THE NITRIC OXIDE METABOLITE NITRATE ANION CONTRIBUTES TO SEPTIC SHOCK AND MULTIPLE ORGAN DYSFUNCTION

par

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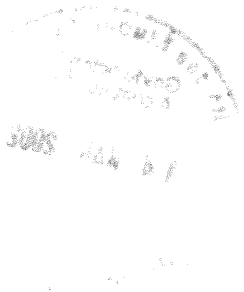

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To my wife, **Pu**;

and my son, **Chao**;

my little daughter, **Annie**;

For their unconditional support, understood and love, c'est la vie!

SUMMARY

Septic shock and the systemic inflammatory response syndrome (SIRS) are very complex clinical syndromes which represent major causes of mortality in intensive care units. Nitric oxide (NO) plays many essential roles in the physiological and pathophysiological processes of sepsis/SIRS and multiple organ dysfunction syndrome (MODS). NO either acts as a direct signaling messenger and cytoprotective molecule, or has an indirect cytotoxic effect, via the formation of various nitrogen species, e.g. peroxynitrite, and ultimately nitrate. It is well documented that NO plays a significant beneficial as well as detrimental roles during sepsis/SIRS/MODS processes. However, nitrate is decidedly the major, most important, and longest lasting end-product of NO *in vivo*. Nitrate itself is a permeant anion that can cross the plasma membrane influencing cell electrophysiological properties, membrane enzyme activity *in vitro*, muscle contractility, and blood pressure *in vivo*. Therefore, we hypothesized that over-accumulation of nitrate may contribute to septic shock/SIRS/MODS during sepsis.

To assess the role of nitrate during sepsis, Sprague-Dawley rats were treated intraperitoneally (I.P.) with 5, 10 and 20 mg/kg lipopolysaccharide (LPS) for 0 (control), 6, 12, or 24 h and then the spatial (organs) distribution and temporal production of LPS-induced nitrate concentration were measured. Following LPS treatment, we found significantly different nitrate concentrations in the heart, lung, kidney, liver, brain, aorta, diaphragm, spleen, thymus, testis or ovary, hind limb muscle, small intestine, adipose tissue (omentum), bone, bladder, plasma and urine. A significant nitrate gradient was evident between organ and plasma, which indicates a nitrate gradient between

intracellular and extracellular compartments. LPS induced a significant increase in nitrate concentration at 12 h in most organs tested, except in brain, adipose and muscle tissue. LPS-dose dependent nitrate concentration response ($P< 0.05$) was only seen in the aorta and lungs at 12 h after treating the rats with LPS. In pigs, exogenous NO, such as inhaled (40 ppm) NO gas or NO donors (nitroglycerin at 90 $\mu g\ kg^{-1}\ min^{-1}$ for 20 min), significantly and transiently increased plasmatic and urinary nitrate concentration. iNOS protein concentration was higher in blood than in the diaphragm and lungs 12 h after LPS administration. Blood iNOS content correlated significantly with nitrate level in plasma (r^2=0.992, P<0.008). Nitrate concentration change in the tissues or organs occurred in parallel with inflammatory responses as was indicated by alterations in myeloperoxidase (MPO) activity (measured in heart, lung, liver, kidney, spleen and plasma), histological modifications (hematoxylin and eosin staining heart, kidney, liver, lung, brain, spleen, intestine, diaphragm, testis, skeletal muscle, bone and adipose) and cell sarcolemmal integrity alterations (Procion Orange 14 fluorescent tracer dye) in the heart, kidney, liver, lung, intestine, spleen, diaphragm, and to a lesser degree in the brain. Diaphragm contractility was reduced significantly by LPS *in vivo* and by nitrate *in vitro*. Respiratory rate was increased significantly in rats 6 and 12 h after 10 mg/kg LPS administration and there was a significant correlation between respiratory rate and nitrate level in the lung (r^2=0.996, P<0.01). LPS induced a significant decrease in the mean arterial blood pressure particularly at 12 h. This was correlated negatively with the nitrate concentration in the aorta (r^2 = -0.96, $P< 0.05$) as well as positively with the aorta/plasma nitrate concentration ratio (r^2 = 0.95, $P< 0.05$). Collectively, we concluded that the nitric oxide metabolite nitrate anion contributes to septic shock/SIRS/MODS during sepsis.

RÉSUMÉ

La présente étude constitue une suite des quatre manuscrits déjà publiés et/ou soumis à un processus de révision. Les résultats trouvés antérieurement démontrent pour la première fois que l'anion nitrate, un métabolite du monoxyde d'azote (NO), contribue de façon significatif au processus de survenue du choc septique et d'atteinte du syndrome du dysfonctionnement d'organes multiples (MODS) observés durant le sepsis.

Le choc septique ainsi que le syndrome de la réponse inflammatoire systémique (SIRS) sont des syndromes complexes qui constituent la majeure cause de mortalité en soins intensifs. Le NO joue un rôle physiopathologique important dans la survenue du sepsis, du MODS et du SIRS. Les résultats expérimentaux et cliniques démontrent que le monoxyde d'azote et/ou le nitrate est (sont) élevé(s) ou produit(s) en grande quantité durant le SIRS et plus particulièrement pendant le sepsis. La sévérité du MODS est aussi associé à la quantité de nitrate présente dans le plasma. Il a été démontré que le NO produit autant d'effets bénéfiques que délétères dans la survenue du sepsis, du SIRS et du MODS. Par contre, le rôle du nitrate dans le processus physiopathologique du NO durant le sepsis n'est pas connu. La production de différentes quantités d'anion nitrate dans plusieurs organes, induite par différentes doses de lipopolysaccharide (LPS) administrées, ainsi que la contribution de l'anion nitrate dans la survenue du choc septique et MODS nous sont aussi inconnus.

Le monoxyde d'azote agit comme messager et comme molécule cytoprotectrice. Il a aussi un effet cytotoxique indirect via la formation de différents oxydes d'azote incluant le péroxynitrite et l'anion nitrate. Bien que l'anion nitrate soit le plus important des dérivés métaboliques du NO in vivo, le rôle des nitrates eux-mêmes dans le sepsis

n'est pas bien etabli. L'anion nitrate traverse aisément la membrane plasmique et influence les propriétés éléctrophysiologiques de la cellule, l'activité enzymatique (ATPase) de la membrane in vitro, la contractilité musculaire ainsi que la tension artérielle in vivo. De ce fait nous proposons comme hypothèse dans cette étude que l'accumulation excessive de l'anion nitrate, induite par l'administration de LPS, pourrait contribuer à la survenue du choc septique et du dysfonctionnement de d'organes multiples pendant le sepsis, le SIRS et le MODS.

Pour évaluer le rôle de l'anion nitrate, nous avons mesuré par chemiluminescence les changements de concentration de nitrates plasmatique et organique suite à un traitement au LPS. Des rats Sprague-Dawley ont été traités par injection intra-péritonéale de 5, 10 et 20 mg/kg de LPS et sacrifiés 0, 6, 12 ou 24 heures suite au traitement. Nous avons trouvé une différence dans les concentrations de nitrate présentes dans les 18 tissus et organes testés (cœur, poumon, reins, foie, cerveau, aorte, diaphragme, rate, thymus, testicules, ovaires, muscle de la cuisse, intestin grêle, tissu adipeux (omentum), os, vessie, plasma et urine). Il est important de noter que cette différence existe aussi dans les tissus et organes des animaux non-traités bien que les concentrations de nitrate présentes soient plus faibles. Le LPS induit donc une augmentation significative de la concentration de nitrate 12 heures post-administration dans la plupart des tissus et organes, excepté le cerveau, le tissu adipeux et le muscle de la cuisse. Cette augmentation est plus élevée dans le plasma que dans les autres organes (ex.: le cœur et le diaphragme). Plus la concentration de nitrate dans le plasma augmente plus le rapport tissu/plasma ou organe/plasma de nitrate est faible (cœur/plasma, aorte/plasma et cerveau/plasma à $p<0.005$). Nous avons pu mettre en evidence une relation dose-réponse entre la dose de

LPS administrée et la concentration du nitrate ($p<0.005$) dans l'aorte et dans le poumon 12 heures après le traitement. Chez le porc, il a été démontré que l'oxyde nitrique exogène (le gaz NO inhalé [40 ppm] ou de donneurs de NO [Nitrogène 90 μg/kg/minute pendant 20 minutes]) augmente significativement mais de façon transitoire la concentration de nitrates plasmatiques et urinaires. Dans le rat, après le traitement au LPS, on a aussi observé une augmentation de la concentration de nitrate dans l'urine prélevée directement dans la vessie de l'aminal. En temps normal, les concentrations de nitrate mesurées dans le plasma, le tissu renal et l'urine, étaient respectivement de 12, 59 et 179 pmol/mg (ou μM) du poids de tissu dans les et 233, 154, 1428 μM durant le sepsis. En comparant la demi-vie du NO (3 à 20 sec), à celle du nitrate (1.5 à 8.0 heures) (270 à 9600 fois plus longue in vivo) on peut considérer que la concentration de nitrate dans le tissu reflète la production tissulaire de NO. Par ailleurs, la concentration de protéine iNOS, déterminée par immunobuvardage, augmente de façon significative dans le diaphragme, le poumon et le sang après administration de 10 mg/kg de LPS. La concentration de iNOS étant plus élevée dans le sang que dans le diaphragme et le poumon 12 heures après le traitment, nous pouvons établir une corrélation statistiquement significative entre l'iNOS sanguin et le niveau du nitrate dans le plasma ($r^2=0.992$, $p<0.008$).

Le changement de concentration de nitrate dans un tissu ou organe est parallèle à la réponse inflammatoire telle qu'indiquée par l'altération de l'activité du myélopéroxydase (MPO) (mesurée au niveau du cœur, poumon, foie, reins, rate et dans le plasma), par des modifications histologique (coupe du cœur, reins, foie, rate, poumon, cerveau, intestin grêle, testicules, muscle squelettique, os et tissu adipeux) et par la

dysfonction du sarcolemme cellulaire (mesuré par procion orange 14 fluorescent tracer dye dans le cœur, les poumon, le foie, les reins, les intestins, la rate, le diaphragme et à un niveau moindre dans le cerveau). Les cellules inflammatoires contribuent de façon significative à la concentration et à l'activité de l'iNOS durant le sepsis et pourraient ainsi modifier la concentration extracellulaire de nitrate. Celle-ci est aussi modifiée parallèlement aux altérations morphologiques dans la plupart des organes (cœur, diaphragme, foie, reins, poumons et intestins) et ce particulièrement 12 heures après l'administration du LPS.

Une réduction de la contractilité du diaphragme évaluée en chambre tissulaire a été observée après injection intra-abdominale de LPS in vivo et après exposition du tissu musculaire à l'anion nitrate in vitro. L'altération de la contractilité du diaphragme, causée par l'anion nitrate seul in vitro, peut être corrélée à l'altération de la fonction respiratoire. Cette altération est induite suite au traitment au LPS des rats durant le sepsis. En effet une tachypnée peut-être associée au traitement 6 à 12 heures suite à l'administration de 10 mg/kg de LPS. Nous avons trouvé une corrélation significative entre la respiration et la concentration de nitrate dans le poumon (r^2=0.996) à $p<0.01$. Le LPS induit aussi une réduction de la tension artérielle moyenne, plus particulièrement 12 heures après son administration. Cette réduction est par contre négativement corrélée avec la concentration du nitrate dans l'aorte (r^2=-0.96, $p<0.05$) et positivement corrélée avec le rapport des concentrations de nitrates aorte/plasma (r^2=0.95, $p<0.05$) après injection intra-abdominale de 10 mg/kg de LPS.

En conclusion, nous pouvons dire qu'il existe des différences de concentration de l'anion nitrate entre les différents tissus ou organes du groupe contrôle. Le LPS induit

une altération significative de la production du nitrate dans plusieurs organes et tissus, excepté dans le cerveau, le tissu adipeux et dans le muscle de la cuisse. Il y a un gradient de concentration en nitrate entre les tissus et/ou organes (cœur, aorte et diaphragme) et le plasma. Ce gradient est modifié durant le sepsis. Le changement de concentration du nitrate dans les tissus et organes est parallèle à la réponse inflammatoire reflétée par les modifications de l'activité de la myélopéroxidase, de l'image histologique, l'intégrité du sarcolemme cellulaire ainsi que par les modifications physiologiques (la fréquence respiratoire du rat, la tension artérielle). L'utilisation de l'anion nitrate seul in vitro cause la dépression de la contractilité du muscle du diaphragme. Nos résultats suggèrent que l'anion nitrate, qui est le métabolite majeur du NO, contribue à la survenue du syndrome de la réponse inflammatoire systémique et du dysfonctionnement d'organes multiples durant le sepsis.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AA	arachidonic acid
ACCP	American College of Chest Physicians
AG	aminoguanidine
ALI	acute lung injury
ANF	atrial natriuretic factor
ANOVA	analysis of variance
APACHE	Acute Physiology And Chronic Health Evaluation
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
CD	clusters of differentiation
cGMP	cyclic guanosine monophosphate
CLP	cecal ligation and puncture
cNOS	constitutive NOS
CNS	central nervous system
CO	cardiac output
COX	cyclooxygenase
CSF	cerebrospinal fluids
CVP	central venous pressure
DAP	diastolic arterial pressures

DIC	disseminated intravascular coagulation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E coli	*Escherichia coli*
EDRF	endothelial derived relaxing factor
ELAM	endothelial leukocyte adhesion molecules
eNOS	endothelial NOS
EPR	electron paramagnetic resonance
ET	endotheline
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
FW	formula weight
GOT	glutaminc-aspartate transamine
GFR	glomerular filtration rate
GPT	glutaminc-pyruvic transamine
GTN	nitroglycerin
H&E	hematoxylin and eosin
HbNO	nitrosohæmoglobin
HPLC	high-performance liquid chromatography
HR	heart rate
HTAB	hexadecyltrimethyl-ammonium bromide
ICU	intensive care units
IFN-γ	gamma interferon

IL	interleukins
iNO	inhaled nitric oxide
iNOS	inducible NOS
LAP	left atrial pressure
LBP	lipopolysaccharide-binding protein
LD	lethal dose
L-NMMA	N^G-monomethyl-L-arginine
L-NNA	N^G-nitro L-arginine
LPS	lipopolysaccharide
LSD_F	Fisher's protected least significant difference
LTC	leukotrienes
MAP	mean arterial pressures
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaI	sodium iodide
nNOS	neuronal NOS
NO	nitric oxide
NO^-	nitroxide
NO^+	nitrosonium
NO_2^-	nitrite
NO_3^-	nitrate

NOS	NO synthase
NOx	nitrite and nitrate
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
$PaCO_2$	partial pressure of arterial carbon dioxide
PAF	platelet-activating factor
PAH	para-amino-hippuric acid
PAP	pulmonary arterial pressure
PCD	programmed cell death
PCV	packed cell volume
PDE	phosphodiesterases
PEHT	phenylephrine-induced hypertension
PG	prostagladins
PK	protein kinases
PMN	polymorphonuclear
PMT	photo multiplier tube
PTPase	protein tyrosine phosphatase
PVR	pulmonary vascular resistances
RBFc	renal blood flow corrected
RPF	renal plasma flow
RR	respiratory rate
SAP	systolic arterial pressures
SCCM	Society of Critical Care Medicine

sGC	soluble guanylate cyclase
SIRS	systemic inflammatory response syndrome
SMT	S-methylisothiourea sulfate
SNK	Student-Newman-Keuls
SOD	superoxide dismutase
SVR	systemic vascular resistances
TLR	toll-like surface receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TXA	thromboxanes
UF	urinary flow
VSM	vascular smooth muscle
WBC	white blood cells

1. INTRODUCTION

1.1. Septic shock

1.1.1. Introduction

Septic shock and the systemic inflammatory response syndrome (SIRS) are very complex clinical syndromes, which are a major cause of mortality in intensive care units (ICU) [1, 2]. Nitric oxide (NO) plays many essential roles in the physiological and pathophysiological processes of SIRS [6, 7, 14]. During the last decade, it has become clear that microbial components, such as endotoxins, namely lipopolysaccharide (LPS), initiate a complex network of mediator cascades [18, 62, 75], and it is these mediators that, directly or indirectly, cause SIRS and the more severe multiple organ dysfunction syndrome (MODS) [77, 78]. Sepsis and/or septic shock are life-threatening complications of an overwhelming infection in which the immune system releases inflammatory mediators resulting in pathophysiological vasodilation, hematological abnormalities, and consequently multiple organ dysfunction/failure [80, 93]. The pathological changes in septic shock, a disease state involving several hemodynamics and metabolic parameters, are not completely understood. It is now recognized that excessive NO is induced by endotoxin and cytokines that may play a central role in infectious disease as well as in hemorrhage, trauma, and ischemia/reperfusion status [53, 56, 80, 86-88, 93, 94, 271]. There is abundant clinical and experimental evidence demonstrating that NO and/or

nitrate anion is/are elevated or over-produced during SIRS, especially sepsis, and that the level of plasma nitrate anion is associated with the severity of this syndrome [318]. However, both beneficial and detrimental effects have been assigned to NO during sepsis or SIRS [121, 306, 374]. Despite basic and clinical efforts, a "magic bullet" to fight sepsis remains elusive, giving rise to a literal explosion of publications on septic shock and/or inflammatory response/SIRS/MODS [38, 39, 134]. There are more than 14 papers published daily for 365 days per year regarding sepsis/SIRS or NO, respectively (Figure 1).

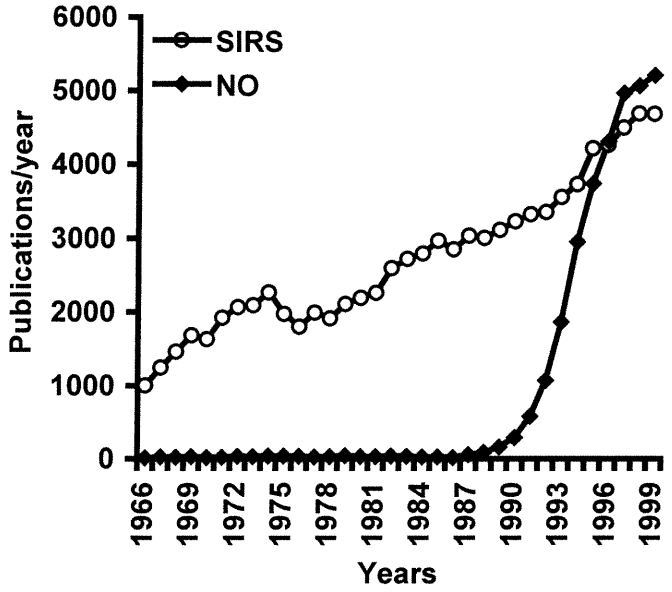


Figure 1. The number of yearly publications cited in Medline from 1966 to 1999 in the fields of septic shock/ inflammatory response/SIRS, and NO respectively.

1.1.2. Definition

The first case of gram-negative bacteremia was described in 1899 by Brill and Libman, and the first indication that endotoxin participates in the genesis of shock arose from the clinical observations of Spink et al. in 1948 [239]. The complexity of the clinical manifestations of this pathophysiological phenomenon has led to past confusion in the definition of sepsis [1, 239, 258]. The term, septic shock, is usually considered to be similar or equal to endotoxin shock, and septicemic shock [239]. Recent international consensus conference efforts in 1994, 1997, and 2000 by the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM), have led to guidelines (Table 1) for identifying patients with sepsis, septic shock, acute lung injury (ALI, $PaO_2/FiO_2 \leq 300$), and acute respiratory distress syndrome (ARDS, $PaO_2/FiO_2 \leq 200$) with the goal of achieving standardization of terminology and improved homogeneity of patient populations in clinical studies [1, 28, 258]. The guideline is still insufficiently specific to address the problem of heterogeneous mechanisms leading to clinical syndromes. Since many non-infection conditions (such as trauma, ischemia, and drugs) can eventually cause the same process of inflammatory response of sepsis, the committee recommends the use of the term SIRS to describe sepsis. It is an important challenge to progress from clinical syndromes to more specific entities that are delineated by alterations in specific physiological, metabolic, immunological or biochemical pathways [239]. Such mechanistic definitions will provide more homogeneous groups of patients who can be identified in the early stages of their clinical course. This approach encourages focused investigation of pathways leading to organ dysfunction and death [1],

Table 1: Definition of sepsis, SIRS and MODS.

Term	Definition
Bacteremia	The presence of viable bacteria in the blood.
Endotoxemia	The absence of viable of bacteria but presence of endotoxin in the blood.
The systemic inflammatory response syndrome (SIRS)	The systemic inflammatory response to various insults, e.g. endotoxin, bacteria, virus, fungi, trauma, burn, and drug overdose.
Sepsis	The systemic response to infectious, manifested by the two or more conditions as a results of infectious, e.g. temperature > 37°C, or < 36°C; HR > 90 beats/min; RR > 20 breaths/min; or $PaCO_2$ < 32 torr; WBC > 12,000 or < 4,000/mm3; or > 10% immature (bands) form.
Septic shock	Sepsis with hypotension or the requirement for vasopressors/inotropes to maintain blood pressure of perfusion abnormabilities that may include, but not limit to, lactic acidosis, oliguria, or acute alteration in mental status.
MODS	Multiple organ dysfunction/failure following SIRS.

Modified from the ACCP/SCCM consensus Conference Committee in 1994, 1997 and 2000 [1, 28, 258]. HR, heart rate; RR, respiratory rate; WBC, white blood cells.

eventually providing an efficient framework for the development of new useful clinical therapies [1, 212, 258].

Septic shock, as a clinical syndrome, is characterized by evidences of systemic inflammation, cardiovascular instability or various organ dysfunctions [1, 19, 238, 239, 263, 376]. Signs of systemic inflammation include fever or hypothermia, tachycardia, hyperventilation, tachypnea, and leukocytosis or leukopenia. Cardiovascular instability is generally characterized by high cardiac output (CO) and high or low systemic vascular resistance (SVC) recognized by hypotension and signs of poor organ perfusion. Even though CO might be maintained above normal levels, impaired myocardial contractility, inadequate distribution of blood flow and disturbance of tissue oxygen consumption are common [238, 239, 346, 383].

1.1.3. Aetiology

Infection is usually the most probable initiating cause of sepsis and subsequently, severe infections may cause systemic symptoms and signs that constitute the clinical syndrome of septic shock [1, 28, 258]. Bacteria, either gram negative or positive, are the most common infectious causes of septic shock [274], but other agents such as viruses, fungi and parasites may also cause this clinical syndrome. Occasionally, non-infectious inflammatory conditions, such as mechanical/chemical tissue damage, ischemia/reperfusion injury, pancreatitis, burns, trauma, and drug overdose, may result in clinical features characteristic of septic shock [1, 2, 28, 258]. Many different mediators derived from host cells are responsible for most of the manifestations of endotoxemia, for

instance: 5-hydroxytryptamine (5-HT), thromboxanes (TXA), endotheline (ET), leukotrienes (LTC), prostaglandins (PG), platelet-activating factor (PAF), vasoactive intestinal peptide, Kallikrein-kinin, vasopressin, endorphins, anaphylatoxin, interleukins (IL), tumor necrosis factor (TNF), myeloperoxidase (MPO, EC 1.11.1.7), proteinases such as caspases, superoxide anion (O_2^-) peroxynitrite ($ONOO^-$), and possibly the nitrate anion as well [1, 28, 258].

1.1.4. Incidence and mortality rate

In 1997, 17.3 of 52.2 million of total global deaths were caused by infectious and parasitic diseases, exceeding the combined deaths caused by circulatory diseases, cancer, and respiratory diseases. Infections thus remain a major problem in the world [1, 28, 38, 39, 258]. Septic shock or SIRS is probably the most common cause of morbidity and mortality in intensive care units. It is estimated that ~500,000 cases of sepsis occur in the USA each year and 75,000 cases in Germany. Of these, approximately half, or more than 250,000, will develop septic shock within days giving rise to the acute mortality rate ranging from 20 to 80%. The variability in mortality rate is due in part to different clinical definitions of the syndrome and patient case-mix differences. Recent reports indicate that about 50 to 70% of well-defined septic shock episodes will be fatal in outcome, resulting in 100,000 or more deaths annually in the USA [1, 28, 258].

After a comparison of all-cause mortality in 12 prospective, controlled, randomized, double-blind, multicentre trials involving more than 6,200 patients, no significant benefit of methylprednisolone, antiendotoxin antibodies, ibuprofen, platelet

activating factor receptor antagonist, IL-1 receptor antagonist, and anti-TNF monoclonal antibody was found [64]. In other words, more than $1 billion has been spent to test these agents, but not a single "magic bullet" has been licensed for clinical use [1, 28, 38, 39]. Therefore, the underlying pathophysiological mechanisms of involved in sepsis or SIRS must be understood more clearly and then manipulated more precisely and appropriately.

1.1.5. Pathophysiology

Gram-negative bacilli are responsible for at least 45-60% of sepsis caused by bacterial infection and are probably the key to understanding how infectious agents precipitate the clinical manifestations of septic shock [274]. *Escherichia coli* (*E coli*) is the most common clinical isolate among gram-negative pathogens, followed by *Klebsiella, Enterobacter, Serratia,* and *Pseudomonas* [274]. LPS, commonly referred to as "endotoxin" which was first isolated approximately 60 years ago, is an integral part of the cell wall of gram-negative bacilli (Figure 2). It can be released from the cell wall to circulate freely, especially after bacterial autolysis. The toxic lipid-A core of the LPS molecule can interact with cellular receptors thereby leading to the induction of a SIRS cascade. The binding of endotoxins to a circulatory protein called lipopolysaccharide-binding protein (LBP) facilitates subsequent interaction of endotoxin with the CD14 or toll-like surface receptor (TLR) of macrophages. The membrane-bound CD14 (mCD14) constitutes a 53-kDa glycoprotein present on the surface of myeloid cells and involves in cellular response including monocytes and macrophages. CD14 also exists in soluble form (sCD14). As such it is present in the circulation (several subtypes, e.g. 48-, 53-, 55-

kDa), at a concentration of 2-6 μg/ml. Soluble CD14 is capable of interacting directly with LPS. The sCD14/LPS complex is capable of binding to CD14-negative cells such as endothelial cells and of activating these to produce cytokines. This interaction is the critical first step in triggering a complex inflammatory cascade (Figure 3) [62, 212]. Macrophages, when activated by endotoxin, release various cytokines, such as TNF, IL-1, IL-6, IL-8, and gamma interferon (IFN-γ). These cytokines have a variety of potent

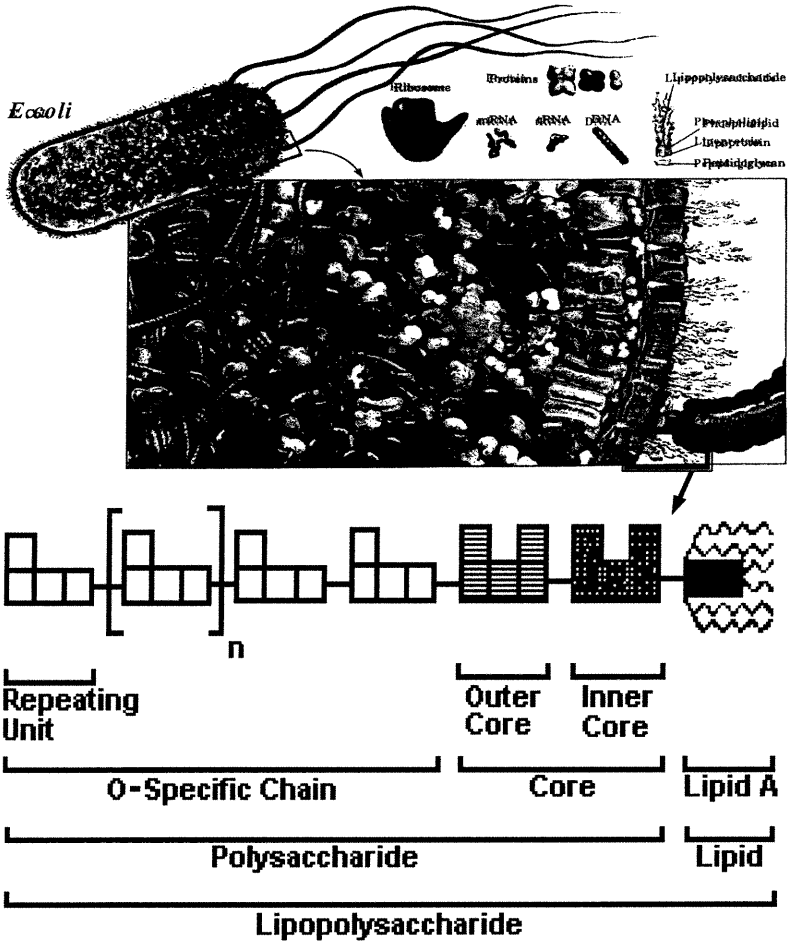


Figure 2: LPS and its component.

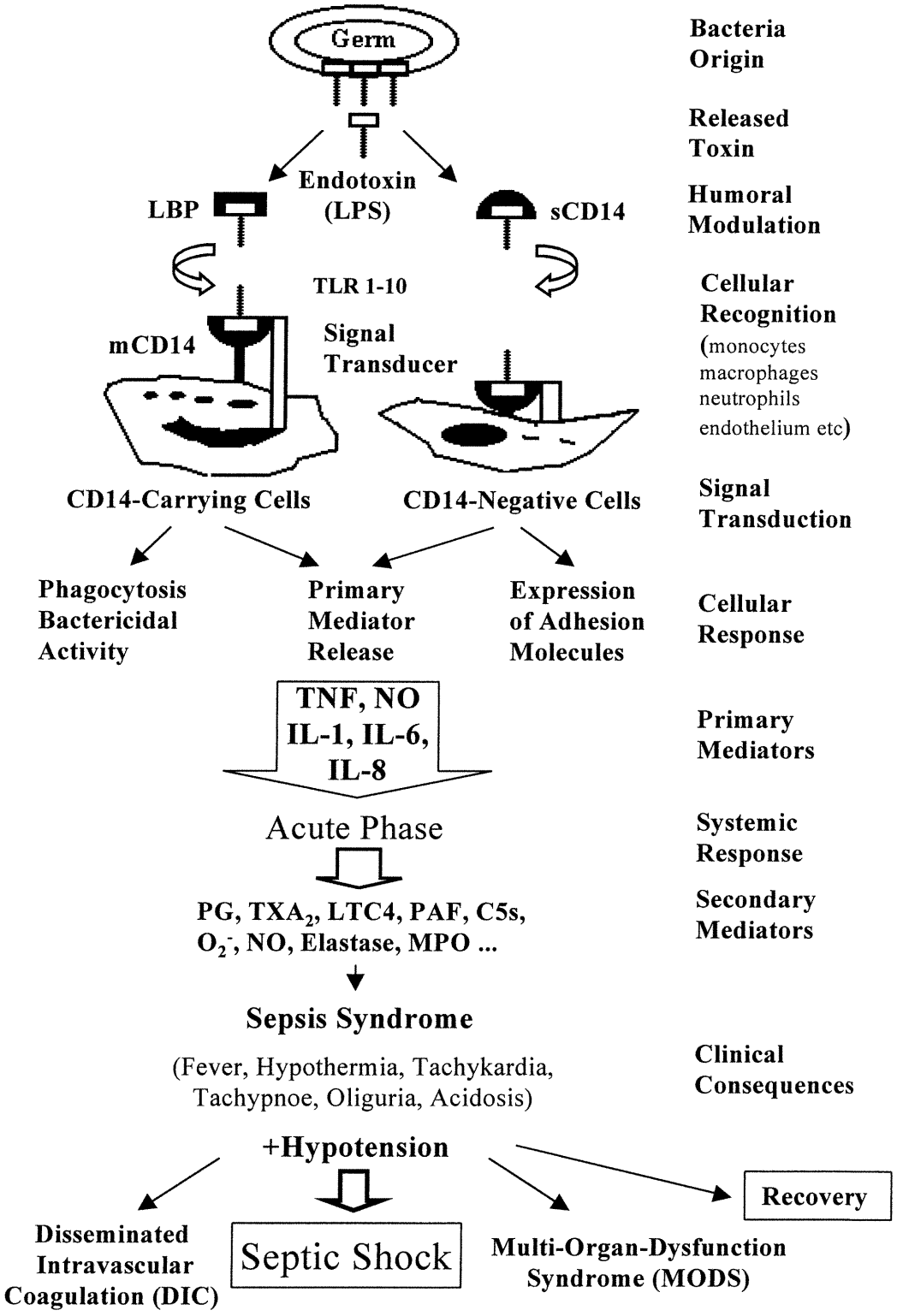


Figure 3: Inflammatory cascade after infections or endotoxins/SIRS.

inflammatory effects and, additionally, the administration of TNF-alpha or IL-1 itself produces immediate similar clinical sequelae of sepsis. They amplify the inflammatory cascade by stimulating polymorphonuclear (PMN) cells, lymphocytes, the coagulation pathway, and the release of other cytokines and other mediators and stimulate over-production of NO. Activation of this inflammatory cascade will facilitate bacterial clearance, but may also proceed in an unregulated fashion to cause alterations of host cell/organ functions, severe host cell/organ injury, MODS and subsequent death [1, 64]. The pathophysiological derangement observed in patients with sepsis has been hypothesized to represent the host inflammatory response to endotoxin derived from gram-negative bacteria. Furthermore, it has been proposed that it is not the bacterial infection that causes the lethality, but rather the uncontrolled host response to infection. At least 4 main reasons support the role of SIRS at the origin of organ injury in sepsis: (I) Endotoxin infusion causes the release of massive quantities of pro-inflammatory cytokines, NO, and superoxide [224]. (II) Recombinant cytokines infusion produces a "septic-like" appearance [142]. (III) Levels of cytokines, NO and superoxide are elevated in the plasma of both septic patients and animal models, the high levels seem to correlate with outcome [274, 300]. (IV) Inhibition of the cytokines or NO may prevent mortality in experimental animals.

1.1.6. Animal models of septic shock

It is encouraging that the complex pathophysiology of bacterial sepsis is gradually becoming better understood as more studies from both clinical trials and animal

models are reported [274, 300]. Although extensive research is being carried out, the mechanism of sepsis and sepsis-induced organ dysfunction and failure remain largely unknown. In view of this, animal models that simulate clinical conditions would be useful, even though the final testing of any results will eventually have to be carried out in patients [1, 64].

During this century, intensive attempts have been made to design animal models of sepsis such as using peritonitis, injection of endotoxin or live organisms, and abscesses in the extremities [28, 62, 239]. Endotoxin can reproduce similar immunological, physiological, metabolic, and biochemical parameter alterations associated with gram-negative sepsis in both small and large animal models. More importantly, the results of many of these studies have been incorporated into clinical treatments and recommendations for patients [1, 64]. Many laboratory animals, such as mice, rats, rabbits, sheep, pigs, dogs, monkeys, and baboons, have been used to reproduce sepsis following several approaches, such as slow or bolus I.V., or I.P. injection or infusion of LPS or live organisms or feces; intramuscular or subdermal placement of live organisms or feces; and surgical cecal ligation and puncture (CLP). The administration of feces and CLP have the disadvantage of an uncontrolled dosage, bacteria strains as well as the presence of surgical damage.

Using small rodents e.g. mice or rats as experimental models of SIRS present many advantages. They are relative small in size, inexpensive, widely available in large numbers of the same age and sex, genetically identical, free of specific pathogens, and on the same diet. This minimizes biological variables that are well matching the criteria of animal model of diseases [28, 39, 64]. Using large animals e.g. dogs, sheep, or pigs, can

also readily match the criteria of animal model, but are more expensive, frequently nonstandardized, and only a small number of animals are available. A number of studies from various animal models of SIRS have shown that levels of various inflammatory mediators, e.g. TNF, IL-1, MPO and NO/nitrate, increase after LPS I.P. or I.V. injection [300]. They also note the appearance of clinical symptoms and signs of sepsis such as fever, hypotension, ALI or ARDS [80, 104]. In this regard, experimental animal models closely approximate the clinical situation.

1.2. Multiple organ dysfunction syndrome in septic shock

1.2.1. Introduction

In the late 1960s, several reports appeared describing remote organ failure as a complication of severe sepsis. Tilney et al. in 1970s [340] observed that severe injury can cause sequential failure of the initially uninvolved organ systems. A editorial paper, entitled "Multiple, Progressive or Sequential Systems Failure: A Syndrome of the 1970s" was formulated as the basis of a "new" clinical syndrome by Baue [27]. Several terms were coined thereafter, but more recently the term MODS has been proposed as a more appropriate description [1, 28, 258]. MODS was defined as a clinical syndrome characterized by the development of progressive but potentially reversible physiological dysfunction in two or more organ or organ systems induced by a variety of acute insults, including sepsis. Marshall et al. in 1995 reviewed more than 30 papers related to MODS showing that seven systems, e.g. cardiovascular, respiratory, renal, hepatic, intestinal,

central nervous and hematological, were most often included [238]. However, the criteria for organ dysfunction varied markedly and it may have been unspecific [238].

Several studies demonstrated that sepsis and septic shock are the most common condition leading to MODS. The mortality of MODS patients is extremely high, ranging from 30% to 100%. Mortality is also correlated with both number and duration of organ dysfunction. Mortality almost uniformly reaches 90%-100% when three or more organ systems fail. Friedman et al. (1995) reviewed all relevant of 131 papers from 1958 to 1997 through a MEDLINE search and from the bibliographies of identified articles, involving a total of 10,694 patients [104]. The overall mortality rate in the 131 studies was 49.7%. Recently, 23 completed clinical trials in sepsis provided a large database (number of patients ranging from 110 to 971). Taken together, the mortality rate in respiratory failure range from 6% to 83%, renal failure range from 9% to 51%, hepatic failure range from 4.5% to 28.0%, neurological failure range from 6.0% to 32.0%, hematological disorder from 7.5% to 49.0%, respectively. Although the definition and criteria of organ failure varied, the conclusion is warranted that the incidence of organ failure in sepsis and particularly septic shock is high [64, 80, 340].

