (PDE3). J.Biol.Chem. 272: 6823-6826, 1997.

57. Dimmeler, S., I. Flemming, B. Fisslthaler, C. Hermann, R. Busse, and A.M. Zeiher. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399: 601-605, 1999.

58. Dimmeler, S., C.K. Kum, C. Troost, M. Auweiler, A. Lechleuthner, M. Nagelschmidt, and E. Neugebauer. H1-antagonism improves intestinal mucosal pH and heart function in porcine hypodynamic endotoxic shock. Shock 5(3): 213-216, 1996.

59. Ducrocq, C., B. Blanchard, B. Pignatelli, and H. Ohshima. Peroxynitrite: an endogenous oxidizing and nitrating agent. Cell Mol.Life Sci. 55: 1068-1077, 1999.

60. Ecky-Mitchel, A., V. Martin, and C. Lugnier. Involvement of cyclic nucleotide-dependent protein kinase in cyclic AMP-mediated vasorelaxation. B.J.Pharmacol. 122: 158-164, 1997.

61. Eizirik, D.L., A. Bjorklund, and N. Welsh. Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-fos induction and depends on gene transcription and protein synthesis. FEBS Lett 317: 62-66, 1993.

62. Evans, T., A. Carpenter, and J. Cohen. Purification of a distinctive form of endotoxin-induced nitric oxide synthase from rat liver. Proc.Natl.Acad.Sci.USA 89: 5361-5365, 1992.

63. Evans, T.G., L.D. Buttery, A. Carpenter, D.R. Springall, J.M. Pollock, and J. Cohen. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. Proc.Natl.Acad.Sci.USA 93: 9553-9558, 1996.

64. Feldman, P.L., O.L. Griffith, and D.J. Stuehr. The surprising life of nitric oxide. Chem.Eng.News 71: 26-38, 1993.

65. Feron, O., L. Belhassen, L. Kobzik, T.W. Smith, R.A. Kelly, and T. Michel. Endothelial nitric oxide synthase targeting to caveolae. J.Biol.Chem. 271: 22810-22814, 1996.

66. Feron, O., L. Belhassen, L. Kobzik, T.W. Smith, R.A. Kelly, and T. Michel. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes. J.Biol.Chem. (271)37: 22810-22814, 1996.

67. Feron, O., J.B. Michel, K. Sase, and T. Michel. Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. Biochemistry 37: 193-200, 1998.

68. Fierro, I.M., V. Nascimiento-DaSilva, M.A. Arruda, M.S. Freitas, M.C. Plotkowski, F.Q. Cunha, and C. Barja-Fidalgo. Induction of NOS in rat blood PMN in vivo and in vitro: modulation by tyrosine kinase and involvement in bactericidal activity. J.Leuko.Biol. 65: 508-514, 1999.

69. Fink, M.P., V. Fiallo, K.L. Stein, and W.M. Gardiner. Systemic and regional hemodynamic changes after intraperitoneal endotoxin in rabbits: development of a new model of the clinical syndrome of hyperdynamic sepsis. Circ.Shock 22(1): 73-81, 1987.

70. Fiscus, R.R. Molecular mechanism of endothelium-enhanced vasodilation. Semin.Thromb.Hemost. 14 Suppl I: 12-22, 1988.

71. Fleming, I., G.A. Gray, G. julou-Schaeffer, J.R. Parratt, and J.C. Stoclet. Incubation with endotoxin activates the L-arginine pathway in vascular tissue. Biochem.Biophys.Res.Commun. 171(2): 562-568, 1990.

72. Florquin, S., Z. Amraoni, C. Dubois, J. Decuyper, and M. Goldman. The protective role of endogenously synthesized nitric oxide in staphyloccocal enterotoxin B-induced shock in mice. J.Exp.Med. 180: 1153-1158, 1994.

73. Forstermann, U., J.-P. Boissel, and H. Kleinert. Expressional control of the constitutive isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J. 12: 773-790, 1998.

74. Forstermann, U., E.I. Closs, J.S. Pollack, M. Nakane, P. Schwarz, I. Gath, and H. Kleinert. Nitric oxide synthase isoenzymes characterization, purification, molecular cloning, and function. Hypertension 23 (part2): 1121-1131, 1994.

75. Forstermann, U. and H. Kleinert. Nitric oxide synthase: expression and expressional control of three isoforms. Naunyn-Schmledebergs Arch Pharmacol 352: 351-364, 1995.

76. Forstermann, U. and B. Neufang. The endothelium-dependent vasodilation effect of acetylcholine: characterization of endothelial relaxing factor with inhibitors of arachidonic acid metabolism. Eur.J.Pharmacol. 103: 65-70, 1984.

77. Forstermann, U., J.S. Pollock, H.H.H.W. Schmidt, M. Heller, and F. Mourad. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. Proc.Natl.Acad.Sci.USA 88: 1788-1792, 1991.

78. Fujii, H. and L.J. Berliner. Ex vivo EPR detection of nitric oxide in brain tissue. Magn.Reson.Med. 42(3): 599-602, 1999.

79. Fulton, D., J.-P. Gratton, T.J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T.F. Franke, A. Papapetropouloa, and W.C. Sessa. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399(10): 597-601, 1999.

80. Furchgott, R.F., P.D. Cherry, J.V. Zawadzki, and D. Jothianandan. Endothelial cells as a mediators of vasodilation of arteries. J.Cardiovas.Pharmacol. 6 Suppl 2: S336-S3431984.

81. Furchgott, R.F. and P.M. Vanhoutte. Endothelium-derived and contracting factors. FASEB J. 3: 2007-2018, 1989.

82. Furchgott, R.F. and J.V. Zawadzki. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373-376, 1980.

83. Garcia-Duran, M., T. deFrutos, J. Diaz-Recasens, G. Garcia-Galvez, A. Jimeriez, M. Monton, J. Farre, L. Sanchez de Minguel, F. Gonzalez-Femandez, M.D. Arriero, L. Rico, R. Garcia, S. Casado, and A. Lopez-Farre. Estrogen stimulates neuronal nitric oxide synthase protein expression in human neutrophils. Circ.Res. 85: 1020-1026, 1999.

84. Garcia-Cardena, G., R. Fan, V. Shah, R. Sorrentino, G. Cirino, A. Papapetropouloa, and W.C. Sessa. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 392: 821-824, 1998.

85. Gardiner, S.M., P.A. Kemp, J.E. March, and T. Bennett. Cardiac and regional haemodynamics, inducible nitric oxide synthase (. B.J.Pharmacol. 116: 2005-2016, 1995.

86. Gaston, B. Nitric oxide and thiol group. Biochim.Biophys.Acta 1411: 323-333, 1999.

87. Gaston, B., J.M. Drazen, J. Loscalzo, and J.S. Stamler. The biology of nitrogen oxides in the airways. Am.J.Respir.Crit.Care Med. 149: 538-551, 1994.

88. Gath, I., E.I. Closs, U. Gödtel-Armbrust, S. Schmitt, M. Nakane, I. Wessler, and U. Forstermann. Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications for contractile function. FASEB J. 10: 1664-1620, 1996.

89. Gath, I., U. Gödtel-Armbrust, and U. Forstermann. Expressional downregulation of neuronal-type NO synthase I in guinea pig skeletal muscle in response to bacterial lipopolysaccharide. FEBS Lett 410: 319-320, 1997.

90. Geng, Y., M. Almqvist, and G.K. Hansson. cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. Biochim.Biophys.Acta 1218: 421-424, 1994.

91. Giuilli, G., A. Luzi, M. Poyard, and G. Guellaen. Expression of mouse brain soluble guanylate cyclase and NO synthase during ontogeny. Brain Res.Dev.Brain Res. 81: 269-283, 1994.

92. Gomez-Jimenez, J., A. Salgado, M. Mourella, M.C. Martin, R.M. Segura, R. Peracaula, and S. Moncada. L-arginine: nitric oxide pathway in endotoxemia and human septic shock. Crit.Care Med. 23: 253-258, 1995.

93. Goren, N., C.P. Leiros, L. Sterin-Borda, and E. Borda. Nitric oxide synthase in experimental autoimmune myocarditis dysfunction. J.Mol.Cell Cardiol. 30: 2467-2474, 1998.

94. Green, L.C., S.R. Tannenbaum, and P. Goldman. Nitrate synthesis in the germ free and conventional rat. Science 212: 56-58, 1981.

95. Gries, A., C. Bode, K. Peter, A. Herr, H. Bohrer, J. Motsch, and E. Martin. Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding in vitro and in vivo. Circulation 97: 1481-1487, 1998.

96. Griscavage, J.M., N.E. Rogers, M.P. Sherman, and L.J. Ignarro. Inducible nitric oxide synthase from rat alveolar macrophage cell line is inhibited by nitric oxide. J.Immunol. 151: 6329-6337, 1993.

97. Groenld, P.H.P., K.M.C. Kwappenberg, J.A.M. Langermans, P.H. Nibbering, and L. Curtis. Nitric oxide (NO) production correlates with renal insufficiency and multiple organ dysfunction syndrome in severe sepsis. Intensive Care Med. 22: 1197-1202, 1996.

98. Guo, F.H., H.R. DeRaeve, T.W. Rice, D.J. Stuehr, F.B. Thunnissen, and S.C. Erzurum. Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. Proc.Natl.Acad.Sci.USA 92: 7809-7813, 1995.

99. Guo, Y., M.E. Ward, S. Beasjours, M. Mori, and S.N.A. Hussain. Regulation of cerebellar nitric oxide production in response to prolonged in vivo hypoxia. J.Neurosci. 49: 89-97, 1997.

100. Hall, A.V., H. Antoniou, Y. Wang, A.H. Cheung, A.M. Arbua, S.L. Olson, W.C. Lu, C.L. Kau, and P.A. Marsden. Structural organization of the human neuronal nitric oxide synthase gene (NOSI). J.Biol.Chem. 269: 33082-33090, 1994.

101. Harada, S., T. Imaki, N. Chikada, M. Naruse, and H. Demura. Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection. Brain Res. 821(2): 322-332, 1999.

102. Hattori, Y., K. Akimoto, N. Nakanishi, and K. Kasai. Glucocorticoid regulation of nitric oxide and tetrahydrobiopterin in a rat model of endotoxic shock. Biochem.Biophys.Res.Commun. 240: 298-303, 1997.

103. Haywood, G.A., P.S. Tsao, E. van der Leyon, M.J. Mann, P.J. Keeling, P.T. Trindade, N.P. Lewis, C.D. Byrne, P.R. Rickenbacher, N.H. Bishopric,

J.P. Cooke, W.J. McKenna, and M.B. Fowler. Expression of inducible nitric oxide synthase in human heart failure. Circulation 93: 1087-1094, 1996.

104. Heck, D.E., D.L. Laskin, C.r. Gardner, and J.D. Laskin. Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of wound healing. J.Biol.Chem. 267: 21277-21280, 1992.

105. Hecker, M., A.T. Bara, J. Banersachs, and R. Busse. Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. J.Physiol. 481 (pt2): 407-414, 1994.

106. Henry, Y., M. Lepoivre, J. Drapier, C. Ducrocq, J. Boucher, and A. Guissani. EPR characterization of molecular targets for NO in mammalian cells and organelles. FASEB J. 7: 1124-1134, 1993.

107. Henry, Y., M. Lepoivre, J.C. Drapier, and C. Ducrocq. EPR characterization of molecular targets for NO in mammalian cells and organelles. FASEB J. 7: 1124-1134, 1993.

108. Hess, D.T., S.L. Atterson, and D.S. Mith. Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. Nature 366: 562-565, 1993.

109. Hevel, J.M., K.A. White, and M.A. Marletta. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. J.Biol.Chem. 266: 22789-22791, 1991.

110. Hibbs, J.B., R.R. Taintor, and Z. Varin. Macrophage cytotoxicity: role of L-arginine deiminase and imino nitrogen oxidation to nitrite. Science 235: 473-476, 1987.

111. Hock, C.E., K. Yin, G. Yue, and P.Y. Wong. Effects of inhibition of nitric oxide synthase by aminoguanidine in acute endotoxemia. Am.J.Physiol. 272: H843-H8501997.

112. Hom, J.G., S.K. Grant, G. Wolf, J. Tom, D. Bach, E. MacIntyre, and N.I. Hutchinson. Lipopolysaccharide-induced hypotension and vascular hyporeactivity in the rat: tissue analysis of nitric oxide synthase mRNA and protein expression in the presence and absence of dexamethazone, N^G-monomethyl-L-arginine or indomethacine. J.Pharmacol.Exp.Ther. 272: 452-459, 1995.

113. Houslay, M.D. and G. Milligan. Tailoring cAMP-signalling response through isoform multiplicity. Trends Biochem.Sci. 22: 217-224, 1997.

114. Ignarro, L.J. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu.Rev.Pharmacol.toxicol. 30: 535-560, 1990.

115. Ignarro, L.J., G.M. Buga, K.S. Wood, R.E. Byrns, and G. Chaudhuri. Endothelium-derived releasing factor produced and released from artery and vein is nitric oxide. Proc.Natl.Acad.Sci.USA 84: 926-9669, 1987.

116. Ignarro, L.J. and C.A. Gruetter. Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. Biochim.Biophys.Acta 631: 221-231, 1980.

117. Ischiropoulos, H. and A.B. Al-Mehdi. Peroxynitrite-mediated oxidative protein modifications. FEBS Lett 364: 279-282, 1995.

118. Ischiropoulos, H., L. Zhu, J. Chaen, M. Tsai, J.C. Martin, C.D. Smith, and J.S. Beckman. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch.Biochem.Biophys. 298(2): 431-437, 1992.

119. Iwai, N., K. Hanasi, I. Tooyama, Y. Kitamura, and M. Kinoshita. Regulation of neuronal nitric oxide synthase in rat adrenal medulla. Hypertension 25: 431-436, 1995.

120. Iyengar, R., D.J. Stuehr, and M.A. Marletta. Macrophage synthesis of nitrite, nitrate, and N-nitrosamine: precursors and role of the respiratory burst. Proc.Natl.Acad.Sci.USA 84: 6369-6373, 1987.

121. Jaffery, S.R., N.A. Cohen, T.A. Rouault, and R.D. Klausner. The iron responsive element binding protein: A novel target for actions of nitric oxide. Proc.Natl.Acad.Sci.USA 91: 12994-12998, 1994.

122. Jaffrey, S.R., A.M. Snowman, M.J. Eliasson, N.A. Cohen, and N.H. Snyder. CAPON:a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 20: 115-124, 1998.

123. Jaffrey, S.R. and S.H. Snyder. PIN an associated protein inhibitor of neuronal nitric oxide synthase. Science 274: 774-777, 1996.

124. Javeshghani, D., D. Sakkal, M. Mori, and S.N.A. Hussain. Regulation of diaphragmatic nitric oxide synthase expression during hypobaric hypoxia. Am.J.Physiol.Lung Cell Mol.Physiol. 279: L520-L5272000.

125. Jeong, Y., J. Won, and J. Yim. Cloning and structure of rabbit protein inhibitor of neuronal nitric oxide synthase (PIN) gene and its pseudogene. Gene 214: 67-75, 1998.

126. Katsuki, S., W.P. Arnold, C. Mittal, and F. Murad. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J.cyclic nucleotide Res. 3: 23-35, 1977.

127. Katsuki, S., W.P. Arnold, and F. Murad. Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and

mechanical activity of various tissues. J.cyclic nucleotide Res. 3: 239-247, 1977.

128. Khatsenko, O.G., S.S. Gross, A. Rifkind, and J.R. Vane. Nitric oxide is the mediator of the disease in cytochrome p450-dependent metabolism caused by immunostimulants. Proc.Natl.Acad.Sci.USA 90: 11147-11151, 1993.

129. Kiechle, F.LK. and T. malinski. Nitric oxide biochemistry, pathophysiology, and detection. Am.J.Clin.Pathol. 100: 567-575, 1993.

130. Kilbourn, R., S. Gross, A. Jubran, J. Adams, O. Griffith, R. Levi, and R. Lodata. N^G -methyl-L-arginine inhibits tumor necrosis factor-induced hypotension. Implication for the involvement of nitric oxide. Proc.Natl.Acad.Sci.USA 87: 3629-3632, 1990.

131. Kilbourn, R.G. and P. Belloni. Endothelial cell production of nitrogen oxides in response to interferon in combination with tumor necrosis factor, interlukin-1 or endotoxin. J.Natl.Cancer Inst. 82: 772-776, 1990.

132. Kilbourn, R.G., A. Jubran, S.S. Gross, O.W. Griffith, R. Levi, J. Adams, and R.F. Lodato. Reversal of endotoxin mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis. Biochem.Biophys.Res.Commun. 172: 1132-1138, 1990.

133. Kirkeboen, K.A. and A. Strand. The role of nitric oxide in sepsis-an overview. Acta Anaesthesiol Scand 43: 275-288, 1999.

134. Kishimoto, J., T. Tsuchiya, P.C. Emson, and Y. Nakayama. Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic-pituitary-adrenal axis in rats. Brain Res. 720: 159-171, 1994.

135. Kleinert, H., C. Euchenhofer, I. Ihrig-Biedert, and U. Forstermann. Glucocorticoids inhibit the induction of nitric oxide synthase II by down regulating cytokine-induced activity of transcription factor nuclear factor-Kappa B. Mol.Pharmacol. 49: 15-21, 1996.

136. Klem.M., R. Dahmann, D. Wink, and M. Feelisch. The nitric oxide / superoxide assay. J.Biol.Chem. 272: 9922-9932, 1997.

137. Klosterhalfen, B., K. Horstmann-Jungemann, P. Vogel, G. Dufhues, B. Simon, G. Kalff, C.J. Kirkpatrick, C. Mittermayer, and P.C. Heinrich. Hemodynamic variables and plasma levels of PGI2, TXA2 and IL-6 in a porcine model of recurrent endotoxemia. Circ.Shock 35(4): 237-244, 1991.

138. Knowles, R.G., M. Merrett, M. Salter, and S. Moncada. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem.J. 270: 833-836, 1990.

139. Kojima, M., T. Morisaki, Y. Tsukahara, A. Uchiyama, Y. Matsunari, R. Mibu, and M. Tanaka. Nitric oxide synthase expression and nitric oxide

production in human colon carcinoma tissue. J.Surg.Oncol. 70: 229-229, 1999.

140. Komalavilas, P. and T.M. Lincoln. Phosphorylation of the inositol 1,4,5-triphosphate receptor. Cyclic GMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in the intact rat aorta. J.Biol.Chem. 271: 21933-21938, 1996.

141. Komori, K. and P.M. Vanhoutte. Endothelium-dervied hyperpolarizing factor. Blood Vessels 27: 238-245, 1990.

142. Kone, B.C. Protein-protein interactions controlling nitric oxide synthases. Acta Physiol.Scand. 168: 27-31, 2000.

143. Kontos, H.A., E.P. Wei, J.T. Povlishock, and C.W. Christman. Oxygen radicals mediate the cerebral arteriolar dilation from arachidonate. Circ.Res. 55: 295-303, 1984.

144. Kosonen, O., H. Kankaanranta, U. Malo-Ranta, and E. Moilanen. Nitric oxide-releasing compounds inhibit neutrophil adhesion to endothelial cells. Eur.J.Pharmacol. 382: 111-117, 1999.

145. Krakaff, T.L. Central actions of nitric oxide in regulation of autonomic functions. Brain Res.Brain Res.Rev. 30: 52-65, 1999.

146. Kurihara, N., M.E. Alfie, D.H. Sigmon, N. Rhaleb, E.G. Shesely, and O.A. Carretero. Role of nNOS in blood pressure regulation in eNOS null mutant mice. Hypertension 32: 856-861, 1998.

147. Lamas, S., P.A. Marsden, G.K. Li, P. Tempst, and T. Michel. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc.Natl.Acad.Sci.USA 89: 6368-6352, 1992.

148. Lefer, D.J., S.P. Jones, W.G. Girod, A. Baines, M.B. Grisham, A.S. Cockrell, P.L. Huang, and R. Scalia. Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. Am.J.Physiol. 276(45): H1943-H19501999.

149. Lefer, D.J., R. Scalia, B.N.T. Campbell, R. Hayward, M. Salamon, J. Grayson, and A.M. Lefer. Peroxynitrite inhibits leukocyte - endothelial cell interactions and protects against ischemia - reperfusion injury in rats. J.Clin.Invest. 99: 684-691, 1997.

150. Liao, J.k., J.J. Zulueta, F.S. Yu, H.B.C.C.G. Peng, and P.M. Hassoun. Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. J.Clin.Invest. 96: 2661-2666, 1995.

151. Liu, J., T.E. Hughes, and W.C. Sessa. The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase

into the Golgi region of cells: a green fluorescent protein study. J.Cell Biol. 137: 1525-1535, 1997.

152. Liu, J. and W.C. Sessa. Identification of covalently bound amino-terminal myristic acid in endothelial nitric oxide synthase. J.Biol.Chem. 269: 11691-11694, 1994.

153. Liu, S., J.S. Beckman, and D.D. Ku. Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. J.Pharmacol.Exp.Ther. 268(3): 1114-1121, 1994.

154. Liu, S.F., I.M. Adcock, R.W. Old, P.J. Barnes, and T.W. Evans. Differential regulation of the constitutive and inducible nitric oxide synthase mRNA by lipopolysaccharide treatment in vivo in the rat. Crit.Care Med. 24: 1219-1225, 1996.

155. Lohmann, S.M., A.B. Vaaandrager, A. Smolenski, U. Walter, and H.R. De Jonge. Distinct and specific functions of cGMP-dependent protein kinases. Trends Biochem.Sci. 22: 307-312, 1997.

156. Lonchampt, M.O., M. Auguet, S. Delaflotte, J. Goulin Schulz, P.E. Chabrier, and P. Braquet. Lipoteic acid: a new inducer of nitric oxide synthase. J.Cardiovas.Pharmacol. 20 Suppl 12: S145-S1471992.

157. Lorente, J.A., L. Landin, E. Renes, R. Depablo, P. Jorge, and D. Liste. Role of nitric oxide in the hemodynamic changes of sepsis. Crit.Care Med. 21(5): 759-767, 1993.

158. Lorsbach, R.B., W.J. Murphy, C.J. Lowestein, S.H. Snyder, and S.W. Russell. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. J.Biol.Chem. 268: 1908-1913, 1993.

159. Lowestein, C.J., J.L. Dinerman, and S.H. Snyder. Nitric oxide: A Physiologic Messenger. Ann.Intern.Med. 120: 227-237, 1994.

160. Luscher, T.F. and M. Barton. Biology of endothelium. Clin.Cardiol. 11 suppl II: 3-10, 1997.

161. MacMicking, J., Q.W. Xie, and C. Nathan. Nitric oxide and macrophage function. Annu.Rev.Physiol. 15: 323-350, 1997.

162. MacMicking, J.D., C. Nathan, G. Hom, N. Chartrain, D.S. Fletcher, M. Trumbauer, K. Stevens, Q.W. Xie, K. Sokol, N.I. utchinson, H. hen, and J.S. udgett. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81: 641-650, 1995.

163. Magder, S. and G. Vanelli. Circuit factors in high cardiac output of sepsis. J.Crit.Care 111: 155-166, 1996.

164. Marietta, M., F. Facchinetti, I. Neri, F. Piccinini, A. Volpe, and G. Torelli. L-arginine infusion decreases platelet aggregation through intraplatelet nitric oxide release. Thromb.Res. 88: 229-235, 1997.

165. Maro, M.A., V.M. Darley-Usmer, D.A. Goodwin, N.G. Reed, R. Zamora-Pino, M. Feelisch, M.W. Radomski, and S. Moncada. Paradoxical fate and biological action of peroxynitrite on human platelets. Proc.Natl.Acad.Sci.USA 91: 6702-6706, 1994.

166. Mashimo, H., X.D. He, P.L. Haung, M.C. Fishman, and R.K. Goyal. Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmitter. J.Clin.Invest. 98: 8-13, 1996.

167. Mayer, B., M. John, and E. Bohme. Purification of a calcium /calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor role of tetrahydrobiopterin. FEBS Lett 277: 215-219, 1990.

168. Mayor, J., L.D. Traber, S. Nelson, C.W. Lentz, H. Nakazawa, D.N. Herndon, H. Noda, and D.L. Traber. Reversal of hyperdynamic response to continious endotoxin administration by inhibition of NO synthesis. J.Appl.Physiol. 73(1): 324-328, 1992.

169. McCall, T.B., N.K. Boughton-Smith, R.M.J. Palmer, B.J.R. Whittle, and S. Moncada. Synthesis of nitric oxide from L-arginine by neutrophils: release and interaction with superoxide anion. Biochem.J. 261: 293-296, 1989.

170. McDonald, L.J. and F. Murad. Nitric oxide and cyclic GMP signalling. Proc.Natl.Acad.Sci.USA 211(1): 1-6, 1996.

171. Metha, S., D. Javeshghani, P. Datta, R.D. Levy, and S. Magder. Porcine endotoxemic shock is associated with increased expired nitric oxide. Crit.Care Med. 27(2): 385-393, 1999.

172. Michel, J.B., O. Feron, S. Socks, and T. Michel. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. J.Biol.Chem. (272)25: 15583-15586, 1997.

173. Michel, T. and S. Lamas. Molecular cloning of constitutive endothelial nitric oxide synthase: evidence for a family of related genes. J.Cardiovas.Pharmacol. 20 suppl.12: S45-S491992.

174. Moncada, S. and A. Higgs. The L-arginine-nitric oxide pathway. New Engl.J.Med. 329: 2002-2012, 1993.

175. Moncada, S., R.M.J. Palmer, and E.A. Higgs. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol.Rev. 43: 109-142, 1991.

176. Moro, M.A., V.M. Darley-Usmer, I. Lizasoain, Y. Su, R.G. Knowles, M.W. Radomski, and S. Moncada. The formation of nitric oxides from peroxynitrite. B.J.Pharmacol. 116: 1999-2004, 1995.

177. Moro, M.A., J. DeAlba, J.C. Leza, P. Lorenzo, A.P. Ernandez, M.L. Entura, L. Bosca, J. Rodrigo, and I. Lizasoain. Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. Eur.J.Neurosi. 10: 445-456, 1998.

178. Morris, S.M.Jr. and T.R. Billiar. New insights into regulation of inducible nitric oxide synthesis. Am.J.Physiol. 266: E829-E8391994.

179. Murthy, K.S. and G.M. Makhlouf. Differential regulation of phospholipase A_2 (PLA_2)-dependent ca^{++}-signaling in smooth muscle by cAMP- and cGMP-dependent protein kinases. J.Biol.Chem. 273: 34519-34526, 1998.

180. Nakane, M., H.H. Schmidt, J.S. Pollock, U. Forstermann, and F. Murad. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett 316: 175-180, 1993.

181. Nakayama, D.K., D.a. Geller, C.J. Lowestein, P. Davies, B.R. Pitt, R.L. Simons, and T.R. Billiar. Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle. Am.J.Respir.Cell Mol.Biol. 7: 471-476, 1992.

182. Nathan, C.F. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-3064, 1992.

183. Nishida, K., D.G.N.J.P. Harrison, A.a. Fisher, S.P. Dockery, M. Urematsu, R.M. Nerem, R.W. Alexander, and T.J. Murphy. Molecular cloning and

characterization of constitutive bovine aortic endothelial cell nitric oxide synthase. J.Clin.Invest. 90: 2092-2096, 1992.

184. Nishijima, M.K., M.J. Breslow, C.F. Miller, and R.J. Traystman. Effect of naloxane and ibuprofen on organ blood flow during endotoxic shock in pig. Am.J.Physiol. 255(1pt2): H177-H1841988.

185. Nisoli, E., C. Tonello, L. Briscini, and M.O. Carruba. Inducible nitric oxide synthase in rat brown adipocytes: implications for blood flow to brown adipose tissue. Endocrinology 138: 676-682, 1997.

186. North, A.J., R.A. Star, T.S. Brannon, K. Ujiie, L.B. Wells, C.J. Lowestein, S.H. Snyder, and P.W. Shaul. Nitric oxide synthase type I and type III gene expression are developmentally regulated in rat lung. Am.J.Physiol. 266: L635-L6411994.

187. Nussler, A.k., M. DiSilvio, Z. Ze-Liu, D.a. Geller, P. Freeswick, K. Dorko, F. Bartolio, and T.R. Billiar. Further characterization and comparison of inducible nitric oxide synthase in mouse, rat, and human hepatocytes. Hepatology 21: 1552-1560, 1995.

188. O'shea, R.D. and A.L. Gundlach. Food or water deprivation modulates nitric oxide synthase activity and gene expression in rat hypothalamic neurons: correlation with neurosecretory activity? J.Neuroendocrinol. 8(6): 417-425, 1996.

189. Ochoa, J.b., A.O. Udekwu, and T.R. Billiar. Nitrogen oxide levels in patients after trauma and during sepsis. Ann.Surg. 214: 621-626, 1991.

190. Okamato, T., A. Schlegel, P.E. Scherer, and M.P. Lisant. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J.Biol.Chem. (273)10: 5419-5422, 98 A.D.

191. Okayama, N., L. Coe, M. Itoh, and S. Alexander. Intracellular mechanisms of nitric oxide plus hydrogen peroxide-mediatyed neutrophil adherence to cultured human endothelial cells. Inflamm.Res. 47: 428-433, 1998.

192. Okayama, N., L. Coe, M. Itoh, and S. Alexander. Exogenous nitric oxide increases neutrophil adhesion to cultured human endothelial monolayers through a protein kinase G dependent mechanism. Inflammation 23(1): 37-50, 1999.

193. Ollerstam, A., J. Pittner, A. Persson, and C. Thorup. Increased blood pressure in rats after long-term inhibition of the neuronal isoform of nitric oxide stnthase. J.Clin.Invest. 99: 2212-2218, 1997.

194. Paakkari, I. and P. Lindsberg. Nitric oxide in the central nervous system. Ann.Med. 27: 369-377, 1995.

195. Palmer, P.M., D.D. Rees, D.S. Asthon, and S. Moncada. L-arginine is the physiological precursor for formation of nitric oxide in endothelium-

dependent relaxation. Biochem.Biophys.Res.Commun. 153: 1521-1556, 1988.

196. Palmer, R.M., A.G. Ferrige, and S. Moncada. Nitric oxide release accounts for biological activity of endothelium-derived relaxing factor. Nature 327: 524-526, 1999.

197. Palmer, R.M., M.S. Hickery, I.G. Charles, S. Moncada, and M.T. Bayliss. Induction of nitric oxide synthase in human chondrocytes. Biochem.Biophys.Res.Commun. 193: 398-405, 1993.

198. Palmer, R.M., D.D. Rees, D.S. Ashton, and S. Moncada. L-arginine is the physiological precursor for formation of nitric oxide in endothelium-dependent relaxation. Biochem.Biophys.Res.Commun. 153: 1251-1256, 1988.

199. Papapetropoulos, A., R.D. Rudic, and W.C. Sessa. Molecular control of nitric oxide synthase in the cardiovascular system. Cardiovasc.Res. 43: 509-520, 1999.

200. Parillo, J.E. Management of septic shock. Present and future. Ann.Intern.Med. 115: 491-493, 1991.

201. Park, C.S., P.K. , C. Gianotti, E. Villegas, and G. Krishna. Human retina expresses both constitutive and inducible isoforms of nitric oxide synthase mRNA. Biochem.Biophys.Res.Commun. 205: 159-164, 1994.

202. Payen, D., C. Bernard, and S. Beloucif. Nitric oxide in sepsis. Clin.Chest.Med. 17(12): 333-350, 1996.

203. Payor, W.A. and G.L. Squadrito. The chemistry of peroxynitrite: a product of the reaction of nitric oxide with superoxide. Am.J.Physiol. 268: L699-L7221995.

204. Petros, A., D. Bennett, and P. Vallance. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 338: 1557-1558. 1991.

205. Pichilue, P., J.c. Chavez, R.J. Przybylski, and J.c. LaManna. Increase of neuronal nitric oxide synthase during chronic hypoxia. Adv.Exp.Med.Biol. 454: 319-323, 1998.

206. Picunio, S., M. Simoni, and M.G. Doni. Platelet activation and modulation of the induction of nitric oxidesynthase in the concious rat. Life Sci. 65(14): 1463-1475, 1999.

207. Pollock, J., U. Forstermann, J.A. Mitchell, T.D. warner, H.H.H.W. Schmidt, M. Nakane, and F. Mourad. Purification and characterization of particulate endothelium-derived relaxing factorsynthase from cultured and native bovine aortic endothelial cells. Proc.Natl.Acad.Sci.USA 88: 10480-10484, 1991.

208. Prabhekar, N.R. NO and CO as second messanger in oxygen sensing in the carotid body. Respir.Physiol. 115(2): 161-168, 1999.

209. Radi, R., J.S. Beckman, K.M. Bush, and B.A. Freeman. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch.Biochem.Biophys. 288: 481-487, 1991.

210. Radi, R., J.S. Beckman, K.M. Bush, and B.A. Freeman. Peroxynitrite oxidation of sulfuhydryls: The cytotoxic potential of superoxide and nitric oxide. J.Biol.Chem. 266: 4244-4250, 1991.

211. Radomski, M.W., R.M. Palmer, and S. Moncada. An L-arginine oxide pathway present in human platelets regulates aggregation. Proc.Natl.Acad.Sci.USA 87: 5193-5197, 1990.

212. Radomski, M.W., T. Zakar, and E. Salas. Nitric oxide in platelets. Methods Enzymol. 269: 88-107, 1996.

213. Rapoport, R. Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. Circ.Res. 58: 407-410, 1986.

214. Reagan, L.P., C.R. Mckittrick, and B.S. McEwen. Cortisone and phenytoin reduce neuronal nitric oxide synthase messenger RNA expression in rat hyppocampus. Neuroscience 91(1): 211-219, 1999.

215. Redi, G., J. Newald, G. Schlag, L.D. Traber, and D.L. Traber. Cardiac function in an ovine model of endotoxemia. Circ.Shock 35(1): 31-36, 1991.

216. Rees, D.D., S. Cellek, R.M. Palmer, and S. Moncada. Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone; an insight into endotoxin shock. Biochem.Biophys.Res.Commun. 173: 541-547, 1990.

217. Reif, D.W. and R.D. Simmons. Nitric oxide regulates iron release from ferritin. Arch.Biochem.Biophys. 283: 537-541, 1990.

218. Reilly, C.M., P. Zamorano, V.S. Stopper, and T.M. Mills. Androgenic regulation of NO availability in rat penile erection. J.Androl. 18: 110-115, 1997.

219. Resta, T.C., L.G. Chicoine, J.L. Omdahl, and B.R. Walker. Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia. Am.J.Physiol. 276(2pt2): H699-H7081999.

220. Romanska, H.M., T.S. Ikonen, A.E. Bishop, R.E. Morris, and J.M. Polak. Up-regulation of inducible nitric oxide synthase in fibroblasts parallels the onset and progression of fibrosis in an experimental model of post-transplant obliterative airway disease. J.Pathol. 191: 71-77, 2000.

221. Rosselet, A., F. Fracoise, M. Markert, G. Alex, C. Perret, and L. Liaudet. Selective iNOS inhibition is superior to norepinephrine in the treatment of rat endotoxic shock. Am.J.Respir.Crit.Care Med. 157: 162-170, 1998.

222. Salvucci, O., J.P. Kolb, B. Dugas, N. Dugas, and S. Chouaib. The induction of nitric oxide by interleukin-12 and tumor necrosis factor alpha in human natural killer cells: relationship with the regulation of lytic activity. Blood 92: 2093-2102, 1998.

223. San, J. Alonso, F. Gonzalez-Fernendez, J. DelaOsada, M. Monton, J.A. Rodriguez-Feo, J.I. Guerra, M.m.R.L. Arriero, S. Casado, and A. Lopez-Farre. Evidence that an endothelial cytosolic protein binds to the 3'-untranslated region of endothelial nitric oxide synthase mRNA. J.Vas.Res. 36: 201-208, 1999.

224. Saura, M., S. Lopez, P.M. Rodriguez, P.D. Rodriguez, and S. Lamas. Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli. Kid.Int. 47: 500-509, 1995.

225. Saura, M., C. Zaragoza, M. Diaz-Cazorla, O. Hemandez-Perera, E. Eng, C.J. Lowestein, D. Perez-sala, and S. Lamas. Involvement of transcriptional mechanisms in the inhibition of NOS2 expression by dexamethasone in rat mesangeal cells. Kid.Int. 53(1): 38-49, 1998.

226. Schirar, A., C. Bonnefond, C. Meusnier, and E. Devinoy. Androgens modulate nitric oxide synthase messenger ribonucleic acid expression in neurons of the major pelvic ganglion in the rat. Endocrinology 138: 3093-3102. 1997.

227. Schmidt, H.H., S.M. Lohmann, and U. Walter. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim.Biophys.Acta 1178: 153-175, 1993.

228. Schmidt, H.H., J.S. Pollock, M. Nakane, L.D. Gorsky, U. Forstermann, and F. Murad. Purification of a soluble isoform of guanylate cyclase-activating factor synthase. Proc.Natl.Acad.Sci.USA 85: 365-369, 1991.

229. Schulz, R., K.L. Dodge, G.d. Lopaschuk, and A.S. Clanachan. Peroxynitrite impaires cardiac contractile function by decreasing cardiac efficiency. Am.J.Physiol. 272: H1212-H12191997.

230. Seidel, B., A. Stanarius, and G. Wolf. Differential expression of neuronal and endothelial nitric oxide synthase in blood vessels of the rat brain. Neurosci.Lett. 239: 109-112, 1997.

231. Sessa, W.C., M.C. Barber, and K.R. Lynch. Mutation of N-myristoylation site converts endothelial cell nitric oxide synthase from a membrane to a cytosolic protein. Circ.Res. 72: 921-924, 1993.

232. Sessa, W.C., G. Garcia-Cardena, J. Liu, A. Keh, J.S. Pollock, J. bradley, s. Thiru, i.M. Braverman, and K.M. Desai. The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. J.Biol.Chem. 270: 17641-17644, 1996.

233. Shaul, P.W., A.J. North, T.S. Brannon, K. Ujii, L.B. Wells, P.A. Nisen, C.J. Lowestein, S.H. Snyder, and R.A. Star. Prolonged in vivo hypoxia enhances nitric oxide synthase type I and type III gene expression in adult rat lung. Am.J.Respir.Cell Mol.Biol. 13: 167-174, 1995.

234. Sheu, J.R., W.C. Hung, C.H. Su, C.H. Lin, L.W. Lee, Y.M. Lee, and M.H. Yen. The antiplatelet activity of Escherichia coli lipopolysaccharide is mediated through a nitric oxide/cyclic GMP pathway. Eur.J.Haematol. 62: 317-326, 1999.

235. Silvago, F., H. Xia, and D.S. Bredt. Neuronal nitric oxide synthase-μ, an alternatively spliced isoform expressed in differentiated skeletal muscle. J.Biol.Chem. 271: 11024-11028, 1996.

236. Singh, S. and T.W. Evans. Nitric oxide, the biological mediator of the decade: fact or fiction? Eur.Respir.J. 10: 699-707, 1997.

237. Stamler, J.S. Redox signalling: Nitrosylating and related target interactions of nitric oxide. Cell 78: 931-936, 1994.

238. Stewart, T.E., F. Valenza, S.P. Riberio, A.D. Wener, G. Volgyesi, J.B. Mullen, and A.S. Slutsky. Increased nitric oxide in exhaled gas as an early marker of lung in a model of sepsis. Am.J.Respir.Crit.Care Med. 151(3pt1): 713-718, 1995.

239. Stoclet, J.C., I. Fleming, G. Gray, G.J. Schaeffer, F. Schneider, C. Schott, and J.R. Parratt. Nitric Oxide and Endotoxemia. Circulation 87: V-77-V-801993.

240. Stuehr, D., H.J. Cho, N.S. Kwon, M.F. Weise, and C.F. Nathan. Purification and characterization of cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc.Natl.Acad.Sci.USA 7773-7777, 1991.

241. Stuehr, D.J. and M. Ikeda-Suito. Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome P-450-like hemoproteins that contain a flavin semiquinolone radical. J.Biol.Chem. 267: 20547-20550, 1992.

242. Stuehr, D.J., N.S. Kwon, C.F. Nathan, O.W. Griffith, P.L. Feldman, and J. Wiseman. N-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J.Biol.Chem. 266: 6259-6263, 1991.

243. Stuehr, D.J. and M.A. Marletta. Mammaliuan nitrate biosynthesis: mouse macrophage produce nitrate and nitrite in response to Escherichia coli lipopolysaccharide. Proc.Natl.Acad.Sci.USA 82: 7738-7742, 1985.

244. Suffredini, A.F., R.E. Fromm, M.M. Parker, M. Brenner, J.A. Kovacs, R.A. Wesley, and J.E. Parrillo. The cardiovascular response of normal humans to the administration of endotoxin. New Engl.J.Med. 321: 280-287, 1989.

245. Sugaya, K. and M. McKinney. Nitric oxide synthase gene expression in cholinergic neurons in the rat brain examined by combined immunohistochemistry and in situ hybridization histochemistry. Brain Res.Mol.Brain Res. 23: 111-125, 1994.

246. Szabo, C. Alteration in nitric oxide production in various forms of circulatory shock. New Horiz. 3: 2-32, 1995.

247. Szabo, C. Low-level expression and limited role for inducible isoform of nitric oxide synthase in the vascular hyporeactivity and mortality associated with cecal ligation and punture in the rat. Shock 6: 248-253, 1996.

248. Szabo, C. The pathophysiological role of peroxynitrite in shock, inflammation, and ischemia-reperfusion injury. Shock 6: 79-88, 1996.

249. Szabo, C. Regulation of the expression of inducible nitric oxide synthse by glucocorticoids. Ann.N.Y.Acad.Sci. 851: 336-341, 1998.

250. Szabo, C., J.A. Mitchell, C. Thimermann, and J.R. Vane. Nitric oxide-mediated hyporeactivity to noradrenaline preceeds the induction of nitric oxide synthase in endotoxin shock. B.J.Pharmacol. 108: 786-792, 1993.

251. Szabo, C., C. Saunders, M. Oconnor, and A.L. Sazman. Peroxynitrite causes energy depletion and increases permeability via activation of poly (ADP-ribose) synthase in pulmonary epithelial cells. Am.J.Respir.Cell Mol.Biol. 16: 105-109, 1997.

