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Evaluation of nitric oxide production by bovine alveolar macrophages

Gary L. Mason

To the Graduate Council:

I am submitting herewith a dissertation written by Gary L. Mason entitled "Evaluation of nitric oxide production by bovine alveolar macrophages." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Philip N. Bochsler, Major Professor

We have read this dissertation and recommend its acceptance:

David Slauson, David Bemix, Roger Carroll

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EVALUATION OF NITRIC OXIDE PRODUCTION BY BOVINE ALVEOLAR MACROPHAGES

A Dissertation Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Gary L. Mason

December 1999

DEDICATION

This dissertation is dedicated to my wife Amy Womack, who sacrificed a significant portion of her life to make this work possible and while supporting me, brought our children into this world, who make it all worthwhile.

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There are many people without whom this work would not have been possible. I would like to thank the members of my graduate committee, Phil Bochsler, David Slauson, David Bemis, and Roger Carroll. Phil Bochsler served not only as chair of my committee but was and remains a mentor and friend in the truest sense of those words. Tim Olchowy, a former committee member, contributed materially to this work and also deserves special recognition. My fellow pathology residents and graduate students contributed as much to my training as the faculty. Among these a special thanks goes to Zhengang Yang who was intimately involved with much of the work presented here.

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Lastly, I wish to acknowledge the people of the state of Tennessee whose financial support allowed me the opportunity to train in the combined program in the Department of Pathology.

ABSTRACT

Bovine respiratory disease complex (BRD) is the most costly disease problem of cattle in North America. BRD is multifactorial with contributions from management practices, environmental factors, and infectious agents including *Pasteurella haemolytica* A1, bovine herpes virus type 1(BHV), parainfluenza type 3 (PI3), and bovine virus diarrhea virus (BVD). Alveolar macrophages are intimately involved in defense of the lower respiratory tract against infectious agents. Expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO·) is a key defensive response of rodent macrophages. Microbicidal activity of rodent macrophage derived-NO· has been demonstrated against many taxonomically diverse infectious agents *in vitro* and *in vivo*. The work presented here was designed to characterize expression of iNOS and production of NO· by bovine alveolar macrophages and evaluate the microbicidal activity of NO· against important bovine pulmonary pathogens.

Bovine alveolar macrophages recovered from the lungs of slaughter cattle were examined in cell culture. Recombinant human and bovine cytokines and endotoxin from *Escherichia coli* and *Pasteurella haemolytica* A1 were used to activate macrophages. NO· production was estimated by measuring nitrite accumulation in cell supernatants using the Greiss reaction. iNOS mRNA and protein expression were detected by Northern and Western blotting respectively. Bovine alveolar macrophages express iNOS mRNA and protein and produce NO· in a time and dose-dependent fashion *in vitro* in response to stimuli known to

be present in pneumonic tissue, including IFN-γ, IL-1β, and endotoxin. Whole killed *P. haemolytica* A1 is also a potent inducer of NO· production. Competitive inhibitors of iNOS restrict nitrite accumulation in cell culture media without affecting iNOS protein expression.

Reactive nitrogen oxides chemically generated by S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) and 3-mopholinosydnonimine (SIN-1) kill wild type *P. haemolytica* A1 in a dose-dependent fashion. Bovine alveolar macrophages kill the leukotoxin-deficient mutant *P. haemolytica* A1 D153, but prior stimulation for NO· production abrogates this effect. Wild type *P. haemolytica* A1 readily kills macrophages.

BHV, PI3, and BVD infection of macrophages depresses NO· production. This effect is due to loss of viability of macrophages infected with BHV and BVD, but is due to an alteration of cell function in PI3 infected macrophages. BVD and PI3 readily replicate in macrophages, but there was only minimal replication of BHV in these cells. Prior stimulation of macrophages for NO· production did not significantly effect the replicative ability of any of these viruses in macrophages.

Bovine alveolar macrophages express iNOS and produce NO· in response to stimuli known to be present in pneumonic lung and in a fashion similar to that previously characterized in rodent macrophages. However unlike findings in rodent model systems, no microbicidal activity of macrophage derived NO· was demonstrated. The role and significance of NO· production by bovine alveolar macrophages in infectious pneumonia remains speculative.

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LIST OF ABBREVIATIONS AND SYMBOLS

BHV	Bovine herpes virus type 1
BRD	Bovine respiratory disease complex
BVD	Bovine virus diarrhea virus
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
cNOS	Constitutive nitric oxide synthase
ecNOS	Endothelial constitutive nitric oxide synthase
GM-CSF	Granulocyte macrophage colony stimulating factor
IFN-γ	Interferon gamma
IFN-α	Interferon alpha
IL-1β	Interleukin-1beta
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide, endotoxin
mRNA	Messenger ribonucleic acid
ncNOS	Neuronal constitutive nitric oxide synthase
NFκB	Nuclear factor kappa B
NO$\cdot$	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
$O_2\cdot^-$	Superoxide anion
PI3	Parainfluenza type 3
RNS	Reactive nitrogen species
SIN-1	3-morphylinosydnonimine
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
TGF-β	Transforming growth factor beta

PART 1

Review of Literature

Overview

Nitric oxide (NO·) is a reactive radical mediator that is generated de novo and can be produced by most mammalian cell types. NO· has received much attention by basic biomedical scientists in recent years due to its involvement in diverse physiologic and pathophysiologic processes in many tissues. NO· has a molecular weight of 30 daltons and due to its small size and lack of charge is freely diffusible in aqueous solutions and biologic membranes. NO· is produced by reduction of L-arginine in the presence of oxygen with L-citrulline as a co-product. These reactions are carried out by the Nitric Oxide Synthase enzyme family (NOS), consisting of a high-output inducible isoform (iNOS) and two low-output constitutive isoforms (cNOS). NO· produced by these enzymes mediates diverse actions dependent on the site and redox state of the local tissue setting. In general, cNOS isoforms intermittently generate small quantities of NO· that functions as a signalling molecule in homeostatic processes, such as the regulation of blood pressure or as a neurotransmitter. Conversely, NO· produced by the high output iNOS isoform functions as a potent oxidizing species in host defense. NO· has an unpaired electron and thus is capable of reactivity with a variety of targets. Many of the actions originally ascribed to NO· are attributable to secondary reactive nitrogen oxide species (RNS).

Characterization of NO· production by bovine alveolar macrophages and an assessment of this molecule's potential contribution to pulmonary host defense in cattle form the purpose for this manuscript, but first a review of

general knowledge regarding nitric oxide is appropriate. The following sections contain an overview of NOS enzymology, regulation of enzyme activity and expression, chemistry and mechanism of action of NO·, and biologic effects of NO· in health and disease.

Nitric Oxide Synthase Enzyme Family

Nomenclature

Three NOS isoforms are known. Attempts to identify others using a homology-based molecular cloning approach have proven fruitless, suggesting that other isoforms, if they exist, probably differ markedly in structure.[1] Three nomenclature systems are used to describe the NOS family.

The first system divides NOS isoforms on the basis of their expression. The isoform first identified in inflammatory cells was detected only after cellular activation and was catalytically active in the absence of elevated intracellular calcium; thus this isoform is designated as the **i**nducible or calcium-**i**ndependent isoform or **i**NOS.[2] The other two isoforms, initially characterized in endothelia and neurons, were expressed as **c**onstitutive enzymes, demonstrated **c**alcium-dependent activity, and were thus designated as **c**NOS enzymes.[2]

The second system designates the isoforms on the basis of the rodent cell types from which they were first identified.[3] In this scheme, iNOS, nNOS and eNOS refer to the isoforms first isolated and cloned from inflammatory cells,[4]

neurons,[5] and endothelial cells.[6;7] A modification of this system also indicates dependence on calcium for activity. In this scheme e- and nNOS become ec- and ncNOS, respectively.[8]

The third system designates NOS isoforms based on the order of their identification and characterization from human tissues. In this system, NOS 1, 2, & 3 refer to isoforms identified in neurons, inflammatory and endothelial cells, respectively.[2]

In this manuscript, the first and second nomenclature systems will be used where best suited to the discussion at hand.

Distribution of Nitric Oxide Synthases

The value of the ability to elaborate NO· is revealed by the identification of NO· production in species as diverse as molds,[9] protozoa,[10] mollusks,[11] and chickens,[12] in addition to mammals.[13] NOS nomenclature systems indicate the cellular origin of each isoform and NOS enzymes play major roles in the physiology of these cell types, however the nomenclature is somewhat misleading because these isoforms are now known to be widely distributed. iNOS may be expressed not only in inflammatory cells, but also in glia, endothelia, many epithelial cell types, cardiac myocytes, and smooth muscle cells.[2;13] ncNOS is expressed in pancreatic islets, neutrophils, endometrium, and skeletal muscle as well as in the nervous system, and ecNOS is found not only in endothelia, but also in platelets, cardiac myocytes, and hippocampus.[2;13] This list

is not exhaustive, but it reveals the ubiquity of NOS enzymes, suggesting that all three isoforms can be expressed in most tissues.

Enzymology

NO· is produced by the five electron reduction of the terminal guanidino nitrogen of the basic amino acid, L-arginine, in a complex reaction that also requires molecular oxygen and NADPH as substrates and heme, FAD, FMN, Ca^{2+}, reduced thiols, calmodulin and tetrahydrobiopterin as cofactors.[8;14] N-ω-hydroxyarginine is the intermediate and L-citrulline is the coproduct.

Nitric oxide synthases exist as homodimers and have structural and catalytic similarities to the P-450 reductase enzyme family.[15] NOS are highly conserved. Among mammals, the interspecies identity between the same isoform averages 90%, while identity between isoforms within a species (man) is 53%.[8] Regions devoted to cofactor binding and catalysis retain the greatest homology.[8] NOS isoforms are composed of a relatively conserved C-terminal reductase domain and a more variable N-terminal oxidase domain.[16] These domains are separated by an intervening, approximately 30 amino acid long, calmodulin recognition sequence.[16] Binding sites for FAD, FMN and NADPH are present in the reductase domain, while L-arginine, tetrahydrobiopterin and heme binding sites and the active site are in the oxygenase domain.[16] The oxidase domain is required for homodimer formation and both heme and tetrahydrobiopterin are incorporated during dimerization.[16] During catalysis,

flavins acquire electrons from NADPH and transfer them to the heme iron allowing O_2 binding and NO· synthesis.[16]

Regulation of Nitric Oxide Synthase Activity

NO· is small, without charge or polarity, freely diffusible, lacks the structural specificity characteristic of classical mediators, and is endowed with the reactivity of an unpaired electron.[17] In many ways, this molecule appears to be a poor choice as a mediator for signaling specific biologic effects. Specificity is provided by regulation of NOS activity, subcellular localization of NOS, and control of NOS expression.

Regulation by Calcium and Enzyme Expression

Regulation of the cNOS isoforms by calcium, and iNOS by transcription and expression were the first forms of regulation recognized and are still considered the major determinants of NOS activity.[16]

All three NOS isoforms require a bound calmodulin prosthetic group for NO· synthesis. Calmodulin binding permits electron transfer between reductase and oxygenase domains in ec- and ncNOS and these isoforms bind calmodulin tightly only when calmodulin is bound by calcium.[16] This feature provides an important regulatory mechanism for these isoforms, resulting in enzyme activity only in response to a perturbation of the cell that results in a flux in cytosolic calcium concentration.[13] Because increases in cytosolic [Ca^{2+}] are typically

transient events in normal cellular activation, these enzymes are temporally regulated by calcium.

Calmodulin binding to iNOS is essentially irreversible and calmodulin is so tightly bound that it co-purifies with iNOS in denaturing gels.[18] This tight association results in activation of iNOS by the low [Ca^{2+}] found within resting cells.[16] Once expressed, iNOS may remain catalytically active as long as the concentrations of substrates and cofactors are not limiting, thus regulation of iNOS appears primarily due to control at the level of expression.

INOS expression is best characterized in macrophages stimulated by cytokines and microbial products. IFN-γ and bacterial endotoxin (LPS) are the classic stimuli, but other cytokines, including Il-1β, Il-2, Il-12, Il-18, TNFα, the type 1 interferons α and β, and bacterial products including peptidoglycan and lipotiechoic acids from the cell wall of gram positive bacteria, lipoarabinomannans from *Mycobacteria*, fungal cell wall components, double stranded RNA and viral coat proteins are also effective.[13;19-26]

INOS expression induced by cytokines and microbial products is transcriptionally regulated. Induction of the murine iNOS gene by IFN-γ and LPS is dependent on several transcription factors including Nuclear factor kappa B (NF-κB),[27] signal transducer and activator of transcription (STAT) proteins,[28] and interferon-regulatory factors (IRF)[29]. These transcription factors also regulate the transcription of other genes involved in inflammatory responses, including cytokines[30] and cell adhesins.[31]

Effective stimuli for expression of iNOS vary between species. The available information suggests this is due to species variation in promoter organization. The murine iNOS promoter contains a γ-interferon activated sequence (GAS),[28] an IRF-1 site,[29] and an NF-κB element[27] within 1.7 kb of the transcriptional start site. In contrast, induction of the human iNOS gene by TNF-α and Il-1β is dependent on four NF-κB enhancer elements in the promoter upstream of 3.8 kb.[32] Maximal expression of human iNOS also requires IFN-γ, but the transcription factors and promoter sites involved have not yet been elucidated.

Effective stimuli for iNOS induction also varies between cell types within a species.[33] LPS alone can induce iNOS in murine macrophages, but a marked increase in expression occurs when IFN-γ is added, while Il-1β has little iNOS expression enhancing activity in this cell type.[34] By contrast, murine hepatocytes, and most other parenchymal cell types, do not respond to LPS as a single stimulus and require the combination of LPS, IFN-γ, Il-1β, and TNF-α for maximal expression.[34;35] The mechanistic basis responsible for this observation is unknown, but may be due to cell-type specific variation in signal transduction pathways, and expression of, or associations between, transcription factors.[36]

Transforming growth factor-β (TGF-β) and glucocorticoids and cyclosporin-A suppress iNOS expression and NO· production by decreasing gene transcription, decreasing mRNA stability, decreasing translation, and

accelerating iNOS protein degradation.[37-41] Interleukins -4, 8, and 10 and macrophage deactivating factor also suppress iNOS expression.[13;39;42;43]

In the lung, iNOS may function as a constitutive enzyme in bronchial epithelial cells which are chronically activated by exposure to endotoxin and other immunoactivating species in inhaled air.[44] iNOS also displays long-term expression in the pregnant uterus. iNOS is undetectable in the nongravid uterus, increases in the myometrium during pregnancy and diminishes rapidly with onset of labor.[45] NO· production in the myometrium likely maintains relaxation of uterine smooth muscle, because contractile activity near term is blocked by nitrovasodilators and enhanced by NOS inhibitors.[45]

Activity of the constitutive NOS isoforms is also regulated to some degree by expression, but these are typically adaptive or perhaps maladaptive responses in disease and will be discussed in sections devoted to the biology and disease of each isoform.

Regulation by Intracellular Targeting

NO·, although a radical molecule, is a relatively stable one which may persist for several seconds and diffuse into cells adjacent it's origin before encountering a suitable target.[17] Even so, localization of NO· generation near the desired target would enhance signaling specificity and efficiency.

In inflammatory cells, iNOS is readily isolated in soluble form, but has also been identified in particulate cell fractions, which may represent lysosomes.[46] In neurons, ncNOS immunoreactivity is present in the rough endoplasmic reticulum

and electron dense post-synaptic vesicles.[47] In skeletal muscle, ncNOS is associated with the sarcollema.[48] The ecNOS isoform requires detergent solubilization for isolation, but the protein contains no recognizable hydrophobic transmembrane domains.[49] Targeting of all three NOS isoforms appears to be due to protein binding motifs in the oxygenase domain.[2] In the cNOS isoforms these motifs allow binding to membrane associated proteins.[2] Membrane targeting of cNOS isoforms likely localizes these enzymes near other signal transduction proteins.