1.2.2. Features of septic organ dysfunction

A variety of pathophysiological responses in various tissues and organ systems occur during sepsis [159]. In particular, circulatory and respiratory dysfunction/failure, leukocyte-induced tissue injury appear to be critical determinants in the development of sequential organ dysfunction/failure. Other mechanisms for the development of MODS in

septic shock should be considered including inhibition of cellular mitochondria respiration and vascular endothelial cell cytotoxicity. The lungs, kidneys, liver, cardiovascular system, central nervous system and coagulation system are commonly involved in septic MODS [197, 200, 201, 219-222, 238, 239, 269, 351]. MODS is often irreversible and organ dysfunction and failure during sepsis can be significantly modified by many variables e.g. preexistent diseases, age, sex, and even antibiotics [1, 300]. In any event, the clinical result of these perturbations is tissue hypoxia which is clinically non-specific. However, cardiovascular instability, increased respiratory rate, peripheries that are either warm and vasodilated, or cold and vasoconstricted, poor urine output, and mental dullness may indicate organ dysfunction [80, 104].

Abnormal hemodynamic responses comprise a primary hallmark of severe endotoxin or septic shock and may be the most impressive initial, or the latest fatal clinical expression of organ failure [55]. They are of great complexity and variability and differ between species. They vary according to the severity, sequential time periods, and duration of shock. This has been related to the release of many mediators, but the release of NO plays an essential role. Alterations in peripheral vascular tone and cardiac function contribute to the cardiovascular manifestations of septic shock. CO and heart rate commonly increase. Systemic vascular resistance is low because of decreased arteriolar tone whereas pulmonary vascular resistance is high, causing an increase in pulmonary arterial pressure. Despite the increase in cardiac output, tissue hypoperfusion is manifested by raised blood lactate concentration, with oxygen extraction at less than maximum [272, 275]. The deficit in oxygen utilization seen in septic shock has been linked to maldistribution of blood flow to regional beds at the level of the

microcirculation, rather than to a defect in the cellular oxygen metabolism [1, 156]. Due to endothelial cell swelling (later necrosis), leukocyte sequestration, and microthrombus formation, consequences of microcirculatory dysfunction during sepsis are (I) the breakdown of endothelial and epithelial barrier function, leading to tissue edema and uncontrolled inflammatory cell infiltration; (II) vasodysregulation, leading to the formation of arteriovenous shunts (bypass microcirculation) and/or the loss of peripheral resistance with severe macrohemodynamic consequences; and (III) disturbance of oxygen transport and utilization by tissue cell [1, 156, 192-194].

The heart serves as the power source for circulation. Myocardial dysfunction is seen in most septic patients or animals. Several substances such as NO, TNF, IL-1 have been identified as potential myocardial depressants [52]. Pulmonary hypertension and the down-regulation of β-adrenergic receptors are other factors that may impair cardiac function in septic shock. Cardiovascular dysfunction and failure during sepsis also complicates invasive bacterial infection in ~40% to 50% of patients and circulatory dysfunction itself can contribute significantly to the development of other organ failure. However, there is a wide variety in the time course and severity of complicating circulatory dysfunction, ranging from mild circulatory instability to severe progressive shock that can be the earliest as well the latest fatal symptom [93, 94, 339].

Respiratory dysfunction mostly appears following septic shock and is the major risk factor of ARDS. This syndrome occurs in 20% to 60% of ARDS cases. Its basic pathological mechanism is pulmonary microvascular injury with increased permeability and exudation of cells and/or protein-rich fluids in alveoli that consequently cause: alveolar collapse, increased intrapulmonary shunting, progressive hypoxemia, decreased

compliance, decreased residual capacity, and increased work of breathing. Tachypnea and hyperventilation occur almost uniformly in septic shock. The increased work of breathing, diminished oxygen supply to respiratory muscle, progressive respiratory muscle fatigue, and metabolic disorders are its pathogenetic basis [107, 158].

Oliguria is a very common initial sign in septic shock. Proteinuria and polyuria is not uncommon. Renal hypoperfusion is the most important pathogenetic mechanisms, Other factors including sepsis or SIRS induced humoral mediators, e.g. TXA_2, ET, NO, $ONOO^-$, can also contribute significantly to the development of renal dysfunction [29, 32, 334].

Changes in mental state may be an early and sometimes the sole initial sign of severe sepsis, ranging from mild to impaired orientation, to confusion, and some cases to delirium followed by coma. Cerebral hypoperfusion, hypoxemia, the blood-brain barrier (BBB) damage, and effects of cytokines, superoxide anion, NO and $ONOO^-$ on brain function have been implicated during sepsis [182, 355]. Central nervous system (CNS) dysfunction may occur very early in the course of septic shock, but is often difficult to evaluate as many patients are mechanically ventilated. However, the mechanism of encephalopathy has not been clarified and is presumably multi-factorial.

Sepsis or SIRS is the most common cause of acute disseminated intravascular coagulation (DIC), a syndrome characterized by intravascular clotting, microvascular fibrin deposition, and consumption of clotting factors and most often DIC is used as a marker of hematological failure. Leukocytosis, a common finding in SIRS, reflects the release of less mature leukocytes from bone marrow storage pools. Varying degree of leukocytosis and leukopenia can be seen in the septic shock [66].

Liver dysfunction is a virtually general occurrence with severe sepsis and SIRS. It may range from minor elevation of liver enzymes, e.g. glutamine-pyruvic transaminase (GPT), glutamine-aspartate transaminase (GOT) and alkaline phosphatase, to pronounced jaundice in the late stages. The pathogenesis of liver function in SIRS or sepsis is clearly multi-factorial and includes such mechanisms as liver ischemia, direct injury from endotoxin, NO, free radicals, and SIRS induced cytokines [33, 53, 320, 321].

The similarity between sepsis-induced and non-infectious-induced MODS in clinical, pathophysiological, and biochemical abnormalities has led to the concept that MODS is the result of SIRS of the host, e.g. activated inflammatory cells (e.g. macrophages, neutrophils) and tissue residence cells (e.g. endothelial cells) contribute significantly to generate mediators, tissue injury, widespread endothelial inflammation and dysfunction, abnormalities in vascular tone, myocardial depression, metabolic derangements, coagulation abnormalities, and resulting cells or tissues or organs damage. These are all hallmarks of severe septic shock. This improved knowledge, concerning both host factors which are activated or formed during sepsis and which are largely responsible for the self-destructive reactions associated with sepsis, on the one hand, and microbial factors which are responsible for the initiation of sepsis on the other, have important therapeutic implications. The modern interventional strategies of sepsis, therefore, should not only aim at toxin factor neutralisation, but also at breaking the synergistic action of the different bacteria- and host-induced harmful factors.

1.2.3. Cellular response in septic organ dysfunction

Various cells such as neutrophils, macrophages, endothelium, and myocardial cells are responsible for the release of a large number of different humoral mediators and products (such as NO, oxygen free radicals, prostaglandins, leukotrienes, platelet activating factor, ET, proteinases and endophins) that are involved in the exaggerated host response and cause most, if not all, of clinical symptoms of SIRS or sepsis and ultimately lead to tissue damage and MODS [212]. Biological effects of these mediator systems are diverse and complex, many of these processes are interrelated and have synergistic effects, many may have different effects in combination than they have alone, and many interactions occur that may either augment or limit the inflammatory response. Moreover, the pathogenesis of the sepsis and SIRS and consequently of MODS is far from elucidated [212].

Interaction between neutrophil and endothelial cells may be the central mechanism in the pathogenesis of SIRS or MODS [12, 349, 365]. In the 1880s Elie Metchnikoff observed that phagocytic cells ingest bacteria, and recognized this phenomenon as a defense mechanism in the hosts. Normally, typical phagocytes such as neutrophils are present in three separate pools within the body: the bone marrow, intravascular, and the tissues pool, without having any deleterious effects on the other organs or underlying tissues [295]. During acute inflammation, more immature polymorphonuclear neutrophils are released into the circulation with effusion and recruitment of neutrophils and monocytes. This process occurs in various organ systems, especially in the lungs. Other pathways may apparently also lead to SIRS and MODS. In

animal models, TNF, IL-1 and endotoxin can induce both endothelial leukocyte adhesion molecules (ELAM-1, ICAM-1) and their ligands neutrophil adhesion molecules (CD11b/CD18, TLR2), which promote adhesion and migration; the phenomenon known as neutrophil rolling to endothelium [189, 193]. There are at least four steps for leukocyte recruitment: (I) tethering or primary adhesion; (II) triggering of strong adhesion; (III) strong adhesion; (IV) transendothelial migration into tissue and at least two types of adhesion molecules are involved in the interaction of endothelium and leukocytes: the selectins (P, L, and E selectin) and the integrines (CD11b/CD18). Interestingly, a primary inflammatory response could affect a secondary inflammatory response as PMN exudation in a primary site reduces an exudation of PMN in a secondary site such as in a skin window reduced by 90% compared to controls. A similar reduction of PMN delivery to Rebuck skin windows was observed more than 30 years ago in burn patients [189-194]. There was no correlation between the circulating white blood cell count and PMN delivery to the skin window. This phenomenon may indicate that, with only limited immune resources in the body following severe insults such as SIRS or sepsis, the immune resources (e.g. immune cells, enzymes, antibodies and proteins) may defend or scavenge the invaders in a "first come first serve" manner.

Activated neutrophils in tissues degranulate and release several mediators such as MPO, elastase, collagenase, caspase, oxygen free radicals, and reactive nitrogen species. A role for MPO as a component of the antimicrobial activity of neutrophils was proposed in 1967 when it was found that MPO was strongly microbicidal and that MPO is a major constituent of the azurophilic cytoplasmic granules and a classic heme peroxidase which can oxidize chloride ions to the strong nonradical oxidant, HOCl [62,

184]. These complex systems can promptly kill many species of bacteria and MPO appears critical for this function. Several studies have demonstrated that the inhibition of neutrophil function or MPO activity dramatically decreases at least by 80% the rate constant for killing bacteria. In experimental septic models, leukostasis in several organs is a prominent histopathological sign of the endothelial-leukocytes interaction. Activated neutrophils *in vivo* or *in vitro* can be assessed by its sensitive biological marker of MPO activity. MPO activity has been found to be elevated and positively related to the development and the severity of SIRS, ALI, ARDS, MODS, and outcome in septic patients [9, 62]. In both septic animals and patients, PMN counts represent more than 80% of the total cells obtained by bronchoalveolar lavage (BAL) and higher counts are associated with more severe lung dysfunction [4]. There is no doubt that increased pulmonary permeability is mediated significantly by the interaction between neutrophils and pulmonary endothelial cells apparently with a collaborative action of other mediators such as TXA_2, PAF, cytokines, NO, superoxide anion, $ONOO^-$, and nitrate anion as well [190-194]. Other cells, e.g. mast cells, resident and infiltrating macrophages, may also play a significant role during endotoxic shock. Macrophages are the principal source of the key mediators of septic shock, such as tumor necrosis factor, IL1, and macrophage migration inhibitory factor (MIF). MIF, in turn, can stimulate the production of pro-inflammatory mediators by macrophages, can activate T-cells, and can act as a physiological antagonist of the anti-inflammatory and immunosuppressive effects of steroids. In addition, macrophages can produce large amounts of NO, a potent vasodilator and cytotoxic agent which is an important cause of the hypotension and tissue damage

seen in endotoxic shock. It is likely that similar mechanisms are functioning in other organ systems, although the exact process may vary.

1.2.4. Morphological evidence in septic tissues and organs

The most important septic or MODS-related pathophysiological alterations known to date have been shown to affect programmed cell death (PCD), namely apoptosis, rates in almost all cell types [1, 6, 62]. Organ cell death involving both parenchymal and microvasculature endothelial cells conceivably underlies organ dysfunction. Apoptosis or PCD occurs commonly under septic shock or SIRS. All of the known mediators of SIRS/MODS potentially enhance apoptosis in organ tissues and endothelial cells. Morphologically, apoptosis is characterized by cell dehydration, which leads to cytoplasmic condensation, compaction of nuclear chromatin, and nuclear fragmentation reflected by the ladder pattern of DNA segments in agarose gel electropheresis. The fas (a death specific receptor) and fasL (fas ligand) system which can be activated by TNF is involved in the PCD of most cell types including thymus, bone marrow, spleen, ileum, colon, lung, kidney and cardiovascular cells both in human and animal models during sepsis [140, 146, 151, 156]. Organ differences in the induction of apoptosis might account for the clinically apparent sequence of MODS that is most commonly initiated in the lungs, followed by the liver, kidney, intestine, and the heart. LPS alone is strikingly sufficient to induce apoptosis. Aortic endothelial cells were more susceptible to endotoxin-induced apoptosis than cells from other organs e.g. myocardial.

This is the first study implying that different organs might exhibit different degrees of susceptibility to endotoxin [160].

The main feature of SIRS and MODS that has emerged is essentially one of persistent and uncontrolled inflammatory response that ultimately causes system-wide effects, shock, and end organ dysfunction/failure which usually are accompanied with histopathological alterations [1, 77, 80]. A current theory suggests that apoptosis contributes to the multiple organ dysfunction of sepsis. However, Hematoxylin and eosin (H&E) examination of tissues from septic species did not necessarily "quantitatively" reflect the severity of organ dysfunction. Conventional light microscopy of H&E stained specimens is highly specific if characteristic morphologic changes are observed, but is one of the least sensitive methods for detecting apoptosis and morphological alterations [6, 13]. Under light microscopy, cell shrinkage with condensed nuclei (pyknosis) and nuclear fragmentation (karyorrhexis) can be seen. Apoptosis is also readily distinguished from necrosis. H&E-stained septic samples demonstrated morphological changes at least in the organs of the lungs, liver, heart, kidney, brain, spleen, pancreas, adrenal, ileum, colon, lymph node, aorta, muscle, from mild to massive, and focal to effusive, both in animal models and in clinical patients. However, histopathological appearances vary widely from case to case and organ to organ [18, 28].

There are strong clinical and animal experimental evidences that histologic alterations do exist in various tissues/organs in septic samples [2, 6, 32, 80]. For example, examination of septic spleens showed that more than half of spleens had histologic changes characteristic of apoptosis and that some were present focally at a high degree in the field of view. These changes were statistically different from control. An additional

finding in septic spleens was a decreased density of lymphocytes in white pulp. There was increased apoptosis in colons from septic samples in which focal regions of columnar epithelial apoptosis occurred in crypts or villi. The degree of apoptosis in the colonic villi and crypts was greater in septic than in nonseptic samples. Examination of ileums of septic samples showed that some had increased focal apoptosis in epithelial cells to varying degrees. At least some of these cells were recognizable as lymphocytes and plasma cells. Of note, a readily apparent increase in the number of sloughed apoptotic cells was observed in the intestinal lumen of septic samples but not in control samples. Foci of hepatocyte necrosis and apoptosis can be observed in septic samples and apopotosis, usually located in close proximity to the necrosis [135]. Histological findings in septic kidneys can be seen, but renal histology did not necessarily reflect the severity of renal injury indicated by the decrease in kidney function. Histological findings in lungs from septic samples revealed pulmonary congestion, exudation in alveoli and extensive inflammation. Fibrosis, and/or hyaline membrane formation can be also observed. Pathological examination of the liver may reveal Kupffer's cell hyperplasia, focal hepatic necrosis, and PCD [33]. There are also electronic microscope evidences that support the above observations [27, 28, 239].

1.3. Nitric oxide

1.3.1. Introduction

In 1980 Furchgott, a 1998 Nobel Prize laureate in medicine, reported that the relaxation of isolated preparations of the aorta and other blood vessels by acetylcholine requires the presence of endothelial cells [108]. They postulated that an endothelial derived relaxing factor (EDRF) causes relaxation of the vascular smooth muscle (VSM). In 1987, two groups of investigators identified NO as EDRF [153, 154, 278]. The key element in all of these studies was the correlation of between the amounts of NO measured by bioassay and those detected by chemiluminescence [109, 110, 153, 154].

1.3.2. Biochemistry of nitric oxide

NO (nitrogen monoxide, N=O, molecular weight 30.01) is a relatively simple, unstable, potentially toxic gaseous free radical [322]. It is the smallest synthetic product of mammalian cells and readily diffuse across cell membranes, a value of 4.8×10^{-5} $cm^2.s^{-1}$ was derived from the Fick's law of diffusion ($\mathbf{x} = 2\sqrt{D \bullet \tau}$, where D represents the diffusion coefficient and τ represents life-time) [30, 199, 322]. It has a very short half-life ($t_{1/2}$ 3 to 20 s) *ex vivo*, and a ~ 2 ms half-life *in vivo* [440], and this lability is due to its rapid conversion to nitrate and nitrite anion by superoxide anion or heme-protein e.g. Hb, or oxygen [155, 370, 371, 380-382]. There are three redox states of NO: radical ($NO^{\cdot}$), nitrosonium (NO^{+}), and nitroxide (NO^{-}) [322]. The radical type represents the

major part of NO's physiological and pathophysiological functions. The functions of NO^+ and NO^- are not well delineated. NO reacts readily with superoxide at near-diffusion-limited rates ($6.7 \times 10^9\ M^{-1}.s^{-1}$) to form the peroxynitrite anion ($ONOO^-$) which rapidly (~ 0.7 s) converts to the nitrate anion *ex vivo* (Figure 4) [30, 199]. This reaction is 3.35 times faster than the reaction of superoxide with Cu,Zn superoxide dismutase (SOD, $2.0 \times 10^9\ M^{-1}.s^{-1}$) and is essentially irreversible, but due to the high concentration of SOD (up to 10 μM in the cytosol and 20 μM in the mitochondria), most of the O_2^- will be channelled towards dismutation products [208, 270]. Accordingly, only high concentrations of NO (above 10 μM) will be able to compete for O_2^- with SOD to yield peroxynitrite [212]. Peroxynitrite is a more powerful oxidant as well as a more complicated molecule. The cross-talk among NO and superoxide, and the consequent production of peroxynitrite, and eventually of nitrate anion, has become a more complex issue and it is far from being completely elucidated.

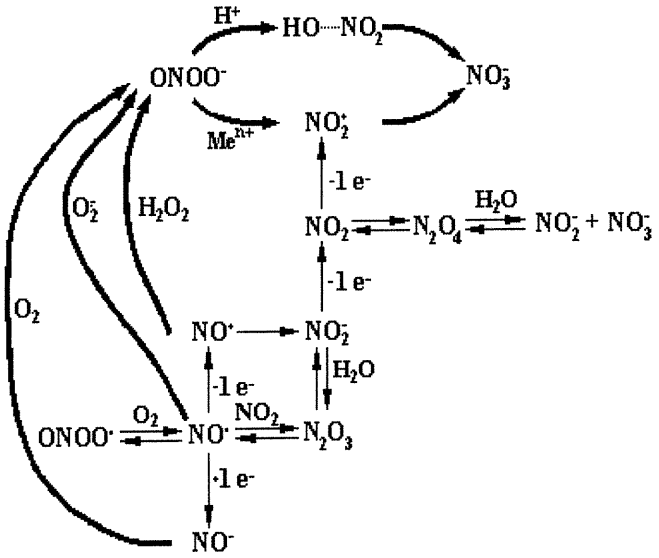


Figure 4. The possible pathways of NO metabolism and its major metabolite nitrate anion formation [30, 291, 319, 400].

1.3.3. Synthesis of nitric oxide

The discovery of NO is still recent but it already has affected current views on many physiological, pathophysiological and therapeutic approaches especially for sepsis, SIRS and MODS. Until 1988, the amino acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells [292]. Definitive mass spectrometry experiments using [^{15}N] L-arginine demonstrated the formation of ^{15}NO or nitrite from the terminal guanidino nitrogen atoms of L-arginine [279-281]. This reaction is L-arginine specific since other amino acids or analogues of L-arginine are not substrates.

Furthermore, it was found that NO production (by measuring nitrate anion) in cultured cells can be inhibited in an enantiomerically specific manner by L-NMMA [279-281], an inhibitor of the generation of nitrate and nitrite anions and citrulline from L-arginine in macrophages [84, 252-254]. All of these studies indicated that NO and citrulline were coproducts of the same enzymatic reaction. Other mass spectrometry evidence using $^{18}O_2$ has shown that this enzyme incorporates molecular oxygen into both NO and citrulline, indicating that it is a dioxygenase [209]. So far, three NO synthases (EC 1.14.13.39) have been identified, namely endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) which is calcium independent [264, 377, 396-399]. Recently NOS was also found in mitochondria. Both eNOS and nNOS also termed constitutive NOS (cNOS), are calcium dependent and are constantly expressed under physiological status. The synthesis of NO requires L-arginine as a substrate and the presence of calmodulin and four cofactors including flavine mononucleotide (FMN), flavine adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4), and reduced nicotinamide adenine dinucleotide phosphate (NADPH). NOS can be expressed in a variety of cells, e.g. endothelium, smooth muscle cells, macrophages, neutrophils, and myocytes [217]. An enormous number of different agents can act as inducers, e.g. proinflammatory cytokines TNF-α, IL-1, and IFN-γ. LPS is an extremely potent activator of iNOS production, both on its own and as an agent synergising with proinflammatory cytokines. Therefore, iNOS may be responsible for most of the increased output of NO during sepsis. Once synthesized, this isoform can produce large amount of NO over a long period time [377, 387].

1.3.4. Role of nitric oxide

NO diffuses readily into nearby cells and it is able to relax vascular smooth muscle by activation of the enzyme soluble guanylate cyclase (sGC) through nitration of its heme group (EC50: 100nM) [14, 44, 174, 248, 400]. sGC catalyzes the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP), which in turn activates several downstream elements, including cGMP-dependent protein kinases (PK), cGMP-regulated phosphodiesterases (PDE) and cGMP-gated channels, resulting basically in a decrease of intracellular free calcium. Overall, the activation of sGC by NO constitutes the major pathway of NO signaling involved in a vast array of physiological functions [130, 260-262, 296]. These include relaxation of vascular and smooth muscle, inhibition of platelet aggregation and leukocyte adhesion to the endothelium, and signal transduction in the nervous system.

NO can modulate the activity of both inducible and constitutive cyclooxygenase (COX) which converts arachidonic acid (AA) into prosglandins, prostacyclins and TXA_2. It also modulates the activity of lipoxygenase, which converts AA into various leukotrienes. It is clear that the NOS and COX systems often co-exist, share a number of similarities and play fundamental roles in similar physiological and pathophysiological situations [212, 401, 402]. An important link between the NOS and COX pathways is that NO can markedly activate the COX 1 and 2 enzymes resulting in an increased production of prostaglandins. The molecular mechanism by which NO activates COX remains to be identified, but it is likely due to (a) antioxidant effect (COX can be auto-inactivated by O_2^- which can be limited by NO), (b) NO nitrosylates cysteine residues in COX, or (c) the

generation of peroxynitrite. The main differences between the effects of NO and PGs are that NO activates the sGC leading to cGMP production while PG activates the adenylate cyclase (AC) leading to cAMP production. Selective iNOS inhibitors have the potential to reduce both NO and simultaneously PG. However, there are also some controversial experimental data on an inhibitory effect of NO on the COX pathway. Nevertheless, the interaction between the NOS and COX systems may play an important modulatory role in both physiological and pathophysiological conditions, and these interactions between the NO and COX pathways provide an important cross-talk in the regulation of the inflammatory response. NO also interacts with the heme moiety of cytochrome P-450, preventing the binding of oxygen to the catalytic site in a reversible inhibition fashion [212].

NO has broad biological effects in relation to almost every organ system (Table 2). NO production, which can be induced by LPS and/or TNF, has been shown to be elevated after sepsis and trauma. It is suggested that excess production of NO may play an essential role in mediating cardiovascular dysfunction in septic shock [297]. The change in plasma nitrate anion concentrations correlated temporally with hemodynamic abnormalities occurring after LPS administration in animals, although some controvercial experimental evidences exist. The excess production of NO can be prevented by NOS inhibitors, such as the non-specific agents NG-monomethyl-L-arginine acetate (L-NMMA) and N-nitro L-arginine (L-NNA), or the partially selective, e.g. aminoguanidine (AG), S-methylisothiourea sulfate (SMT), and mercaptoethylguanidine, or the highly selective, e.g. 1400W, GW273629 and GW274150 during septic shock [38, 50, 81, 127, 180, 181, 396, 397]. However, NOS inhibitors at the same time show significant

detrimental effects *in vivo* [63, 119, 265, 266]. Therefore, it is proposed that a combination of several therapies or multifunctional agents, directed to various phases of SIRS or associate-molecules, might be more successful [64, 286, 399]. Inhaled NO may be a right agent for SIRS, especially SIRS-associate-ARDS [106], since inhaled NO distributes preferentially to those areas with greater ventilation [286, 343, 399]. NO would stimulate localized vasodilation and enhanced blood flow to well-ventilated lung units while simultaneously "stealing" [286, 343] perfusion from more poorly ventilated areas. The net effect is improved ventilation/perfusion (V/Q) matching and reduced hypoxemia (↑ PaO_2).

Table 2. Some known effects of nitric oxide

Reactions	Molecular Targets	Effects	Physiologic Consequences
1) Metals			
Heme iron	Guanylate cyclase	Activation	Vasodilation, reduced adhesion of platelets and leukocytes; signal transduction in the nervous system
	Cytochrome P-450	Inhibition	Regulation of steroid and eicosanoid metabolism; altered drug metabolism
	Cyclooxygenase	Activation/inhibition	Modulation of the inflammatory response
	Catalase	Inhibition	Potentiation of H_2O_2 -mediated cytotoxicity
	Cytochrome oxidase	Inhibition	Physiologic control of cell respiration
	NO synthase	Inhibition	Autoregulation of NO production; iNOS-mediated endothelial dysfunction (impaired relaxation)
	Hemoglobin	Formation of nitrosyl-Hb	Allosteric regulation of O_2 release by Hb
Non-heme iron	Thromboxane synthetase	Inhibition	Reduced formation of proinflammatory leukotrienes
Redox reactions	Hemoglobin	NO scavenging	Formation of metHb; NO catabolism; formation of nitrate
Hypervalent metals	Reduction	Reduced oxidant stress	
Fe-S clusters	Mitochondrial aconitase	Inhibition	Reduced mitochondrial respiration
Cytoplasmic aconitase	Inhibition	Reduced ferritin synthesis; increased transferrin receptor synthesis	
2) Free radicals	Lipid hydroperoxides	Scavenging	Reduced oxidant stress
Superoxide radical (O_2^-)	Formation of $ONOO^-$	Indirect effects (oxidation and nitration)	Formation of nitrate
3) Oxygen	Formation of N_2O_3	Indirect effects (nitrosation)	Formation of nitrite or nitrate

Adapted from references 18, 212.

1.4. Nitrate anion

1.4.1. Introduction

As early as 1890, the nitrate anion (but not nitrite) was identified as a constituent of saliva [350], and in 1916, Mitchell et al demonstrated that humans were capable of synthesizing nitrate anion [235, 236]. In 1981, the seminal experiments, carried out by Tannenbaum et al., demonstrated that nitrate anion synthesis was indeed a mammalian process [122-125, 335-338]. They found that excreted nitrate anion concentration surpassed the ingested amount in different experimental models e.g. in human, and in conventional and germ-free rats [358-362]. The latter rules out gut microflora participation in the reaction. Later studies in 1985 by Marletta et al. showed that LPS induced inflammatory cells, primarily macrophages, can synthesize nitrate anions both *in vitro* and *in vivo* [330-333]. Soon thereafter, evidences began to appear that NO is the precursor of nitrate anion [204-206, 235].

1.4.2. Potential role of the nitrate anion

The role of the nitrate anion in cell physiology/pathophysiology *in vivo* or *in vitro* is much more elucidated. However, many controversies remain. There are several reports suggesting that ingestion of nitrate anion or nitrated foods can cause clinically

toxic symptoms and signs such as methemoglobin, cardiovascular and respiratory instability and, in severe case, a fatal outcome [10, 21, 95, 120, 138, 164, 178, 226].

There is no clear cut line yet for the role of the different concentration of nitrate anion in different cell types, species, as well as its role in the physiologic and pathophysiological situations. Generally speaking, nitrate anions from μM to mM ranges show significant effects on the cellular membrane fatty acid composition (37°C for 60 min from 200 to 1000 μM) [176]. Nitrate anion at 5.9 μmol/ml per hour can significantly increase the muscle permeability as well as decrease muscle contractility from 27.8 (control) to 15.8 (*g*). The mechanism of this effect is not fully understood [145]. The Ca^{2+}, Mg^{2+}-ATPase activity in the membrane of rat red blood cells decreased depending on the sodium nitrate concentration from 20 to 200 μM and even further to 50 mM both under *in vivo* and *in vitro* experimental conditions [195]. The properties of high affinity nitrate transporter in a heterologous expression system are significantly modulated by changes in the membrane potential (e.g. 1 to 200 μM external nitrate anion), external pH and both intracellular and extracellular nitrate concentration. This transporter is very sensitive to the concentration gradient of nitrate anion across the plasma membrane, and the transporter belongs to a multigene family with members that have been identified in bacteria, fungi, plants and animals such as in rat brain and retina [387]. A relative wide nitrate anion concentration (from 5 μmol to 400 mM) had been used in the conventional electrophysiological experiments [16, 187]. Nitrate anion infused intravenously at 10, 20, 30, 40 μg/kg/min or 50 mg/kg/min can significantly increase the arterial pressure of dogs (117, 385). Other effects of the nitrate anion were also documented, such as its effects on the energy metabolism, lipid composition and membrane integrity etc. The above results

show that the effect of the nitrate anion on the cells in both physiological and pathophysiological situations is complex, and may depend on the type of cells or species, the pH of physiological solution, the time of exposure to nitrate, the concentration of nitrate or the compartment e.g. intra or extracellular site. Although the different nitrate concentrations were applied to different experimental models, the conclusion may be warranted that there must be a role for the nitrate anion in both the physiological and pathological conditions of the cells, tissues and organs.

Overall, several physiological and/or pathophysiological roles can be assigned to the nitrate anion, which include, but is not limited to, the effects of the nitrate anion on the cellular membrane potential [16, 162, 187, 213, 249, 387], ATPase activity [83, 243, 255, 345], membrane permeability [145], alteration of the growth of cells or animals, neuronal activity, depressed muscle contractility [16, 145], increased blood pressure [117, 333, 385] and/or impaired active calcium uptake by the sarcoplasmic reticulum [145]. Recently, experiments have suggested that the nitrate anion may pass through a distinct set of channels, or cotransportation of nitrate-H^+ in mammalian cells [35, 60]. NO also functions as a messenger molecule in both plant and microbes [54, 76, 79, 240, 244, 268, 285] in which nitrate anion channels/transporters were well documented [278, 285, 387]. Nitrate (NO_3^-), a trigonal oxyanion, is isosteric with the planar PO_3 moiety expected in a phosphotransfer transition state of protein tyrosine phosphatase (PTPase). It binds within the P-loop of PTPase and effects the closed conformation of the active site of PTPase [96]. Nonetheless, the nitrate anion itself (from nmol to mmol concentrations) do/may cause:

- Disturbance of energy metabolism as a result of the depression and the uncoupling of oxidation and phosphorylation in the mitochondria of the liver, kidney and heart of rats in a dose-dependent manner [186, 202, 229, 230];
- Disturbance of the absorption of glucose and glycine; decrease chloride and sodium reabsorption in the distal nephron [170, 267];
- Damaged cellular membrane integrity [195]; selective changes in the fatty acid composition of phosphatidylserine erythrocyte membrane [176, 185]; changes in nuclei, mitochondria [305], and secretory granules of the gastric endocrine cells [308];
- A major causative factor in the case of palindromic rheumatism [111, 363];
- Blocks both directly evoked muscle contraction and the neuromuscular transmission; reduces the norepinephrine-induced contractile response by 75%; impairs calcium uptake by sarcoplasmic membrane [145];
- Increased the toxicity of nitrate and nitrite due to exercise [128, 129, 165, 169];
- Significantly increase or decease transmembrane electrical potential [16, 59, 113, 213, 249, 387]; significantly decrease of the potassium influx in vessels [214];
- An increase in the blood pressure after cerebral-ventricular administration in rats [333] or I.V. in dogs [117, 385];
- A reduction in the activity of digestive enzymes in the small intestine mucosal layer and in the post-epithelium [301, 341, 345]; the response of the enzyme systems of the liver and kidney was heterogeneous [341]; induce ovulation disorders [356, 388];
- Effects on growth, erythrocytic count, liver and kidney functions, humoral and cell mediated immune responses in cockerels [116, 161, 241];

- Cytogenetic and histopathologic effects [292, 347]; i.e. induce chromosomal aberrations and micronuclei in rat and mouse bone marrow cells [342]; induction of liver tumors in Wistar rats [228]; a positive correlation between exposure to nitrate and gastric cancer death rates [40, 196, 203, 288, 327, 328 384].

1.4.3. Nitrate anion concentration: the best marker of sepsis?

The majority of evidence concludes that NO metabolites, especially the nitrate anion, are significantly increased in the different organs (e.g. plasma, heart and lung) of septic animals and patients [3, 12, 31]. NO itself is also significantly increased after LPS administration such as in the lung parenchyma (rats) and in the exhaled air of lungs (dog and human) [133, 150].

NO released from rat endothelial cells range from control levels being hardly detectable, to ~50 nM to ~950 nM (stimulated with LPS), which was the highest at the cell membrane (950 nmol), decreasing exponentially with distance from the cell, and becoming undetectable at distances beyond 50 μm [46, 47]. The amount (peak rate) of NO release in bovine aortic endothelial cells increased from ~0.08 to ~3.80 pmol/s under shear stress conditions, and the kinetics of shear stress-induced NO release is ~2.23 nmol/L per second measured with a porphyrinic microsensor [31, 36, 65, 114]. An acute burst of NO release (measured by a porphyrinic sensor via a catheter/needle which was inserted into the lung parenchyma approximately 3 to 5 mm) from cNOS was observed in the experimental septic model of rats, peaking at 10 to 15 minutes after LPS I.V. infusion, mirroring a coincident peak drop in arterial pressure. NO concentration declined

over the next hour to twice the pre-LPS infusion levels, where it remained until the rats died, 5 to 6 hours after LPS infusion. However, the chronic drop in arterial pressure observed from 70 minutes to 6 hours after the start of LPS infusion was not convincingly mirrored by a chronic increase in NO concentration, even though indirect NO assay (Griess method, assaying NO metabolites nitrate and nitrite anions) showed that NO production was increasing as a result of continuous NO release by iNOS [132, 344]. These data show that NO levels ranging from the undetectable level (< ~1 nM) to ~50 nM to ~950 nM *in vivo* or *in vitro* can be released from different species e.g. rats, rabbits, dogs, or humans as well as from different cells e.g. myocytes, endothelial and smooth muscle cells [148, 149, 231-233]. Additionally, at least 37% of NO produced can be consumed in chemical reactions in the site of tissues e.g. aorta [32]. Nevertheless, the NO level does not necessarily reflect the level of its metabolites, such as nitrate and nitrite anions, during endotoxemia. In other words, NO is one thing, nitrate anion is another thing.

The nitrate anion is the major metabolite of NO *in vivo* and its half-life is 1.5 to 8.0 hours [61, 101-103, 256, 257, 353, 385]. In comparison to the half-life of NO (3 to 20 s [108, 109, 304]; recently an experiment has documented that the half-live of NO is ~ 2 ms *in vivo* [400]), the half-life of nitrate anion is at least 270 to 9,600 times longer than NO *in vivo*. Therefore, nitrate anion concentrations in tissues indicate only past NO production. In other words, the nitrate anion concentration may be 270 to 9,600 times higher than NO at transient time points *in vivo* depending on half-life factors of both molecules. Other factors, such as the rate of production and elimination, superoxide anion concentration, and NOS activity, could also contribute to further affect this NO-nitrate

anion concentration difference [277, 282]. There is experimental evidence that support this view as NO concentrations vary from undetectable (~pM) to ~50 nM to ~950 nM, and nitrate anion concentrations vary from ~μM to ~mM *in vivo* depending on experimental conditions such as under normal or sepsis/SIRS/MODS.

Like NO, peroxynitrite has recently been associated with both deleterious and beneficial effects [223]. Peroxynitrite is formed almost exclusively at equimolar concentration of NO and superoxide [30]. Thus, the maximally achievable concentration of peroxynitrite is limited by the highest amount of either NO or superoxide produced *in vivo*. Physiological concentrations of NO occur in the range from undetectable level to ~ 1-5 nM, and pathophysiological concentrations of NO occur in the range from 50 to 950 nM, which is measured directly by NO sensors in the site of cell membranes [226-233]. Accordingly, maximal concentration of peroxynitrite formed *in vivo* may probably be very hard to exceed the 2-5 μM range [229]. *In vitro* experiments have shown that 3-morpholinosydnonimine (SIN-1), which simultaneously produces equimolar amounts of NO and superoxide, generates peroxynitrite at a rate roughly equivalent to 1% of the SIN-1 concentration at pH 7.4 and 37°C, e.g. 1 mM SIN-1 produces 10 μM peroxynitrite [30]. In addition, nitrotyrosine formation, which was thought to be the "footprint" of *in vivo* formation of $ONOO^-$, is now clearly shown to be primarily due to neutrophil myeloperoxidase-driven nitryl chloride and nitrogen dioxide formation in disease where neutrophils are present at sites of inflammation [270]. Therefore, the presence of nitrotyrosine can no longer be considered a reliable index of $ONOO^-$ production *in vivo*. Furthermore, several recent studies have showed that peroxynitrite at concentrations from 3 to 30 μM have significant beneficial/protective effects on the endothelium and

myocardium *in vivo* [208, 223, 229, 270]. Moreover, nitration-denitration has been proposed similar to the phosphorylation-dephosphorylation process of cell as a post-translational modification of protein [172]. Protein nitrotyrosine residue has been found in many tissues and cells under control conditions, as well as displaying dynamic processes in septic rats, e.g. LPS can induce an increase or a decrease in nitrotyrosine content of some select proteins *in vivo* or *in vitro* [171, 172]. This reaction may be metabolized by a putative "nitrotyrosine denitrase" in tissues or cells and is perhaps inducible. This "enzyme" reverses selectively protein nitration [172].