252. Taylor, B.S., L.H. Alarcon, and T.R. Billiar. Inducible nitric oxide synthase in the liver: regulation and function. Biochemistry (Mosc) 63(7): 766-781, 1998.

253. Thanos, D. and T. Maniatis. NF-kappa B : a lesson in family values. Cell 80: 529-532, 1995.

254. Thimermann, C. and J. Vane. Inhibition of nitric oxide synthesis reduces hypotension induced by bacterial lipolysaccharides in the rat in vivo. Eur.J.Pharmacol. 182: 591-595, 1990.

255. Thomas, S.R., D. Mohr, and R. Stocker. Nitric oxide inhibits 2,3-dioxygenase activity in interferon-primed mononuclear phagocytes. J.Biol.Chem. 269: 14457-14464, 1994.

256. Titheradge, M.A. Nitric oxide in septic shock. Biochim.Biophys.Acta 1411: 437-455, 1999.

257. Tojo, A., S.S. Gross, L. Zhang, C.C. Tisher, H.H. Schmidt, C.S. Wilox, and K.M. Madsen. Immunocytochemical localization of distinct isoforms of nitric oxide synthase in the juxtaglomerular apparatus of normal rat kidney. J.Am.Soc.Nephrol. 4: 1438-1447, 1994.

258. Tracey, W.R., J. Tse, and G. Caster. Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentration in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. J.Pharmacol.Exp.Ther. 272: 1011-1015, 1995.

259. Tzeng, E., T.R. Billiar, P.D. Robbins, M. Loftus, and D.J. Stuehr. Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H_4B)-deficient cell line: H_4B promotes assembly of enzyme subunits into an active dimer. Proc.Natl.Acad.Sci.USA 92: 11771-11775, 1995.

260. Ueta, Y., A. Levy, S.L. Lighman, Y. Hara, R. Serino, M. Nomura, I. Shibuya, Y. Hattori, and H. Yamashita. Hypovolemia upregulates the expression of neuronal nitric oxide gene in the paraventricular and supraoptic nuclei of rats. Brain Res. 790(1-2): 25-32, 1998.

261. Vanden Berg, C., J.G.C. Amsterdam, A. Bisschop, J.J. Piet, J. Wermer, and D.J. de Wildt. Septic shock: no correlation between plasma levels of nitric oxide metabolites and hypotension or lethality. Eur.J.Pharmacol. 270: 379-382, 1994.

262. Vander Vliet, A., C.A. O'Neill, B. Halliwell, C.E. Cross, and H. Kaur. Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. FEBS Lett 339: 89-92, 1994.

263. Vasquez-Vivar, J., P. Martasek, H. Karoui, K.A. Pritchard, and B. Kalyanaraman. Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. J.Biol.Chem. 274: 26736-26742, 1999.

264. Vejlstrup, N.G., A. Bouloumie, S. Boesgaard, C.B. Andersen, J.E. Nielsen-Kudsk, S.A. Mortensen, J.D. Kent, D.G. Harrison, R. Busse, and J. Aldershvile. Inducible nitric oxide synthase (iNOS) in the human heart: Expression and localization in congestive heart failure. J.Mol.Cell Cardiol. 30: 1215-1223, 1998.

265. Venema, V.J., H. Ju, R. Zou, and R.C. Venema. Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin. J.Biol.Chem. (272)45: 28187-28190, 1997.

266. Villa, S.M., E. Salas, V.M. Darley Usmar, M.W. Radomski, and S. Moncada. Peroxynitrite induces both vasodilation and impaired vascular relaxation in the isolated perfused rat heart. Proc.Natl.Acad.Sci.USA 91: 12383-12387, 1994.

267. Vodovotz, Y. Modified microscopy for serum nitrite and nitrate. Biotechniques 20: 390-394, 1996.

268. Vos, I.H., J.A. Joles, M. Schurink, G. Weckbecker, T. Stojanovic, T.J. Rabelink, and H.J. Grone. Inhibition of inducible nitric oxide synthase improves

graft function and reduces tubulointerstitial injury in renal allograft rejection. Eur.J.Pharmacol. 391: 382000.

269. Wakabayashi, I., K. Hatake, E. Kakishita, and K. Nagai. Diminution of contractile response of the aorta from endotoxin-injected rats. Eur.J.Pharmacol. 141: 117-122, 1987.

270. Wallerath, T., I. Gath, W.E. Aulitzky, J.S. Pollack, H. Kleinert, and U. Forstermann. Identification of NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets. Thromb.Haemost. 77: 163-167, 1997.

271. Wallerath, T., I. Gath, W.E. Aulitzky, J.S. Pollock, H. Kleinert, and U. Forstermann. Identification of NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocyte, and platelets. Thromb.Haemost. 77: 163-167, 1997.

272. Wang, G.R., Y. Zhu, P.V. Halashka, T.M. Lincoln, and M.E. Mendelsohn. Mechanism of platelet inhibition by nitric oxide: In vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. Proc.Natl.Acad.Sci.USA 95: 4888-4893, 1998.

273. Wang, Y. and P.A. Marsden. Nitric oxide synthases. Gene structure and regulation. Adv.Pharmacol. 34: 71-90, 1995.

274. Weitzberg, E., A. Rudehill, A. Modin, and J.M. Lundberg. Effect of combined nitric oxide inhalation and NG-nitro-L-arginine infusion in porcine endotoxic shock. Crit.Care Med. 23(5): 909-918, 1995.

275. Wong, H.R. Potential role of the heat shock response in sepsis. New Horiz. 6(2): 194-200, 1998.

276. Wong, H.R., M. Ryan, and J.R. Wispe. The heat shock response inhibits inducible nitric oxide synthase gene expression by blocking I Kappa-B degradation and NF-Kappa B nuclear translation. Biochem.Biophys.Res.Commun. 231(2): 257-263, 1997.

277. Wu, C.C., S.J. Chen, C. Szabo, C. Thimermann, and J.R. Vane. Aminoguanidine attenuates the delayed circulatory failure and improves survival in rodent models of endotoxic shock. B.J.Pharmacol. 114: 1666-1672, 1995.

278. Wyler, F., J.M. Neutze, and A.M. Rudolph. The effect of endotoxin on distribution of cardiac output in unanesthetized rabbits. Am.J.Physiol. 219: 246-251, 1970.

279. Xia-quing, W., I.G. Charles, A. Smith, J. Ure, G.-J. Feng, F.-P. Huang, D. Xu, W. Muller, S. Moncada, and F.Y. Liew. Altered immune response in mice lacking inducible nitric oxide synthase. Nature 375: 408-411, 1995.

280. Xia, Y., V.L. Dawson, T.M. Dawson, S.H. Snyder, and J.L. Zweier. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells

leading to peroxynitrite-mediated cellular injury. Proc.Natl.Acad.Sci.USA 93: 6770-6774, 1996.

281. Xia, Y. and J.L. Zweier. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc.Natl.Acad.Sci.USA 94: 6954-6958, 1997.

282. Xie, Q.W., H.J. Cho, J. Calaycay, R.A. Murnford, T. Troso, and C. Nathan. Cloning and characterization of inducible nitric oxide synthase from mouse macrophage. Science 256: 225-228, 1992.

283. Yoshimoto, H., A. Snehiro, and E. Kakishita. Exogenous nitric oxide inhibits platelet activation in whole blood. J.Cardiovas.Pharmacol. 33: 109-115, 1999.

284. Yoshizumi, M., M.A. Perrella, J.c. Burnett, and M.E. Lee. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening of its half-life. Circ.Res. 73: 205-209, 1993.

285. You, Y. and C. Kaur. Expression of induced nitric oxide synthase in amoeboid microglia in postnatal rats following an exposure to hypoxia. Neurosci.Lett. 279: 101-104, 2000.

286. Yousif, M.H., M.A. Oriowo, and K.I. Williams. Sodium nitropruside-induced cGMP-independent vasodilation responses in the perfused rabbit ovarian vascular bed. Pharmacol.Res. 38(5): 381-386, 1998.

287. Yu, Y.A., M.G. Frid, L.A. Shimodo, C.M. Wiener, K. Stenmark, and G.L. Semenza. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am.J.Physiol. 275(4pt1): L818-L8261998.

288. Zelzer, E., Y. Levy, K. Chaim, B.Z. Shilo, M. Rubistein, and B. Cohen. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J. 17(17): 5085-5094, 1998.

289. Zembowics, A. and J.R. Vane. Induction of nitric oxide synthase activity by toxic shock syndrome toxin 1 in a macrophage-monocyte cell line. Proc.Natl.Acad.Sci.USA 89: 2051-2055, 1992.

290. Zhang, J. and S.H. Snyder. Nitric oxide in nervous system. Annu.Rev.Pharmacol.toxicol. 35: 213-233, 1999.

291. Zhang, Z.G., M. Chopp, C. Zaloga, J.S. Pollock, and U. Forstermann. Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. Stroke 24: 2016-2021, 1993.

292. Zhu, L., C. Gunn, and J.S. Beckman. Bactericidal activity of peroxynitrite. Arch.Biochem.Biophys. 298(2): 452-457, 1992.

293. Ziesche, R., V. Petkov, J. William, S.M. Zakeri, W. Mosgoller, M. Knofler, and L.H. Block. Lipopolysaccharide and interlukine-1 augment effects of hypoxia and inflammation in human pulmonary arterial tissue. Proc.Natl.Acad.Sci.USA 93: 12478-12483, 1996.

294. Zingarelli, B., M. O'Connor, A.L. Salzman, and C. Szabo. Peroxynitrite-mediated DNA strand breakage activates poly-adenosine diphosphate ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipopolysaccharide. J.Immunol. 156: 350-358, 1996.

295. Zweier, J.L., P. Wang, and P. Kuppusamy. Direct measurement of nitric oxide generation in ischemic heart using electron paramagnetic resonance spectroscopy. J.Biol.Chem. 270: 304-307, 1995.

Link to chapter two:

Inducible NOS has been hypothesized to be the major factor in the vascular abnormalities of sepsis. This hypothesis is based on the observation that NOSII is induced in rats and mice. However when we started this study there was no evidence of NOSII induction in human sepsis. Investigators use ηg of LPS in humans, μg of LPS in pigs, and mg of LPS in rats to make them septic. Therefore, septic pigs are closer models than septic rat to human. Thus, this study was designed to address the role of NOSII in porcine model of sepsis.

CHAPTER TWO

PRESENCE OF NITROTYROSINE WITH MINIMAL NOSII INDUCTION IN ENDOTOXEMIC PIGS

Abstract

Objective: The induction of the inducible form of nitric oxide synthase (NOSII), production of large amounts of nitric oxide (NO) and subsequent production of peroxynitrite ($OONO^-$) are believed to be major factors in the hemodynamic abnormalities of sepsis. However, most of the data comes from studies on rats and mice. We examined the role of NOSII in endotoxemic pigs which have a hemodynamic pattern more similar to humans than rats. *Method*: Pigs were anesthetized and ventilated and given endotoxin (n=12), 20 μg/kg over 2 hours, or saline (n=7). They were sacrificed after 2 (n = 8 endotoxin, 7 control) or 4 hours (4 endotoxin). We measured cardiac output (CO), mean arterial (P_{art}), pulmonary and central venous pressures, expired NO and plasma nitrate/nitrite concentration. We measured NOS activity in lung tissue by the L-[2,3, ^{3}H]-arginine to L-[^{3}H]-citrulline assay and NOSII protein by Western analysis in multiple tissues. We probed for NOSII by immunohistochemistry in lung and liver. We measured NOSII mRNA by Northern analysis in liver and lung. We also measured nitrotyrosine as evidence of $OONO^-$ production by slot blot, Western analysis and immunohistochemistry in multiple tissues. *Results:* By 2 hours, P_{art} fell and CO did not change so that systemic vascular resistance decreased from 21.5 ± 2.9 to 12.7 ±3.1 mmHg · L^{-1} · min ($p < 0.05$) and remained at 11.3 ± 1.7 mmHg · L^{-1} · min in the animals followed for 4 hours. Plasma nitrate/nitrite, and NOS activity did not change. There was also no difference of expired NO at zero time and at 4 hours in (n=4) We found no NOSII by Western analysis with 5 different antibodies in multiple tissues. However, we found a small amount of NOSII by immunohistochemistry in inflammatory cells and small vessels with two antibodies. There was a small increase in NOSII mRNA in liver and lung.

Despite the minimal increase in NOSII, we found increased nitrotyrosine by slot blot, Western analysis and immunohistochemistry in small vessels and in inflammatory cells. *Conclusion:* In contrast to what is seen in rats and mice, there is only minimal NOSII present in endotoxic pigs with low SVR, but there is still production of nitrotyrosine which indicates probable $OONO^-$ production. This suggests a role for constitutive NOS in the vascular abnormalities of sepsis and caution should be used when extrapolating the septic response in rodents to larger animals.

Introduction

Increased production of nitric oxide (NO) by the inducible form of nitric oxide synthase (NOSII) has been proposed to be a major factor in the pathophysiology of sepsis (44, 52, 54). In particular, NOSII is believed to be responsible for the hypotension and loss of vascular tone that occurs in sepsis. This hypothesis is based on a number of observations. Infusion of endotoxin results in the production of large amounts of NOSII in rats and mice (23, 52, 23). The presence of NOSII is associated with large increases in plasma nitrate/nitrite concentrations (29) and expired NO (51) and a marked increase in calcium independent NOS activity which is typical of NOSII induction. Increased NO production is associated with an increase in smooth muscle cyclic GMP which mediates vascular dilatation (8). Increased NO is also believed to affect the vasculature by interacting with superoxide O_2^- (4, 5) and producing peroxynitrite ($OONO^-$). $OONO^-$ is a reactive oxygen species (4, 46) which can cause diffuse cellular damage by nitrating important cellular proteins and by breaking DNA strands which activates poly(ADP-ribose) synthetase (50, 56). Furthermore, inhibition of

NOS restores arterial pressure in endotoxic dogs (27, 28), rats (57), pigs (36) and septic humans (45). The benefit of a NOS inhibitor is even greater when the inhibitor has specificity for NOSII (48). Finally, mice which have a genetically altered non-functional NOSII gene (knockout model) survive longer than wild type mice when given endotoxin or bacteria (34, 65). Based on these observations, clinical trials have been organized to study the therapeutic effects of NOS inhibitors in septic patients (16).

On the other hand, a lot of evidence is inconsistent with the hypothesis that NO from NOSII is responsible for the hemodynamic abnormalities of sepsis. Hypotension in rodents occurs before the induction of NOSII (55) and does not correlate with the concentration of NO (58, 60). Although, some NOSII knockout mice survive an endotoxin infusion better than wild type mice, no benefit was demonstrated in one model (34). Cardiac output is generally decreased in rodents given endotoxin (13), whereas cardiac output is increased in humans (53). Rodents also require doses of endotoxin (13) which are orders of magnitude greater than those used in human studies (53). Although the presence of NOSII mRNA in human tissue is well documented, there are much fewer reports of NOSII protein in human tissue. Furthermore, although plasma nitrate/nitrite concentrations are elevated in septic patients, the concentrations are much lower than in rodents (15, 43). The role of NOSII in species larger than mice and rodents is thus not clear.

We have been studying a porcine model of sepsis which has many of the hemodynamic features of septic humans. We found that endotoxin infusion increased expired NO, but there was no evidence of NOSII production by Western analysis in different tissues (38). This, however, could have been due to a lack of specificity of the antibodies.

The objective of the present study was to look further for evidence of NOSII in endotoxemic pigs. We performed Northern blotting for NOSII mRNA, Western analysis for NOSII protein, NOS activity assay with the L-[2,3, ^{3}H]-arginine to L-[^{3}H]-citrulline assay, measured changes in plasma nitrate/nitrite and performed immunohistochemistry on multiple tissues. We found that NOSII mRNA is induced, but there was only a minimal amount of NOSII protein and no significant increase in NOS activity or plasma nitrate/nitrite. However, to our surprise, we found evidence of increased nitrotyrosine which indicates that peroxynitrite was likely formed. This suggests that production of NO from constitutive NOS may be important in the pathophysiology of sepsis. In a companion paper, we found increased endothelial NOS (NOSIII) and neuronal NOS (NOSI) protein and increased NOS activity in the aorta and vena cava. Our results indicate that murine and rodent data may be very misleading for the understanding of the role of NO in other species, including humans, and far too much emphasis may have been placed on NOSII.

Materials and Methods

Animal Preparation: The experiments were performed in juvenile pigs weighing 25-30 kg. They were allowed to have access to water but no food one day before the experiments. After premedication with intramuscular injection of ketamine hydrochloride (20 mg/kg) (Ayerst), xylazine (2 mg/kg) (Bayer), and 0.4 mg/kg atropine sulphate (Sigma), animals were anesthetized with a bolus of sodium thiopental (10 mg/kg). They were placed in the recumbent position in a V-shaped support, intubated orotracheally with a cuffed endotracheal

tube, and ventilated with a volume ventilator at 10-15 ml/kg and 12-18 b/min and 5 cmH_2O positive end expiratory pressure.

The left femoral vein was cannulated and used for administration of fluid and drugs. A catheter was advanced into the thoracic portion of the inferior vena cava to record central venous pressure. A balloon-tipped flotation catheter (Swan-Ganz, Edward Laboratory, Santa Anna, CA) was inserted through the right jugular vein and positioned in the pulmonary artery for measurement of right atrial pressure (Pra), pulmonary artery (Ppa) and pulmonary occlusion pressures (Pcw), as well as cardiac output (CO) by thermodilution. The right common carotid artery was cannulated to record arterial pressure (P_{art}). Transducers were referenced to the mid-point of right atrium. $PaCO_2$ was maintained between 35-40 mmHg by adjustment of respiratory rate and PO_2 was kept above 90 mmHg with supplemental inspired oxygen as necessary.

Measurement of Nitrate/Nitrite: To measure nitrate/nitrite concentration in plasma blood, samples were collected in EDTA containing tubes, centrifuged at 4000 rpm for 20 minutes and then the separated plasma was frozen at -20°C until analyzed. When ready to process, samples were thawed and 25 μl of 30% (w/v) $ZnSO_4$ were added to 200 μl of sample to precipitate plasma proteins of samples and spun at 12,000 rpm for a few minutes. After incubation with cadmium fillings to reduce nitrate to nitrite at room temperature for 16-17 hours, the supernatant was separated. Triplicates of each sample (100 μl/tube) were prepared, diluted to 1 ml with double distilled water, and 100 μl fresh Griess reagent (1% sulfanilamide, .1% naphtyethylenediaminedihydrochloride, 2.5% concentrated H_3PO_4 acid;

Sigma) were added. Nitrite concentration was measured at 550 nm against a standard curve of sodium nitrite.

Expired NO: At time zero and then each hour, gas was collected from the expiratory port of the ventilator and vigorously mixed manually for 10 seconds before the NO content of a 50 ml aliquot was determined by chemiluminescence (NOA 270B NO analyzer, Sievers Medical Instruments Inc., Boulder, CO) as previously described (39). Briefly, a photomultiplier tube produces an electrical signal proportional to the light generated from a chemical reaction between NO in the sample and ozone in the reaction chamber. The NO analyzer was calibrated using a reference gas of known NO concentration (Medigas Inc., Montreal, Quebec, Canada). Expired NO is expressed as the rate of pulmonary NO excretion ($V_E NO$) in pmol/kg/min which was calculated as the product of the expired NO concentration at body temperature and minute ventilation (V_E).

Homogenization: Frozen tissues were homogenized at a ratio of 1 gram/3-6 ml of ice-cold homogenization buffer. Homogenization was first done with pestle and mortar and then with a homogenizer in ice and salt-cold environment. The homogenate was centrifuged at 10,000-11,000 rpm for 15-20 minutes at 4°C. Supernatants were aliquoted and frozen at -80°C. Homogenization buffer (pH = 7.4) had the final concentration of 50 mM Hepes, 100 μM dithiothreitol (DTT), .55 μM leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1.4 μM pepstatin A, 2.5% glycerol in double distilled water.

NOS assay: Supernatant (50 μl) from the homogenate was added to 10 ml pre-warmed (37°C) tubes which contained 100 μl of reaction buffer of the following composition: 50 mM KH_2PO_4, 60 mM valine, 1.5 mM NADPH, 10 mM FAD, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml BSA, 1 μg/ml calmodulin, 10 μM BH_4, and 25 μl of 120 μM stock L-[2,3 ^{3}H] arginine (150-200 cpm/pM). The samples were incubated for 30 min at 37°C, and the reaction was terminated by the addition of cold (4°C) stop buffer (pH = 5.5), 100 mM HEPES, and 12 mM EDTA. Dowex 50W resin (8% cross-linked, Na^+ form) was added to eliminate excess L-[2.3 ^{3}H] arginine. Enzyme activity was expressed in pmole of L-citrulline produced x mg total protein^{-1} x min^{-1}. Protein was measured by the Bradford technique with bovine serum albumin as a standard (BioRad Inc. Ca). The supernatant was assayed for L-[^{3}H] citrulline by liquid scintillation counting. NOS activity was also measured in the presence of 1.5 mM of each EGTA and EDTA which replaced $CaCl_2$-calmodulin in the reaction buffer and in the presence of 1 mM of N^G-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor). Ca^{2+}/calmodulin-independent NOS activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and those measured in the presence of L-NAME.

Western blot: Frozen tissue extracts were thawed on ice and mixed with equal volume of sample buffer (composed of 4 ml distilled water, 1 ml of .5 M Tris-HCl, pH = 6.8, .8 ml glycerol, 10% SDS (w/v), .05% (w/v) bromophenol blue, .4 ml of 2-beta-mercaptoethanol) followed by heating at 95°C for 5 minutes. They were centrifuged and loaded on 8% or 4-12% Tris/glycine/SDS-polyacrylamide gel for fractionation. Predetermined MW standards (Novex Inc.) were used as markers. Protein on the gel was blotted onto nitrocellulose or PVDF membranes at 4°C at 25-30V and 370-380 mA for 150 minutes. After transfer, the

membranes were incubated with 7% skim milk, and 1% heat-inactivated (60°C) fetal calf serum (Sigma) in wash buffer for 2 hrs, or overnight, at a cold temperature. NOSII protein was detected by incubating with primary antibodies at concentrations of 1:500 to 1:1000. Two monoclonal antibodies were used. The major one we used was directed against a 21 KDa protein fragment from the mouse macrophage NOSII (Signal Transduction Laboratory #N32020). The other was provided by Dr. Hattori (21). We used three polyclonal antibodies, Signal Transduction #32030, the antibody in reference Saleh et al (49) and No53 (provided by John Humes at Merck, Rahway, NJ). Nitrotyrosine was detected with anti-nitrotyrosine polyclonal antibody. The membranes were washed three times with wash buffer and then incubated with the appropriate secondary antibody for the primary mono- or polyclonal antibody at concentrations of 1:500 to 1:1000. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit) according to the specifications of the manufacturer (Amersham). Blots were scanned with an imaging densitometer, and the optical densities of protein bands were quantitated with software (Sigma Gel, Jandel Scientific, San Rafael, CA).

Immunopreciptation: Immunoprecipitation of NOSII protein was performed on tissue from lungs of pigs treated with endotoxin for four hours. Lung tissue from endotoxic rats was used as a control for the technique. Tissues were ground in immunoprecipitation lysis buffer in a liquid nitrogen cooled mortar, until a homogenous mixture was obtained. This mixture was homogenized in an ice-cold environment and centrifuged at 10,000 - 11,000 rpm for 15 minutes at 4°C. Supernatant was aliquoted and the protein concentration was determined. We then mixed 500 μg of tissue extract, with 2 μl of monoclonal antibody (21) and 20 μl of

both protein A and protein G (beads) which was washed three times (wash buffer had 20 mM HEPES, 150 mM, NaCl .1% Triton X-100, and 10% glycerol pH of 7.5). The mixture was incubated at 4°C for 2 hrs or overnight. Following incubation and 5 minutes centrifugation, 100 μg of protein was taken from the supernatant of immunoprecipitation. The beads were washed three times with wash buffer, spun and the supernatant was discarded. The proteins of the precipitate were solubilized from beads by addition of 1X Bio-Rad sample buffer (composed of 4 ml distilled water, 1 ml of .5 M Tris-HCl pH = 6.8, .8 ml glycerol, 10% SDS (w/v), .05% (w/v) bromophenol blue, 0.4 ml of 2 beta-mercaptoethanol), heated fro 5 minutes at 95° C, and then subjected to SDS-PAGE electrophoresis (8% Novex gel) in the same condition as above. Proteins were transferred to PVDF membrane blocked for 2 hrs or overnight. NOSII protein was detected by incubating with the Hattori anti NOSII monoclonal antibody (1:1000 dilution) (21) and anti-mouse antibody as secondary antibody (1:1000 dilution) on the shaker at cold temperature for 2 hrs. Immunoreactive bands were visualized by chemiluminescence.

Detection of NOSII in porcine neutrophiles

To determine if our antibody could detect NOSII, we needed to find a porcine tissue which expressed it. To do so, we treated a pig with 10 μg/kg/hr LPS for 2 hours and supported it with volume as in our previous studies. After a total of 4 hours, we injected carrageenan (Sigma type IV) into the pleural space and waited another 5 hours (total = 9 hours) to allow neutrophils to migrate to pleural space and then sacrificed the animals. Pleural fluid was collected. The cells were spun down and washed three times with 17 mM

Tris buffer which had .75% NH_4Cl to lyse the red cells. A Wright stain of the sample showed predominantly neutrophils. These were then lysed and frozen for further analyses including western blot as above.

Slot blot: Sample buffer was added to 100 μg of protein of tissue extract and loaded on a slot blot manifold with a nitrocellulose membrane soaked with transfer buffer or a PVDF membrane soaked with methanol, followed by soaking in transfer buffer. After suction for 3-5 minutes, the membranes were blocked with blocking solution at cold temperature for 2 hours, or overnight, and then incubated with primary anti-nitrotyrosine polyclonal antibody (1:1000). They were then washed, incubated with secondary anti-rabbit antibody (1:1000), washed again and bands were visualized by chemiluminescence and the densities measured as above. For negative control 10mM nitrotyrosine was prepared in phosphate -buffer saline and incubated with primary anti-nitrotyrosine antibody for two hours at room temprature to block the antibody.

RNA Extraction and Northern Analysis

Total RNA was extracted from lung and liver tissues with TriZol reagent following the manufacturer's instructions. RNA content was determined by optical density and the quality was examined on a 1% agarose gel stained with ethidium bromide.

Polymerase Chain Reaction

To obtain probes for Northern analysis, we first performed reverse transcriptase PCR on tissue from porcine lungs. We reverse transcribed 1 μg of total RNA to the complementary DNA (cDNA) with random hexamers and then cloned it with Moloney murine leukemia virus reverse transcriptase (MMLV RDA, Gibco, BRL). Samples were incubated at 42°C for 60 minutes and 10 minutes at 95°C and quickly chilled on ice. The total volume of the reverse transcriptase mixture was 20 uL. We used 5 uL of the reverse transcriptase product for polymerase chain reaction (PCR) with oligonucleotides for porcine NOSII based on a sequence kindly provided by Michael P. Murtaugh (University of Minnesota, St. Paul, MN, USA) (forward 5' GTL CAA CCT GCA GGT CTT CG3' and reverse 5' CCA TGA TGG TCA CAT TCT GC3'). The sample volume was 50 uL to which TAQ polymerase (GIBCO-BRAL) were added. PCR was performed in a programmable thermocontroller (MJ Research Inc.). We began with a denaturation step at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension step at 72°C for 1 minute and 30 seconds for 35 cycles.

The polymerase chain reaction product of NOSII cDNA was cleaved out with the restriction enzyme and ligated with T-4 DNA ligase into bluescript KS^+ vector which was then transformed into competent E-coli cells. The culture was grown overnight at 37°C and spread on agar plates (containing 1.5% Agar, 0.2% ampicillin, 0.5% isopropropol-thiogalacto-peranozide and 0.2% xgal for a blue-white selection). White colonies, containing the vector, were picked and the plasmids were isolated using standard procedures. After cleavage with the restriction enzyme, the samples were separated by gel electrophoresis and bands of appropriate size cut out for sequencing. The sequence was compared with the available rat

and human NOSII , and pig NOSIII sequences in Genebank . This comparison showed 57% and 72% homology with rat and human NOSII respectively, and 34% with pig NOSIII . Once it was established that it was indeed NOSII, large scale plasmid preparation was performed. After plasmid isolation and enzymatic cleavage, the product was separated by gel electrophoresis and the insert was elluted into 0.7% low melting point agarose and purified with phenol-chloroform extraction. The probe was then labelled with alpha[^{32}P] deoxycytidine triphosphates by Nick translation.

For Northern analysis, total RNA (50 μg) samples were fractionated by electrophoresis in a denaturing formaldehyde 1% agarose gel. The RNA was transferred to Hybond-N nylon membrane by capillary diffusion with 20 × SSC. After RNA immobilization by bathing under vacuum for 2 hrs at 80°C, the membranes were pre-hybridized for 3 hrs at 42°C with 5 × SSPE, 5 × Denhrd's solution, 0.5 % SDS, 58% formamide and 10 μg/ml of a heat denatured salmon sperm DNA. Hybridization was done overnight at the same temperature in a solution containing ^{32}P labelled probe for NOSII which was randomly labelled. The membranes were washed with 2 × SSPE/0.1% SDS for 5 minutes at room temperature 3 times, followed by 2 more washes at 42°C with 0.2 × SSPE/0.1% SDS for 15 minutes and, finally, 1 hour at 65°C, with a 1 × SSPE/0.5% SDS solution. The membranes were exposed to a KODAK film at -80° C for 70 days. Integrity and equal loading of RNA samples was assessed by hybridization of the membranes using a ^{32}P labelled olignucleotide specific for 18S ribosomal RNA .

Immunohistochemistry

Tissue samples for immunohistochemistry were immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.01 M phosphase buffer at pH 7.2 with 0.15 M NaCl) for 3 hours then transferred to PBS containing 15% sucrose and 0.1% sodium azide and stored at 4°C. Immunohistochemistry was performed using avidin-biotin-peroxidase complex (ABC) method (24) with Vectastain Elite kit (Vector Laboratories, Burlingame, CA) using a polyclonal or monoclonal antibody (Signal Transduction) for NOSII and polyclonal to nitrotyrosine as previously described (49) . Cryostat sections (10 μm thick) were cut from tissues, picked up on poly-lysine coated slides and dried at 37°C overnight. The sections were then washed in PBS three times for 5 minutes each and incubated in 10% normal goat serum for 30 minutes at room temperature followed by incubation with antisera overnight at 4° C. After 3 more washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG anti-serum for 45 minutes, washed in PBS and incubated with ABC for 45 minutes. Immunoreactive sites were developed by immersion of the section in a solution containing 0.01 % hydrogen peroxide and 0.025% 3,3diamino benzidine. Sections were counterstained with Harris' hematoxylin dehydrated, cleared in toluene and mounted. For negative controls, sections were incubated with 10% normal goat serum instead of primary antisera, or with antisera preabsorbed with respective antigens prior to the addition to sections. Immunohistochemistry was performed in paired tissues in 3 control and 3 endotoxic animals.

Protocol

After insertion of the lines, the animals were stabilized. Baseline hemodynamics were obtained and animals randomly received either 10 μg/kg/hr for 2 hours of endotoxin (n=8) or normal saline (n=7). Animals were sacrificed between the second and third hours. An additional 4 animals were given endotoxin for 2 hours and followed for 2 more hours (4 hours total). An infusion of 6% dextran was given as necessary to maintain the central venous pressure between 4 and 5 mmHg during the full course of the experiment. Generally, not much fluid was required in the septic animals in the first hour, but the amount increased significantly in the second hour. Only small amounts of fluid were required in the control animals. Animals were sacrificed by an overdose of KCl. Tissue samples from the aorta, vena cava, pulmonary artery, left ventricle, lung, kidney cortex, kidney papillae, spleen, diaphragm and liver were rapidly frozen in liquid nitrogen and stored at -80°C for further analysis. Samples were also placed in paraformaldehyde for immunohistochemistry studies.

Statistical Analyses:

All data are presented as mean ± SD. Significance was accepted at $p < 0.05$. Repeated measures over time were analyzed with a two-way ANOVA for repeated measures (Sigma Stat - Jandel Scientific) and, where significant differences were found, post hoc analysis was performed with the Student Newman Keul's test. Comparison of unpaired single repeat data was performed by unpaired t-test.

RESULTS

Blood gases: At the start of the experiment, the pO_2 in the control animals was 125 ± 12 mmHg and remained at 125 ± 11mmHg after two hours of observation. In the endotoxin treated animals, the pO_2 started at 139 ± 25 mmHg before receiving endotoxin and was 120 ± 21 mmHg after two hours of endotoxemia, and 113 ± 22 mmHg in the 4 animals treated for four hours. The initial pH in the control group was 7.48 and did not change during the experiment. In the endotoxin treated animals, the pH began at 7.48 ± .03 and was 7.47 ± .03 after two hours of endotoxin, and 7.13 ± .04 after four hours. The initial pCO_2 in the control group was 39 ± 3 mmHg and did not change during the experiment. In the endotoxin treated animals, the initial pCO_2 was 42 ± 4 mmHg and was 48 ± 9 mmHg following two hours of endotoxemia and 55 ± 9 mmHg in the animals treated for four hours. The hematocrit in the control animals started at 25 ± 4% and remained at 19 ± 6% after two hours. In the endotoxin treated animals, the hematocrit started at 27±4 and remained 23 ± 7 at two hours and 23 ± 11 in the animals treated for four hours.

Hemodynamics: The cardiac output, mean arterial blood pressure (P_{art}) and systemic vascular resistance (SVR) are shown in Figure 1 for the full course of the experiment. The cardiac output started at 5.38 ± 1.17 L/min. It fell significantly during the first 30 minutes of endotoxemia and then returned to baseline by 60 minutes and stayed at this level for the rest of the experiment. P_{art} started at 117 ± 13 mmHg. It rose slightly during the first 30 minutes and then began to fall. The decrease from baseline was significant by 90 minutes of endotoxin infusion and reached 71 ± 13 mmHg at 2 hours. It changed little after that. There

was no change in the control group. The SVR rose in the first 30 minutes and was back to baseline by 60 minutes of endotoxemia. By 90 minutes, it was significantly below baseline. P_{art} remained stable after 120 minutes of endotoxemia and was less than half the initial value. There was no change in the control group. Changes in pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) are shown in Figure 2. There was an immediate rise in PAP with endotoxemia which then decreased by 60 minutes and remained stable during the rest of the experiment. There was no change in the control group. The PVR thus increased during the first 30 minutes and declined to a stable but elevated level after the first 60 minutes.

Nitrate/Nitrite and Expired NO: Plasma nitrate/nitrite concentrations are shown in Figure 3. There was no increase in plasma nitrite in the endotoxin-treated animals for up to 4 hours of observation; if anything, there was a tendency for a decrease at 4 hours. The mean of the control group (n = 5) at 2 hours was 33 ± 22 μM. It was 28 ± 17 μM in the endotoxin treated animals (n = 8) and remained at 9 ± 3 μM at four hours (n = 4). There was no change in expired NO during the four hours of study in four animals (38 ± 29 pmol/min/kg at time zero and 34 ± 22 at pmol/min/kg at 4 hours).

NOS activity: The measurement of NOS activity in the lung is presented in Figure 4. These measurements were performed on the lungs of animals treated for 4 hours of endotoxemia (n = 4). There was no change in total NOS activity or calcium independent NOS activity in the endotoxin treated animals when compared to the control animals.

Western Analysis:

Western blots for NOSII were performed with five different antibodies on multiple tissues. The principal antibody was a monoclonal antibody to mouse macrophage lysate (Signal Transduction N32020). We analyzed lung (n = 7), kidney (n = 7), liver (n = 7), left ventricle (n = 7), aorta (n = 5, plus 3 pooled samples), vena cava (n = 5), pulmonary artery (n = 4) and diaphragm (n = 4) from animals treated for two hours and failed to detect NOSII in any of these tissues. An example is shown in Figure 5 a. We also analyzed lung, liver, aorta, vena cava, pulmonary artery and spleen from 4 animals, which were followed for four hours and found no NOSII (Figure 5 b). We analyzed lung and liver with the polyclonal used for immunohistochemistry (49), liver, lung, aorta, vena cava, pulmonary artery and spleen with the polyclonal from Dr. R. Hattori (21), lung, liver aorta, vena cava with the polyclonal antibody from Signal Transduction (32030), lung (n=5), liver (n=4) and pooled samples (n = 4) from liver, lung, pulmonary artery, vena cava, spleen, aorta with polyclonal antibody (No 53) from Merck Frost.

The immunoprecipitation of NOSII from porcine and rat lung with monoclonal antibody (Hattori) is shown on the left of Figure 6. NOSII was immunoprecipitated (IP) from rat lung (Lane 4) and there was only a small amount of NOSII in the supernatant (sup-Lane 5) . The crude extract also showed an NOSII band (Lane 6). No bands were demonstrated in crude sample (Lane 1), supernatant (Lane 2) or immunoprecipitate from the pig lung, although clear bands could be seen for the proteins used for the immunoprecipitation. The

right hand side of the figure shows Western blot of neutrophils obtained from the pleural space of the pig treated for 9 hours. The signal transduction antibody was used for these studies. The large arrow shows that NOSII was evident even in the crude sample and a very large signal was seen in the immunoprecipitate. Thus, this antibody is able to detect porcine NOSII.

Nitrotyrosine:

Figure 7 shows the slot blot analysis of the lung tissue with an anti-nitrotyrosine antibody for 5 control and 8 endotoxic animals after 2 hours of endotoxemia. There was a significant increase in density in endotoxic versus control animals ($p < 0.05$). Figure 8 shows a slot-blot analysis with anti-nitrotyrosine antibody of liver homogenates. The signal was very faint in all control samples. Three of the septic animals had a strong signal, whereas the fourth did not. Figure 9 shows a slot-blot analysis of the aorta of 4 control and 7 septic animals. The density of the bands was significantly greater in endotoxic animals than control animals.

A Western blot for nitrotyrosine from aorta and lung tissue from animals followed for 4 hours is shown in Figure 10. No bands were seen in the two aortic samples or lung sample from control animals. A faint band could be seen in the aorta of endotoxemic animal (S), and a stronger band in the two samples from lungs. In the lung, bands were identified at 87, 186 and 204 KDa.

Northern Analysis

Northern analysis of the lung (top) and liver (bottom) for NOSII mRNA are shown in Figure 11. The 18 S rRNA was used to control for loading. One control animal showed an increased signal for NOSII mRNA, whereas the other 3 showed faint staining. All 4 septic animals showed evidence of NOSII mRNA. However, overall NOSII signals were weak and were only obtained after 70 days of exposure. No control animals showed evidence of NOSII in the liver, but NOSII was present in all 4 endotoxic animals.

Immunohistochemistry:

Immunohistochemical analysis of NOSII in lung and liver is shown in Figure 12. Two antibodies were used to detect NOSII. Staining was stronger with the polyclonal antibody (49) than with the monoclonal antibody (Signal Transduction, #N32020) (49) . Bronchial epithelial cells and inflammatory cells stained positive for NOSII in both control and endotoxemic animals, but inflammatory cells were more prevalent in endotoxemic animals, and thus there was more NOSII. NOSII staining was also present in glandular tissue in the lungs of endotoxemic animals as well as some small vessels. There was NOSII staining of endothelium of small vessels in both the lung and liver of endotoxemic animals but not in control animals.

Immunohistochemical analysis for nitrotyrosine is shown in Figure 13. Nitrotyrosine was evident in endothelial cells as well inflammatory cells in liver and lung. It was particularly evident in the vasa-vasorum of the aorta and vena cava and subepicardial vessels of the heart. There was no staining of bronchial epithelial cells despite NOSII being present

in these cells. It was also was present in renal tubules of endotoxemic animals and even control animals.

DISCUSSION

The major observations of this study are that endotoxemic pigs with a low systemic vascular resistance have only a small induction of NOSII messenger RNA (mRNA) and a small amount of NOSII protein which could only be detected by immunohistochemistry during four hours of sepsis. We found no increase in NOSII activity nor an increase in plasma nitrate/nitrite. However, by both immunoblotting and immunohistochemistry technique, we found that nitrotyrosine was increased in multiple tissues. These results indicate that large amounts of NOSII and NO are not necessary for the production of the hemodynamic abnormalities of endotoxemia, nor for the production of nitrotyrosine and challenges the prevailing hypothesis that NOSII and NO accounts for the vascular dysfunction of septic shock.

We were concerned that our failure to detect NOSII on Western analysis may have been related to the specificity of the antibodies, and the short period of observation. In the present study, we therefore included animals which were studied for up to 4 hours which allowed more time for the induction of NOSII. We also used 5 different antibodies, 2 monoclonal and 3 polyclonal. However, we still failed to detect NOSII by immunoblotting, even when we loaded large amounts of protein and when we tried to immunoprecipitate NOSII. To confirm that our NOSII antibody worked, we obtained a solution rich in neutrophils by producing pleural inflammation in pigs pre-treated with endotoxin. In this

sample, we were indeed able to detect NOSII which indicates that the antibody works in the pig. We finally obtained evidence by immunohistochemistry that some NOSII is indeed present. There was a small amount of constitutive expression in pulmonary epithelial cells as seen in humans (18, 30, 33) and NOSII appeared in small vessels after two to three hours of endotoxemia. However, the quantity of NOSII was very small for up to four hours of endotoxemia.

That the amount of NOSII was very small is also supported by the very small induction of NOSII mRNA, lack of change in NOS activity in the lung, the lack of change in plasma nitrate/nitrite and lack of change in expired NO. Yet, these animals had a marked decrease in systemic vascular resistance. In another study, with the same protocol, we have also observed decreased responsiveness to norepinephrine as occurs in human sepsis (10). Thus, the pattern of NOS in pigs is very different from rodents and the NOSII hypothesis needs to be re-evaluated.

There are a number of problems with the NOSII hypothesis for sepsis even in rats. Rats become hypotensive and have decreased reactivity to catecholamines much before the induction of NOSII (55). Nitrite concentration does not correlate with hypotension (58). In one NOSII knockout model (32), survival was not improved after endotoxin injection, and even in one of the studies in which survival was improved, animals were still hypotensive (34). In the cecal perforation model of sepsis in rodents which has a hemodynamic picture much more similar to humans, NOSII induction is much less than in rats injected with endotoxin (60).