The details of this regulatory process are best documented with the ecNOS isoform.[50] This isoform undergoes a variety of covalent modifications including myristolation, acylation, and palmitoylation. Post-translational modification of ecNOS determines not only intracellular targeting to caveolin signal transducing membrane microdomains, but also enzyme function. Myristolation of ecNOS on a glycine residue near the N-terminus is required for targeting to the membrane; myristolation of ecNOS is an irreversible modification. In contrast, palmitoylation, which occurs on 2 cysteines also near the N-terminus, are readily reversible modifications. Depalmitoylation of ecNOS occurs secondary to activation of endothelial cells by agonist binding of surface receptors. Agonist binding results in release of ecNOS from the membrane, an increase in cytosolic calcium and enzyme activation. NO· production is terminated by repalmitoylation with loss of calmodulin and reassociation of ecNOS with caveolin.[2] Additionally, ecNOS rapidly associates with the molecular chaperone, Hsp90, in endothelial cells activated by shear stress, vascular

endothelial growth factor or histamine and this association markedly increases enzyme activity.[51] The increase in ecNOS activity secondary to Hsp90 binding is significant, because prior to this discovery the demonstrated vmax of ecNOS was many fold less than that of the other NOS isoforms.[13]

Targeting of cNOS isoforms is also tissue-specific. ecNOS associates with different caveolin isoforms when expressed in endothelial cells versus cardiac myocytes.[52] Alternate splicing of ncNOS mRNA in skeletal muscle yields the tissue-specific, muscle isoform designated, ncNOSμ.[48] ncNOSμ is localized to the sarcolemma by association with the dystrophin complex via PDZ motifs near the N-termini of both ncNOSμ and the dystrophin complex protein, syntrophin.[53] In neurons, ncNOS is targeted to N-methyl-D-aspartate (NMDA) receptors by association of the NH_2-terminal PDZ domain to PSD-95 protein[47] which is physically associated with the NMDA receptor at postsynaptic densities.[54] The alternatively spliced ncNOS isoforms, ncNOS-β and ncNOS-γ which represent a minority fraction of ncNOS catalytic activity, lack the PDZ domain and do not associate with membranes.[55]

Regulation by Phosphorylation

NOS enzymes may also be regulated by phosphorylation. All NOS isoforms can be phosphorylated by purified protein kinases and can be isolated as phosphoproteins[2] and phosphorylation of tyrosine (tyr) residues increases iNOS activity.[56] In intact endothelial cells, shear stress results in phosphorylation of serine (ser) residues on ecNOS and a marked increase in enzyme activity,[57]

however phosphorylation of ser and tyr residues in in the oxygenase domain of ncNOS markedly diminishes its activity.[2] Phosphorylation is temporally associated with enzyme translocation to the cytosol.[58] These changes are not replicated with agonists that increase cytoplasmic calcium concentration, suggesting phosphorylation mediated by agonist dependent signal transduction pathways provides a calcium-independent level of regulation of ecNOS activity.[57]

Control at the Level of Substrate and Cofactors

L-arginine is the only amino acid that can be utilized by NOS to produce NO·. [59] L-arginine is a product of the urea cycle and is present in human blood at approximately 100 μm.[60] Active transport maintains cellular concentrations at an even higher level; a process that can be modulated by cytokines.[61] Intracellular arginine concentrations are certainly in excess of the Km of 5μm required for enzyme activity.[62] Nevertheless, control of substrate availability is a potential control point for regulation of NOS activity and this may be particularly important in cells expressing the high output iNOS isoform.

Endothelial cells[63] and macrophages[64] are capable of recycling L-citrulline back into L-arginine via induction of the urea cycle enzymes, argininosucinate synthetase and argininosuccinate lyase. In macrophages, these enzymes are co-induced with iNOS apparently to prevent L-arginine concentrations becoming rate limiting for NO synthesis *in vivo.*[64] Counterbalancing these enzymes, these cells also contain arginase enzymes which convert arginine to ornithine and urea. Arginase I is constitutive, while Arginase II is induced by IFN-γ and endotoxin.[65]

N-ω-hydroxyarginine, an intermediate in the NOS hydrolytic pathway is an inhibitor of arginase.[65] The role of arginase in modulating NO· production remains undetermined.[66]

NO· may inactivate NOS during enzyme synthesis when tetrahydrobiopterin levels are subsaturating. However, GTP cyclohydrolase 1, the enzyme necessary for tetrahydrobiopterin synthesis is co-induced with iNOS by IFN-γ, presumably to prohibit this outcome.[8] Tetrahydrobiopterin bound to one NOS subunit markedly decreases affinity of the other subunit for tetrahydrobiopterin,[16] and enzymes containing only one bound tetrahydrobiopterin generate NO· from that subunit and superoxide anion from the other, conceivably setting the stage for generation of the potent oxidizing and nitrosating NO· congener, peroxynitrite.[67] Apparently low BH4 levels can be encountered in cells expressing cNOS isoforms, but the physiologic significance of mixed oxide production by the low output cNOS isoforms remains unresolved.[16]

Autoregulation by Nitric Oxide

NOS are auto-regulated by reversible binding of NO· to heme. Binding occurs rapidly, inactivates the enzyme, and markedly limits the potential rate of catalysis.[68] The rate of spontaneous formation and dissociation of NO-heme complexes are determined by, and proportional to, the concentration of NO· scavengers and O_2, indicating the redox state of the cell potentially regulates

NOS catalytic activity.[69] NO· may also inhibit iNOS during enzyme assembly by blocking heme insertion and dimerization.[16]

Molecular and Cellular Targets of Nitric Oxide and Reactive Nitrogen Intermediates

NO· is a relatively stable radical which may persist for several seconds and whose rate of diffusion exceeds the rate at which it interacts with intracellular targets.[17] Because of this, the quantity of NO· in a cell's environment is determined by the number of cells in the area that produce NO· and the amount being produced. Because NO· is freely diffusible and nonhydrolyzable, deactivation requires interaction with another molecular target. In settings in which homeostasis is not disturbed, NO· is typically deactivated by oxidation in the presence of oxyhemoglobin or oxymyoglobin to produce the benign anion, nitrate, which is filtered in the glomerulus and excreted in the urine.[69] Bioactive NO· is also stored by forming stable adducts, particularly with thiols.[70;71] Many of the actions ascribed to NO· are duplicated, or mediated, by secondary reactive nitrogen oxide species (RNS) formed by the reaction of NO· with other species. RNS is a general term which includes the redox forms of NO·, nitrosonium (NO^+), and nitroxyl anion (NO^-); as well as secondary products, including; peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2·), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), S-nitrosothiols, and dinitrosyl-iron complexes.[72] Many of these compounds are more potent oxidizing and nitrosating species that NO· itself.

The unpaired electron possessed by NO· and RNS interact with a variety of molecular targets, including heme iron, iron-sulfur clusters, thiols, cysteine (cys) and tyr residues, reactive oxygen species, such as superoxide anion, lipids and DNA.[72;73] Reaction with these targets provides the mechanistic basis for the biological effects of NO·. NO· mediated biologic reactions are paradoxical in that NO· has benign regulatory signaling, antioxidant, and anti-inflammatory functions and is also involved in destructive oxidative, nitrosative and pro-inflammatory reactions. This dichotomy seems difficult to resolve at first, but is likely related to the quantity and site at which NO· is produced. The relative rates of production and concentrations of NO· and other reactive oxygen species, particularly superoxide anion ($O_2\cdot^-$), may influence the balance between beneficial and destructive actions.[69;72]

As a framework for the following discussion, the actions of NO· can be divided into direct and indirect effects.[72] Direct effects are defined as those in which NO· interacts directly with a target. This is the setting typically encountered in cNOS mediated signaling processes where small quantities of NO· are produced intermittently and only trace quantities of reactive oxygen species are produced, typically in the form of superoxide anion ($O_2\cdot^-$) generated by mitochondria. In this setting NO· interactions typically result in deactivation of oxidative radicals and cytoprotection.[72] Indirect effects are reactions mediated by secondary RNS generated from the interaction of NO· and $O_2\cdot^-$ or O_2. This is thought to occur when large quantities of NO· are produced, either secondary to expression of iNOS, or perhaps in tissue injury, where inability of the cell to

regulate cytoplasmic [Ca^{2+}] results in high output NO· production from cNOS isoforms. Indirect effects typically dominate in settings of tissue inflammation and are characterized by oxidative and nitrosative cellular stress that alters protein function and gene expression resulting in cytostasis or cytotoxicity and tissue injury.[69;72]

Examples of NO· mediated signaling, cytostatic effects, and both pro-and anti-oxidative and pro- and anti-inflammatory roles of NO· and RNS are described in the following section.

Nitric Oxide Mediated Signaling

NO· produced by ec- and ncNOS isoforms mediates signaling in the cardiovascular and nervous systems, respectively, largely by modulation of the activity of hemoprotein containing enzymes, such as Guanylyl cyclase. Guanylyl cyclase (GC) is a heterodimer that catalyzes the conversion of guanosine 5'-triphosphate to the second messenger, cyclic guanosine 3',5'-monophosphate(cGMP).[14] NO· mediated cGMP production plays important roles in regulation of vascular tone,[74] inhibition of platelet aggregation,[75] and inhibition of leukocyte-endothelial interactions.[76] Regulation of vascular tone illustrates this classic NO· mediated homeostatic-signaling mechanism. Exposure of endothelial cells to acetylcholine, bradykinin, histamine and other agonists results in vasodilation.[33] Vasodilation in response to these agonists is dependent on an intact endothelium which releases endothelium-derived relaxing factor(s) now known to be NO· or a secondary nitrosothiol. Agonist ligation of endothelial cell

surface receptors produces an increase in cytoplasmic [Ca^{2+}] and activation of ecNOS. Newly synthesized NO· diffuses into the subjacent smooth muscle cell where it interacts with the heme iron of GC forming an iron-nitrosyl complex that facilitates a change in enzyme structure permitting cGMP formation.[14] Cyclic GMP acts as a second messenger, activating cGMP-dependent protein kinase, which in turn phosphorylates the inositol 1,4,5-triphosphate receptor, producing a decrease in cytoplasmic [Ca^{2+}], relaxation of the smooth muscle cell and dilation of the vascular bed.

Other heme containing enzymes targets of NO· are cGMP-dependent phosphodiesterases which control the intracellular balance of cyclic nucleotide second messengers,[77] cGMP-gated ion channels which transduce visual and olfactory signals,[78] and cytochrome oxidase. NO· produced by NOS within mitochondria appears to serve as a physiologic oxygen sensor, regulating respiration by competition with oxygen for the heme of cytochrome oxidase.[79]

NO· and RNS interact with reactive thiol groups in proteins to form S-nitrosothiols.[71] S-nitrosothiol species have longer half-lives than NO· and increase the duration of NO· mediated signals by acting as a reservoir for bioactive NO·.[69;70] S-nitrosothiols are thought to be key intermediates in the action of nitrovasodilators, such as nitroglycerin and sodium nitroprusside.[80] S-nitroso-albumin is present in the blood at concentration reaching 5 μm and may be the bioactive factor that mediates endothelium and NO· dependent vasodilation.[73] S-nitrosothiols and other stable NO· adducts are redox sensitive

and can deliver bioactive NO· at remote sites. The following hypothetical example illustrates this concept. Airway epithelium and some alveolar macrophages express iNOS in an almost constitutive pattern,[44] probably due to chronic low-grade exposure to ozone and bacterial products present in inhaled particulate matter.[81] NO· elaborated in the airways likely forms stable S-nitrosothiol adducts in the alveolar fluid which would exist in equlibrium with alveolar cellular nitrosothiols.[82] Cysteine 93 in the beta chain of hemoglobin in blood circulating through the lung obtains a nitroso group presumably from alveolar S-nitrosothiols.[83] This stable adduct is thought to donate NO· in the microvasculature where decreased oxygen tension indicates a need for vasodilation and increased blood flow.[83]

Role of Nitric Oxide and Reactive Nitrogen Species in Cytostasis and Inflammation

As suggested above, pro-oxidative and -inflammatory actions of NO· occur in situations characterized by production of large quantities of NO· and reactive oxygen species. Superoxide dismutase or NOS inhibitors ameliorate much of the vascular and tissue injury in inflammatory models, revealing roles for $O_2^{\cdot -}$, NO· and RNS.[82;84] Peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3)are the predominate RNS formed in inflammation and these contribute to nitrosative and oxidative cellular stress, altering enzyme function, signal transduction pathways and gene expression. These alterations may result in cytostasis, apoptosis, necrosis or contribute to tissue inflammation.[85]

Peroxynitrite is formed by the reaction of NO· and O_2·⁻. Peroxynitrite generation is optimized when the same or adjacent cells produce similar amounts of both NO· and O_2·⁻.[72] Although differentially controlled, both iNOS and NADH/NADPH oxidases may be induced simultaneously by the same stimuli in macrophages and endothelial cells.[72;86] Neutrophils, on the other hand produce 100-1000 times more O_2·⁻ than NO·, therefore these cells are poor sources of $ONOO^-$.[72] Peroxynitrite production from O_2·⁻ and NO· is estimated at the nearly diffusion limited rate of 6.7 X 10^9 M^{-1}/s^{-1}; three times faster than the known dismutation rate of O_2·⁻ catalyzed by either Mn or Cu/Zn superoxide dismutase.[87] Peroxynitrite nitrosates and deactivates superoxide dismutase, thus promoting the availability of O_2·⁻.[88] Peroxynitrite also forms from the interaction of nitroxyl (NO^-) and O_2, or within the confines of an acidic phagolysosome, from hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2) or nitrite (NO_2^-).[89]

Peroxynitrite is a potent oxidizing species that readily reacts with iron-sulfur clusters and zinc-fingers, oxidizes protein thiols, nitrosates protein cys and tyr residues, produces strand breaks in DNA and initiates lipid peroxidation.[69;72] Peroxynitrite has a half-life of < 1 second, but is sufficiently stable to diffuse to another area within the cell before interacting.[69;72] Peroxynitrite exists in equilibrium with the uncharged conjugate acid, ONOOH (pKa = 6.8) which can diffuse through membranes to cause damage to the contents of membrane bound compartments in the cell of origin or diffuse through the cell membrane and release peroxynitrite in adjacent tissue.[69;72]

Auto-oxidation of NO· forms the potent nitrosylating agents N_2O_3 and N_2O_4, as well as the potent oxidizing radical NO_2·.[69;72] Auto-oxidation of NO· with O_2 occurs rapidly in hydrophobic environments, such as biologic membranes, where hemoproteins and other preferred targets are absent.[90] NO_2· is also formed from the oxidation of NO_2^- by myeloperoxidase and hydrogen peroxide (H_2O_2) which may occur within leukocyte phagolysosomes. S-nitrosothiols, such as S-nitrosoglutathione result from interaction of secondary RNS with reduced thiols in the presence of an electron acceptor.[91] Nitrosonium is a potent nitrosating species that readily forms S-nitrosothiols by this mechanism.[72] N_2O_3 is the pre-imminent member of this group of RNS which are potent nitrosating species. N_2O_3 nitrosylates a variety of molecular targets, particularly amines and thiols.[85] Nitrosamines are markers of cellular nitrosative stress and are implicated in the malignant transformation that may follow chronic inflammation.[92]

The interaction of RNS with heme-iron prosthetic groups, and iron-sulfur clusters may cause cellular energy depletion and cytostasis or necrosis.[73] NO· reversibly inhibits cytochrome oxidase by competing with O_2 for heme.[79] RNS, particularly peroxynitrite, irreversibly inhibit iron-sulfur complex containing enzymes in the electron transport chain, including NADH ubiquinone/oxidoreductase and NADH succinate/oxidoreductase as well as the tricarboxylic acid cycle enzyme, *cis*-aconitase.[73] Peroxynitrite oxidizes mitochondrial NAD(P)H leading to permeability transitions, which further depresses ATP synthesis and leads to loss of ability of mitochondria to sequester Ca^{2+}.[93] RNS-mediated cytotoxicity is associated with cellular iron depletion[94] and

a component of RNS inactivation of iron-sulfur complex containing enzymes may be restriction of available cellular iron stores.[95]

Iron and hemoprotein-catalyzed oxidative reactions likely mediate damage in inflammation by producing oxidative stress that enhances leukocyte and endothelial cell adhesion molecule expression, injures cells, induces apoptosis, damages DNA, promotes inflammatory mediator synthesis and regulates gene expression.[69;72] Many of the actions of NO· on these hemoproteins result in opposing effects, depending on the experimental model, redox conditions, quantity of NO· produced, and the presence and concentrations of other reactive species.