There is compelling evidence that nitrate anion concentrations are significantly increased in different pathophysiological conditions, such as sepsis, ischemia/reperfusion, cardiac failure, trauma or burn (Table 3). However, some controversial evidences also exist [159]. Nonetheless, several recent investigations demonstrated that nitrate anion concentration was a positive predictive value of 96% for septic patients and this was a better predictor than TNF-alpha, IL-6, or Acute Physiology And Chronic Health Evaluation (APACHE) II score in both adult and pediatric patients, respectively [165, 173].

Table 3. The comparison of some properties of NO, Peroxynitrite and nitrate:

	Nitric Oxide	Peroxynitrite	Nitrate
Chemical structure	NO•	$ONOO^-$ or ONOOH	NO_3^-
Substrate	L-arginine and O_2	$NO + O_2^-$	ONOO- and/or NO + O_2, NO_2^-
Catalyst	NOSs	? Peroxynitritase	?
Targets	Enzyme, proteins, amino acids etc.	Enzyme, protein, DNA, Lipids etc.	Enzyme, protein, Lipids and DNA
Normal Plasma Concentration	1 to 5 nM	Undetectable ?	20 to 100 μM
Intracellular concentration	? 1 to 5 nM or or undetectable	? 1 to 5 nM or undetectable	20 to 200 μM
Sepsis conditions	5 to 950 nM	? 5 to 950 nM	100 to 2000 μM
Half-life *in vivo*	~2 ms [400]	~2 μs	1.5 to 8 hours
Anion exchange 1	yes	yes	yes
Beneficial effects	yes	yes	?
Detrimental effects	yes	yes	yes

1.4.4. Measurement of nitric oxide and nitrate anion

NO diffuses readily into nearby cells [199] and decomposes spontaneously and quickly to nitrate anion *in vivo* [30]. The potent biological effects and short half-life of NO make its detection and direct measurement difficult.

Understanding the role of NO in physiology and pathophysiology has been hindered by the difficulties in measuring the very low concentrations (~pM to ~nM) of the short-lived NO [346]. Several assays are available that can directly or indirectly measure NO, such as by chemiluminescence [70, 379], electron paramagnetic resonance (EPR) [11], gas chromatography [179, 209], high-performance liquid chromatography (HPLC) [188, 210, 251], mass spectroscopy, microelectrode, or measuring nitrite or nitrate anions (e.g. Griess reagent) [41, 67, 68], citrulline [57], cGMP, methemoglobin [372], and biological activity of NO via bioassay [24]. However, the advantages and disadvantages of each technique should be considered carefully before selecting a specific and suitable method for different applications [11, 179]. Nonetheless, chemiluminescence remains the most sensitive and suitable technique in the detection of NO, nitrite and nitrate anion for a variety of samples e.g. gas (inhaled or expired), liquid (plasma, urine, cerebrospinal fluids [CSF]) or tissue samples (supernatant of homogenates) [379, 385]. Exogenous nitrate may contribute to total amount of nitrate concentration measured *in vivo* and this effect may vary depending on the amount of nitrate in diet [99, 143, 293, 299, 358, 351, 364, 366].

Briefly, the detection of NO, as well as nitrate and nitrite anions, by chemiluminescence are based on the fact that ozone interacts with NO to generate light "chemiluminescence", which is measured by a sensitive photomultiplier tube (PMT), the light being directly proportional to NO levels [11, 70]. If the specimen is gaseous, one can simply inject the sample into the reaction purge vessel. If the specimen is a fluid, following injection of the sample into the purge vessel, the NO in the sample is purged into the detector by bubbling the solution with an inert gas, e.g. nitrogen, under vacuum conditions. Nitrite or nitrate anions in samples can also easily be converted to NO in the purge vessel which contain a reducing reagent solution such as 1% KI or NaI for selective conversion of nitrite anion to NO at room temperature, and V_3Cl or Ti_3Cl for conversion of nitrite plus nitrate anions to NO at high temperature (85 to 100°C), respectively. (Figure 1 on page 50 shows a schematic representation of a chemiluminescence NO analyzer). Chemiluminescence is one of the most sensitive (1 pmol), specific and reproducible techniques to determine NO concentration. Some disadvantages may exist (such as the cost of the chemiluminescence analyzer). After EDRF has been identified as NO by this technique [154, 278], many applications have been made using chemiluminescence to measure NO, nitrite and nitrate anion concentrations in different samples.

1.5. One more question and one more hypothesis

At the turn of a new millennium, a number of questions regarding NO biology still remain unanswered, the most challenging and confusing problem being set by the

ambivalent character of NO [97, 252-254]. It has always been challenging and controversial to ascribe a causal role for reactive nitrogen species in SIRS [63, 98]. The emergence of NO as an essential messenger molecule in physiological conditions and as a critical mediator in pathophysiological states have both complicated and shed new light on understanding key mechanisms of SIRS and MODS, as well as generated new controversies. Nitrogen reactive species and the action of these molecules are much more multifaceted than previously thought [212, 306].

NO either acts as a direct signaling messenger and cytoprotective molecule, or as an indirect cytotoxic effect, via the formation of various nitrogen species, e.g. peroxynitrite and ultimately nitrate anion [30, 316, 317, 324]. It is certain that NO plays a significant defensive/damage role during the process of sepsis/SIRS/MODS. However, the nitrate anion is absolutely the major and longest-lasting end-product of NO *in vivo* and accordingly this phenomenon may raise one more question: does the nitrate anion represent another facet of NO *in vivo*? Since the nitrate anion itself is a (at least partially) permeant anion that can cross the plasma membrane and influence cell electrophysiological properties, membrane enzyme (e.g. ATPase) activity *in vitro*, depressed muscle contraction, and increased blood pressure *in vivo,* we therefore propose one more hypothesis: over-accumulated nitrate anions may contribute to septic shock and multiple organ dysfunction during LPS-induced sepsis/SIRS/MODS. The redox products of NO, and their chemical reactions with biological molecules have been studied extensively in the last few years. Interestingly, as the chemistry of NO reveals its richness, the pharmacological and pathophysiological roles of NO become more diverse as well as confusing. To date, there is no satisfactory explanation as to why

supplementation of exogenous NO as well as inhibition of endogenous NO both attenuate tissue injury and a great number of questions in the field of NO and its metabolites remain to be answered. Understanding the molecular mechanisms of one of the most common as well as important NO products in the pathogenesis of sepsis - nitrate anion, perhaps also the most underestimate molecule during sepsis, - may contribute to innovative and more effective therapies. It is likely that an interplay between NO, O_2^-, $ONOO^-$ and nitrate anion takes place during sepsis and that the phenomena of synergism, priming and sensitisation are of great clinical relevance. Molecular mechanisms of septic or endotoxic shock, therefore, should not only aim at NO, O_2^-, $ONOO^-$, but also at breaking the synergistic action of the different bacteria- and host-derived harmful factors, probable including nitrate anion. The results of the present study may offer an alternative explanation for some apparently contradictory results on the role of NO in sepsis/SIRS/MODS.

A series of four manuscripts are included in this thesis to address the above-mentioned hypothesis: (1). to establish the state of art technique for quantifying nitrate concentration in different tissues and organs such as plasma, aorta and heart in the 1st manuscript. (2). to explore whether or not exogenous NO e.g. NO donor and inhaled NO would alter the concentration of nitrate anion in different tissues and organs such as in the plasma, diaphragm and lungs and its potential role in the 2nd manuscript. (3). to study that LPS-induced endogenous NO-derived nitrate anion may synergistically contribute to the detrimental effects of NO and/or peroxynitrite on the impairment of respiratory function during endotoxic shock in the 3rd manuscript. (4). to investigate further that LPS-induced endogenous NO-derived nitrate anion may synergistically contribute to the

detrimental effects of NO and/or peroxynitrite on the impairment of multiple organ function during endotoxic shock in the 4th manuscript. The evidence presented here shows that LPS induced a significant increase in nitrate anion concentration in organs and plasma as well as impairing diaphragm force generation and cell membrane integrity. The nitrate anion itself also caused the impairment of diaphragm force generation *in vitro* that did mimic, to some extent, LPS-induced impairment of respiratory function *in vivo*. The levels of nitrate anion in the heart, lung, diaphragm, aorta and other organs were paralleled with the alteration of hemodynamic and respiratory functions, tissue morphology and cellular permeability, and tissue MPO activity after endotoxin treatment in rats. From these results, we conclude that the nitrate anion may contribute synergistically to the detrimental effects of NO and/or peroxynitrite on the impairment of multiple organ function during endotoxic shock. Thus, it is reasonable to assume that cellular function modified by NO-derived nitrate anion may contribute to the multiple-organ functional alterations observed in SIRS and MODS during endotoxic shock.

2. MATERIALS AND METHODS AND RESULTS (MANUSCRIPTS)

2.1. *Manuscript 1*: Effects of reducing reagents and temperature on conversion of nitrite and nitrate to nitric oxide and detection of NO by chemiluminescence. Fan Yang, Eric Troncy, Martin Francœur, Bernard Vinet, Patrick Vinay, Guy Czaika, and Gilbert Blaise. (*Clinical Chemistry*, 1997, 43(4):657-662.)

Nitric oxide (NO) plays a pivotal role in both physiological and pathophysiological states such as cardiovascular and respiratory functions as well as inflammation, immunity and neurotransmission. NO is a small, gaseous, paramagnetic, relatively active molecule as well as in low concentration (~pmol to ~nmol) *in vivo* and is rapidly destroyed by O_2 or O_2^- or other molecules, and consequently NO produces its major metabolite, namely nitrate anion, particularly *in vivo*.

Measurement of NO and of selectively its metabolites such as nitrite (NO_2^-) and (or) nitrate (NO_3^-) concentrations in biological systems is a challenging analytical problem. Although several techniques (Spectrophotometry, HPLC, Mass spectrometry) are available, chemiluminescence is highly sensitive, selective, and accurate method for measurement of NO, especially at low concentration. Several reducing reagents have been tested for the reduction of NO_2^- and NO_3^- to NO, however, a systemic evaluation of different reducing agents and optimal temperature condition for the conversion of NO_2^- and (or) NO_3^- to NO has not performed as well as the recovery of NO_2^- and NO_3^- from plasma. In this study, we

examined the efficiency of five reducing agents: [V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III)] to reduce a different amount (100-500 pmol or 0.4-2 μmolar) of NO_2^- and (or) NO_3^- to NO, and the effect of temperature from 20, 30 ... to 90°C on the conversion of a fixed amount of NO_2^- or NO_3^- (400 pmol or 1.6 μmolar) to NO as well as the recovery of NO_2^- or NO_3^- from plasmas of pig and of dog.

Abstract

To measure the concentration of nitrites and nitrates by chemiluminescence, we examined the efficiency of five reducing agents: [V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III)] to reduce nitrite (NO_2^-) and (or) nitrate (NO_3^-) to nitric oxide (NO). The effect of each reducing agent on the conversion of different amounts of NO_2^- and (or) NO_3^- (100 to 500 pmol, representing concentrations of 0.4 to 2 μmolar) to NO was determined at 20°C for NO_2^- and at 80°C for NO_3^-. The effect of temperature from 20 to 90°C on the conversion of a fixed amount of NO_2^- or NO_3^- (400 pmol or 1.6 μmolar) to NO was also determined. These five reducing agents are similarly efficient for the conversion of NO_2^- to NO at 20°C. V(III) and Mo(VI) + Fe(II) can completely reduce NO_3^- to NO at 80°C. NaI and Cr(III) were unable to convert NO_3^- to NO. Increased temperature facilitated the conversion of NO_3^- to NO, rather than that of NO_2^- to NO. We evaluated the recovery of NO_2^- and NO_3^- from plasma of pig and of dog. Recovery from plasma of both animals was reproducible and near quantitative.

INDEXING TERMS: endothelium-derived relaxing factor • nitric oxide synthase • free radical • vasodilation • inflammation • thrombosis • immunology • neurotransmission

Nitric oxide (NO) is a free radical that reacts rapidly with several molecules *in vitro* or *in vivo* to form mainly nitrite (NO_2^-) and nitrate (NO_3^-). Interest in NO, NO_2^-, and NO_3^- measurements has increased exponentially with the discovery that NO or a chemically related compound plays a major role in vasodilation, inflammation, thrombosis, immunology, and neurotransmission *[73]*. Measurements of NO_2^- and NO_3^- are also important in clinical chemistry as markers of nitric oxide synthase activity *[256]*.

Measurement of NO concentration in biological systems is a challenging analytical problem *[11,179]*. The chemiluminescence detector-based method for trace NO_2^- and (or) NO_3^- in aqueous samples was first reported by Cox *[70]* and was later widely applied *[144, 303, 310]*. This earliest and most commonly applied method is based on the conversion of NO_2^- to NO at room temperature by an acetic acid-sodium iodide [NaI] mixture. Ammonium molybdate [Mo(VI)] with ferrous ammonium sulfate [Fe(II)] in hot, 50% concentrated sulfuric acid, was used for the reduction of both NO_2^- and NO_3^- to NO. NO_3^- was then determined as the difference of results obtained by the two methods. Vanadium(II) [V(II)] was mentioned as a possible reducing agent in the initial work by Cox *[5]*, who reported that it reduces NO_3^- to NO. Later work by Braman and Hendrix *[43]* indicated that it is V(III), not V(II), that reduces NO_3^- to NO. Stronger reducing agents such as chromium(II) [Cr(II)] and titanium(III) [Ti(III)] could also reduce NO_3^- to NO *[71, 216]*. However, a systematic evaluation of different reducing agents and temperature conditions for the conversion of NO_2^- and NO_3^- to NO has not been performed. We compared the efficiency of V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III) at different temperature conditions (20, 30, ... to 90°C) for the conversion of NO_2^- and (or) NO_3^- to NO. We also evaluated the recovery of NO_2^- and NO_3^- from plasma of pig and of dog.

Materials and Methods

The NO concentration in samples was detected by chemiluminescence with a Sievers 270B NO analyzer (Sievers Instruments, Boulder, CO). The detector is based on the reaction of NO with ozone (O_3) to give nitrogen dioxide in an excited state (NO_2*) plus molecular oxygen. The excited state of NO_2* decays to give a weak infrared chemiluminescence above 600 nm. A microreaction purge vessel coupled with a condenser and heating jackets permitted introduction of the sample directly into the reduction solution via a gas-tight syringe (Hamilton, Reno, NV). The condenser jacket temperature was controlled by a continuous flow of cold water while the temperature of the heating jacket was controlled by a continuous flow of warm water regulated by a Haake constant-temperature circulating bath model D1-L (Fisher Scientific, Montréal, QC, Canada). Nitrogen, at a rate of 100 ml/min, was used as the carrier gas of NO to the NO analyzer. The flow into the reaction chamber of the NO analyzer could be adjusted with a needle valve placed between the filter (Nupro Co., Willoughby, OH) and the NO analyzer. A MacLab data acquisition system (ADInstruments Pty, Castle Hill, NSW, Australia) was used to collect and report the data as area under the curve response from baseline to baseline *[43]* (Fig. 1). We did not detect any NO_2^- or NO_3^- in the blanks used in this study.

REAGENTS

NO gas and nitrogen were purchased from Air Liquide (Canada), Montréal, QC, Canada. Vanadium trichloride, and sodium nitrite and nitrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chromium trichloride, titanium trichloride, ferrous ammonium sulfate, ammonium molybdate, sodium iodide and other reagents were purchased from Fisher

Scientific. All were reagent-grade quality and used without further purification. High-purity distilled water was used in the preparation of all solutions.

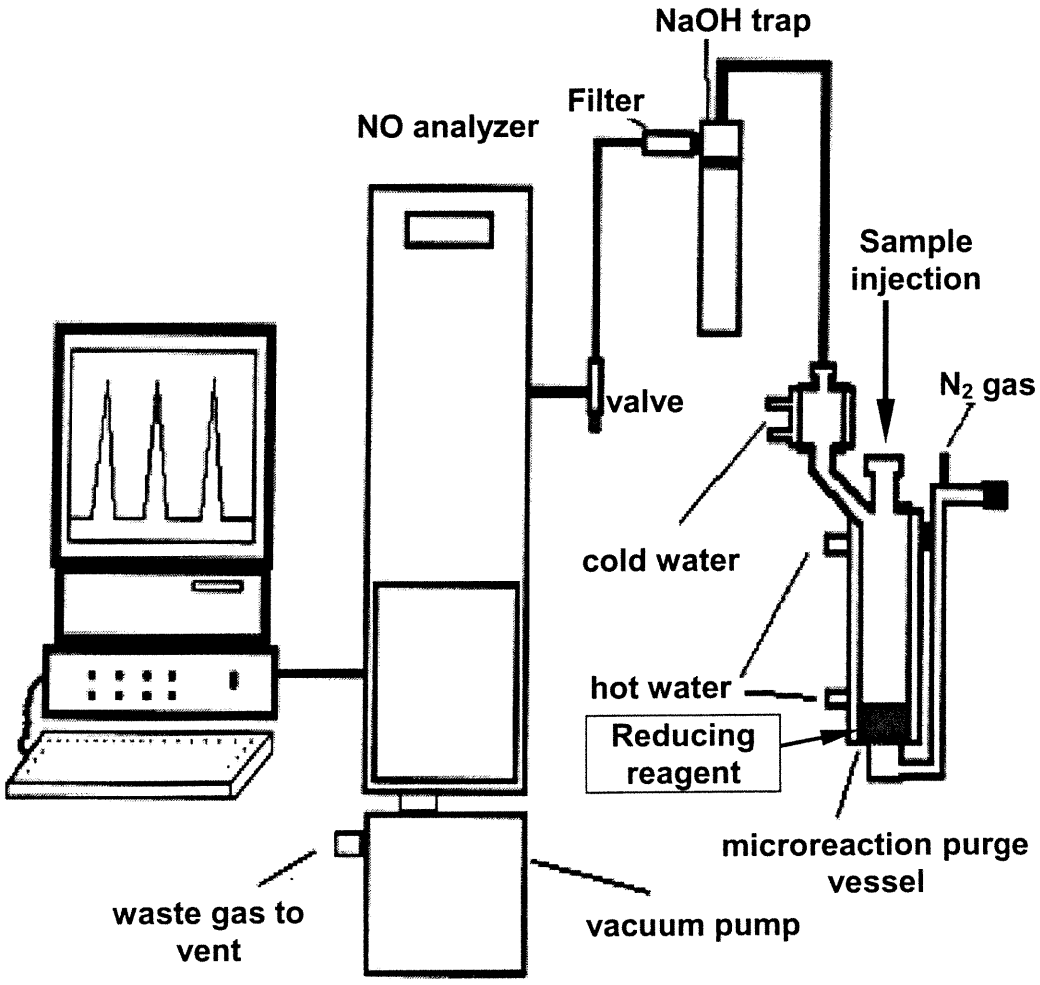


Fig. 1. Schematic of the analysis system

RECOVERY FROM AQUEOUS SOLUTION

V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(II) have been reported as reducing agents for the conversion of NO_2^- and (or) NO_3^- to NO *[43, 70, 71, 90, 136, 144, 216, 303]*. Except for Cr(II) for which we substituted Cr(III) with its three valences, all of these reducing agents were prepared as reported. Cr(III) was prepared in the same conditions as the other two reducing agents with three valences. Samples of inorganic NO_2^- ($NaNO_2$) or NO_3^- ($NaNO_3$) in 250 µl of water (100, 200, ... to 500 pmol) were injected into the microreaction purge vessel containing 5 ml reducing agent solution, and the quantity of produced NO was measured after conversion by each reducing agent at 20°C for NO_2^- and at 80°C for NO_3^-. The effect of temperature on the conversion of 400 pmol of NO_2^- and (or) NO_3^- (in a volume of 250 µl) to NO with each reducing agent was determined by changing the temperature scale from 20, 30, ... to 90°C. The chemiluminescence analyzer was calibrated by injecting known amounts of NO gas (100, 200, ... to 500 pmol) through the microreaction purge vessel heated at different temperatures in the absence of reducing agent solution. Data were collected as area under the curve response from baseline to baseline and divided by the mean standard response of NO gas for each concentration. From this we obtained a recovery factor (R) expressed in percentage. Serial measurements can be performed for each NO_2^- or NO_3^- concentration without changing the reducing agent solution in the microreaction purge vessel, since the volume of added samples is very small compared with the volume of the reducing agent solution.

RECOVERY FROM THE PLASMA

We added known amounts (100, 200, ... to 500 pmol) of inorganic NO_2^- or NO_3^- in 0.1 ml of

pig and dog plasma and then measured baseline amounts of NO_2^- and (or) NO_3^- to evaluate their recovery. Excessive foaming in the microreaction purge vessel caused by plasma proteins interfered with the reduction process. Therefore, all determinations in plasma samples were performed after deproteinization. Plasmas were diluted 10-fold with distilled water and deproteinized by addition of $1/20^{th}$ volume of zinc sulfate to a final concentration of 15g/L. After centrifugation at 1000*g* for 15 min, 0.1 ml of supernatant was applied to the microreaction purge vessel containing NaI solution at 30°C for the conversion of NO_2^- to NO, or V(III) solution at 80°C for the conversion of $NO_3^- + NO_2^-$ to NO. Samples of NO_2^- or NO_3^- added in plasma were compared with those prepared in distilled water in the same condition. This method only detects NO_2^- and NO_3^- that can readily pass into the gas phase to react with the O_3 in the microreaction purge vessel. Thus, any of the NO formed *in vivo* that would react with thiol groups in low-molecular-mass compounds is not detected here.

STATISTICAL ANALYSIS

Unless otherwise stated, all data are expressed as the mean ± SE of R. Global mean indicates the mean of all values of R calculated for each of the five agents with all concentrations of inorganic NO_2^- or NO_3^- used. The statistical analyses were done with the SAS statistical analysis program. The significance level was set at 0.05. The analysis of variance (GLM procedure) was used to compare values of global means (P_1) for the five reducing agents. The analysis was repeated for each amount of samples used (100-500 pmol). The *post hoc* analysis between the five reducing agents was realized by using the Student-Newman-Keuls (SNK) tests. The effect of temperature and agents on R values was analyzed using the two-

way analysis of variance (P_2). The SNK *post hoc* analysis was used to compare R values for each agent at different temperatures and R values for different agents at the same temperature. The optimal reduction temperature(s) was (were) determined as the temperature(s) with the highest R value significantly different from the other R values. Separate paired *t*-tests were used for dog and pig plasma when the recovery of NO_2^- was compared with that of NO_3^- (P_3).

Results

EFFECT OF DIFFERENT REDUCING AGENTS ON CONVERSION OF NO_2^- TO NO AT 20°C

Analysis of variance indicated a difference on the global mean of the five agents (P_1=0.0001). The *post hoc* analysis distinguished two groups: one, comprising Mo(VI) + Fe(II) and Ti(III), had higher recovery rates than the second, comprising V(III), NaI, and Cr(III). The same two groups were identified for each amount (100-500 pmol) of nitrite and nitrate. However, the difference between both groups was not statistically significant when the global mean values for the five reducing agents were compared. This analysis is summarized in Table 1.

EFFECT OF TEMPERATURE ON CONVERSION OF 400 PMOL OF NO_2^- TO NO

Temperature had no effect on the detection of NO gas by chemiluminescence (data not shown; $P_1 = 0.63$). Recovery of NO from NO_2^- ($P_2 = 0.01$) by the five reducing agents was affected by temperature (Fig. 2). For NaI, the lowest R value (89.1%) was obtained at 20°C (SNK $P<0.05$) and no significant difference was found between the other temperature values.

Table 1. Effect of different reducing agents on conversion of NO_2^- to NO at 20 °C.

NO_2^-, pmol	V(III) (n = 5)	Mo(VI) + Fe(II) (n = 4)	NaI (n = 4)	Ti(III) (n = 5)	Cr(III) (n = 5)
100	92.3% ± 1.9%	94.7% ± 1.7%	89.2% ± 1.2%	96.5% ± 1.3%	86.2% ± 1.6%
200	91.6% ± 0.7%	94.8% ± 0.7%	92.2% ± 0.8%	94.4% ± 0.7%	91.2% ± 1.0%
300	92.9% ± 1.2%	97.3% ± 0.7%	93.4% ± 0.9%	96.4% ± 0.6%	93.1% ± 1.1%
400	93.3% ± 1.5%	98.2% ± 0.6%	95.1% ± 0.9%	97.5% ± 0.7%	94.6% ± 1.1%
500	93.9% ± 1.8%	98.8% ± 0.9%	97.1% ± 0.8%	98.3% ± 0.8%	95.5% ± 1.0%
Global mean	92.8% ± 0.6%	96.7% ± 0.5%	93.4% ± 0.5%	96.6% ± 0.4%	93.5% ± 0.6%

n = number of separate experiments (triplicate measurements of each sample).

Recoveries are expressed as relative percentages calculated from the area under the response curve from baseline to baseline of NO gas as 100.0% recovery (mean ± SE).

For other agents, the optimal reduction temperature was: Mo(VI) + Fe(II) at 50-60°C; V(III) at 60-80°C; Cr(III) at 20-70°C; and Ti(III) at 20-60°C. The comparison between agents showed that the R values with NaI and Mo(VI) + Fe(II) were higher than the R values of the three other agents at 50-60°C (SNK $P<0.05$).

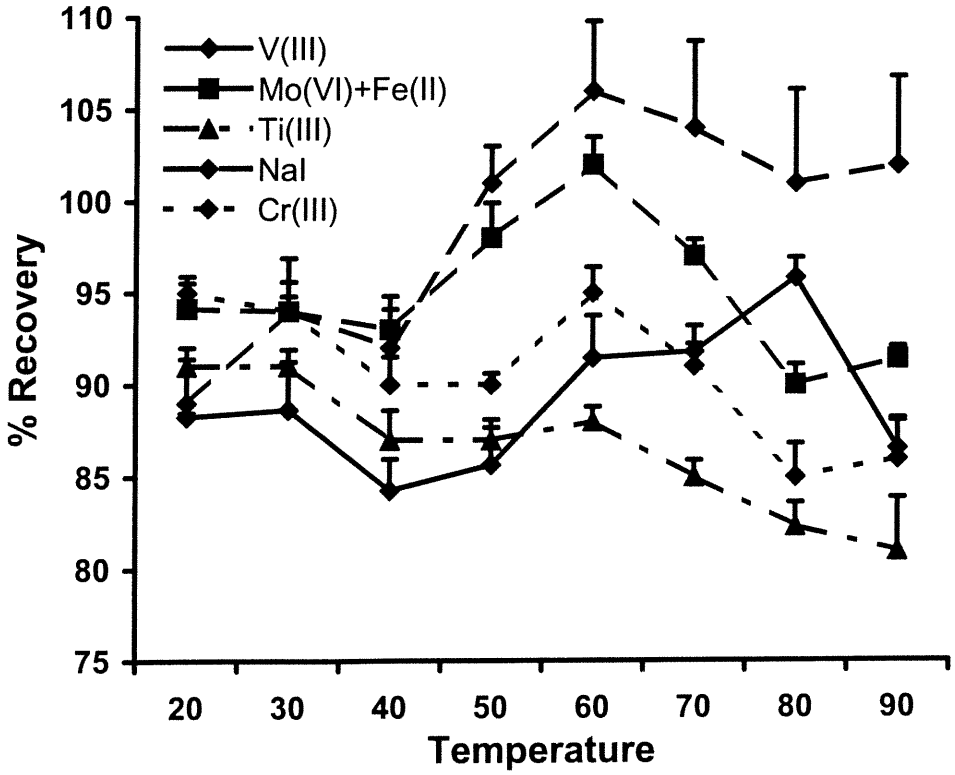


Fig. 2. Effect of temperature on conversion of 400 pmol of NO_2^- to NO by the five reducing agents. Each point was determined from three separate experiments and triplicate measurements were made for each sample.

EFFECT OF DIFFERENT REDUCING AGENTS ON CONVERSION OF NO_3^- TO NO AT 80°C.

The recovery of NO from different amounts of NO_3^- (100-500 pmol) was <1.7% when using NaI and Cr(III) as reducing agents. Often, no conversion was detected. The global mean of the R value for Ti(III) was 82.3% ± 1.8% whereas those for V(III), and Mo(VI) + Fe(II) were respectively105.8% ± 1.6% and 101.1% ± 2.8%. The R values obtained for each amount of NO_3^- are summarized in Table 2.

EFFECT OF TEMPERATURE ON CONVERSION OF 400 PMOL OF NO_3^- TO NO

Temperature affected the recovery of NO from NO_3^- by V(III), Mo(VI) + Fe(II), and Ti(III) ($P_2 = 0.0001$; Fig. 3). The optimal reduction temperature was 80-90°C for V(III) and 70-90°C for Mo(VI)+Fe(II) and Ti(III). Temperature did not affect the low recovery (<1.7% at any temperature tested) of NO from NO_3^- by NaI or Cr(III).

Table 2. Effect of different reducing agents on conversion of NO_3^- to NO at 80 °C.

NO_3^-, pmol	V(III) (n = 4)	Mo(VI) + Fe(II) (n = 3)	Ti(III) (n = 3)	NaI (n = 3)	Cr(III) (n = 3)
100	88.6% ± 3.0%	73.3% ± 3.1%	77.4% ± 3.5%	ND	ND
200	94.4% ± 2.6%	92.2% ± 5.5%	75.7% ± 4.5%	ND	ND
300	101.9% ± 2.7%	101.5% ± 5.4%	76.1% ± 5.6%	ND	ND
400	109.2% ± 1.5%	106.7% ± 5.7%	84.1% ± 1.3%	0.8% ± 0.1%	1.0% ± 0.2%
500	114.5% ± 0.6%	106.5% ± 4.2%	88.5% ± 3.0%	1.5% ± 0.3%	1.7% ± 0.3%
Global mean	105.8% ± 1.6%	101.1% ± 2.8%	82.3% ± 1.8%	1.5% ± 0.2%	1.6% ± 0.3%

n = number of separate experiments (triplicate measurements of each sample).

Recoveries expressed as percentage based on the area under the response curve from baseline to baseline of NO gas as 100.0% recovery (mean ± SE). ND = not detectable.

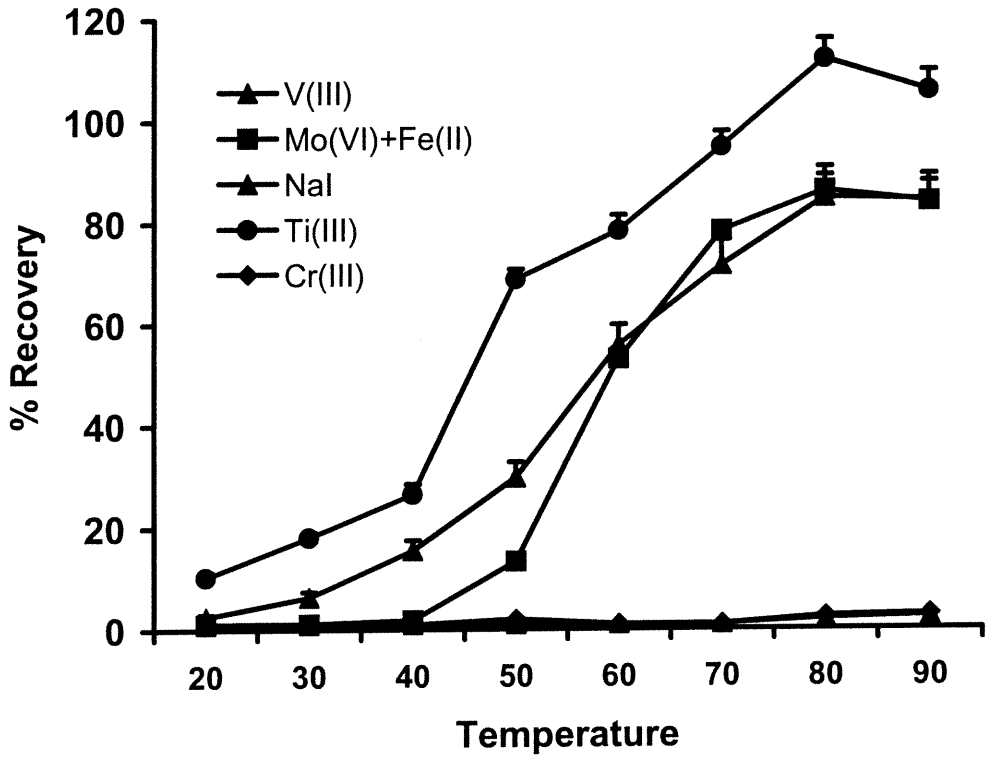


Fig. 3. Effect of temperature on conversion of 400 pmol of NO_3^- to NO by the five reducing agents. Each point was determined from three separate experiments and triplicate measurements were made for each sample.

RECOVERY FROM PLASMA

For pig plasma, the recovery of NO_2^- was 96.4% ± 1.9% (n = 20) and the recovery of NO_3^- was 104.3% ± 4.9% (n = 20) over the entire concentration range tested (100-500 pmol). There was no significant difference between the recovery of NO_2^- and NO_3^- ($P_3 = 0.21$). For dog plasma, the recovery of NO_2^- was 89.6% ± 2.0% (n = 20) and the recovery of NO_3^- was 107.4% ± 2.4% (n = 20) over the entire concentration range tested (100-500 pmol). The difference between the recovery of NO_2^- and NO_3^- was significant ($P_3 = 0.0008$).

Discussion

NO is a highly reactive messenger molecule that readily diffuses through plasmalemma to exert its biological activity in a variety of cells *[70]*. Several biological actions are attributed to NO via its activation of soluble guanylate cyclase, which catalyzes the transformation of GTP to cGMP. This transformation in turn activates a classical second messenger system that relays signals from the cell exterior to cell interior. Determination of increased NO formation is therefore of the utmost interest *[11, 43, 310]*.

Determination of NO in itself is difficult because of its free-radical nature and short half-life. NO reacts rapidly with oxygen to form NO_2^- and with superoxide or with oxyhemoglobin to form NO_3^-. In most cell culture systems *[256]*, NO will be oxidized primarily to NO_2^-, whereas in animal models and human samples, NO is oxidized both to NO_2^- and NO_3^-. Nitrite and nitrate are both stable in frozen plasma for at least 1 year *[256]*. Therefore, determination of the stable end products of the NO radical is most often used to measure its concentration. NO_3^- is the major metabolite of NO in blood *[256]*; Thus,

determination of NO_2^- alone as a marker for NO concentration is meaningless, even if some previous studies had reported NO_2^- as the major byproduct of NO in blood *[147]*. The availability of a quantitative assay for both NO_2^- and NO_3^- can facilitate further elucidation of some of the physiological, pathophysiological, pharmacological, and therapeutic roles of NO.

Compared with other analytical methods *[11, 179, 256]* (e.g., spectrophotometry, electron paramagnetic resonance, gas or liquid chromatography, mass spectrometry), chemiluminescence is highly sensitive, selective, and accurate for NO_2^- and (or) NO_3^-, especially at the low concentrations in complex matrices found in water, food, and biological fluids. The key point in this procedure is to choose the appropriate reducing agent/temperature combination to selectively and completely reduce NO_2^- or NO_3^- to NO.

Several reducing agents have been tested for the reduction of NO_2^- and (or) NO_3^- to NO, such as NaI for the conversion of NO_2^- to NO, and V(III), Mo(VI) + Fe(II), Ti(III), and Cr(II) for the conversion of NO_3^- to NO *[43, 70, 71, 90, 136, 216]*. NO_2^- can be reduced to NO by using most reducing agents at room temperature, whereas conversion of NO_3^- to NO requires both a strong reducing agent and high temperature. V(III) and Ti(III) with three valences can reduce most of NO_3^- to NO at high temperature. We compared the efficiency of Cr(III), also with three valences, with V(III) and Ti(III) for the conversion of NO_2^- or NO_3^- to NO.

Our work revealed that the five reducing agents have a similar efficiency for the conversion of NO_2^- to NO at 20°C, with a slight advantage for Mo(VI) + Fe(II) and Ti(III) over the other three agents. The recovery of NO from NO_2^- was almost complete, compared with the known amount of NO gas standard [lower R value: 86.2% ± 1.6% for Cr(III) for the recovery of 100 pmol NO_2^-].

V(III) and Mo(VI) + Fe(II) were equally efficient in converting NO_3^- to NO at 80°C and recovery of NO was nearly complete. However, recovery with Ti(III) was lower. NaI and Cr(III) were unable to reduce NO_3^- to NO, as only trace amounts of NO were recovered from NO_3^- regardless of the amount of NO_3^- and the temperature used. NaI and Cr(III) can thus be considered selective for reducing NO_2^- to NO. To our knowledge, this is the first report to show that Cr(III) can selectively reduce NO_2^- to NO. Enzymatic reduction of NO_3^- by using an immobilized *Escherichia coli* nitrate reductase column *[115]* converts ~30% of NO_3^- to NO_2^-. Another assay based on the coupled oxidation of NADPH during the enzymatic conversion of NO_3^- to NO_2^- by *Aspergillus* nitrate reductase only yields ~64% of serum NO_3^- to NO_2^- and is unsatisfactory for NO_3^- analysis in serum samples. Furthermore, though many reports claim a possible recovery of 100%, commercial nitrate reductases are rather expensive *[311]*. Powerfull chemical reducing agents such as V(III), Mo(VI) + Fe(II), and to a lesser degree Ti(III) are more efficient than nitrate reductases for converting NO_3^- to NO_2^- in biological samples.