Support for the NOSII hypothesis is even weaker when higher order species than rats and mice are considered. Although plasma nitrite is elevated in septic humans, the increase is much smaller than what is observed in rats, and the increase may be due to decreased clearance of nitrite (15). Endotoxic rats have a large production of expired NO, whereas in a previous study, we found only a small increase in expired NO after two hours of endotoxemia in pigs (38) and in this study we found no change at 4 hours. To date, no study has demonstrated increase expired NO in septic humans.

There is evidence of constitutive expression of NOSII in humans (18, 30, 33), but the evidence for physiologically significant NOSII induction in septic humans is weak. NOSII can be found in human macrophages and endothelial cells of inflamed tissues (40), but the evidence for NOSII in circulating monocytes is inconsistent (1). NOSII mRNA is found in atherosclerotic lesions (9), multiple cell types (26, 37, 42, 44) and various tumor cells (47)(3). There are also reports of NOSII in dilated human hearts (12, 19, 22). NOSII mRNA has been found in human smooth muscle cells but no NOSII protein or NO production have been reported in these studies (6, 35).

Evidence of NOSII protein in humans by Western analysis has only been found in three studies to our knowledge. Haywood et al (22) found NOSII in the hearts of patients with dilated cardiomyopathies. Recently, Lanone et al (31) found NOSII by Western analysis in the rectus abdominis muscle of septic patients. The clearest evidence of NOSII protein in humans is that of Wheeler et al (62) who isolated neutrophils from the urine of patients and were able to demonstrate NOSII by Western analysis in immunoprecipitates from neutrophils of patients with urinary tract infections (62).

An important part of the proposed pathophysiology of tissue injury in the NOSII hypothesis is the production of $OONO^-$ by the reaction of superoxide (O_2^-) with NO in a reaction which is essentially diffusion limited (5). The high concentrations of superoxide dismutase in tissues normally keeps this reaction to a minimum. However, when NO production is increased, as occurs with NOSII induction in rodents, $OONO^-$ production is markedly increased. Once $OONO^-$ is formed, the reaction is essentially irreversible (4). $OONO^-$ has a relatively long half-life and therefore can diffuse and attack distant targets (5, 46). It is stable in alkaline solution, but has a pKa of 6.6 at 0° C. Once protonated to peroxynitrous acid, a potent oxidant, it rapidly dissociates and reacts with proteins and lipids. Since acidosis is common in sepsis, this would tend to increase the proportion of peroxynitrous acid.

Targets of $OONO^-$ are tyrosine residues on proteins. These are nitrosated to form 3-nitrotyrosine residues which are said to be a "footprint" of prior attack of peroxynitrate (25). Despite not finding a large increase in NOSII, we found nitrotyrosine in multiple tissues, both by immunoblotting and immunohistochemistry. What then are the potential sources?

Even though NOSII was present in small amounts, it is still possible that it was the source of $OONO^-$ and nitrotyrosine. Both NOSII and nitrotyrosine were present primarily in endothelial cells and inflammatory cells. This distribution is similar to what has been observed in the hearts of rats treated with cytokines (2). If the location of NO production by NOSII was strategically matched to regions with increased O_2^- production, $OONO^-$ could be preferentially produced without a detectable increase in plasma nitrite. O_2^- for the reaction could come from inflammatory cells or from NADH/NADPH oxidase in vessel wall (14, 39).

It is even possible that NOSII itself could produce both O_2^- and NO, for this occurs when the substrate L-arginine, or its co-factor tetrahydrobiopterin (BH-4), are in limited supply (63, 64). The possible role of NOSII as the source of the nitrotyrosine is supported by the work of El-Dwairi et al (11). They found that endotoxin induced only a small increase in NOSII in skeletal muscle of endotoxic rats, but induced a large increase in constitutive NOSs. Yet the time course of the appearance of nitrotyrosine followed that of NOSII rather than constitutive NOSs. Of note, the molecular size of the nitrated proteins they saw on the Western analysis were the same as we observed.

$OONO^-$ could also come from the reaction of NO from constitutive NOS with increased production of O_2^-. Based on the rate of production of NO by endothelium of the rabbit aorta, Beckman et al (4), calculated that the intraluminal rate of NO production could reach 8 mM/min (4). The increase in plasma concentrations of various agonists in endotoxemia, including endothelin and catecholamines, could increase constitutive NO production even more. Indeed, a four-fold increase in local NO production by direct measurement has been found *in vivo* (7, 17). Since there are many plasma stores of NO, this local production might not translate into an increase in plasma nitrite, especially if the NO reacts with O_2^- to form $OONO^-$. $OONO^-$ formation by this mechanism, however, would also require an increase in O_2^-. This, too, has been shown to occur in endotoxemia (7). As discussed with NOSII, possible sources of O_2^- are increased production by inflammatory cells as well as production of O_2^- by endothelial or smooth muscle NADH/NADPH oxidase. Alternatively it is possible that NOSIII produces both NO and O_2^- because of inadequate substrate or cofactors (63). In rodent studies, NOSIII has been found to be decreased in

endotoxemia. However, in the companion paper, we show that NOS activity and constitutive proteins (NOSIII and NOSI) are increased in the aorta and vena cava of septic animals.

Besides coming from reactions with $OONO^-$, nitrotyrosine could also be produced by the reaction of tyrosine residues with other reactive molecules (20). These include perchloric acid which could come from infiltrating neutrophils (59, 61) and hydroxyl radical from myeloperoxidase. However, it is argued that the concentration of these molecules is likely too small in biological systems for this to be an important factor (46) and, although these are reactive molecules, their biological activity also differs from peroxynitrite. For example, perchloric acid is more potent than peroxynitrite as a blocker of prostaclyclin synthesis but much slower (66).

No matter what the source, the presence of nitrotyrosine in small vessels is a sign of oxidative injury in a potentially strategic location for vascular regulation, for these are resistance vessels. Lipid peroxidation and nitrosation of proteins of the walls of endothelial cells could alter transmembrane potential, normal channel function and receptor activation and thus explain the lack of responsiveness to agonists as well as hypotension. The diffuseness of the injury is also consistent with the widespread evidence of nitrotyrosine.

If nitrotyrosine is related to production of NO, whether from NOSII or NOSIII, then it is rational to inhibit NOS function. However, NO is also important for normal vascular function, including the matching of flow to metabolic needs and preventing the adhesion of inflammatory cells. Thus, inhibition of NO could have potentially harmful effects (17). In most studies, (17) NOS inhibition raises arterial pressure but decreases cardiac output (36,

41). If, however, $OONO^-$ is produced by dysfunctional NOS, because of inadequate substrate or cofactors, perhaps some NOS inhibition might be beneficial.

The markedly different response of pigs to endotoxin when compared to rats and mice, raises questions about the significance of data from rodents and mice. The usual doses of endotoxin in rodents is 10-20 mg/kg; we used 20 μg/kg in our pigs, for larger doses were fatal. In human studies, nanogram quantities of endotoxin have been used (53). Rats have a marked generalized increase in NO production, which is far different from the pathological picture seen in pigs and what has been observed in humans. It is possible that the large increase in NO which is seen in rodents masks more physiological changes that are the true culprits in sepsis.

REFERENCES:

1. Albina, J.E. On the expression of nitric oxide synthase by human macrophages. Why no NO? J.Leukoc.Biol. 58: 643-649, 1995.

2. Balligand, J.-L., D. Ungureanu-Longrois, W.W. Simmons, L. Kobzik, C.J. Lowenstein, S. Lamas, R.A. Kelley, T.S. Smith, and T. Michel. Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1b and IFN-Y. Am.J.Physiol. 268: H1293-H13031995.

3. Bani, D., E. Masini, M.G. Bello, M. Bigazzi, and T.B. Sacchi. Relaxin activates the L-arginine-nitric oxide pathway in human breast cancer cells. Cancer Res 55: 5272-5275, 1995.

4. Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall, and B.A. Freeman. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc.Natl.Acad.Sci.USA 87: 1620-1624, 1990.

5. Beckman, J.S. and W.H. Koppenol. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am.J.Physiol. 40: C1424-C14371996.

6. Bellan, J.A., R.K. Minkes, D.B. McNamara, and P.J. Kadowitz. NG-nitro-L-arginine selectively inhibits vasodilator responses to acetylcholine and bradykinin in cats. Am.J.Physiol. 260: H1025-H10291991.

7. Brovkovych, V., S. Patton, S. Brovkovych, F. Kiechle, I. Huk, and T. Malinski. In situ measurement of nitric oxide, superoxide and peroxynitrite during endotoxema. J.Physiol.& Pharmacol. 48: 633-644, 1997.

8. Busse, R. and A. Mulsch. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Letter 275: 87-90, 1990.

9. Buttery, L.D.K., D.R. Springall, A.H. Chester, T.J. Evans, N. Standfield, D.V. Parums, M.H. Yacoub, and J.M. Polak. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab.Invest. 75: 66-85, 1996.

10. Datta, P. and S. Magder. Hemodynamic response to norepinephrine with and without inhibition of nitric oxide synthase in porcine endotoxemia. Am.J.Resp.Crit.Care Med. 160: 1987-1993, 1999.

11. El-Dwairi, Q., A. Comtois, Y. Guo, and S.N.A. Hussain. Endotoxin-induced skeletal muscle contractile dysfunction: contribution of nitric oxide synthases. Am.J.Physiol. 274: C770-C7791998.

12. Fukuchi, M., S.N.A. Hussain, and A. Giaid. Heterogeneous expression and activity of endothelial and inducible nitric oxide synthases in end-stage human heart failure. Their relation to lesion site and b-adrenergic receptor therapy. Circulation 98: 132-139, 1998.

13. Gonzalez, P.K., J. Zhuang, S.R. Doctrow, B. Malfroy, P.F. Benson, M.J. Menconi, and M.P. Fink. EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. JPET 275: 798-806, 1995.

14. Griendling, K.K., C.A. Minieri, J.D. Ollerenshaw, and R.W. Alexander. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ.Res. 74: 1141-1148, 1994.

15. Groeneveld, P.H.P., K.M.C. Kwappenberg, J.A.M. Langermans, P.H. Nibbering, and L. Curtis. Nitric oxide (NO) production correlates with renal insufficiency and multiple organ dysfunction syndrome in severe sepsis. Int.Care Med. 22: 1197-1202, 1996.

16. Grover, R., D. Zaccardelli, G. Colice, K. Guntupalli, D. Watson, and J.L. Vincent. An open-label dose escalation study of the ntiric oxide synthase inhibitor, N(G)-methyl-L-arginine hydrochloride (546C88), in patients with septic shock. Glaxo Wellcome International Septic Shock Study Group. Crit.Care Med. 27: 913-922, 1999.

17. Gryglewski, R.J., P.P. Wolkow, W. uracz, E. Janowski, J.B. Bartus, O. Balbatun, S. Patton, V. Brovkovych, and T. Malinski. Protective role of pulmonary nitric oxide in the acute phase of endotoxemia in rats. Circ.Res. 82: 819-827, 1998.

18. Guo, F.H., H.R. De Raeve, T.W. Rice, D.J. Stuehr, F.B. Thunnissen, and S.C. Erzurum. Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal

human airway epithelium in vivo. Proc Natl Acad Sci USA 92: 7809-7813, 1995.

19. Habib, F.M., D.R. Springall, G.J. Davies, C.M. Oakley, M.H. Yacoub, and J.M. Polak. Tumour necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. The Lancet 347: 1151-1154, 1998.

20. Halliwell, B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? FEBS Letters 411: 157-160, 1997.

21. Hattori, R., K. Kosuga, H. Eizawa, K. Sase, R. Inoue, M. Sunamoto, Y. Ichimori, K. Sato, T. Mori, K. Takahaski, C. Kawai, S. Sasayama, and Y. Yui. Stabilization of inducible nitric oxide synthase by monoclonal antibodies. Hybridoma 12: 763-769, 1993.

22. Haywood, G.A., P.S. Tsao, H.E. von der Leyen, M.J. Mann, P.J. Keeling, P.T. Trindade, N.P. Lewis, C.D. Byrne, P.R. Rickenbacher, N.H. Bisopric, J.P. Cooke, W.J. McKenna, and M.B. Fowler. Expression of inducible nitrix oxide synthase in human heart failure. Circulation 93: 1087-1094, 1996.

23. Hom, G.J., S.K. Grant, G. Wolfe, T.J. Bach, D.E. MacIntyre, and N.I. Hutchinson. Lipopolysaccharide-induced hypotensiona nd vascular hyporeactivity in the rat: Tissue analysis of nitric oxide synthase mRNA and protein expression in the presence and absence of dexamethason, N^G-monomethyl-L-arginine or indomethacin. JPET 272: 452-459, 1995.

24. Hsu, S.M., L. Raine, and H. Fanger. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J.Histochem.Cytochem. 29: 577-580, 1981.

25. Ischiropoulos, H., L. Zhu, J. Chen, M. Tsai, J.C. Martin, C.D. Smith, and J.S. Beckman. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Archives of Biochemistry and Biophysics 298: 431-437, 1992.

26. Kasai, K., Y. Hattori, N. Nakanishi, K. Manaka, N. Banba, S. Motohashi, and S. Shimoda. Regulation of inducible nitric oxide production by cytokines in human thyrocytes in culture. Endocrinology 136: 4261-4270, 1995.

27. Kilbourn, R., S. Gross, A. Jubran, J. Adams, O. Griffith, R. Levi, and R. Lodata. N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. Proc.Natl.Acad.Sci.U.S.A. 87: 3629-3632, 1990.

28. Kilbourn, R.G., A. Jubran, and S.S. Gross. Reversal of endotoxin-mediate shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. Biochem.Biophys.Res.Commun. 172: 1132-1138, 1990.

29. Knowles, R.G., M. Merrett, M. Salter, and S. Moncada. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem.J. 270: 833-836, 1990.

30. Kobzik, L., D.S. Bredt, C.J. Lowenstein, J. Drazen, B. Gaston, D. Sugarbaker, and J.S. Stamler. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am.J.Respir.Cell.Mol.Biol. 9: 371-377, 1993.

31. Lanone, S., A. Mebazaa, D. Henin, D. Payen, M. Aubier, and J. Roczkowski. Inducible nitric oxide synthase (iNOS) expression and activity in skeletal muscle from septic patients. Am J Resp Crit Care Med 157: A1021998.(Abstract)

32. Laubach, V.E., E.P. Garvey, and P.A. Sherman. High-level expression of human inducible nitric oxide synthase in Chinese hamster ovary cells and characterization of the purified enzyme. Biochem.Biophys.Res.Commun. 218: 802-807, 1996.

33. Lundberg, J.O., T. Farkas-Szallasi, E. Weitzberg, J. Rinder, J. Lidholm, A. Anggaard, T. Hokfelt, J.M. Lundberg, and K. Alving. High nitric oxide production in human paranasal sinuses. Nature Medicine 1: 370-373, 1995.

34. MacMicking, J.D., C. Nathan, G. Hom, N. Chartrain, D.S. Fletcher, M. Trumbauer, K. Stevens, X. Qiao-wen, K. Sokol, N. Hutchinson, H. Chen, and J.S. Mudgett. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81: 641-650, 1995.

35. MacNaul, K.L. and N.I. Hutchinson. Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and

inflammatory conditions. Biochem.Biophys.Res.Commun. 196: 1330-1334, 1993.

36. Magder, S. and G. Vanelli. Circuit factors in the high cardiac output of sepsis. J.Crit.Care 111: 155-166, 1996.

37. McLay, J.S., P. Chatterjee, A.G. Nicolson, A.G. Jardine, N.G. McKay, S.H. Ralston, P. Grabowski, N.E. Haites, A.M. MacLeod, and G.M. Hawksworth. Nitric oxide production by human proximal tubular cells: a novel immunomodulatory mechanism? Kidney International 46: 1043-1049, 1994.

38. Mehta, S., D. Javeshghani, P. Datta, R.D. Levy, and S. Magder. Porcine endotoxaemic shock is associated with increased expired nitric oxide. Crit.Care Med. 27: 385-393, 1999.

39. Mohazzab-H, K.M. and M.S. Wolin. Sites of superoxide anion production detected by lucigenin in calf pulmonary artery smooth muscle. Am.J.Physiol. 267: L813-L8221994.

40. Moilanen, E., T. Moilanen, R. Knowles, I. Charles, Y. Kadoya, N. Al-Saffar, P.A. Revell, and S. Moncada. Nitric oxide synthase is expressed in human macrophages during foreign body inflammation. Am.J.Pathol. 150: 881-887, 1997.

41. Nava, E., M.J. Palmer, and S. Moncada. Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? Lancet 338: 1555-1557, 1991.

42. Niemann, A., A. Bjorklung, and D.L. Eizirik. Studies on the molecular regulation of the inducible form of nitric oxide synthase (iNOS) in insulin-producing cells. Mol.Cell.Biochem. 106: 151-155, 1994.

43. Ochoa, J.B., A.O. Udekwu, and T.R. Billiar. Nitrogen oxide levels in patients after trauma and during sepsis. Ann.Surg. 214: 621-626, 1991.

44. Park, C.-S., R. Park, and G. Krishna. Constitutive expression and structural diversity of inducible isoform of nitric oxide synthase in human tissues. Life Sci 59: 219-225, 1996.

45. Petros, A., D. Bennett, and P. Vallance. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 338: 1557-1558, 1991.

46. Pryor, W.A. and G.L. Squadrito. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am.J.Physiol. 268: L699-L7221995.

47. Rosbe, K.W., J. Prazma, P. Petrusz, W. Mims, S.S. Ball, and M.C. Weissler. Immunohistochemical characterization of nitric oxide synthase activity in squamous cell carcinoma of the head and neck. Otolaryngology-Head and Neck Surgery 113: 541-549, 1995.

48. Rosselet, A., F. Feihl, M. Markert, A. Gnaegi, C. Perret, and L. Liaudet. Selective iNOS inhibition is superior to norepinephrine in the treatment of rat endotoxic shock. Am.J.Respir.Crit.Care Med. 157: 162-170, 1998.

49. Saleh, D., P.J. Barnes, and A. Giaid. Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. Am.J.Respir.Crit.Care Med. 155: 1763-1769, 1997.

50. Squadrito, G.L. and W.A. Pryor. The formation of peroxynitrite in vivo from nitric oxide and superoxide. Chemico-Biological Interactions 96: 203-206, 1995.

51. Stewart, T.E., F. Valenza, S.P. Ribeiro, A.D. Wener, G. Volgyesi, J. Brendan, M. Mullen, and A.S. Slutsky. Increased nitric oxide in exhaled gas as an early marker of lung inflammation in a model of sepsis. Am.J.Respir.Crit.Care Med. 151: 713-718, 1995.

52. Stoclet, J., I. Fleming, and G. Gray. Nitric oxide and endotoxemia. Circulation 87: V-77-V-801993.

53. Suffredini, A.F., R.E. Fromm, M.M. Parker, M. Brenner, J.A. Kovacs, R.A. Wesley, and J.E. Parrillo. The cardiovascular response of normal humans to the administration of endotoxin. New Engl.J.Med. 321: 280-287, 1989.

54. Szabo, C. Alterations in nitric oxide production in various forms of circulatory shock. New Horiz 3: 2-32, 1995.

55. Szabo, C., J.A. Mitchell, C. Thiemermann, and J.R. Vane. Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. Br.J.Pharmacol. 108: 786-792, 1993.

56. Szabo, C., C. Saunders, M. O'Connor, and A.L. Salzman. Peroxynitrite causes energy depletion and increases permeability via activation of poly (ADP-Ribose) synthetase in pulmonary epithelial cells. Am.J.Respir.Cell Mol.Biol. 16: 105-109, 1997.

57. Thiemermann, C. and J. Vane. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. Eur.J.Pharmacol. 182: 591-595, 1990.

58. van den Berg, C., J.G.C. Amsterdam, A. Bisschop, J.J. Piet, J. Wemer, and D.J. de Wildt. Septic shock: no correlation between plasma levels of nitric oxide metabolites and hypotension or lethality. Eur.J.Pharmacol. 270: 379-382, 1994.

59. van der Vliet, A., J.P. Eiserich, B. Halliwell, and E.C. Cross. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. J.Biol.Chem. 272: 7617-7625, 1997.

60. Vromen, A., M.S. Arkovitz, B. Zingarelli, A.L. Salzman, V.F. Garcia, and C. Szabo. Low-level expression and limited role for the inducible isoform of nitric oxide synthase in the vascular hyporeactivity and mortality associated with cecal ligation and puncture in the rat. Shock 6: 248-253, 1996.

61. Weiss, S.J. Tissue destruction by neutrophils. N.Engl.J.Med. 320: 365-376, 1989.

62. Wheeler, M.A., S.D. Smith, G. Garcia-Cardena, C.F. Nathan, R.M. Weiss, and W.C. Sessa. Bacterial infection induces nitric oxide synthase in human neutrophils. J.Clin.Invest. 99: 110-116, 1997.

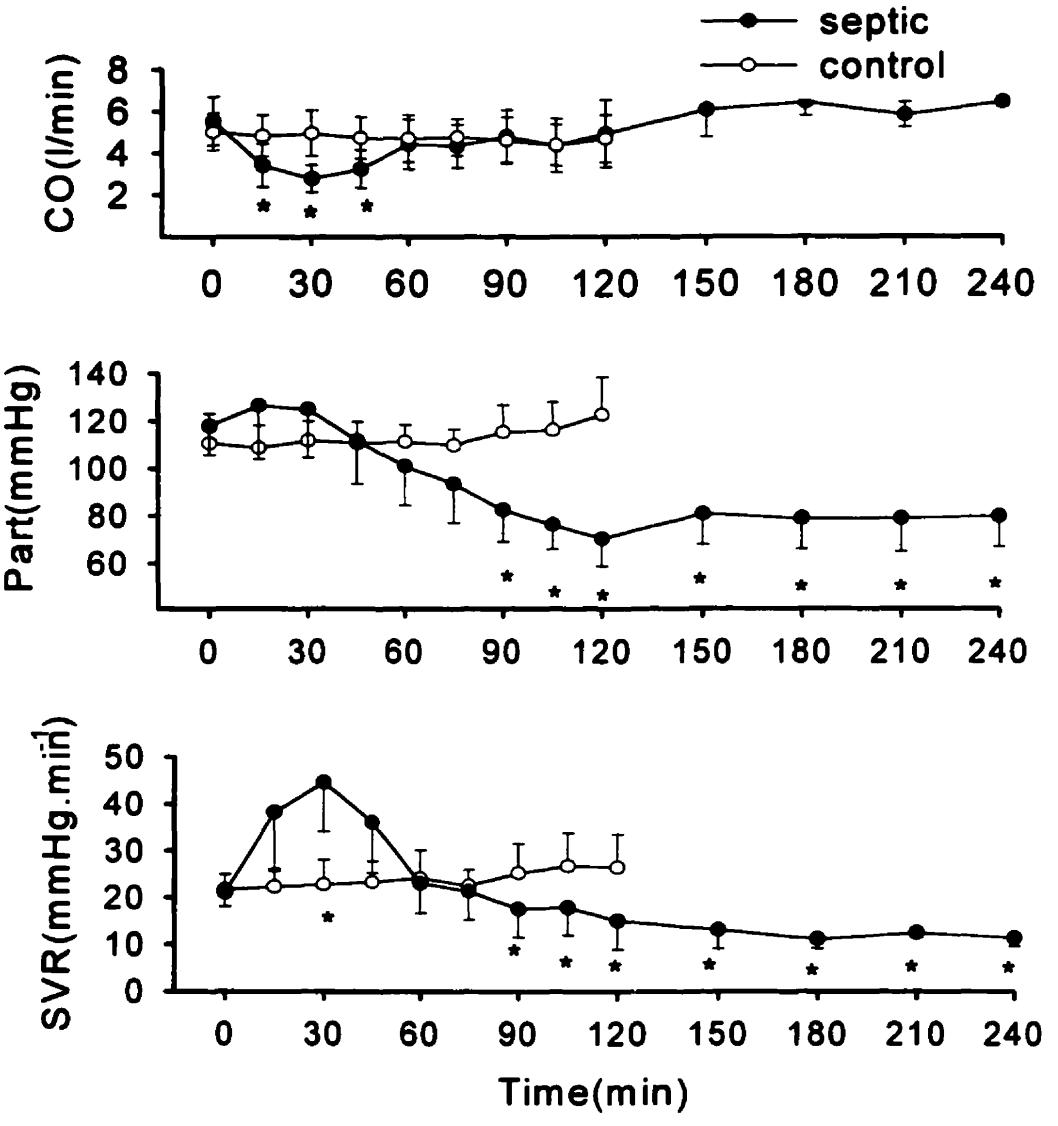
63. Xia, Y., V.L. Dawson, T.M. Dawson, S.H. Snyder, and J.L. Sweier. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc.Natl.Acad.Sci.U.S.A. 93: 6770-6774, 1996.

64. Xia, Y. and J.L. Zweier. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc.Natl.Acad.Sci.U.S.A. 94: 6954-6958, 1997.

65. Xiao-qing, W., I.G. Charles, A. Smith, J. Ure, G.-J. Feng, F.-P. Huang, D. Xu, S. Moncada, and F.Y. Liew. Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 375: 408-411, 1995.

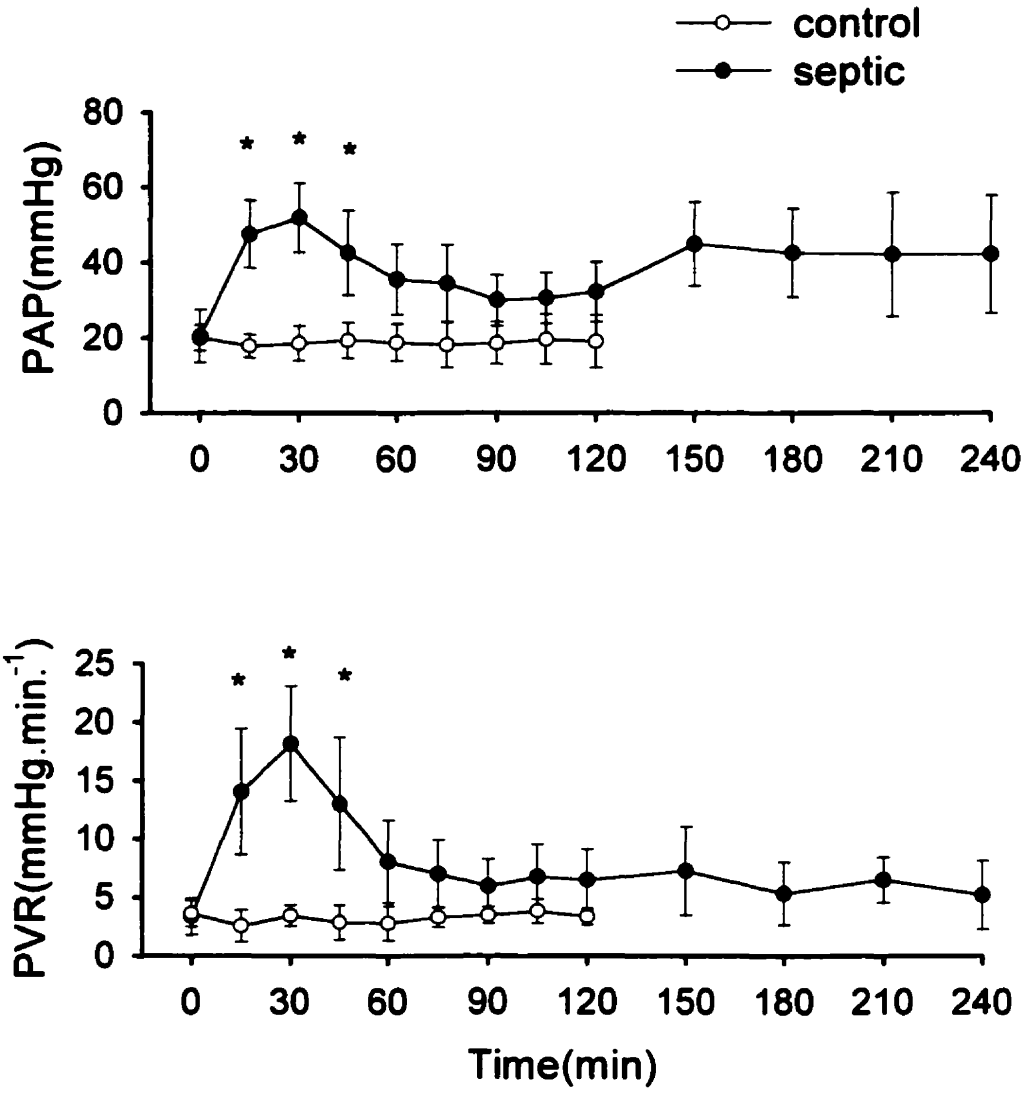
66. Zou-M.-H. and V. Ullrich. Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibts bovine aortic prostacyclin synthase. FEBS Letters 382: 101-104, 1996.

Figure 1



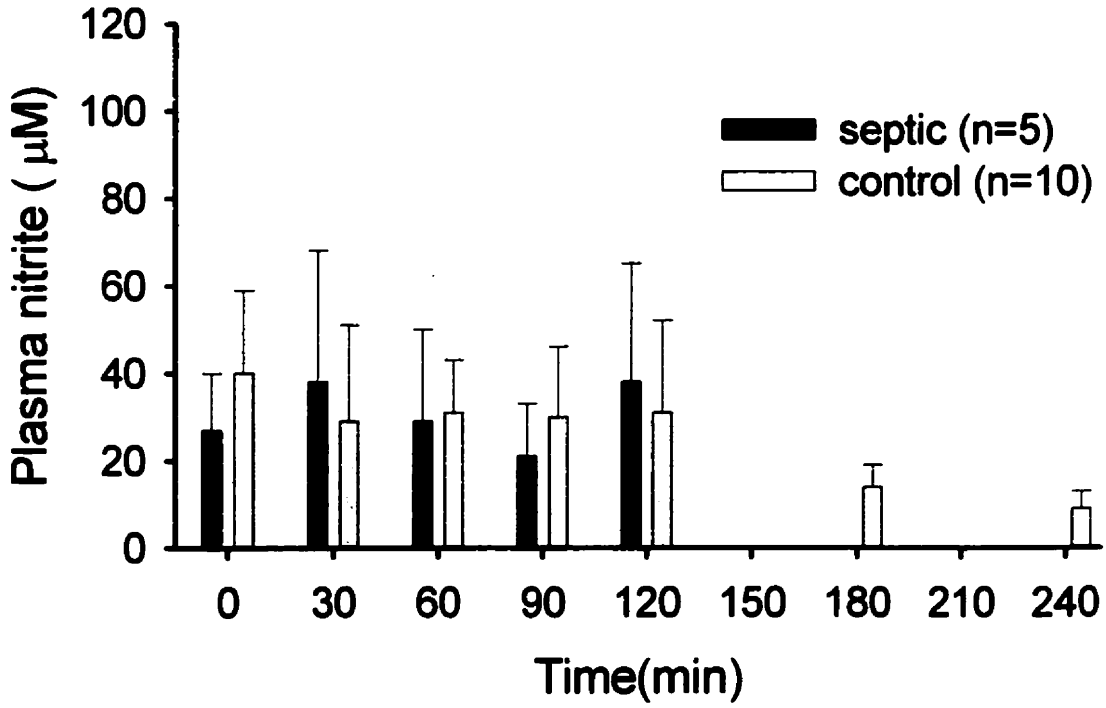
Time course of cardiac output (CO,L/min), arterial pressure (P_{art} mmHg) and systemic vascular resistance (SVR, mmHg.min.L^{-1}) for 2 hours in control (o) and endotoxemic animals (●) for 2 hours (n=12) and 4 hours (n=4). There was an initial fall in cardiac output but by 60 minutes. It was back to baseline and did not change further. The arterial pressure, P_{art} began to fall after 30 minutes and did not change significantly between 2 and 4 hours. The SVR rose in the first 30 minutes but was back to baseline by 60 minutes and below baseline by 90 minutes. There was no change in any of the control values. (Mean ± SD) (* represents $p<0.05$ compared to time zero)

Figure 2



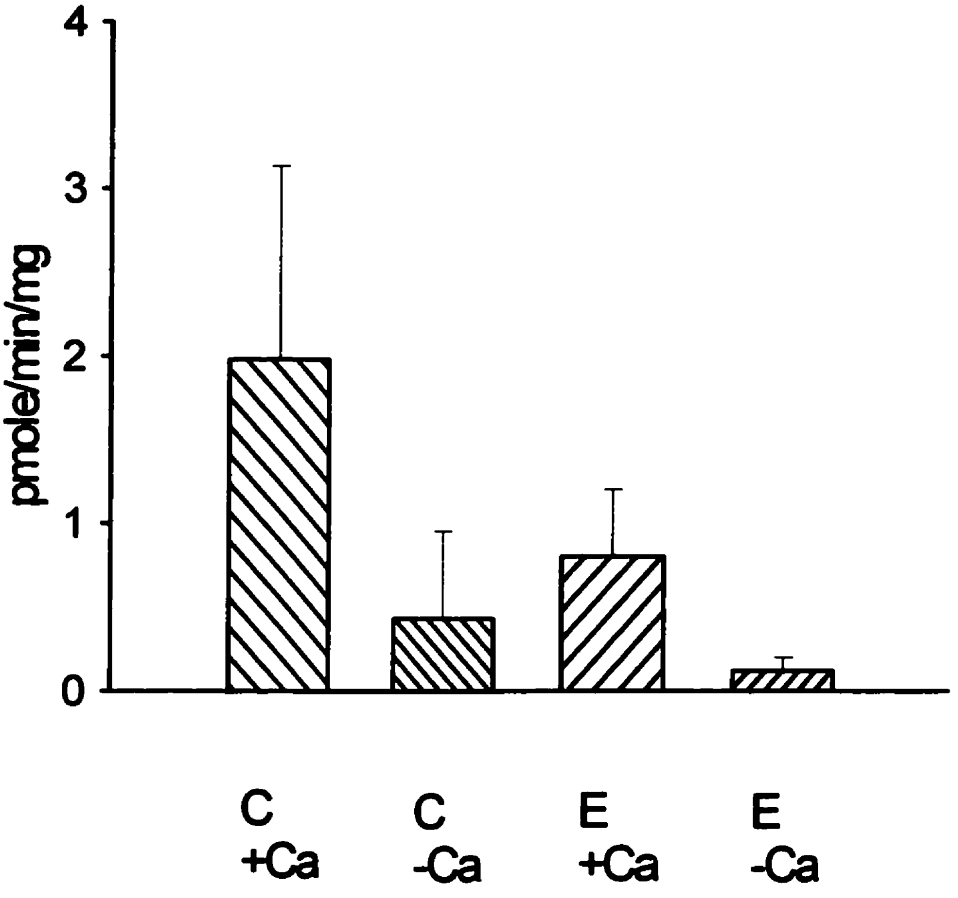
Time course of pulmonary artery (PAP, mmHg) and pulmonary artery vascular resistance (PVR, mmHg.min.L^{-1}) in control (o) and endotoxemic animals (●). PAP and PVR rose in endotoxemic animals in the first 30 minutes. The level came down by 60 minutes but remained above baseline level in the control group (Mean ± SD) (* represents $p<0.05$ compared to time zero)

Figure 3



Time course of plasma nitrate/nitrite in 5 control and 6 endotoxemic animals for 2 hours, and 4 endotoxemic animals at 3 and 4 hours. There was no significant change in plasma nitrate/nitrite over time and no significant difference between groups.

Figure 4



NOS activity in the lung of animals with 4 hours of endotoxemia (n=4). There was no change in total NOS activity or calcium independent NOS activity in endotoxin treated animals.

Figure 5 (a)

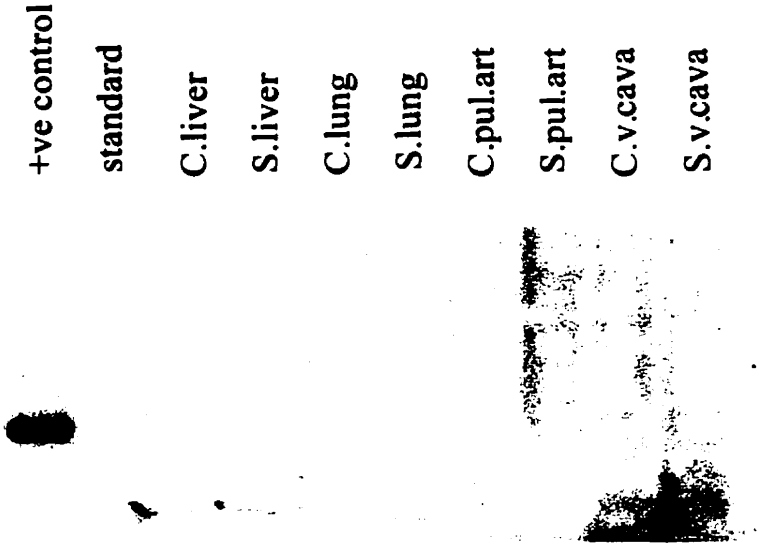
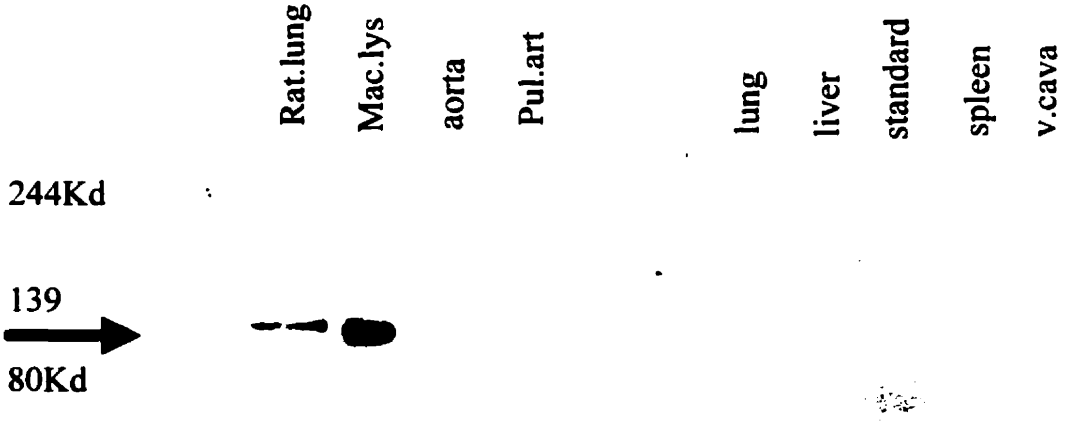
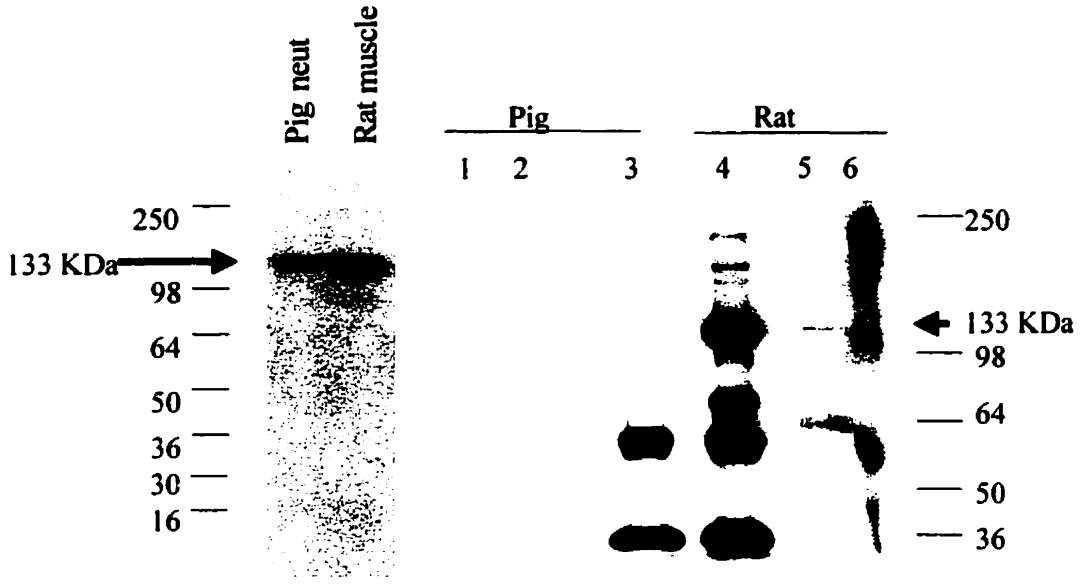


Figure 5 (b)



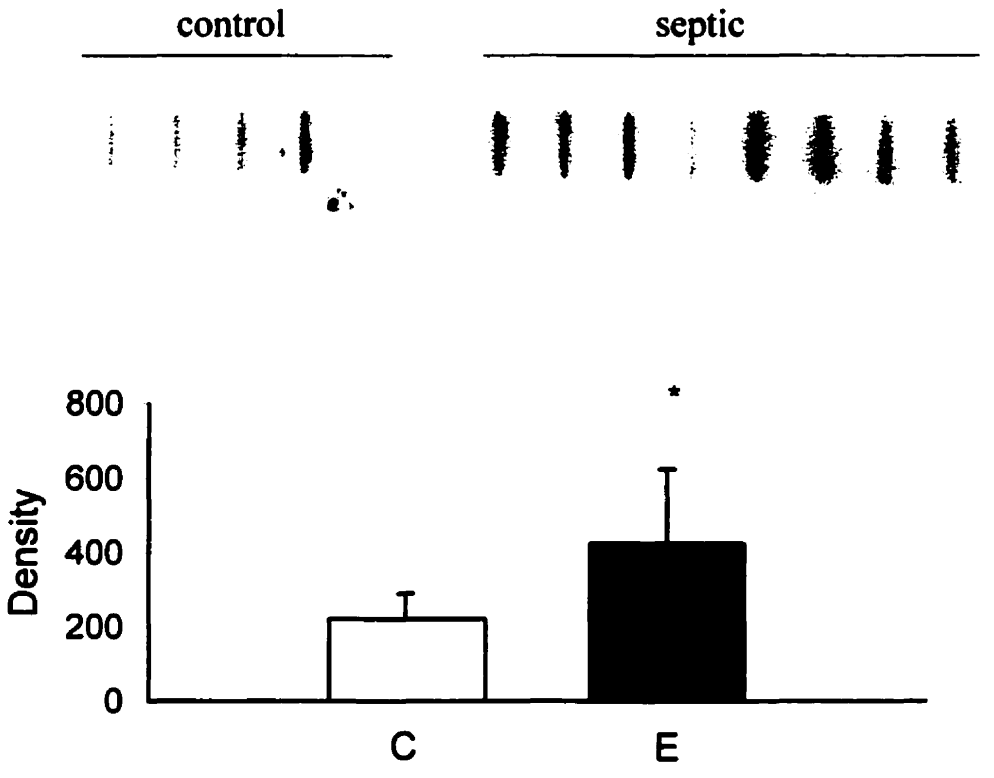
Examples of a Western blot analysis with anti-NOSII monoclonal antibody on pooled extracts of control (C) and endotoxemic pigs (S) after 2 hours (n=4) (a) and 4 hours (n=4) (b). The appropriate size band was seen in macrophage lysates (mac lys) and lung tissue from endotoxic rats. However, there was no evidence of iNOS in any of the porcine tissue; pul art=pulmonary artery; v cava= vena cava (standard is lane used for size by using stained protein with known molecular weight.