NO· may modulate iron-catalyzed reactions such as the $O_2\cdot^-$ driven Fenton reaction, resulting in generation of the potent oxidizing species hydroxyl radical (OH·). Addition of NO· *in vitro*, markedly attenuates the Fenton reaction.[72] The mechanism is not clear but may be due to inactivation of the reactant H_2O_2, or the product OH·. NO· can liberate iron from ferritin, and reduce Fe, enhancing the iron catalyzed Haber-Weiss reactions, however NO· may limit the availability of iron for catalysis of oxidative reactions by forming iron-nitrosyl complexes.[96]

H_2O_2 reacts with hemoproteins to produce oxidative radical species,[97] however NO· may act as an antioxidative cytoprotectant by reducing heme iron in these complexes, yielding nitrite or nitrate and preventing accumulation of this oxidizing species.[97] NO· binds the heme group in catalase, inhibiting this enzyme, thus preserving hydrogen peroxide for reaction.[98;99]

Low concentrations of NO· stimulate prostaglandin production.[100] $O_2^{\cdot -}$ inhibits cyclooxygenase by reducing heme to its inactive ferrous (Fe^{2+}) form. NO· may scavenge $O_2^{\cdot -}$, maintaining the heme in its active ferric form and enabling the formation of prostaglandins. If NO· and $O_2^{\cdot -}$ are overproduced in settings of tissue inflammation, peroxynitrite may be formed which inactivates prostacyclin synthetase by heme-thiolate-catalyzed tyrosine nitration of the enzyme.[101]

RNS inhibition of many enzymes is due to nitrosylation of thiol groups. RNS mediated nitrosylation inactivates ribonucletide reductase, the rate limiting enzyme in deoxyribonucleotide synthesis.[102] This is thought to be an important component of NO· dependent tumor cell cytostasis[103] and virostasis.[104] Glyceraldehyde-3-phosphate dehydrogenase and γ-glutamylcysteinyl synthetase are inactivated by S-nitrosylation of thiols.[71;96] NO· also may nitrosylate thiols in creatine kinase, disrupting supply of ATP by mitochondria.[71] Thiol nitrosylation predisposes proteins to secondary modifications, such as ADP ribosylation, and may modify secondary structure by enhancing disulfide bonding.[70]

RNS species including NO·, dinitrogen trioxide, nitrite radical, and peroxynitrite can oxidatively damage DNA resulting in deamination and strand breaks.[105-107] Poly(ADP-ribose) polymerase (PARP) is activated by damaged DNA. PARP polyribosylates nuclear proteins by transferring ADP-ribose moieties from NAD^+. The resulting polyADP-ribose polymers are degraded by glycohydrolases. This initiates a futile cycle, resulting in ATP consumption in the

regeneration of NAD^+. By this mechanism, RNS-mediated DNA damage may contribute heavily to depletion of cellular energy reserves.[69]

S-nitrosylation of protein thiols by NO· or RNS may act as a redox-sensitive molecular switching mechanism which transduces changes in nitrosative and oxidative cellular stress to regulate cell functions, including signal transduction pathways, gene expression and apoptosis. S-nitrosylation regulates the activities of a variety of molecules including, the transcription factors, NF-kB, CREB, and AP-1,[69;108] G-proteins, [109] protein kinase C,[110] ion channels,[111] caspase,[112] tyrosine phosphatases,[113] and several kinases, including members of the Janus and mitogen-activated protein kinase cascades.[69;114;115]

NO· modulates the expression of several genes involved in inflammation, healing and repair, however the mechanisms are poorly understood and a NO· responsive promoter element has not yet been identified.[36] Apart from modulation of redox sensitive transcription factors like AP-1 and NF-κB, NO· or RNS may modulate gene transcription by disruption of zinc finger-type transcription factors.[116] NO· may scavenge other radical species, thus inhibiting activation of NF-κB.[36] This is the case for NO· modulation of monocyte chemoattractant protein 1[117] and macrophage colony stimulating factor 1.[118] Vascular cell adhesion molecule 1expression is modulated by NO· mediated increases in the expression of the inhibitor IκBα.[119] Expression of the chemokines IL-8 and macrophage inflammatory protein 1α, as well as TNF-α appear to be induced by NO· mediated control of gene transcription.[120-122] In the

case of TNF-α formation, nitrosative stress influences the signal transduction pathway leading to gene expression. Nitrosylation of a cys residue on p21 Ras in lymphocytes is critical for guanosine nucleotide exchange and signaling that results in TNF-α formation.[109] Expression of matrix metalloproteinase, extracellular matrix protein, and growth factor genes, including vascular endothelial growth factor are also modulated by NO· or RNS. [123-125]

Oxidative and nitrosative stress may lead to apoptosis. In macrophages, apoptosis secondary to expression of iNOS or exogenously generated NO· follows accumulation of p53, due to a decreased rate of degradation.[126] Accumulation of p53 likely reflects DNA damage due to nitrosative and oxidative cellular stress. Caspaces are activated following elevation of p53, however NO· mediated nitrosylation of caspase proteins prevents their activation.[127] Although NO· is a potent inducer of apoptosis in macrophages,[127] in many other cell types, including lymphocytes, B-cells, eosinophils, endothelial cells and hepatocytes, NO· protects against apoptosis.[128] These differences may be due to the quantity of NO· present. Anti-apoptotic effects of NO· are observed at concentrations only 2-10% of those that initiate apoptosis experimentally.[129]

Lipid peroxidation is a critical player in oxidant mediated cell injury, and peroxynitrite is known to initiate lipid peroxidation.[69] The unpaired radical in NO· reacts rapidly with alkoxyl (RO·) and alkyl hydroperoxyl (ROO·) radicals at diffusion limited rates.[130] NO· acts to terminate rather than propagate lipid peroxidation chain reactions by a mechanism that remains undefined.[131]

Nitric Oxide in Health and Disease

The preceding sections provided an overview of NO· enzymology, chemistry and molecular mechanisms of action. In this section, the relevance of nitric oxide to biological processes at the organ level are considered in health and in disease. Much of what we know about the physiologic relevance of NO· production stems from studies in which the actions of NOS are blocked by non-hydrolyzable L-arginine analogs. Because L-arginine analog inhibitors specific for individual NOS isoforms are not available, much of the most definitive information assessing the role of individual NOS isoforms derives from studies using mice in which specific isoforms have been knocked out by homologous recombination. Although NOS isoforms are expressed in a wide variety of cell types, this overview will be delineated, in general, by examples of processes involving the individual NOS isoforms.

Nitric Oxide in the Nervous System

NO· mediated-processes in the Central nervous system (CNS) were first discovered in the late 1980s. Garthwaite et. al. demonstrated that ligation of N-methyl-D-Aspartate (NMDA) receptors in brain tissue explants by the excitatory amino acid glutamate, resulted in elaboration of a mediator with the physical characteristics of NO·.[132] This mediator was capable of raising [cGMP] in cerebellar cells that did not themselves respond to excitatory amino acids and also induced relaxation of smooth muscle.[132] Additionally, stimulation of non-adrenergic, non-cholinergic autonomic neurons led to the generation of a labile

mediator that stimulated cGMP formation, relaxed smooth muscle and whose synthesis could be blocked by N^{ω}-substituted L-arginine analogs.[133] These findings ultimately lead to the identification of NO· as a neurotransmitter and cloning of the ncNOS isoform.[5]

ncNOS serves major signaling roles not only in the CNS, but also in the autonomic nervous system and in skeletal muscle and has demonstrated or hypothesized roles in genetic, inflammatory and degenerative diseases.

NO· produced by ncNOS in the peripheral nervous system controls gastrointestinal motility, neuroendocrine secretory function, and regional blood flow through release at autonomic nonadrenergic, noncholinergic postganglionic nerves terminals.[134] Action potentials in these myenteric nerves are mediated by voltage-dependent calcium channels resulting in elevation of cytoplasmic [Ca^{2+}] which in turn activates ncNOS.[135] NO· produced at the nerve terminus diffuses into adjacent smooth muscle cells where it activates GC resulting in cyclic GMP accumulation which in turn results in smooth muscle relaxation.[14] ncNOS knockout mice have markedly enlarged stomachs[136]that closely resemble a similar congenital condition in man referred to as infantile hypertrophic pyloric stenosis.[137] It is tempting to speculate that a similar pathogenesis may be responsible for the idiopathic pyloric muscular hypertrophy occasionally encountered in young canines.

NO· mediates penile erection by altering penile blood flow. This action is mediated by ncNOS present in nerves of the pelvic plexus[138] and can be blocked

by NOS inhibitors.[139] In intact animals, NOS inhibitors block penile erection, while ncNOS knockout mice retain normal erectile function, presumably due to actions of other NOS isoforms.[140] This hypothesis is supported by the observation that administration of NOS inhibitors also inhibits penile erection in ncNOS knockout mice.[140]

In skeletal muscle, ncNOSμ mediates myotube fusion during development,[141] contractile force in fast-twitch rodent myofibers,[142] and exercise-induced, but not insulin-dependent glucose uptake.[143] Deranged signaling by NO· may contribute to muscle pathology in Becker and Duchenne muscular dystrophy.[53;144]

In the CNS, ncNOS is activated by calcium influx at the synapse mediated by ligation of the NMDA receptor by the excitatory amino acid mediator, glutamate.[132] NO· then diffuses in a retrograde fashion to modulate neurotransmitter release at the presynaptic terminus.[145] In the CNS, NO· appears to mediate synaptic plasticity, particularly long-term potentiation in the hippocampus[146] and long-term depression in the cerebellum.[147] NMDA receptors are critically involved in learning and memory, suggesting a physiologic role for NO· in synaptic plasticity.[55] ncNOS appears to regulate cerebral blood flow. ncNOS occurs in high levels in large cerebral blood vessels[148] and the local increase in blood flow associated with neuronal activity is inhibited by NOS inhibitors.[149]

Long-term quantitative changes in ncNOS content in neural tissue follows changes in gene transcription. Tissue injury results in elevated ncNOS protein expression in nociceptive nerves and ncNOS expression appears to mediate prolonged pain sensations associated with the injury.[150] ncNOS induced by injury to the nervous system may itself produce further injury. ncNOS induction in motor neurons precedes cell death following rootlet avulsion and cell death can be prevented by NOS inhibitors.[151]

Excitotoxic damage may result from excess NO· release at synapses secondary to glutamate binding of NMDA receptors; this pathogenesis is important in stroke. Brain parenchymal NO· production is markedly increased following transient ligation of the middle cerebral artery.[152] NOS inhibition decreases stroke volume *in vivo*,[153] and prevents neuronal cell toxicity in response to glutamate *in vitro*.[154] All three NOS isoforms can be expressed in brain tissue, however only the constitutive isoforms are normally present. The relative roles of these isoforms in CNS damage in animal models of stroke are illustrated by findings with knockout mice. Following transient ischemia produced by ligation of the middle cerebral artery, ncNOS knockout mice have 38% smaller infarct volumes than wild type mice, but display similar post-ischemic blood flow patterns.[155] ecNOS knockout mice have decreased blood flow peripheral to the focus of infarction and demonstrate increased infarct volume.[156] iNOS protein is detectable in glia and infiltrating neutrophils within 24 hours of a focal ischemic event, peaking at 48 hours, thus iNOS contributes to damage in the late reperfusion period.[157] Infarct volume is 28% smaller in iNOS deficient mice than

wild type mice.[158] Cu/Zn SOD inactivates $O_2^{\cdot -}$, thus preventing the formation of peroxynitrite. Transgenic mice that overexpress Cu/Zn SOD have reduced infarct volumes compared with wild type mice following cerebral ischemia.[159] These mice bred with ncNOS knockout mice are even more resistant to cerebral infarction than the parent strains.[160]

Apart from stroke, NO· also play roles in other degenerative and inflammatory disease of the CNS.

NADPH-diaphorase positive staining neurons (NOS containing) are spared in the striatum of patients with Huntington's disease, while neurons that do not contain NOS undergo degeneration.[134] These neurons have high cytoplasmic levels of superoxide dismutase, apparently providing resistance to RNS-mediated cytotoxicity.[161] Three-nitrotyrosine immunoactivity (a marker of peroxynitrite oxidative activity) is prominent in affected striatal neurons in animal models of this disease, suggesting a role for RNS mediated damage.[162]

MPTP (l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) contaminated batches of illicit drugs in the 1970's producing a Parkinson's like disease in those exposed.[134] MPTP induced animal models of Parkinson's disease are characterized by accumulation of 3-nitrotyrosine and loss of neurons in the nigrostriatial pathway.[163] Both neuronal loss and 3-nitrotyrosine accumulation are prevented by inhibition of NOS.[163] Furthermore, Cu/Zn overexpressing transgenic mice and nNOS knockout mice do not develop nigrostriatial lesions

after exposure to MPTP providing evidence for NOS mediated damage in this disease model.[164]

iNOS may be expressed in the CNS in response to infectious agents. iNOS induction in the CNS functions as an important component of the non-specific immune response to *T. gondii* infection. iNOS knockout mice cannot limit *T. gondii* infection of the CNS following peripheral inoculation.[165] Although this role is protective, iNOS expression in the brain is also associated with cytotoxicity. High levels of iNOS are present in astrocytes at demyelinating lesions in patients with multiple sclerosis[166] and in glial cells of patients with AIDS dementia.[26] HIV gp41 protein induces iNOS expression in neuron cell cultures and expression results in cell death that can be blocked by NOS inhibitors.[26]

Nitric Oxide in the Vascular System

The vasoactive properties of nitroglycerin have been known and exploited therapeutically since the time of Pasteur,[55] however the physiologic basis for nitrovasodilation was not revealed until the late 1980s.[80] In 1980, Furchgott et. al. demonstrated that agonist-stimulated endothelial cells produce a labile factor that produced vasodilation.[167] This mediator was named endothelium derived relaxing factor (EDRF) and was definitively identified as NO· in 1987.[168;169] Isolation and cloning of ecNOS followed.[6;7]

Regulation of vascular tone was the first defined role of ecNOS and remains this isoform's major known physiologic function. NO· mediates basal resting vascular tone. Inhibition of nitric oxide synthase utilizing non-

hydrolyzable analogues of L-arginine dramatically increases mean arterial blood pressure in normal subjects.[170] Knockout mice that lack ecNOS are hypertensive and have markedly depressed endothelium dependent vasodilator responses to acetylcholine.[171] Transgenic mice that overexpress bovine ecNOS have significantly lower blood pressures, higher plasma nitrate levels, and also exhibit reduced vasodilator responses, perhaps due to saturation of GC by NO·.[172] ecNOS expression in endothelial cells *in vitro* is modulated by shear stress,[173] cyclic strain,[174] oxidized low density lipoproteins,[175] and cyclic GMP analogues.[176] Chronic exercise training in dogs upregulates endothelial ecNOS expression as well as vascular responsiveness to nitrogenous vasodilators.[177] ecNOS is downregulated by stimuli including hypoxia, TNF alpha, high concentrations of oxidized low density lipoproteins and in endothelial cells overlying atheromatous plaques.[178;179]

NO· produced by ecNOS modulates responses to other reactive oxygen species. Vascular endothelial cells can produce superoxide via xanthine oxidase,[180] as well as via a membrane bound NADH/NADPH oxidase that resembles the neutrophil enzyme.[181] Oxidative radical production by endothelial cells is modulated by angiotensin II,[182] cytokines,[86] chronic nitro-vasodilator therapy,[183] and hypercholesterolemia.[180] In comparison to neutrophils, endothelial cells produce only small quantities of superoxide,[86] however alterations in endothelial $O_2\cdot^-$ and NO· production may underlie some of the vasomotor dysregulation that occurs in vascular diseases such as, atherosclerosis, diabetes, and hypertension.[180;184;185]

Endogenous NO· appears to play a major role in protecting the microvasculature from injury. Beneficial effects of NO· donors and the deleterious effects NOS inhibition are revealed in models of ischemia/reperfusion injury.[186-189] The vasculoprotective effects of NO· appear largely due to modulation of leukocyte adhesion. Nitrosothiols appear to be intimately involved in regulation of leukocyte adhesion to the endothelium. Exogenous NO· donors inhibit neutrophil adhesion, *in vitro*.[190] Adhesion of white blood cells is mediated by critical thiol groups and nitrosation of these groups may abolish adhesion.[190] In inflammatory settings, $O_2\cdot^-$ may limit formation of endogenous nitrosothiols by NO·, including those on cellular adhesins, thus promoting leukocyte adhesion.[191] Endogenously produced NO· from both cNOS isoforms, but from ecNOS in particular, limits leukocyte rolling and adhesion by depressing adhesion molecule expression[76;192]. P-selectin and VCAM-1expression are both down-regulated by NO·. VCAM-1 by NO· mediated inhibition of NF-κB activation[193] and P-selectin by an undetermined mechanism.[192] NO· also inhibits platelet adhesion and aggregation[75], limits cytokine-dependent endothelial cell activation[193] inhibits vascular smooth muscle proliferation[194] and regulates microvascular permeability mediated by both leukocyte-dependent and leukocyte-independent pathways.[195] Some of these effects may be due to scavenging and deactivation of reactive oxygen species by NO·.[97]

iNOS is expressed in endothelial cells as part of the host defense response. High output production of NO· by this isoform contributes to vascular

and tissue damage and is important in the pathophysiology of septic shock.[42] This topic is addressed in the following section.