The temperature affected the conversion of both NO_2^- and NO_3^- to NO, as the reduction process is facilitated and more rapid at higher temperatures. This is particularly true for the conversion of NO_3^- to NO. However, increasing temperature had several technical drawbacks on the conversion of NO_2^- and NO_3^- to NO. The first is that the measurement of NO by chemiluminescence is influenced by humidity *[11, 173]*. As temperature increased, the heat evaporated more water, which quenched NO_2* produced by the O_3 reaction. The second problem encountered is that the reducing solution has a tendency to move from the microreaction purge vessel to the condenser and even to the NO analyzer itself at high temperatures. We therefore propose that 60°C would be the really appropriate

temperature for converting NO_2^- to NO by these five reducing agents. In the case of NaI, the most efficient NO_2^- reducing agent at any temperature, increasing the temperature had the undesirable effect of increasing the variability of the results (larger SE) and lowering the reproducibility, because of the above-mentioned reasons. The same situation was observed for the conversion of NO_3^- to NO. It would thus be very useful to find and select a reducing agent which can reduce NO_3^- to NO at low temperatures.

It has been suggested that strong reducing agents, such as V(III), can be used at different temperatures to achieve different goals: at low temperatures for the conversion of NO_2^- to NO, and at high temperatures for the conversion of $NO_2^- + NO_3^-$ to NO. NO_3^- would then be determined by the difference between analysis of the same sample by both assays. Fig. 3 shows that V(III), Mo(VI) + Fe(II), and Ti(III) can also reduce NO_3^- to NO at low temperature, albeit at a low degree. Use of only two different temperatures cycling for a strong reducing agent to selectively reduce NO_2^- and (or) NO_3^- to NO can be a cause of overestimated NO_2^- and underestimated NO_3^- measurements.

Our results indicate that the most accurate procedure is to use NaI or Cr(III) as a reducing agent to selectively convert NO_2^- to NO at low temperatures and then use a strong reducing agent to convert all $NO_2^- + NO_3^-$ to NO at 80°C or 90°C.

Proteins contained in most biological samples can cause excessive foaming in the microreaction purge vessel and interfere with the reduction process. Deproteinization is therefore essential for the analysis of such samples. Investigating the recovery of both NO_2^- and NO_3^- in biological samples with particular attention given to the reproducibility of the assay and the occurrence of artifacts is important. In this study, recovery of NO_2^- and NO_3^- in deproteinized plasma was 93.0% ± 1.6% and 105.9% ± 2.7% respectively. Although the

recoveries of NO_2^- and NO_3^- were similar and complete for the pig plasma, we discovered a small but significant difference in the case of the dog plasma. The residual protein environment in the dog plasma seemed to interfere more with NO_2^- than with NO_3^-. Though part of the NO formed in vivo may react with thiol groups in low-molecular-mass compounds, this method does not detect these low-molecular-mass nitroso compounds, as NO must be in the gas state to react with the O_3 in the reaction chamber.

Compared with the same amount of NO gas, V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III) are similarly efficient reducing agents for the conversion of NO_2^- to NO at 20°C. V(III) and Mo(VI) + Fe(II) can also completely reduce NO_3^- to NO at high temperatures. However, Cr(III) and NaI were unable to convert NO_3^- to NO. Cr(III) and NaI can specifically reduce NO_2^- to NO. We recommend the use of NaI or Cr(III) at room temperature to selectively and completely reduce NO_2^- and the use of V(III) or Mo(VI) + Fe(II) at 80-90°C to reduce $NO_2^- + NO_3^-$ to NO. Recovery of both NO_2^- and NO_3^- in experimental animal plasma was reproducible and near quantitative, albeit to a lesser degree in the case of the dog plasma. These results also highlight the need for a relatively large-scale study with human subjects to establish proper baseline measurements for clinical assays of plasma nitrite and nitrate concentrations.

We estimate that a properly organized clinical laboratory could process ~30 samples/h for NO_2^- measurements and ~15 samples/h for the measurement of NO_3^- concentrations.

2.2. *Manuscript 2*: Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine. Eric Troncy, Martin Francœur, Igor Salazkin, Fan Yang, Marc Charbonneau, Guy Leclerc, Patrick Vinay, Gilbert Blaise. (*British Journal of Anaesthesia* 1997; 79: 631-640).

In this study, we have compared the effects of inhaled nitric oxide (iNO) and i.v. nitric oxide (NO) donor e.g. nitroglycerin (ivGTN) on pulmonary and systemic haemodynamics, gas exchange as well as its metabolite, both nitrite (NO_2^-) and nitrate (NO_3^-) anions, concentration in plasma and urine (pNO_x^- and uNO_x^-), respectively. In comparison with the baseline values of pNO_x^-(~30 μM) in acute respiratory distress syndrome (ARDS) of pig model, surgical stress (or trauma) evoked by our surgical procedures in this study may have induced release of endogenous NO (~85.5 μM). Exogenous NO, such as iNO 40 ppm and NO donor (ivGTN, 92.43 (13.45) $\mu g\ kg^{-1}\ min^{-1}$) for 20 min induced considerable alteration of renal function as well as partially reversed the passive pulmonary hypertension. Both iNO and ivGTN administration to pigs linearly and transiently increase in the concentration of pNO_x^- and uNO_x^-, and particularly decreased on the cessation of iNO. This result may indicate that administration of a small (limited) amount of exogenous NO for a short period of time *in vivo* in pigs induced only a transient alteration of extracellular NO metabolites concentrations (in comparison with endotoxin can induce a large amount of NO, which is intracellularly generated by endothelium, myocytes, macrophages etc., and consequently its metabolites lasting for a relatively longer period of time in both intracellular and extracellular compartments [see next 2 manuscripts]). The diuretic effect of exogenous NO (both iNO and ivGTN) was at least explained partially by the effects of its major metabolite, NO_3^- in the range of ~μM.

Summary:

We have compared the effects of inhaled nitric oxide (iNO) and i.v. nitroglycerine (ivGTN) on the haemodynamic response to phenylephrine-induced hypertension (PEHT) in anaesthetized pigs. PEHT did not change either pulmonary vascular resistance or gas exchange throughout all experiments. Both treatments lowered pulmonary arterial pressure to the same extent (-12.4% iNO; -13.7% ivGTN) and passively via an effect on left atrial pressure (-26.3% iNO; -31.4% ivGTN). Both treatments failed to reverse the decrease in renal blood flow (RBFc) induced by PEHT, but both increased urinary flow (UF)(+128% iNO; +148% ivGTN). IvGTN significantly increased plasma concentration of nitrite and nitrate during (+22.7% arterial blood; +26.2% venous blood) and beyond the period of infusion (iNO: +6.4% and +4.9%, respectively). In four control pigs (no PEHT), iNO markedly increased RBFc (+109%), glomerular filtration rate (+72.5%), and UF (+68.7%). We conclude that iNO may have direct cardiac and renal effects, probably via intervention of NO carrier forms such as *S*-nitroso compounds.

Key words: Gases non-anaesthetic, nitric oxide. Pharmacology, nitric oxide. Pharmacology, nitroglycerine. Sympathetic nervous system, phenylephrine. Pig. Kidney, function. Kidney, diuresis.

I.v. endothelium-independent nitrovasodilators such as sodium nitroprusside and nitroglycerine (ivGTN) produce their vasodilator effects by providing nitric oxide (NO) [152]. In contrast with i.v. medication, inhaled nitric oxide (iNO) is used commonly as a selective pulmonary vasodilator in experimental [368] and clinical [106] conditions. Inhaled NO is "microselective" in that it dilates only the vessels directly adjacent to the alveolar units being ventilated. Therefore, in a hypoxæmic patient, iNO can improve oxygenation by improving ventilation/perfusion matching with redistribution of blood flow from unventilated shunted areas to ventilated but underperfused areas [273]. Some authors also explain the improved oxygenation with iNO by the fact that it produces local bronchodilation [273, 368], decreases vascular permeability and the appearance of pressure-driven pulmonary oedema [273], and exerts a platelet antiaggregating effect [368].

A swine-model has been used in the past to study the treatment of experimental pulmonary hypertension induced by hypoxia [343], a thromboxane analogue [352], oleic acid-induced ARDS [314] or sepsis [273, 368]. Phenylephrine is a sympathomimetic amine commonly used during *in vitro* studies of the L-arginine: NO pathway [34] or to counteract clinical hypotension [8]. We have developed a phenylephrine-induced hypertension (PEHT) model in anaesthetized pigs. We wished to test the hypothesis that iNO does not affect the systemic circulation. Accordingly, we compared the effects of iNO and ivGTN on pulmonary and systemic haemodynamics and gas exchange, in addition to plasma and urinary concentrations of nitrite (NO_2^-) and nitrate (NO_3^-), stable metabolites of nitrogen oxides and related species (NO_xS), this is pNO_x^-, and uNO_x^-, respectively. In order to evaluate potential extra-pulmonary effects of iNO, we have

compared the effects of iNO and ivGTN on diuresis and renal haemodynamics in pigs in the presence or absence of PEHT.

Materials and methods

This study was approved by the Institutional Research and Animal Welfare Committee. The animals were treated according to the Canadian Council on Animal Care guidelines. We studied 28 female pigs, mean weight 22.84 (SEM 0.27) kg.

The pigs were premedicated with azaperone 2 mg kg^{-1} i.m., ketamine 15 mg kg^{-1} i.m. and atropine 4 µg kg^{-1} i.m.. After induction of anaesthesia with fentanyl 5 µg kg^{-1} i.v. and pentobarbitone 6.5 mg kg^{-1} i.v., the trachea was intubated and the lungs ventilated using a volume-controlled ventilator (Ventilator 7200 AE, Puritan Bennett, Carlsbad, CA, USA). Tidal volume was set at 15 ml kg^{-1}, FIO_2 at 0.30 (Oxygen Monitor 5590, Hudson RCI, Temecula, CA, USA) and ventilatory frequency adjusted to maintain the partial pressure of arterial carbon dioxide ($PaCO_2$) at 4.6-6.0 kPa (Blood gases analyzer IL 1620, Coulter Electronics Ltd., Ville Saint -Laurent, Qc, Canada). Anæsthesia was maintained with a continuous infusion of fentanyl 3.8 µg kg^{-1} h^{-1} and pentobarbitone 9.8 mg kg^{-1} h^{-1}. Neuromuscular block was produced with a mixture of tubocurarine 0.2 mg kg^{-1} h^{-1} and pancuronium 0.15 mg kg^{-1} h^{-1} . Lactated Ringer's solution was infused at a rate of 15 ml kg^{-1} h^{-1} to meet the maintenance needs during open-chest surgery.

After placing the electrocardiographic leads, we inserted an arterial pressure catheter (carotid artery), two venous infusion cannulae (external jugular vein, auricular vein), a pulmonary artery thermodilution catheter (for cardiac output), a core temperature

probe and central venous catheter. Median sternotomy was performed, the pericardium opened and two catheters (12-gauge) inserted, one into the main pulmonary artery and the other into the left atrial appendage for pressure monitoring in addition to arterial and venous blood sampling for blood-gas analysis. The pericardium and chest wall were closed with sutures and metallic wires. Via a laparotomy, the left renal vein (renal venous blood sampling site) and both urethers were catheterized, and the left ovarian vein was ligated because of drainage into the renal vein. After the instrumentation, 45 minutes were allowed to elapse to obtain a stable physiological state.

Each experiment was divided into four stages (I-IV) of 20 minutes (table 1). Animals were enrolled in the experiments in a random order.

Table 1 Study Design.

* no intervention. † PE = phenylephrine infusion at mean 15.75 (SEM 0.85) μg kg^{-1} min^{-1}; ‡PE = phenylephrine infusion at 14.68 (1.36) μg kg^{-1} min^{-1}; iNO = inhaled nitric oxide 40 ppm; ivGTN = nitroglycerine infusion at 92.43 (13.45) μg kg^{-1} min^{-1}.

	Stage and time					
Arm	Protocol	**I** 30-50'	**II** 50-70'	**III** 70-90'	**IV** 90-110'	Sample size
Control	**A**	Nil*	Nil	Nil	Nil	n = 4
	B	Nil	PE†	PE†	Nil	n = 4
	C	Nil	iNO	iNO	Nil	n = 4
Experimental	**D**	Nil	PE‡	PE‡-ivGTN	Nil	n = 8
	E	Nil	PE‡	PE‡-iNO	Nil	n = 8

Control arm. In control studies we evaluated the effect of time (experiment A), PEHT alone (experiment B), and iNO alone (experiment C).

Experimental arm. The four stages in the experiments were: (I) baseline measurements, (II) induction of PEHT by continuous infusion of phenylephrine diluted in saline (mean 14.68 (SEM 1.36) $\mu g\ kg^{-1}\ min^{-1}$), (III) intervention to treat PEHT with an infusion of ivGTN (experiment D) or 40 ppm of iNO (experiment E), and (IV) a recovery period where both the hypertensive stimulus and treatment were discontinued.

ADMINISTRATION OF IVGTN

The dose of ivGTN (Nitroject, Omega Laboratories Ltd., Montréal, Qc, Canada) was started at 10 $\mu g\ kg^{-1}\ min^{-1}$ and increased progressively (3-min interval) to reduce mean pulmonary arterial pressure to the level observed after iNO. The period of measurement (stage III) was then started. The mean final dose of ivGTN used in experiment D was 92.43 (SEM 13.45) $\mu g\ kg^{-1}\ min^{-1}$.

ADMINISTRATION OF INO

A mixture of NO-N_2 972 ppm (Cylinder 972 ppm NO, < 5 ppm NO_2, Vitalaire Canada, Montréal, Qc, Canada) was injected cyclically into inspiratory limb of the ventilator system using a method developed in our institution [84]. Concentrations of gaseous NO and NO_xs were monitored continuously using a chemiluminescence analyzer (CLD700AL NO/NO_x analyzer, Ecophysics Tecan AG, Dürten, Switzerland). Based on our previous works [343, 352], we chose to study the effects of iNO 40 ppm. This dose was shown in several studies [106, 273, 314, 343, 352] to have a significant pulmonary

vasodilator effect while ensuring an inspired fraction of NO_2 below 1 ppm with our system [84].

RENAL MONITORING

Thirty minutes before the beginning of baseline measurements (stage I), a bolus of inulin 2 g and para-amino-hippuric acid (PAH) 2 g dissolved in 50 ml of glucose was administered, immediately followed by an infusion of inulin (2 g) and PAH (4 g) dissolved in 1000 ml glucose at a constant rate of 1 ml min^{-1}. Blood samples (4 ml) were obtained simultaneously from left renal vein and carotid artery in order to measure packed cell volume (PCV), PAH and inulin concentrations, in addition to arterial and venous pNO_x^- concentrations. Blood samples were collected at the beginning of stage I and at the end of each 20- min period. Urine was collected separately in graduated cylinders from both urethers at 5-min interval during the whole procedure to measure urinary flow (UF), PAH and inulin concentrations, and uNO_x^-. The concentration of PAH in both urine and blood was measured using the technique described by Bratton and Marshall. Renal plasma flow (RPF) was taken as the PAH clearance ($Cl_{PAH} = C_u \times [UF \div C_a]$, where C_u = PAH concentration in urine and C_a = PAH concentration in arterial blood plasma). Renal plasma flow was corrected (RPFc) for PAH extraction coefficient ($E_{PAH} = [C_a - C_v] \div C_a$, where C_v = PAH concentration in renal vein blood plasma), and renal blood flow corrected for PAH extraction (RBFc) was taken as RPFc/(1-PCV). Because PAH acetylation was observed with some pigs, RBFc was calculated for each 5-min interval using PAH and inulin clearances and extractions. Inulin concentration was measured by

an anthrone colorometric technique [267], and whole kidney glomerular filtration rate (GFR) was calculated for each 5-min interval by the standard clearance formula.

MEASUREMENTS OF pNO_X^- AND uNO_X^-

Plasma samples obtained by centrifugation were diluted 10-fold with distilled water and deproteinized by adding 5% volume of zinc sulphate to a final concentration of 15 g $litre^{-1}$ [380]. After centrifugation at 1000X*g* for 15 min at room temperature, 100 µl of supernatant was applied to a microreaction purge vessel chamber (270B NO analyzer, Sievers Research Inc., Boulder, CO, USA) equipped with a temperature regulator and a condenser, which allowed direct introduction of prepared plasma and urine samples into the reducing solution. pNO_x^- and uNO_x^- concentrations were measured by conversion to NO using hot acidic vanadium (III) chloride. NO was eluted in a stream of nitrogen, and detected by an ozone-induced chemiluminescence reaction. The NO/NO_x analyzer was connected directly to a data acquisition system (Mac Lab, Lamont Scientific Ltd., Downsview, Ont, Canada). Each sample was analyzed in duplicate, and measured once at the end of each 20-min period, as described previously [380].

CARDIORESPIRATORY VARIABLES

Systolic (SAP), mean (MAP) and diastolic (DAP) systemic arterial pressures, mean pulmonary arterial pressure (PAP), left atrial pressure (LAP), central venous pressure (CVP), heart rate (HR), and cardiac output (CO; 5 ml of cold normal saline were injected in triplicate for each measurement) were recorded every 5 minutes in each period.

Arterial and mixed venous blood-gas tension were measured once at the end of each 20-min period.

DATA ANALYSIS

In the control arm, the sample size was n = 4 for each protocol (table 1). In the experimental arm, the sample size was n = 8 for each experiments. Half of the pigs in experiemnts D and E were used to evaluate renal effects and the other half to measure pNO_x^- and uNO_x^- concentrations.

Data distribution of each variable was Gaussian. For each stage, mean values of four measurements (obtained every 5 min) or a single measurement (obtained only once during each stage) were chosen as the values for the period. Pulmonary (PVR) and systemic (SVR) vascular resistances were calculated using standard formulae. All values are reported as mean (SEM) for each variable in each stage (I-IV) of the experiments.

Data were analyzed using SuperANOVA (V 1.11, Abacus Concepts Inc. 1989/1991, Berkeley, CA, USA) and SAS (Statistical Analysis System V6.11 for Windows 95, SAS Institute Inc. 1995, Cary, NC, USA) software.

For each experiment, the effect of stage was evaluated with the one-factor repeated measures analysis of variance (F-ANOVA), and when necessary, multiple comparisons were made using the Fisher's protected least significant difference (LSD_F) test.

Between the five experiments, for the difference between two stages (*e.g.* stages-difference [**III-I**] = between stages III and I), one-way analysis of variance (W-ANOVA)

was used and multiple comparisons were made using the Fisher's protected least significant difference (LSD_W) test.

P <0.05 was considered significant, except for the multiple comparisons where the global significance level of 0.05 was adjusted for the number of null hypotheses tested (P<0.008 for multiple comparisons within group LSD_F and P<0.005 for multiple comparisons between the five groups LSD_W).

Results

For each experiment, the cardiovascular, renal, and metabolic (pNO_x^-, uNO_x^-) effects observed during each stage are presented.

CONTROL ARM

In experiment **A** on the effect of time, there was no change in measured variables, except for a decrease in renal haemodynamics and diuresis: stage IV was different from the three preceding periods for RBFc (-22.9%; fig. 1A), GFR (-20.8%; fig. 2A), and UF (-19.5%, fig. 3A).

During experiment B, phenylephrine significantly increased PAP by 40% (LSD_F comparison of stage II with stage I: P=0.0001), but PVR remained unchanged (F-ANOVA: P= 0.42). PEHT significantly increased MAP and SVR: +65.9%, and +64.5%, respectively. LAP and CVP exhibited similar changes: +67.9%, and +49%, respectively. Phenylephrine had no effect on HR and CO (F-ANOVA, P=0.17 and P=0.33 respectively). The haemodynamic changes were stable during stage III. Compared to

stage I, Phenylephrine induced a similar decrease in RBFc for stage II (-34%, LSD_F: P= 0.001) (fig. 1A) and III (-37%, LSD_F: P=0.001) (fig. 1A) but other renal variables remained unchanged (fig. 2A and 3A). The vasoconstrictor did not significantly modify the arterial (92.6 (4) to 96 (5.1) μmol $litre^{-1}$) and venous (89.8 (5.5) to 94.1 (8.3) μmol $litre^{-1}$) pNO_x^-, or uNO_x^- (988.2 (73.4) to 1132.4 (102.6) μmol $litre^{-1}$) concentrations.

In experiemnt C, iNO alone did not significantly alter cardiorespiratory variables. However, renal function was affected: RBFc increased by +94% (LSD_F: P=0.0046) (fig. 1A), GFR by +63% (LSD_F: P=0.0001) (fig. 2A) and UF by +34% (LSD_F: P=0.006) (fig. 3A) during stage II; the changes in the three variables remained constant during stage III (+109%, +72.5%, and +68.7%, respectively) (fig. 1A to 3A). UF remained high during stage IV (LSD_F: P=0.001 compared with stage I) (fig. 3A). The concentration of pNO_x^- was slightly modified by iNO in arterial (109.2 (3.1) to 119.8 (3.3) μmol $litre^{-1}$) and venous (116.4 (4.7) to 124.3 (4.8) μmol $litre^{-1}$) blood, and uNO_x^- increased from 691.2 (113.8) to 1011.3 (157.3) μmol $litre^{-1}$ during stage II. ΔpNO_x^- increased continually during stage III compared with stage I (+22.1 and +21.4 μmol $litre^{-1}$ in arterial and venous blood), and decreased on cessation of iNO. The value of uNO_x^- remained at the same high level during stages III and IV.

EXPERIMENTAL ARM

Infusion of phenylephrine in experiments D and E produced the same cardiovascular (table 2), renal (fig. 1B to 3B), and metabolic (table 3) effects as in experiment B (W-ANOVA: P>0.1 on difference [**II-I**] between experiments B, D and E).

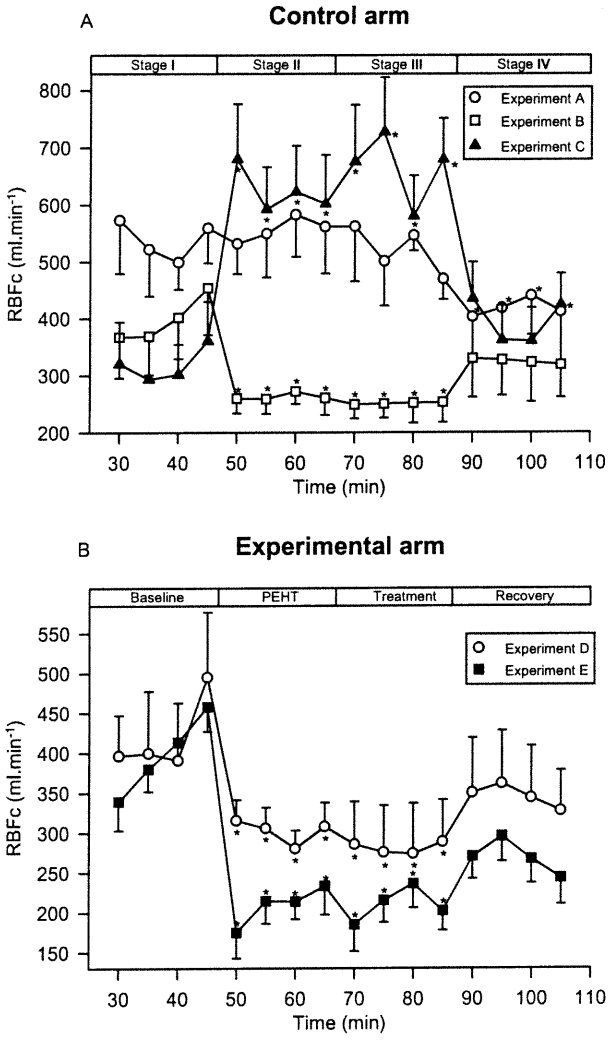


Figure 1 Variation in renal blood flow corrected for PAH extraction (RBFc).
A. Control arm: mean (SEM) values for RBFc for each sampling time in each stage (I-IV) for experiment A (no intervention during the four stages), experiment B (infusion of phenylephrine 15.75 (0.85) $\mu g\ kg^{-1}\ min^{-1}$ during stages II and III), and experiment C (iNO 40 ppm during stages II and III). RBFc decreased with time in experiment A. Infusion of phenylephrine significantly reduced RBFc while iNO considerably increased RBFc. * Significant difference for F-ANOVA ($P<0.05$) and for the LSD_F test of the stage compared with stage I ($P<0.008$).
B. Experimental arm: mean (SEM) values of RBFc for each sampling time in each stage (I-IV) for experiment D (PEHT + ivGTN treatment) and experiment E (PEHT + iNO treatment). Infusion of phenylephrine significantly reduced RBFc, and nitrergic treatments failed to reverse the decrease.* Significant difference for F-ANOVA ($P<0.05$) and for the LSD_F test of the stage compared with stage I ($P<0.008$). (See text for further details)

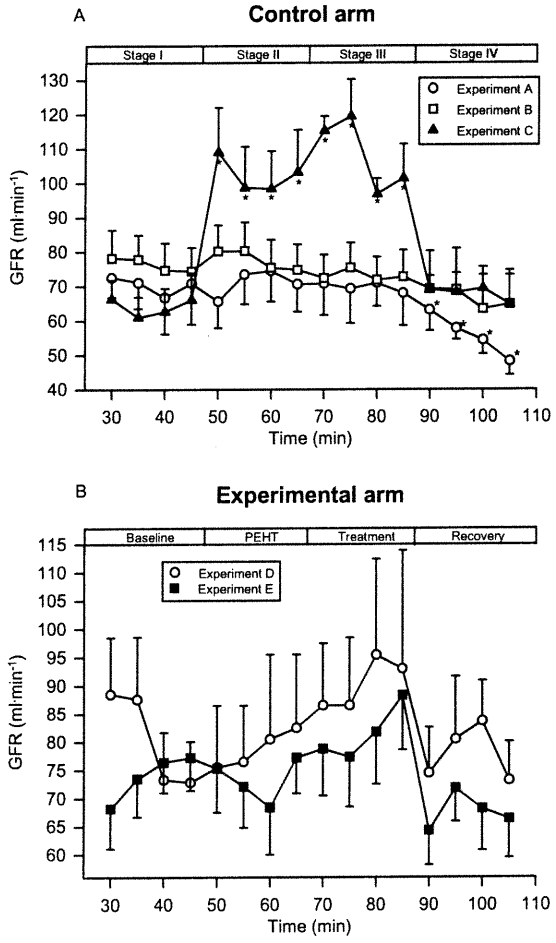


Figure 2 Variation in glomerular filtration rate (GFR).
A. Control arm: mean (SEM) values for GFR for each sampling time in each stage (I-IV) for experiment A (no intervention during the four stages), experiment B (infusion of phenylephrine 15.75 (0.85) μg kg^{-1} min^{-1} during stages II and III), and experiment C (iNO 40 ppm during stages II and III). GFR decreased with time in experiment A. Infusion of phenylephrine had no effect on GFR while iNO considerably increased GFR. * Significant difference for F-ANOVA ($P<0.05$) and for LSD_F test of the stage compared with stage I ($P<0.008$).
B. Experimental arm: mean (SEM) values of GFR for each sampling time in each stage (I-IV) for experiment D (PEHT + ivNTG treatment) and experiment E (PEHT + inhNO treatment). Infusion of phenylephrine and nitrergic treatments did not modify GFR. (See text for further details).

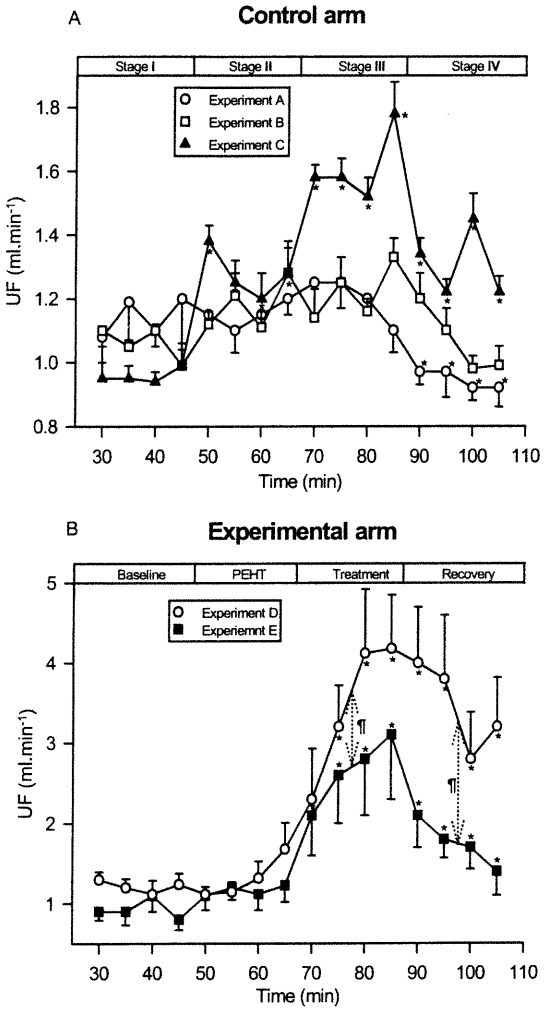


Figure 3 Variation of urinary flow (UF).
A. Control arm: mean (SEM) values for UF for each sampling time in each stage (I-IV) for experiment A (no intervention during the four stages), experiment B (infusion of phenylephrine 15.75 (0.85) $\mu g\ kg^{-1}\ min^{-1}$ during stages II and III), and experiment C (iNO 40 ppm during stages II and III). UF decreased with time in experiment A. Infusion of phenylephrine had no effect on UF while iNO considerably increased UF. * Significant difference for F-ANOVA (P<0.05) and for the LSD_F test of the stage compared with stage I (P<0.008).
B. Experimental arm: mean (SEM) values of UF for each sampling time in each stage (I-IV) for experiment D (PEHT + ivNTG treatment) and experiment E (PEHT + inhNO treatment). Infusion of phenylephrine did not modify UF and both nitrergic treatments significantly increased UF, ivNTG more than iNO. * Significant difference for F-ANOVA (P<0.05) and for the LSD_F test of the stage compared to stage I (P<0.008). ¶ Significant difference between groups for W-ANOVA (P< 0.05) and for the LSD_W test (P<0.005) on the difference [**III-II**] and [**IV-III**]. (See text for further details)

Table 2 Effects of phenylephrine (PEHT) and its treatment by inhaled NO (iNO) or i.v. nitroglycerine (ivGTN) on pulmonary and systemic haemodynamic variables (mean (SEM)) in anaesthetized pigs. **[III-II]** = mean (SEM) value of the percentage variation between stages III (treatment) and II (PEHT). **P* value <α. †*P* value of the one-factor repeated measures analysis of variance for the within-group analysis on the four periods was not significant. P_1= *P* value of Fisher's protected LSD_F test for comparison of stages II and III within group (α = 0.008); P_2= *P* value of Fisher's protected LSD_W test for comparison of the difference **[III-II]** between groups (α = 0.005).

		Baseline (I)	PEHT (II)	Treatment (III)	[III-II] (%)	Recovery (IV)	P_1
PAP (mm Hg)	iNO	22 (0.97)	29.1 (1.17)	25.5 (1.06)	-12.4 (0.81)	23.06 (1.1)	*0.0001**
	ivGTN	21.5 (1.23)	29.1 (1.52)	25.1 (0.64)	-13.7 (1.34)	23.8 (0.83)	*0.001**
					$P_2 = 0.51$		
PVR (dyn s cm^{-5})	iNO	382.9 (16)	341.8 (23.37)	357.3 (18.27)	+4.5 (3.63)	416.3 (20)	*NS*†
	ivGTN	309.4 (17.3)	313.7 (23)	309.3 (20.7)	-1.4 (2.82)	336.5 (29.5)	*NS*
					$P_2 = 0.92$		
MAP (mm Hg)	iNO	104.9 (7.8)	172.5 (4.56)	161.5 (4.35)	-6.3 (3.07)	100.4 (5.32)	*0.004**
	ivGTN	89.8 (3.4)	160.1 (1.52)	97.1 (4.84)	-39.4 (4.52)	84.5 (2.8)	*0.0001**
					$P_2 = 0.00001$*		
SVR (dyn s cm^{-5})	iNO	3020.8 (149.1)	4924.3 (217.2)	4494.3 (159.2)	-8.7 (7.7)	3133.5 (123)	*0.068*
	ivGTN	2318.9 (85.7)	4480.3 (253)	2263.9 (146.8)	-49.3 (9.96)	2292.6 (136)	*0.00001**
					$P_2 = 0.001$*		
SAP (mm Hg)	iNO	116.2 (6.96)	199.7 (4.7)	178.2 (4.63)	-10.8 (2.22)	111.6 (3.4)	*0.0001**
	ivGTN	109.4 (3.64)	196.9 (2.54)	122.2 (5.5)	-37.9 (4.77)	104.6 (3.2)	*0.0001**
					$P_2 = 0.000001$*		
DAP (mm Hg)	iNO	91.31 (5.3)	147.6 (4.87)	140.5 (4.56)	-4.8 (1.8)	84.55 (4)	*0.04*
	ivGTN	75.82 (4.92)	138.5 (5.4)	79.6 (3.92)	-42.5 (5.55)	67.96 (3.2)	*0.0001**
					$P_2 = 0.000001$*		
LAP (mm Hg)	iNO	9.06 (0.9)	17.1 (1.1)	12.6 (1.27)	-26.3 (3.78)	10.06 (1.07)	*0.0001**
	ivGTN	10.5 (1.13)	18.8 (1.52)	12.9 (0.46)	-31.4 (6.1)	12.43 (0.72)	*0.0001**
					$P_2 = 0.1$		
CVP (mm Hg)	iNO	5.72 (0.4)	9.9 (0.92)	8 (0.64)	-19.1 (2.76)	6.14 (0.5)	*0.0001**
	ivGTN	7.7 (0.37)	10.5 (0.46)	7.8 (0.39)	-25.7 (3.43)	7.86 (0.31)	*0.0001**
					$P_2 = 0.09$		
HR (beat min^{-1})	iNO	122.6 (7.51)	120.6 (7.1)	132.2 (9.36)	+9.06 (2.6)	129.4 (5.6)	*NS*
	ivGTN	132.43 (9)	141.3 (10.3)	158.1 (9.75)	+11.9 (3.85)	144.6 (8)	*NS*
					$P_2 = 0.1$		
CO (litre min^{-1})	iNO	2.73 (0.18)	2.76 (0.18)	2.81 (0.14)	+1.8 (2.05)	2.47 (0.16)	*NS*
	ivGTN	2.87 (0.15)	2.95 (0.35)	3.1 (0.28)	+5.1 (2.76)	2.86 (0.26)	*NS*
					$P_2 = 0.37$		

Table 3 Plasma nitrite and nitrate (pNO_x^-) concentrations (mean (SEM)) during phenylephrine-induced hypertension (PEHT), and its treatment with superimposed inhaled NO (iNO) or i.v. nitroglycerine (ivGTN) in anaesthetized pigs. There was no difference between arterial and venous pNO_x^- values. *P value $<\alpha$. $\boldsymbol{P_1}$= P value of the one-factor repeated measures analysis of variance for the within-group analysis on the four periods ($\alpha = 0.05$); $\boldsymbol{P_2}$= P value of Fisher's protected LSD_F test for multiple comparison of stages within group ($\alpha = 0.008$). $\boldsymbol{P_3}$= P value of Fisher's protected LSD_W test for comparison of the stages-difference between experiments D and E ($\alpha = 0.005$).

		Baseline (I)	[II-I]	PEHT (II)	[III-II]	Treatment (III)	[IV-III]	Recovery (IV)	[IV-I]	P_1
Arterial pNO_x^- (μmol litre^{-1})	iNO	86.4 (2.1)		90.6 (1.3)		96.4 (2.7)		82.7 (2.6)		*0.002**
	$\boldsymbol{P_2}$		*0.16*		*0.057*		*0.0001**		*0.21*	
	ivGTN	98.7 (8.3)		102 (11)		124.6 (12)		122 (9.5)		*0.004**
	$\boldsymbol{P_2}$		*0.90*		*0.003**		*0.90*		*0.001**	
	$\boldsymbol{P_3}$		*0.71*		*0.002**		*0.0048**		*0.001**	
Venous pNO_x^- (μmol litre^{-1})	iNO	93 (6.29)		101 (7.1)		105.9 (4.1)		90.1 (3.5)		*0.04**
	$\boldsymbol{P_2}$		*0.39*		*0.63*		*0.004**		*0.76*	
	ivGTN	85.5 (6.9)		92.5 (10)		117 (11.2)		113 (10)		*0.02**
	$\boldsymbol{P_2}$		*0.78*		*0.004**		*0.89*		*0.001**	
	$\boldsymbol{P_3}$		*0.17*		*0.002**		*0.004**		*0.003**	

Both nitrergic treatments decreased LAP (-31.4% ivGTN group = experiment D; -26.3% iNO group = experiment E), and CVP (-25.7% and -19.1%, respectively), achieving the same stages-difference **[III-II]** between experiments D and E (LSD_W: P=0.1 for LAP, and P=0.09 for CVP) (table 2). The effect on pulmonary hypertension was the same in both groups (table 2): PAP decreased by 13.7%, and 12.4%, respectively. IvGTN decreased MAP (-39.4%) and SVR (-49.3%), and iNO induced a much smaller decrease in the systemic haemodynamic variables (table 2). HR, CO, and blood-gas values remained unchanged throughout the experiment.