Figure 6



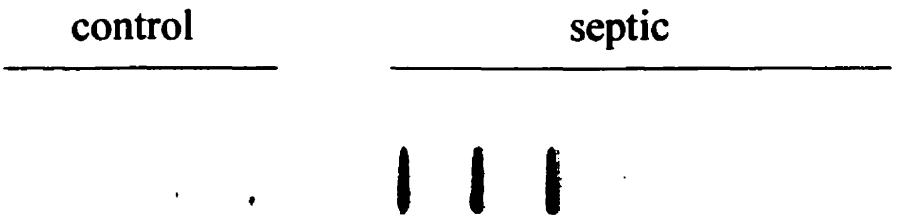
Immunoprecipitation of NOSII from LPS-treated porcine and rat lung on 4-12% gel electrophoresis (Right figure). Lane 1, crude pig lung extract. Lane 2, supernatant of porcine lung. Lane 3, immunoprecipitation of porcine lung. Lane 4, immunoprecipitation of rat lung. Lane 5, supernatant of rat lung. Lane 6, crude rat lung extract. The arrow marks the expected size of NOSII protein. There was no evidence of NOSII in any of the samples from the pig (lane 1-3). The two bands in lane 3 represents the immunoprecipitated proteins. A dark band was seen (lane 4) in the immunoprecipitate of the rat lung , and there was only a faint band in the supernatant of the sample (lane 5). NOSII was also seen in the crude extract from rat lung (lane 6). Left figure shows the result of electrophoresis of 40μg of endotoxic rat skeletal muscle and 40μg of endotoxemic pig neutrophils on 14% gel. Protein size (KDa) is shown next to it.

Figure 7



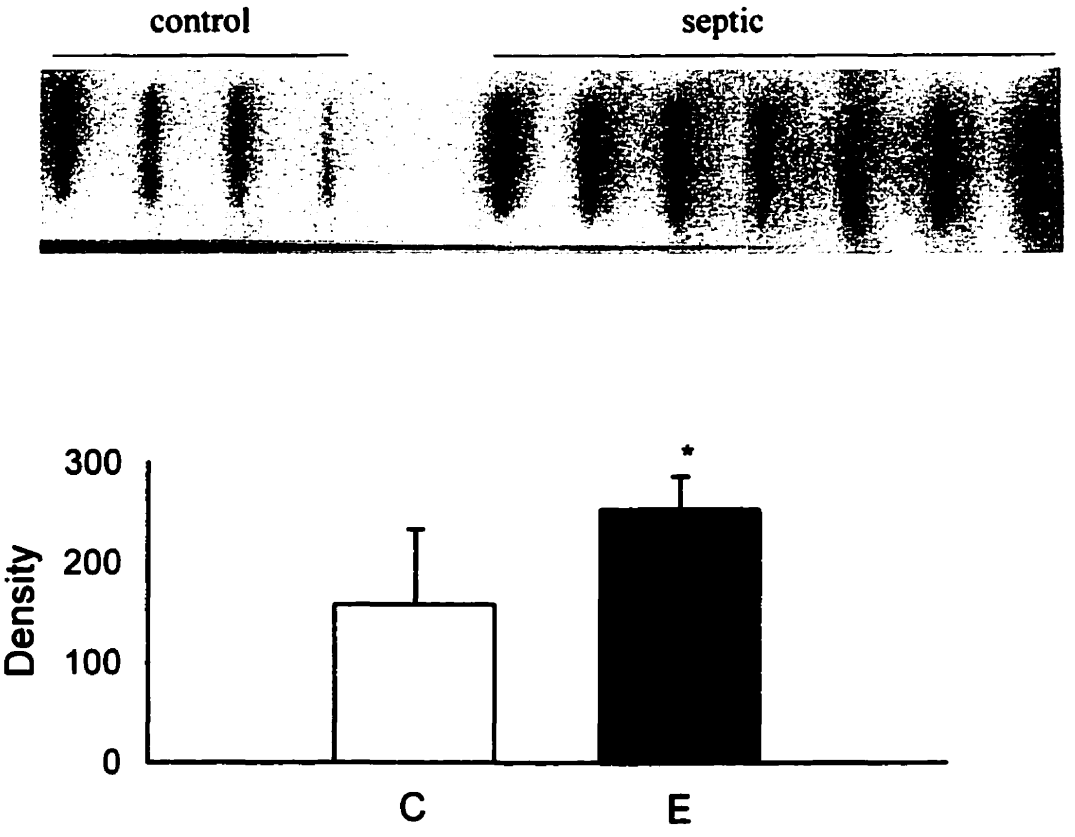
Example of a slot blot analysis of lung tissue with antinitrotyrosine antibody for 5 control (C) and 8 endotoxic (E) animals after 2 hours of endotoxemia. Densitometric analysis showed a significant increase in endotoxemic (septic) animals.

Figure 8



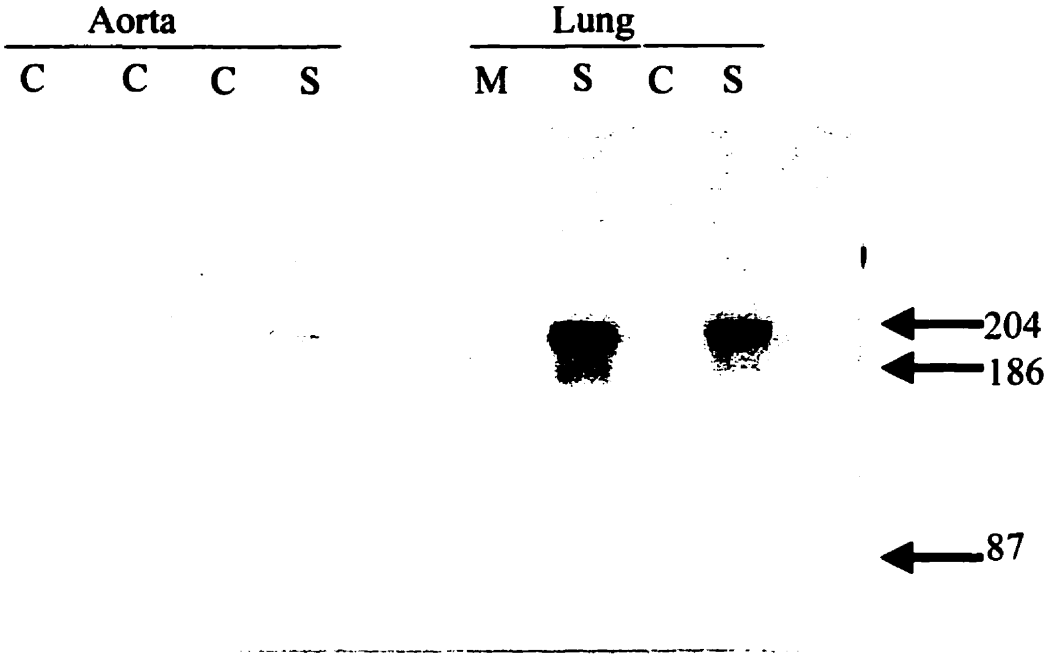
Slot blot analysis of liver homogenates with nitrotyrosine antibody in 4 control and 7 endotoxemic animals (septic). A strong signal was seen in 3 septic animals but not in the other 5.

Figure 9



Slot blot analysis of aortic tissue with nitrotyrosine antibody in 4 control and 7 endotoxemic animals (septic). By densitometric analysis there was a significant increase in nitrotyrosine in the aorta of endotoxemic animals.

Figure 10



Examples of Western blot analysis with nitrotyrosine antibody on samples of aortic and lung tissue. No bands were seen in the control samples. There was a faint band at approximately 204 kDa in the aorta as well as the lungs of endotoxemic animals. Fainter bands were also at 186 and 87 KDa in the lung from endotoxemic animals.

Figure 11(a)

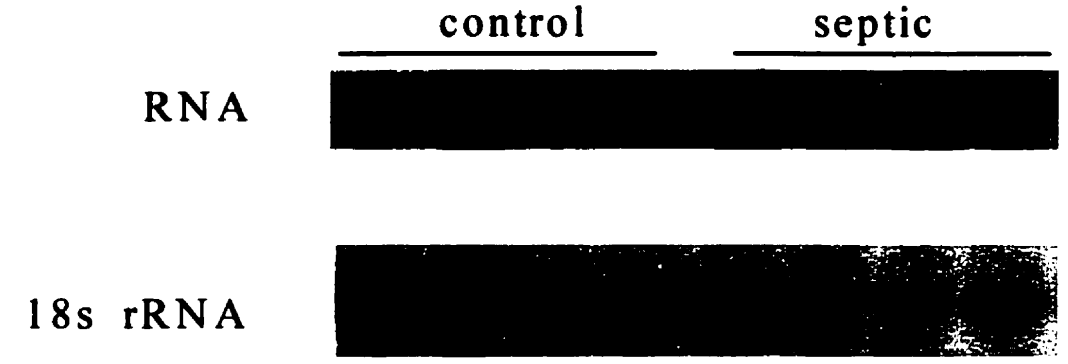
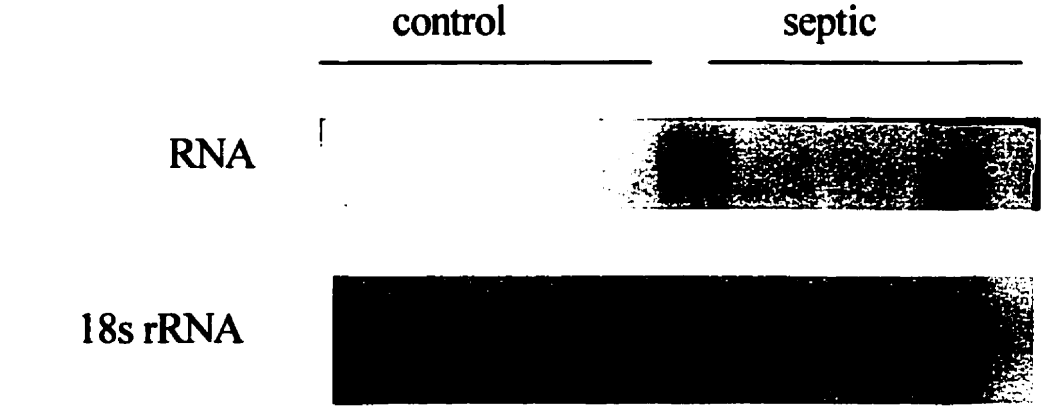


Figure 11(b)



Examples of a Northern blot analysis of lung tissue (a), and hepatic tissue (b) for NOSII mRNA. The 18s RNA was used as a control for loading. Exposure was for 70 days. NOSII was evident in 1 of 4 control lungs samples and in all 4 endotoxemic animals. There was no IINOS RNA detected in any of the control liver samples, although the loading, based on the 18s RNA, appeared a little less. NOSII was evident in the 4 endotoxemic animals.

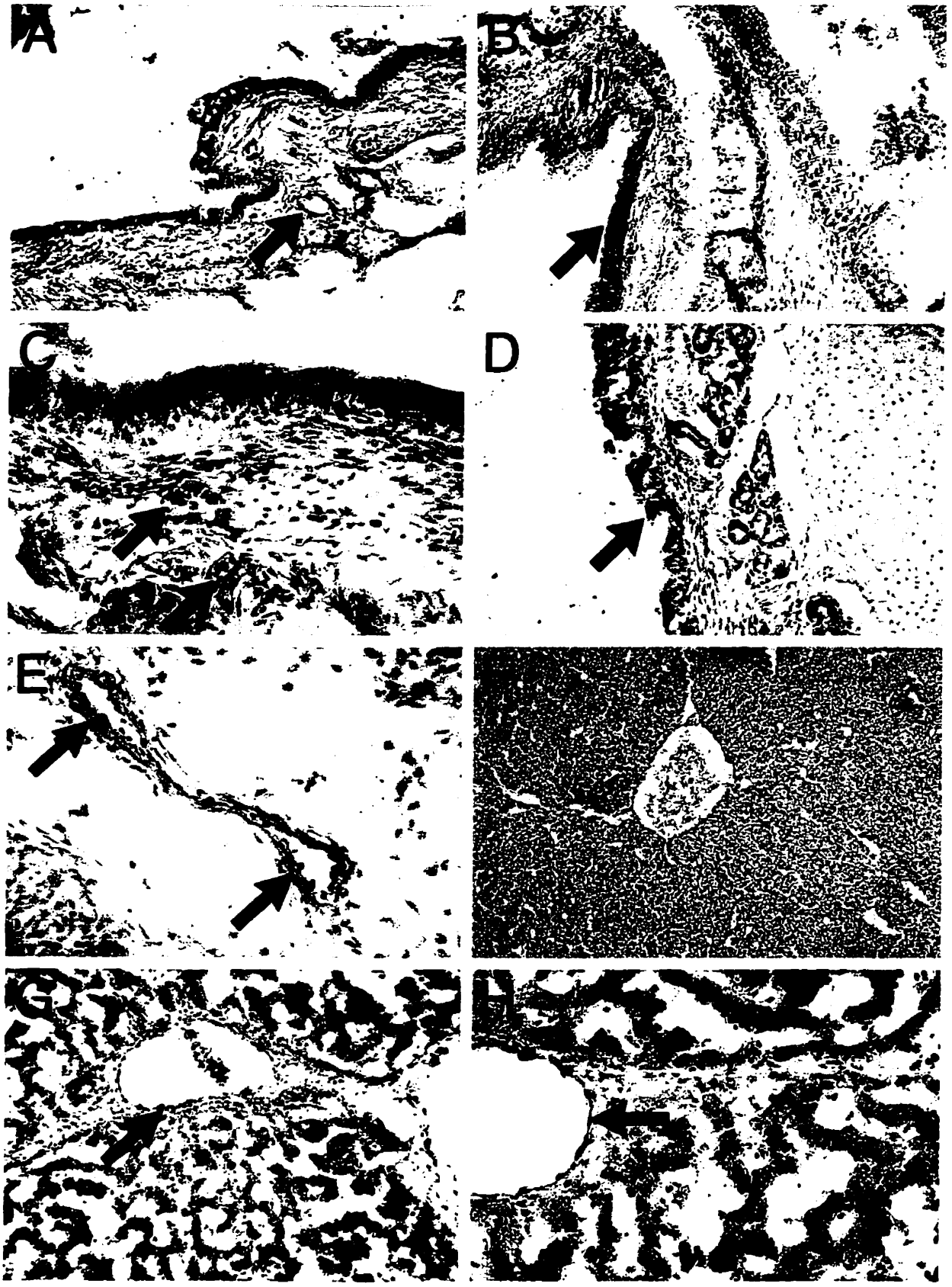

A
B
C
D
E

Figure 12 on page 147 (previous page)

Photomicrograph of lung and hepatic tissue. Figures (a) and (b) represent bronchial wall from control animals. The monoclonal antibody (mAb) (ST #N32020) was used in (a) and the polyclonal antibody (poly Ab) (Saleh, Ref. #50) was used in (b). NOSII is evident in epithelial cells with the polyclonal antibody (arrow in b) and in occasional small vessels in (a), but not in endothelial cells. (c) and (d) represent lung from endotoxic animals with the monoclonal antibody (c) and polyclonal antibody (d). There was more NOSII evident in small vessels and inflammatory cells (arrows, c). There was no NOSII present in epithelial cells with the monoclonal antibody, whereas it was evident with the polyclonal antibody in epithelial cells and glandular tissues. (e) shows evidence of NOSII in the endothelial layer of a small pulmonary vessel (poly Ab). (f) shows control hepatic tissue with no NOSII. There was a very small amount of NOSII in the lining of the central vein (g and h) (poly Ab) and in some inflammatory cells in the liver parenchyma. Magnification: a, b, d, f and g x 100, c, e, h x 200.

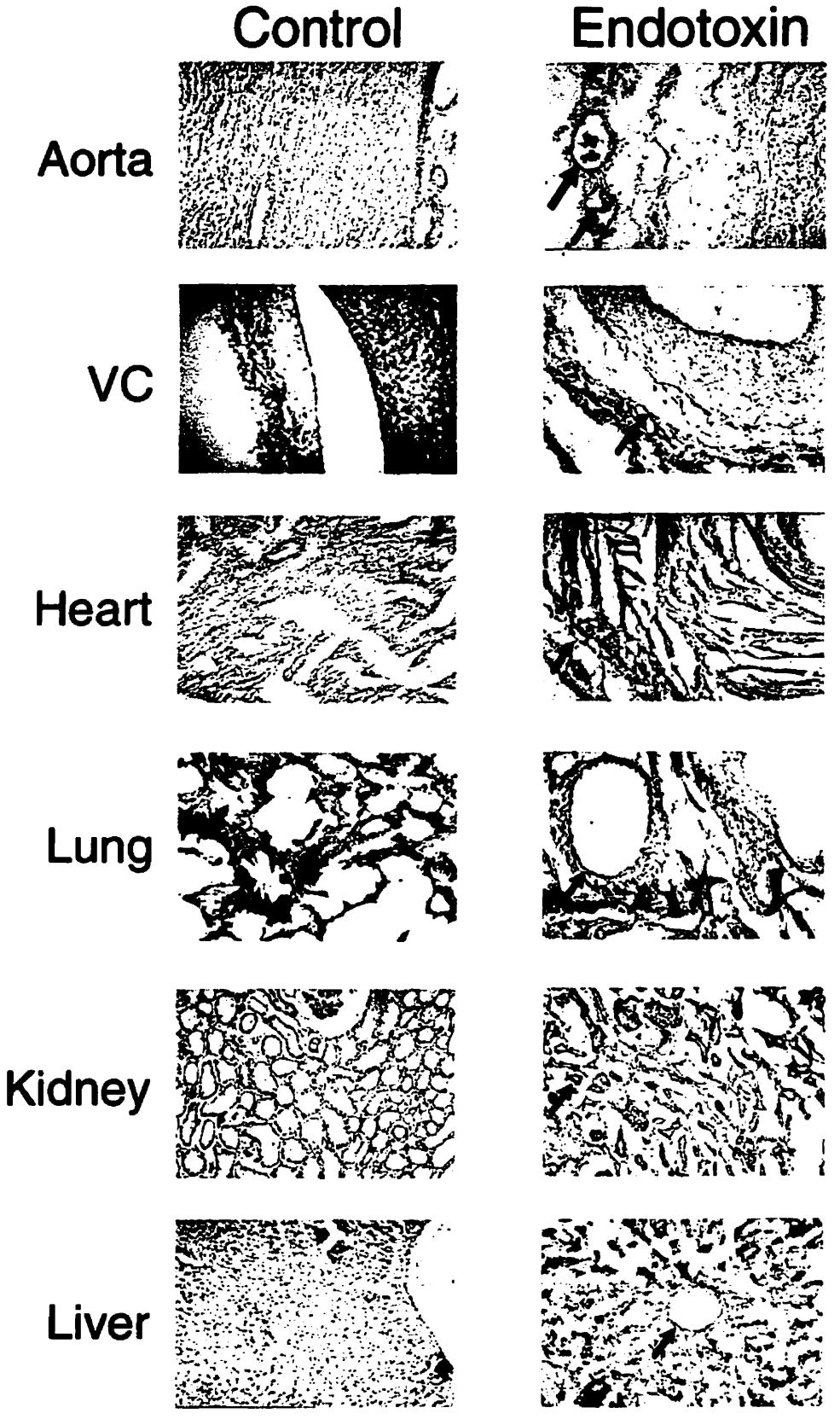

Control
Endotoxin
Aorta
VC
Heart
Lung
Kidney
Liver

Figure 13 on page 149 (previous page)

Photomicrograph of tissues from control and endotoxic animals. Nitrotyrosine was evident in the small vessels and inflammatory cells in the adventitia of the aorta, vena cava and small vessels of the heart (arrows), but there was none seen in control animals. It was also evident in the lining of some bronchial vessels and the macrophages in the lung. There was patchy staining for nitrotyrosine in the renal tubules of both control and endotoxic animals. No nitrotyrosine was seen in the liver of control animals but there was slight staining in cells lining the central vein and in some inflammatory cells in the liver.Magnification: x100

Link to chapter three:

Because the reports regarding the regulation of constitutive NOSIII are contradictory. I investigated this in our porcine model of sepsis. This is important because the production of peroxynitrite requires the increase in either NO or O_2^- or both. Because I detected no NOSII activity, I hypothesized constitutive NOS activity is increased to produce NO.

CHAPTER THREE

REGIONAL CHANGES IN CONSTITUTIVE NOS AND THE HEMODYNAMIC CONSEQUENCES OF ITS INHIBITION IN PORCINE ENDOTOXEMIA

ABSTRACT

The role of constitutive nitric oxide synthases in sepsis remains controversial. Part of the problem is that many of the studies have been performed in rats which respond differently than larger animals. Our objective, therefore, was to determine if constitutive nitric oxide synthases, i.e. eNOS (NOS-III) and nNOS (NOS-I) are still active, in vessels of hyperdynamic, endotoxemic pigs. We also characterized the dose-response relationship of the NOS inhibitor N^G-nitro-L-arginine-methyl-ester (L-NAME) in the arterial, venous and pulmonary circuits as a reflection of NO production. We anesthetized and ventilated 14 pigs which were instrumented for measurements. We measured mean circulatory filling pressure (MCFP) and resistance to venous return (RVR) by transiently arresting the circulation with a balloon in the right atrium. Animals were given with 20 μg/kg of endotoxin (n = 8) or saline (n = 6) over 2 hours. They were then given progressively increasing doses of L-NAME (0.5 to 16 μg/kg). We injected 20 μg boluses of norepinephrine (NE) at baseline, after 2 hours, and after 0.5, 4 and 16 μg of L-NAME to test the pressor response. Tissue was obtained from 6 control animals followed for 2 hours, 8 animals treated for 2 hours and then sacrificed and 4 animals treated for 2 hours and sacrificed after 2 more hours. Cardiac output did not change and the systemic vascular resistance (SVR) fell in endotoxin animals. By Western analysis eNOS was increased at 2 and 4 hours in the aorta and vena cava and this was paralleled by changes in nNOS in the vena cava. In contrast, eNOS decreased in the pulmonary artery and nNOS did not change. Calcium-dependent NOS activity increased in the aorta and vena cava, but decreased in pulmonary artery at 4 hours. The dose-response relationships to L-NAME for SVR, RVR and cardiac output were shifted to the left in

support of increased sensitivity. L-NAME decreased cardiac output and 90% of the fall was reached by 4 mg/kg. The pressor response to NE was depressed in endotoxemic animals and was partially restored with 4 mg/kg of L-NAME. In conclusion, in contrast to data from rats, cNOS activity is present in the systemic vessels of septic pigs and could play a role in the pathophysiology of sepsis.

Key Words: sepsis, cardiac output, nitric oxide, venous resistance, pulmonary artery

INTRODUCTION

The septic syndrome is characterized by a loss of vascular tone, decreased responsiveness of arterial vessels to catecholamines and an increase in cardiac output (1). Increased production of nitric oxide (NO) has been proposed to be a factor in these vascular abnormalities.(2;3) The potential role of NO gained strength with the identification of a form of nitric oxide synthase (NOS) which can be induced *in vivo* by bacteria or their lipopolysaccharide coating (LPS) which is referred to as endotoxin (4). Inducible NOS (iNOS, NOSII) produces large amounts of NO and can be induced outside the endothelium so that it can have diverse hemodynamic effects (3). However, induction of iNOS protein takes more than three hours (5), yet the loss of vascular tone and hyporesponsiveness occurs within the first hour of endotoxemia (6). For example, tumor necrosis factor-α (TNF-α) produces hypotension in dogs within 30-40 minutes, and this is reversed by an inhibitor of NOS activity (7). We also found that endotoxemic pigs have hypotension and a loss of vascular responsiveness to catecholamine which is reversible with a NOS inhibitor (8-10), despite the lack of major iNOS induction. Furthermore, LPS stimulates an immediate release of NO from bovine endothelial cells (11;12). These observations support a role for constitutive NOS in the hemodynamic abnormalities of endotoxemia. On the other hand, NO release from endothelial cells and eNOS expression have been shown to be decreased by LPS (13-16) or treatment with the cytokine, TNF-α (13). Thus, the role of endothelial NOS in LPS induced shock needs to be clarified, for it has important implications for the appropriate management of NOS activity in sepsis.

The objectives of this study were therefore: 1) to determine whether the amount of the constitutive NOSs, eNOS and nNOS, are changed in the vessels of LPS treated pigs versus control animals, 2) to determine whether there is a change in calcium dependent NOS activity in the vessels of LPS treated animals versus control animals, 3) to characterize the dose-response relationship of the NOS inhibitor, N^G-nitro-L-arginine-methyl-ester (L-NAME) in normal and LPS treated pigs, 4) to determine if there is a dose of L-NAME that will restore vascular responsiveness without decreasing cardiac output in endotoxemic animals, and 5) to determine whether there is a differential effect on eNOS and the hemodynamic response to a NOS inhibitor in the arterial, venous and pulmonary circuits. We predicted that there would be no change or an increase in eNOS in LPS treated animals. This would argue that eNOS is still available to play a role in the early hemodynamic abnormalities seen in endotoxemic animals.

MATERIALS AND METHODS

General Methods

All procedures were performed according to the guidelines of the animal care committee of McGill University. Domestic pigs (n = 14) weighing approximately 30 kg were sedated with ketamine 30 mg · kg^{-1}, atropine 1.0 mg · kg^{-1} and xylazine 2 mg · kg^{-1}. Twenty minutes later they were anesthetized with 10 to 15 mg ·kg^{-1} of sodium thiopental; anesthesia was maintained with a continuous intravenous infusion of sodium thiopental at 5 mg · kg^{-1} · hr^{-1}. The animals were placed in the supine position in a V-shaped support, intubated with a cuffed endotracheal tube, and ventilated with a volume respirator at a tidal

volume of 12 ml · kg^{-1} and a frequency of 12 to 15 breaths · min^{-1} and 5 cm water positive end-expiratory pressure. Through a midline incision in the neck, the left common carotid artery was isolated and cannulated with a polyethylene catheter for pressure measurements. The right internal jugular vein was isolated and a Swan Ganz flow-directed catheter was passed into the pulmonary artery. A 12F balloon-tipped catheter with a 50-cc capacity (Prewitt aortic occlusion catheter #10, Pramel Inc., Longueuil, Québec, Canada) was placed in the right atrium through the external jugular vein. When inflated to 40 cc, this balloon transiently obstructed the circulation and was used to stop venous return and measure the mean circulatory filling pressure (MCFP). The right femoral vein was cannulated with a polyethylene catheter (Cole Palmer, Anjou, Québec) for the administration of drugs. Blood gases were monitored, and we tried to keep PCO_2 between 30 and 40 mmHg by adjusting the ventilator, and PO_2 at greater than 90 mmHg by giving supplemental oxygen. Cardiac output was measured by the thermodilution (Abbott 3300, North Chicago, Chicago, IL) method by injecting 3 ml of 5% dextrose in water at room temperature into the right atrial port of the Swan Ganz catheter.

Measurement of MCFP

To measure MCFP, the balloon in the right atrium was rapidly inflated with 40 cc of air for 15 to 20 seconds (8). This transiently arrests venous return, and the venous pressure measured in the central vein rises and reaches a plateau at a pressure which is equal to the pressure upstream in the compliant region of the venous system. This procedure could be repeated frequently without an effect on the hemodynamic parameters or general condition

of the animal. It was reproducible with <0.5 mmHg difference on repeated measurements under the same conditions. The measurement is obtained after 15 seconds because this avoids reflex changes, although the arterial plateau pressure (APP) remains above the venous plateau pressure (VPP). The APP remains higher than VPP because volume continues to drain through the high arterial resistance and also because there is an arterial critical closing pressure, which traps volume in the arterial vessels (17). This volume is accounted for by the formula *MCFP - VPP + (APP - VPP)* (arterial compliance/venous compliance), where the ratio of arterial to venous compliance is assumed to be 1:30.

Hemodynamic Calculations

The SVR was calculated as $SVR = (\text{mean } P_{art} - P_{ra})/CO$, where P_{art} is arterial blood pressure. Pulmonary vascular resistance (PVR) was calculated from $PVR = (\text{mean } PAP - \overline{P_{cw}})/CO$, where *PAP* is pulmonary artery pressure. Resistance to venous return (RVR) from $RVR = (MCFP - P_{ra})/CO$. All these are in the units of mmHg $\cdot$ L^{-1} $\cdot$ min.

Western Blot

Frozen tissue extracts were thawed on ice and mixed with equal volume of sample buffer (composed of 4 ml distilled water, 1 ml of .5 M Tris-HCl, pH = 6.8, .8 ml glycerol, 10% SDS (w/v), .05% (w/v) bromophenol blue, 0.4 ml of 2-beta-mercaptoethanol) followed by heating at 95°C for 5 minutes. Samples were centrifuged and loaded on 8% or 4-12% Tris/glycine/SDS-polyacrylamide gel for fractionation. Predetermined MW standards (Novex Inc.) were used as markers. Protein on the gel was blotted on to nitrocellulose or

PVDF membranes at 4°C at 25-30V and 370-380 mA for 150 minutes. After transfer, the membranes were incubated with 7% skim milk, and 1% heat-inactivated (60°C) fetal calf serum (Sigma) in wash buffer for 2 hrs, or overnight, at a cold temperature. ENOS, and nNOS proteins were detected by incubating with primary antibodies at concentrations of 1:500 to 1:1000. ENOS was detected with a monoclonal antibody (Signal Transduction Laboratory #N30020) and human endothelial cell lysate (Signal Transduction Laboratory #E15900) was used as positive control. nNOS was detected with a monoclonal antibody (Signal Transduction Laboratory #N31020). Rat pituitary tumor cell line (Signal Transduction Laboratory #G28900) was used as positive control for nNOS. The membranes were washed three times with wash buffer and then incubated with the appropriate secondary antibody for the primary mono or polyclonal antibody at concentrations of 1:500 to 1:1000. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL kit) according to the specifications of the manufacturer (Amersham). Blots were scanned with an imaging densitometer, and the optical densities of protein bands were quantitated with software (Sigma Gel, Jandel Scientific, San Rafael, CA).

NOS Activity

Supernatant (50 μl) was added to 10 ml pre-warmed (37°C) tubes which contained 100 μl of reaction buffer of the following composition: 50 mM KH_2PO_4, 60 mM valine, 1.5 mM NADPH, 10 mM FAD, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml BSA, 1 μg/ml calmodulin, 10 μM BH_4, and 25 μl of 120 μM stock L-[2,3,^{3}H]- arginine (150-200 cpm/pM). The samples were incubated for 30 min at 37°C, and the reaction was terminated by the

addition of cold (4°C) stop buffer (pH = 5.5), 100 mM HEPES, and 12 mM EDTA. Dowex 50W resin (8% cross-linked, Na^+ form) was added to eliminate excess L-[2,3,^{3}H]- arginine. Enzyme activity was expressed in pmole of L-citrulline produced/mg/min total protein. Protein was measured by the Bradford technique with bovine serum albumin as a standard (BioRad Inc. Ca). The supernatant was assayed for L-[^{3}H] citrulline by liquid scintillation counting. NOS activity was also measured in the presence of 1.5 mM each of EGTA and EDTA which replaced $CaCl_2$-calmodulin in the reaction buffer, and in the presence of 1 mM of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Ca^{2+}/calmodulin-independent NOS activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and those measured in the presence of L-NAME.

Polymerase chain reaction

Total RNA was obtained from 50 - 100 μg of liver, lung and aorta, by homogenizing the tissue with TriZole, (Gibco-Brl). For the aorta we used the modification of the procedure made by Chomczynski (18). The reverse transcriptase (RT) was performed on 1 μg of total RNA. Polymerase chain reaction was then carried out in an automated thermal cycler (M.J. Research) on 200 ng of the RT product. The sequence of the oligonucleotides used for primers are as follows (19):

forward: 5' GAA CAL GAG ACG CTG GTG CTG GTG G-3'

and reverse: 5' GGA GCC GAG CCC GAA CAC ACA GAA C-3'

The sample was cycled 35 times with an annealing temperature of 55°C. RNA quantity and PCR reaction were controlled by performing PCR for GAPDH on the RT product with

porcine specific primers. The reaction for GAPDH was cycled 24 times which we previously found to be on the linear portion of cycle number vs GAPDH amplification. We also loaded six concentrations of the RT product ranging from 12.5 ng/ml to 250 ng/ml to confirm that the eNOS reaction was still in the linear phase of the concentration/reaction relationship and we confirmed that 200 ng was within the linear phase.

Northern Analysis

To create an eNOS probe for Northern analysis, the polymerase chain reaction product of eNOS cDNA was cleaved out with the restriction enzyme and ligated with T-4 DNA ligase into PGEM-T vector (Promega Inc. Madison) which was then transformed into competent E-coli cells. The culture was grown overnight at 37°C and spread on agar plates (containing 1.5% Agar, 0.2% ampicillin, 0.5% isopropyl-thiogalacto-pyranaside and 0.2% Xgal for a blue-white selection). White colonies, containing the vector, were picked and the plasmids were isolated using standard procedures. After cleavage with the restriction enzyme, the samples were separated by gel electrophoresis and bands of appropriate size cut out for sequencing. Once it was established that it was indeed eNOS, large scale plasmid preparation was performed. After plasmid isolation and enzymatic cleavage, the product was separated by gel electrophoresis and the insert was elluted into 0.7% low melting point agarose and purified with phenol-chloroform extraction. The probe was then labelled with alpha[^{32}P] deoxycytidine triphosphates by random priming.

For Northern analysis, total RNA (50 μg) samples were fractionated by electrophoresis in a denaturating formaldehyde 1% agarose gel. After RNA was transferred

to Hybond-N nylon membrane by capillary diffusion with 20 x SSC. After RNA immobilized by bathing under vacuum for 2 hrs at 80°C, the membranes were prehybridized for 3 hrs at 42°C with 5 x SSPE, 5 x Denhard's solution, 0.5% SDS, 50% formamide and 10 μg/ml of a heat denatured salmon sperm DNA. Hybridization was done overnight at the same temperature in a solution containing ^{32}P labelled probe for eNOS which was randomly labelled. The membranes were washed with 2 x SSPE/0.1% SDS for 5 minutes at room temperature 3 times, followed by 2 more washes at 42°C with 0.2 x SSPE/0.1% SDS for 15 minutes and, finally, 1 hour at 65° C, with a 1 x SSPE/0.5% SDS solution. The membranes were exposed to a KODAK film at -80°C. CNOS mRNA was semiquantitated by comparing the density of the eNOS fragment to labelled GAPDH.

Protocol

The animals were stabilized for 30 minutes and baseline hemodynamics, including measurement of the MCFP, were obtained. We then gave a 20 μg bolus of norepinephrine (NE) and measured the change in arterial pressure (P_{art}). In 6 animals, we infused sufficient normal saline (average of 25-30 ml/kg) to maintain the right atrial pressure at approximately 3 mmHg for two hours (control). In the other 8 animals, we infused 20 μg/kg of E. Coli endotoxin over two hours. After the two hours, we repeated the 20 μg bolus of NE in both groups. We then gave both groups of animals 0.5, 1, 2, 4 and 16 μg/kg of L-NAME, in progressive 10 minute steps, and repeated the hemodynamic measurements at each dose. We also repeated the norepinephrine bolus after the 0.5, 4 and 16 μg doses of L-NAME.

For western and activity analyses we obtained tissue from 6 control animals sacrificed after 2 hours, 8 animals treated for 2 hours with endotoxin and then sacrificed and 4 animals treated for 2 hours with endotoxin and then followed for 2 more hours (total equals 4 hours).

Statistics

All values represent mean ± SD. A two-way ANOVA for repeated measures was used to evaluate significant differences between control and septic animals and, where significance was found, post hoc analysis was performed with a Student-Newman Keul's test. Densitometric data were analyzed by a "t" test or the Mann-Whitney test. The statistical significance was considered at $p < 0.05$ for all tests.

RESULTS

In the animals that underwent the dose-response study with L-NAME, the PO_2 in the control group started at 134 ± 19 mmHg and 161 ± 22 mmHg in the endotoxemic group. It did not change significantly over the course of the experiment in either group (control 153 ± 24 mmHg, endotoxin 156 ± 55 mmHg). The PCO_2 began at 40 ± 3 mmHg in the control and 41 ± 5 mmHg in endotoxemic animals. PCO_2 also did not change significantly over the course of the experiment (control 38 ± 3 mmHg, endotoxin 38 ± 7 mmHg). The pH began at 7.49 in the control and 7.44 in the endotoxemic group and did not change significantly (control 7.48, endotoxin 7.35). The initial hemoglobin in the control animals was 10.4 ± 0.7 g/dl and remained at 10.4 ± 1.0 g/dl. In the endotoxemic group, it started at 10.8 ± 1.0 g/dl and was 12.0 ± 1.1 g/dl at the end (p = ns).

The hemodynamic values before endotoxin (time zero) and at 120 min, which was just before the L-NAME infusion, are given in Table 1. There was no significant change in cardiac output in control or endotoxemic animals over this time period. Arterial pressure (Part) fell in endotoxemic animals and so did the SVR, but SVR did not change in control animals. The PAP was increased in endotoxemic animals. The hemodynamics in the animals from which the tissues were obtained for Western analysis and activity analysis had a similar hemodynamic profile and are reported in the companion paper (20).

Western Blotting

Immunoblotting (Western analysis) was performed on tissue samples from individual animals as well as pooled samples from animals studied for two hours and four hours. An example of the Western analysis at four hours is shown in Figure 1 (n = 4, control 4 and endotoxin 4). Density was greater in endotoxemic animals. There was no significant difference in iNOS with endotoxin (not shown).

Western analysis was performed on individual and pooled samples for eNOS in the vena cava. An example of a Western blot on pooled samples (n = 3) from vena caval tissue at 40, 80 and 120 μg of protein under control and endotoxemic conditions is shown in Figure 2. The density of staining was greater for endotoxemic animals at each dose. An example of nNOS in the vena cava is shown in Figure 3. The density in endotoxemic animals was also increased ($p<0.05$).

An example of a Western analysis for eNOS in the pulmonary artery is shown in Figure 4 for 3 control and 3 septic animals treated for 2 hours. By densinometric analysis

eNOS was decreased in endotoxemic animals at 4 hours. Western analysis performed on pooled samples and individual samples for nNOS in the pulmonary artery showed no change in endotoxemic animals (Figure 5).

NOS Activity

NOS activity in the aorta was significantly higher at two hours in endotoxemic animals (n = 8) (Figure 6 , top figure). Unfortunately, the tissues at 4 hours were not adequate for analysis. Activity was also increased in the vena cava (n = 4) at two hours, compared to control animals (n = 3) and remained increased at 4 hours (n = 4) (Figure 6, middle figure). NOS activity in the pulmonary artery was unchanged two hours of endotoxemia (n = 6) compared to controls (n = 4) but was decreased at four hours (n = 4) (Figure 6, bottom figure). NOS activity was predominantly calcium dependent and there was no increase in the calcium independent portion over time.

Polymerase Chain Reaction and Northern Analysis

After establishing the linear range for the PCR reaction by loading different amounts of cDNA, we found no significant regulation of eNOS (data not shown) in aorta, lung and liver at two hour, and at four hours in lung and liver. (The RNA from the aorta at 4 hours was degraded.) PCR of eNOS on RNA in lung and liver from an animal studied after 6 hours of endotoxemia also did not appear decreased. Northern analysis on RNA from the lungs of 3 animals showed no regulation of eNOS mRNA (Figure 7).

Hemodynamic Response to L-NAME

The dose responses to L-NAME after two hours in control and endotoxemic animals are shown in Figure 8. Cardiac output began to fall with the first dose of L-NAME in both control and endotoxemic animals (Figure 8 a). The dose-response curve of the change in cardiac output in the endotoxemic group was shifted to the left of the control group indicating increased sensitivity. By 4 mg/kg of L-NAME, 86% of the maximal fall in cardiac output was reached in endotoxemic animals. P_{art} rose with L-NAME in both groups and the relationship was shifted to the right in endotoxemic animals 8 b). SVR increased in both groups with L-NAME; the response in endotoxemic animals was shifted to the left (Figure 8 c) indicating increased sensitivity to the L-NAME. L-NAME increased the pulmonary artery pressure in both groups, but the overall dose effect was not significant by the two-way ANOVA (Figure 8 d). Separate analysis of the control group showed a dose effect. The effect of L-NAME on the pulmonary artery pressure was significantly different by ANOVA in endotoxemic vs. control group. The MCFP increased in the control group with L-NAME and decreased slightly in the endotoxemic group (Figure 8 e). There was no dose effect. RVR increased with L-NAME in both groups and, as with SVR, the dose response curve was shifted to the left in the endotoxemic animals (Figure 8 f) indicating increased sensitivity.