Inducible Nitric Oxide Synthase in Host Defense

The antibacterial activities of nitrogen-oxygen containing ionic species such as nitrites, nitrates, and their derivatives have been known for centuries and are commonly applied in the practice of curing of meats.[196] The first indication that nitrogen oxides might be endogenously generated in host defense reactions was the observation by Prout in 1818 that a febrile patient excreted large amounts of nitrate in the urine.[197] This finding was followed, 163 years later, by the observation that germ-free rats excreted more nitrate in their urine than they consumed[198] and the quantity excreted could be increased by exposure to agents that induced an inflammatory response.[199] Endotoxin-activated macrophages were subsequently identified as a source of nitrogen oxides[200] and L-arginine was identified as the amino acid that supported their synthesis and contributed to macrophage cytostatic activity.[201] This work was followed by the demonstration that endotoxin-stimulated macrophages elaborate nitric oxide which produces cytostasis by a mechanism resulting in target cell energy and iron depletion.[202] Isolation and cloning of the iNOS isoform was achieved in 1992.[4]

iNOS expression secondary to microbial products and cytokines is an important component of inflammatory and immune responses bridging both innate and adaptive responses. Hepatocytes, macrophages, vascular smooth

muscle and endothelial cells are cell types that express iNOS prominently in immune responses, but iNOS can be expressed in cells representing all branches of the histogenic tree.

Several lines of investigation suggest the role and importance of iNOS expression in infectious disease.

First, iNOS expression is associated with infectious disease. iNOS is induced by bacterial products and cytokines expressed as part of the response to infection.[96] Infected subjects have elevated blood and urinary nitrate and nitrite levels indicating accentuated NO· production[203;204] and display evidence of increased nitrosative tissue stress.[205] Second, iNOS and production of NO· can be demonstrated in tissues at infected sites.[205-209] Third, activated phagocytic cells exert cytostatic or cytotoxic effects against a variety of microbial targets in an L-arginine dependent fashion, *in vitro*.[94;210-212] Fourth, *in vivo* inhibition of iNOS results in recrudescence of previously silent infections and dramatic increases in tissue burden of some infectious agents.[206;213;214] Fifth, *in vivo* inhibition of NOS ameliorates the microbicidal effect of NOS expression.[215] Sixth, compounds that spontaneously donate NO· have microbicidal effects against a range of pathogens including protozoa, fungi, bacteria, and viruses,[216] and finally studies using iNOS knockout mice reveal iNOS expression is essential for, or contributes to, the control of some infectious agents.[213;217]

NO· mediated cytostatic or cytotoxic activities have been demonstrated against numerous taxonomically diverse infectious agents *in vitro* or *in vivo*.

Susceptible eukaryotic agents include organisms in the genera *Entamoeba,*[218] *Leishmania,*[219] *Naegleria,*[220] *Plasmodium,*[221] *Schistosoma,*[222] *Toxoplasma,*[165;223] *Candida,*[224] *Cryptococcus,*[225] *Histoplasma,*[226] and *Pneumocystis*[227]. Prokaryotes susceptible to NO· mediated cytostatic/toxic activities include both Gram positive and negative bacteria and Rickettsiae. This list includes; *Brucella,*[228] *Ehrlichia,*[229] *Escherichia,*[211;230] *Francisella,*[231] *Legionella,*[232] *Listeria,*[233;234] *Mycobacteria,*[213] *Rickettsia,*[235] *Salmonella,*[212] and *Staphylococcus*[236] species. Viral infectious agents susceptible to replication inhibition by NO· include: Coxsackie,[208] Ectromelia,[180;237] Epstein-Barr,[238] Herpes simplex,[239] and Vaccinia.[240]

NO· mediated microbiostatic and microbiocidal mechanisms are poorly characterized, but are thought to be due to interaction of NO· and RNS with many of the molecular targets previously described, including heme and iron sulfur containing proteins, cys and tyr groups, membranes and DNA.[96;241] Although mechanistic details are sketchy, *in vivo* studies using a variety of infectious agents reveal iNOS expression has both positive and negative effects in host defense and, in some settings may be irrelevant.

The iNOS gene has been designated as an innate resistance locus for *Mycobacterium tuberculosis* and Ectromelia virus in mice and appears to be essential for control of *Leishmania* species.

Mycobacterium tuberculosis replication is controlled by activated macrophages by an L-arginine-dependent mechanism, *in vitro.*[242] Progression of this infection is accelerated by NOS inhibition.[213] Mice in which genes for CD8 T

cell development, T cell receptors, IFN-γ, or receptors for IFN-γ and TNF-α receptor have been disrupted are no more susceptible than iNOS knockout mice.[243] Mice with severe combined immunodeficiency have a small degree of resistance to *M. tuberculosis* which can be abolished by gluocorticoid treatment, which also inhibits iNOS, but glucocorticoid treatment of iNOS knockout mice does not further increase their susceptibility to this agent.[243] Because all of the gene disruptions described are involved in initiation of iNOS expression and because iNOS expression is inhibited by glucocorticoids, iNOS is considered to be an innate monogenetic resistance locus to *M. tuberculosis*.[217] Ectromelia virus is also controlled by iNOS.[237] iNOS knockout mice have increased susceptibility to this agent without evidence of depression of other characterized anti-ectromelia immune responses.[237] These findings suggest iNOS is an innate resistance locus to Ectromelia in mice.

Leishmania species are also controlled by macrophages in an L-arginine-dependent fashion.[219] INOS deficient mice are remarkably susceptible to *Leishmania* infection and reveal roles for iNOS in both innate and adaptive responses. During early infection, iNOS is expressed as part of the innate immune response mediated by the type 1 interferons, IFN-α and -β. At this stage, prior to dissemination of the parasite, iNOS expression is essential for NK cell responsiveness to IL-12, for release of IFN-γ by NK cells, and IFN-γ dependent suppression of the iNOS expression inhibitor, TGF-β.[19;20] Later in the

disease course iNOS induction via Il-12 stimulated, IFN-γ secreting, CD4+ T cells is required for microbicidal activity against parasites.[19;20]

In contrast to the essential expression of iNOS for controlling *M. tuberculosis*, *Lesihmania* spp. and Ectromelia virus, iNOS expression is an important component, but not the sole contributor to control of *Toxoplasma gondii* and *Listeria monocytogenes.*

iNOS expression is essential for control of cerebral infection by *Toxoplasma gondii*, but the presence or absence of iNOS in other body compartments has no influence on parasite burden. This is true even though explanted murine peritoneal macrophages require iNOS expression for parasite killing.[165] In this disease, iNOS appears to mediate a tissue-specific role in host defense. INOS deficient mice display increased susceptibility to infection by *Listeria monocytogenes.*[233] Mice which are deficient in the transcription factor interferon consensus binding protein, are even more susceptible, yet these mice are capable of wild type levels of iNOS expression, indicating iNOS expression is an important component of host defense against this agent, but is not the sole determinant of resistance.[244]

The inability of knockout mice to express iNOS *in vivo* appears to be irrelevant to control of Legionella *pneumophila*, *Trypanosoma cruzi*, generative stages of several *Plasmodium* species, *Pseudomonas aeruginosa*, and *Chlamydia trachomatis.*[82] *Legionella pneumophila* growth in alveolar macrophages is restricted by NO· *in vitro*, however activated macrophages also

can kill this organism by an IFN-γ dependent, but NO· independent mechanism.[245] Similarly, NO· can kill *Plasmodium* species *in vitro*, while control *in vivo* is dependent on IFN-γ, but not iNOS.[246]

Expression of iNOS results in detrimental effects in infections caused by Influenza A virus, Herpes simplex, and *Mycobacterium avium*. Intranasal inoculation of influenza A virus produces morbidity and mortality by inciting a pulmonary interstitial inflammatory response.[247] iNOS deficiency markedly improves survival in this infection, probably because of a reduced inflammatory response.[247] Similar findings are reported in mice infected with Herpes simplex virus. Inhibition of NOS, results in an increased viral titer in the lung, but markedly reduced mortality and decreased the histologic severity of pneumonia.[205] Again, it appears that iNOS produces disease primarily by accentuating the inflammatory response. iNOS deficient mice infected with *M. avium* have similar bacterial loads in visceral organs as do wild-type mice, but differ in having normal splenic lymphocyte mitogenic responses as opposed to the depressed response characteristic of wild type mice. The primary effect of iNOS expression in this disease appears to be induction of immunosuppression.[248] These findings reveal that iNOS is essential for control of some agents, is an important, but not sole contributor to control of others, is ineffective against another group of pathogens, and contributes to host impairment in yet another. As might be expected, iNOS also has complex roles in inflammatory diseases. Shock and the attendant pulmonary injury are examples.

Sepsis is defined as the systemic response to infection. Sepsis is characterized by tachycardia, high cardiac output, low systemic vascular resistance, hypoxemia, oliguria and lactic acidosis.[249] This hyperdynamic state often progresses to hypotension with vasoplegia and multiple organ failure.[249] Sepsis is induced by bacterial products including endotoxin from Gram negative bacteria and peptidoglycan and lipotiechoic acids from Gram positive bacteria.[249;250] These bacterial products signal production of cytokines by interaction with lipopolysaccharide binding protein and CD 14 which transmit signals via the toll-like receptors.[251] This results in expression of IFN-γ, Il-1β, TNF-α and Il-6 and subsequent expression of iNOS in most organs in the body, including marked upregulation in endothelial and vascular smooth muscle cells.[204] Systemic high output NO· production contributes to systemic vasodilation, microvascular injury, and inhibition of cellular energy metabolism, all of which conspire to produce mortality.[42]

The role of high output NO· production by iNOS in the pathogenesis of shock is revealed by the ability of L-arginine analog inhibitors of iNOS to restore blood pressure and vasopressor responsiveness in animal models of shock.[252] Additionally iNOS knockout mice maintain blood pressure and survive an iv dose of LPS which is lethal in wild type mice.[233] However iNOS expression is just one of the factors that leads to mortality in septic shock and in clinical trials, inhibition of NOS has not resulted in improved survival.[42;253]

In man, the lung is the most common site of infection leading to sepsis and pulmonary injury is a common and clinically important sequelae of shock.[254] iNOS plays important roles in modulating inflammatory and immune responses in the lung.

Shock induced by hemorrhage and resuscitation is characterized by pulmonary injury secondary to neutrophil influx, which in turn follows activation of NF-κB and Stat-3 transcription factors. Activity of these factors is markedly decreased in iNOS knockout mice indicating iNOS modulates the pulmonary inflammatory response in this model.[255] iNOS deficient mice have decreased eosinophil infiltration and pulmonary damage in a model of experimental allergic airway disease induced by aerosolized ovalbumin.[256] In this model, iNOS appears to promote airway inflammation by down-regulating IFN-γ production by T cells.

Endotoxin induced lung injury is characterized by neutrophil infiltration, procoagulant activity, elaboration of inflammatory mediators, loss of compliance and vascular leakiness leading to edema. Inhibition of iNOS decreases damage in animal models of LPS-induced pulmonary injury.[257] However the role of iNOS is unclear. LPS-dependent lung injury measured by lung weight and enzyme release was markedly reduced in iNOS deficient mice,[258] suggesting iNOS promotes LPS-dependent pulmonary inflammation, while in another study iNOS knockout mice had exaggerated pulmonary neutrophil influx apparently due to an anti-adhesive change in the wild type leukocytes mediated by NO·, suggesting

iNOS protects the lung from LPS-induced neutrophil influx.[259] Dichotomous results of this type are common in experiments designed to assess the role of NO· in inflammatory disease.

Summary

This introductory chapter provides a broad overview of the breadth and scope of biologic processes in which the NOS enzyme family and reactive nitrogen oxides play a role. We now turn our attention to an assessment of the potential role of iNOS expression by alveolar macrophages in defense of the lower respiratory tract of cattle. In chapter 2, iNOS expression and NO· production by bovine alveolar macrophages is characterized, while in chapters 3 and 4 findings of experiments designed to assess the potential role of NO· in infectious bovine respiratory disease are presented. In chapter 5, this work is placed in perspective and future studies are suggested.

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PART 2

Characterization of Nitric Oxide Production by Bovine Alveolar Macrophages

Abstract

Alveolar macrophages play a central role in defense of the lower respiratory tract. Production of the reactive intermediate nitric oxide, via expression of inducible nitric oxide synthase, is an important microbicidal effector mechanism possessed by macrophages. In this study, cytokine regulation of expression of iNOS mRNA and protein and NO· production by bovine alveolar macrophages was evaluated. Bovine alveolar macrophages were exposed to one or more of the following: recombinant human (rh) and recombinant bovine (rb) IFN-γ, rbIFN-α, rh- & rbIL-1β, rbGM-CSF, rhTNF-α, rhIL-4, endotoxin (LPS), fetal bovine serum (FBS), mitogen-stimulated bovine splenic supernatant (SS), and purified human TGFβ-1. LPS alone, or in combination with SS, rbIFN-γ, or rbIL-1β stimulated production of NO· in a time and dose-dependent fashion. Recombinant bovine IFN-γ, rbIL-1β, and rhTNF-α in combination produced maximal stimulation which was not further enhanced by the addition of LPS. Recombinant human IFN-γ, rhIL-1β, rbGM-CSF and rbIFN-α had minimal effect either as single stimuli, or in combination with LPS, rbIFN-γ, rbIL-1β, or rhTNF-α. Nitric oxide production was inhibited by rhIL-4, and the L-arginine analogue antagonists of iNOS, N^G-monomethyl-L-arginine (N^GMMA) and aminoguanidine (AG). Purified human TGFβ-1 did not inhibit NO production. Messenger RNA for iNOS was maximally expressed by 8 hours and remained detectable for at least 48 hours. iNOS protein expression was demonstrated by western blot and

NADPH diaphorase histochemistry. Expression of iNOS mRNA and protein induced by cytokines and LPS varied with strength of the stimulus as determined by nitrite production in culture supernatant.