The two nitrergic treatments did not reverse the decrease in RBFc induced by phenylephrine (fig. 1B), but they both increased UF (+148%, LSD_F: P=0.0005 for experiment D; +128%, LSD_F: P=0.0004 for experiment E) (fig. 3B). Comparison between experiments D and E on the stages-difference **[III-II]** showed that ivGTN exerted more effect on UF than iNO (LSD_W: P=0.003). Infusion of ivGTN increased pNO_x^- concentrations compared with stage II (table 3): +22.8% for arterial blood, +26.2% for venous blood. The values in experiment E were +6.4%, and +4.9% for arterial and venous blood, respectively (table 3). Both nitrergic treatments markedly increased uNO_x^- concentrations (+65.4% for experiment D, and +46.3% for experiment E).

At the end of the experiment, cardiovascular, respiratory, and renal (except UF for experiments D and E, LSD_F: P=0.001 for comparison of the stages IV to equivalent stages I; fig. 3B) variables essentially returned to their initial values. In experiment D, the increases in arterial and venous pNO_x^- (table 3) and uNO_x^- concentrations observed during stage III were still present during stage IV. Twenty minutes after cessation of iNO,

pNO_x^- concentration significantly decreased (compared to stage III) in experiment E (table 3), whereas uNO_x^- remained high (1206.4 (108.9) μmol $litre^{-1}$). Comparison between experiments D and E on stages-differences **[IV-III]** and **[IV-I]** confirmed pNO_x^- return to the baseline values in the iNO group, but not in the ivGTN group.

Discussion

To our knowledge, this is the first report of a PEHT in swine treated with nitrergic therapies and the first study of the haemodynamic extra-pulmonary effects of iNO.

The model used in these studies is well established. There were no mortality during the surgical preparations and we demonstrated with experiment **A** that cardiorespiratory variables remained stable during the period of the study and there were only minimal changes in renal function.

EXPERIMENT B

Cardiovascular effects

Phenylephrine acts on both α_1- and α_2-adrenoceptors [246] and induces hypertension (major α_1-effect) by constricting resistance and, to a lesser degree, capacitance vessels [8]. W observed no signs of tachyphylaxis with phenylephrine during the experiments. The observed passive post-capillary pulmonary hypertension with increased central filling pressures is secondary to the left ventricular haemodynamic effects of the acute increase in after-load induced by systemic hypertension. During the experiment, PVR did not change, in agreement with a heterogenous [386] α-adrenergic innervation of large (5-

7 mm in diameter) and small (2-3 mm in diameter) porcine pulmonary arteries and sparse or non-existant [137] α-adrenergic innervation of swine pulmonary resistance vessels.

Renal effects

The kidney reacted to phenylephrine-induced increase in SVR by a decrease in RBFc [8], while GFR and UF remained unchanged. This suggests that phenylephrine constricted mainly the efferent arteriole and that GFR was maintained, despite a decrease in RBFc, by an increase in the glomerular filtration fraction [225].

Metabolic effects

The baseline values of pNO_x^- reported were higher than those measured in a porcine model of ARDS (30 μmol $litre^{-1}$) [314]. However, the techniques of measurements were different (chemiluminescence *versus* high-performance liquid chromatography based on the Griess reaction [314]) and the surgical stress of our procedure might have stimulated release of endogenous NO. Furthermore, reported values for basal concentration of pNO_x^- (mainly NO_3^-) in human varied greatly [123, 369, 370]. Our basal values for uNO_x^- were in accordance with those published previously in humans [123, 369, 370].

EXPERIMENT C

Inhaled NO alone induced no cardiorespiratory modifications, but considerably altered renal function.

Transport of EDRF-NO

Several authors have suggested that EDRF-NO interacts with circulating molecules to regulate its activity, allowing its transport (NO carriers) [163, 322] or promoting its degradation (NO scavengers) [200]. Metal nitrosylation with haem and non-haem proteins and the NO reaction with nucleophil groups (*e.g.* sulfhydryl, amine) [82] of amino acids, peptides, and proteins lead to the potential interaction and formation of many nitrosyl-haem adducts, *S*-non-protein-nitrosothiols (*S*-nitroso-L-cysteine), *S*-nitrosylated proteins (*S*-nitrosoalbumins, [322] *S*-nitrosohaemoglobins [163, 200]), nitrosylated iron -sulphur clusters, dinitrosyl-iron-complexes [258], nitrosoamines, and others.

Renal effects

Renal haemodynamics and diuresis were altered by iNO (experiment **C**) strongly supporting the presence of extra-pulmonary effects. It is now widely accepted that EDRF-NO is an important modulator of RBF and that regional microcirculation [20] may be mediated by endogenously produced EDRF-NO. The pharmacological blockade of EDRF-NO results in an increase in MAP and renal vascular resistance, and decrements in RBF, GFR, UF and sodium excretion [48]. There is some controversy regarding a selective effect of the EDRF-NO blockade on either afferent or efferent arteriolar tone [20]. It would seem that EDRF-NO primarily alters afferent vascular tone [157], thereby modifying the ability of the preglomerular vasculature to autoregulate glomerular capillary pressure. Several studies have emphasized an effect of EDRF-NO not only on glomerular, but also on tubular function and the medullary circulation [157, 295]. McKee, Scavone and Nathanson [246] showed that EDRF-NO, through generation of

cGMP and stimulation of cGMP-dependent protein kinase(s), mediates the actions of several intercellular messengers to regulate renal tubular Na-K-ATPase. Inhibition of this sodium pump (*e.g.* by acetylcholine, bradykinin) would decrease transcellular water and sodium reabsorption from the tubular lumen, resulting in diuresis and natriuresis. Moreover, low doses of NO synthase inhibitors, that have no effect on either RBF or GFR, present antidiuretic and antinatriuretic effects [242]. Here is the first report of exogenous iNO modulating renal function. Renal haemodynamics state might be influenced during iNO admnistration by circulating NO metabolites (*e.g.* NO_x^-) [385], renal NO delivery through release from carrier *S*-nitrosocompounds (*e.g. S*-nitrosothiols) [175] after nitrosylation in the pulmonary circulation, or a local renal effect owing to unknown paracrine influence stimulated by iNO. *In vitro* studies have shown that $NaNO_2$ did not influence renovascular tone in concentrations up to 0.1 mmol $litre^{-1}$ [175]. The diuretic effect of $NaNO_3$ was observed with a plasma NO_3^- concentration of 38 μmol $litre^{-1}$ in dogs [385]. In view of the rapid renal response to application and discontinuation of iNO, we postulate that iNO may be accompanied by non selective, extra-pulmonary effects due to local delivery of NO on peripheral territories that include the renal bed.

Metabolic effects

Nitric oxide reaching the circulation can be metabolized *via* three pathways [199]: (i) interaction with dissolved oxygen in blood to form NO_2^-, (ii) reaction with oxyhaemoglobin to form methaemoglobin which is in turn reduced back to haemoglobin and NO_3^- mainly by the NADPH-methaemoglobin reductase and peroxynitrite pathways, and (iii) combination with deoxyhaemoglobin to form the rather stable

nitrosohæmoglobin (HbNO) or with carrier molecules to form *S*-nitrosothiols among others (*see above*). However, to date, the mechanisms by which NO is inactived and eliminated are not fully known.

The concetration of pNO_x^- increased almost linearly throughout the inhalation, and decreased on cessation of iNO. This result is in agreement with those of previous reports [369, 370] which have shown a linear temporal increase in pNO_x^-. Approximately 40 μmol of uNO_x^- were excreted in urine during the 40-min inhalation period, a result similar to that of Wennmalm and colleagues [369], and the rate of elimination was maintained over the following 20 min. This implies that 23.6% of the retained NO was excreted in urine as uNO_x^- within 1 h after the start of inhalation. This early and fast iNO elimination corroborates with a previous study [370] that showed 69% of the inhaled ^{15}NO was excreted into the urine as $^{15}NO_3^-$ within 24 h, and another 4% from 24 to 48 h after the start of inhalation. Moreover, the decrease in pNO_x^- and maintenance of diuresis and high uNO_x^- level after cessation of iNO suggest that NO_x is stored in packaged forms before its release without vasoactive but with diuretic effect, transformation and elimination as NO_x^-.

EXPERIMENTAL ARM

Cardiovascular effects

Inhaled and i.v. nitrergic treatments partially reversed the passive pulmonary hypertension induced by phenylephrine. With the PEHT swine model, iNO 40 ppm was less effective in reducing PAP (50% reversal of the increase) than during hypoxia

(108.7% at 40 ppm) [343], thromboxane-mimetic infusion (61% at 40 ppm) [352], experimental ARDS (57.1% at 40 ppm) [314] or sepsis (84% at 10 ppm [365]; 64.8% at 40 ppm [273]). PVR remained unchanged throughout the experiment suggesting that the pulmonary resistance vasculature was not constricted and that both treatments did not act at this level in the PEHT swine-model. Because the pulmonary vascular tone was unchanged by nitrergic treatments superimposed on PEHT, the baseline gas exchange variables also remained unchanged.

The increase in PAP induced by phenylephrine was adjusted to comparable levels in both groups. Both treatments induced similar decreases in LAP, and consequently PAP and CVP. The decrease in LAP can be explained by a decrease in left atrial filling, increase in left compliance, decrease in left ventricular after-load, or a combination of these effects. The first hypothesis is unlikely because CO was unchanged and there was no clinical sign of increase in lung water as gas exchange variables remained constant. The second assumption implies, from the formed cGMP [152], the block of α_1 effects on cardiac contraction and relaxation [312], and a positive lusitropic effect increasing the relaxation and the diastolic distensibility of the heart [49]. A direct cardiac effect of endothelium-independent nitrovasodilators, such as depressed myocardial contractility [45], improved ventricular relaxation and diastolic distensibility [283] is accepted. The decrease in systemic afterload (third postulate) was manifest with ivGTN infusion which induced a substantial decrease in SVR. An endogenous EDRF-NO signaling pathway has been found to regulate the cardiac myocyte function [23]. The paracrine actions of EDRF-NO stimulated by substance P or bradykinin, particularly on left ventricular relaxation, are also well established [177]. The second and third hypotheses imply extra-

pulmonary effects with iNO. In a previous study [343], we suggested that iNO has a direct cardiac effect. There was a slight modification of left ventricular afterload with iNO, as the observed decrease in SAP and MAP were statistically significant (LSD_F: P=0.0001 and P=0.004, respectively) (table 2). We propose that iNO had a direct cardiac effect to explain the similar action of ivGTN and iNO on PAP, LAP, and CVP. Our data indicate a larger decrease in SAP and MAP than that for DAP, suggesting that factors other than aortic compliance or SVR are modified by iNO. These other factors are usually related to changes in the mechanical performance of the heart as a pump [100]. A trend of iNO to decrease SVR or MAP was observed in several publications [106, 314, 368].

Renal effects

In experiments **D** and **E**, nitrergic treatments superimposed on phenylephrine infusion resulted in increases in UF without reversing the phenylephrine-induced decrease in RBFc. The primary phenylephrine site of action in the kidney seems to be the efferent arteriole in contrast with the afferent arteriole for EDRF-NO. This could explain the lack of reversibility for the decrease in RBFc induced by phenylephrine for both nitrergic treatments. Our findings also support direct diuretic (and natriuretic?) effect of exogenous NO. Another hypothesis is the intervention of regulatory systems, either intrarenal such as kallikrein-kinin system or the prostaglandins (PGE_2), or extrarenal such as atrial natriuretic factor (ANF) or the oubain-like endogenous factor that will increase diuresis [22]. However, EDRF-NO released from the endocardium or from the endothelial cells of the coronary vasculature, directly or through a decrease in LAP, or both, inhibits the

release of ANF [218]. The results of experiment C and those from another study [176] give greater credence to intervention of exogenous NO in packaged forms.

Inhaled NO had a major effect on the kidney and a slight (on SAP and MAP) or no statistically apparent (on DAP and SVR) systemic effect. The kidney could be more sensitive to exogenous NO than the systemic circulation as demonstrated by the high sensitivity of RBF to NO synthase inhibition [112]. Indeed, the renovasculature showed significant modifications with low quantities of NO. Another hypothesis is that the kidney could more easily concentrate the NO carriers or recuperate NO from its transport forms (local catabolism of *S*-nitrosocompounds such as *S*-nitrosoglutathion, etc). The persistent diuresis after discontinuing PEHT and nitrergic treatments (stage IV) was in agreement with previous data [175, 385]. It correlated with the persistent high pNO_x^- and uNO_x^- concentrations for experiment D or previous hypotheses such as intervention of other regulatory systems or a particular renal sensitivity to NO carriers for experiments C and E. It would imply that NO transported *via* GTN and that transported when NO is inhaled have similar (on the heart and kidney) and different (on the systemic circulation) effects. These could be explained by the nature of the implied packaged forms and their respective metabolism, particularly biotransformation of GTN to NO.

Metabolic effects

Because we deproteinized the plasma samples, NO (from iNO or released by GTN)-derived metabolites (NO_2^- and NO_3^-) and GTN-derived metabolites (1,3- and 1,2-glyceryl dinitrate, and glyceryl mononitrate) were potentially measured as pNO_x^-. At the end of stage III, infusion of ivGTN significantly increased arterial and venous pNO_x^-

concentrations (table 3), and we observed only a statistical trend of iNO to increase pNO_x^- concentrations. This difference could be explained by a greater NO_x intake for ivGTN than for iNO and the larger number of metabolites of GTN. Other studies found significant difference in pNO_x^- concentrations between baseline and iNO treatment [7, 19, 20] with a longer period of inhalation and larger sample size.

During stage IV, pNO_x^- concentration remained high in experiment D (ivGTN), but decreased in experiment E (iNO), whereas uNO_x^- concentration remained high in all experiments. NO might be released during stage IV from its carrier-proteins with diuretic and without vasoactive effect, and metabolized in NO_x^-. Moreover, GTN-derived metabolites could be present at the end of stage IV and measured as NO_x^-. The absence of haemodynamic effects of these compounds during stage IV could be explained by their low potency or nitrate tolerance phenomenon. The explanation for pNO_x^- decrease in experiment E is the disruption of iNO administration.

In summary, previous studies have shown clearly a marked effect of iNO on pulmonary hypertension in pulmonary vasoconstriction models. Furthermore in the passive PEHT model without pulmonary vasoconstriction, iNO maintained its pulmonary antihypertensive property. These results suggest that iNO may have direct cardiac effects. This study also demonstrates a marked effect of iNO on renal function, similar to that of ivGTN. We conclude that iNO has extra-pulmonary effects in an *in vivo* swine model and consequently that iNO cannot be considered as a pure selective agent on the pulmonary vasculature.

2.3. *Manuscript 3:* Nitrate anion contributes to the deleterious effect of nitric oxide in respiratory dysfunction during endotoxic shock. Fan Yang, Alain S. Comtois, Li-wei Fang, Yves Gingras, Neil G. Hartman, Gilbert Blaise. *Intensive Care Medicine* 2000 (submitted).

In vivo nitrate anion ($t_{1/2}$, 1.5 to 8.0 h) is the major metabolite of nitric oxide (NO, $t_{1/2}$, 3 to 20 sec) and nitrate ion self is a permeant anion which can cross plasma membrane, affect cell electrophysiological properties and membrane enzyme (e.g. ATPase) activity, depress muscle contractility, facilitate vasopressin secretion, enhance sympathetic activity and increase vascular tone *in vivo*. Both beneficial and detrimental effects of NO in endotoxic shock have been elucidated. Therefore, we hypothesized that nitrate anion may contribute to the deleterious effect of NO in respiratory dysfunction during endotoxic shock *in vivo*.

In this study, we have demonstrated that there is a significant nitrate anion gradient between the lungs, diaphragm and plasma in control rats. Different doses (5, 10 or 20 mg/kg) of endotoxin (lipopolysaccharide, LPS) induced a significant increase in nitrate concentration both in tissues (lung and diaphragm) and in plasma. Alteration of nitrate concentration is paralleled by modification of histology and cell sarcolemmal permeability in the lungs and diaphragm, and respiratory rate is significantly correlated with nitrate concentration in the lungs during 24 h endotoxic shock. Nitrate concentration increased in a LPS dose-dependent manner was seen only in the lungs at 12 h. Diaphragm contractility can be reduced significantly by endotoxin *in vivo* or by nitrate anion alone *in vitro*. Collectively, these results indicate that nitrate anion contributes to the deleterious effect of NO in the impairment of respiratory function during endotoxic shock.

Abstract

Objective: To elucidate the role of nitrate anion, the major nitric oxide metabolite, in endotoxin-associated respiratory dysfunction.

Design: Prospective, comparable, experimental study.

Setting: Laboratory at a university hospital.

Subjects: Sprague-Dawley rats.

Interventions: Rats were injected intraperitoneally (i. p.) with 5, 10, or 20 mg/kg of lipopolysaccharide (LPS) or saline.

Measurements and results: Nitrate concentration and iNOS protein in the lungs, diaphragm, and plasma (or blood), respiratory rate during 24 h, histological and cell sarcolemmal integrity of the lungs and diaphragm, diaphragm contractility *ex vivo* were evaluated. A significant nitrate gradient was evident between the lungs, diaphragm, and plasma in control rats. Nitrate increased significantly in the lungs, diaphragm, and plasma at 12 h in septic rats. As nitrate concentration rose profoundly in plasma, the lung/plasma and diaphragm/plasma nitrate concentration ratio was decreased significantly. iNOS protein was increased in the lungs, diaphragm and blood. Nitrate concentration modification was paralleled by histological and cell sarcolemmal permeability changes in the lungs and diaphragm, and respiratory rats was significantly correlated with nitrate concentration in the lungs during endotoxic shock. Diaphragm contractility was reduced significantly by LPS *in vivo* or by nitrate anion alone *in vitro*.

Conclusions: These results, collectively, indicate that the nitrate anion contributes to the deleterious effect of NO on the impairment of respiratory function during endotoxic shock.

Key words Rat, nitric oxide metabolite, Endotoxic Shock, Acute Respiratory Distress Syndrome and Systemic Inflammatory Response Syndrome

Introduction

Nitric oxide (NO) is an active message molecule ($t_{1/2}$, 3 to 20 sec) synthesized by three isoforms of NO synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Growing lines of evidence suggest that NO plays a significant role in the physiological conditions and pathogenesis of respiratory dysfunctions in septic shock [4, 298]. NO react with superoxide in diffusion-limited rates, producing peroxynitrite, which (pKa 6.8, $t_{1/2} < 1$ sec) is rapidly converted to nitrate [30, 132].

Nitrate ($t_{1/2}$, 1.5 to 8.0 h) represents the major end-product of NO *in vivo* [256, 353] and nitrate itself is also a permeant anion which can cross plasma membrane [60, 187] and influence cell electrophysiological properties [162, 249], membrane enzyme (e.g. ATPase) activity [255], depress muscle contraction *in vitro* [16, 145], and facilitate vasopressin secretion, enhance sympathetic activity [313] and increase vascular tone *in vivo* [117, 385]. Therefore, we hypothesize that the modification of nitrate concentration, the major NO metabolite, in the lungs, diaphragm and plasma, may contribute to the deleterious effect of NO in respiratory function during sepsis. Despite evidence of the important role of NO in physiological functions and the pathogenesis of respiratory dysfunction in septic shock, the involvement of its major and long-lasting metabolite, nitrate anion, during septic shock remains unclear. Therefore, this study was designed to elucidate whether or not nitrate anion contributes to the deleterious effect of NO in respiratory dysfunction during endotoxic shock.

Materials and methods

Animal preparation

All experimental procedures were approved by the Animal Care Committee of the centre hospitalier de l'Université de Montréal. Sprague-Dawley rats (275–325 g) were injected intraperitoneally (i. p.) with a bolus LPS (5, 10 or 20 mg/kg, *Escherichia coli* Serotype 026:B6) or saline, and sacrificed, exsanguinated at 0 (control group), 6, 12 and 24 h after LPS treatment. Plasma, the lungs and diaphragm were harvested. Tissues were homogenized for 15 sec at 4°C and 0.1 ml of the supernatant of homogenized tissue was collected for nitrate assay.

Nitrate determination

Nitrate concentrations in the lungs, diaphragm and plasma were measured by a chemiluminescence NO Analyzer (Sievers Instruments, Boulder, CO) as described previously in detail [379, 385]. Briefly, plasma and supernatants of homogenized tissues were diluted 10-fold with distilled water and deproteinized by adding 1/20 volume of zinc sulfate. After centrifugation at 4,000 rpm for 15 min, 0.1 ml of the supernatants was measured. There was no detectable NO gas and only traces of nitrite in samples. Each sample was analyzed in duplicate.

Immunoblotting

Blood, crude diaphragm and lung homogenate proteins (80 μg) from each group (0, 6, 12 and 24 h) of rats were heated for 5 min at 95°C, then loaded onto 10% SDS-PAGE, and after electropheresis, proteins were transferred from the gels to the PVDF membranes, which were blocked overnight with 5% nonfat dry milk at 4°C and then incubated for 1 h at room temperature with primary monoclonal anti-iNOS (1:2500) antibodies (Transduction Laboratories, Lexington, KY). Specific proteins were detected with horseradish peroxidase-conjugated anti-mouse secondary antibodies and ECL reagents.

The blots were scanned and optical densities of protein bands were quantified (Alpha Imager, Alpha Innotech Inc., San Leandro, CA). High molecular weight standards served as markers. Protein concentration was measured by BCA kits from Bio-Rad [37, 89].

Histology and sarcolemmal integrity

Lung and diaphragm tissues were collected at designated experimental time points and fixed immediately in 10% buffered formalin overnight at room temperature, then embedded in paraffin. 5-μm thick sections were stained with routine hematoxylin and eosin. The slides were viewed in a blinded fashion under a Nikon ECLIPSE E600 light microscope and digitized with an MetaMorph image system (Universal Image, West Chester, PA) [30, 215].

The diaphragm myofiber sarcolemmal integrity was assessed by procion orange 14 (Sigma, St. Louis, MO) staining, as described previously [215]. Briefly, 10-μm tissue cross-sections were obtained by cryostat from diaphragm samples that had been placed in experimental Kreb's solution containing 0.2%(w/v) procion orange 14 for 60 min. Sarcolemmal lesions were detected by microscope and digitized with the MetaMorph system. Muscle fibers that demonstrated a clear increase in cytoplasmic fluorescence were counted, and the percentage of dye-positive fibers on each diaphragm section was measured.

Respiratory rate evaluation

Two additional groups of rats were used to evaluate respiratory rate at 0, 6, 12, and 24 h after sepsis induction. The animals were anesthetized with 50 mg/kg pentobarbital i. p. and the right carotid artery was cannulated with polyethylene tubing that was connected to a pressure transducer (Abbott Laboratories, North Chicago, IN) to measure blood

pressure, and respiratory rate was measured from the blood pressure trace, which showed a harmonic related to intra-thoracic pressure swings (Fig. 5, insert). The animals were allowed to stabilize for 30 min after surgery, then treated i. p. with 10 mg/kg LPS or normal saline (control). The animals were allowed to breathe spontaneously and kept in a prone position Normal saline (2.5 ml/kg/h) was infused continually i. p. and pentobarbital (2 mg/kg/h) was infused i. p. as required, via Harvard syringe pumps. Respiratory rate was recorded continually in a 24-h period with a Dataq data acquisition system.

Diaphragm strip preparation

A muscle strip (2-3-mm wide) was dissected from control and 12-h LPS-treated rats. The strip was mounted in a muscle chamber, placed vertically, and a double thread used to secure the central tendon to an isometric force transducer (Kent Scientific Instruments, Nagashigi, Japan). Muscle contractility was evaluated by stimulating the muscle at 5, 10, 20, 30, 50, 100 and 120 Hz while maintaining constant supramaximal current and stimulation duration (600 msec). Tetanic contractions were digitized at a frequency of 1 kHz with a computer and stored on hard disk for later analysis. Isometric forces were normalized for the muscle cross-sectional area by 1.056 g/cm^3 for muscle density. Peak tension (N/cm^2) was measured for each contraction within the force-frequency curve [37, 89]. To further evaluate the nitrate effect on diaphragm contractile function, diaphragm force generation was measured in control rats *ex vivo* under 10 mM sodium nitrate treatment for 60 min.

Statistical analysis

All values are expressed as means ± SEM. The data was assessed by two-way analysis of variance (two-way ANOVA) with the SAS program (SAS Institute Inc., Cary, NC). Any differences detected were evaluated *post hoc* by the Student-Newman-Keuls test. $p<0.05$ was considered significant.

Results

Fig. 1 shows nitrate concentration in the diaphragm, lung and plasma. In the diaphragm, all LPS doses tested (5, 10 and 20 mg/kg) induced a significant maximum increase in nitrate at 12 h ($p<0.01$), which returned to the time 0 (control) value at 24 h (Fig. 1A). Nitrate levels in the lungs (Fig. 1B) were significantly augmented after LPS treatment ($p<0.01$), exhibiting a peak value at 6 to 12 h. They increased significantly in a LPS dose-dependent manner ($p<0.05$) at 12 h (Fig. 1, B, insert). Plasma nitrate rose markedly at 6 and 12 h, but no significant differences were evident at these same time points after different dosages (5, 10, 20 mg/kg) of LPS administration (Fig. 1C).

Nitrate concentrations in plasma (μM or pmol/mg plasma), lungs and diaphragm (pmol/mg wet weight tissue or μM) were calculated in terms of the same mass unit (mg) and, subsequently, the lung/plasma and diaphragm/plasma nitrate concentration ratios were evaluated. A marked difference between tissue and plasma nitrate in control rats was evident in the lungs (8.2 times, $p<0.01$) and diaphragm (7.4 times, $p<0.01$). These diaphragm/plasma (Fig. 1, A, insert) and lung/plasma (data not shown) nitrate concentration ratios decreased significantly after LPS treatment, with a minimum being reached at 12 h for both ($p<0.05$).

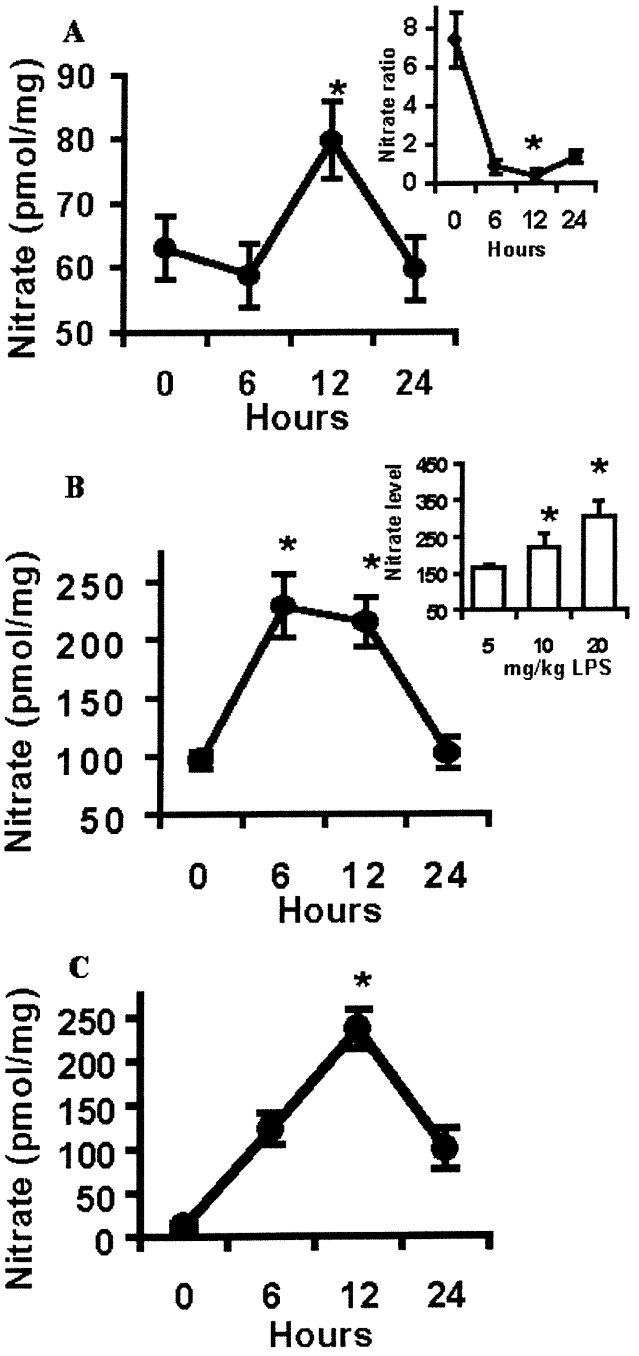


Fig. 1. Temporal production of nitrate in the diaphragm (A), lungs (B) and plasma (C) during 24 h (for clarity, data are the mean of three groups of 5, 10, and 20 mg/kg LPS-treated rats, n=10 (control) to 20 rats per data point). The inserts respectively show alteration of the diaphragm/plasma nitrate concentration ratio and nitrate concentration increased in the lungs in a LPS dose-response manner at 12 h. * $p<0.05$.

iNOS protein concentration in the diaphragm, lungs and blood increased significantly after 10 mg/kg LPS administration (Fig. 2). iNOS protein concentration was higher in blood than in the diaphragm and lungs at 12 h after LPS administration.

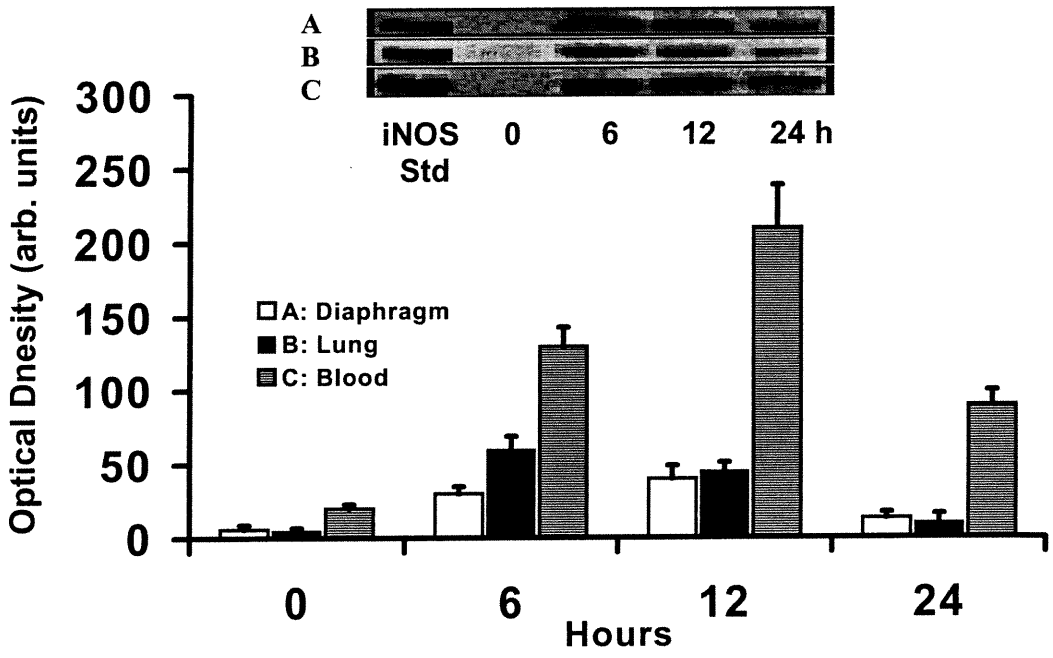


Fig. 2. iNOS protein concentration alterations in the diaphragm (A), lungs (B), and blood (C) at time 0 (control), 6, 12 and 24 h after 10 mg/kg LPS administration to rats.

Histology of the lungs and diaphragm revealed that (Fig. 3), in comparison with control tissue, there was a significant amount of inflammatory cell infiltration into these tissues in LPS-treated rats, particularly, lung histology revealed that there was a significant amount of exudation, including fluids, inflammatory cells, and debris in both alveoli and bronchioli, edematous and marked congestion of the alveolar walls with granulocytes distributed almost equally across the section after 12 h LPS treatment in rats. As seen in Fig. 4, cell sarcolemmal lesions in the lungs and diaphragm were markedly visualized by fluorescent dye staining, and there were 22.8% ± 4.5% and 1.8% ± 0.3% ($p<0.01$) injured diaphragm myofibers in 10 mg/kg LPS treated and control rats, respectively.

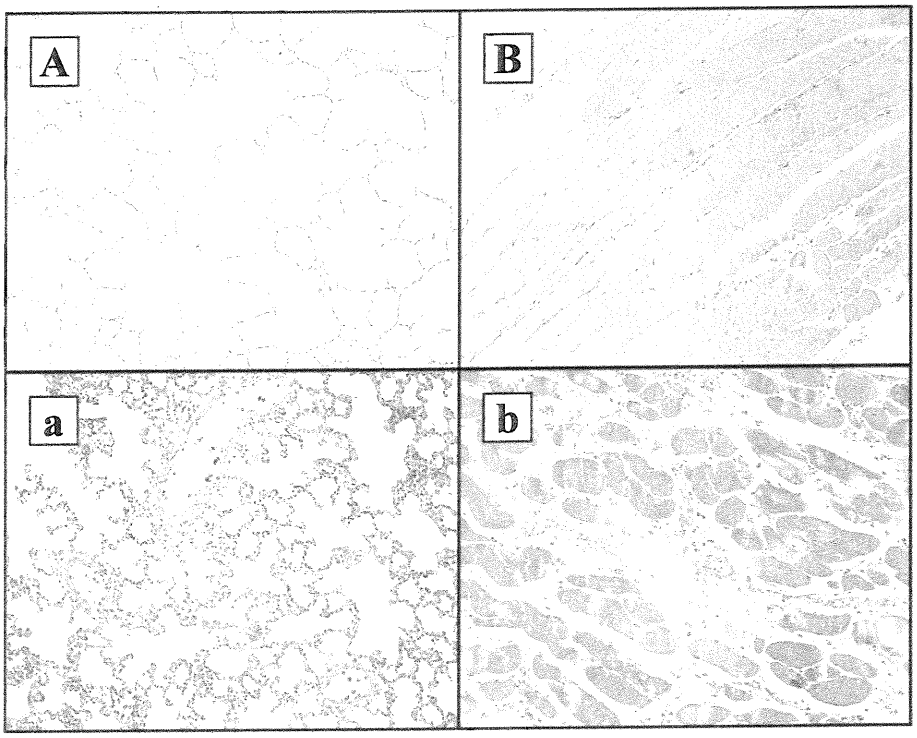


Fig. 3. Histology in the lungs and diaphragm from control rats (A to B) and septic rats (a to b), respectively. Note inflammatory cells infiltrating septic lung and diaphragm. Light microscopy x 200.

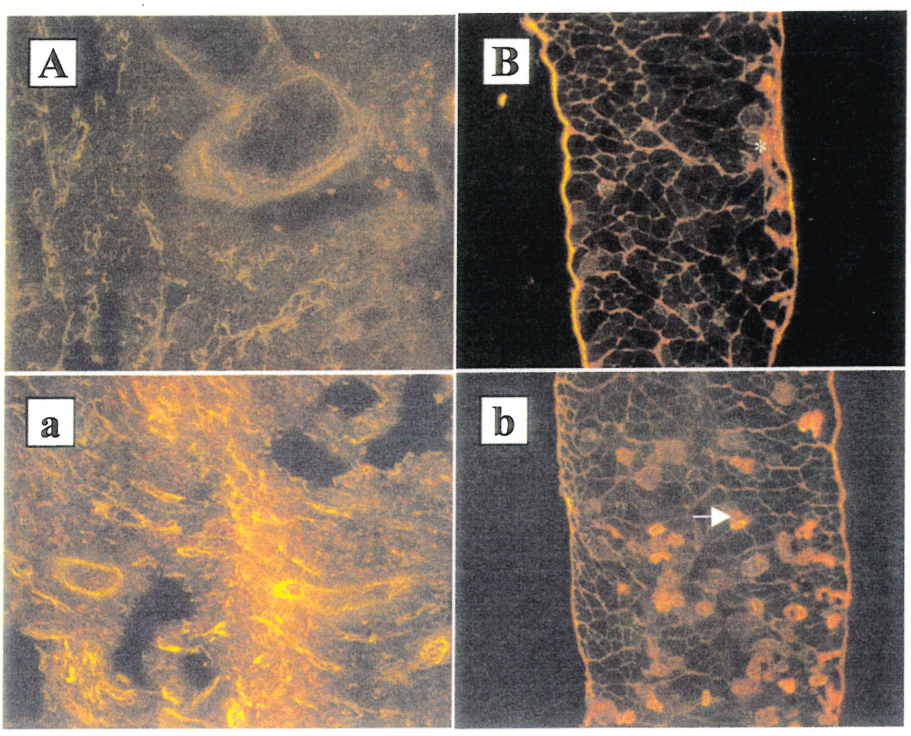


Fig. 4. Sarcolemmal lesions in the lungs and diaphragm from control rats (A and B) and septic rats (a and b). Note the example of injured fibers (arrows) and artifact (stars). Fluorescent microscopy x 200.

Respiratory rate was increased significantly at 6 and 12 h after LPS administration (Fig. 5). There was a significant correlation between the respiratory rate and nitrate level in the lungs (r^2=0.996, $p<0.01$).