Pressor Response to Norepinephrine

Figure 9 shows the change in arterial pressure in response to norepinephrine at baseline and after two hours of saline or endotoxin and then after selected doses of L-NAME

for each group. The pressor response to norepinephrine (NE) was significantly decreased after two hours of endotoxemia compared to baseline and compared to the response in saline treated time control animals. Infusion of 1 mg/kg of L-NAME had no effect on the decreased response to NE in endotoxemic animals, but the pressor response was partially restored after 4 mg/kg of L-NAME and there was no greater benefit at 16 mg/kg (Figure 9). L-NAME had no significant effect on the NE response in saline treated animals.

DISCUSSION

The major observations of this study are that eNOS protein and calcium-dependent NOS activity were increased after two hours of endotoxemia when a low SVR state and decreased vascular responsiveness were fully established. This challenges a commonly held belief that eNOS is decreased in sepsis in the systemic circulation (16). However, eNOS was decreased in the pulmonary artery by Western analysis and activity analysis at four hours, indicating the presence of regional differences. We also found that endotoxin infusion shifted to the left of the dose-response effect of L-NAME on SVR, RVR and cardiac output. Finally, the dose of L-NAME which restored the pressor response to norepinephrine caused a large fall in cardiac output.

Once it was recognized that NO from eNOS is essential for normal regulation of vascular function, its potential role in the vascular dysregulation of sepsis was considered. However, conclusions about the role of eNOS are contradictory because of differences in

experimental models, species, levels of analysis and the time of exposure. Furthermore, at the time of the first studies, it was not known that there is an inducible isoform of NOS (21).

The role of NO in sepsis was first examined by testing agonist induced vasodilation and release of endothelium-derived relaxing factor (EDRF) from vascular rings from animals treated *in-vivo* with tumor necrosis factor-alpha (TNF-α) (13) or endotoxin (14;15) or vessels treated *in-vitro* with TNF-α (22). A consistent observation was a shift to the right of endothelial dependent dilators, such as acetycholine, whereas endothelial independent agonists were not affected (13;14;15;22). The decrease in agonist response occurs as early as 30 minutes (22) and as late as 18 hours (15). The decreased responsiveness to acetylcholine is prevented by treatment with cyclohexamide (13) which indicates that protein synthesis is required for this process. Pertussus toxin also prevents the decrease in responsiveness, suggesting a role for G-coupled proteins (22). The effect on the acetylcholine receptor is selective for endotoxin and does not alter the action of another endothelial agonist, A23185 (22). Decreased vascular responsiveness to acetylcholine is also present in animals made septic by cecal perforation (23) and in hemorrhaged rats (24).

The observation of decreased dilatory response to acetylcholine seems to be in conflict with our observation that there is an increase in eNOS protein and calcium-dependent NOS activity, as well as a shift to the left of the response to the NOS inhibitor, L-NAME. However, as pointed out by Wylam et al (14), decreased vascular responsiveness to an agonist, does not rule out increased basal release of NO. Furthermore, the prevention of the loss of function with a protein inhibitor and pertussus toxin suggests that eNOS

function itself is still normal. Of note, the loss of responsiveness is not simply due to a blood borne product, because loss of responsiveness occurs in bloodless systems.

The role of NO was also examined by assessing the release of NO by endothelium. An immediate release of NO from bovine endothelial cells was suggested by increased anti-aggretory effect of NO on platelets (25). Endothelial cells also release more nitrite in response to cytokines (12;26). Recently, an immediate increase in NO release from vessels after endotoxemic exposure has been demonstrated *in-vivo* with an *in-situ* electrode which rules out iNOS induction as the mechanism (27).

On the other hand, decreased release of NO was observed in cultured bovine aortic endothelial cells after one hour, but not ten minutes of exposure to cytokines (28). and decreased NOS activity was found in porcine pulmonary arterial endothelial cells (29).

The role of eNOS in the vascular dysfunction of sepsis has also been examined by assessing eNOS mRNA. Multiple cytokines (30) and TNF (31-34) decrease eNOS in endothelial cells. In most studies, high doses of TNF were used, but physiological levels of TNF-α still significantly decreased ec-NOS half-life (31). eNOS mRNA is also decreased in the aorta, lung and hearts of endotoxemic rats treated for four hours with endotoxin and this decrease is suppressed by pre-treatment with dexamethasone (35). These results are in conflict with our observation in pigs of increased protein by Western blotting, increased NOS activity and no change in mRNA by PCR and Northern analysis. However, four of the previous studies were done on cultured cells (30;31;34) and the one *in-vivo* study was on rats (35) which, in contrast to pigs, are large iNOS producers.

An increase of eNOS protein could come from increased production or decreased breakdown. Increased production could come from increased transcription or increased translation. Our PCR analysis of the aorta, lung and liver, and Nothern analysis of the lung did not show a change in eNOS mRNA but this still does not rule out a small increase in transcription or small increase in translation.

An increase in eNOS protein in the aorta and vena cava does not mean necessarily that more NO is produced. Indeed, we previously found no change in plasma nitrite/nitrate with endotoxemia (10). This could be because NO is converted to nitrosothiols (36) or peroxynitrate (37) but it is also possible that eNOS produces superoxide instead of NO in septic animals (38). This could occur because of the lack of substrate or cofactors *in-vivo*. The consequence of this is that NO and superoxide could react to form peroxynitrite (37) which can produce severe tissue damage (39). Alternatively, normal or increased NO could interact with increased superoxide from smooth muscle or endothelial cells and produce peroxynitrite. Indeed, we found increased peroxynitrite in pigs treated with the same protocol as in this study (see companion paper), and part of the rationale for studying eNOS in this study was to determine whether there is a sufficient amount of eNOS to contribute to the process. It appears that there is.

The dose-response to L-NAME for SVR and RVR were shifted to the left, whereas the change in Part was shifted to the right. These results reflect the difficulties of interpreting *in-vivo* dose-response relationships. Part is dependent upon summation of effects of all regional resistances (TSVR) and total cardiac output. If cardiac output decreases proportionally more than TSVR increases, then Part will decrease as we observed.

We previously found that L-NAME decreases cardiac output by depressing cardiac function and increasing RVR (8;40).

In control animals, L-NAME increased MCFP at the lowest dose, and produced no further change with higher doses. In endotoxemic animals, L-NAME actually produced a decrease in MCFP. MCFP is dependent upon the volume in the capacitant region and its compliance. It is likely that increased capillary leak from sepsis resulted in a greater volume loss and lower MCFP in endotoxemic animals, especially when the RVR, i.e. the venous outflow resistance, was increased by L-NAME.

In contrast to what was observed in the aorta and vena cava, eNOS protein by Western anlaysis was decreased at two hours in the pulmonary artery, although activity was still preserved, perhaps because nNOS was not changed. By four hours, the activity was also decreased. Zhang et al (29) also found a decrease in NOS activity and mRNA in pulmonary arteries. This shows the importance of tissue differences as well as species differences. Despite the decrease in eNOS, NOS still seems to play a role in the pulmonary hemodynamic abnormalities in endotoxemic animals, for L-NAME had a greater pressor effect in the pulmonary vasculature of endotoxemic animals compared to controls (Figure 8).

The greater effect of L-NAME on RVR and SVR in endotoxemic animals compared to control animals may not only reflect increased NO production, but could be due to the production of more vasoconstrictors which are counterregulated by NO. In particular, endothelin (41) and thromboxane A-2 (42) are increased in sepsis, and NO controls their physiological effects and production (43).

Inhibition of NOS restores responsiveness to pressors (6), even in animals that do not have induction of iNOS (9) but, lowers cardiac output. We wanted to know if there is a dose of L-NAME that could restore the vasopressor response without decreasing cardiac output. Unfortunately, the pressor response to norepinephrine was only restored at 4 mg/kg of L-NAME which produced close to 90% of the maximum decrease in cardiac output.

In conclusion, endotoxemic pigs have either no change or increased eNOS protein by Western analysis and activity analysis in the aorta and vena cava and no change in eNOS mRNA, whereas eNOS is decreased in the pulmonary artery. All vascular regions showed a greater sensitivity to NOS inhibition. Thus, in contrast to what has been observed in rats, eNOS could still play a significant role in the vascular dysfunction of septic pigs.

REFERENCES:

1. Magder S: Heart-lung interactions in sepsis. In: Dantzker and Scharf eds. Cardiopulmonary Critical Care, Third edition. W.B. Saunders, Philadelphia, 1998, pp. 435-448.

2. Busse R, Mulsch A: Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Letter 275: 87-90, 1990.

3. Moncada S, Higgs A: The L-Arginine-nitric oxide pathway. N Engl J Med 329: 2002-2012, 1993.

4. Xie Q, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Torso T, Nathan C: Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256: 225-228, 1992.

5. Bateson AN, Jakiwczyk OM, Schulz R: Rapid increase in inducible nitric oxide synthase gene expression in the heart during endotoxemia. Eur J Pharmacol 303: 141-144, 1996.

6. Szabo C, Mitchell JA, Thiemermann C, Vane JR: Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. Br J Pharmacol 108: 786-792, 1993.

7. Kilbourn R, Gross S, Jubran A, Adams J, Griffith O, Levi R, Lodata R: N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. Proc Natl Acad Sci U S A 87: 3629-3632, 1990.

8. Magder S, Vanelli G: Circuit factors in the high cardiac output of sepsis. J Crit Care 111: 155-166, 1996.

9. Datta P, Magder S: Hemodynamic response to norepinephrine with and without inhibition of nitric oxide synthase in porcine endotoxemia. Am J Resp Crit Care Med 160: 1987-1993, 1999.

10. Mehta S, Javeshghani D, Datta P, Levy RD, Magder S: Porcine endotoxaemic shock is associated with increased expired nitric oxide. Crit Care Med 27: 385-393, 1999.

11. Salvemini D, Korbut R, Anggard E, Vane J: Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by Escherichia coli lipopolysaccharide. Proc Natl Acad Sci USA 87: 2593-2597, 1990.

12. Lamas S, Michel T, Brenner BM, Marsden PA: Nitric oxide synthase in endothelial cells - evidence for a pathway inducible by TNF-a. Am J Physiol 261: C634-C641, 1991.

13. Aoki N, Siegfried M, Lefer AM: Anti-EDRF effect of tumor necrosis factor in isolated, perfused cat carotid arteries. Am J Physiol 256: H509-H512,1989.

14. Wylam ME, Samsel RW, Umans JG, Mitchell RW, Leff AR, Schumacker PT: Endotoxin In Vivo impairs endothelium-dependent relaxation of canine arteries In Vitro. Am Rev Respir Dis 142: 1263-1267, 1990.

15. Parker JL, Adams HR: Selective inhibition of endothelium-dependent vasodilator capacity by Escherichia coli endotoxemia. Circ Res 72: 539-551, 1993.

16. Traber DL: Presence and absence of nitric oxide synthase in sepsis. Crit Care Med 24: 1102-1105, 1996.

17. Magder S: Starling resistor versus compliance. Which explains the zero-flow pressure of a dynamic arterial pressure-flow relation? Circ Res 67: 209-220, 1990.

18. Chomczynski P, Mackey K: Modification of the TR1 reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques 19: 942-945, 1995.

19. Zhang J, Patel JM, Block ER: Molecular cloning, characterization and expression of a nitric oxide synthase from porcine pulmonary artery endothelial cells. Comp Biochem,Physiol 116B: 485-491, 1997.

20. Javeshghani D, Neculcea J, Magder S: Induction of iNOS mRNA without expression of iNOS protein in porcine endotoxemia. Am J Resp Crit Care Med 157: A690. 1998.

21. Stoclet J, Fleming I, Gray G: Nitric oxide and endotoxemia. Circulation 87: V-77-V-80, 1993.

22. Greenberg S, Jianming X, Wang Y, Baiqiang C, Kolls J, Nelson S: Tumor necrosis factor-alpha inhibits endothelium-dependent relaxation. J Appl Physiol 75: 2394-2403, 1993.

23. Zhou M, Wang P, Chaudry IH: Endothelial nitric oxide synthase is downregulated during hyperdynamic sepsis. Biochimica et Biophysica Acta 1335: 182-190, 1997.

24. Wang P, Ba ZF, Chaudry IH: Endothelium-dependent relaxation is depressed at the maco- and microcirculatory levels during sepsis. Am J Physiol 269: R988-R994, 1995.

25. Villa M, Salas E, Darley-Usmar M, Radomski MN, Moncada S: Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. Proc Natl Acad Sci USA 91: 12383-12387, 1994.

26. Kilbourn RG, Belloni P: Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst 82: 772-776, 1990.

27. Brovkovych V, Patton S, Brovkovych S, Kiechle F, Huk I, Malinski T: In situ measurement of nitric oxide, superoxide and peroxynitrite during endotoxemia. J Physiol Pharmacol 48: 633-644, 1997.

28. Myers PR, Wright TF, Tanner MA, Adams HR: EDRF and nitric oxide production in cultured endothelial cells: direct inhibition by E. coli endotoxin. Am J Physiol 262: H710-H718, 1992.

29. Zhang J, Patel JM, Li YD, Block ER: Proinflammatory cytokines downregulate gene expression and activity of constitutive nitric oxide synthase in porcine pulmonary artery endothelial cells. Research Communications in Molecular Pathology and Pharmacology 96: 71-87, 1997.

30. MacNaul KL, Hutchinson NI: Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions. Biochem Biophys Res Commun 196: 1330-1334, 1993.

31. Yoshizumi M, Perrella MA, Burnett JC, Lee ME: Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res 73: 205-209, 1993.

32. Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T: Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett 307: 287-293, 1992.

33. Venema RC, Nishida K, Alexander RW, Harrison DG, Murphy TJ: Organization of the bovine gene encoding the endothelial nitric oxide synthase. Biochimica et Biophysica Acta 1218: 413-420, 1994.

34. Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP: Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest 90: 2092-2096, 1992.

35. Liu SF, Adcock IM, Old RW, Barnes PJ, Evans TW: Differential regulation of the constitutive and inducible nitric oxide synthase mRNA by lipopolysacchride treatment in vivo in the rat. Crit Care Med 24: 1219-1225, 1996.

36. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, Loscalzo J: Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. Proc Natl Acad Sci USA 89: 7674-7677, 1992.

37. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620-1624, 1990.

38. Xia Y, Dawson VL, Dawson TM, Snyder SH, Sweier JL: Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci U S A 93: 6770-6774, 1996.

39. Szabo C, Saunders C, O'Connor M, Salzman AL: Peroxynitrite causes energy depletion and increases permeability via activation of poly (ADP-Ribose) synthetase in pulmonary epithelial cells. Am J Respir Cell Mol Biol 16: 105-109, 1997.

40. Magder S, Kabsele K: Evidence for constitutive release of nitric oxide in the venous circuit of pigs. J Cardiovas Pharmacol 32: 366-372, 1998.

41. Weitzberg E: Circulatory responses to endothelin-1 and nitric oxide with special reference to endotoxin shock and nitric oxide inhalation. Acta Physiol Scand 611: 1-72, 1993.

42. Hellyer PW, Johnson LW, Olson NC: Effect of N^G-nitro-L-arginine-methyl-ester on cardiopulmonary function and biosynthesis of cyclooxygenase products during porcine endotoxemia. Crit Care Med 25: 1051-1058, 1997.

43. Curzen NP, Griffiths MJD, Evans TW: Role of the endothelium in modulating the vascular response to sepsis. Clin Sci 86: 359-374, 1994.

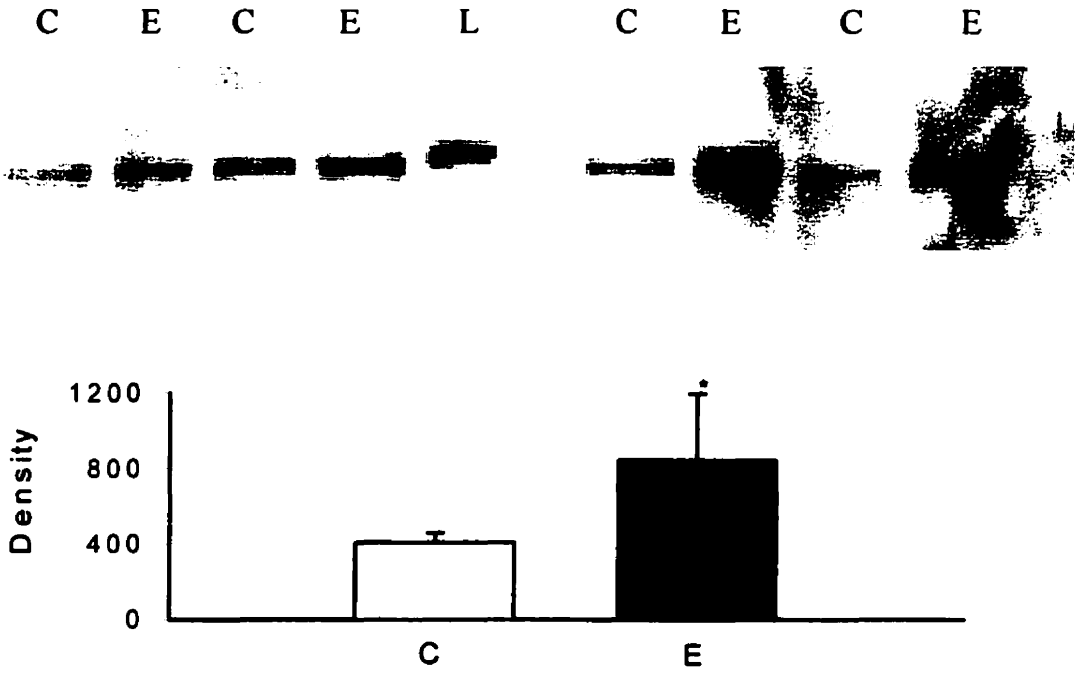
Table 1

Hemodynamics at the start and after 120' in control and endotoxemic animal

	Control (n=6)		Endotoxin (n=8)	
Time (min)	**0**	**120**	**0**	**120**
Cardiac output (L/min)	3.7 ± .25	3.54 ± .41	3.14 ± .52	3.32 ± .91
P_{art} systolic (mmHg)	107 ± 7	104 ± 9	90 ± 20	72 ± 14 *
SVR (mmHg.L^{-1}.min)	27.5 ± 2.1	29 ± 3.6	28.9 ± 9.1	21.8 ± 6.3 *
PAP (mmHg)	13 ± 3.6	14.5 ± 2.5	14.6 ± 3.5	28.1 ± 5.3 *
RVR (mmHg.L^{-1}.min)	1.83 ± .22	2.00 ± .29	2.29 ± .56	2.48 ± .66
MCFP (mmHg)	9.3 ± 1.1	9.5 ± 1.3	9.4 ± 1.4	11.2 ± 2.4 *

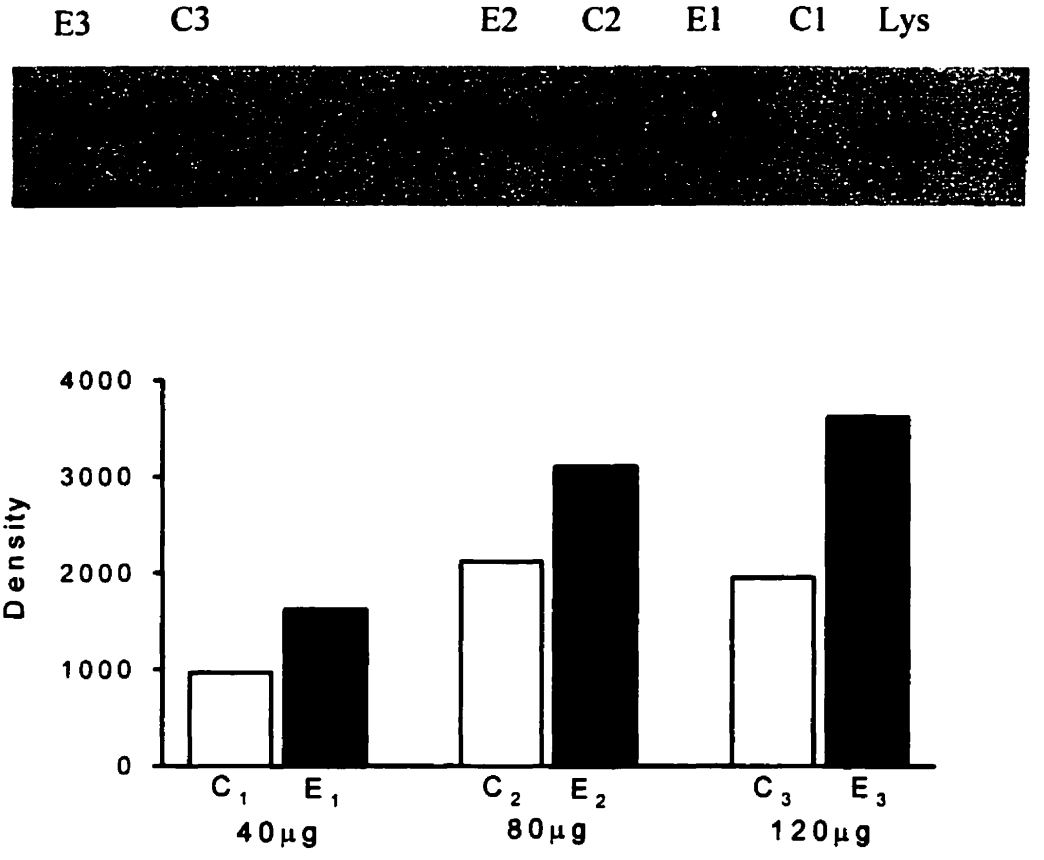
P_{art}= mean arterial pressure, SVR = systemic vascular resistance, PAP = mean pulmonary arterial pressure, RVR=resistance to venous return, MCFP = mean circulatory filling pressure, * = $p < 0.05$

Figure 1



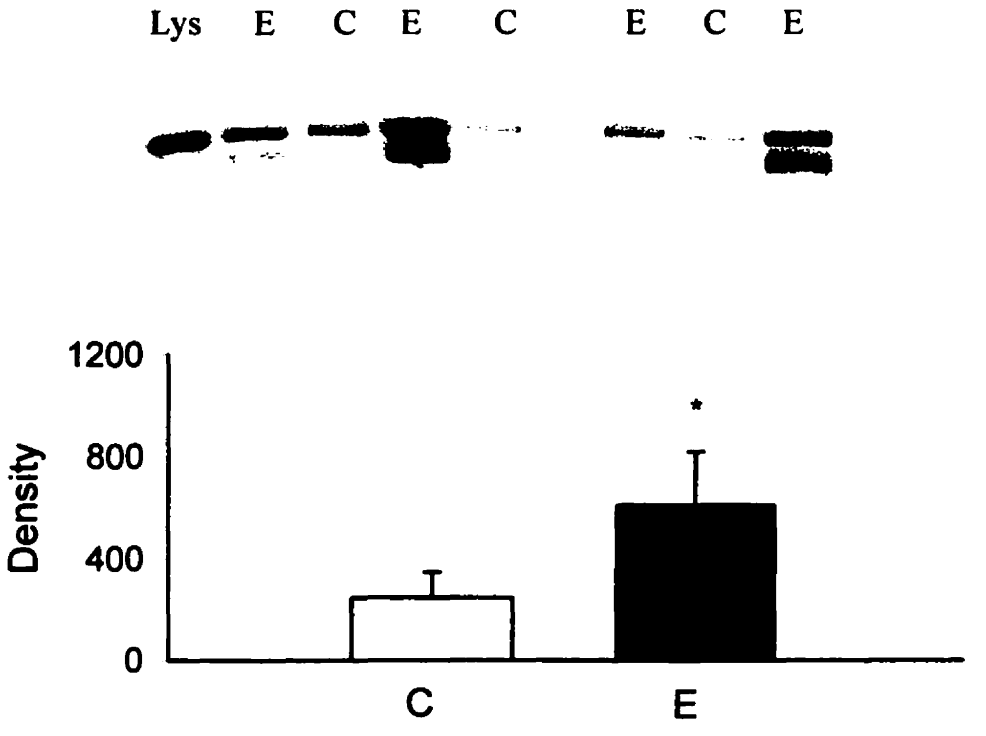
Example of Western blot analysis of NOSIII in aorta from animals at 4 hours. The figure shows 4 control (C) and 4 endotoxemic (E) animals. (L represents positive control from human endothelial cells). Densitometric analysis showed greater density for endotoxin treated animals than controls.

Figure 2



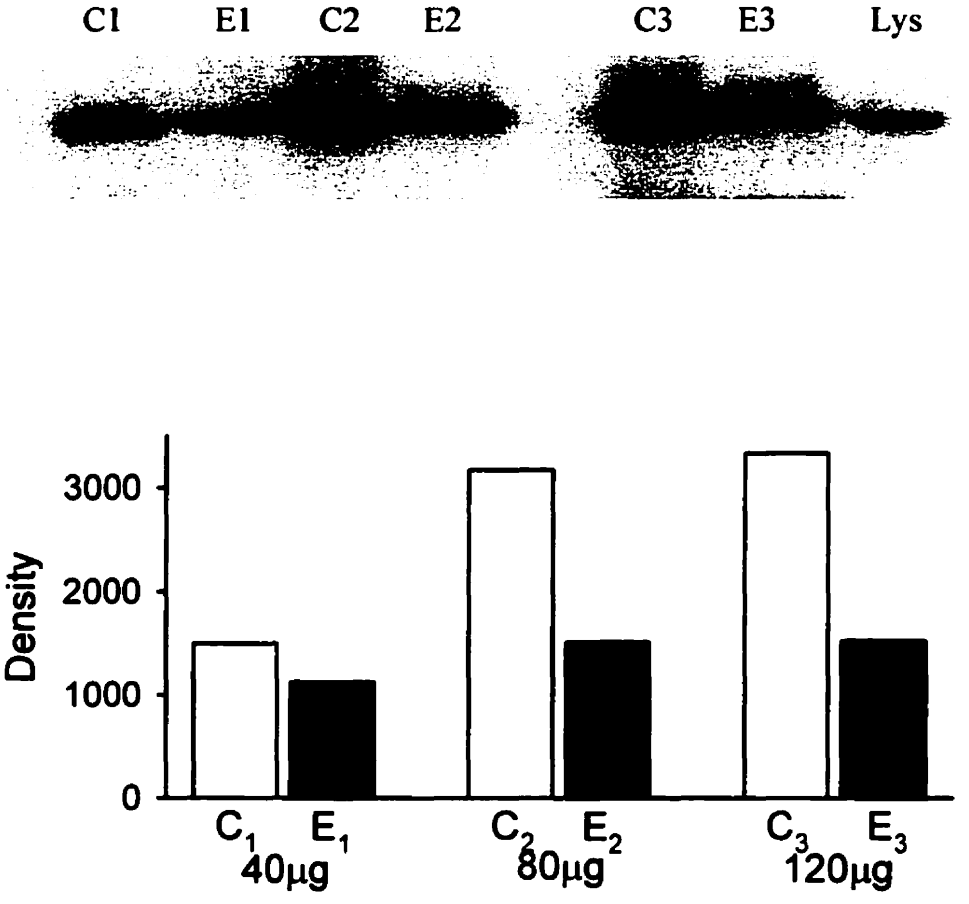
Example of Western blot analysis for NOSIII on vena caval tissue. Samples were pooled from 3 control (C) and 3 endotoxic animals (E). The controls are shown in the lighter columns and the endotoxic animals in the darker columns. The results are shown for 3 different loading concentrations of protein 40 (1), 80 (2), and 120 (3) μg per lane). (Lys = positive control from human endothelial cells for NOSIII).

Figure 3



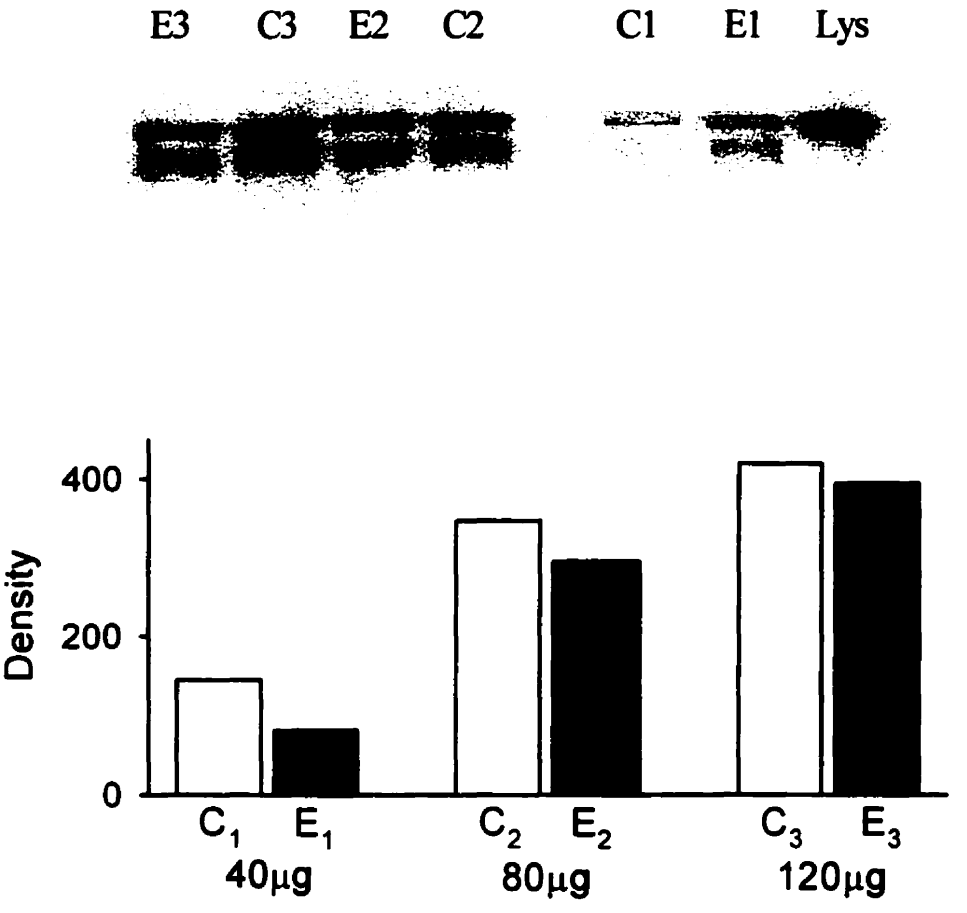
Example of Western blot analysis for NOSI in vena caval tissue. Equal amount of tissue samples from three control (C) and four endotoxemic (E) animals were loaded. An increase in NOSI was seen in septic animals vs. controls by densitometric analysis. Lys= positve control from rat pituitatary tumor cell line for NOSI.

Figure 4



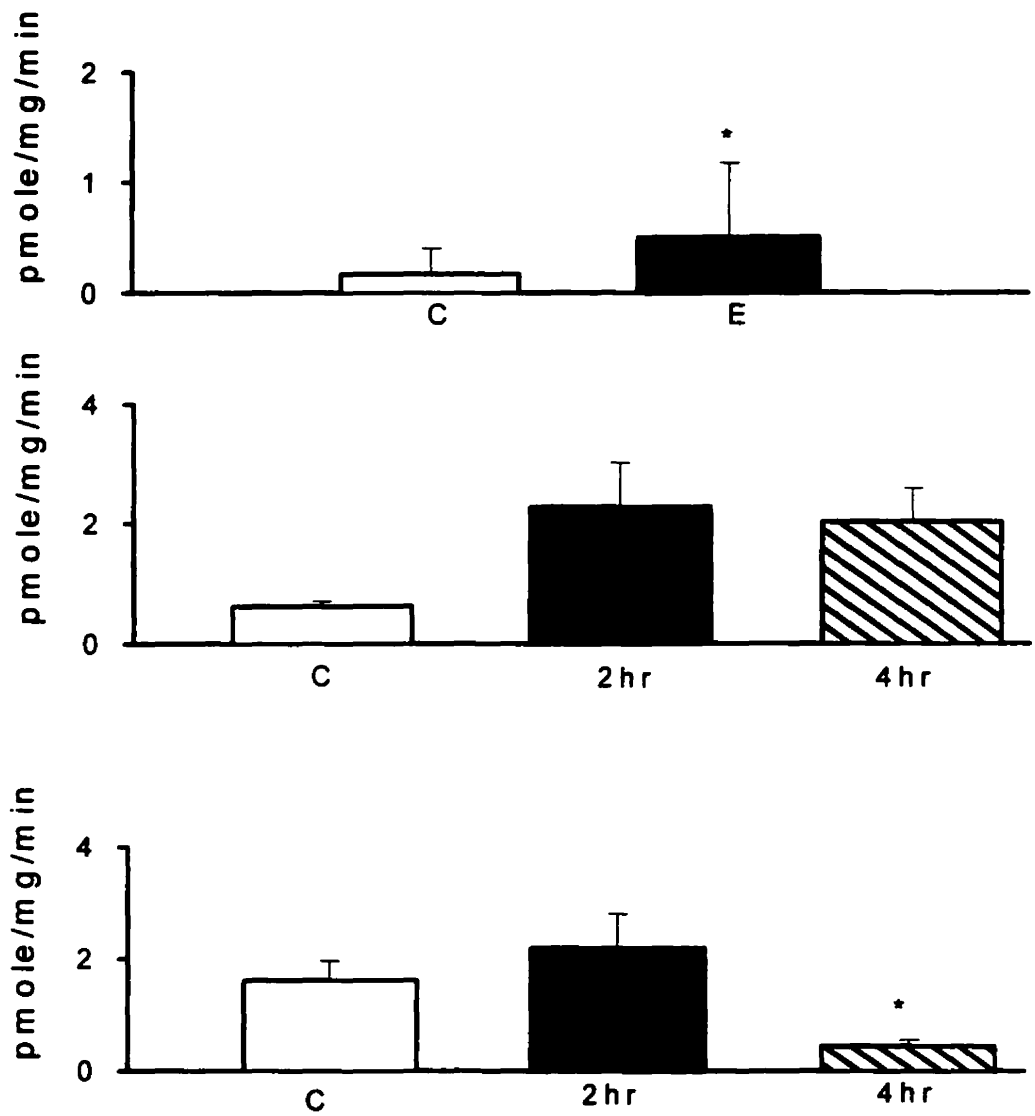
Example of Western blot analysis for NOSIII in the pulmonary artery. The samples were pooled from 3 control (C) and 3 endotoxemic (E) animals. We loaded 40 (1), 80 (2) and 120 (3) μg. Density was always higher in the control animals when compared to endotoxemic animals. (lys= positive control from human endothelial cell for NOSIII)

Figure 5



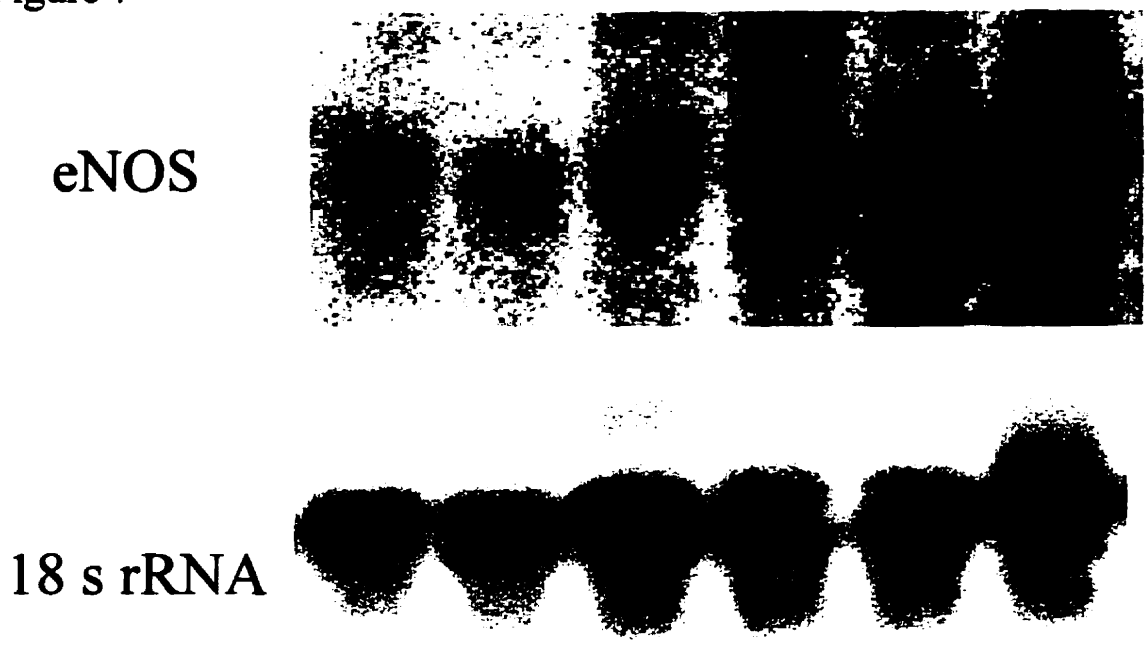
Example of a Western blot analysis for NOSI in the pulmonary artery. The samples were pooled from 3 control (C) and 3 endotoxemic (E) animals. We loaded 40 (1), 80 (2), 120 (3) μg. Lys = positive control from rat pituitary tumor cell line for NOSI. There was no change in NOSI in endotoxemic animals compared to control animals.

Figure 6



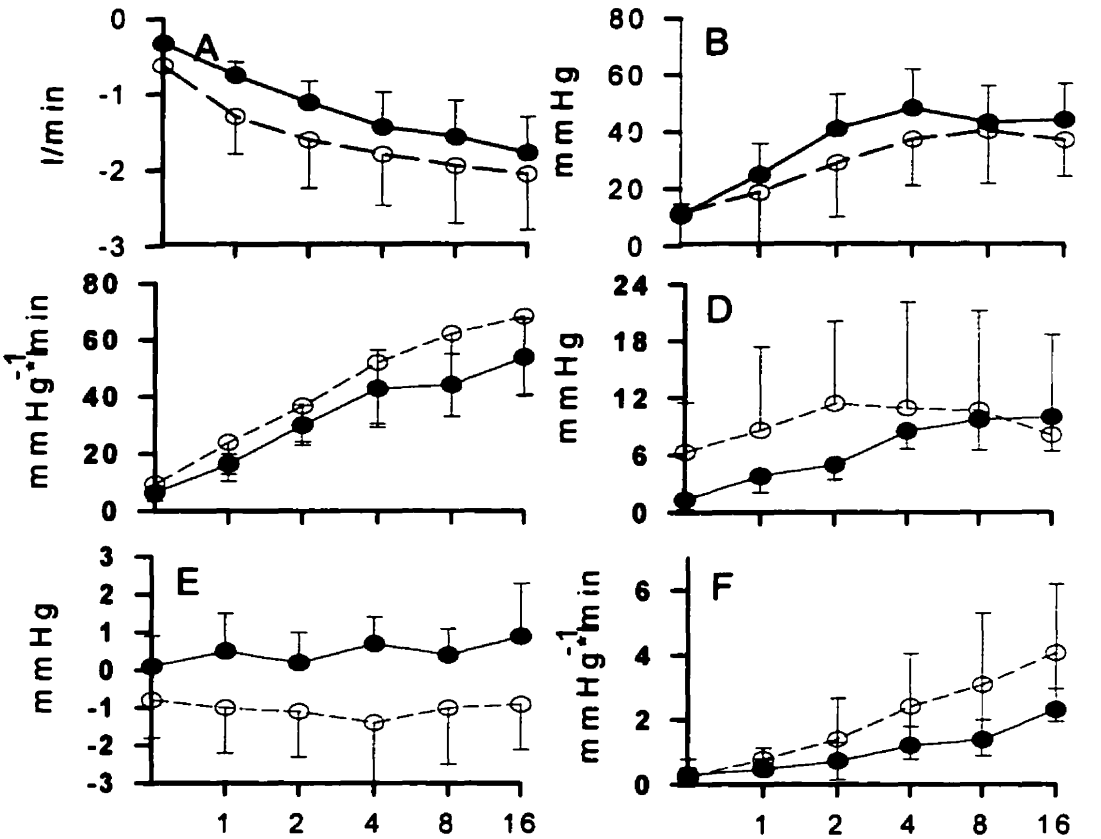
NOS activity in the aorta, vena cava and pulmonary artery. Top figure represents NOS activity in aorta of control (C) and endotoxemic (E) animals treated for 2 hours (n = 8 in each group). Activity was significantly increased in the aorta of endotoxemic animals (Mann-Whitney). Middle figure represents total NOS activity in the vena cava of control (n = 3), 2 hours of endotoxin (n = 4) and 4 hours of endotoxin (n = 4); Bottom figure represents total NOS activity in the pulmonary artery of control (n = 6), 2 hours (n = 6) and 4 hours (n = 4) of endotoxemia. NOS activity was significantly reduced at 4 hours ($p < 0.05$ by ANOVA). NOS activity was almost entirely calcium dependent. (Mean - SD).

Figure 7



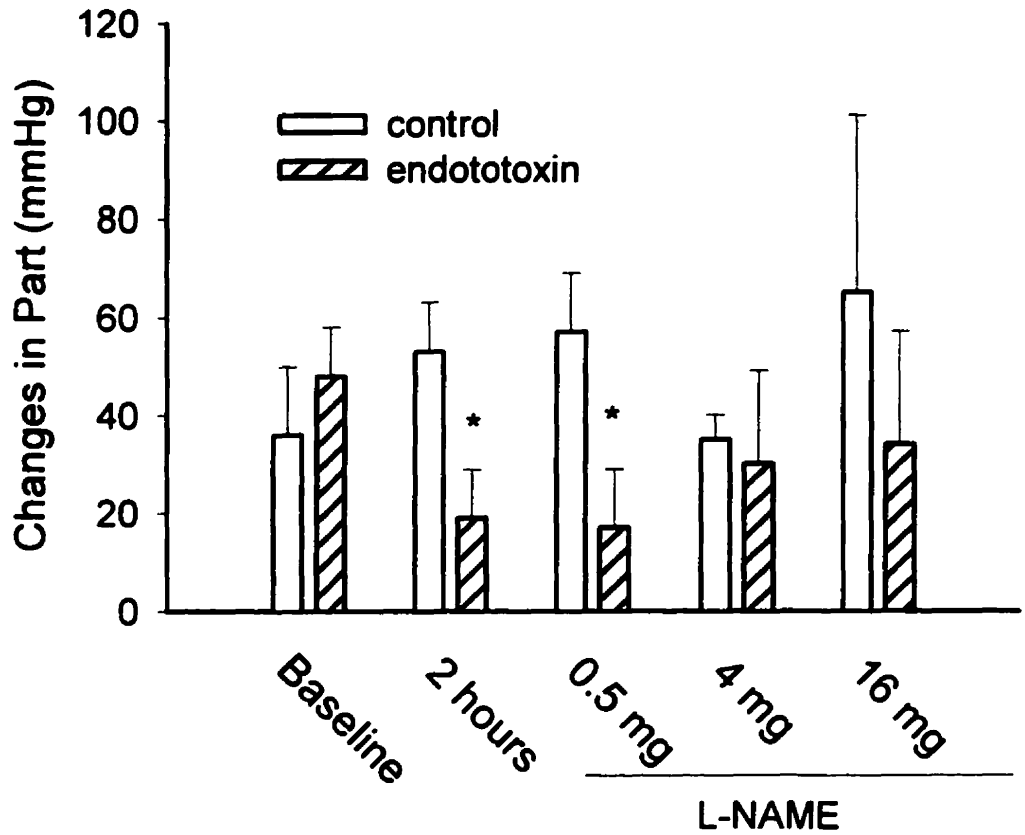
Northern analysis on lung tissue for NOSIII (eNOS) mRNA in 3 control (C), 3 animals treated with endotoxin (E) for 4 hours (e). 18 s rRNA is shown in the bottom part of the figure to control for loading and the ratio of the density of NOSIII to 18S rRNA is shown in the bar graph on the right. There was no change in NOSIII mRNA with endotoxin treatment.