Introduction

The respiratory tract presents a broad tissue interface between the internal environment of the body and the external environment. This interface is continuously exposed to a variety of potentially harmful substances and organisms present in inspired air. Fortunately, the threat from the vast majority of inhaled foreign material is negated by both specific and non-specific mechanisms in the upper respiratory tract. Turbulent airflow and a sticky mucus coat in the upper respiratory tract trap particulate matter with dimensions greater than 2 μm. Entrapped particulate material is transported out of the respiratory tract by the coordinated beating action of cilia on the surface of epithelial cells lining the upper respiratory tract. This material is expectorated and swallowed, however if the volume is great enough, forcible expulsion occurs by coughing. The majority of adaptive immunologic responses that occur in the respiratory tract occur in the upper airways.[1] The sol and mucus coat lining the upper respiratory tract contain secreted antibodies as well as non-specific antimicrobial factors, such as lactoferrin, lysozyme, and tracheal antimicrobial peptide.[1;2]

By far the largest surface area presented by the respiratory tree is in the lower respiratory tract, which is also the thinnest segment anatomically. At this site the inspired air is separated from the bloodstream by a thin film of alveolar fluid, a flattened type 1 pneumocyte, a basement membrane and a flattened endothelial cell. Maintenance of the structural integrity of the alveolar duct and alveolus is essential for life and the alveolar macrophage plays a critical role in defense of this structure; acting in both innate and adaptive immune responses.[1;3;4] Alveolar macrophages can phagocytose, kill and digest microbes, present antigen in the context of MHC I and/or II, promote fibrin elaboration, secrete hydrolytic enzymes, synthesize and express protein and lipid mediators including pro- and anti-inflammatory cytokines, arachidonic acid metabolites, chemokines, and growth factors, and produce nitrogen and oxygen based radicals.[4;5] These responses are oriented toward host survival, but may in themselves contribute to host damage.

In order for alveolar macrophages to elaborate a defensive response, the presence of a pathogen must first be perceived. Microbial signals sensed by macrophages are often products of pathogen exterior cell wall or coat. These include lipopolysaccharide (LPS, endotoxin) from the outer coat of Gram negative bacteria, lipoarabinomannans from Mycobacteria, and peptidoglycans and lipotheichoic acids from Gram positive bacteria.[6-8] Responsiveness of macrophages to these microbial components is mediated and enhanced by

specific proteins and surface receptors, including soluble and surface bound CD 14, lipopolysaccharide binding protein, and the toll-like receptors.[9-11] Pathogen recognition is also aided by opsonization with complement, antibody or surfactant. Surfactants produced by type II cells are important in antibody-independent defense of the lower respiratory tract.[12] Surfactants can agglutinate pathogens, including virus, bacteria and fungi via Ca^{2+}-dependent binding of their C-type lectin domains to surface carbohydrates on these organisms, enhance phagocytosis by interaction with phagocyte surface surfactant receptors, and initiate cytokine synthesis via interaction with LPS and CD14[12;13] Macrophages that sense microbial products by these pathways become "activated" with enhanced antimicrobial effector functions and changes in gene expression leading to synthesis of iNOS and cytokines.[4;6-8;12]

Microbial products, xenobiotics, aeroallergens and immune complexes induce expression of both pro- and anti-inflammatory cytokines by macrophages, T and NK cells in the respiratory tract.[3;4] Among the many cytokines involved, TNF-α, IL-1β, chemokines, and IFN-γ regulate leukocyte chemoattraction and activation, while IL-12 enhances inflammatory reactions by inducing expression of TNF-α and IFN-γ. Il-4 and -10 act to down regulate inflammatory reactions by directly inhibiting leukocyte antimicrobial function and inhibiting pro-inflammatory cytokine expression.[3;4] These actions are probably designed to limit excessive inflammation, but may impair the host defense response.[3;4]

The importance of these mediators in pulmonary inflammatory or immune responses is revealed by numerous studies that demonstrate both positive and negative effects of cytokine expression on survival, pulmonary pathogen clearance, and lung injury. The contributions of individual cytokines are experimentally delineated by strategies that include inhibition of cytokine action by application of antibodies specific for that cytokine or its receptor or through removal of components of the receptor, the signal transduction pathway, or the cytokine itself via homologous recombination. Additionally, in some experiments, the cytokine is overexpressed by supplemental administration or overexpression via a transgene. The following paragraphs reveal evidence for significant roles of several cytokines in defense of the respiratory tract to infection.

TNF-α and Il-1β are potent inducers of leukocyte activation. These cytokines activate both neutrophils and macrophages, leading to enzyme secretion, stimulation of the respiratory burst and enhanced phagocytosis and killing[14]. TNF-α and Il-1β enhance recruitment of leukocytes by enhancing expression of adhesive molecules on endothelial cells and phagocytes and induce expression of chemokines via NF-κB[14;15]. TNF-α and Il-1β are expressed in pneumonic lung and inhibition of TNF-α or overexpression of Il-1β receptor antagonist impair pathogen clearance.[15-17]

IFN-γ is produced primarily by T cells and NK cells and is instrumental in cell mediated immunity against a wide range of pulmonary pathogens. IFN-γ

activates alveolar macrophage effector functions including stimulation of the respiratory burst, iNOS expression, priming for TNF-α secretion, enhanced antigen presenting ability, and enhancement of microbial killing.[18] IFN-γ depletion reduces pulmonary clearance of bacteria and fungi.[18] IFN-γ knockout mice have enhanced susceptibility to bacterial pneumonia, while overexpression of IFN-γ, via an adenoviral vector, increased resistance to bacterial pneumonia in mice.[19;20]

The colony stimulating factor GM-CSF is produced by myeloid and stromal cells and promotes proliferation and maturation of both neutrophils and macrophages, but has prominent activating activity only for macrophages.[21] GM-CSF enhances production of TNF-α in LPS-stimulated macrophages and enhances macrophage-dependent killing of *Cryptococcus neoformans*, *in vitro*.[21;22] Parenteral administration of GM-CSF reduced the severity of *Pneumocystis carinii* pneumonia in mice.[22]

Interleukin 12 is a heterodimer that is the major inducer of IFN-γ expression by T cells and NK cells.[23] Il-12 promotes Th1 responsiveness and depresses Th2 responses and is an important resistance factor to infection by pathogens, particularly intracellular organisms.[23] IL-12 administration enhances resistance against pulmonary fungal and bacterial infections and inhibition of Il-12 decreases survival in bacterial pneumonia.[24;25]

Il-4 and Il-10 oppose the actions of IFN-γ and Il-12 by promoting Th2

responsiveness, inhibiting Th1 responses and deactivating phagocytes by downregulating expression of TNF-α, Il-1β, IFN-γ, and chemokines.[18;26;27] Additionally, Il-4 stimulates expression of Il-1β receptor antagonist.[27] These cytokines appear to be critical in limiting the development of inflammation and may act to prevent systemic proinflammatory cytokine over production and the sepsis syndrome.[26;28] Il-10 expression may be detrimental to the host in infections such as pneumonia where a vigorous inflammatory response is required for pathogen clearance.[29] Il-10 is expressed early in bacterial pneumonia and inhibition of Il-10 enhances bacterial clearance from the lung, reduces hematogenous dissemination, increases TNF-α and IFN-γ expression and enhances survival.[30] Administration of Il-10 in bacterial pneumonia significantly impairs pulmonary bacterial clearance and increases mortality.[29]

Chemokines are cytokines that chemoattract and activate white blood cells. The chemokines are divided into C-C (α) and C-X-C (β) groups. In general, C-C chemokines are chemotactic for neutrophils, while the C-X-C group are chemotactic for macrophages and other white blood cells. Interleukin-8, which chemoattracts neutrophils, and monocyte chemoattractant protein (MCP) are examples of C-C and C-X-C family members.[3] Chemokines are expressed in pneumonic lung and inhibition of chemokines markedly reduces inflammatory cell influx and diminishes survival.[31]

Interleukin-6 is produced by many cell types and is involved in the acute

phase response, B cell differentiation, antibody production, and control of acute local and systemic inflammatory reactions.[18;32] Il-6 is produced locally in pneumonic lung tissue and Il-6 knockout mice demonstrate increased susceptibility to bacterial pneumonia and septicemia.[33;34]

Several of the microbial products and cytokines shown to be important in pulmonary defense against infectious agents or regulation of pulmonary inflammation are also recognized as stimuli that induce or regulate iNOS expression in macrophages.[35-38] iNOS expression and NO· production are known to be important for protection of rodent species against a variety of infectious agents, including primary pulmonary pathogens. iNOS is efficiently induced in rodent macrophages by IFN-γ and LPS, however stimuli necessary for iNOS expression vary among species and cell types.[37;39-41] Several of the stimuli discussed above, including LPS, Il-1β, TNF-α and IFN-γ are known to be present in bovine pneumonic lung tissue.[42-46] In order to assess the potential role of iNOS expression and NO· production in defense of the bovine lower respiratory tract, stimuli that regulate iNOS expression in bovine alveolar macrophages must be characterized. That is the aim of the material presented in this part.

Materials and Methods

Isolation of Alveolar Macrophages

Bovine alveolar macrophages were collected from fresh lungs obtained at an abattoir. Lungs from slaughter cattle were inspected and those free of gross pathology were chosen for lavage. Lungs were lavaged with 1-3 liters of Hanks balanced salt solution (HBSS) without calcium, pH 7.25 (GIBCO, Grand Island,NY). Lavage fluids were filtered through sterile gauze and spun at 200 *x*g. for 8 minutes at 4 C°. Cell pellets were gently resuspended and washed twice with 50 ml ice cold HBSS. They were then resuspended in Dulbecco's Minimal Essential Medium (DMEM) (Whittaker Bioproducts, Walkersville,MD) and enumerated with a hemocytometer. Lavage isolates yielding greater than 90 % macrophages (Wright-Giemsa stained cytocentrifuge preparations) with greater than 90% viability (trypan blue dye exclusion) were used for studies.

Culture of Alveolar Macrophages

Bovine alveolar macrophages were cultured at $1x10^6$ cells/ml ($5x10^5$ cells/well) in 48 well flat bottom polystyrene plates (Costar, Cambridge, MA). Culture media was DMEM with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml fungizone, and 2mM L-glutamine (all from Whittaker Bioproducts). Cells were incubated with various concentrations of LPS (E. coli O55:B5, Sigma, St. Louis, MO); rhTNF-α, rhIFN-γ, & rhIL-1β (R&D Systems, Minneapolis, MN); rbIFN-α,

rbIFN-γ, & rbGM-CSF (Ciba-Geigy, Basel, Switzerland); rbIL-1β (American Cyanamid, Princeton, NJ); 100 ng rhIL-4/ml (R&D Systems); 50 ng TGF-β/ml (R&D Systems); 2% fetal bovine serum (Hyclone, Logan, UT); and the NOS inhibitors; 2 mM N^G-monomethyl-L-arginine (N^GMMA; Calbiochem, San Diego, CA); or 2mM Aminoguanidine (AG; Sigma).

Five percent mitogen-conditioned splenic supernatant was used as a source of bovine cytokines in some experiments. Splenic supernatant was derived from mitogen stimulated bovine splenocyte preparations. Splenocytes were isolated by grinding fresh bovine spleen between sterile glass slides, followed by hypotonic lysis of red blood cells, and two centrifuge washes with ice cold HBSS. Splenocytes were incubated at $5x10^6$/ml in 75 cm^2 flasks (Costar) with 5% FBS and 5 μg/ml phytohemagglutinin (Sigma) for 4 hours, followed by washing of the cells three times in HBSS, 48 hours of additional culture, and harvest of cell free supernatant by centrifugation.

Macrophage cultures were incubated at 37^{0}C in the presence of 5% CO_2 for 24, 48, and 72 hours. Cell free supernatants were collected after centrifugation (10 minutes @ 300xg), and were assayed for nitrite immediately or frozen at -20°C until assay.

Nitrite Assays

Nitric oxide production was estimated by measuring nitrite in cell free supernatants by the Griess reaction.[47] In this assay, nitrite reacts with the Griess

reagent to form a purple dye. Briefly, 100 μl Griess reagent (aqueous solution of: 0.5% sulfanilamide, 0.05% naphthylethylene-diamine dihydrochloride, & 2.5% phosphoric acid; Sigma) was added to an equal volume of supernatant in 96 well flat bottom microtiter plates (Costar, Cambridge, MA). Plates were incubated at room temperature for five minutes. Absorbance at 550 nm was measured with a microplate reader (Bio-Tek; Winooski, VT). A standard curve for each assay was generated using dilutions of sodium nitrite (Sigma) in DMEM in the concentration range 0-100 μM.

The relative quantities of nitrite and nitrate produced by macrophages were quantified by measuring total nitrite in cell supernatants with the Griess reaction, before and after reduction of nitrate to nitrite by nitrate reductase (Cayman's Nitrate/Nitrite Assay Kit, Alexis, San Diego,CA).

Cytotoxicity Assay

Cytotoxic effect of selected treatments on cultured bovine alveolar macrophages was assessed using the MTT assay as described.[48] In this assay, mitochondrial dehydrogenases in viable cells reduce the tetrazolium salt, [3(4,5-dimethyl thiazole-2-yl)2,5 diphenyltetrazolium bromide] (MTT) to a purple formazan, which can be measured by absorbance at 570 nm. Briefly, MTT solution (5mg ml^{-1} in PBS, Sigma) is added to cell wells and incubated at 37 C° for 4-6 hours. Ten and 25 μl of MTT solution is used for 96 and 48 well plates respectively. Following incubation, the supernatant is aspirated and 100 μl of

acidified 2-propanol (0.04N HCl in isopropyl alcohol) and 20 μl of sodium dodecyl sulfate (3% w/v in water) are added to each well to disrupt cells and solubilize the colored product. The contents of each well are mixed by aspirating up and down with a micropipette and the plate contents mixed on an orbital shaker for 5-10 minutes. Absorbance is measured on a microplate reader (Bio-Tek; Winooski, VT). at 570 nm. Determinations were performed on triplicate wells.

Northern Blot Analysis

Total cellular RNA from macrophages stimulated for periods ranging from 0-48 hours was extracted using TRIZOL™ (Life Technologies, Gaithersburg, MD) according to the manufacturers suggested protocol. Twelve μg of RNA was loaded in each lane of 1% agarose gels, fractionated by electrophoresis, transferred by capillary action to nylon membranes, and immobilized by UV irradiation with a UV cross linker (Hoefer, San Francisco,CA). Blots were pre-hybridized for 3 hours at 42°C in 50% formamide, and hybridized overnight at 39°C with a ^{32}P-labeled, 827 bp fragment of murine iNOS cDNA (murine iNOS cDNA was kindly provided by Drs. Qiao-wen Xie and Carl Nathan, Cornell University Medical College, NY, NY). The hybridized blot was washed twice in 2 X SSC at room temperature, followed by three washes in 0.1 X SSC/0.1% SDS at 50°C (Life Technologies). Blots were then incubated with preflashed Kodak X-Omat™ XAR-5 film and an intensifying screen at -70°C for 24 hours.

NADPH Diaphorase Histocytochemistry

Demonstration of iNOS by NADPH diaphorase histocytochemistry was performed on macrophages stimulated in 48 well plates as previously described.[49] NADPH Diaphorase activity is a marker of NOS enzyme presence as revealed by the ability of NOS enzymes to transfer electrons from NADPH and reduce tetrazolium salts to a colored formazan dye.[49] Briefly, supernatants were aspirated and cells were fixed by the incubation with 300 μl of 2% paraformaldehyde for 30 minutes at 37 C°. After aspiration of fixative, cells were rinsed twice with 200 μl of 0.01 M PBS (pH 7.3). 300 μl of reaction (staining) solution was added to each well and incubated for I hour at 37 C°. Reaction solution was prepared fresh, by combining equal volumes of 0.6mM nitroblue tetrazolium, 0.6% Triton X-100, and 3 mM NADPH, all dissolved in 0.1M Tris (pH 8.0) (all from Sigma). The reaction was stopped by aspirating the reaction mixture and rinsing wells with 200 μl of 0.01M PBS. Stained cells were photographed using inverted microscopy (Olympus, Melville,NY) and Kodak 2415 Technical Pan film.