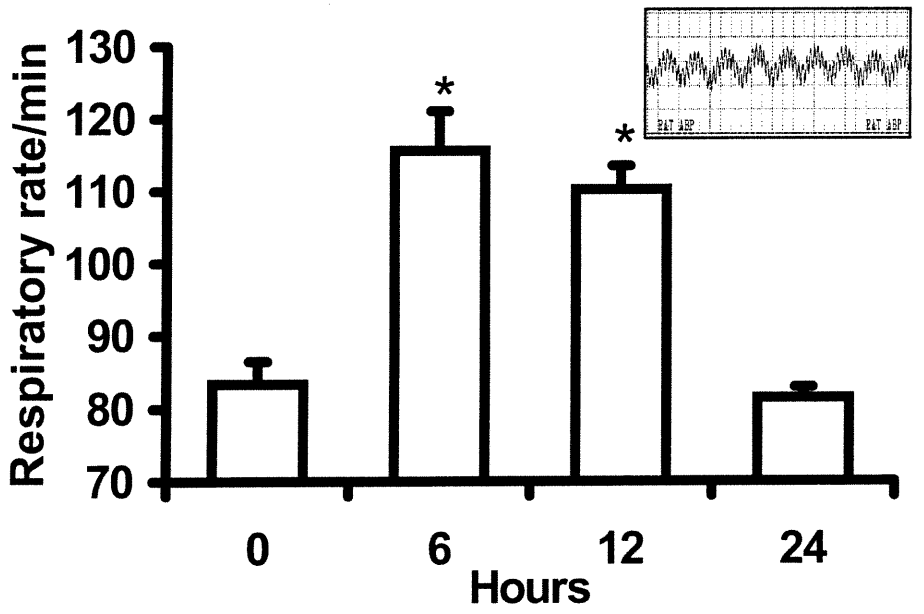


Fig. 5. Change in the respiratory rate in response to 10 mg/kg LPS i. p. administration. * $p<0.01$. Insert: representative tracing of the respiratory rate recorded at 6 h with continuous 24-h monitoring.

The diaphragm force-frequency relationship was significantly shifted down ($p<0.05$) at all frequencies measured (except at 5 Hz) after LPS *in vivo* treatment (Fig. 6, A). Diaphragm contractility was also depressed significantly by *in vitro* 10 mM sodium nitrate for 60 min, but at frequencies greater than 30 Hz ($p<0.05$) (Fig. 6, B).

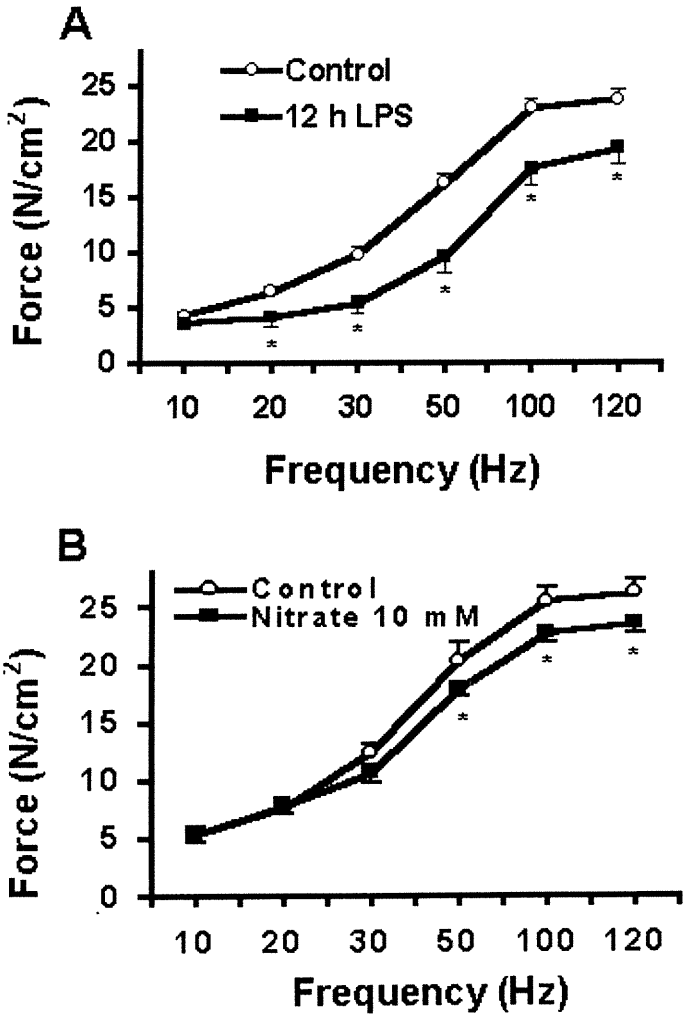


Fig. 6. Change in diaphragm contractility in response to 10 mg/kg LPS i. p. injection (A) *in vivo* and 10 mM sodium nitrate (B) *in vitro*. * $p<0.05$.

Discussion

The present study have showed that: 1) There is a nitrate gradient between the lungs, diaphragm and plasma of normal rats; 2) Nitrate concentrations are increased not only in plasma but also in the lungs and diaphragm after LPS administration to rats, and they are elevated more in plasma than in tissue. As a consequence, lung/plasma and diaphragm/plasma nitrate concentration ratios are significantly decreased; 3) Nitrate anion alone *in vitro* can significantly decrease diaphragm contractility. From these findings, we conclude that nitrate anion may synergistically enhance the detrimental effects of NO and/or peroxynitrite on the impairment of respiratory function during endotoxic shock.

The results we obtained for plasma nitrate are consistent with those in which it was found to be increased after LPS administration [132, 256]. Still, little is known about nitrate concentration in the diaphragm and lungs under normal conditions and during septic shock. Nitrate is the major metabolite of NO *in vivo*, and its half-life is ~1.5 to 8.0 h. In comparison to the half-life of NO (~3 to 20 sec), nitrate's half-life is at least 270 to 9,600 times longer *in vivo*, which, therefore, indicates that nitrate anion is only past NO production, and experimental evidence supports this view as NO reaches ~nM levels and nitrate ~uM levels as well as a chronic drop in blood pressure after LPS infusion is not convincingly mirrored by NO concentration (relatively constant amount of NO), measured directly by NO sensor, but is paralleled by indirect NO assay (nitrite + nitrate) [30, 132]. In agreement with previous reports that iNOS is elevated after LPS

administration [37, 89], our data also show that iNOS was much more abundant in blood than in the lungs and diaphragm.

Our results are consistent with previous findings that LPS induces significant inflammatory cell infiltration into the diaphragm as well as increased diaphragm myofiber membrane permeability and significant granulocyte infiltration in the lungs with exudation in alveoli and bronchioli [132, 215]. Since inflammatory cells contribute significantly to iNOS concentration and activity during sepsis [37, 89], they could alter extracellular nitrate concentration. SIRS induced by endotoxins can also produce a significant amount of superoxide and peroxynitrite that could, at the same time, contribute to cell membrane injury as well as cell dysfunction [30, 37, 89]. Therefore, several direct and indirect LPS-induced factors could potentially act in parallel or synergistically to impair diaphragm myofiber membrane and respiratory functions of the lung during sepsis. SIRS or endotoxins can also trigger an acute respiratory distress syndrome (ARDS), both in animals and humans. As shown in Fig. 5, increase in respiratory rate is an additional response contributing to lung and diaphragm injury [4,30, 37, 89]. In agreement with this observation, our data show, additionally, there was a significant correlation between nitrate concentration in the lungs and the respiratory rate in septic rats, which may indicate that respiratory rate changes have an intrinsic association with nitrate concentration alteration during sepsis.

It has been shown that muscle contractility is depressed following LPS induced sepsis [37, 89], and muscle contraction can be depressed by nitrate anion alone [16, 145], which impairs the active calcium uptake by the sarcoplasmic reticulum and also increases the permeability of amphibian muscle cell *in vitro* [145, 162]. Similarly, our results show

that nitrate anion alone *in vitro* affects only the high frequency component of tension development when compared to the septic diaphragm. The discrepancy observed in our results (between LPS and nitrate force frequency curves) may indicate two different mechanisms contributing to force loss, one which involves mostly loss of force through attenuated excitability and the other which may involve force loss through NO, superoxide anions and peroxynitrite [4, 30, 132]. Thus, our results provide additional experimental evidence that nitrate can affect the force developed in the diaphragm of rats *ex vivo*. This effect is possibly additive or synergestic to the effects of NO, superoxide anions and peroxynitrite during sepsis.

Direct comparisons of intracellular and extracellular nitrate concentration under normal and septic shock conditions have not been elucidated, particularly between the diaphragm and lungs. Diaphragm tissue is mainly composed of muscle proteins. Therefore, nitrate concentration in homogenized diaphragm tissue represents mainly the intracellular component. Extracellular liquids are mainly plasmatic and interstitial, and electrolyte composition in these two compartments is identical (anion and cation) [198]. We, therefore, assumed that nitrate concentration in plasma and the interstitium are very similar. NO is constantly synthesized by NOS under physiological conditions, and its synthesis can be altered in a variety of pathophysiological situations [4, 30, 132, 298]. Nevertheless, nitrate is the major end-product of NO metabolism [256, 353]. Our results provide the first evidence that there is a nitrate gradient between tissue of the lungs, diaphragm (mostly intracellular) and plasma (extracellular), which was altered significantly in septic rats. Over-accumulation of plasma nitrate and, subsequently, a change in this intra/extracellular nitrate concentration ratio may represent a deleterious

effect of NO on respiratory functions during sepsis. Thus, it is reasonable to assume that cell function modified by nitrate anion may provide one of multiple-mechanisms for the functional alterations of lung and diaphragm observed during septic shock.

In conclusion, either LPS or nitrate anion alone affects diaphragm force generation and there is a nitrate gradient between the diaphragm, lungs and plasma, which was significantly altered during sepsis. From these findings, we consider that nitrate anion may synergistically enhance the detrimental effects of NO and/or peroxynitrite on the impairment of respiratory function during endotoxic shock.

2.4. *Manuscripts 4:* The nitric oxide metabolite nitrate anion contributes to septic shock and multiple organ dysfunction. Fan Yang, Alain S. Comtois, Liwei Fang, Neil G. Hartman, and Gilbert Blaise. *Critical Care Medicine* 2000 (submitted).

It has always been challenging and controversial to ascribe a causal role for NO and its metabolites (e.g. $ONNO^-$ and NO_3^-) in process of sepsis/SIRS/MODS. The emergence of NO as a essential messenger molecule in physiological condition and critical mediator in pathophysiological states have both complicated and shed new light on the understanding key mechanisms of sepsis/SIRS/MODS, as well as generating new controversies. NO and its metabolites and the action of these molecules are much more multifaceted than previously thought. The redox products of NO and their chemical reactions with biological molecules have been studied extensively in the last few years. Interestingly, as the chemistry of NO reveals its richness, the pharmacological and pathophysiological roles of NO become more diverse as well as confusing. To date, there is no satisfactory explanation as to why supplementation of exogenous NO as well as inhibition of endogenous NO both attenuate/cause tissue injury. Since nitrate anion is absolutely the major and long-lasting end-product of NO *in vivo* and accordingly this phenomenon may raise additional question: does nitrate anion represent another facet of NO *in vivo*? As it mentioned in previous manuscripts, nitrate anion (in the range of μM to mM) itself can affect enzyme activity, cell and organ functions. Therefore, we hypothesized that over-accumulated nitrate anion may contribute to the deleterious effect of NO *in vivo,* and consequently to LPS-induced sepsis/SIRS/MODS. The results of present study may offer an alternative explanation for some apparently contradictory results on the role of NO and its metabolites in sepsis/SIRS/MODS.

Abstract

Objective: To assess the role of nitrate anion, the major nitric oxide (NO) metabolite, in septic shock and multiple organ dysfunction Syndrome (MODS).

Design: Prospective, comparable, experimental study.

Setting: Laboratory at a university hospital.

Subjects: Sprague-Dawley rats.

Interventions: Rats were injected intraperitoneally (i. p.) with 5, 10, or 20 mg/kg of lipopolysaccharide (LPS) or saline for 0, 6, 12, or 24 h.

Measurements and Main results: There were significant differences between nitrate concentrations in the heart, lung, kidney, liver, brain, aorta, diaphragm, spleen, thymus, testis or ovary, hind limb muscle, intestine, adipose tissue, bone, bladder, urine and plasma tested, which imply that there is a nitrate gradient between intracellular and extracellular compartments. LPS induced a significant increase in nitrate concentration at 12 h in most tissues and organs, except the brain, adipose tissue and muscle. Nitrate concentration in plasma rose more than in tissues. The LPS dose-dependent nitrate concentration was observed only in the aorta and lungs. The nitrate concentration change was paralleled by systemic inflammatory response syndrome which was indicated by alterations of myeloperoxidase activity and by impaired histological and sarcolemmal integrity in tissues and organs. Mean arterial pressure was negatively correlated with nitrate concentration modifications in the aorta during 24 h sepsis.

Conclusions: These results, collectively, indicate that the major NO metabolite nitrate anion contributes to the deleterious effect of NO in septic shock and MODS during sepsis.

***Key words*:** rat; nitric oxide; nitrate; metabolite; free radical; myeloperoxidase; endotoxic shock; Intensive care; systemic inflammatory response syndrome; multiple organ dysfunction syndrome.

Introduction

Sepsis and septic shock, the most serious sequelae of bacterial infections, are caused mostly by gram-positive or gram-negative bacteria. Bacterial lipopolysaccharide (LPS), the outer membrane of gram-negative bacteria, plays a key role in the pathogenesis of septic shock by activating the systemic inflammatory response, releasing a number of mediators and cytokines from various cells and organs, including the endothelium, vascular smooth muscle cells, cardiac myocytes, astrocytes, Kupffer and inflammatory cells. Many mediators, such as tumor necrosis factor, reactive oxygen species, and more recently nitric oxide (NO), have been speculated to cause the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [17, 30, 126].

NO plays an important role in many physiological and pathophysiological conditions. It is derived from oxidation of the terminal guanidino nitrogen atom of L-arginine by NO synthase (NOS), of which 3 isoforms have been identified as being distributed ubiquitously in the body. Two of these isoforms are neuronal NOS (nNOS) and endothelial NOS (eNOS), respectively, both constitutive NOS (cNOS). The third isoform (inducible NOS or iNOS) is evoked upon stimulation by LPS and/or cytokines, leading to the production of large quantities of NO. Numerous investigations have confirmed that NO is generated in significantly elevated concentrations in several tissues and organs of endotoxemic animals and humans [103, 215, 284], and growing lines of evidence suggest that it plays a significant role in the pathogenesis of SIRS and MODS during sepsis [89, 132, 315]. NO itself is a relatively simple radical gas and a short-lived, highly-reactive molecule ($t_{1/2}$, 3 to 20 s), as exemplified by its diffusion-limited reaction

with superoxide to produce peroxynitrite (pKa 6.8, $t_{1/2} < 1$ s), which is rapidly converted to nitrate *in vivo* [30, 103, 132].

Nitrate ($t_{1/2}$, 1.5 to 8.0 h) represents the major end-product of NO *in vivo* [30, 103, 132, 353]. Nitrate itself is a permeant anion that can cross the plasma membrane and significantly affect cell electrophysiological properties [162, 187], potassium transport in microvessels [214] and changes in cell membrane functions to facilitate calcium uptake [145], membrane enzyme (e.g. ATPase) activity [255] and muscle contractility *in vitro* [16]. Nitrate anion can facilitate vasopressin secretion and function synergistically in the central nervous system (CNS) to enhance sympathetic activity [313] and increase blood pressure *in vivo* [117, 385]. We, therefore, hypothesized that alteration of nitrate concentration, the major NO metabolite, may contribute to SIRS and MODS during sepsis. Despite tremendous evidence demonstrating that LPS can raise NO production in different cell types *in vitro* and in plasma of both humans and animal models of sepsis *in vivo*, the systemic evaluation of nitrate concentration in organs and tissues under normal or septic conditions has not been done. Furthermore, it remains to be elucidated whether or not there is a dose response to LPS-induced nitrate production alteration in tissues and organs, and the potential role of nitrate in septic shock has not yet been determined.

The present study shows that there are significantly different baseline nitrate anion concentrations in tissues and organs of normal rats. The different nitrate concentrations between tissues, organs and plasma indicate a nitrate gradient between intracellular (tissues) and extracellular (plasma) sites. This nitrate concentration change in tissues and organs is paralleled by the inflammatory response, indicated by alterations of myeloperoxidase (MPO) activity with impaired histological and sarcolemmal integrity

in tissues and organs. Hemodynamic changes are negatively correlated with nitrate concentration modification in the aorta during sepsis. Taken together, our results indicate that over-modification of this major NO metabolite's concentrations *in vivo*, namely nitrate anion, contributes to the deleterious effect of NO in septic shock and MODS during sepsis.

Methods

Reagents

LPS (from *Escherichia coli* Serotype 026:B6), tetramethylbenzidine (TMB), 3% H_2O_2, vanadium(III) chloride (VCl_3) and sodium nitrate were obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO). All samples and solutions were prepared in purified distilled water, which contained no detectable NO or its metabolites (detection limit = 1 pmol), as measured by a Sievers 270B Chemiluminescence NO Analyzer (Sievers Instruments, Boulder, CO).

Animal preparation

Sprague-Dawley rats weighing 275-325 g (Charles River, St-Constant, QC, Canada) were acclimatized for one week in a quarantine room under a 12-h light-dark cycle with free access to standard pellet chow and water. All experimental procedures were approved by the Animal Care Committee of the Centre hospitalier de l'Université de Montréal.

NO metabolite determination

The NO metabolite (nitrate) concentrations in samples were measured by chemiluminescence, as described previously in detail [379]. The rats were injected ip

with a single dose of LPS (5, 10 or 20 mg/kg body weight in saline) or normal saline (control). They were sacrificed by decapitation and exsanguinated at 0 (control), 6, 12 or 24 h after experimental treatment. Blood was collected in heparinized tubes. Plasma was prepared by centrifuging whole blood at 4,000 rpm at ambient temperature for 15 min, and was stored at -36°C until analyzed. The heart, lungs, liver, kidneys, brain (cerebrum), aorta, diaphragm, spleen, thymus, testes or ovaries, skeletal muscle (adduct magnus), intestine (small), adipose tissue (omentum), and bone (sternum) were harvested. Urine was collected from the intact bladder, after which the bladder was dissected surgically, and harvested. Adherent connective tissues were removed carefully from the different organs. The tissues and organs were rinsed externally 3 times with cold saline (pH 7.4, 4°C), blotted, dried, and weighed. They were then immersed in saline solution and stored in test tubes at -36°C for further measurement. When necessary, they were homogenized in a Polytron tissue homogenizer for 15 s. Sonication was repeated 3 times for 15 s at 4°C. After centrifuging the homogenized cell extracts at 4,000 rpm at ambient temperature for 15 min, a 0.1 ml fraction of the supernatant was collected for nitrate assay.

Briefly, plasma and the supernatant of homogenized tissues were diluted 10-fold with distilled water and deproteinized by adding 1/20 volume of zinc sulfate to a final concentration of 15 g/L. After centrifugation at 4,000 rpm for 15 min, 0.1 ml of the supernatant was applied to the microreaction purge vessel containing about 5 ml 1.0% VCl_3 solution at 90°C for the conversion of nitrate to NO, which was measured by a chemiluminescence NO analyzer with a Dataq data acquisition system (Dataq Instruments, Akron, OH). A known concentration of nitrate solution served as standard.

There was no detectable NO gas and only trace amounts of nitrite in the samples. Each sample was analyzed in duplicate.

Myeloperoxidase measurement

MPO was extracted from homogenized tissues (heart, lung, liver, kidney, spleen, and plasma, respectively) by suspending the material in 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0. The specimens were sonicated in an ice bath for 15 s, then freeze-thawed 3 times. The suspensions were centrifuged at 4,000 rpm for 15 min, and the resulting supernatant assayed. The reaction buffer, consisting of 1.6 mM TMB, 0.3 mM H_2O_2 and 50 mM potassium phosphate (pH 6.0), was incubated for 5 min at 37°C. A blank reading was taken at 652 nm in a 1-cm cuvette, with a spectrophotometer (Model 8542A, Hewlett-Packard, Palo Alto, CA), and 50 μl of the supernatant solution to be assayed was added in 2 ml total volume. The initial increase in absorbance at 652 nm served to calculate the amount of TMB oxidation product. One unit of MPO activity was defined as the amount of enzyme that utilizes 1 μmol of H_2O_2 per min at 37°C [9].

Histology

The heart, kidney, liver, lung, brain, spleen, intestine, diaphragm, muscle, adipose tissue, bone and testis were collected randomly (total of 24 rats) at designated experimental time points and fixed immediately in 10% buffered formalin, kept overnight at room temperature, then embedded in paraffin. The samples were cut into 5-μm thick sections by microtome and stained with routine hematoxylin and eosin. The slides were viewed in a blinded fashion under a Nikon ECLIPSE E600 light microscope digitized with a MetaMorph image-processing system (Universal Image, West Chester, PA).

Cellular sarcolemmal permeability evaluation

To analyze cellular sarcolemmal integrity, heart, kidney, liver, lung, brain, spleen, intestine and diaphragm tissues were collected after euthanasia in 2 additional groups of rats (control and 10 mg/kg LPS at 12 h, n = 5 per group) at designated experimental time points for perfusion with low-molecular weight (FW = 631) fluorescent tracer dye (Procion Orange 14, Sigma Chemical Co.) to which the sarcolemma of normal cell is impermeable [215]. The tracer dye (1% in Kreb's solution, total volume 10 ml) was slowly infused into the femoral vein for 10 min, and the samples were then surgically dissected and submerged immediately in oxygenated 1% Procion dye/Kreb's solution at room temperature for an additional 60 min. They were subsequently snap-frozen in isopentane pre-cooled with liquid nitrogen, and preserved at -80°C. Serial sections (10-μm thick) were cut at -20°C with a cryostat microtome (Leica Cryocut 1800, Heidelberg, Germany).

To assess cellular sarcolemmal integrity, serial sections of tissue samples were selected randomly in a blinded manner and digitized with an epifluorescence microscope (Nikon ECLIPSE E600 light microscope, using the fluorescein filter setting and magnification level of X200) and a MetaMorph Image system. Sepsis-induced cellular sarcolemmal injury was identified by the inability to exclude the tracer dye from the cytoplasm. Cells that demonstrated a clear increase in cytoplasmic fluorescence were considered to have impaired cellular sarcolemmal integrity. Areas with sectioning artifacts (folds and tears) were excluded, as were the edges of sections, to avoid areas potentially damaged by tissue dissection.

Hemodynamic monitoring

Mean arterial pressure (MAP) was monitored in 2 additional groups of rats (n = 10 rats per group). The animals were anesthetized ip with 50 mg/kg sodium pentobarbital and allowed to breathe spontaneously. The right carotid artery was cannulated with polyethylene tubing that was tunneled subcutaneously and connected to a pressure transducer (Transpac IV, Abbott Laboratories, North Chicago, IN) with a Hewlett-Packard 54S monitor. The animals were allowed to stabilize for at least 30 min after surgery. They were then treated ip with 10 mg/LPS or normal saline (control). MAP was recorded continually in a 24-h period using a Dataq data acquisition system with an IBM computer. The animals were kept in a prone position on a heating pad to maintain their rectal temperature between 37 and 38°C. Temperature was monitored with a HP electronic rectal probe. Normal saline (2.5 ml/kg/h) was infused continually ip, with pentobarbital (2 mg/kg/h) ip as required, using Harvard syringe pumps (Harvard Apparatus, St. Laurent, QC, Canada).

Data analysis

All values are expressed as means ± SEM. The data were analyzed by two-way analysis of variance (two-way ANOVA) and Pearson's correlation coefficients, when applicable, with the SAS program (SAS Institute Inc., Cary, NC). Any differences detected were evaluated *post hoc* by the Student-Newman-Keuls test. $P<0.05$ was considered to be significant.

Results

Based on the assumption that 0.1 ml of plasma weighs 0.1 g, we used the same mass unit (mg) to calculate NO metabolite concentration in plasma (pmol/mg plasma or equivalent in μM), tissues and organs (pmol/mg wet weight tissue). Subsequently, the intracellular/extracellular nitrate concentration ratio was evaluated.

Baseline nitrate concentration in urine, plasma, tissues and organs. Nitrate concentration was significantly different in the tissues and organs studied, the highest being in urine and the lowest in plasma ($P< 0.001$) respectively, in terms of equivalent mass units in normal rats (Fig. 1 A). Significantly different nitrate concentrations between organs and plasma, such as in heart/plasma (7.7 times, $P< 0.01$) and aorta/plasma (11.2 times, $P< 0.01$) were evident in control rats. Nitrate concentration increased more in plasma (equal to or even higher) than in most tissues, except the aorta, bladder and urine, at 12 h after LPS treatment (Fig. 1 B and C).

Alteration of nitrate concentration in urine, plasma, tissues and organs during sepsis. LPS induced a significant rise in plasma nitrate concentrations at 6 and 12 h ($P< 0.01$), which did not fully recover at 24 h in 5, 10 and 20 mg/kg LPS-treated rats. Nitrate concentrations in most tissues and organs (Fig. 2A, B, C and D) were increased significantly after LPS treatment in rats ($P< 0.01$), and exhibited peak values at 12 h, but did not change significantly in the brain, adipose tissue and skeletal muscle during 24 h after LPS treatment. Nitrate levels in the aorta and lungs (lung data not shown) were significantly elevated in a LPS dose-dependent manner at 12 h (Fig. 2 insert, $P< 0.05$). As plasma nitrate concentration increased markedly, the tissue or organ/plasma nitrate concentration ratio was significantly decreased, including the brain, skeletal muscle, and

adipose tissue, after LPS treatment, with the minimum ratio in the heart being reached at 12 h (Fig. 2 insert, $P< 0.05$) and recovering partially at 24 h. Nitrate concentration in the aorta was negatively correlated with MAP alterations ($r^2 = -0.96$, $P< 0.05$) while the aorta/plasma nitrate ratio (data not shown) correlated positively with MAP changes ($r^2 = 0.95$, $P< 0.05$) after 10 mg/kg LPS ip injection into rats. Following LPS treatment, nitrate concentration was also significantly increased in urine taken directly from the bladder. Active nitrate secretion from plasma to kidney to urine was evident, progressing from 12 pmol/mg (or μM) to 59 to 179 pmol/mg under normal conditions, and from 233 to 154 to 1,428 pmol/mg under septic conditions, respectively.

Temporal MPO activity in tissues and organs during sepsis. MPO activity was significantly increased in the heart, lung, kidney, and liver 12 h after LPS treatment in rats (Fig. 3 A), but was not significantly changed in plasma, and decreased in the spleen. This parameter also increased in a LPS dose-dependent manner in the lungs during sepsis (Fig. 3 B).

Histological changes in tissues and organs during sepsis. The heart, kidney, liver, lung, brain, spleen, intestine, diaphragm, muscle, adipose tissue, bone and testis (6 to 20 sections) from 8 septic rats at 12 h (3 rats at 5 mg/kg LPS, 3 rats at 10 mg/kg LPS, 2 rats at 20 mg/kg LPS) as well as from 6 controls (6 to 12 sections) were evaluated. Hematoxylin-eosin staining of the heart, kidney, liver, lung, brain, spleen, intestine, diaphragm, muscle, adipose tissue, bone and testis revealed that (Fig. 4 a to l), in comparison with control tissues (Fig. 4 A to L), there was a minimal to severe inflammatory response with an increase in inflammatory cells infiltrating these tissues in LPS-treated rats.

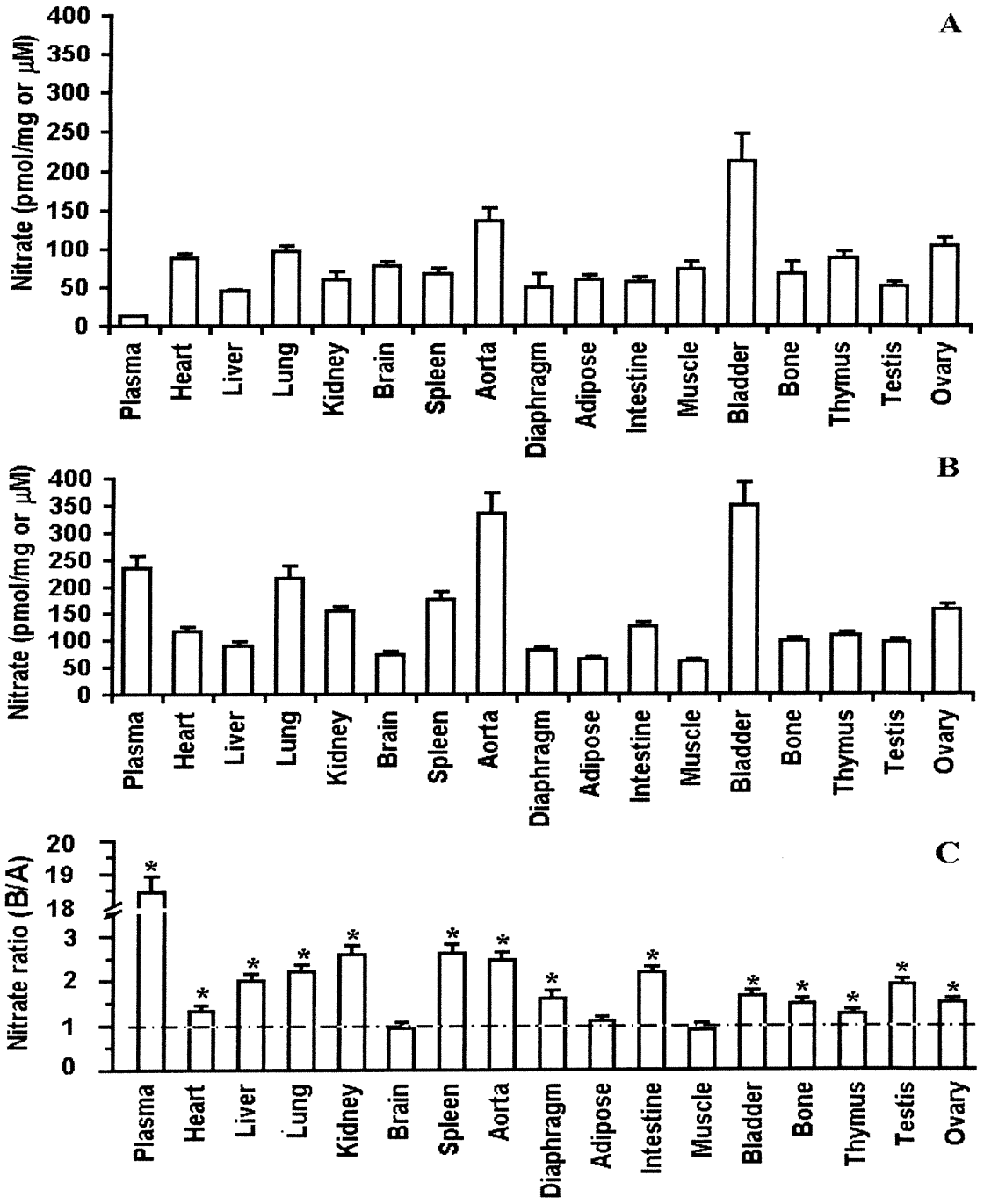


Figure 1. Spatial distribution of NO metabolite (nitrate) in the tissues and organs of normal (A) and septic rats (B) with its ratio (C) (B/A, namely septic/control) at 12 h. (For clarity, data are the means of 3 groups of 5, 10, and 20 mg/kg LPS-treated rats; n=10 (control) to 22 rats per data point.) * $P < 0.05$.

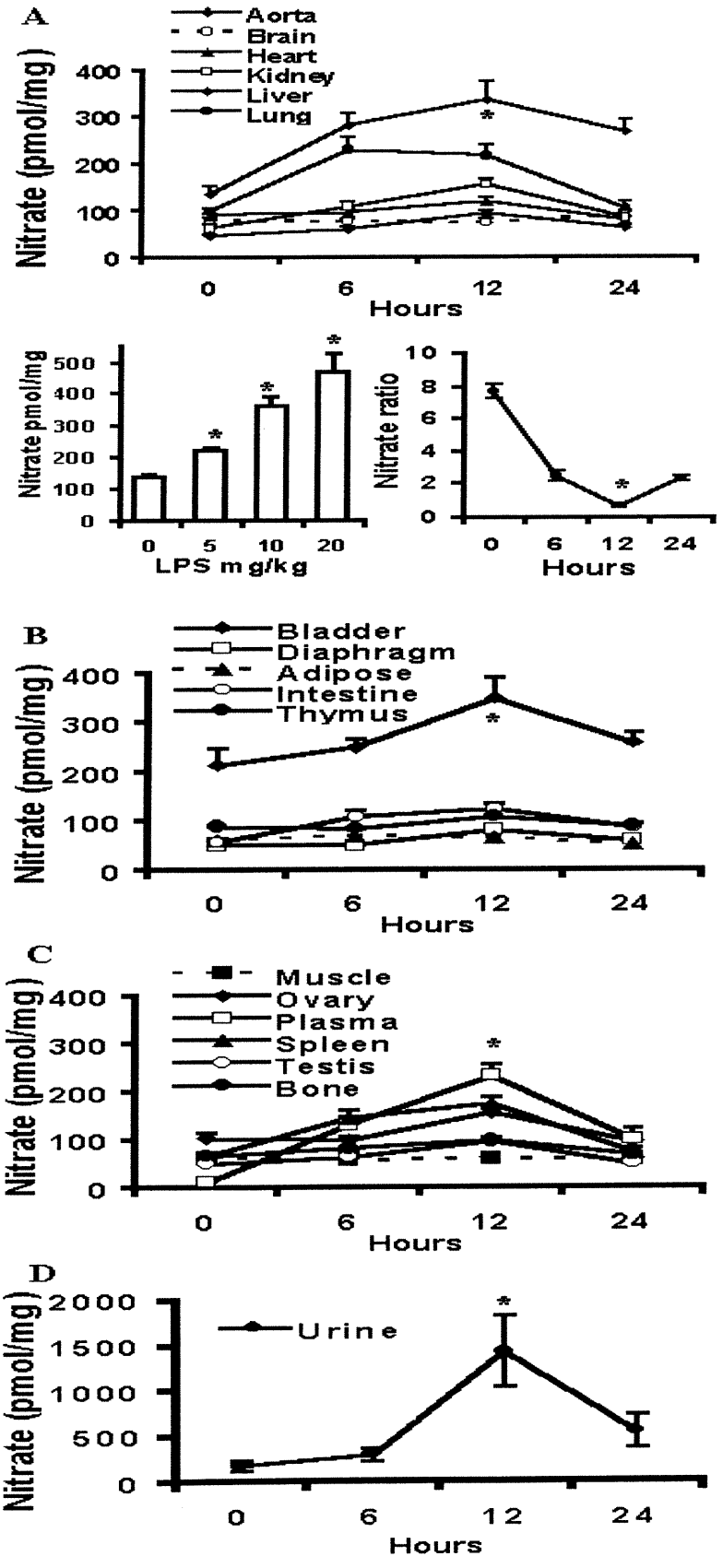

A
Aorta
Brain
Heart
Kidney
Liver
Lung
Nitrate (pmol/mg)
400
300
200
100
0
0
6
12
24
Hours
Nitrate pmol/mg
500
400
300
200
100
0
0
5
10
20
LPS mg/kg
Nitrate ratio
10
8
6
4
2
0
0
6
12
24
Hours
B
Bladder
Diaphragm
Adipose
Intestine
Thymus
Nitrate (pmol/mg)
400
300
200
100
0
0
6
12
24
Hours
C
Muscle
Ovary
Plasma
Spleen
Testis
Bone
Nitrate (pmol/mg)
400
300
200
100
0
0
6
12
24
Hours
D
Urine
Nitrate (pmol/mg)
2000
1500
1000
500
0
0
6
12
24
Hours

Figure 2. Temporal production of NO metabolite (nitrate) in the heart, liver, lung, kidney, aorta and brain (A), bladder, diaphragm, adipose tissue, intestine, and thymus (B), skeletal muscle, ovary, plasma, spleen, testis, bone (C), and urine (D), respectively at times 0, 6, 12 and 24 h. (For clarity, data are the means of 3 groups of 5, 10, and 20 mg/kg LPS-treated rats; n=10 (control) to 22 rats per data point.) Inserts show that nitrate concentration in the aorta increased in a LPS dose-dependent manner and alteration of the heart/plasma nitrate concentration ratio during sepsis, respectively. * $P < 0.05$.

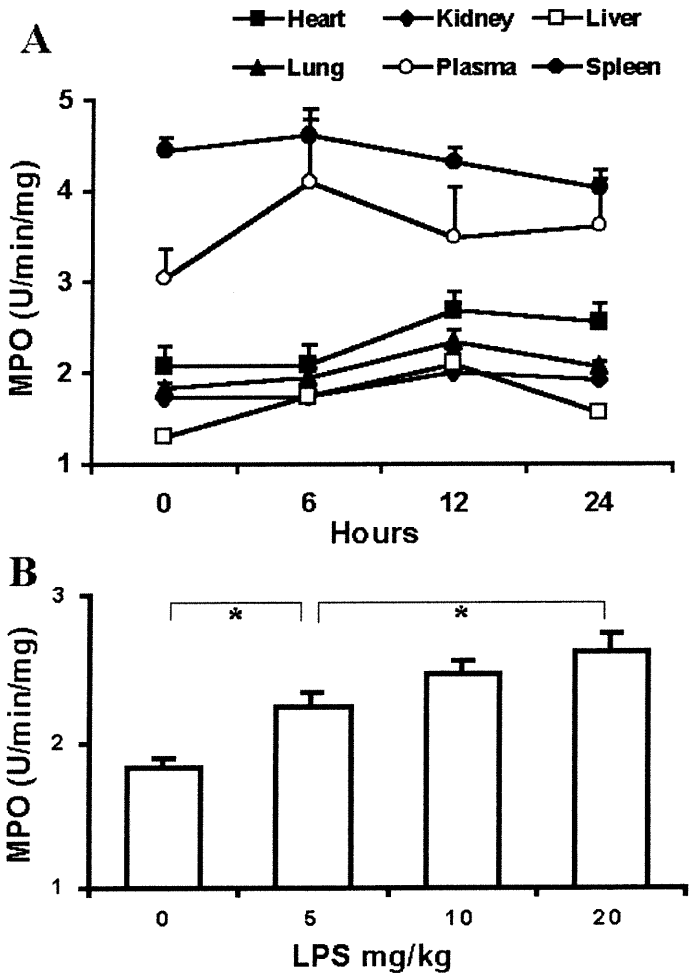


Figure 3. MPO activity in the heart, lung, kidney, plasma, liver and spleen at times 0 (control), 6, 12, and 24 h after LPS treatment in rats (A). MPO activity in the lungs showed a LPS dose-dependent response (B) at 12 h. (For clarity, data are the means of 3 groups of 5, 10, and 20 mg/kg LPS-treated rats; n=10 (control) to 22 rats per data point.) * $P < 0.05$.