Figure 8



Logarithmic dose-response to L-NAME in control and endotoxin treated animals for two hours. (A) = cardiac output, (B) = arterial pressure (Part), (C) = systemic vascular resistance (SVR), (D) = pulmonary artery pressure (PAP), (E) = mean circulatory filling pressure (MCF), (F) resistance to venous return (PVR) (mean± SD). Closed circles and solid lines indicate control animals, and open circles and dashed line indicate endotoxin. Endotoxin shifted the dose response curves for cardiac output (A), systemic vascular resistance (C), and resistance to venous return (F) to the left, indicating increased sensitivity to NOS inhibition ($p < 0.05$) for endotoin condition vs. control condition. The mean arterial pressure response (B) was shifted to the right ($p < 0.05$). The pulmonary pressure response (D) was shifted upward ($p < 0.05$ for endotoxin condition vs. control). MCFP (E) was shifted downward in the endotoxemic animals.

Figure 9



Change in pressure in response to 20 μg bolus of norepinephrine. This is shown at baseline (t = 0) after 2 hours (t = 120) and after 1, 4 and 16 mg/kg of L-NAME in saline treated control animals (open bars) and endotoxin treated animals (hatched bars). The pressor response to NE was reduced after 120 minutes of endotoxemia ($p < 0.05$). The response was partially restored with 4 and 16 mg/kg of L-NAME. There was no significant change in the response to NE in control animals.

Link to chapter four:

In endotoxemic pigs, in contrast to rats there was no significant increase in plasma nitrite. Yet similar to the reports of studies on endotoxic rats, they had nitrotyrosine formation which, has been used as a footprint of peroxynitrite. Peroxynitrite is the product of NO and O_2^-. Since I found normal or mildly elevated NOS activity, we next asked whether O_2^- production could produce peroxynitrite by interacting with NO.

CHAPTER FOUR

VASCULAR PRODUCTION OF SUPEROXIDE ANION BY NAD(P)H OXIDASE AND INTERACTION WITH NITRIC OXIDE SYNTHASE IN ENDOTOXEMIA

Abstract: Sepsis is associated with increased reactive oxygen species (ROS). We hypothesized that the non-phagocytic NAD(P)H oxidase system could be a source of the ROS, superoxide (O_2^-). We tested this in rats which, are NOSII producers and pigs, which are non-NOSII producers, because of the potential interaction with NO. Sprague-Dawley rats (n=5) were given intraperitoneal (IP) injection of 10-12mg/kg of LPS, followed for 18 hr, then sacrificed, and the aorta removed. Pigs (n=7) were treated intravenously (IV) with 20μg/kg of endotoxin, and followed for 4hr. A carotid artery was resected prior to endotoxemia and the other one after 4 hours. Lucigenin-enhanced chemiluminesence was used to measure O_2^- with and without stimulation with NADH and NADPH. We also tested specificity of the reaction with superoxide dismutase (SOD), Tiron, a O_2^- scavenger, Diphenyleneiodonium (DPI) and the NOS inhibitor L-NAME. In rats the basal O_2^- concentration (1.18±. 23 nmole O_2^-/mg/10min) was higher in septic than in aortic segments (.38±. 07 nmole O_2^-/mg/10min) from untreated rats (P=0.01). Addition of either Tiron or SOD to NADH-treated septic rat aortic rings, decreased O_2^- from 113±6 nmole O_2^-/mg/10min to 49±4 nmole O_2^-/mg/10min and 56± 8 respectively (P=. 001). The addition of L-NAME to NADH-treated rat aortic rings, increased O_2^- in septic but not in control rats (113±7 nmole O_2^-/mg/10min vs. 75 ± 3 nmole O_2^-/mg/10min). On immunohistochemical examination there was $P22^{phox}$, in rat aortic adventitia. In pigs, Carotid rings from pigs had a basal O_2^- concentration of .63 ± .05 nmole O_2^-/mg/10min which was increased in sepsis to 1.5 ± 1.6 nmole O_2^-/mg/10min (P= .001). NADH stimulation increased the O_2^- concentration of 43 ± 8 nmole O_2^-/mg/10min in control and 63 ± 4.3 nmole O_2^-/mg/10min in endotoxemic pigs (P<0.05). The O_2^- concentration was

decreased with O_2^- scavengers, Tiron, and SOD ($P<0.01$) and flavin-containing enzyme inhibitor DPI ($P< 0.01$) in endotoxemic rings but not control rings. However in contrast to septic rats, L-NAME decreased the NADH stimulated O_2^- concentration from 63 ± 4 nmole O_2^-/mg/10min to 33 ± 5.6 nmole O_2^-/mg/10min ($P<0.01$). On immunohistochemistry $p22^{phox}$ was seen in endothelial cells of pig carotid. Additionally, Luminol-enhanced chemiluminesence was increased in arterial rings from endotoxemic pigs from .44 ± .1 nmole O_2^-/mg/10min to 1.1 ± .23 nmole O_2^-/mg/10min which is consistent with increased peroxynitrite formation. These results indicate a different behavior of NAD(P)H oxidase in NOSII producing and non-NOSII producing species.

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Introduction: Based on the observations in rats and mice it is commonly argued that NO causes organ injury and hemodynamic abnormalities in sepsis (33) (34) (11). In rats NOSII is expressed and the nitrotyrosine is produced. Nitrotyrosine formation has been used as a footprint of peroxynitrite (13) (12). Peroxynitrite is the product of O_2^- and NO. Increase of either or both of these reactants increases the formation of peroxynitrite (26) (32) (37). However pigs produce minimal NOSII, yet they still produce nitrotyrosine (15). We hypothesized this peroxynitrite could be due to increase in O_2^- which reacts with normal concentration of NO.

Recently it has been appreciated that O_2^- can be produced by a number of sources including mitochondria, NADH oxidoreductases, xanthine oxidase, cyclooxygenase, nitric oxide synthase, or NAD(P)H oxidase (4). NAD(P)H oxidase has been shown to be present in phagocytes as well as nonphagocytes, such as fibroblasts (25), smooth muscle

cells (6) glomerular mesangial cells (27) (14), and endothelial cells (16) (2). NAD(P)H oxidase complex is composed of at least five components in phagocytes. However the significance of O_2^- production from non-phagocytes and the source of its production in sepsis is not well known. In phagocytes NAD(P)H system has to utilize NADPH to produce O_2^- which is in the manner of rapid large short bursts of O_2^-. In non-phagocytes mostly it is NADH which is the preferred substrate, and the production of O_2^- is longer lasting, but the level of production is lower (8). We hypothesized that the non-phagocytic NAD(P)H oxidase system could be a source of O_2^-. Support for this comes from a recent study by Brandes et al (17) who found increased O_2^- concentration in endotoxic rats. They showed expression of NOSII, increased formation of both peroxynitrite, and nitrotyrosine. These findings were associated with increased level of the expression of NAD(P)H oxidase subunits, $p22^{phox}$ transcript, and the proteins of $p67^{phox}$, and $gp91^{phox}$ and decrease in O_2^- production by DPI in endotoxic rats(3). Increased NO may decrease the concentration of O_2^-. However the interaction of O_2^- and NO in sepsis has not been shown yet. Our objectives therefore were to I) confirm O_2^- increase in sepsis II) examine the interaction between O_2^- and NO in septic rats. Finally to determine if in pigs which are non-NOSII producers, O_2^- production by NAD(P)H oxidase is increased as in rats.

Methods

Preparation of pigs: The experiments were performed in seven juvenile pigs weighing 18-22 kg. They premedicated by intramuscular injection of ketamine hydrochloride ($30mg.kg^{-1}$) (Ayerst), xylazine $2mg.kg^{-1}$ (Bayer), and .4 $mg.kg^{-1}$ atropine sulphate

(sigma). They were anesthetized 10-15 minutes later with a 10-15 $mg.kg^{-1}$ sodium thiopental and anesthesia was maintained with a continuous intravenous infusion of sodium thiopental at 3-5$mg.kg^{-1}$.The animals were positioned on a V-shaped support, intubated with a cuffed endotracheal tube, and ventilated with a volume respirator at a tidal volume 12$ml.kg^{-1}$ and a frequency of 12-18 $breaths.min^{-1}$ and 5 cm water positive end-expiratory pressure. The right internal jugular vein was isolated and a Swan Ganz flow-directed catheter was passed into the pulmonary artery for measurement of right atrial pressure (Pra), pulmonary artery (Ppa) and the pulmonary occlusion pressures (Pcw), as well as cardiac output by thermodilution. One of the superficial branches of femoral artery was used to administer endotoxin and fluid. The position of transducers on stand was adjusted to midpoint of right atrium. Arterial blood gas samples were obtained at intervals of 30 minutes to keep the $PaCO_2$ between 35 to 45 mmHg by adjustment of respiratory rate and PO_2 was kept 90-150 mmHg with supplemental inspired oxygen as necessary. Lipopolysaccharide (Sigma) administration was $10ug.kg^{-1}.hr^{-1}$ for two hours. To maintain central venous pressure between 2-5, dextran was infused.

Vascular rings preparation and Chemiluminescence

Preparation of rats and their thoracic aortic rings: Male Sprague-Dawley rats weighing 250-300g were injected with .5 ml saline with (n=5) or without LPS (10mg/kg) (n=4), and after 18 hrs they were anesthetized with intraperitoneal injection of (90 mg/kg) of sodium pentobarbital. Aorta was removed immediately and put in ice-cold Krebs-HEPES (mM content: Nacl, 99.01; Kcl, 4.69; Cacl2, 1.87; MgSO4, 1.20; K2PO4, 1.03; NaHCO3, 25; Na-HEPEPS, 20; Glucose, 11.1; pH 7.4) (24). Aortic lumen was flushed with a

syringe that had a blunt pointed needle. Then extra-adventitial tissues were removed carefully, and aorta was cut into 2-3 mm segments. They remained in air-equilibrated Krebs-HEPES for about 30 minutes until they were used for lucigenin-enhanced chemiluminesence.

Preparation of rings from carotid artery of pigs: Through an incision parallel to midline in the neck, one of the carotid arteries was isolated and a segment of 2-3 cm length was resected prior to endotoxemia, and transferred into ice-cold Krebs-HEPES. Then this carotid was cannulated with a polyethylene catheter for pressure measurements, and a piece of 2-3 cm length of it was removed for chemiluminesence. Four hours after the start of LPS, the remaining carotid was removed for chemiluminesence. After removal of the carotid segments they were prepared similar to rat aortic preparation and used for lucigenin or luminol-enhanced chemiluminesence.

Detection of chemiluminesence in vascular rings:

Aortic segments of rats (already placed in ice-cold Krebs-HEPES buffer) were assigned to three different groups. One group (basal O_2^- production) was placed in glass tubes and incubated in the dark for a period of 10 minutes in Krebs-HEPES buffer at 37° C). The tubes were then transferred to the luminometer and lucigenin (230 μM) was then added and luminometer output was then measured in a luminometer (Lumat LB 9501, Berthold Inc.). We collected the chemiluminescence data every 15-20 seconds for a period of 10 minutes. The background signal was subtracted from luminometer output for and then the total area under the curve was calculated and by using the standard curve obtained from xanthine/xanthine oxidase (see chapter 5) the amount of O_2^- was calculated.

In the second and third groups of aortic rings, NADH or NADPH-stimulated O_2^- production were measured by incubating the rings with either NADH (100μM) or NADPH (100μM) in glass tubes in the dark for a period of 10 minutes at 37° C. Lucigenin-chemiluminescence was then measured as mentioned above.

Effects of various inhibitors on lucigenin-enhanced chemiluminescence: To evaluate the molecular sources of O_2^- production in aortic rings, we incubated vascular rings for a period of 10 minutes at 37° C with either Tiron (10 mM, cell permeable non-enzymatic O_2^- chelator), diphenyleneiodonium (DPI, 1.85 μM, inhibitor of flavin-containing enzymes), SOD (0.5 U/ml, selective O_2^- scavenger), N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM, inhibitor of nitric oxide synthases). Rings were then exposed to NADH (100 μM) for an additional 10 minutes and lucigenin chemiluminescence was then measured as above.

O_2^- detected in pig carotids in the same way as in rat aorta, but we used longer per-incubation period of 20-30 minutes for DPI. In addition we used luminol (230μM) (2-electron reduction) to assess peroxynitrite formation by luminol chemiluminescence as previously described (28).

Immunohistochemistry: After isolation of vessels from either pigs or rats, some sections after washing with Krebs-HEPES buffer were embedded in embedding medium (Histo Prep cat# SH75-125D; Fisher Scientific Inc.) and then frozen in liquid nitrogen-cooled isopentane. The tissues were kept at -80°C untill they were used. Frozen tissues were cryo-sectioned at 5-10μm and adsorbed to slides (cat# 12-550-15 Fisher) and dried. The sections were fixed with acetone for 1 minute followed by rehydration with PBS

containing 1% BSA (pH=7.4) for 5 minutes and were then blocked with 1% normal donkey serum for another 20 minutes at room temperature. The sections were incubated for 1 to 2 hours at room temperature with primary monoclonal raised against human NADPH oxidase subunits including $p22^{phox}$ subunit. For negative control, the primary antibody was replaced with mouse or rat IgG. After three rinses with PBS, sections were incubated with Cy3-labelled anti-mouse or anti-rabbit secondary antibodies (Jackson Immunoresearch labratories, Inc.) under dark condition in a closed humid chamber overnight at room temperature. Sections were then examined with a Nikon fluorescence microscope and photographed with a 35 mm camera (Nikon Inc.).

Statistical analysis: Mean ± SEM values of O_2^- levels were compared (Sigma Stat-Jandel Scientific) by one way ANOVA followed by Tukey test for multiple comparison. In some cases average of 2-3 measurement was used as n=1. Significance was considered present when P<. 05.

Results: Rat aorta (figure 1) shows typical chemiluminescence because of reaction of O_2^- with lucigenin. Addition of lucigenin to non-endotoxic rat aortic rings resulted in a minimal rise in first minute followed by a gradual decline in light emission (basal). In the presence of NADH light emissions curve peaked at around 2 minutes, which declined after (top curve). Addition of NADPH resulted in a chemiluminescence output curve similar to that of NADH, but with lower amplitude (middle curve).

The mean basal concentration of O_2^- production in rings from non-endotoxic rats was .38 ± .07 nmole / mg / 10 minutes. Incubation with NADH increased this

concentration to 93.9 ± 11.1 nmole / mg / 10 minutes, but with NADPH it only increased to (17 ± 2 nmole/mg/10 minutes) in non-endotoxic rats (figure 2 P<0.01). Pre-incubation with L-NAME did not alter the production of O_2^- in NADH treated aortic rings of non-endotoxic rats (75 ± 3 nmole / mg/10 minutes). The addition of SOD to NADH treated rings also did not decrease the O_2^- production (78 ± 3 nmole / mg /10 minutes), but Tiron decreased it to 21.6 ± 5 nmole /mg /10 minutes) (figure 2 P<0.01).

The basal O_2^- concentration increased in endotoxic rats to 1.18 ± .23 nmole /mg /10 minutes which was significantly higher than in non-endotoxic rats (figure 3 P=0.01). The addition of NADH to aortic rings from endotoxic rats increased the O_2^- concentration to 67.34 ± 15.8 nmole /mg /10 minutes which was not different from rats not treated with endotoxin (figure 3). The O_2^- scavenegers, SOD and Tiron in endotoxin treated rings decreased the O_2^- level to 56 ± 8 nmole /mg /10 minutes and 49 ± 4 nmole /mg /10 minutes (P<0.01) respectively (not shown in figure 3). Addition of L-NAME to NADH-treated rings of non-endotoxic rats, did not change O_2^- production (75 ± 3 nmole O_2^- /mg/10 minutes), but it increased production to 113 ± 7 nmole O_2^- /mg/10 minutes in rings of endotoxin treated rats higher than both NADH-treated endotoxic (67± 15 nmole O_2^- /mg/10 minute; P=<0.05) and L-NAME -pre-incubated NADH-treated rings from non-endotoxin treated rats (75± 3 nmole O_2^- /mg/10 minute; P<0.01) (figure 3).

Figure 4 shows the immunohistochemistry of rat aortic segment with staining for $p22^{phox}$. Staining was evident in the rat adventitia but not detected in endothelial cells of aorta with monoclonal antibody against phagocytic component, $p22^{phox}$.

Figure 5 and 6 show that the basal level of O_2^- production in carotid arteries from

the pigs. The basal O_2^- production was .63 ± .05 nmole O_2^- /mg/10 minutes in normal and increased to 1.55 ± .16 nmole O_2^- /mg/10 minutes in rings from pigs treated for four hours with endotoxin ($P<0.001$, n=7). Figure 5 shows that the addition of NADH raised O_2^- production in control and endotoxemic pig vascular rings to 43 ± 8.1 nmole O_2^- /mg/10 minutes and 63 ± 4.2 nmole O_2^- /mg/10 minutes respectively. These amounts were significantly different in the rings from endotoxemic and non-endotoxemic pigs ($P<0.05$). O_2^- scavengers, Tiron and SOD decreased the O_2^- level to 18 ± 4 nmole O_2^- /mg/10 minutes ($P<0.01$; n=5) and 34 ± 6.9 nmole O_2^-/mg/10 minutes ($P<0.01$; n=6) respectively in the presence of NADH in rings endotoxemic pigs. Similarly DPI decreased O_2^- level in NADH-stimulated endotoxemic group to 38.6 ± 6.4 nmole O_2^- /mg/10 minutes ($P<0.01$; n=5). In non-endotoxemic rings Tiron, SOD, and DPI lowered O_2^- production in NADH-stimulated rings from 43 ± 8.11 nmole O_2^-/mg/10 minutes to 36.1± 8.72 nmole O_2^-/mg/10 minutes, 28.9 ± 2.7 nmole O_2^-/ mg/10 minutes, and 32.7 ± 3.29 O_2^-/mg/10 minutes, but these decreases were not significant.

To determine if peroxynitrite is formed, we used luminol to enhance chemiluminesence. There was a significant increase from 47586 ± 11296 RLU /mg/10 minutes in control to 118282 ± 25743 RLU /mg/10 minutes in rings of endotoxemic pigs ($P<0.05$; n=7) (figure 6).

Immunohistochemical staining of carotid sections of pig revealed $p22^{phox}$ in endothelial cells of these vessels (figure 7).

Discussion: The major observations in this study are that: I) O_2^- is increased in endotoxic

rats. ii) Increase in O_2^- production is counterbalanced by increased NO production in endotoxic rats. iii) O_2^- generation is also increased in vessels of endotoxemic pigs and appears to be NOS-dependent iv) luminol-chemiluminesence is increased in endotoxemic pigs, which is suggestive of increased peroxynitrite formation.

Methods problem

Before discussing the results a number of methodological issues need to be reviewed.

Lucigenin chemiluminescence for the measurement of O_2^- has been criticized because of its ability to undergo redox cycling at high concentrations (31) (19) and the production of O_2^-. However we observed that in the absence of tissue, the chemiluminescence signal was small compared to the difference in concentration of O_2^- that was seen between endotoxin and non-endotoxin treated animals. Thus, the production of O_2^- by lucigenin can obscure differences but would not account for a difference between conditions.

Luminol has been widely used for detection of radicals. It undergoes a two-electron oxidation and makes an unstable compound that decomposes to aminophtallate. This substance emits light upon relaxation from excited to ground states (20) (18). H_2O_2 and peroxynitrite anion can oxidize luminol. The tissue production of H_2O_2 can be inhibited by catalase and the production of H_2O_2 can be detected by the decrease in luminol-derived chemiluminescence. We used catalase in a few rings of animals and observed no appreciable change in chemiluminescence signal which indicates that the luminol signal was not due to H_2O_2

In this paper we investigated the increase in O_2^- in 18 hours of endotoxemia in rats to reproduce the results of Brandes et al (3). However, we used 4 hours endotoxemia in pigs, because we previously observed nitrotyrosine formation in these pigs at that time (15).

We tested the specificity of the reaction to lucigenin with blockers and scavengers of O_2^- production. SOD did not lower the signal (figure 2) in rings from non-endotoxic rats. This could be because the O_2^- was intracellular and SOD is a non-permeable O_2^- scavenger. There was no significant decrease in the chemiluminescence signal from NADH-treated rings that were pre-incubated with DPI. This may have been been due to insufficient pre-incubation with DPI. For longer pre-incubation period, DPI decreased O_2^- production in carotid rings from endotoxemic pigs, significantly.

Preliminary measurements were done to detect O_2^- in our system. We observed that the peak of lucigenin chemiluminescence signal is within the first 5 minutes and therefore felt that ten minutes collection of data would be sufficient to compare normal and endotoxin-treated animals.

We used carotids in pigs, because one piece of carotid vessel from each pig could be used as control for that pig. But in rats the carotids are too small for adequate detection, so we decided to use aorta from rats and used placebo treated animals as others did (3).

O_2^- can be produced by a number of sources including mitochondria, xanthine oxidase, cyclooxygenase, nitric oxide synthase, or NAD(P)H oxidase (4).We can not rule out mitochondrial and cyclooxygenase sources of O_2^- production in septic rats. Brandes et

al (3) showed decreased O_2^- production by DPI in aorta of rats, treated with endotoxin for 12 hours which, indicates flavin-containing enzymes, including NAD(P)H oxidase, contributes to O_2^- production. They observed that oxypurinol, an inhibitor of O_2^- had effect at 30 hours in aorta of endotoxemic rats which, indicates that xanthine played no role. Since we only studied rats for 18 hours, it is unlikely that xanthine is the source of O_2^-.

There is an important interaction between NO and O_2^-. Furthermore there is an indirect effect of NO on O_2^-. For example, NO prevents the assembly of NAD(P)H oxidase subunits in phagocytes (5). However the interaction of these two in sepsis is not known. Brandes et al (3) did not use LNAME (N^G-nitro-L-arginine methyl ester), an inhibitor of NOS, to assess this interaction. L-NAME did not have a significant effect on the O_2^- concentration in control aortic rings of rats, however, interestingly, it significantly increased O_2^- concentration in endotoxic rats. A possible mechanism is that NOSII is increased in endotoxemic rats and this leads to an increase in NO production. This increase in NO would then decrease the O_2^- concentration.

A source of O_2^- production is the NAD(P)H oxidase system which is found in phagocytes (1) and more recently in non-phagocytes (8). We used NADH and NADPH as substrates for NAD(P)H oxidase to assess the behavior of NAD(P)H oxidase in aortic rings of rats and obtained higher lucigenin chemiluminescence with the same concentration of NADH than NADPH. This observation of higher O_2^- production with NADH than NADPH in this study argues against the O_2^- being produced by phagocytes. This is because it has been shown that NAD(P)H oxidase in phagocytes has a higher

affinity for NADPH than NADH as substrate, whereas in phagocytes it is the opposite (1) (9) (8) (7) (30) (23). Moreover as we demonstrated (figure1) the pattern of the release of O_2^- in nonphagocytes (9) is not as rapid and as short as it happens in neutrophils (9), further supporting the non-phagocytic source of O_2^- release in aorta of rats. In immunohistochemical sections of rat aorta we found evidence of p22phox. Therefore non-phagocytes NAD(P)H system was present in the aorta of rats and could have been the source of O_2^-.

Brandes et al (3) also argued that NAD(P)H oxidase is the major source of O_2^- production in endotoxic rats. Increased O_2^- was associated with increased expression of p22phox, p67phox, and gp91phox components of NAD(P)H oxidase in aortic tissue. We assessed production of O_2^- in both control and endotoxic rat, and observed increased basal production of O_2^- in endotoxin treated rats as they did (3).

We also found that O_2^- production was increased in the carotids of endotoxin treated pigs. Previously we did not find increased NO concentration in blood of endotoxemic pigs, but still, found increased peroxynitrite as indicated by increase nitrotyrosine. We therefore speculated that there might be an increase in O_2^- that reacts with normal NO to produce peroxynitrite. Indeed we observed an increased O_2^- concentration in carotid after 4 hours of endotoxemia. As shown in figure 6 the luminol-enhanced chemiluminescence also was increased in carotid rings from endotoxemic pigs at basal condition which indicates possible formation of peroxynitrite, and supports this interaction. We also used catalase in a few rings, to assess the contribution of H_2O_2 release from carotid rings in luminol-derived chemiluminescence. Catalase did not

change the chemiluminescence signal in rings from endotoxemic pigs. This indicates that H_2O_2 is not the major factor reacting with luminol in rings of endotoxemic pigs.

The increase in luminol and nitrotyrosine in our previous study (15) occurred despite minimal induction of NOSII in endotoxemic pigs. It is possible that the NO from constitutive NOS (NOSI and NOSIII) reacts with increased O_2^- NAD(P)H oxidase in pigs to produce peroxynitrite. Peroxynitrite has been shown to result in nitration of protein, which affect the contractile function of vessel (29) (22) (21) (12) and could be a factor in the hypotension of sepsis.

Our demonstration of increased O_2^- concentration in porcine model of sepsis which is inhibited with L-NAME in NADH stimulated rings, indicates that in contrast to rats, O_2^- formation is NOS-dependent. The mechanism is not obvious. A possible explanation is as follows. Peroxynitrite has been reported to activate protein kinase C (PKC) in vascular smooth muscle and mesangial cells (14) (35). PKC activation in turn increases NAD(P)H oxidase activity (10). It appears that L-NAME blocks substrate availability of NO, which in turn inhibits the formation of peroxynitrite formation. It can be speculated in these septic pigs that increased peroxynitrite activates PKC which in turn increases NAD(P)H oxidase activity.

L-arginine is the substrate for NOSs, and tetrahyrobiopterin (BH_4) is the cofactor for these enzymes. It has been shown in few in vitro studies that deficiency of either L-arginine, tetrahyrdobiopterine (BH_4) or both (36) (38) result in O_2^- formation, however, others have shown (39) that addition of L-arginine did not decrease O_2^- production, but supplying the cells with BH_4 decreased this production from recombinant NOS. It is

possible that constitutive NOS is the source of increased O_2^- formation in endotoxemia, however, we observed that addition of NADH increases O_2^- concentration several fold higher than the basal concentration of O_2^- in pigs. NADH is a substrate for NAD(P)H oxidase, but NOS uses NADPH to synthesize NO. We therefore, believe NOS is probably not the source of O_2^- formation in endotoxemic pigs.

In summary, first, there is O_2^- production in septic rats and pigs. Second, there is an important interaction between NO and O_2^- which, needs to be taken into account when either of these radicals are measured. Third, in pigs, O_2^- production appears to be NOS-dependent.

REFERENCES:

1. Babior, M.B. NADPH Oxidase: An Update. Blood 93 (5): 1464-1476, 1999.

2. Bayraktutan, U., N. Draper, D. Lang, and A.M. Shah. Expression of a functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. Cardiovasc.Res. 38: 256-262, 1998.

3. Brandes, R.P., G. Koddenberg, W. Gwinner, D. Kim, H. Kruse, R. Busse, and A. Mugge. Role of increased production of superoxide anions by NAD(P)H oxidase and xanthine oxidase in prolonged endotoxemia. Hypertension 33: 1243-1249, 1999.

4. Cross, A.R. and O.T.G. Jones. Enzymatic mechanisms of superoxide production. Biochim.Biophys.Acta 1057: 281-298, 1991.

5. Fujii, H., K. Ichimori, K. Hoshiai, and H. Nakazawa. Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process. J.Biol.Chem. 272: 32773-32778, 1997.

6. Fukui, T., B. Lassegue, H. Kai, W. Alexander, and K.K. Griendling. Cytochrome b-558 α-subunit cloning and expression in rat aortic smooth muscle cells. Biochim.Biophys.Acta 1231: 215-219, 1995.

7. Griendling, K.K., C.A. Minieri, J.D. Ollerenshaw, and R.W. Alexander. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ.Res. 1141-1148, 1994.

8. Griendling, K.K., D. Sorescu, and M. Ushio-Fukai. NAD(P)H oxidase. Role in cardiovascular biology and disease. Circ.Res. 86: 494-501, 2000.

9. Griendling, K.K. and M. Ushio-Fukai. NADH/NADPH oxidase and vascular function. Trends Cardivasc.Med. 7: 301-307, 1997.

10. Heitzer, T., U. Wenzel, U. Hink, D. Krollner, M. Skatchkov, R.A.K. Stahl, R. Macharzina, J.H. Brasen, and T. Meinertz. Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: Evidence for an involvement of protein kinase C. Kid.Int. 55: 252-260, 1999.

11. Hom, J.G., S.K. Grant, G. Wolf, J. Tom, D. Bach, E. MacIntyre, and N.I. Hutchinson. Lipopolysaccharide-induced hypotension and vascular hyporeactivity in the rat: tissue analysis of nitric oxide synthase mRNA and protein expression in the presence and absence of dexamethazone, N^G-monomethyl-L-arginine or indomethacine. J.Pharmacol.Exp.Ther. 272: 452-459, 1995.

12. Ischiropoulos, H. and A.B. Al-Mehdi. Peroxynitrite-mediated oxidative protein modifications. FEBS Lett 364: 279-282, 1995.

13. Ischiropoulos, H., L. Zhu, J. Chaen, M. Tsai, J.C. Martin, C.D. Smith, and J.S. Beckman. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch.Biochem.Biophys. 298(2): 431-437, 1992.

14. Jaimes, E.A., J.M. Galceran, and L. Raij. Angitensin II induces superoxide anion production by mesangial cells. Kid.Int. 54: 775-784, 1998.

15. Javeshghani, D. and S. Magder. Induction of iNOS mRNA without expression of iNOS in porcine endotoxemia.. Am.J.Respir.Crit.Care Med. 157: A6901998.(Abstract)

16. Jones, S.A., V.B. O'Donnell, J.D. Wood, J.P. Broughton, E.J. Hughes, and O.T.G. Jones. Expression of NADPH oxidase components in human endothelial cells. Am.J.Physiol. 271: H1626-H16341996.

17. Katsuki, S., W.P. Arnold, C. Mittal, and F. Murad. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J.cyclic nucleotide Res. 3: 23-35, 1977.

18. Kooy, N.W. and J.A. Royall. Agonist-induced peroxynitrite production from endothelial cells. Arch.Biochem.Biophys. 310: 352-359, 1994.

19. Li, Y., H. Zhu, P. Kuppusamy, V. roubaud, J.L. Zweier, and M.A. Trush. Validation of lucigenin (Bis-N-methylacridinium) as a chemiluminescence

probe for detecting superoxide anion radical production by enzymatic and cellular systems. J.Biol.Chem. 273: 2015-2023, 1998.

20. Li, Y., H. Zhu, and M.A. Trush. Detection of mitochondria-derived reactive oxygen species production by the chemiluminogenic probes lucigenin and luminol. Biochim.Biophys.Acta 1428: 1-12, 1999.

21. Lin, K.T., J.Y. Xue, M. Norman, B. Spur, and P.Y.K. Wong. Peroxynitrite-induced apoptosis in HL-60 cells. J.Biol.Chem. 270: 16487-16490, 1995.

22. Liu, S., J.S. Beckman, and D.D. Ku. Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. J.Pharmacol.Exp.Ther. 268(3): 1114-1121, 1994.

23. Mohazzab-H, K.M. and M.S. Wolin. Properties of superoxide anion-generating microsomal NADH oxidoreductas, a potential pulmonary artery Po_2 sensor. Am.J.Physiol. 267: L823-L8311994.

24. O'hara, Y., T.E. Peterson, and D.G. Harrison. Hypercholestrolaemia increases endothelial superoxide anion production. J.Clin.Invest. 91: 2546-2551, 1993.

25. Pagano, P.J., J.K. Clark, M.F. Cifuentes-Pagano, S.M. Clark, G.M. Callis, and M.T. Quinn. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc.Natl.Acad.Sci.USA 94: 14438-14488, 1997.

26. Payor, W.A. and G.L. Squadrito. The chemistry of peroxynitrite: a product of the reaction of nitric oxide with superoxide. Am.J.Physiol. 268: L699-L7221995.

27. Radeke, H.H., A.R. Cross, J.T. Hancock, O.T.G. Jones, M. Nakamura, V. Kaever, and K. Resch. Functional expression of NADPH oxidase components (α– and β– subunits of cytochrome b_{558} and 45-Kda flavoprotein) by intrinsic human glomerular mesangial cells. J.Biol.Chem. 266 (31): 21025-21029, 1991.

28. Radi, R., T.P. Cosgrove, J.S. Beckman, and B.A. Freeman. Peroxynitrite-induced luminol chemiluminesence. Biochem.J. 290: 51-57, 1993.

29. Radi, R., M. Rodrigguez, L. Castro, and R. Telleri. Inhibition of mitochondrial electron transport by peroxynitrite. Arch.Biochem.Biophys. 308: 89-95, 1999.

30. Rajagopalan, S., S. Kurz, T. Munzel, M. Tarpey, B.A. Freeman, K.K. Griendling, and D.G. Harrison. Angiotensin II mediated hypertension in the rat increases vascular superoxide production in membrane NADH/NADPH oxidase activation: contribution to alterations of vascular tone. J.Clin.Invest. 97: 1916-1923, 1996.

31. Skatchkov, M., D. Springall, U. Hink, A. Mulsch, D.G. Harrison, I. Snidermann, T. Meinertz, and T. Munzel. Validation of lucigenin as a chemiluminescence

probe to monitor vascular superoxide as well as basal vascular nitric oxide production. Biochem.Biophys.Res.Commun. 254: 319-324, 1999.

32. Squadrito, G.L. and W.A. Pryor. The formation of peroxynitrite in vivo from nitric oxide and superoxide. Chem.Biol.Interact. 96: 203-206, 1995.

33. Stoclet, J.C., I. Fleming, G. Gray, G.J. Schaeffer, F. Schneider, C. Schott, and J.R. Parratt. Nitric Oxide and Endotoxemia. Circulation 87: V-77-V-801993.

34. Szabo, C. Alteration in nitric oxide production in various forms of circulatory shock. New Horiz. 3: 2-32, 1995.

35. Tepperman, B.L., Q. Chang, and B.D. Soper. The involvement of protein kinase in nitric oxide-induced damage to rat isolated colonic mucosal cells. B.J.Pharmacol. 128: 1268-1274, 1999.

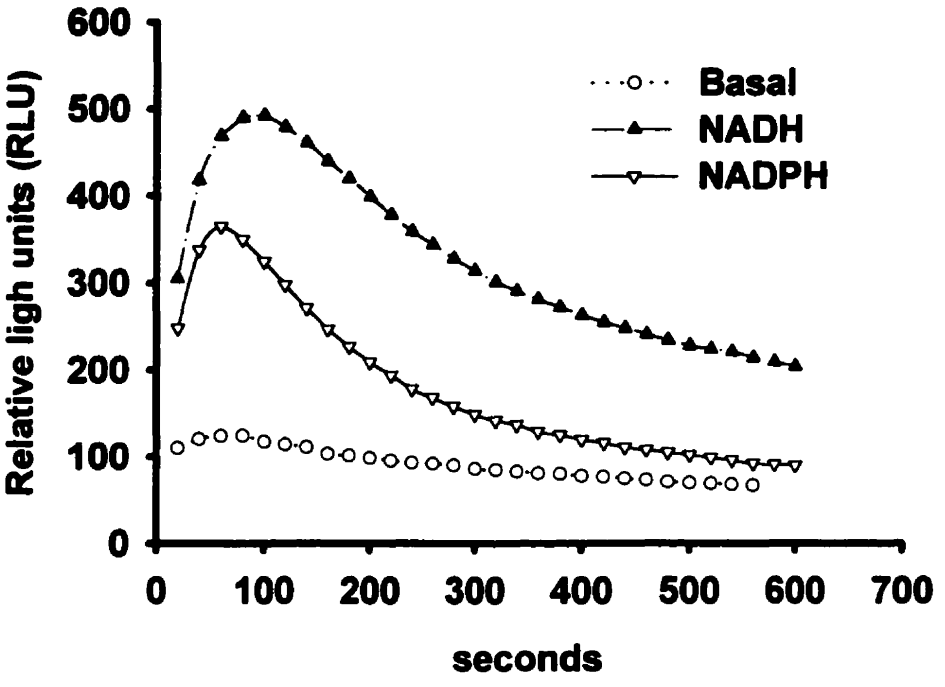
36. Vasquez-Vivar, J., B. Kalyanaraman, P. Martasek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo, and K.A.J. Pritchard. Superoxide generation by endothelial nitric oxide synthase: The infulence of cofactors. Proc.Natl.Acad.Sci.USA 95: 9220-9225, 1998.

37. Villa, S.M., E. Salas, V.M. Darley Usmar, M.W. Radomski, and S. Moncada. Peroxynitrite induces both vasodilation and impaired vascular relaxation in the isolated perfused rat heart. Proc.Natl.Acad.Sci.USA 91: 12383-12387, 1994.

38. Wallerath, T., I. Gath, W.E. Aulitzky, J.S. Pollack, H. Kleinert, and U. Forstermann. Identification of NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets. Thromb.Haemost. 77: 163-167, 1997.

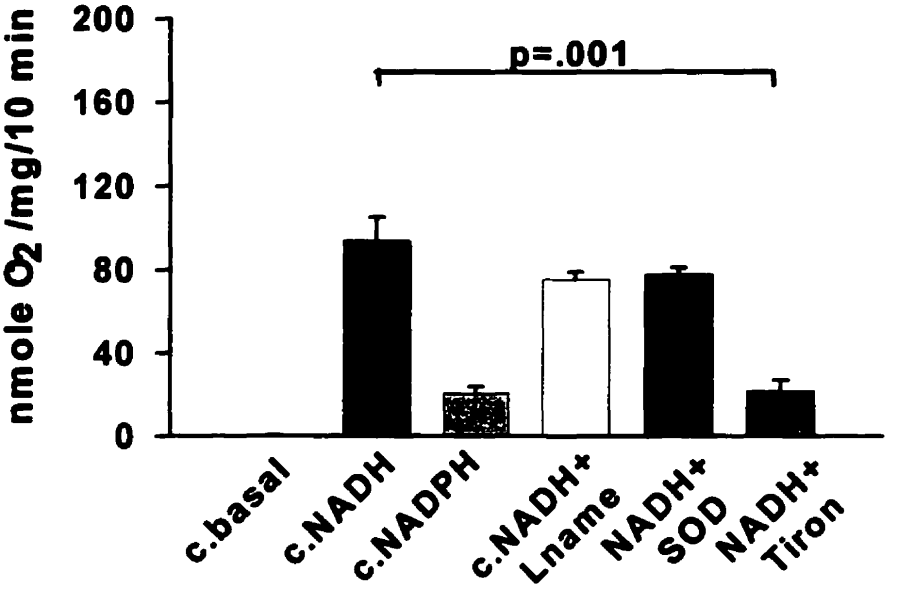
39. Wever, R.M.F., T. van Dam, H.J.M. van Rijin, F. de Groot, and T.J. Rabelink. Tetrahyrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem.Biophys.Res.Commun. 237: 340-344, 1997.

Figure 1



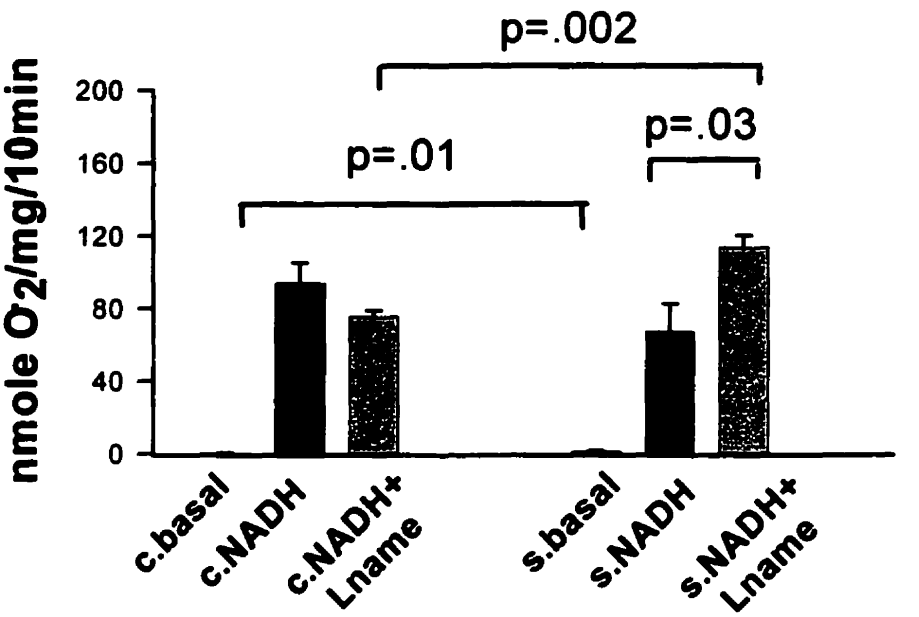
Patterns of enhanced-lucigenin chemiluminescence in control aorta

Figure 2



Behavior of NAD(P)H oxidase in response to stimulation with NADH, NADPH, inhibitor of NOS (LNAME), and superoxide inhibitors (Tiron and SOD).