Western Blot Analysis

Total cellular protein from bovine alveolar macrophages and murine Raw 264.7 cells cultured with and without stimuli for 24 hours was isolated using RIPA lysis buffer containing protease inhibitors. RIPA stock solution contains: 1% Deoxycholic acid, 1% NP 40, 1% Triton X-100, 0.1% SDS, 50 mM Tris(pH 7.4), 0.3 mM EDTA, 1 mM EDTA, 50 mM Na Fluoride and 10 mM Na Pyrophosphate. The

following protease inhibitors are added to every 10 ml of this stock solution immediately before use: 100 μl each of 100 mM Na orthovalanate and 20 mM AEBSF, and 200 μl of a cocktail containing 300 μg/ml benzamidine HCl and 500 μg/ ml each of phenanthroline, leupeptin and pepstatin A(all from Sigma). Protein concentration in cellular lysates was determined by the BCA technique according to manufacturers instructions(Pierce, Rockford, IL). Lysates were denatured for 1 minute at 40 C° and 20 μg of total protein loaded in each lane. Proteins were separated by electrophoresis in 8% SDS gels. Gels were transblotted onto nitrocellulose membranes with a semidry blotter(Bio-Rad, Hercules, CA) and blocked overnight at 4 C° on a rocker plate with 50 ml PBS-10% nonfat dry milk. Membranes were washed at room temperature in 50 ml of PBS, 0.1% Tween 100 for 15, 5 and 5 minutes before application of antibody. Membranes were rinsed with a 1:750 dilution of anti-murine iNOS antibody(Upstate Biotechnology, Lake Placid, NY) in PBS, 10% nonfat dry milk for 1 hour at room temperature followed by a single 15 minute wash and 2, 5 minute washes in 50 ml PBS, 0.1% Tween at room temperature. Second antibody (HRP-labelled rabbit, anti-murine antibody, Amersham, Princeton,NJ) was diluted 1:500 in PBS, 10% nonfat dry milk and incubated with the membrane for 1 hour at room temperature. The membrane was then rinsed twice for 15 minutes in 50 ml PBS,.01% Tween. Detection was performed using ECL detection reagents and Hyperfilm-ECL™ (Amersham) according to manufacturers instructions. Duplicate gels were silver stained using

Silver Stain Plus reagents (Bio-Rad) according to the manufacturers instructions.

Statistical Analysis

Where applicable, differences between treatments were compared by ANOVA and student's t-test.

Results

In this model system, macrophages produce nitrite as the predominant nitrogen oxide secondary to NO· synthesis with only negligible quantities of nitrate appearing in the supernatants. A small quantity of nitrite was elaborated in wells containing unstimulated bovine alveolar macrophages and the quantity increased in a time dependent fashion (Fig. 1)[†]. There was marked variation between animals, with peak nitrite accumulation in identical treatments ranging from 11 to 39 μM at 72 hours of incubation. Nitrite accumulated in wells treated with LPS in a time and dose-dependent fashion (Fig. 1). Responsiveness to LPS was enhanced approximately 100-fold by the presence of bovine serum, with equivalent nitrite accumulation in wells containing 2% FBS and 10 ng LPS/ml versus wells containing 1000 ng LPS/ml without serum (Figs. 1 & 2).

[†] All figures and tables in this volume are in an Appendix following each part.

Recombinant bovine IL-1β, rbIFN-γ, or 10 ng LPS/ml as single stimuli resulted in minimal accumulation of nitrite (Figs. 3 & 4). Dose-dependent increases in nitrite accumulation were observed with increasing concentrations of rbIL-1β or rbIFN-γ when co-stimulated with a constant dose of LPS (Figs. 3 & 4). A dose-response relationship was also present for increasing concentrations of LPS with a constant concentration of rbIFN-γ (data not shown). Nitrite accumulation was enhanced by some combinations of cytokines with maximum levels achieved after stimulation with rbIFN-γ, rbIL-1β, & rhTNF-α. Addition of LPS to this trio of cytokines did not further increase nitrite accumulation (Fig. 5). Stimulation of bovine alveolar macrophages with splenic supernatant and LPS resulted in peak nitrite accumulation similar to that seen with other maximal stimuli (data not shown). Other cytokines including rhTNF-α, rhIFN-γ, rhIL-1β, and rbGM-CSF were minimally effective as single stimuli or did not significantly enhance nitrite accumulation in the media when in combination with rbIFN-γ, rbIL-1β, or LPS (data not shown). The addition of 2 mM N^{G}MMA or 2 mM AG to media restricted nitrite accumulation induced by rbIFN-γ and LPS to basal levels at 48 hours(Fig. 6) Recombinant human IL-4 was a partial inhibitor and human platelet TGF-β had no effect (Fig. 6). Table 1 summarizes relative potency of selected stimuli for production of nitrite in media.

An optimal dose of rbIFN-γ and LPS for nitrite accumulation resulted in

cumulative loss of stimulated cells over time, with a 28% average reduction in stimulated vs. control cell numbers at 72 hours of incubation (Fig.7).

An 837 bp fragment of murine iNOS cDNA hybridized with an approximately 4.5 kb mRNA from bovine alveolar macrophages stimulated with 5%-SS & 100 ng LPS/ml. This mRNA was maximally expressed at 8 hours and persisted for at least 48 hours post-stimulation (Fig. 8). A dose-response relationship between potency of the stimulus for nitrite production and quantity of message induced was found for LPS, and LPS in combination with rbIFN-γ, or rbIL-1β, and the combination of both cytokines and LPS (Figs. 9 & 10). Message was apparent using rbIL-1β as a single stimulus, and also for the combination of rbIL-1β and rbIFN-γ without LPS (Figs. 9 & 10).

An antibody to the N-terminus of murine iNOS recognized an approximately 130 kd protein in lysate of Raw 264.7 cells stimulated with murine IFN-γ and LPS for 24 hours. A similar sized protein was detected in lysates from bovine alveolar macrophages stimulated for 24 hours. No iNOS protein was detected in lysates of bovine alveolar macrophages stimulated with LPS, rbIl-1β, and rbIFN-γ as single stimuli. The quantity of iNOS protein detected in bovine alveolar macrophages exposed to combinations of stimuli correlated with the strength of stimulus as revealed by iNOS mRNA and nitrite production (Fig.11). The presence of the NOS inhibitor, aminoguanidine, did not affect iNOS expression (Fig. 11).

NADPH Diaphorase activity was present in macrophages at 24, 48, and 72

hours, with maximum activity at 48 hours. Between 10 and 20% of unstimulated macrophages exhibited diaphorase activity at all time points. Macrophages stimulated with SS and 1000ng LPS/ml had the most intense staining, with 80-100% staining in some wells. Positive staining was also associated with a more angular cellular profile, indicating cellular activation. Photomicrographs of macrophages exhibiting NADPH diaphorase-dependent staining are presented in Figure 12.

Discussion

The purpose of this study was to characterize the requirements for NO· production by bovine alveolar macrophages. Key findings include: i) bovine alveolar macrophages produce NO· in response to LPS in a time and dose dependent fashion, ii) nitrite is the major oxidized end product of NO· production by bovine alveolar macrophages, iii) NO· production by bovine alveolar macrophages is inhibited by L-arginine analogues, iv) the presence of FBS enhances LPS-induced NO· production by bovine alveolar macrophages, v) rbIFN-γ and rbIL-1β are minimally effective single stimuli for NO· production by bovine alveolar macrophages, vi) combinations of rbIFN-γ, rbIL-1β, rhTNF-α, and LPS enhance NO· production in a synergistic fashion, and vii) rhIFN-γ, rhIL-1 and rbIFN-α are poor inducers of NO· by bovine alveolar macrophages as single stimuli, or in combination with other cytokines and LPS. Furthermore, NO· production by

bovine alveolar macrophages is regulated by expression of iNOS mRNA and protein.

In the absence of bacteria, accumulation of oxides of nitrogen in mammalian cell culture systems is considered to be due to elaboration of NO·.[35] Nitrite was the predominant terminal oxidation product representing NO· production by bovine alveolar macrophages in this study and was also the predominant product identified in other studies using both murine and bovine cells.[39;50]

An 837 bp fragment of the murine iNOS cDNA hybridized with an approximately 4.5 kb mRNA in Northern blots of total cellular RNA from stimulated but not control alveolar macrophages. This mRNA is in the size range found for murine and bovine iNOS mRNA and displayed a time course of induction similar to that previously reported.[50;51] Immunoblotting of bovine alveolar macrophages lysates with an antibody to the N-terminus of murine iNOS revealed expression of an approximately 130 kD protein in stimulated but not unstimulated bovine alveolar macrophages. A similar sized protein was recognized in lysates from Raw 264.7 cells stimulated with IFN-γ and LPS. This protein is the same size as that previously reported for both bovine and murine iNOS.[50] iNOS mRNA and protein expression varied with potency of the stimulus for NO· production by macrophages in culture, suggesting that induction of the enzyme controls NO· production in bovine macrophages in a fashion similar to that shown for other species and cell types.[52]

Nitrite accumulation in cell supernatants was decreased to baseline levels by relatively low concentrations of the nonhydrolizable L-arginine analogue, AG and N^{G}MMA. These inhibitors are known to compete with L-arginine for the active site of iNOS, and add further evidence that nitrite accumulation is due to the enzymatic action of iNOS.[53] Additionally, the presence of aminoguanidine did not affect iNOS protein accumulation in stimulated bovine alveolar macrophages, supporting an inhibitory effect at the level of enzyme action.

The cytokine induction pattern for iNOS expression in bovine alveolar macrophages closely resembles that described for murine macrophages, but differs from some other reports that describe iNOS expression in bovine mononuclear phagocytes.[39;50;54-56] This heterogeneity among bovine mononuclear phagocytes in their ability to express iNOS is thought to be dependent on the stage of differentiation.[55] IFN-γ was a weak stimulus for NO· production by bovine alveolar macrophages in this study as revealed by minimal nitrite accumulation in supernatants and low levels of mRNA and protein expression, a finding also documented by others working with bovine alveolar macrophages.[57] In contrast, bovine monocytes readily respond to IFN-γ as a single stimulus, but lose this ability in a time-dependent fashion as they differentiate into cells with the morphologic characteristics of macrophages, *in vitro*.[50;54;55] IFN-γ and LPS display marked synergism for expression of iNOS in bovine alveolar macrophages and monocyte derived macrophages, but the addition of LPS to IFN-γ only weakly enhances

iNOS expression in bovine monocytes.[55] Additionally bovine alveolar macrophages express iNOS mRNA and protein in response to combinations of IFN-γ and TNF-α or Il-1β, while macrophages derived by in vitro differentiation from bovine monocytes or bone marrow cells fail to express iNOS in response to combinations of cytokines.[50]

Both monocytes and monocyte-derived macrophages expressed iNOS in response to LPS, heat-killed *Salmonella dublin*, or IFN-γ in combination with heat killed *Listeria monocytogenes*, but monocyte-derived macrophages contained more iNOS mRNA and elaborated much more NO· in response to these stimuli than monocytes.[55] Additionally, another group found IFN-γ and TNF-α were effective stimuli in freshly isolated, adherent bovine monocytes *in vitro*, but that the ability to respond to these stimuli waned rapidly as a function of time in culture.[54] In this study, an increasing proportion of mononuclear phagocytes became positive for IL-10 protein by histochemistry and a temporal increase in message for Il-10 was inversely correlated with Il-1β and TNF-α message, suggesting an Il-10-mediated autoinhibition of bovine macrophages occurs during differentiation from monocytes, *in vitro*.[54]

Il-4, Il-10 and TGFβ are known to inhibit iNOS expression in murine macrophages by actions at transcriptional and post-transcriptional levels, *in vitro*.[58] In the work presented here, recombinant human Il-4 effectively inhibited nitrite accumulation while human TGF-β1 was ineffective. Similar results are reported in

experiments using bovine monocyte-derived macrophages and both recombinant bovine and human IL-4 and TGF-β.[50;56] The bioactivity of human TGF-β for bovine macrophages has been revealed by its ability to down-regulate procoagulant activity and TNF-α secretion in response to Gram negative bacteria.[50] Thus, differences in the iNOS gene promoter region may underlie the noted species differences in the activity of TGF-β on iNOS expression. Several stimuli that might reasonably be expected to promote iNOS expression were not effective. Among these were rbGM-CSF and rbIFN-α, both of which enhance iNOS expression in murine macrophages, but were ineffective in this study and in others using bovine cells.[39;56] Again, species-specific differences in the iNOS gene promoter are postulated to underlie this disparity. The lack of iNOS inducing activity of rhIFN-γ on bovine alveolar macrophages is likely due to the well-documented species-specificity of interferons.[59] Species-specificity may also underlie the apparent lack of responsiveness of bovine alveolar macrophages to rhIL-1β.[60]

Lipopolysaccharide is a potent inducer of NO· production in both rodent and bovine macrophages.[39;50] The enhancement of LPS-induced NO· production by FBS shown in this study is likely due to the presence of serum factors, such as soluble CD14 and lipopolysaccharide binding protein, which are known to enhance iNOS expression in murine macrophages and enhance LPS-induced effector functions in bovine macrophages.[10;11] Bacterial pneumonias in cattle are often characterized by a tremendous vascular exudative response, so the ability of

serum proteins to enhance responsiveness of bovine alveolar macrophages to LPS is significant.

Although it is apparent that iNOS expression by bovine alveolar macrophages is regulated by factors known to be present in pneumonic lung, the relevance of iNOS expression by bovine alveolar macrophages in defense of the respiratory tract is undetermined. Chapters 3 and 4 contain results of studies designed to assess the potential role of iNOS expression by bovine alveolar macrophages in infectious bovine pneumonia.