Figure 4 (Heart, Kidney, Liver, Lung)

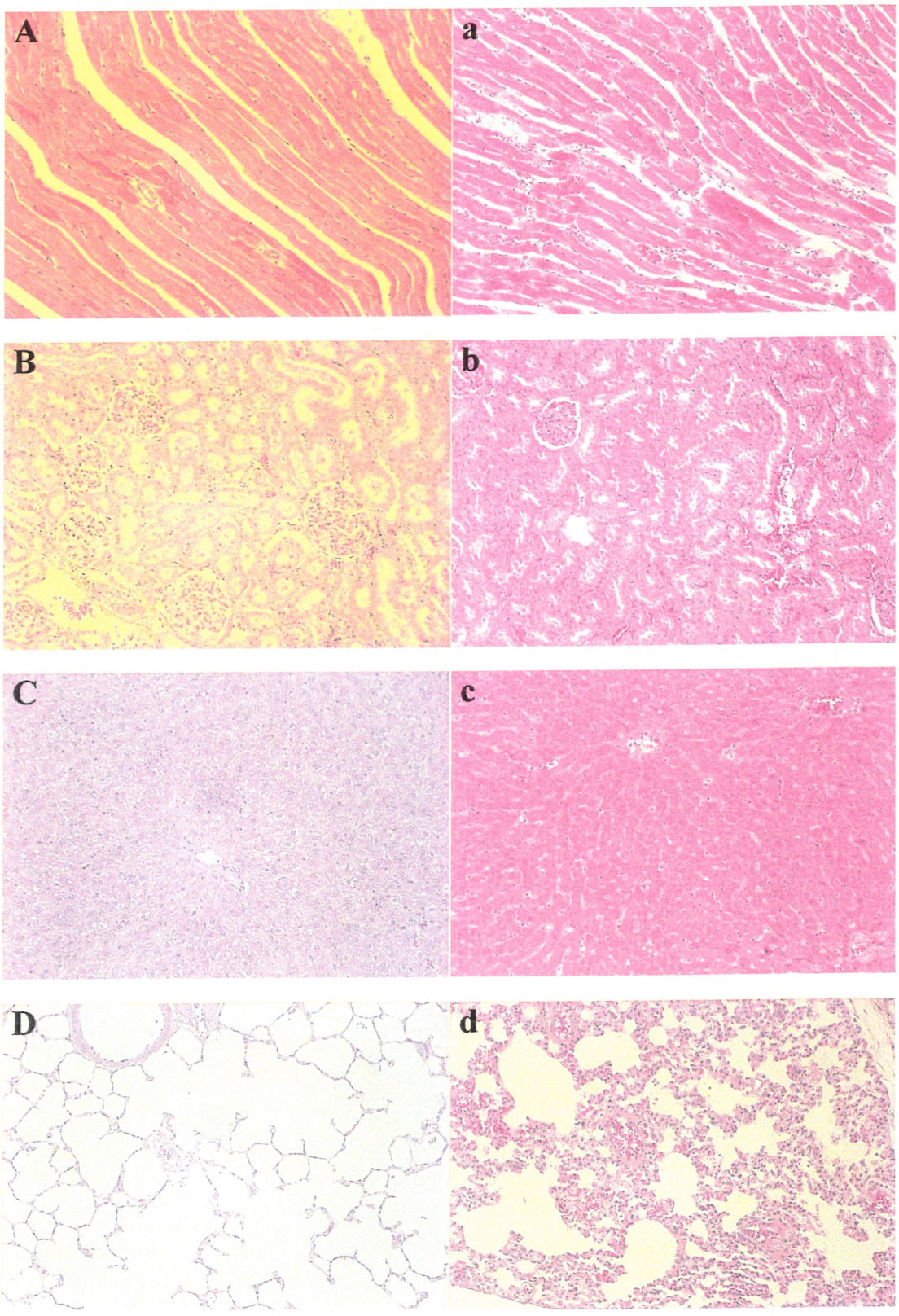


Figure 4 (Bone, Spleen, Intestine, Diaphragm)

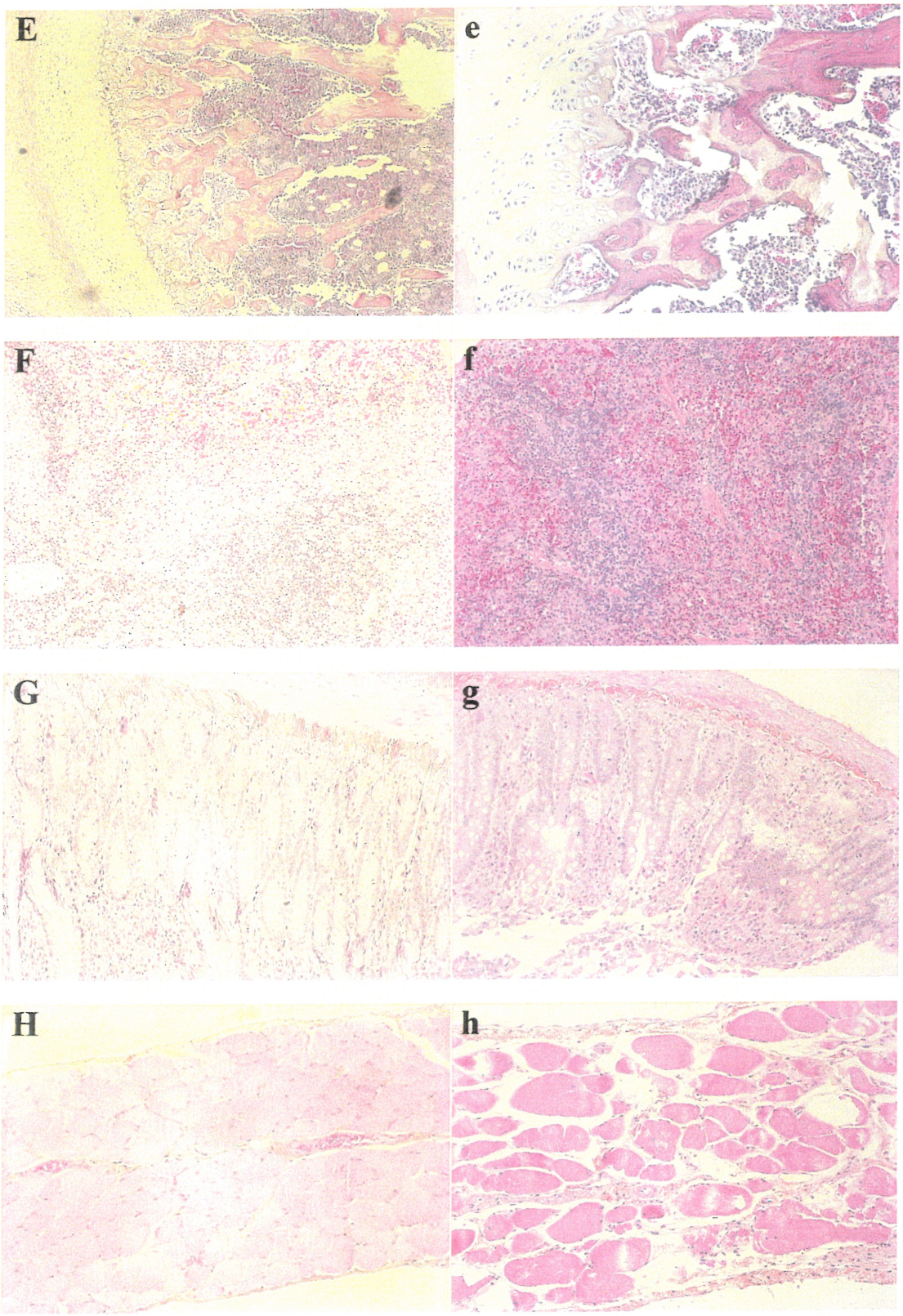


Figure 4 (Testis, Muscle, Adipose, Brain)

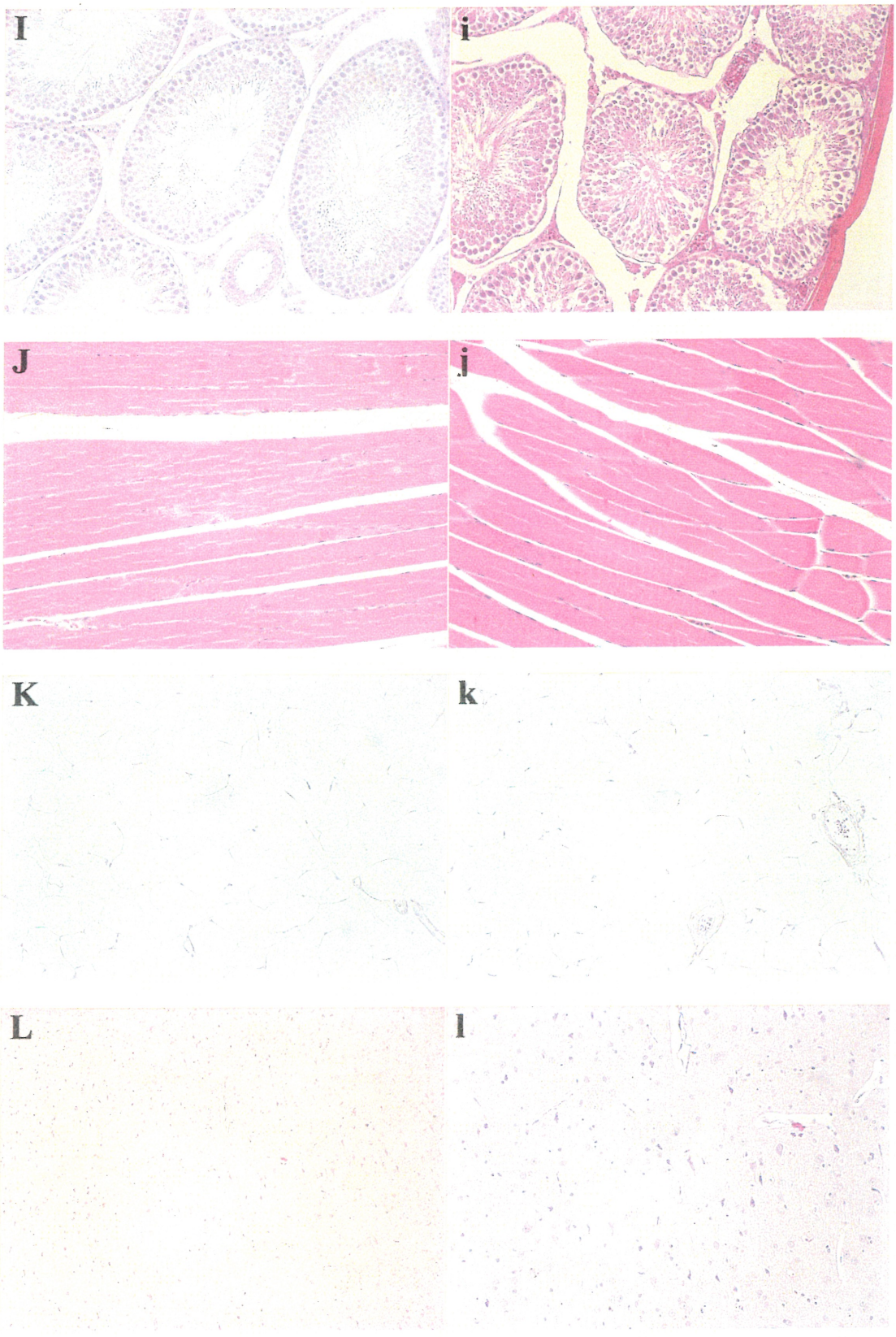


Figure 4. Histological evaluation of tissues and organs during LPS-induced sepsis. Capital letters (A to H) and small letters (a to h) show representative control and septic samples, respectively, of the heart, kidney, liver, lung, brain, spleen, intestine and diaphragm, respectively. Light microscopic amplification x 200.

Figure 5. Evaluation of cellular sarcolemmal integrity of tissues and organs after LPS-induced sepsis. Capital letters (A to H) and small letters (a to h) show representative control and septic samples, respectively, of the heart, kidney, liver, lung, brain, spleen, intestine and diaphragm. Epifluorescent microscopic amplification x 200.

Figure 5 (Heart, Kidney, Liver, Lung)

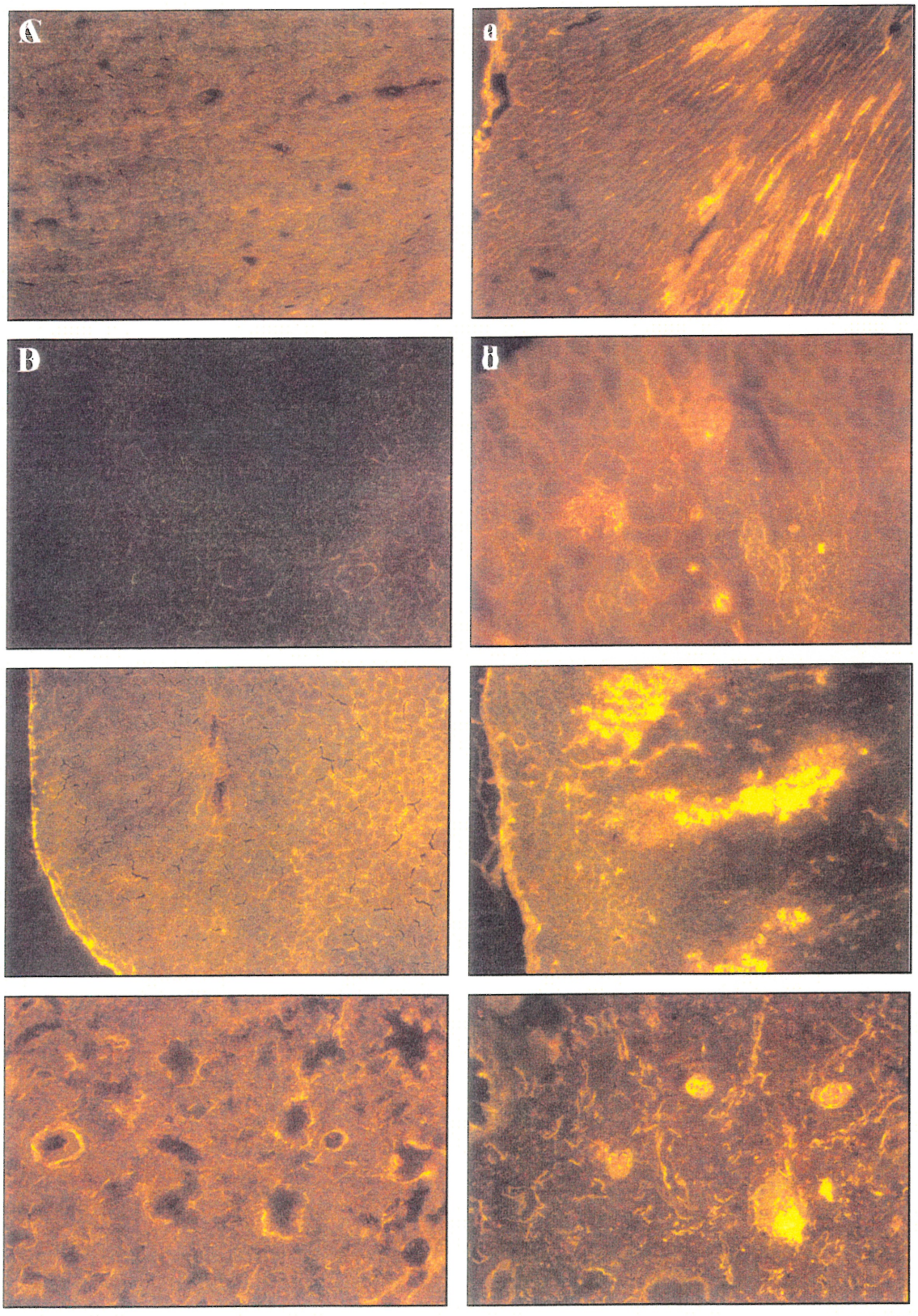
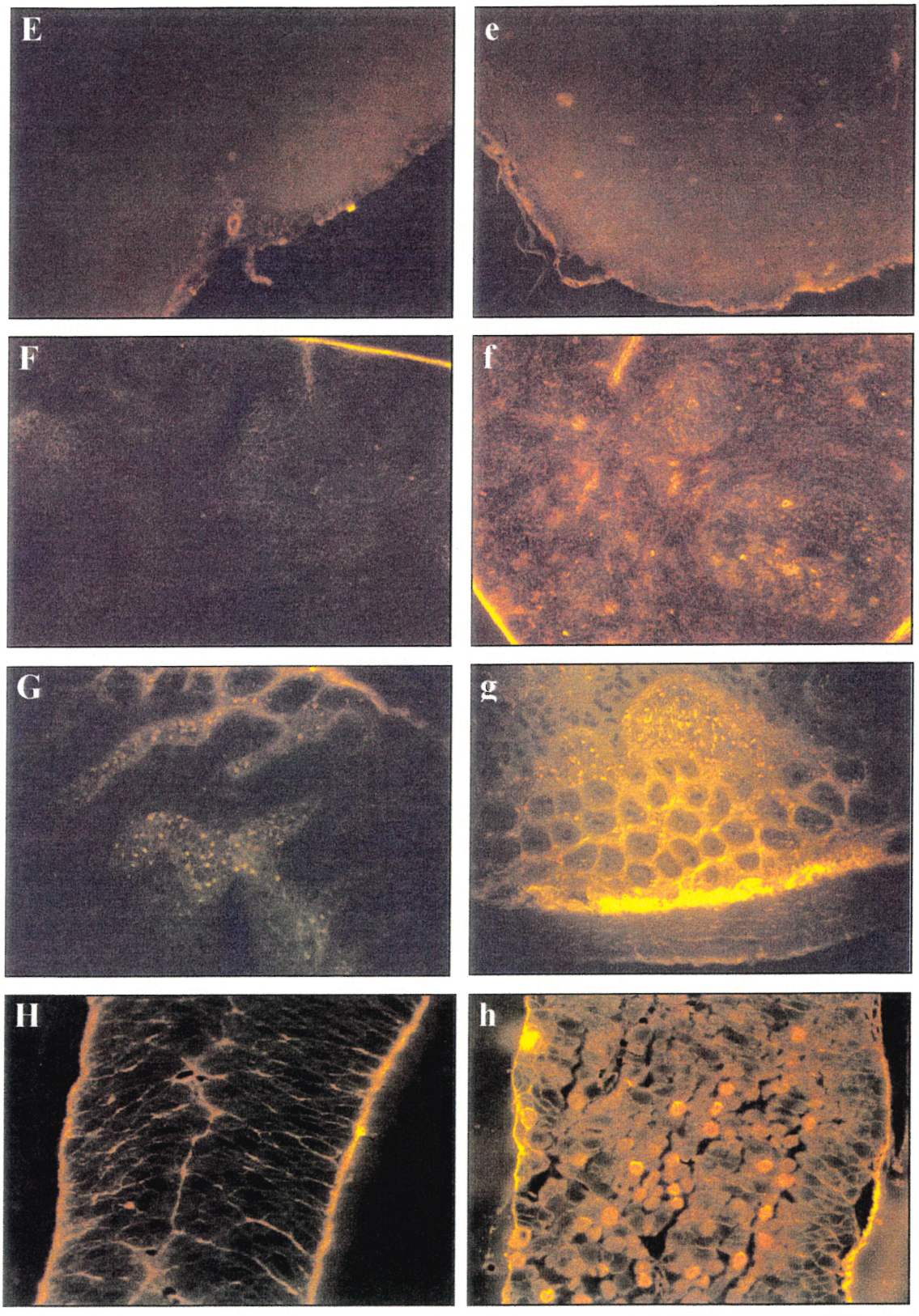


Figure 5 (Brain, spleen, intestine, diaphragm)



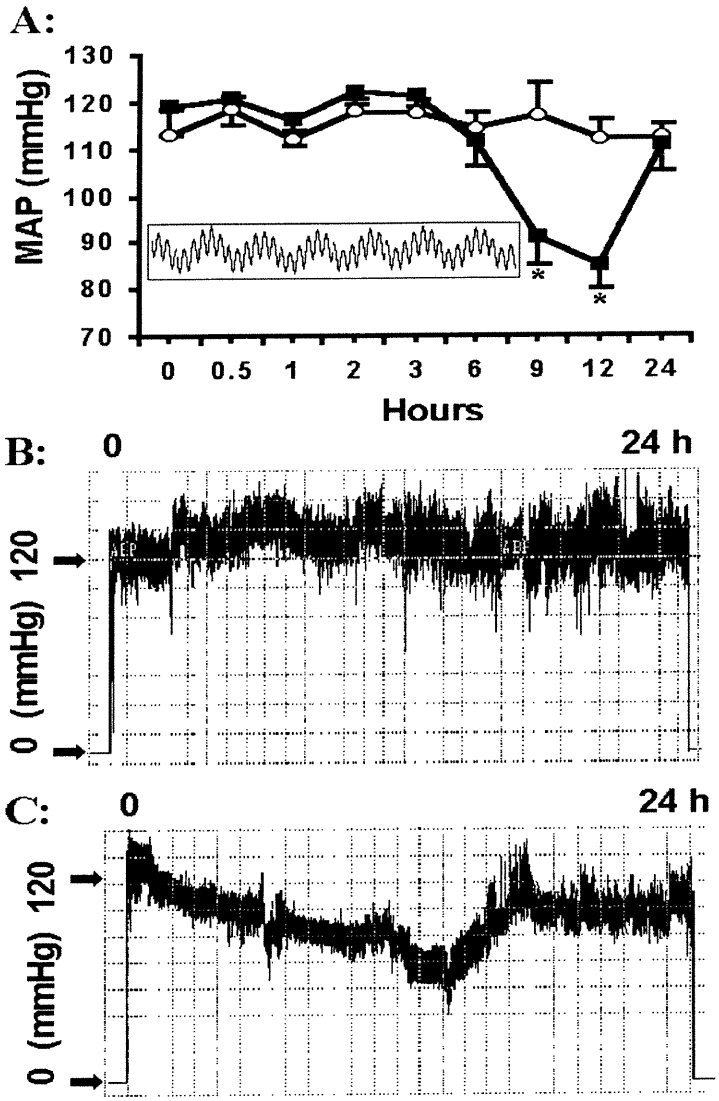


Figure 6: Time course of MAP across 24 h in control and 10 mg/kg LPS-treated rats (A) with representative compressed tracings showing its time course in (B) control and (C) 10 mg/kg LPS-treated rats over a 24-h period. The insert presents detailed tracing of continuous MAP recording. * $P< 0.05$.

Briefly, the most notable histological changes were seen in all 20 sections from 8 septic lungs at 12 h with exudations ranging from moderate to severe, including fluid, inflammatory cells, and cell debris in alveoli and bronchioli, and an almost even distribution throughout the sections. In the lungs of control animals, a focal inflammatory response (2 of 8 sections) ranging from minimal to moderate was observed incidentally, but it was much less severe in comparison with septic lungs. Inflammatory cell infiltration was found in the heart, kidney, liver, spleen, intestine, diaphragm, bone, testis, and, to a lesser degree, in the brain, skeletal muscle, and adipose tissues.

Cell permeability changes during sepsis. Procion Orange 14 staining of septic samples (18 to 23 sections) revealed that, in comparison to control tissues (10 to 20 sections) (Fig. 5 A to H), there were significant cellular sarcolemmal lesions in the heart, kidney, liver, lung, spleen, intestine, diaphragm, and to a lesser degree in the cerebrum (Fig. 5 a to h) 12 h after LPS administration, but this was not evaluated quantitatively. As can be seen in Figure 5 a and c, myocytes and hepatocytes with sarcolemmal injury were easily visualized by Procion Orange 14 (fluorescent cytoplasm) staining in the heart and liver after 12 h of sepsis induced by ip LPS injection. This type of damage was never noted in control tissues.

MAP alteration during sepsis. 10 mg/kg LPS induced hypotension at 12 h ($P < 0.05$), which was reversed at 24 h. Rats treated with 20 mg/kg LPS, but not those given 5 or 10 mg/kg LPS, expired within 24 h. Most of them died of severe hypotension, and MAP was already decreased significantly at 9 to 12 h at this higher LPS dose. Figure 6 A shows the time course of MAP changes across 24 h in control and 10 mg/kg LPS-treated

rats. Figure 6 (B and C) presents representative tracings of the time course of MAP through 24 h in control and 10 mg/kg LPS-treated rats.

Discussion

In this study, we have demonstrated that: 1) There was a significantly different spatial distribution of nitrate anion, the major NO metabolite, in the body of normal rats, and this spatial distribution was significantly altered during LPS-induced sepsis. 2) Nitrate concentrations were increased not only in plasma but also in tissues and organs such as the heart, aorta, liver, lung, kidney, diaphragm, intestine, bone, spleen, thymus, testis, ovary, bladder and urine (but not in the brain, adipose tissue and skeletal muscle) after LPS treatment of rats. Nitrate concentration was elevated even higher in plasma than in tissues and organs; consequently, the tissue and organ/plasma nitrate concentration ratio was decreased significantly. 3) The LPS-induced NO metabolite increased in a dose-dependent manner only in the lungs and aorta at 12 h after LPS treatment. 4) These nitrate concentration changes in tissues and organs were paralleled by the inflammatory response which was indicated by alterations of MPO activity as well as impaired histological and sarcolemmal integrity in tissues and organs. 5) MAP modification was negatively correlated with nitrate concentration changes in the aorta during sepsis. From these results, we believe that nitrate anion may synergistically enhance the detrimental effects of NO and/or peroxynitrite on the impairment of multiple organ function during endotoxic shock. Thus, it is reasonable to assume that cellular function modified by NO-derived nitrate anion may contribute to the multiple-organ functional alterations observed in SIRS and MODS during endotoxic shock.

NO and nitrate

We show, for the first time, the spatial (organ) distribution and temporal production of nitrate in normal and septic rats. Plasma, cardiac and lung NO metabolite concentrations are consistent with those in rats in which plasma, cardiac and lung NO production was found to be increased after LPS administration [132, 284, 315]. NO is constantly generated by NOS in physiological situations, and its production can be altered in a variety of pathophysiological conditions, such as SIRS and sepsis. Nitrate is the major metabolite of NO *in vivo,* and its half-life is 1.5 to 8.0 h. In comparison to the half-life of NO (3 to 20 s), nitrate's half-life is at least 270 to 9,600 times longer *in vivo;* therefore, nitrate concentrations in tissues may only indicate past NO production. In other words, nitrate may be 270 to 9,600 times higher than NO at transient timepoints *in vivo,* considering the half-life of both molecules. Other factors, such as superoxide anion concentration and NOS activity, could also contribute to further affect this NO-nitrate concentration difference. There is experimental evidence that supports this view as NO reaches ~nM levels [132] and nitrate ~μM levels *in vivo* [284, 315]. Our results show similar nitrate concentrations in plasma and cardiac tissue as reported previously [315, 375].

Our data indicate that nitrate concentration is significantly increased in tissues and organs such as the heart, lung, liver, kidney, aorta, diaphragm, intestine, bone, spleen, testis, ovary and plasma, but somewhat surprisingly not in the brain, adipose tissue, and skeletal muscle after LPS-induced sepsis in rats. The reason why nitrate concentration in the brain (as well as in adipose tissue and skeletal muscle) is not significantly altered after endotoxin treatment ip is not well understood [58]. NOS (iNOS, nNOS and eNOS)

has been detected in the brain, and NOS activity is increased in this organ during infections. Peroxynitrite, produced from NO reaction with superoxide, is exceedingly neurotoxic, and nitrate anion in tissue originates, at least partly, from peroxynitrate *in vivo,* especially under septic conditions [30].

Recent investigations show that brain NOS and NO are transiently increased to be then consistently decreased within 5 min to ~50% below baseline after controlled cortical impact injury, and last for at least 24 h [58]. NO production in cerebrospinal fluid is known to be increased during sepsis, but nitrate concentration in the brain is not well elucidated [58]. Other experimental evidence suggests that nitrate anion, injected cerebroventricularly, can induce a significant elevation of blood pressure [313]. Our results demonstrating baseline nitrate concentration in the brain indicate basic NO production as well as stabilized nitrate concentration in the brain during sepsis. This suggests that homeostasis (nitrate anion) of the brain is quite stable, and this organ is relatively resistant to remote site infection and SIRS. Similarly to the brain, nitrate concentration in adipose tissue and skeletal muscle is not significantly altered.

Intra- vs extracellular nitrate concentration

We found a significant difference in organ to plasma nitrate concentration in both normal and septic rats. Direct comparison of intracellular and extracellular nitrate concentrations under normal and septic conditions has not been done, particularly for the heart and aorta. Cardiac tissue is mainly composed of cardiac myocytes, which represent about 85% of its total mass, and vascular cells (endothelium and vascular smooth muscle) represent about 5% [234]. Inflammatory cells are rarely found in cardiac and aortic tissue under normal conditions. Accordingly, nitrate anion in homogenized cardiac and aortic

tissues comes mainly from the intracellular compartment that represents at least 90% of its total value. Extracellular compartments are mainly composed of plasma (or blood) and interstitium, and their electrolyte compositions are identical (cation and anion) [198]. We, therefore, assumed that nitrate concentrations in plasma and the interstitium are very similar. Hence, nitrate concentrations in organs such as the heart and aorta, and plasma (or blood) represent the major part, if not the whole, of intracellular and extracellular compartments, respectively.

To the best of our knowledge, our results provide the first evidence that there is a sizeable difference between intracellular and extracellular nitrate concentrations, particularly, in the heart and aorta of control rats. This intracellular vs extracellular nitrate concentration difference suggests that NO might not diffuse freely, as previously thought, *in vivo* between the intracellular and extracellular milieus. After LPS induction in rats, both intracellular and extracellular nitrate concentrations increased significantly, but more prominently in the extracellular compartment (plasma). Plasma nitrate concentration was found to be approximately equal to (or even higher than) organ nitrate concentration, especially at 12 h. As a consequence, the intra/extracellular nitrate concentration ratio was significantly decreased after LPS administration to rats, and this ratio in the aorta was well paralleled by MAP alteration in LPS-treated rats. Experimental evidence has shown that a chronic drop in arterial pressure after LPS infusion is not convincingly mirrored by a chronic increase in NO concentration (relatively constant amount of NO), measured directly by a NO sensor, but is paralleled by indirect NO assay (nitrite + nitrate) [132]. Therefore, modulation of this intra- or extracellular nitrate

concentration, and subsequently its ratio, may be an important factor affecting cell functions, impacting on cardiovascular, respiratory and other organs.

LPS, nitrate, SIRS and MODS

Infections, trauma, and septic shock could cause SIRS and, more severely, MODS in both experimental animal models and clinically [17, 30, 89, 103, 126, 132, 215, 315]. LPS plays a vital role in these diseases. NO is rapidly produced and quickly destroyed at the same time that nitrate is formed, which, however, is slowly eliminated from the body during sepsis. The measurement of nitrate concentration in different tissues and organs could provide a longitudinal view of the role of NO as well as of nitrate in septic shock. The different doses (5, 10, 20 mg/kg) of LPS induced significantly different nitrate concentration changes at 12 h in the aorta and lung, but not in other tissues and organs tested in this study. These results indicate that the aorta and lungs were more sensitive to different LPS doses or infections. In contrast, the brain, adipose tissue and skeletal muscles were more resistant to LPS-induced inflammation since only minor alterations of nitrate production and morphology in these tissues were observed.

Our results are consistent with previous findings that LPS-induced lung injury produces a typical systemic inflammatory response, revealing significant granulocyte infiltration with exudation in alveoli [132, 215]. Lung injury could be more severe because the decrease in compliance (pulmonary edema), combined with the heightened respiratory rate (data not shown), enhances tissue stress, and eventually results in a vicious circle for both organs: the more difficult it is for the diaphragm to contract (more injury), the more hypoxia and edema occur in the lungs and diaphragm, as can be seen by

marked morphological alterations and impaired sarcolemmal integrity in both the lungs and diaphragm (Figs. 4 and 5).

Additionally, we show that LPS induces significant SIRS and alterations of cellular sarcolemmal integrity in other organs such as the heart, liver, kidney and intestine, and to a lesser degree the brain, adipose and skeletal muscle tissues. The inflammatory response was further supported by alteration of MPO activity in organs as well as a LPS dose-dependent response seen only in the lungs. MPO activity is heightened in the heart, kidney and liver, but not in the spleen and plasma. Since inflammatory cells contribute significantly to iNOS concentration and activity during sepsis [89, 126], they could influence and alter extracellular nitrate production. SIRS can also produce a significant amount of superoxide and peroxynitrite, two powerful oxidants, that could, at the same time, contribute to cell membrane injury, cell dysfunction, and, eventually, MODS [30, 126, 375]. Therefore, several direct and indirect LPS-induced factors could potentially act in parallel or synergistically to impair cell and organ (such as cardiovascular and respiratory) functions during sepsis.

We have reported previously, as have many other investigators, that muscle contractility is depressed 12-24 h after LPS-induced sepsis [89, 215]. Similarly, muscle contraction can be depressed by the nitrate anion itself, which impairs active calcium uptake by the sarcoplasmic reticulum and also increases the permeability of muscle cells to methyl-glucose *in vitro* [145]. This effect is possibly additive or synergistic to the actions of NO, superoxide anions and peroxynitrite accumulation in muscle tissues during sepsis. Nitrate anion can cross the plasma membrane [60, 172, 208] and significantly affect cell electrophysiological properties [16, 162, 350], cellular pH [208, 350],

membrane enzyme (e.g. ATPase) activity [255], and potassium transport [214] *in vitro*. It also synergistically facilitates vasopressin secretion and function in the CNS to enhance sympathetic activity and increase blood pressure *in vivo* in rats [313] and dogs [117, 385]. Recent studies have shown that the nitrate anion may pass through a distinct set of channels or undergo nitrate-H^+ cotransportation in mammalian cells [60]. NO functions as a signal in plants and microbes, in which the nitrate channel is well-documented [79, 249]. Our results establish that the intra/extracellular nitrate concentration ratio is decreased significantly after LPS administration to rats, and this ratio in the aorta is well correlated with MAP alteration. Over-accumulation of plasma and tissue nitrate concentration and, subsequently, a change in the intra/extracellular nitrate concentration ratio may have synergistic deleterious effects on cardiovascular (heart and aorta) and respiratory (lung and diaphragm) functions during sepsis. Thus, it is reasonable to assume that cellular function modified by nitrate may provide one of multiple-mechanisms for the organ functional alterations observed in SIRS and MODS.

3. DISCUSSION

3.1. Quantification of nitric oxide and nitrate anion *in vivo* and *in vitro*:

NO is an important mediator of both physiological and pathophysiological processes, such as cardiovascular and respiratory functions as well as inflammation, immunity and neurotransmission. NO is a small, gaseous, paramagnetic, relatively active molecule and found in low concentrations (~pmol to ~nmol) *in vivo*, and is rapidly destroyed by O_2 or O_2^- or other molecules. Consequently, NO produces its major metabolite, the nitrate anion *in vivo* [396, 400, 403-408].

Considering the functions of NO and its metabolites (nitrite and nitrate anion) and the wide range of pathophysiological conditions in which it has been implicated, interest in measuring NO production has, not surprisingly, obtained huge consideration. Direct measurements of NO concentrations and selectively of its metabolites (e.g. nitrite or nitrate) in biological systems, particularly intracellularly, remain a challenging analytical barrier in the field of NO research. Few experiments have been reported where NO was measured directly *in vivo* or *in vitro* in the cells, tissues or organs. Several techniques, e.g. spectrophotometry, HPLC, electron spin resonance spectroscope, flow cytometry, mass spectrometry and electrochemical micro-sensors, have been introduced to measure NO or its metabolites (nitrite and/or nitrate) concentration. Measuring NO and its metabolites by spectrophotometry is the most commonly cited technique in the literature [11, 179]. However, enzymatic reduction of nitrate by using an immobilized *Escherichia*

coli nitrate reductase column [115] converts only ~30% of nitrate to nitrite. Another assay based on the coupled oxidation of NADPH during the enzymatic conversion of nitrate to nitrite by *Aspergillus* nitrate reductase only yields ~64% of serum nitrate to nitrite and is also unsatisfactory for nitrate analysis in biological samples such as in serum. Although many reports claim a possible recovery of 100% nitrate in the biological samples, commercial nitrate reductases are rather expensive [311] and their activity may vary from different sources as well as different lot. As can be seen in the literature, nitrate concentrations in plasma and tissues vary greatly and can range from 1 to few hundreds μM in control conditions even in the same species.