Figure 3



Lucigenin-chemiluminesence of aortic rings from control (C) and septic (S) rats at basal or NADH-stimulation in absence or presence of LNAME.

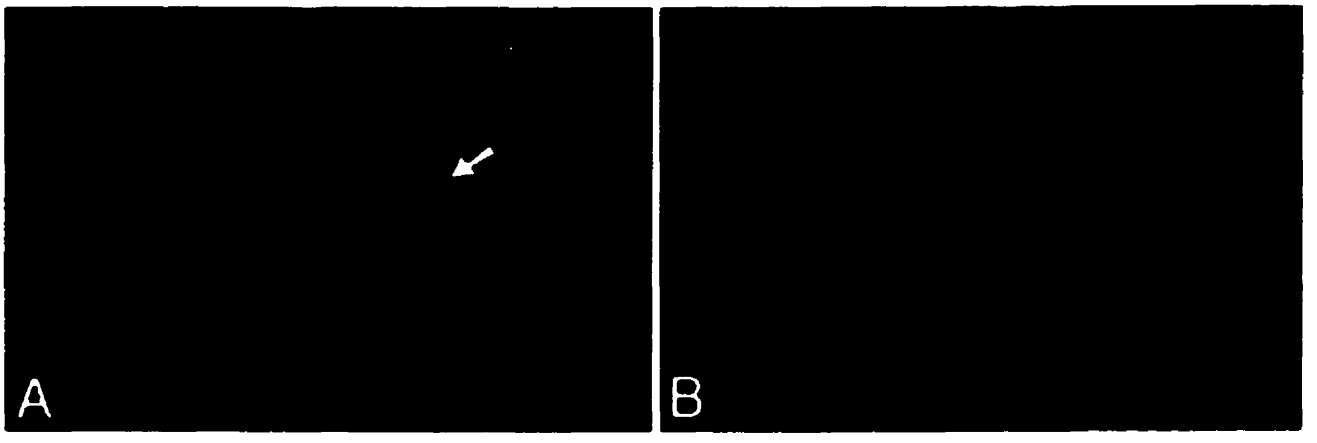
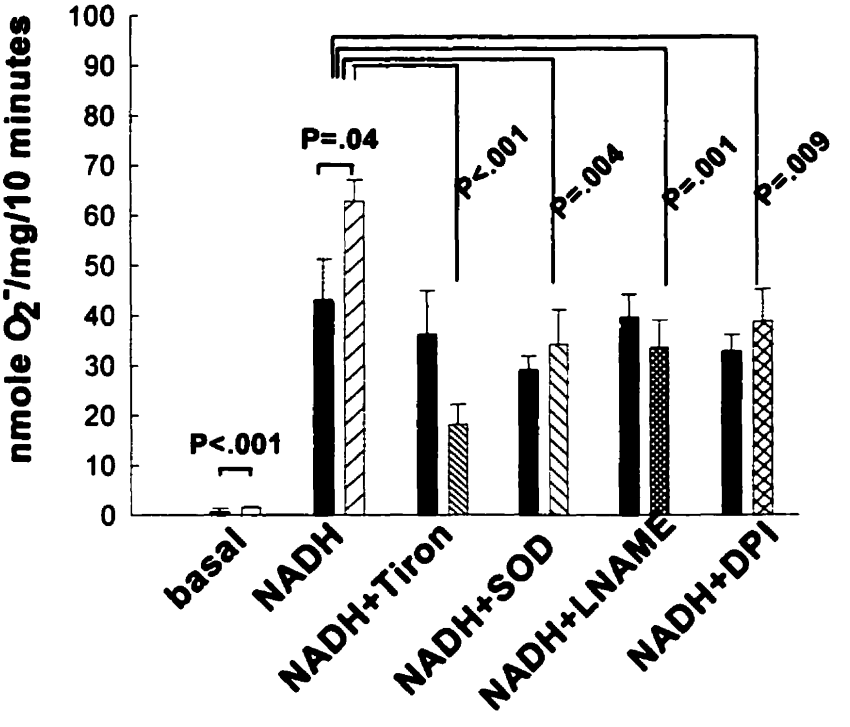

A
B

Figure 4 (previous 217):

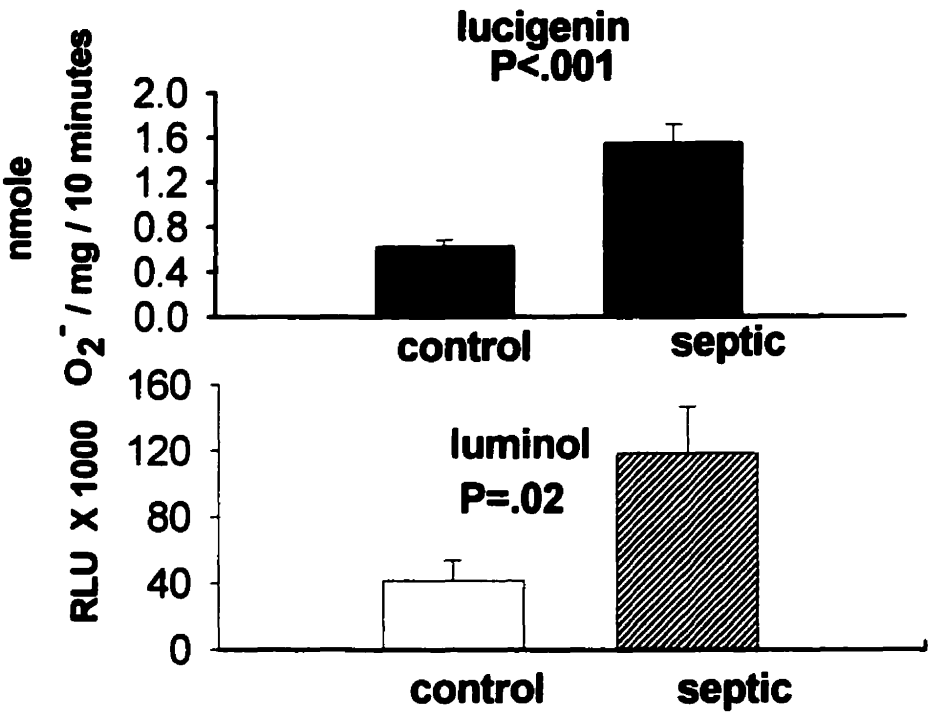
Immunohistochemistry of rat aortic sections with monoclonal antibodies against NAD(P)H oxidase component $p22^{phox}$ .Magnification: x100

Figure 5



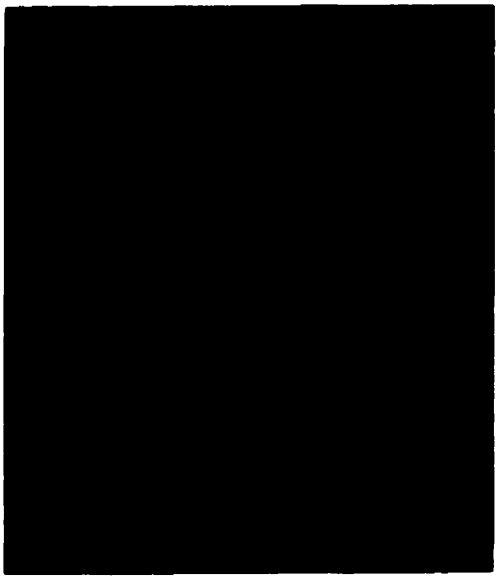
Control or septic carotids shown as pairs (left=control, right= septic) for each treatment without NADH or with NADH stimulation with different inhibitors.

Figure 6



Lucigenin and Luminol chemiluminesence in Porcine carotid Arteries

Figure 7



Immunohistochemistry of pig carotid section with monoclonal antibody against NAD(P)H oxidase components $p22^{phox}$. Magnification: x100

Link to chapter five:

More than 60% of body, is skeletal muscle tissue. They are vascularized tissues that recruit more vessels for their contraction. Therefore production of $O_2^{\cdot}$ by this tissue may be important in sepsis. $O_2^{\cdot}$ can be generated from phagocytes and non-phagocyte sources. Recent studies have shown activation of NAD(P)H oxidase complex in several non-phagocytes. However the presence and the role of this oxidase complex in skeletal muscle is not known. I addressed these matters in skeletal muscle.

CHAPTER FIVE

PRODUCTION OF SUPEROXIDE RADICALS IN THE VENTILATORY MUSCLE BY NAD(P)H OXIDASE

Abstract: Although reactive oxygen species play important roles in the pathogenesis of muscle fatigue, the enzymatic sources of these radicals remain unknown. In this study we investigated the existence, subunit composition and contribution of phagocytic NADPH oxidase to O_2^- production in the ventilatory muscles. O_2^- production in muscle strips was measured with lucigenin chemiluminesence, whereas subunit composition and localization was measured with RT-PCR, western blotting and immunohistochemistry. Basal O_2^- production in normal rat diaphragmatic strips averaged 1.4 nmoles/ mg/ 10 min. Production rose to 18 nmoles/mg/10min in the presence of 100 μM NADH, and to a lesser extent, in the presence of NADPH. O_2^- production was inhibited by Tiron, superoxide dismutase (SOD), apocynin or Diphenyleneiodonium (DPI) but not by nitric oxide synthase (NOS) inhibitors. Rt-PCR revealed the presence of mRNA of $P22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits in rat and human muscle. Western blotting detected the presence of only p^{22phox}, gp^{91phox} and p^{47phox} subunits and they were localized at the endothelium, fibroblasts and inside skeletal muscle fibers. Lucigenin-enhanced chemiluminesence revealed increased level of O_2^- production. These results document for the first time the existence of NADPH oxidase enzyme in the ventilatory muscles, and its increased activity in sepsis.

Introduction:

Reactive oxygen species (ROS)(O_2^-, H_2O_2 and HO^-) are normally produced at a relatively low rate inside resting skeletal muscle fibers, however, ROS levels undergo

significant elevation when muscle fibers become active (30) (32) (7). ROS exert substantial effects on the contractility of skeletal muscle fibers. In unfatigued muscles, the relatively low levels of ROS actually facilitate muscle contractility. Administration of ROS scavengers in fresh muscles causes a significant decline in both twitch and tetanic forces (31) On the other hand, a modest increase in ROS production as occurs during exogenous ROS exposure, strong muscle contractions or in sepsis, a state of oxidative stress develops and leads to reduction in muscle force. The rise in skeletal muscle ROS levels in septic animals coincides with an increase in lipid peroxidation, protein oxidation and a decline in muscle antioxidant capacity (10;35) . The observations that pre-treatment of septic animals with antioxidant enzymes and ROS scavengers reverses the decline in muscle contractility have confirmed that ROS play a significant role in determining muscle contractile performance (37) (35) (39).

Although several sites inside muscle fibers are influenced by ROS, sarcolemmal-related processes such as excitation-contraction coupling are particular targets for ROS actions. ROS oxidize regulatory proteins involved in Na^+-K^+ pump and Na^+-K^+-Cl^- transporter (34). In addition, oxidative modifications of Ca^{++} release channels of the sarcoplasmic reticulum influence channel opening and binding of ryanodine to the channels (11). Moreover, sarcoplasmic reticulum Ca^{++}-dependent ATPase pump activity is also inhibited by ROS as a result of protein thiol modifications and nitrotyrosine formation (20). Numerous studies have attributed the molecular sources of ROS in skeletal muscle fibers to the activities of the electron carriers on the inner mitochondrial membrane, membrane-bound oxidoreductases, the cyclooxygenase pathways and xanthine oxidase (36) (17). Recent studies indicate, however, that a significant portion of

ROS production in response to increased muscle activity is not derived from the mitochondria and is dependent on the presence of NADPH (4). These results suggest that NADPH oxidase may exist inside skeletal muscle fibers and contribute to ROS production in response to increased muscle activity. NADPH oxidase is a complex membrane-associated enzyme system which catalyzes the production of O_2^- by one electron reduction of O_2 using NADPH or NADH as the electron donor (2). The O_2^- generated by NADPH oxidase serves as the starting material for the production of a variety of reactive oxidants. The core component of NADPH oxidase in phagocytes includes 2 membrane-bound subunits ($p22^{phox}$ and $gp91^{phox}$) and 3 cytosolic subunits ($p47^{phox}$, $p40^{phox}$ and $p67^{phox}$) which upon activation associate with the membrane-bound subunits (15). During the past few year, NADPH oxidase has been identified as a major source of ROS in non-phagocytes such as fibroblasts, chondrocytes, mesangial, microglial, epithelial, endothelial and vascular smooth muscle cells (24) (27) (26) (9) (40) (19). NADPH oxidase in these cells differs significantly from that of phagocytes because it is constitutively active, uses NADH preferentially, and does not require the whole five subunits for its activity (4). The existence and the contribution of this enzyme system to ROS production inside normal skeletal muscle fibers has not yet been determined. Also not known is whether NADPH oxidase participates in sepsis-induced oxidative stress in skeletal muscle fibers.

The main objectives of this study is to: 1) document the existence, subunit composition, localization and substrate dependence of NADPH oxidase enzyme complex system in normal skeletal muscles; 2) assess the contribution of NADPH oxidase to ROS

system in normal skeletal muscles; 2) assess the contribution of NADPH oxidase to ROS production in normal and septic skeletal muscles and determine the nature of the interaction between NADPH oxidase and nitric oxide synthases (NOS).

Materials: Lucigenin, Aprotinin, Leupeptin, Trypsin inhibitor, Pepstatin A, Phenylmethylsulphonyfluoride, Diphenyliodonium chloride (DPI), β-Nicotinamide adenine dinucleotide (β-NADH), β-Nicotinamide adenine dinucleotide phosphate (β-NADPH), Tiron (4,5 dihydroxy-1,3-benzene-disulfonic acid), N^G-nitro-l-arginine methyl ester (L-NAME), superoxide dismutase (SOD), Cytochrome C acetylated, Antimycin, Rotenone and oxypurinol, were purchased from Sigma Chemicals Inc. (St. Louis, MO). Potassium cyanide (KCN), Apocynin (Acetovanillone) were purchased from Aldrich Inc. Monoclonal and polyclonal antibodies raised against $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ subunits of human neutrophil NADPH oxidase were provided by both Dr. M.T.Quinn (University of Montana) and Genentech Inc. (San Francisco, CA). Reagent for protein measurement was purchased from Bio-Rad Inc. (Hercules, CA) Gels and loading buffer for immunoblotting was obtained from Novex Inc. (San Diego, CA).

METHODS:

General animal preparation: Male Sprague-Dawley rats weighing 250-300g were killed with an overdose of pentobarbital sodium and the ventilatory (diaphragm) and limb (gastrocnemius and soleus) muscles were exposed through surgical incisions, excised, cleaned of connective tissues, and quickly frozen in liquid nitrogen. In an another set of experiments (measurements of superoxide production and NADH utilization), the

cold Krebs-HEPES buffer equilibrated with room air. The buffer (pH 7.4) has the following composition (in mM): NaCl 99.01; KCl 4.69; $CaCl_2$ 1.87; $MgSO_4$ 1.20; K_2PO_4 1.03; $NaHCO_3$ 25; Na-HEPEPS 20; and Glucose 11.1. Radials strips (2 mm wide) along the muscle fibers extending from the central tend to the rib cage were then excised and placed in the same buffer for a period of 30 minutes.

To evaluate the influence of sepsis on NADPH oxidase activity and expression in skeletal muscles, two groups (n=6 in each group) of male Sprague-Dawley rats were designated as either a control group and injected i.p. with 0.3 ml normal saline, whereas the second group (septic) was injected i.p. with 10-12 mg/kg *E.coli* lipopolysaccharide (LPS, serotype 055:B5, Sigma Chemicals Inc.). Both groups were sacrificed after 18 hours of either saline or LPS injection. The diaphragm was then excised and was either quickly frozen in liquid nitrogen or placed in Krebs-HEPES buffer for O_2^- production and NADH utilization.

Measurement of O_2^- production with lucigenin-enhanced chemiluminescence:

Preparation of standard curve: Increasing levels of xanthine ranging from 1 to 64 nmoles were incubated with cytochrome C (80 μM) and 8 units of xanthine oxidase at 37°C in the presence and absence of superoxide disumutase (SOD) (1.5 U/ml). The change in the absorbance at 550 nm was measured using Spectronic Genesys spectrophotometer (Milton Roy Inc.) and levels of SOD-inhibitable O_2^- produced by the reaction of xanthine and xanthine oxidase were calculated using the formula:

Δ absorbance (550 nm)= 21.1 X concentration O_2^- (25).

The amount of O_2^- calculated was then used to calibrate the lucigenin-enhanced chemiluminescence signal obtained by mixing the same amounts of xanthine and xanthine oxidase in the presence or absence of the same concentration of SOD in a luminometer (Lumat LB 9501, Berthold Inc.). Luminometer output was printed and the area under the curve for a total of 10 minutes of recording output was calculated. We obtained a highly linear relationship between luminometer output and amount of O_2^- produced by the reaction of xanthine and xanthine oxidase during the calibration curve ($r=0.99$). Similarly, luminometer output was linearly related to the amount of xanthine used during the calibration curve (ranging between 1 to 64 nmoles)($r=0.99$, figure 1).

Detection of O_2^- radicals in muscle strips: Isolated diaphragmatic muscle strips (already placed in ice-cold Krebs-HEPES buffer) were assigned to three different groups. One group (basal O_2^- production) was placed in glass tubes and incubated in the dark for a period of 10 minutes in Krebs-HEPES buffer at 37° C). The tubes were then transferred to the luminometer and lucigenin (230 μM) was then added and luminometer output was measured for the next 10 minutes. In the second and third groups of muscles strips, NADH or NADPH-stimulated O_2^- production were measured by incubating the strips with either NADH (100μM) or NADPH (100μM) in glass tubes in the dark for a period of 10 minutes at 37° C. Lucigenin-enhanced chemiluminescence was then measured as mentioned above.

Effects of various inhibitors on lucigenin-enhanced chemiluminescence: To evaluate the molecular sources of O_2^- production in diaphragmatic muscle strips, we incubated muscle strips for a period of 30 minutes at 37° C with either Tiron (10 mM, cell permeable non-

enzymatic O_2^- chelator), Diphenyleneiodonium (DPI, 1.85 μM, inhibitor of flavin-containing enzymes), SOD (0.5 U/ml, selective O_2^- scavenger), N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM, inhibitor of nitric oxide synthases). Strips were then exposed to NADH (100 μM) for an additional 10 minutes and lucigenin chemiluminescence was then measured as above.

Measurement of NADH utilization: Frozen muscle samples were homogenized in 6 vol/wt ice-cooled homogenization buffer with the following composition: HEPES 50 mM; DTT 0.1 mM; leupeptin 2μg/ml; PMSF 100μg/ml; aprotinine 2μg/ml; pepstatin A 1 mg/100ml; Glycerol 5%; pH=7.4. Samples were then centrifuged at 5000g for 45 minutes to remove unhomogenized tissues. The supernatant was collected and then centrifuged at 14,000g for 20 minutes. The pellet contained the mitochondria, while the supernatant was collected and centrifuged at 100,000 g for 1hour to separate the soluble (supernatant) and membrane (pellet) fractions of muscle tissue. Protein concentrations of both fractions were measured according to the Bradford technique (BioRad Inc.). Utilization of NADH was then measured according to Thannickal and Fanburg (40). In brief, various aliquots of membrane and cytosolic fractions (12.5, 25, 50 and 100 μg protein) were incubated with NADH (100μM) at 37° C and the rate of NADH consumption was monitored by the decline in absorbance every 15 minutes at λ=340 nm in a spectrophotometer (40). The procedure was repeated in the presence of Tiron (10 mM), DPI (1.85 μM), SOD (0.5 U/ml), L-NAME (1 mM), apocynin (500 μM, inhibitor of NADPH oxidase), indomethacin (100 μM, cyclooxygenase inhibitor) and oxypurinol

(1 mM, xanthine oxidase inhibitor). In addition, we have assessed whether mitochondrial enzymes are involved in NADH utilization by repeating the NADH utilization measurement in the presence of KCN (inhibitor of mitochondrial complex IV, 1 mM), Rotenone (inhibitor of mitochondrial complex I, 250 μM) and Antimycin A (inhibitor of mitochondrial complex III, 10 μM).

Reverse Transcription polymerase chain reaction (RT-PCR): Total RNA was extracted from tissue samples following the method described by Chomczynski and Sacchi (8). Total RNA (1 μg) was reverse transcribed by using random hexamers and MMLV reverse transcriptase (Life Technologies, Gaithersburg, MD). RT-generated cDNA encoding rat $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (both as an internal standard and positive control) were amplified by using PCR. RNA with no clear GAPDH band in the RT-PCR products (35 cycles) was discarded from further studies. Oligonucleotide primers (synthesized in McGill University DNA synthesis facility) used to amplify mRNA transcripts of rat skeletal muscle NADPH oxidase subunits are listed in Table 1. While primers for $p22^{phox}$ and $gp91^{phox}$ are based on known rat sequences, $p47^{phox}$ and $p67^{phox}$ primers were designed on the basis on both mouse and human sequences of these genes. Experimental conditions for all PCR reactions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min). This was followed by a final 10-min 72°C extension. Ethidium bromide-stained 2% agarose gels were used to separate PCR products that were visualized under UV light. Optical densities of DNA bands were scanned with a densitometer (see above) and quantified by

using SigmaGel software (Jandel Scientific Inc.). To verify the accuracy of the amplified sequence, PCR products were cloned in PCRII cloning vector (Invitrogen, San Diego, CA) and sequenced in the McGill University DNA sequencing facility.

Immunoblotting: Diaphragmatic, gastrocnemius and soleus protein samples (50 to 100 μg of either crude homogenate, membrane or soluble fractions) were mixed with Tris-Glycin-sample buffer and boiled for 5 minutes at 95° C and then loaded onto 8 or 10% 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) membrane, and then blocked with 7% non-fat dry milk containing 1% fetal calf serum (FCS) at 4°C overnight. The PVDF membranes were subsequently incubated with primary monoclonal or polyclonal antibodies raised against human NADPH oxidase subunits. After three washes of 10 minutes with wash buffer on rotating shaker the PVDF membranes were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies. Specific proteins were detected with ECL kit (Amersham Canada). The blots were scanned with an imaging densitometer and optical densities of various protein bands were quantified with SigmaGel software (Jandel Scientific Inc.)

Immunohistochemistry. In few rats, the diaphragm, soleus and gastrocnemius muscles were immediately removed from the animals, embedded in embedding medium (Histo Prep cat# SH75-125D; Fisher Scientific Inc.) and then frozen in liquid nitrogen-cooled isopentane. The tissues were kept at -80°C till they were used. Frozen tissues were cryo-sectioned at 5-10μm and adsorbed to slides (cat# 12-550-15 Fisher) and dried. The

sections were fixed with acetone for 1 minute followed by rehydration with PBS containing 1% BSA (pH=7.4) for 5 minutes and were then blocked with 1% normal donkey serum for another 20 minutes at room temperature. The sections were incubated for 1 to 2 hours at room temperature with primary monoclonal or polyclonal antibodies raised against human NADPH oxidase subunits including $p40^{phox}$ subunit. For negative control, the primary antibody was replaced with mouse or rat IgG. After three rinses with PBS, sections were incubated with Cy3-labelled anti-mouse or anti-rabbit secondary antibodies (Jackson Immunoresearch labratories, Inc.) under dark condition in a closed humid chamber overnight at room temperature. Sections were then examined with a Nikon fluorescence microscope and photographed with a 35 mm camera (Nikon Inc.).

Statistical analysis: Values of O_2^- levels and NADH utilization are presented as means± SEM. Differences between basal, NADH- and NADPH-stimulated O_2^- production in the presence and absence of various inhibitors were compared with one way ANOVA followed by Tukey test for multiple comparison. Linear regression analysis was used to quantify the rate of NADH utilization in the absence and presence of various inhibitors. Differences in the rate of NADH utilization were detected by student t-test. Significance was considered present when $P<.05$.

RESULTS:

Production of O_2^- radicals in normal skeletal muscles: Figure 1 illustrates the generation of O_2^- radicals by a cell-free xanthine/xanthine oxidase system. O_2^- radicals were measured by lucigenin-enhanced chemiluminescence in the presence of increasing levels of xanthine and 8 units of xanthine oxidase. Lucigenin-enhanced chemiluminescence (expressed as relative light units) was linearly related to the levels of xanthine present in the reaction tubes. The addition of SOD resulted in a signal that was not different from background confirming that O_2^- radicals are responsible for the luminescence.

Figure 2 illustrates representative examples of lucigenin-enhanced chemiluminescence signals obtained from normal rat diaphragms. Basal luminescence was detectable above background signals in all muscle strips. However. chemiluminescence signals were significantly higher in the presence of NADH than basal values. Signals reached peak values 100 seconds of the addition of lucigenin and declined progressively thereafter (figure2). Chemiluminescence signals in the presence of NADPH were significantly lower than those generated by NADH but were higher than basal values (figure 2).

Figure 3 shows absolute O_2^- radical levels (normalized per mg wet muscle weight) in normal rat diaphragmatic muscle strips. O_2^- radical production was preferentially elevated in the presence of NADH compared with NADPH. Pre-incubation of muscle strips with Tiron significantly reduced NADH-stimulated O_2^- production $P<0.05$). Significant reduction in O_2^- production was also noticed in response to pre-incubation with either SOD or DPI, whereas inhibition of nitric oxide synthases had no effect on NADH-stimulated O_2^- production (figure 3).

NADH utilization: Figure 4 illustrates the rate of NADH utilization of both soluble and membrane fractions of normal rat diaphragm. The inserted numbers refer to the amounts of sample protein used to measure NADH utilization. No significant NADH utilization was observed at increasing protein levels of the soluble fraction, whereas NADH utilization increased linearly with respect to time at membrane fraction protein levels of 12.5 and 25 μg. At 50 and 100μg, NADH utilization reached peak values after 60 and 10 minutes of reaction time, respectively (figure 4).

To investigate the molecular sources of NADH utilization in the membrane fraction, various inhibitors were incubated throughout the reaction time with 50 μg total protein sample. Diaphragmatic NADH utilization was significantly inhibited by KCN, Antimycin and DPI (figure 5, $P<0.001$ compared with NADH only), whereas Rotenon was without effects (figure 5). Figure 6 shows that diaphragmatic NADH utilization was partially inhibited by oxypurinol or apocynin ($P<0.001$ compared with NADH alone), whereas no effects were noticed with L-NAME and indomethacin (figure 6).

NADPH oxidase subunit structure and localization: Amplification of mRNA expression of various neutrophil NADPH oxidase subunits in rat skeletal muscles with RT-PCR revealed the presence of mRNA of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits (figure 7). Sequencing of RT-PCR products revealed that muscle partial cDNA of $p22^{phox}$ and $gp91^{phox}$ are identical to those amplified from other organs (13) (3). In addition, partial cDNAs of rat muscle $p47^{phox}$ and $p67^{phox}$ subunits appear to have relatively high homology with mouse neutrophil sequences than that of human sequences (appendices 1 and 2).

Immunoblotting with various antibodies raised against neutrophil NADPH oxidase subunits revealed the presence of abundant $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$ proteins in normal rat muscles (figure 8). Interestingly, while rat muscle $gp91^{phox}$ and $p47^{phox}$ proteins appear to be similar in apparent molecular weight to that of human neutrophil, rat muscle $p22^{phox}$ protein has an apparent mass of about 30 kDa (figure 8). This difference is not related to species differences between human and rat since anti-$p22^{phox}$ antibodies detected a 22 kDa protein in rat neutrophil lysate. In addition to the above mentioned subunits, we detected weak expression of $p67^{phox}$ protein in rat muscle samples but we were unable to detect $p40^{phox}$ protein. We should emphasize that $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ proteins were detected mainly in the membrane fraction of normal muscles.

Figure 9 illustrates immunoreactivity of various NADPH oxidase subunits in normal rat muscles detected with monoclonal and polyclonal antibodies. Abundant positive $p22^{phox}$ immunostaining was detected both inside skeletal muscle fibers in close proximity to the sarcolemma and inside blood vessels draining and supplying skeletal muscle fibers (figure 9 top). Interestingly, nerve fibers did not positively stain with anti-$p22^{phox}$ antibodies. Similar pattern of muscle fiber and blood vessel specific immunoreactivity were detected with anti-$gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ antibodies (figure 9 middle and bottom). No apparent differences were detected in the distribution of positive immunoreactivity of various NADPH oxidase subunits between diaphragm, gastrocnemius and soleus muscle. Unlike the above mentioned subunits, positive p40phox immunoreactivity was only detectable in blood vessels traversing muscle fibers (figure 9 bottom).

Effects of sepsis on muscle NADPH oxidase and O_2^- production: Injection of *E.coli* LPS elicited a significant increase in basal diaphragmatic O_2^- production measured with lucigenin-enhanced chemiluminescence ($p<0.01$ compared with control diaphragm, figure 10). However, NADH-stimulated O_2^- production was not different between control and septic diaphragms (figure 10). Interestingly, while pre-incubation with L-NAME had no effect on NADH-stimulated O2- production in control diaphragm, this production was significantly elevated with pre-treatment with L-NAME in septic diaphragm ($P<0.05$ compared with NADH alone).

Figure 11 illustrates the influence of sepsis on protein expression of $p22^{phox}$ and $p47^{phox}$ subunit in rat diaphragm. In both septic and control rats, these subunits were detected mainly in the membrane fraction of muscle samples. Injection of LPS was not associated with significant alterations in protein levels of these two subunits. Similar results were also obtained with $gp91^{phox}$ and $p67^{phox}$ antibodies (not shown).

DISCUSSION:

The main finding of this study is that NADPH oxidase enzyme complex is present in skeletal muscles and is localized inside skeletal muscle fibers and endothelial cells. The subunit structure of this enzyme in skeletal muscle fibers consists of four subunits, $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$, whereas an additional subunit ($p40^{phox}$) is present in endothelial cells but was not identified inside muscle fibers. This enzyme system prefers NADH vs NADPH as a substrate and is responsible in part for O_2^- radical

production in skeletal muscles. Our study also indicates that basal O_2^- production in skeletal muscles increases significantly in sepsis and that inhibition of nitric oxide synthases in septic muscles causes a further augmentation of O_2^- production in the presence of NADH. This finding suggests that NO exerts an inhibitory effects on muscle O_2^- production as a result of either scavenging of O_2^- radicals or inhibition of NADPH oxidase enzyme activity.

NADPH oxidase in non-phagocytes: It became evident over the past few years that in vascular and cardiac cells, ROS are derived from NAD(P)H oxidases rather than from xanthine oxidase, arachidonic acid metabolites and mitochondrial enzymes. These oxidases are membrane-associated enzymes, which catalyze the one electron reduction of molecular oxygen using either NADH or NADPH as the electron donor. In vascular smooth muscle cells, both NADH and NADPH are used by NAD(P)H oxidase to produce O_2^- radicals, whereas NADH-stimulated O_2^- radical release predominates in endothelial cells. Our study confirms the existence of NADPH oxidase enzyme system in normal skeletal muscles. We also found that this enzyme system is located both in blood vessels as well as inside skeletal muscle fibers and that it prefers NADH as compared with NADPH as an electron donor.

Non-phagocyte NADPH oxidase differs in many respects from that of phagocytes. One of these major differences is related to orientation of the enzyme. In phagocytes, the assembled oxidase complex span the membrane and utilizes intracellular NADPH or NADH to transfer electrons to extracellular oxygen resulting in extracellular release of O_2^- radicals. Extracellular release of O_2^- radicals by NAD(P)H oxidase has

been documented in endothelial cells and fibroblasts (23) (43), whereas intracellular ROS radical production has been attributed to NAD(P)H oxidase activity of vascular smooth muscles (14) (42). However, the consistent observation in non-phagocytes including skeletal muscles in the current study that exogenous NADH or NADPH stimulate the formation of O2- radicals questions the classical inside-out proposal of NAD(P) oxidase orientation.

Another important difference between phagocyte and non-phagocyte NAD(P)H oxidases is the rate and time course of O_2^- radical production. Non-phagocyte enzymes produces lower rates and slower-release of O_2^- radicals than that of phagocytes. It has been estimated that vascular smooth muscle NAD(P)H oxidase has one-third the capacity of O_2^- radical production of that of phagocytes (16). In the current study, we found that skeletal muscle O_2^- radical production continues over several minutes, which contrast with almost instantaneous activation of phagocyte enzyme. This finding suggests that the kinetics of muscle-specific NAD(P)H oxidase is similar to that described in vascular smooth muscles, endothelial cells and fibroblasts.

Significant differences in the structure of phagocyte and non-phagocyte NAD(P) oxidases have also been described. In phagocytes, NADPH oxidase consists of five components, a plasma membrane spanning cytochrome b558 ($p22^{phox}$ and $gp91^{phox}$) and 3 cytosolic components ($p47^{phox}$, $p67^{phox}$ and p40phox) which associate with the cytochrome b558 upon activation of phagocytes. Other proteins including rac2 and rap1A may also be involved in the assembly of phagocyte NADPH oxidase. By comparison, mRNA of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and p67phox have been identified in endothelial and adventitial cells, whereas $p22^{phox}$ and $p47^{phox}$ but not $gp91^{phox}$ have been localized in

vascular smooth muscles (with the exception of pulmonary arterial smooth muscles) and mesangial cells (28) (19) (41). Jones et al. (19) described the presence of $p67^{phox}$ subunit as well in mesangial cells. We detected in the current study the mRNA and protein expression of four subunits (p22phox, gp91phox, p67phox and p47phox) in skeletal muscle fibers with an additional subunit (p40phox) being localized in the blood vessels (figure 9). Our study also indicate that, in like phagocyte NADPH oxidase, all of the four subunits of muscle-specific NAD(P)H oxidase constitutively associate with cell membranes. To our knowledge, our study is the first to document the presence of both mRNA and proteins of the four subunits of NADPH oxidase in non-phagocytes.

An important question which need to be answered is whether or not NAD(P) oxidase contributes to ROS production in skeletal muscle fibers. We attempted to answer this question be measuring both the rate of production of NAD(P)H oxidase product (O_2^- radicals) and the rate of NAD(P)H substrate utilization (NADH consumption). Both of these measurements were repeated in the presence of inhibitors of various biochemical sources of ROS inside muscle fibers. Our results indicate that both NADH utilization and O_2^- radical production were inhibited by DPI, thus confirming that heme-containing enzymes are involved. Interestingly, inhibition of NO production and prostaglandin metabolism by indomethacin had no effects on O_2^- production in normal muscle fibers, thereby excluding the contribution of NOS and cyclooxygenases. We also found that oxypurinol, an inhibitor of xanthine oxidase, reduced the rate of NADH utilization of normal muscle samples (figure 6). This result can be explained by the recent study of Sanders and Harrison (33) who demonstrated that the reductase domain of xanthine oxidase is capable of producing O_2^- radicals from NADH.

We should emphasize that endothelial NADPH oxidase may have contributed to total muscle O_2^- radical production in our experiments since this enzyme was also detected in blood vessels supplying and draining muscle fibers (figure 9). Interestingly, endothelial –specific NADPH oxidase differs from that of skeletal muscle fibers enzyme by the presence of $p40^{phox}$ subunit, which was not detected inside muscle fibers (figure 9). To our knowledge, our study is the first to confirm the presence of $p40^{phox}$ subunit in the vascular tissues.

Production of O_2^- radicals in sepsis: It is well established that sepsis causes oxidative stress in skeletal muscle fibers. Peralta *et al.* (29) reported a doubling of limb ROS levels in septic rats which coincided with a significant decline in antioxidant capacity. Indirect rise in ROS levels has also been found in limb muscles of septic rats (10). Lipid peroxidation has been reported to rise by 200% in the diaphragm of LPS-injected animals (35) (1). Many reports have confirmed that pre-treatment of septic animals with antioxidant enzymes and scavengers reduces lipid peroxidation in the ventilatory muscles and partially reverses poor contractility of these muscles (38) (35) (6). While these results confirm the involvement of ROS in sepsis-induced oxidative stress, the sources of ROS generation in septic muscles remain unclear.

Our results indicate that basal O_2^- radical production in the diaphragm of septic rats was significantly higher than that of control animals, confirming that ROS production rises in septic muscles. Interestingly, NADH-stimulated O_2^- production were similar among septic and control muscles. We attribute this finding to the possibility that NADH-stimulated O_2^- production represents the maximum rate of muscle-specific

NAD(P)H oxidase enzyme activity. The notion that we could be measuring the V_{max} of muscle NAD(P)H oxidase activity in our experiment is supported by the study of De Keulenaer *et al.* (9) which described that the Km for NADH by non-phagocyte NADPH oxidase is about 10 μM (about 1 order of magnitude lower than the level of NADH used in our study).

An interesting finding in our study is that inhibition of NOS activity while having no effects on normal muscles, it resulted in a significant elevation of NADH-stimulated O_2^- radical production in septic muscles. This finding suggests that NOS activity exerts an inhibitory influence on muscle O_2^-radical production in septic animals. In normal muscle fibers, NO is synthesized mainly by the neuronal (nNOS) and the endothelial (ecNOS) isoforms (21) (22). However, in septic animals, larger levels of NO is synthesized inside skeletal muscle fibers by the inducible (iNOS) isoform of NOS (18) (5). Our observation that L-NAME increased NADH-stimulated O_2^- production in septic animals suggests that iNOS is primarily responsible for the inhibitory effects of NO on NADPH oxidase activity. The mechanisms through which NOS activity influences muscle O_2^- radicals remain under investigation. We speculate, however, that NO modulates O_2^- radical levels through two different pathways. 1) Recent studies have shown that NO prevents the assembly of neutrophil NADPH oxidase subunits (12). 2) NO is capable of inducing heme oxygenase-1 expression, an enzyme responsible for the conversion of heme into bilirubin and carbon monoxide. Induction of heme oxygenase-1 is likely to reduce ROS levels in the muscle primarily because of the anti-oxidant properties of its products (bilirubin and carbon monoxide). However, we believe that induction of heme oxygenase 1 does not explain our finding because our muscle strips

(both control and septic) were exposed to L-NAME for about 30 minutes, an exposure period which is not likely to affect muscle heme oxygenase 1 expression.