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Appendix

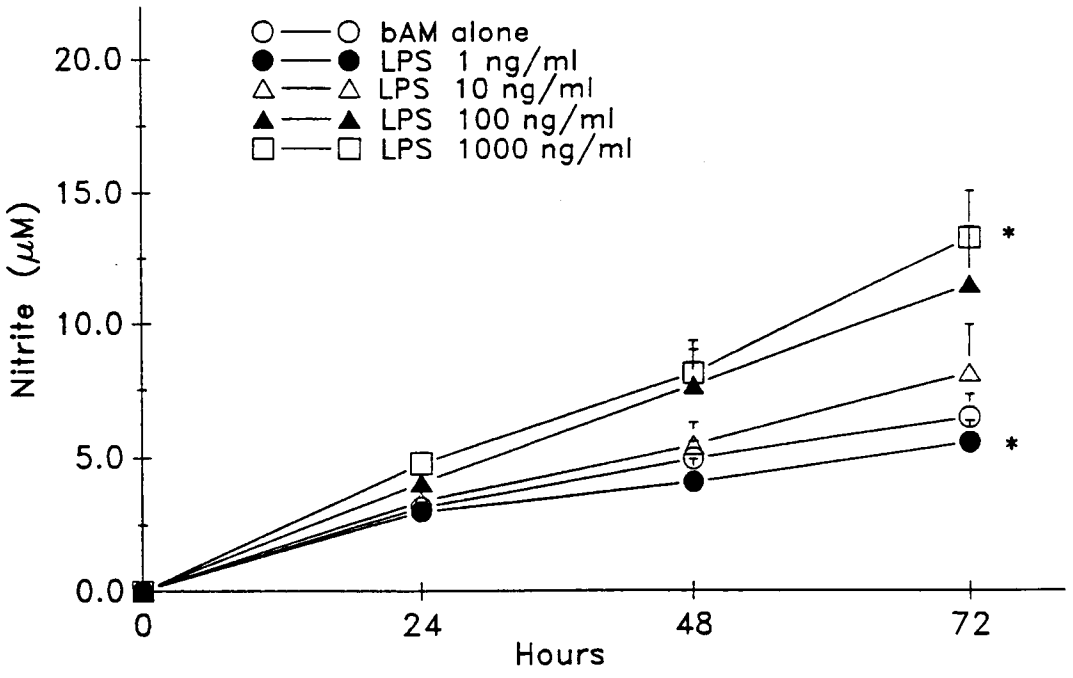


Figure 1. LPS-induced nitric oxide production by bovine alveolar macrophages. Macrophages ($5x10^5$ per well) were cultured with varying concentrations of LPS for 24, 48, and 72 hours. Data are presented as the means and standard errors from six experiments. * ($p < 0.05$)

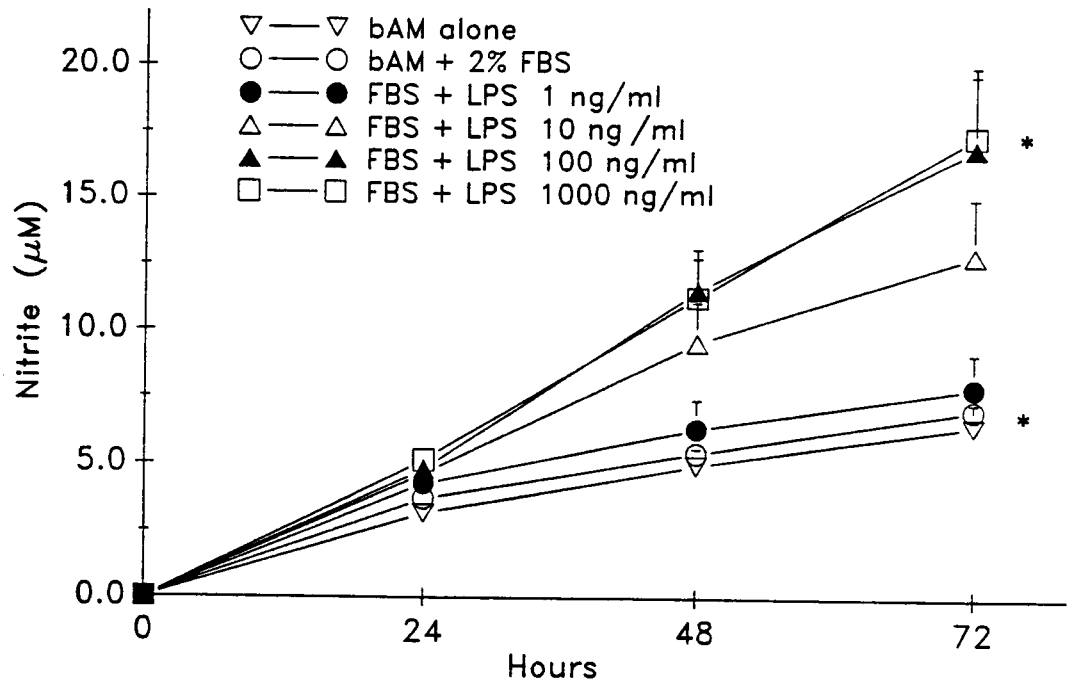


Figure 2. Effect of serum on nitric oxide production by LPS-stimulated bovine alveolar macrophages. Data are presented as the means and standard errors from six experiments. * ($p < 0.01$)

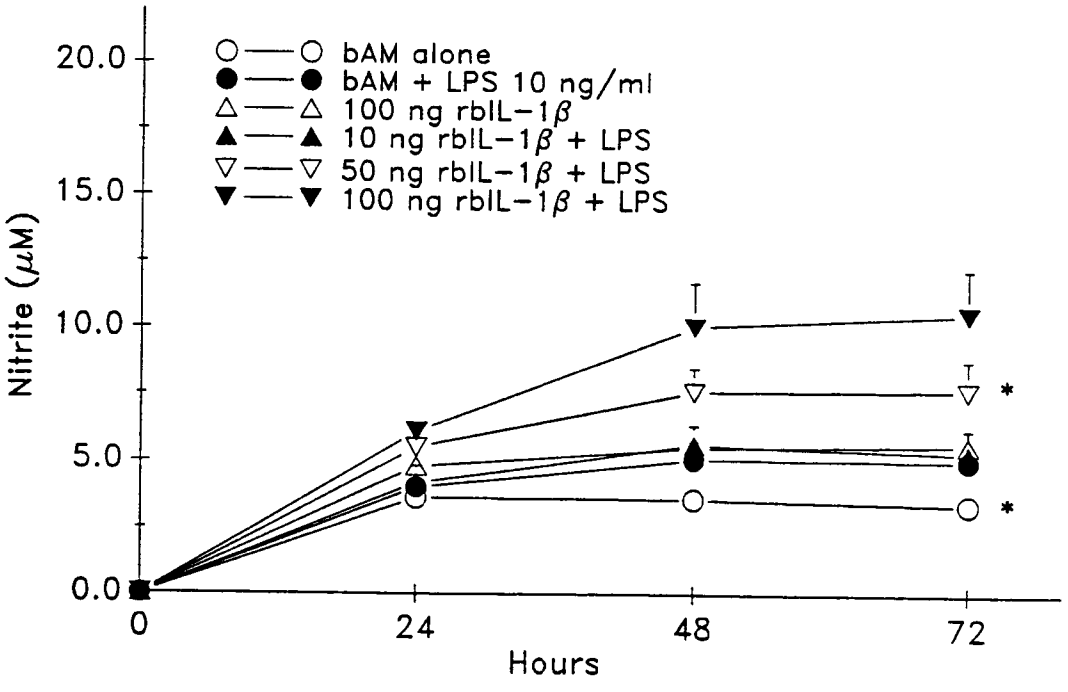


Figure 3. Dose response of nitric oxide secretion by bovine alveolar macrophages stimulated with LPS and varying concentrations of rbIL-1β. Data are presented as the means and standard errors from four experiments. * ($p < 0.05$)

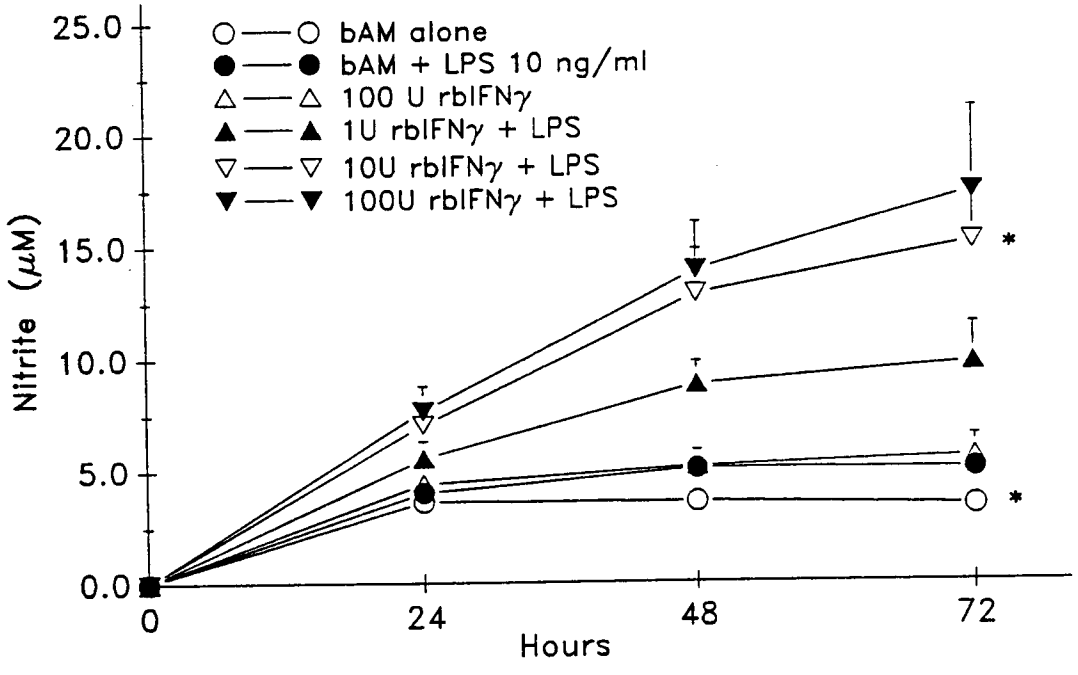


Figure 4. Dose response of nitric oxide secretion by bovine alveolar macrophages stimulated by LPS and varying concentrations of rbIFN-γ. Data are presented as the means and standard erors from four experiments. * ($p < 0.0001$)

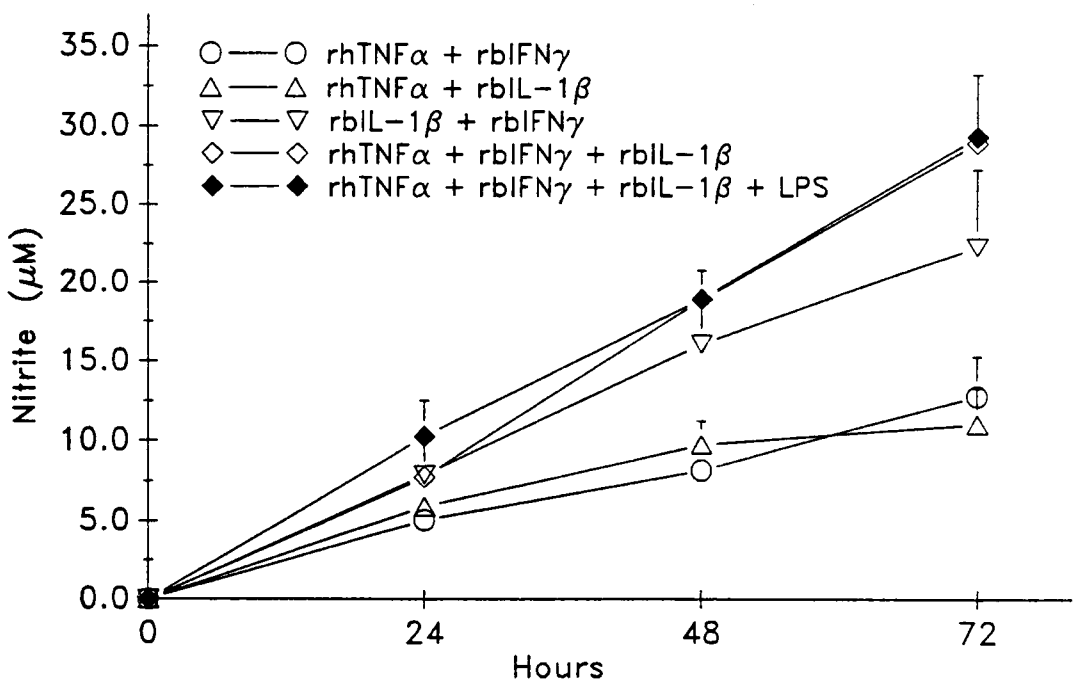


Figure 5. Effect of combinations of cytokines on nitric oxide production by bovine alveolar macrophages. rbIFN-γ (100 U ml^{-1}), rbIL-1β (100 ng ml^{-1}), rhTNF-α (100 ng ml^{-1}), LPS (10 ng ml^{-1}) Data are presented as the means and standard errors from four experiments.

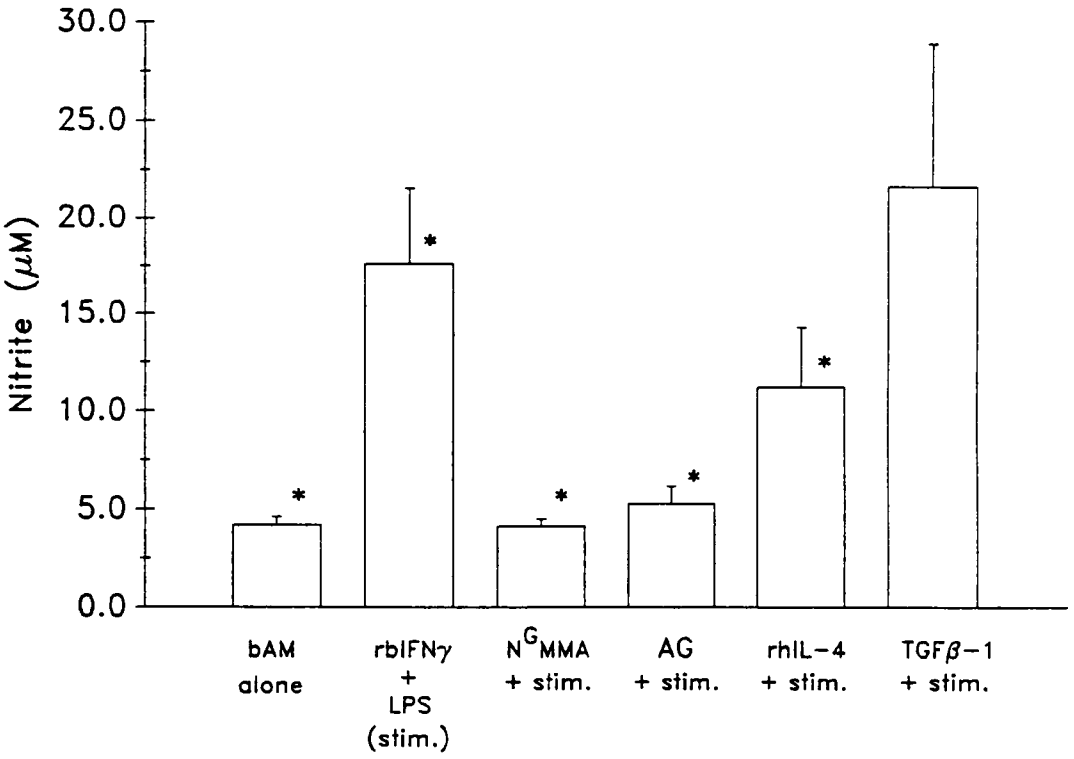


Figure 6. Effect of inhibitors on nitric oxide production by bovine alveolar macrophages stimulated by rbIFN-γ and LPS. rbIFN-γ (100 U ml^{-1}), LPS (10 ng ml^{-1}), N^GMMA (2 mM), AG (2 mM), rhIL-4 (100 ng ml^{-1}), TGF-β (50 ng ml^{-1}) Data are presented as the means and standard errors from five experiments.
* versus stim. ($p < 0.05$)

Table 1. Relative potency of stimuli for nitric oxide production by bovine alveolar macrophages

Treatments *	μM Nitrite **
TNF-α + IFN-γ + IL-1β + LPS	29.4 ± 6.8
TNF-α + IFN-γ + IL-1β	28.9 ± 7.5
TNF-α + IFN-γ + LPS	23.5 ± 8.8
IL-1β + IFN-γ + LPS	23.5 ± 5.1
Il-1β + IFN-γ	22.3 ± 9.9
IFN-γ + LPS	17.4 ± 7.5
TNF-α + IL-1β + LPS	16.1 ± 6.5
TNF-α + IFN-γ	12.8 ± 4.3
TNF-α + IL-1β	11.1 ± 3.9
Il-1β + LPS	10.6 ± 3.4
TNF-α + LPS	7.7 ± 3.3
FBS + LPS	7.5 ± 1.9
Il-1β	5.6 ± 0.8
IFN-γ	5.6 ± 1.9
LPS	5.4 ± 0.8
FBS	3.7 ± 0.5
TNF-α	3.5 ± 0.3
bAM alone	3.4 ± 0.4

* Treatments: rhTNF-α (100 ng ml^{-1}), rbIFN-γ (100 U ml^{-1}), rbIL-1β (100 ng ml^{-1}), FBS (2% v/v), LPS (10 ng ml^{-1}).