Chemiluminescence is a highly sensitive, selective, and accurate method for measuring NO, especially at low concentrations [11, 179]. Several reducing reagents have been used for the reduction of nitrite and nitrate to NO [11, 71, 216, 303, 310]. The efficiency of the different reducing reagents for the conversion of nitrite and nitrate to NO is not clear. Especially under different temperatures, the conversion rate, particularly for nitrate anion, may vary and may be not complete. Therefore, it is necessary to choose a suitable reducing reagent as well as the optimal analytical conditions to quantify NO or its metabolites concentration in different samples. In order to selectively measure NO and its metabolite nitrite and nitrate, we examined the efficiency of five reducing agents [namely V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III)] to reduce different amounts (100-500 pmol or 0.4-2 μmolar) of nitrite and (or) nitrate to NO, and the effect of temperature from 20, 30 … to 90°C on the conversion of a fixed amount of nitrite or nitrate (400 pmol or 1.6 μmolar) to NO as well as the recovery of nitrite or nitrate from plasmas of pig and of dog.

We found that the most accurate procedure is to use NaI or Cr(III) as a reducing agent in order to selectively convert nitrite to NO at low temperatures (NaI and Cr(III) were unable to convert nitrate to NO). Alternatively, one can use a strong reducing agent to convert all nitrite + nitrate to NO at 80°C or 90°C. The difference between these measurements (total NO metabolites value minus nitrite value) is the concentration of nitrate. Recovery of nitrite and nitrate from plasma of both dog and pig was reproducible and near quantitative. We then applied this established technique to measure selectively NO, nitrite, and nitrate anion in different samples (such as plasma, urine and homogenates) in our subsequent experiments.

3.2. Nitric oxide and nitrate anion

NO is a ubiquitous messenger molecule [11, 30, 377] that is constantly synthesized by NOS in physiological situations [264, 377], and its production can be altered in a variety of pathophysiological conditions, such as sepsis, trauma and ischemia [53, 80, 94, 271]. Nitrate is the major metabolite of NO with a half-life of 1.5 to 8.0 hours *in vivo* [61, 101-103, 256, 353, 385]. In comparison to the half-life of NO (3 to 20 s) [108-110, 304], the nitrate half-life is at least 270 to 9,600 times longer *in vivo*. Therefore, nitrate concentrations in tissues may consequently only indicate past NO concentration. In other words, nitrate may be 270 to 9,600 times higher than NO at time points *in vivo* if only the half-life factors of both molecules are considered. Other factors, such as the concentration of the superoxide anion, and NOS activity, could also further contribute to this NO-nitrate concentration difference [277, 282]. Moreover, in chemical

terms, this significant difference between the substrate (NO) and its product (NO_3^-) suggests that their functions maybe quite different. Experimental evidence supports this view as NO levels reach ~nM and nitrate ~μM levels *in vivo.* Indeed, the nitrate anion reveals its functional role in the cells of plants, microbes as well as mammalian [35, 60, 244, 378], affecting cell membrane potential [16, 113, 162, 187, 213, 215, 249, 387], ATPase [83, 243, 255, 345] or PTPase [96] activity, muscle contractility, and calcium, potassium, and sodium ion transportation [145, 214, 313]. Therefore, the nitrate anion should not be considered only as a non-functional molecule or by-product of NO *in vivo*.

We have shown for the first time the spatial (organ) distribution and temporal production of nitrate in normal and septic rats. The results that we have obtained for plasma, cardiac and lung NO metabolite concentrations are consistent with those in rats in which plasma, cardiac and lung NO productions were found to be increased after LPS administration [315, 329, 339]. Our results show that the nitrate concentration is significantly increased in the tissues and organs of the heart, lung, liver, kidney, aorta, diaphragm, intestine, bone, spleen, testis, ovary and plasma, but somewhat surprisingly not in the brain, adipose tissue, and hind limb skeletal muscle after LPS-induced sepsis in rats. The mechanism by which nitrate concentrations in the brain (as well as adipose tissue and hind limb skeletal muscle) are not significantly altered after I.P. endotoxin treatment, is not well understood [58]. NOS (iNOS, nNOS and eNOS) have been detected in the tissue of the brain, and NOS activity is increased in the brain during infections or sepsis [264, 377]. In the central nervous system, NO is an important regulator of glutamate release which plays a role in the neurodegenerative processes. Peroxynitrite, produced from NO's reaction with superoxide, is extremely neurotoxic [30, 58]. Nitrate

anions in tissues are derived at least partly from peroxynitrite *in vivo*, especially under septic conditions [30]. Recent investigations show that brain nitric oxide is transiently increased and then consistently decreased within 5 min to about 50% below the baseline after controlled cortical impact injury and this lasts for at least 24 h. In the mean time, NOS activity is transiently increased and then returned to normal after 30 min, and is decreased from 1 to 7 days [58]. In our results, the nitrate concentration in the brain during I.P. sepsis-induced by LPS was not significantly changed, indicating that the brain is relatively resistant to remote site infection and SIRS. Other experimental evidence shows that nitrate anions directly injected into cerebral-ventricular compartment can induce a significant increase in blood pressure [313]. Our results show that there was a basic nitrate concentration in the brain, indicating the presence of a basic NO production as well as stabilized nitrate concentration in the brain during sepsis. This homeostasis (nitrate anion) of the brain is quite stable. Several studies have shown that LPS induced an iNOS activity in the diaphragm that was significantly increased, but not in the hind limb skeletal muscles (soleus and extensor digitorium longus) [37, 89]. As in the case of the brain and hind limb skeletal muscles, nitrate concentrations in adipose tissue were not significantly changed during LPS-induced sepsis.

It was reported that LPS induced iNOS activity was increased in the diaphragm but not in the abdominal, intercostals, soleus, and extensor digitorium longus (EDL) muscle [37, 89]. There were also controversial reports that NOS activity of the soleus muscle did not increase after LPS injection, although NOS activity was increased in the diaphragm and intercostals muscles. The reasons behind these controversies are not clear

[37, 89]. Our results suggest that NO metabolites were increased in the diaphragm muscle but not in hind limb muscles.

Similarly, iNOS induction had been investigated in different tissues or organs such as in the adipose, diaphragm, lung and brain but NO metabolites in adipose tissue have not been quantified [37, 89, 389-395]. Whether iNOS induction in adipose tissue is positive or negative after LPS treatment both *in vitro* and *in vivo* conditions remain a controversial issue, and LPS alone may not induce NO *in vitro* in adipose tissue [394]. LPS-induced expression of iNOS mRNA in adipose tissue may increase to higher levels than in the liver and the kidney [394], but, its level was not detectable in white epididymal and perirenal adipose tissues after 8 hours LPS treatment. The author claims that adipose tissue is a major site of iNOS expression in endotoxemia [394]. Our results partially support these studies as NO metabolites in adipose tissue of the rat, during 24 hours endotoxic shock, were not increased after 6 hours (controversial) as well as 12 hours (consistent) LPS treatment.

All three isoforms of NOS in the brain have been implicated in a number of physiological and pathophysiological functions [389-393]. It has been reported that LPS-induced iNOS mRNA was increased in the brain, especially in the vascular-rich area and particularly localized in the areas such as the paraventricular nucleus during endotoxemia. This result is not supported by a later experiment performed by other groups, since only eNOS mRNA was increased in response to LPS. No apparent induction of iNOS mRNA was observed in the brain while the its induction was obvious in the lung. The LPS treatment had no effect on nNOS mRNA levels in the brain. Therefore, eNOS is a major form of NOS in the brain during endotoxemia [389, 390].

eNOS was also localized in endothelial cells and astrocytes, two major components of the blood-brain barrier that may protect the brain against systemic bacterial infection.

Finally, it was reported that the estimated concentrations of NO were 11.6, 8.3 and 17.0 nmol/g in the brain tissues of the cerebellum, cortex and hippocampus, respectively, detected by EPR with (DETC)2-Fe(II), but not with (MGD)2-Fe(II), and the concentration of NO in the brain was < 20 % of that in the liver in endotoxemic conditions [392]. We have found ~ 100 μM nitrate concentration in the brain homogenate and its concentration was not significantly altered after LPS treatment in rats. There are probably several reasons for this result: (a) iNOS is not globally and evenly distributed in the brain tissue, therefore NO metabolites in the brain may not be globally increased during endotoxemia, (b) as iNOS or eNOS may located predominantly in endothelial cells, NO may easily efflux into the blood flow and its metabolites may not increase in the brain, and (c) as the alteration of NO concentration was in the 8.3 to 17.0 nmol/g or pmol/mg range, it may be impossible to distinguish these values using our technique.

3.3. Nitrate anion in intracellular and extracellular sites

We found a significant difference in the organ to plasma nitrate concentrations in both control and septic rats. Cardiac tissue is mainly composed of cardiac myocytes and endothelium, representing about 85% and 5% of its total mass, respectively [234]. Inflammatory cells are rarely found in cardiac and aortic tissue under control conditions. Accordingly, nitrate anions in homogenized cardiac and aortic tissues come mainly from the intracellular compartment that represents at least 90% of their total value.

Extracellular compartments are mainly composed of plasma (or blood) and interstitium, where electrolyte compositions (cation and anion) are identical [198]. Hence, the nitrate concentrations in organs such as the heart, aorta and plasma (or blood) represent the major part, if not all, of intracellular and extracellular compartments.

Our results provide the first evidence that there is a sizeable difference between intracellular and extracellular nitrate concentrations in control and septic rats. This intracellular vs. extracellular nitrate concentration difference suggests that NO and its metabolite (nitrate) might not diffuse freely *in vivo*, as previously thought, between the intracellular and extracellular milieus. After LPS treatment, both intracellular and extracellular nitrate concentrations increased significantly, but more prominently in the extracellular compartment (plasma). As a consequence, the intra/extracellular nitrate concentration ratio was significantly decreased after LPS administration to rats, and this ratio in the aorta was well paralleled by MAP alteration in LPS-treated rats. Therefore, the modulation of this intra- and extracellular nitrate concentration, and subsequently its ratio, may be an additional factor affecting cellular functions, impacting on cardiovascular, respiratory and other organs.

Both exogenous (such as inhaled NO and NO donors) [343] and endogenous NO (such as LPS or cytokine-induced NO) [330, 333, 336] could contribute to extracellular and intracellular NO and nitrate concentration. Exogenous nitrate may also contribute to nitrate concentration *in vivo* in tissues and organs [326, 366, 373]. As a stable metabolite of NO, the nitrate anion concentration in solution does not change after boiling it for 24 h [74], or in biological samples (stored at -20°C) for at least one year [256]. Additionally, under normal daily life in North America, ~100 mg/day nitrate per person is consumed in

the average diet which contributes to only ~5-15 μM in blood (as compared normal levels of ~20-40 μM) [111]. In other words, daily exogenous nitrate in the diet under normal conditions contributes less than endogenous NO to the blood nitrate concentration, and in most conditions, exogenous nitrate is transiently consumed or administered and may be only distributed to extracellular compartments. As shown in porcine experiments, inhaled NO and I.V. nitroglycerin (a NO donor) can significantly and transiently increase blood nitrate concentrations. The experimental studies have documented that the half-life of NO *in vivo* is as short as ~2 ms [400]. Furthermore, it has been shown that the reaction rate of exogenous NO with oxygen is greatly occurred in cellular lipid membrane, so it is that much of the exogenous NO reacts in the cellular membrane sites and never enters the cellular cytosol such as the erythrocyte [403-408]. However, endogenous NO is constantly synthesized by NOS under physiological conditions, and its synthesis can be altered in a variety of pathophysiological situations such as sepsis/SIRS/MODS [1, 28]. As a consequence, nitrate is formed intracellularly and eliminated slowly.

3.4. Nitrate anion and muscle Contractility

We show that the force-frequency muscle tension relationship *in vitro* is depressed by both 12 hours of LPS treatment *in vivo* and the application of nitrate anion *in vitro*. Many investigators have shown that muscle contractility is depressed following 12-24 hours of LPS induced sepsis [89, 215]. Traditionally, respiratory insufficiency is attributed to lung injury and is manifested in the early stages of septic shock as hypoxemia, elevated pulmonary arterial pressure, increased pulmonary shunting and

decreased lung compliance [1, 4]. In addition to lung injury, there is growing evidence that septic shock is also associated with ventilatory pump failure [89, 150, 215, 298]. Measurements of ventilatory muscle contractility and fatigue resistance *in vitro* and *in vivo* have confirmed that endotoxemia and septic shock lead to a significant decline in contractility and fatigue resistance. Failure of ventilatory muscle contractility in septic shock have never been attributed to a single factor, but two groups of factors are likely to be involved (I) increased ventilatory muscle metabolite demands due to augmentation of ventilation, hypoxemia, and increased pulmonary impedance; and (II) specific cellular, metabolic, immune and hemodynamic defects which interfere with several processes necessary for normal force generation [1, 4, 89, 298]. These defects are mediated by complex interaction between several local and systemic mediators such as LPS, cytokines, reactive oxygen species and NO, and the exact role of each of these molecules in the pathogenesis of depressed muscle performance remains to be determined [89, 298]. Similarly, muscle contraction can be depressed by nitrate anions, which impair the calcium uptake by the sarcoplasmic reticulum and also increase the permeability of amphibian muscle cells to methyl-glucose *in vitro* [145, 162]. Depressed muscle contraction may also be due to the disturbance of cell membrane (as well as damaged cellular membrane) integrity [195], and the disturbance of the energy metabolism by nitrate as a result of the depression and the uncoupling of oxidation and phosphorylation in the mitochondria of liver, kidney and heart of rats in a dose-dependent manner [186, 202, 229, 230]. Our results show that nitrate affects only the high frequency component of tension development of the septic diaphragm. This is consistent with the effect of membrane potential depolarization, where the action potential firing frequency at high

stimulation frequencies becomes partially attenuated [162, 187, 215, 249]. The discrepancy observed in our results (between LPS and nitrate force frequency curves) may thus indicate two different mechanisms contributing to force loss, one which involves mostly loss of force through loss of excitability (nitrate depolarization) and the other which may involve force loss through NO, superoxide anions and peroxynitrite accumulation in muscle tissue [4, 30, 132]. Thus, our results provide additional evidence that nitrate can affect the force developed of the rat diaphragm *ex vivo*. This effect is possibly additive or synergistic to the effects of NO, superoxide anions and peroxynitrite accumulation in muscle tissue.

Extracellular nitrate alone can significantly affect cell-resting membrane potential *in vitro* [16, 59, 60, 113, 213, 378]. In fact, recent studies have shown that sepsis induced by cecal ligation perforation or LPS infusion produces a diaphragm-myofiber membrane depolarization [215]. This is also consistent to what is observed by other investigators in non-vertebrate muscle tissue [60, 162, 378]. There is also experimental evidence that sufficient amounts of sodium nitrate infusion (i.e. changing plasma nitrate from 20 to 60 μM) can significantly increase *in vivo* MAP in (conscious and anesthetized) dogs as well as in humans [117, 385], which show a significant linear correlation between plasma nitrate and MAP (r^2=0.99, $P < 0.001$). The mechanism by which nitrates can cause an increase in MAP is not fully elucidated and this elevation may be mediated: (A) via the activation of voltage-dependent calcium channels (L-type) of smooth muscle cells by the effects of nitrate depolarization [113, 162, 213, 378] and (B) via the renin-angiotensin stimulatory effects of nitrate in the kidney [307] or (C) via nitrate channels itself [79, 249, 285, 378]. Also under such experimental conditions (by nitrate anions), there is no

or, if any, only a minimal effects of induced NO comparing with septic condition in which NO is over produced that may compromise nitrate effect in MAP. Nonetheless, there are numerous studies indicating that extracellular nitrate accumulation can affect cell membrane potential.

A number of experiments have shown that nitrate has a significant role on various cell functions through their affect on plasma membrane enzyme (ATPase) activity [83, 243, 255, 345], intracellular pH [35, 60, 249], and the electrochemical gradient and/or electrophysiological properties of the cell [16, 162, 387]. However, the mechanism underlying these responses to the nitrate anion is not fully elucidated. Recent experiments have suggested that the nitrate anion in mammalian cells may pass through a distinct set of channels, or cotransportation of nitrate-H^+ which may be related, though not identical, to the nitrate-H^+ cotransportation described in *Arabidopsis* and *Aspergillus* [35, 60, 249]. Moreover, NO functions as a signalling molecule in *Arabidopsis* and microbes, in which a nitrate transporter/channel has been well documented [79, 249, 285, 387]. The transporters, that are able to transport a range of different nitrogen-containing substrates, including the nitrate anion, belong to a multigene family with members that have been identified in bacteria, fungi, plants, and animals, and two types of transporters, high-affinity and low affinity nitrate transporters, have so far been reported. Their full-length cDNAs have very recently been cloned and the high-affinity nitrate transporter from *Aspergillus* has been characterized and expressed in *Xenopus* oocytes [387]. Interestingly, a novel peptide/histidine transporter in the rat brain and retina has also been cloned and expressed in *Xenopus* oocytes [378], and it is characterized as a new member in the growing superfamily of proton-coupled peptide/nitrate transporters since the amino acid

sequence has moderate homology with a nonspecific peptide transporter found in plant. The properties of the transporter can be modified by changes in membrane potential, external pH and external or cytosolic nitrate concentration [387]. This heterogeneous system shows a very sensitive response to the concentration gradient of nitrate anions (1 to 200 μM) across the plasma membrane that is not adequately described by Michaelis-Menton kinetics equation applied to the transporter of the nitrate anion [387].

3.5. LPS, NO/nitrate, SIRS and MODS

LPS or cytokines alone can evoke iNOS expression which produces a large amount of NO. This implies that there is a need for NO during sepsis and this may represent the beneficial effect of NO. However, nitrate is concurrently formed and eliminated slowly from the intracellular compartment, organ and body, and this may present the detrimental effect of NO during sepsis. Different doses (5, 10, 20 mg/kg) of LPS induced significantly different nitrate concentration changes at 12 h only in the aorta and lung. These results indicate that the aorta and lungs were more sensitive to different LPS doses, and these results may explain why LPS administered intra-vascular (arterial or venous) can induce a rapid NO production as well as an acute hypotension (within minutes), while LPS via intraperitoneal administration induces a sub-acute or chronic NO production as well as a chronic hypotension (after hours) [2, 3, 132, 233]. In contrast, the brain, adipose tissue and hind limb muscles were more resistant to LPS-induced inflammation since only minor alterations of nitrate production and morphology in these tissues were observed. Like NO, peroxynitrite may have both detrimental (~>100 μM)

and beneficial (~3-30 μM) effects on different proteins, cells and organs. As well, the nitration-denitration process may be mediated by a putative "nitrotyrosine denitrase" [172, 208, 270]. Therefore, several direct and indirect LPS-induced factors, likely including nitrate anion, could potentially act in parallel or synergistically to impair cell and organ (such as cardiovascular and respiratory) functions during sepsis.

Our results are consistent with previous findings that LPS induces significant SIRS in the tissues or organs of the heart, lung, liver, kidney and diaphragm, as well as causing increased cell membrane permeability. In particular, a significant lung injury showed a typical systemic inflammatory response which reveals a significant granulocyte infiltration with exudation in alveoli and bronchioli [89, 107, 132]. MPO activity was also significantly increased in the lungs in a LPS-dose-dependent manner. MPO activity was increased in the heart, kidney and liver, but not in the spleen and plasma. Since inflammatory cells contribute significantly to iNOS content and activity during sepsis [107], they could contribute and alter extracellular nitrate production. The systemic inflammatory response induced by bacteria or endotoxins can also produce a significant amount of superoxide and peroxynitrite, two powerful oxidants that could, concurrently, contribute to cell membrane injury, cell and eventually multiple organ dysfunction [30]. Moreover, impaired myocardial contractility, inadequate distribution of blood flow and distribution of tissue oxygen use are common and contribute to the development of MODS during sepsis [18, 28]. Therefore, several direct and indirect LPS-induced factors could potentially act in parallel or synergistically to impair cell and organ (such as cardiovascular and respiratory) functions during sepsis. The systemic inflammatory response or endotoxins can also trigger the acute respiratory distress syndrome, in both

animals and humans [4, 222]. Tachypnea is notable in our septic rats, and the increase in respiratory rate may be an additional response contributing to lung and diaphragm injury as shown in the lung and diaphragm histology. Several studies have documented that the amount of sarcolemmal injury in diaphragm *in vivo* and *in vitro* is related to the level of tension time [(TTi, where TTi = Tension/Tension_{max})*(duty cycle)], suggesting that increase in respiratory rate in intense and prolonged exercise and sepsis contributes to sarcolemmal injury [4, 89, 169, 298]. The lung injury could be more severe because of the decrease in compliance (pulmonary edema) combined to the increase in respiratory rate, which causes additional tissue stress, and eventually leading to a vicious circle affecting both organs since the more stress to the lungs, the more difficult it is for the diaphragm to contract (from more fatigue to more injury). This can be seen as histological alterations in both the lungs and diaphragm which exhibited the worst muscle disorder with edema, infiltrate, atrophy and segmental necrosis. In agreement with this observation, our data also shows that there was a significant correlation between nitrate concentration in the lungs and the respiratory rate in the septic rats. These results may indicate that respiratory rate changes have an intrinsic association with NO product alteration during sepsis, and the mechanisms underlying this response needs to be further investigated.

Many studies have shown that the concentration of nitrate anion, which may not represent the concentration of NO, in plasma and tissues is significantly increased during sepsis, endotoxemia, SIRS and MODS [1-3]. Whether or not the nitrate anion contributes to or plays a role in those conditions is indistinct. We have shown that the nitrate anion is significantly increased in most tissues and organs, especially after 12 hours of LPS

treatment in rats. As the nitrate concentration may only indicate past NO production, and NO may have a protective function during sepsis [37, 334, 398], we considered that NO-derived nitrate anions may synergistically enhance the detrimental effects of NO and/or peroxynitrite on the impairment of multiple organ function during endotoxic shock. Thus, it is reasonable to assume that cellular function modified by NO-derived nitrate anions may contribute to the multiple-organ functional alterations observed in SIRS and MODS during endotoxic shock.

There is also experimental evidence that exogenous NO, e.g. inhaled NO or NO donors, can increase plasma NO-derived metabolites such as the nitrate anion [343]. Whether or not exogenous NO or its-derived nitrate anion can cause cellular or organ dysfunction are not documented. We have shown that the administration of exogenous NO can increase nitrate concentration in both plasma and urine [343]. Recently, we also find that exogenous NO, such as the administration of 20 ppm NO for 45 minutes, for 4 or 24 hours, does not statistically increase nitrate concentrations in the lungs, diaphragm or hind limb muscle of pigs, but that it increases plasma nitrate concentrations (~ 3.7 times higher than control from 19.7 ± 4.4 to 73.1 ± 17.9 μM) (see Appendix for detail). However, the nitrate concentration increased 18.5 times in endotoxic compared to control plasma of rats after 12 hours LPS treatment. Furthermore, NO was administered at a relative low concentration (5 to 20 ppm) as well as under normal (control) conditions. In other words, there should be much less pathophysiological stress, if any, in the inhaled NO condition than in sepsis and endotoxemic conditions. Therefore, it is very hard to detect any acute toxicity after the inhalation of 20 ppm NO in those experimental

animals. On the contrary, there are many more pathophysiological, metabolic and immunological alterations under sepsis and endotoxemia conditions [274, 300, 351].

Like other vascular mediators, i.e. ET and angiotensin [1, 77, 93, 346, 348, 398], several experimental evidences have documented that I.V. infusion of nitrate anion (changing plasma nitrate concentration from 20 to 60 μM) alone can raise the mean arterial blood pressure in dogs under aseptic conditions [117, 385]. However, the exact mechanism is not yet fully explored. Also, under such experimental conditions, there are no or minimal effects of induced NO, compared with endotoxic conditions in which NO is over-produced and can compromise the nitrate effect in MAP. However, under endotoxic conditions, the more nitrate over-accumulates, especially in the intracellular compartment, and the longer it lasts, the more damage is caused to the cells and the more stress may ensue to the cardiovascular and other organ systems, consequently leading to multiple organ injury/dysfunction. It was assumed that NO must be synthetized by the diaphragmatic myocytes themselves in order to impair its force generation during endotoxemic shock [37]. This also suggests that intracellular nitrate anions may eventually contribute to impair diaphragmatic force generation if NO and peroxynitrite present their protective role in the acute phase of endotoxemia in rats [37, 132, 398]. Actually, it has been shown that the chronic drop in arterial pressure observed from 70 minutes to 6 hours after the start of LPS infusion is not convincingly mirrored by a chronic increase in NO concentrations, which were monitored by a direct continuous assay of NO concentrations (porphyrinic sensor) in the lung parenchyma. However, plasma NO metabolites concentrations, monitored by indirect NO assay (such as Griess reagent), were negatively associated with MAP [132]. Similarly, we also found that aortic

nitrate anion concentrations were increased significantly and were associated with an alteration of MAP during 24 hours endotoxic shock.

Despite all of the documented experimental evidence that nitrate anion alone plays a significant role in the physiology and pathophysiology of different cell types from bacteria, plant and mammalian cells [35, 54, 76, 240, 244, 268, 285, 378], most previous reports may underestimate or even disregard the role of the nitrate anion in the physiology and pathophysiology of septic shock of experimental animal and humans. Indeed, nitrate itself is at least a partly diffusible anion that can cross the plasma membrane and affect significantly cell electrophysiological properties [16, 162, 187, 213, 249, 387], potassium transport in microvessels [214], as well as cell membrane functions to impair active calcium uptake [145], membrane enzyme (e.g., ATPase) activity [255] and muscle contractility *in vitro* [16], inhibit the energy metabolism of cells, and damage cell membrane integrity [176, 195]. Nitrate anions can facilitate vasopressin secretion, enhance sympathetic activity [313], and increase blood pressure when given intravenously [117, 385].

We have previously shown, as have many other investigators, that muscle contractility is depressed following 12-24 hours of LPS induced sepsis [37, 89, 215]. Similarly, muscle contraction can be depressed by the nitrate anion itself [16, 145], as nitrate anions can impair the active calcium uptake by the sarcoplasmic reticulum and can also increase the permeability of muscle cells to methyl-glucose *in vitro* [145]. The permeability of nitrate anion may quite vary in the different cell types or muscles. The effects of nitrate on the muscle membrane is complex, and may depend on (I) the species; (II) the pH and composition of physiological solution; (III) the time and duration of

exposure to nitrate; and (IV) the type of muscle (such as skeletal, diaphragm, smooth and cardiac muscle) used in the experiments [16, 145]. This effect is possibly additive or synergistic to the effects of NO, superoxide anions and peroxynitrite accumulation in muscle tissue during sepsis. Nitrate anions can cross the plasma membrane and significantly affect cell electrophysiological properties, cellular pH, membrane enzymes (e.g. ATPase, PTPase) activity, and potassium transport *in vitro*. The Ca^{2+},Mg^{2+}-ATPase and Na^{+},K^{+}-ATPase activity decreased in a concentration of 20 to 100 to 500 μM nitrate both *in vitro* (37°C for 60 minutes) and *in vivo* (for 1 to 5 hours) [195]. The nitrate anion can also facilitate vasopressin secretion and function synergistically in the CNS to enhance sympathetic activity, and increase blood pressure *in vivo* in rats [313], dogs and humans [117, 385]. Nitrate is one of chaotropic agents, which cause perturbation of membrane structure and nitrate at 1 mM *in vitro* affects fatty acid turnover of phosphatidylethanolamine and phosphatidylserine in rat erythrocyte due to stimulation of acyltransferase and/or phospholipase [176]. As an oxidizing agent, nitrate can promote the formation of disulfide bonds between proteins in the membrane [83]. Furthermore, recent studies have shown that the nitrate anion may pass through a distinct set of channels. And different types of nitrate transporters have been identified in bacteria, plants and animals [35, 60, 378, 387]. Interestingly, NO also functions as a signal molecule in plant and microbial [240, 244, 268, 285], in which nitrate channel is well documented [35, 387]. Our results show that the intra/extracellular nitrate concentration ratio is decreased significantly after LPS administration to rats, and this ratio in the aorta is well correlated with alteration of MAP during sepsis. Over-accumulation of plasma and tissue nitrate concentrations and a subsequent change in this intra/extracellular nitrate

concentration ratio may have synergistic deleterious effects on cardiovascular (heart and aorta) and respiratory (lung and diaphragm) functions during sepsis. Thus, it is reasonable to assume that the cellular functions modified by nitrate may provide one of many mechanisms in SIRS and MODS for those organ functional alterations observed in septic shock.

Several studies have suggested that NO demand is increased in both the early and late stages of endotoxemia or sepsis [273, 286, 399]. More interestingly, exogenous NO donors or inhaled NO could improve the beneficial/protective effects of NO during endotoxemia or sepsis in rats [132, 273, 399]. Furthermore, one study has shown that iNOS transferred to endothelial cells makes them more resistant to LPS-induced apoptosis. Since vasoconstrictor (i.e., endothelin and angiotensin), platelet activators (i.e., platelet-activating factor, thromboxane A_2) and, similarly, nitrate production are maintained or even increased *in vivo* during the course of endotoxemia [126, 284, 132, 398], and nitrate itself *in vitro* or *in vivo* could interfere with cell energy metabolism, enzyme activity as well as cell electrophysiological properties and even damage cell membrane integrity [19, 176, 195], it is reasonable to consider that nitrate may contribute to the detrimental effect of NO.

Our results indicate, similarly, that nitrate content was significantly increased in plasma and tissues such as the heart, aorta, lung, diaphragm, etc. In addition, this nitrate concentration was inversely related to MAP during endotoxemia, and nitrate content in tissues was also paralleled by cell membrane injury, as shown in Figure 6 of our manuscript. Therefore, under endotoxic conditions, the more nitrate is over-accumulated, especially in the intracellular compartment, and the longer it lasts, the more damage to

the cells and the more stress may ensue to the cardiovascular and other organ systems, consequently, leading to multiple organ injury/dysfunction. NO certainly plays an important role during endotoxemia, SIRS and MODS. Moreover, not only NO, but NO-derived nitrate anions (like endothelin, angiotensin, platelet activating factor, and thromboxane A_2) may also contribute significantly to SIRS and, ultimately, MODS during endotoxemia. All-in-all, we conclude that it is reasonable to assume that over-accumulation of the nitrate anion, the major NO metabolite, contributes to SIRS and MODS during endotoxemia.

3.6. Future studies

As the limitation of this study, several studies can be continued. NO definitely plays an essential role in physiological and pathophysiological response of cells and organs. Both beneficial and detrimental roles have been assigned to NO (and also to peroxynitrite) that may only be dependent on the threshold or on physiological and pathophysiological conditions such as sepsis/SIRS/MODS. As mentioned in the introduction, the confusion between the roles of NO and peroxynitrite remains.

NO is an intermediate between an organic molecule (L-arginine) and inorganic molecule (nitrate). The importance of the L-arginine-NO-nitrate pathway, the cross-talk (balance) between redox status (NO-peroxynitrite-nitrate), metabolism (L-arginine-NO-nitrate), and function (NO-nitrate-membrane potential, enzyme activity) alterations in cells (from bacteria, fungi, plant and animal) may explain some of the controversial roles of NO and peroxynitrite, as well as that of nitrate.

The nitrate anion is a permeable anion in plant, microbial and mammalian cells. Although NO is found to play an important role in plant cells, NO's role in the context of plant and mammalian cells still elusive. Furthermore, a relationship between NO and its metabolite, nitrate anion, in plants is not well elucidated. Since NO plays an important role in both mammalian and plant cells, the link of NO between two species may further uncover the role of nitrate anion in both species.

Nitrate anion transporters/channels in plants/microbes are well documented. However, there is only very limited information concerning nitrate anion transporters/channels in mammalian cells. It has recently been suggested that the nitrate

channels do exist in mammalian cells, but the electrophysiological properties and the role of these channels in the cells and organs in physiological as well as in sepsis/SIRS/MODS conditions remain to be seen.

To further reveal the role of nitrate anion in septic or endotoxic shock, SIRS and MODS, and to distinguish its role from that of NO and peroxynitrite, it is necessary to directly and simultaneously monitor the concentrations of NO, peroxynitrite and the nitrate anion in arterial vessels, cardiac tissue and MAP, as well as in other tissues or organs.

To investigate the role of the exogenous NO donors and nitrate anion in the SIRS, septic or endotoxic shock and MODS, different dosages of both reagents, especially at high doses, may be needed in these animal models.

More interestingly, the role of intracellular nitrate anion in the physiological and pathophysiological functions of different cells and organs need to be further elucidated.

4. CONCLUSIONS

We conclude that the nitrate concentrations in the heart, lung, kidney, liver, brain, aorta, diaphragm, spleen, thymus, testis or ovary, hind limb muscle, intestine, adipose tissue, bone, bladder, plasma and urine are significantly different. A significant nitrate gradient was evident between organs and plasma, indicating a nitrate gradient between intracellular and extracellular compartments.

LPS induced a significant increase in nitrate concentration at 12 h in most organs, except in the brain, adipose and muscle tissue. A LPS-dose dependent nitrate concentration response was only seen in the aorta and lungs after 12 h of treating the rats with LPS.

Exogenous NO, such as inhaled 20 ppm NO gas or NO donors (nitroglycerin), significantly and transiently increased nitrate concentration in arterial and venous plasma as well as in urine, but not in the tissues of the lungs, diaphragm and hind limb muscles.

iNOS protein concentration was higher in blood than in the diaphragm and lungs 12 h after LPS administration. iNOS content in blood correlated significantly with nitrate level in plasma.

Nitrate concentration changes in the tissues and organs occurred in parallel with inflammatory responses which was indicated by alterations in MPO activity, histological modifications, cell sarcolemmal lesions and organ function. LPS-dose-dependent MPO activity was only seen in the tissue of septic lungs.

Diaphragm contractility was reduced significantly by LPS *in vivo* and by nitrate *in vitro*. The respiratory rate was increased significantly at 6 and 12 h after LPS administration and there was a significant correlation between respiratory rate and nitrate levels in the lungs. LPS induced a significant decrease in MAP particularly at 12 h and MAP was correlated negatively with the nitrate concentration in the aorta as well as positively with the aorta/plasma nitrate concentration ratio.

Collectively, we conclude that nitric oxide-derived nitrate anion contributes to endotoxic shock/SIRS/MODS during sepsis.

5. Appendix:

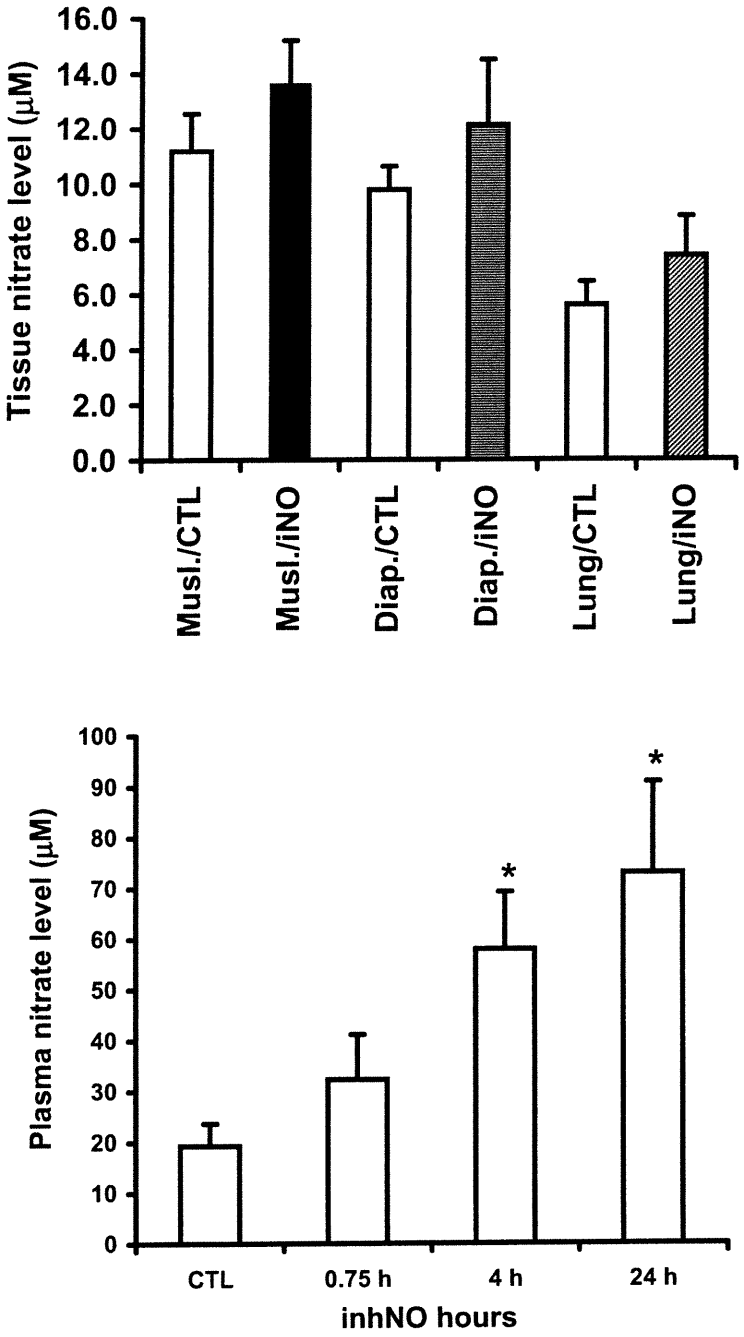


Figure 1. Nitrate anion concentration in plasma and the tissues of the hind limb muscle, diaphragm and lung of pigs after the exogenous NO administration. Nitrate concentrations were significantly increased in plasma of pigs (~ 3.7 times higher than control after 24 hours), but not in the lungs, diaphragm and limb muscle after inhalation of 20 ppm NO for 0.75, 4 and 24 hours. * $P< 0.05$.

6. REFERENCES

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