Table 1

Table 1: **Molecular sequence of RT-PCR oligonucleotide primers used to amplify mRNA of various NADPH oxidase subunits in rat skeletal muscles.**

	Forward	*Reverse*
$p22^{phox}$	*5'-GTTTGTGTGCCTGCTGGAGT-3'*	*5'-TGGGCGGCTGCTTGATGGT-3'*
$gp91^{phox}$	*5'-GCTGTTCAATGCTTGTGGCT-3'*	*5'-TCTCCTCATCATGGTGCACA-3'*
$p47^{phox}$	*5'-ACCCAGCCAGCACTATGTGT-3'*	*5'-GCCGGTGATATCCCCTTTCT-3'*
$p67^{phox}$	*5'-CGAGGGAACCAGCTGATAGA-3'*	*5'-CATAGGCACGCTGAGCTTCA-3'*
GAPDH	*5'-CCCTTCATTGACCTCAACTACATGGT-3'*	*5'-GAGGGGCCATCCACAGTCTTCTG-3*

Appendix1

Deduced amino acid sequence of rat P47^{phox} partial cDNA. Amino acid sequence is aligned with that of human and mouse P47^{phox} amino acid sequences.

```
Human p47phox     MGDTFIRHIALLGFEKRFVPSQHYVYMFLVKWQDLSEKVV     40
mouse p47phox     ------------------i--------------------     40
rat   p47phox     ......................--------------     14

Human p47phox     YRRFTEIYEFHKTLKEMFPIEAGAINPENRIIPHLPAPKW     80
mouse p47phox     --k---------m----------e-ht---v-------r-     80
rat   p47phox     --k---------m----------e-ht---v-------r-     54

Human p47phox     FDGQRAAENRQGTLTEYCSTLMSLPTKISRCPHLLDFFKV    120
mouse p47phox     --------s--------fng--g--v--------------    120
rat   p47phox     y-------s--er----fns--g--v---------n----     94

Human p47phox     RPDDLKLPTDNQTKKPETYLMPKDGKSTATDITGPIILQT    160
mouse p47phox     ----------s-a-------v-----nnva----------    160
rat   p47phox     --------n-s-v-------ta----nnva----------    134

Human p47phox     YRAIADYEKTSGSEMALSTGDVVEVVEKSESGWWFCQMKA    200
mouse p47phox     ---------s--t--tva-----d---------------t    200
rat   p47phox     ---------g-kt--tva-----d---------------t    174

Human p47phox     KRGWIPASFLEPLDSPDETEDPEPNYAGEPYVAIKAYTAV    240
mouse p47phox     ----v---y---------a---d---------t----a--    240
rat   p47phox     ----v---y---------a---d---------t----a--    214

Human p47phox     EGDEVSLLEGEAVEVIHKLLDGWWVIRKDDVTGYFPSMYL    280
mouse p47phox     -e--m--s----i------------v--g-i---------    280
rat   p47phox     -e-.....................................    217

Human p47phox     QKSGQDVSQAQRQIK.RGAPPRRSSIRNAHSIHQRSRKRL    319
mouse p47phox     --a-eeit------rg--------t----q----------    320

Human p47phox     SQDAYRRNSVRFLQQRRRQARPGPQSPGSPLEEERQTQRS    359
mouse p47phox     ---t--------------pg---ql-tdgtkdnps.-p-v    359

Human p47phox     KPQPAVPPRPSADLILNRCSESTKRKLASAV             390
mouse p47phox     -----------s----h--t-------t---             390
```

Appendix II

Deduced amino acid sequence of rat P67phox partial cDNA. Amino acid sequence is aligned with that of human and mouse P67phox . Dashes indicate sequence homology between the three sequences.

```
human p67phox     MSLVEAISLWNEGVLAADKKDWKGALDAFSAVQDPHSRIC     40
mouse P67phox     ---a---r-----------------e---e---------     40

human p67phox     FNIGCMYTILKNMTEAEKAFTRSINRDKHLAVAYFQRGML     80
mouse p67phox     -----vn---e-lqa--q---k-------s----------     80

human p67phox     YYQTEKYDLAIKDLKEALIQLRGNQLIDYKILGLQFKLFA    120
mouse p67phox     --rm--------------t--------------------    120
rat   p67phox     ........................-----------      12

human p67phox     CEVLYNIAFMYAKKEEWKKAEEQLALATSMKSEPRHSKID    160
mouse p67phox     --------l-h-----------------n-----------    160
rat   p67phox     --------l-h-----------------n-----------     52

human p67phox     KAMECVWKQKLYEPVVIPVGKLFRPNERQVAQLAKKDYLG    200
mouse p67phox     ----si-----f--------r------------------    200
rat   p67phox     ----si-----f--------r------------------     92

human p67phox     KATVVASVVDQDSFSGFAPLQPQAAEPPPRPKTPEIFRAL    240
mouse p67phox     ---------h--n----------s----------------    240
rat   p67phox     ---------h--n----------s----------------    132

human p67phox     EGEAHRVLFGFVPETKEELQVMPGNIVFVLKKGNDNWATV    280
mouse p67phox     ---------------p-----------------s------    280
rat   p67phox     ---------------p-----------------s------    172

human 67phox      MFNGQKGLVPCNYLEPVELRIHPQQQPQEESSPQSDIPAP    320
mouse p67phox     ------------------------s----dt--e----p-    320
rat   p67phox     ---------l--------------s----dt-le----p.    211

human p67phox     PSSKAPGKPQLSPGQKQKEEPKEVKLSVPMPYTLKVHYKY    360
mouse p67phox     -n-sp--rl-----h----.---l--------m-------    359

human p67phox     TVVMKTQPGLPYSQVRDMVSKKLELRLEHTKLSYRPRDSN    400
mouse p67phox     ----e-rl------l-n------a-sp--------r---h    399

human p67phox     ELVPLSEDSMKDAWGQVKNYCLTLWCENTVGDQGFPDEPK    440
mouse p67phox     --ll---e-------------------h------li---i    439

human p67phox     ESEKADANNQTTEPQLKKGSQVEALFSYEATQPEDLEFQE    480
mouse p67phox     qr-ns--sk------p-e-t--v-i-----a-------v-    479

human p67phox     GDIILVLSKVNEEWLEGECKGKVGIFPKVFVEDCATTDLE    520
mouse p67phox     --v-----h-------------------a---g--akn--    519

human p67phox     STRREV                                      526
mouse p67phox     gip---                                      525
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REFRENCES:

1. Arias-Diaz, J., E. Vara, J. Torres-Melero, C. Garcia, J. Hernandez, and J.L. Balibrea. Local production of oxygen free radicals and nitric oxide in rat diaphragm during sepsis: effects of pentoxifylline and somatostatin. Eur.J.Surg. 163: 619-625, 1997.

2. Babior, M.B. NADPH Oxidase: An Update. Blood 93 (5): 1464-1476, 1999.

3. Bayraktutan, U., N. Draper, D. Lang, and A.M. Shah. Expression of a functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. Cardiovasc.Res. 38: 256-262, 1998.

4. Bejma, J. and L.L. Ji. Aging and acute exercise enhances free radical generation in rat skeletal muscle. J.Appl.Physiol. 87: 465-470, 1999.

5. Boczkowski, J., S. Lanone, D. Ungureanu-Longrois, G. Danialou, T. Fournier, and M. Aubier. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats: role on diaphragmatic contractile dysfunction. J.Clin.Invest. 98: 1550-1559, 1996.

6. Boczkowski, J., C. Pasquier, Y. Du, E. Frannzini, and M. Aubier. Effects of N-acetylcysteine on diaphragmatic function and malondialdehyde content in Escherichia coli endotoxemic rats. Am.Rev.Respir.Dis. 146: 730-734, 1998.

7. Borzone, G., B. Zhao, A.J. Merola, L. Berliner, and T.L. Clanton. Detection of free radicals by electron spin resonance in rat diaphragm after resistive loading. J.Appl.Physiol. 77: 812-818, 1994.

8. Chomczynski, P. and N. Sacchi. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal.Biochem. 162: 156-159, 1987.

9. DeKeulenaer, G.W., R.W. Alexandre, M. Ushio-Fukai, N. Ishizaka, and K.K. Griendling. Tumour necrosis factor □ activates $p22^{phox}$-based NADH oxidase in vascular smooth muscle. Biochem.J. 329: 653-657, 1998.

10. Fagan, J.M., M. Ganguly, G. Tiao, J.E. Fischer, and P.O. Hasselgren. Sepsis increases oxidatively damaged proteins in skeletal muscle. Arch.Surg. 131: 1326-1332, 1996.

11. Favero, T.G., A.C. Zable, and J.J. Abramson. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. J.Biol.Chem. 263: 18750-18758, 1995.

12. Fujii, H., K. Ichimori, K. Hoshiai, and H. Nakazawa. Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process. J.Biol.Chem. 272: 32773-32778, 1997.

13. Fukui, T., B. Lassegue, H. Kai, W. Alexander, and K.K. Griendling. Cytochrome b-558 □-subunit cloning and expression in rat aortic smooth muscle cells. Biochim.Biophys.Acta 1231: 215-219, 1995.

14. Griendling, K.K., C.A. Minieri, J.D. Ollerenshaw, and R.W. Alexander. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ.Res. 1141-1148, 1994.

15. Griendling, K.K., D. Sorescu, and M. Ushio-Fukai. NAD(P)H oxidase. Role in cardiovascular biology and disease. Circ.Res. 86: 494-501, 2000.

16. Griendling, K.K. and M. Ushio-Fukai. Redox control of vascular smooth muscle proliferation. J.Lab.Clin.Med. 132: 9-15, 1998.

17. Hellsten, Y., U. Frandsen, N. Orthenblad, B. Sjodin, and E.A. Richter. Xanthine oxidase in human skeletal muscle following eccentric exercise: a role in inflammation. J.Physiol. 498: 239-248, 1997.

18. Hussain, S.N., Q. el-Dwairi, D. Sakkal, R. Hattori, and Y. Guo. Expression of nitric oxide synthases and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. Am.J.Respir.Cell Mol.Biol. 17: 173-180, 1997.

19. Jones, S.A., J.T. Hancock, O.T.G. Jones, A. Neubauer, and N. Topley. The expression of NADPH oxidase components in human glomerular mesangial

cells: Detection of protein and mRNA for P47phox, P67phox and P2phox. J.Am.Soc.Nephrol. 5: 1483-1491, 1995.

20. Klebl, B.M., A.T. Ayoub, and D. Pette. Protein oxidation, tyrosine nitration and inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase in low-frequency stimulated rabbit muscle. FEBS Lett 422: 381-384, 1998.

21. Kobzik, L., M.B. Reid, D.S. Bredt, and J.S. Stamler. Nitric oxide in skeletal muscle. Nature 372: 546-548, 1994.

22. Kobzik, L., B. Stringer, M.B. Reid, and J.S. Stamler. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem.Biophys.Res.Commun. 211: 375-381, 1995.

23. Meier, B., A.R. Cross, J.T. Hancock, F.J. Kaup, and O.T.G. Jones. Identification of a superoxide-generating NADPH oxidase system in human fibroblas. Biochem.J. 275: 241-245, 1991.

24. Mohazzab-H, K.M., P.M. Kaminski, and M.S. Wolin. Lactate and PO_2 modulate superoxide anion production in bovine cardiac myocytes.. Potential role of NADH oxidase. Circulation 96: 614-620, 1997.

25. O'hara, Y., T.E. Peterson, and D.G. Harrison. Hypercholestrolaemia increases endothelial superoxide anion production. J.Clin.Invest. 91: 2546-2551, 1993.

26. Pagano, P.J., J.K. Clark, M.F. Cifuentes-Pagano, S.M. Clark, G.M. Callis, and M.T. Quinn. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc.Natl.Acad.Sci.USA 94: 14438-14488, 1997.

27. Pagano, P.J., Y. Ito, K. Tornheim, P.M. Gallop, A.I. Tauber, and R.A. Cohen. An NADPH oxidase superoxide-generating system in the rabbit aorta. Am.J.Physiol. 268: H2274-H22801995.

28. Patterson, C., J. Ruef, N.R. Madamanchi, P. Barry-Lane, Z. Hu, C. Horaist, C.A. Ballinger, A.R. Brasier, C. Bode, and M.S. Runge. Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin: evidence that P47 (phox) may participate in forming this oxidase in vitro and in vivo. J.Biol.Chem. 274: 19814-19822, 1999.

29. Peralta, J.G., S. Liesuy, P. Evelson, M.C. Carreras, B.G. Flecha, and J.J. Poderoso. Oxidative stress in skeletal muscle during sepsis in rats. Circ.Shock 39: 153-159, 1993.

30. Reid, M.B., K.E. Haack, K.M. Francik, P.A. Volberg, L. Kobzik, and M.S. Wes. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro . J.Appl.Physiol. 73: 1797-1804, 1992.

31. Reid, M.B., F.a. Khawli, and M.R. Moody. Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. J.Appl.Physiol. 75: 1081-1087, 1993.

32. Reid, M.B., T. Shoji, M.R. Moody, and M.L. Entman. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. J.Appl.Physiol. 73: 1805-1809, 1992.

33. Sanders, S.A., R. Eisenthal, and R. Harrison. NADH oxidase activity of human xanthine oxidoreductase. generation of superoxide anion. Eur.J.Biochem. 245: 541-548, 1997.

34. Sen, C.K., I. Kolosova, O. Hanninen, and S.N. Orlov. Inward potassium transport systems in skeletal muscle derived cells are highly sensitive to oxidant exposure. Free Radic.Biol.Med. 18: 795-800, 1995.

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

36. Sjodin, B., Y. Hellsten-Westing, and F.S. Apple. Biochemical mechanisms for oxygen free radical formation during exercise. Sport 10: 236-254, 1990.

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

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

39. Surell, C.V., J. Boczkowski, C. Pasquier, Y. Du, E. Franzini, and M. Aubier. Effects of N-Acetylcysteine on diaphragmatic function and malondialdehyde content in Escherichia coli endotoxemia in rats. Am.Rev.Respir.Dis. 146: 730-734, 1998.

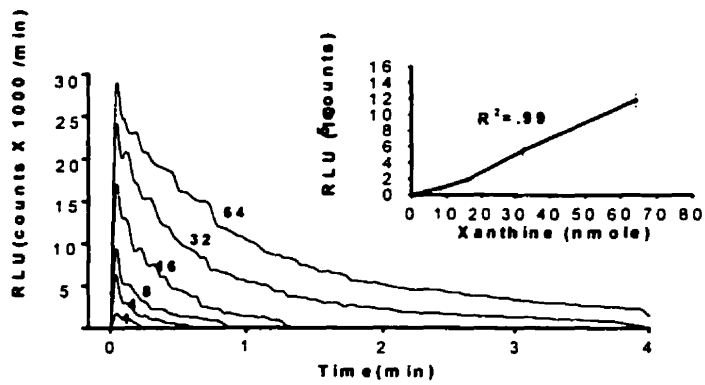
40. Thannickal, V.J. and B.L. Fanburg. Activation of an H_2O_2-generating NADH oxidase in human lung fibroblasts by transforming growth factor □1. J.Biol.Chem. 270(51): 30334-30338, 1995.

41. Ushio-Fukai, M., A.M. Zafari, T. Fukui, N. Ishizaka, and K.K. Griendling. P22 is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. J.Biol.Chem. 271: 23317-23321, 1996.

42. Zafari, A.M., M. Ushio-Fukai, M. Akers, Q. Yin, A. Shah, D.G. Harrison, W.R. Taylor, and K.K. Griendling. Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. Hypertension 32: 488-495, 1998.

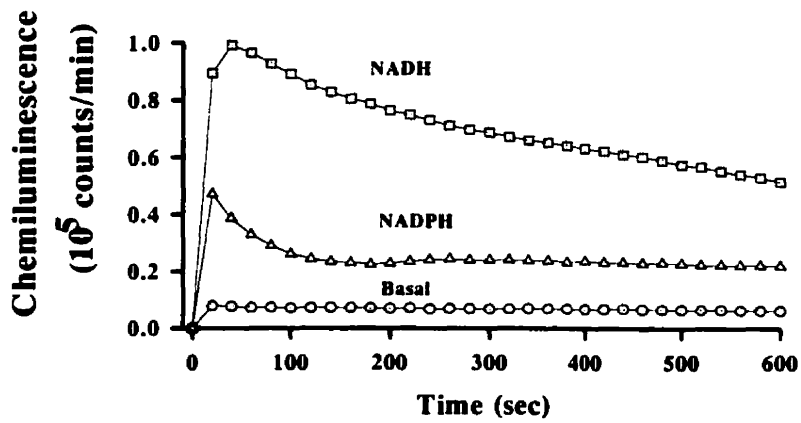
43. Zulueta, J.J., F.S. Yu, I.A. Hertig, V.J. Thannickal, and P.M. Hassoun. Release of hydrogen peroxide in response to hypoxia-reoxygenation: role of an NAD(P)H oxidase-like enzyme in endothelial cell plasma membrane. Am.J.Respir.Cell Mol.Biol. 12: 41-49, 1995.

Figure 1



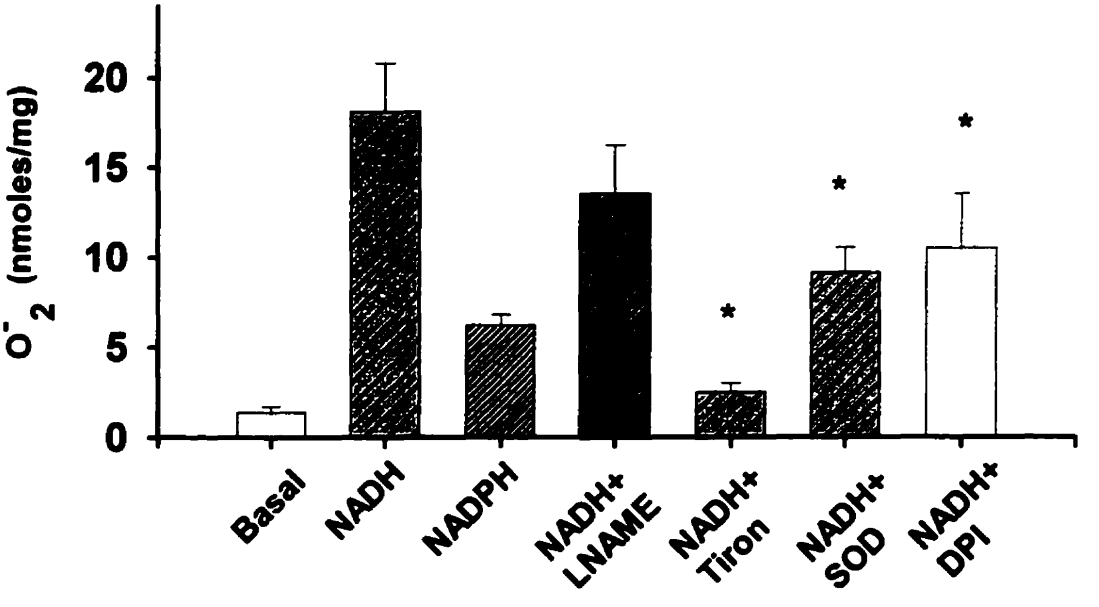
Lucigenin-enhanced chemiluminescence of O_2^- produced from Xanthin/xanthin oxidase (Standard curve)

Figure 2



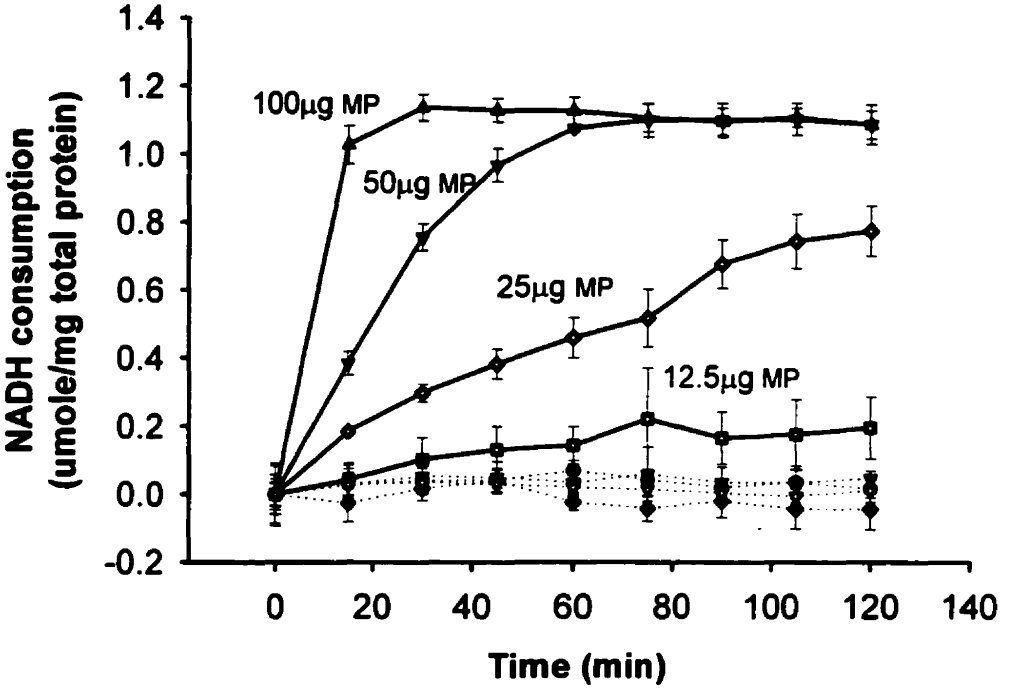
Pattern of lucigenin-enhanced chemiluminescence in control diaphragm strips

Figure 3



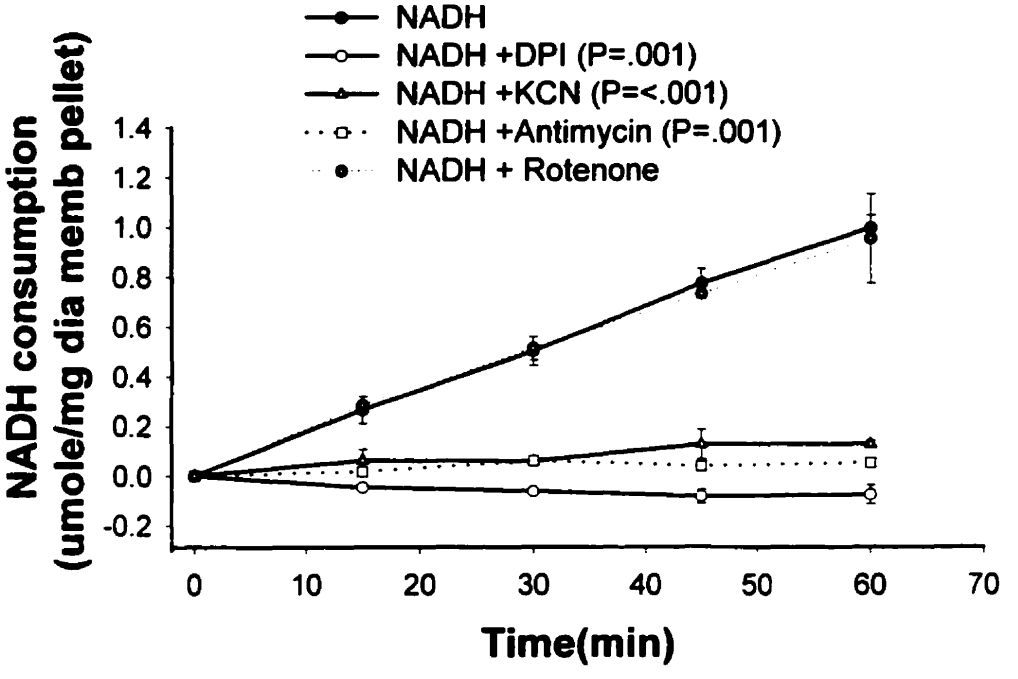
Behavior of NAD(P)H oxidase in response to stimulation with NADH, NADPH, inhibitor of NOS (LNAME), flavin-containing enzyme inhibitor (DPI) and O_2^- scavenger (Tiron and SOD) in control rat diaphragm strips.

Figure4



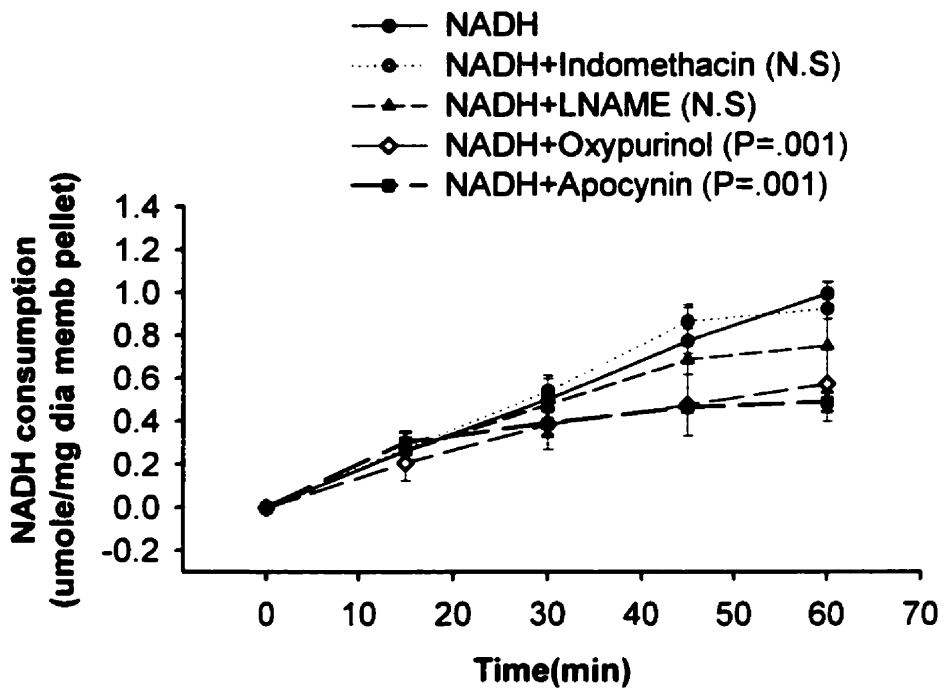
NADH consumption was increased by increasing amounts (12.5, 25, 75, 100 µg) of membrane pellet (solid lines), but not by same amounts (12.5, 25, 75, 100 µg) of cytosolic fractions (dashed lines)

Figure 5



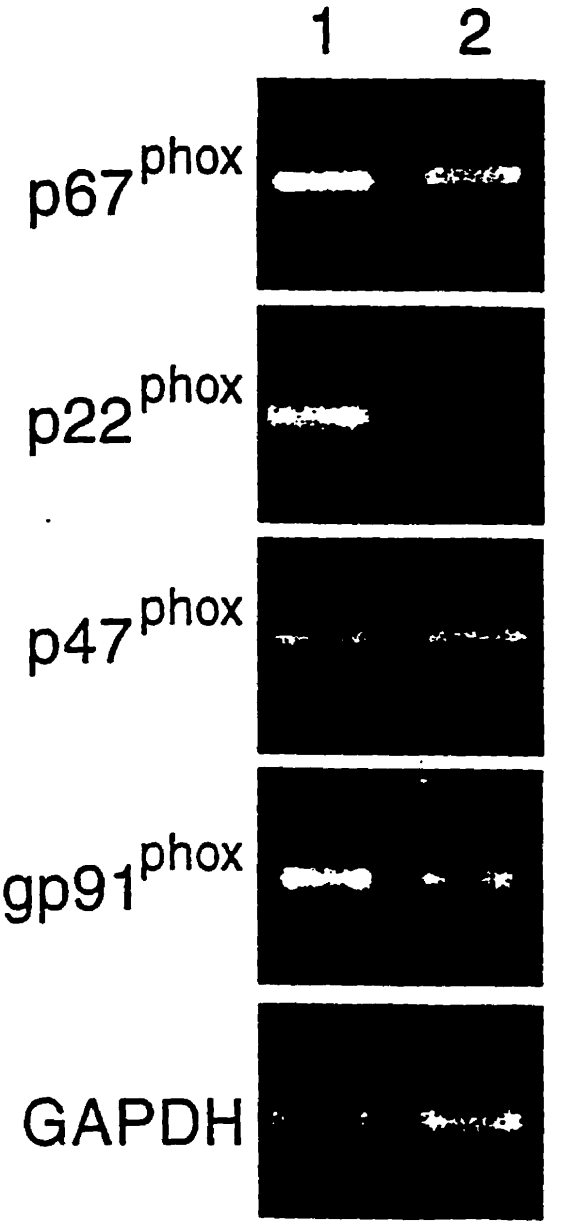
Utilization of NADH in diaphragm membrane pellet was decreased by inhibitors of cytochrome b (KCN & Antimycin), inhibitor of flavin-containg enzymes (DPI), but not by mitochondrial inhibitor.

Figure 6



Utilization of NADH in diaphragm membrane pellet was decreased by NAD(P)H oxidase inhibitor (Apocynin) and Xanthin inhibitor (Oxypurinol), but not by inhibitor of NOS or inhibitor of cyclooxygenase.

Figure 7



Rt-PCR results of NAD(P)H oxidase subunits in rat diaphragm (lane 1) and gastrocnemius muscles (lane 2)

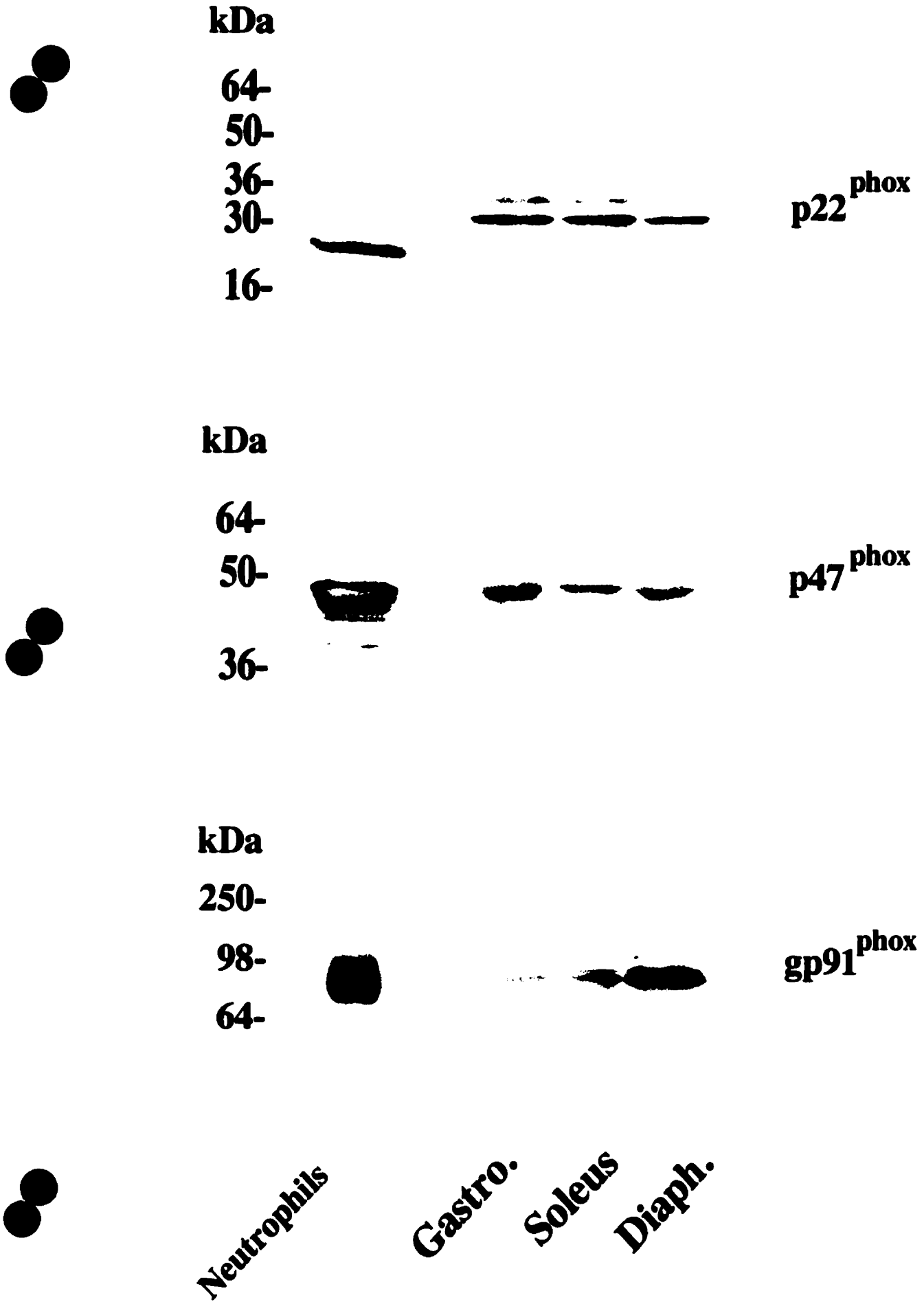

kDa
64-
50-
36-
30-
16-
p22phox
kDa
64-
50-
36-
p47phox
kDa
250-
98-
64-
gp91phox
Neutrophils
Gastro.
Soleus
Diaph.

Figure 8 (previous page)

Immunoblotting of $p22^{phox}$, $p47^{phox}$, and $gp91^{phox}$ in homogenates of Gastrocnemius (Gastro), Soleus, and Diaphragm (Diaph) muscles of rat.

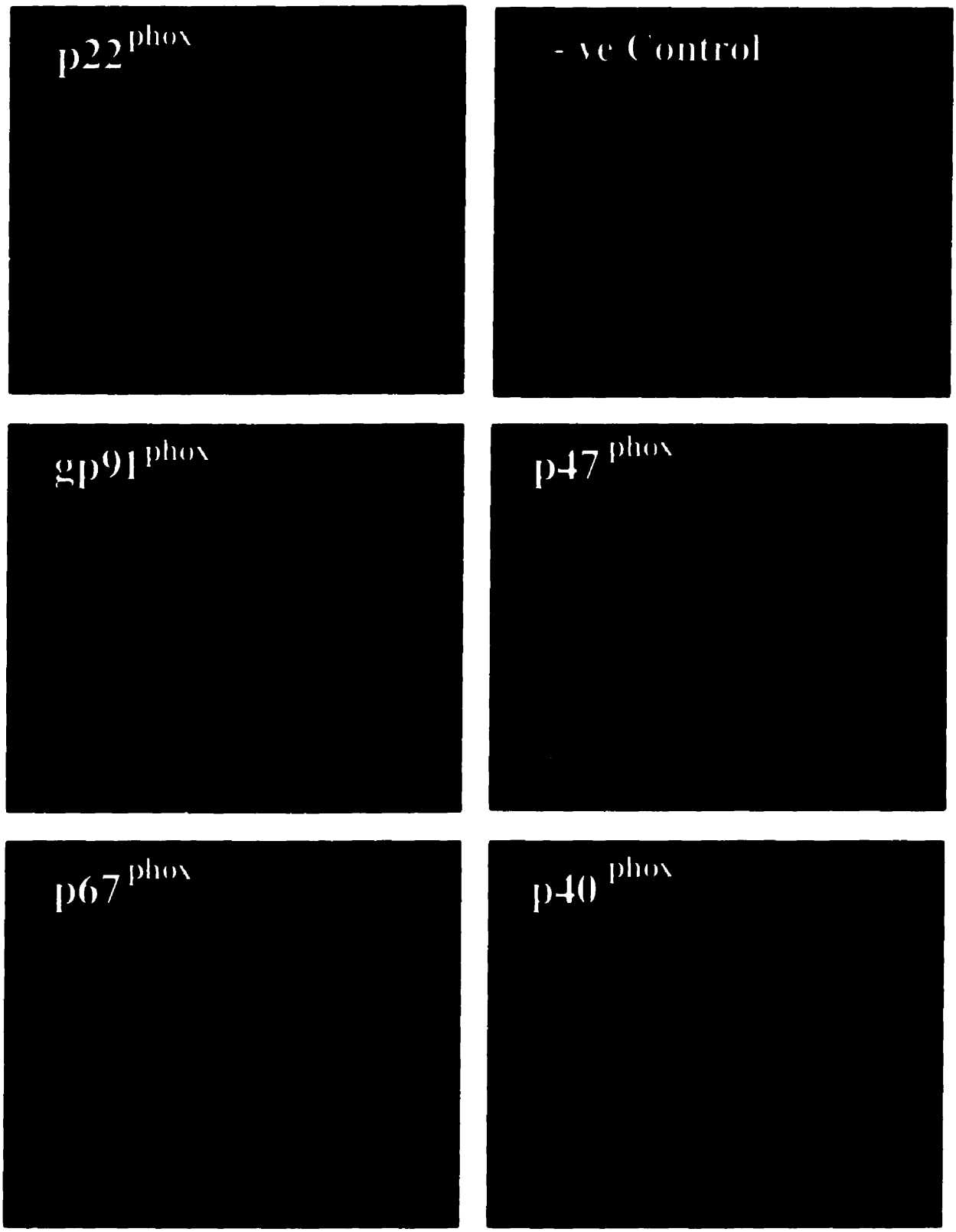
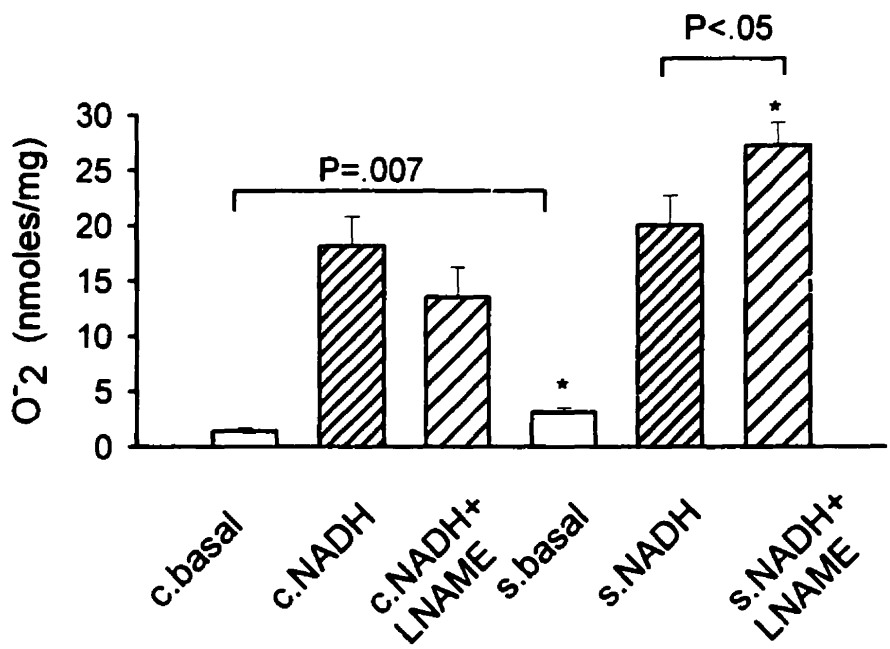

p22 phox
- ve Control
gp91 phox
p47 phox
p67 phox
p40 phox

Figure 9 (previous page)

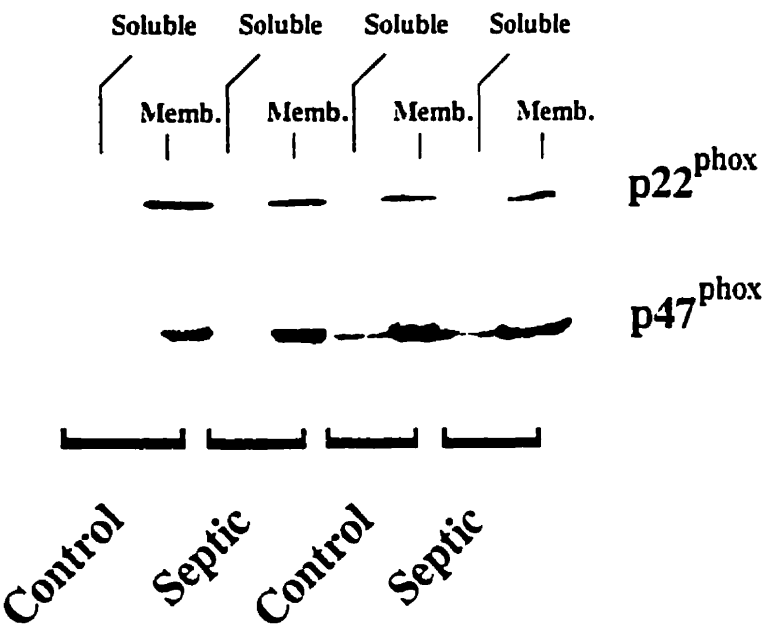
Localization of NAD(P)H oxidase proteins in sections from normal rat skeletal muscle. Magnification: top and middle rows x100, bottom left x200, bottom right x400.

Figure 10



O_2^- production in diaphragm strips from control and endotoxic rats in the basal, or incubation with NADH, or NADH with LNAME

Figure 11



Immunoblotting of p22phox, p47phox in membrane (Memb), and soluble fractions of nonendotoxic (control) and endotoxic (septic) rats

CHAPTER SIX

GENERAL CONCLUSIONS

General conclusions:

Increased production of NO from NOSII has been proposed to be a major factor for hypotension and loss of vascular tone. However, this is based on the observations obtained from rodents. Inducible nitric oxide is not easily expressed in higher species. Therefore the role of NOSII is not well understood in higher species.

In chapter 2, I investigated the induction of NOSII in endotoxemic pigs. Plasma nitrate/nitrite was not increased significantly, and expired NO at 4 hour was not different from time zero in endotoxemic pigs. NOS activity was not changed and NOSII protein was not detected by western blotting in multiple tissues. Northern blot analysis revealed weak induction of NOSII mRNA in lung and liver and there was minimal NOSII protein in small vessels and inflammatory cells. Despite the minimal increase in NOSII we found increased nitrotyrosine formation by slot blot, western blot, and immunohistochemistry. Therefore in contrast to rats and mice, NOSII is only minimally expressed in endotoxemic pigs with low systemic vascular resistance, but there is still production of nitrotyrosine, which indicates the formation of peroxynitrite. This suggests a role for constitutive NOS in the vascular abnormalities of sepsis in pigs and higher species.

In chapter 3, I investigated the role of constitutive NOS in sepsis. NOSIII protein and NOS activity were increased after 2 hour of endotoxemia when a low systemic vascular resistance and decreased vascular hyporesponsiveness to cathecolamines were fully established. This is in contrast to the downregulation of NOSIII that others have observed in bovine endothelial cells in culture. Analysis of vena caval homogenates showed increases in NOSIII and NOSI protein. However NOSIII protein and activity were decreased in pulmonary arteries. The dose of the NOS inhibitor, L-NAME which

restored the pressor response to norepinephrine caused a large fall in cardiac output of endotoxemic pigs. Thus in contrast to rats, constitutive NOS could still have important role in the vascular dysfunction of endotoxemic pigs and possibly humans.

In chapter 4, I investigated the interaction of nitric oxide with superoxide in septic rats. I also measured superoxide and peroxynitrite in endotoxemic pigs. I reproduced the observation that O_2^- formation is increased in septic rats. In addition I found an important interaction between O_2^- and NO in septic rats. The O_2^- concentration in carotid arteries was increased after 4-hours of endotoxemia in pigs. Moreover luminol-enhanced chemiluminesence was increased in these pigs which is consistent with the formation of nitrotyrosine. The addition of L-NAME to NADH-treated vascular rings of septic rats increased O_2^- formation, but it decreased it in the control rings from endotoxemic pigs. Thus O_2^- formation is increased in septic rats, but it is decreased by the increase in NO from NOSII. O_2^- formation is also increased in endotoxemic pigs, but it is NOS-dependent.

In chapter 5, I investigated the existence of NAD(P)H oxidase, as a source of O_2^- formation in normal skeletal muscles of rats. I was able to detect the components gp91 ^{phox}, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ of phagocytic NAD(P)H oxidase in diaphragm muscle. The pattern of activity of this oxidase complex is different from phagocytes and in contrast to phagocytes, NADH is the preferred substrate for this oxidase complex. I also detected increased basal O_2^- formation and increased NAD(PH) oxidase activity in muscle strips from endotoxic rats. Thus increased activity of NAD(P)H oxidase can be important as a source of O_2^- in pathophysiological condition such as sepsis.

Claims to originality

I have made the following original contributions towards understanding the role of nitric oxide synthase in vascular function.

In chapter 2, the new knowledge added to the literature by the work comprising this chapter includes the following

1- That in contrast to rodents the degree of NOSII induction in a low systemic vascular resistance porcine model of sepsis is very low.

2- Hypotension in a porcine model of sepsis is associated with

   a- a minimal increase in NOSII mRNA

   b- no obvious NOSII protein by western except in neutrophils

   c- a small increase by immunohistochemistry

3- With endotoxemia, pigs, have a similar increase in nitrotyrosine formation and nitrated proteins as seen in endotoxic rats.

In chapter 3, the new knowledge added to the literature by the work comprising this chapter includes the following points:

1- The NOSIII mRNA, its protein, and its activity is not downregulated in the aorta of endotoxemic pigs.

2- NOSI protein is upregulated in the vena cava of endotoxemic pigs.

3- Changes in RVR and SVR with L-NAME are greater in entoxemic pigs.

In chapter 4, the work included in this chapter contributes the following knowledge to the literature:

1- In septic rats O_2^- production by NAD(P)H oxidase is increased and counterbalanced by increased NO production.

2- In endotoxemic pigs, O_2^- production also is increased.

3- In endotoxemic pigs, luminol-enhanced chemiluminesence is increased indicating that peroxynitrite formation is likely increased.

4- Activity of vascular NAD(P)H oxidase is in endotoxemic pigs.

5- This increased activity appears to be NO-dependent.

6- Detection of $p22^{phox}$ in endothelial cells of carotids of pig by immunohistochemistry

In chapter 5, the work included in this chapter contributes the following knowledge to the literature:

1- The NAD(P)H oxidase system exists in rat skeletal muscle.

2- The $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ $p67^{phox}$ components of phagocytic NAD(P) H oxidase are found in rat skeletal muscle.

3- Detection of p40phox in endothelial cells of vessels supplying rat skeletal muscle

4- NADH is the preferred substrate for NAD(P)H oxidase in rat skeletal muscle.

5- Skeletal muscle NAD(P)H oxidase is constitutively active.

6- O_2^- production is increased in endotoxic diaphragm

7- Increased O_2^- production in skeletal muscle in sepsis is counterbalanced by increasaed NO