** Data are means and standard errors of μM [NO_2] in culture supernatants measured at 72 hours, (n = 4).

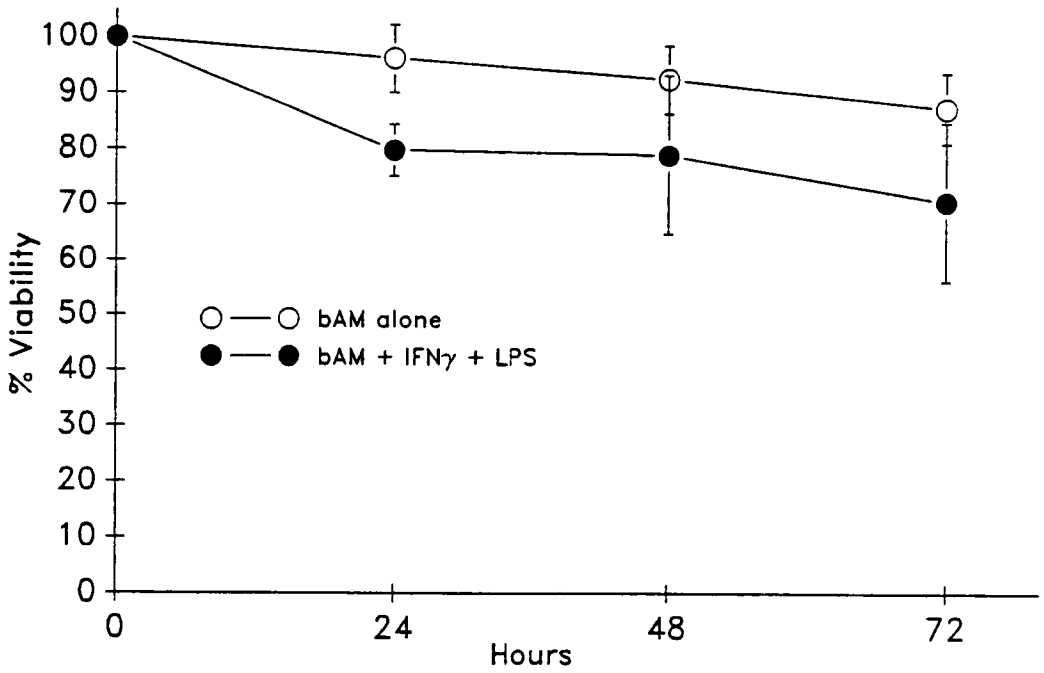


Figure 7. Viability of control and stimulated macrophages in culture. Viability was determined at the time points indicated. Stimulated macrophages were cultured with rbIFN-γ (100 U ml^{-1}) and LPS (10 ng ml^{-1}). Data are presented as the means and standard errors from 5 experiments.

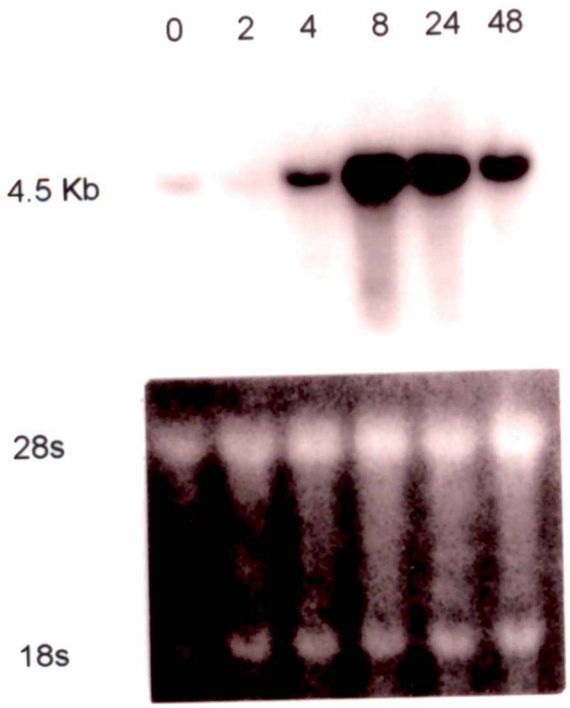


Figure 8. Time course of iNOS mRNA expression by bovine alveolar macrophages stimulated by mitogen-stimulated splenic supernatnant (5% v/v) and LPS (100 ng ml^{-1}). RNA was harvested at the times indicated. The blot was probed with an 837 bp fragment of the murine iNOS cDNA that hybridized with an approximately 4.5 kb mRNA. Photograph of gel at bottom shows fluorescence intensity of 28S and 18S ribosomal RNA to indicate comparative loading of total RNA in respective lanes.

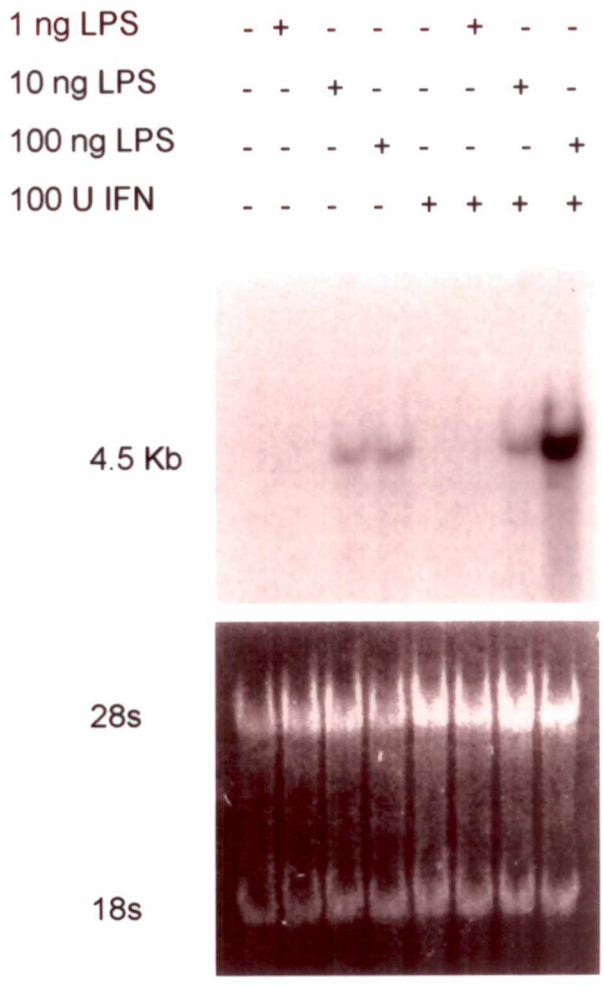


Figure 9. Dose response relationship of iNOS mRNA expression by bovine alveolar macrophages stimulated by varying concentration of LPS, as single stimuli or in combination with rbIFN-γ. The table at the top indicates treatments in lanes 1-8 designated by '+' (presence of stimulus) or '-' (absence of stimulus). RNA was isolated at 8 hours post-stimulation. The blot was probed with an 837 bp fragment of the murine iNOS cDNA. Photograph of gel at bottom shows fluorescence intensity of 28s and 18s ribosomal RNA to indicate comparative loading of total RNA in respective lanes.

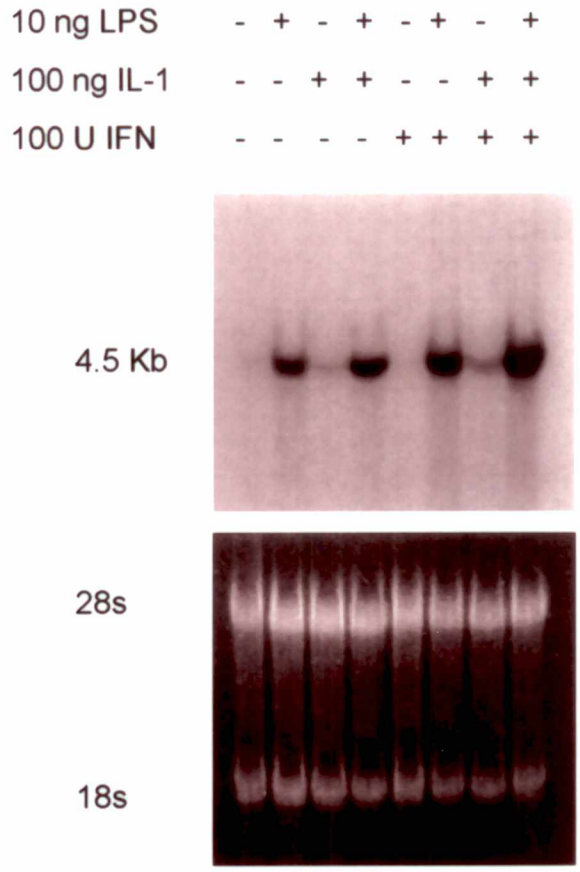


Figure 10. Dose response relationship of iNOS mRNA expression by bovine alveolar macrophages stimulated by LPS, rbIL-1β, and rbIFN-γ as single or combined stimuli. The table at the top indicates treatments in lanes 1-8 designated by presence (+) or absence of stimuli (-). RNA was isolated at 8 hours post-stimulation. The blot was probed with an 837 bp fragment of the murine iNOS cDNA. Photograph of gel at bottom shows fluorescence intensity of 28s and 18s ribosomal RNA to indicate comparative loading of total RNA in respective lanes.

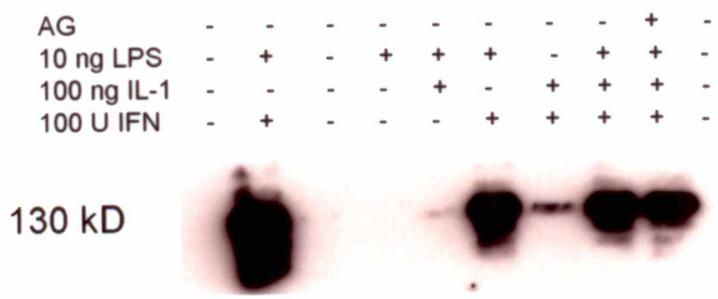
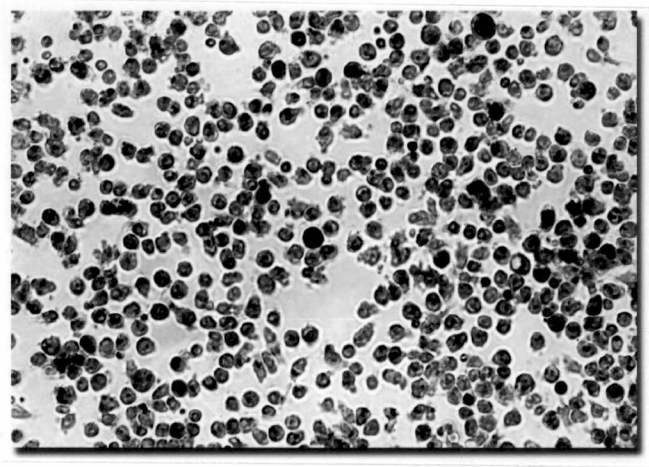


Figure 11. Dose response relationship of iNOS protein expression by bovine alveolar macrophages stimulated by rbIFN-γ, rbIL-1β, and LPS as single or combined stimuli. Cellular lysates were harvested after 24 hours of culture. Table at top indicates the presence (+) or absence of stimuli (-) in respective lanes. The blot was probed with an antibody to the C-terminus of murine iNOS that recognized an approximately 130 kd protein. Lane 2 contains lysate from the murine macrophage cell line Raw 264.7 cells collected 24 hours after stimulation with rmIFN-γ and LPS.

A.



B.

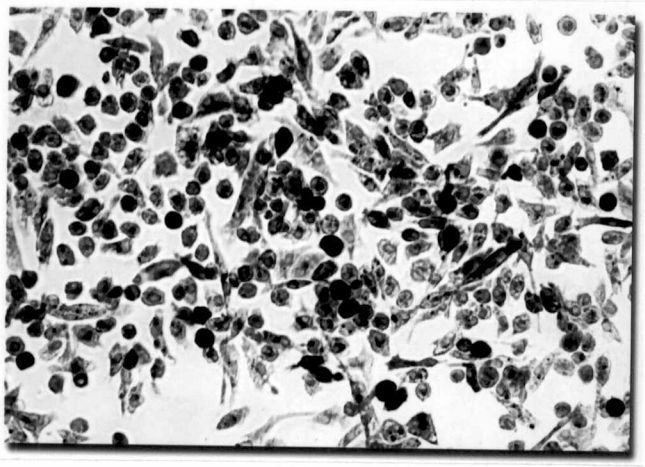


Figure 12. Immunohistochemical demonstration of NADPH Diaphorase expression in cultured macrophages. A. Photomicrograph of unstimulated macrophages stained after 48 hours in culture. B. Photomicrograph of stained macrophages exposed to rbIL-1β (100 ng ml^{-1}) and LPS (10 ng ml^{-1}) for 48 hours. (200 x magnification)

PART 3

Evaluation of Nitric Oxide Mediated Microbicidal Activity of Bovine Alveolar Macrophages

Abstract

Bovine alveolar macrophages play an integral role in the pathogenesis of pneumonia caused by *Pasteurella haemolytica*, but little is known about the potential role of macrophage-derived NO· in this process. This study was designed to assess the effectiveness of *P. haemolytica* as a stimulus for NO· generation from bovine alveolar macrophages and measure the microbicidal activity of NO· against *P. haemolytica*, *in vitro*. Endotoxin from *E. coli* and *P. haemolytica* are equipotent inducers of NO· production by macrophages, exhibiting a dose response effect as single stimuli and enhancement of the response to rbIFN-γ and rbIL-1β. Heat-killed leukotoxin deficient bacteria induced NO· production by macrophages as a single stimulus. This response was further augmented by rbIFN-γ. The nitrogen oxide generating compounds, SNAP and SIN-1 killed *P. haemolytica* in a dose dependent fashion. Macrophages killed leukotoxin deficient *P. haemolytica*, but prior treatment for iNOS expression abrogated this effect. Wild type *P. haemolytica* readily killed macrophages.

Introduction

Bovine respiratory disease complex (BRD) is the most costly disease problem of cattle in North America.[1] BRD associated costs are attributed to medical and labor costs associated with morbidity, losses due to mortality, decreases in gain and feed efficiency, and a higher proportion of low grading carcasses in cattle with a history of pneumonia.[1] This disease complex primarily

affects stocker and feeder cattle and BRD associated expenditures account for 7% of the total production costs from weaning to slaughter.[1] BRD remains a significant problem despite ongoing efforts to develop better vaccines, better management and treatment protocols, and despite advances in our understanding of the pathogenesis of this disease complex.[1;2]

BRD has a complex, multifactorial etiology with contributions due to stress and both viral and bacterial agents.[3] Outbreaks of BRD are sporadic, but often follow a period of stress such as weaning, castration, commingling, exposure to climactic extremes, and transport (hence, "shipping fever pneumonia"). Viral agents involved in BRD include Bovine herpes virus type 1 (BHV), Bovine virus diarrhea virus (BVDV), Parainfluenza virus type 3 (PI3) and Bovine respiratory syncytial virus (BRSV).[3-6] Within the BRD complex, disease outbreaks are characterized by concurrent bacterial infection. Bacterial isolates include *P. haemolytica* followed by *P. multocida* and *Haemophilus somnus*.[1;3] Limited evidence suggests bovine coronavirus, *Mycoplasma bovis, M. dispar,* and *Ureaplasma diversum* may contribute to BRD.[1;4] *Pasteurella haemolytica* biotype A serotype 1 (*P. haemolytica* A1) is the most common and important bacterial agent in BRD and as a single agent is capable of producing the fibrinopurulent bronchopneumonia that characterizes BRD.[3;7]

Pasteurella haemolytica A1 is a non-enteric, fermenting, Gram negative organism that is a commmensal inhabitant of the bovine tonsil and sinus passages.[8] *Pasteurella haemolytica* can be isolated from the upper respiratory tract of clinically normal cattle, but the majority of these isolates are the

nonpathogenic *Pasteurella haemolytica* serotype 2.[9] In times of physical distress or following viral infection, *P. haemolytica* A1 proliferates, is inhaled in droplet nuclei, and colonizes the lower respiratory tract producing disease.[9-12]

The factors contributing to stress induced bacterial colonization and overgrowth in the upper respiratory tract are unknown.[9] In the case of concurrent virus infection, virus-induced changes in host cell surface fibronectin and receptor expression, disruption of mucociliary escalator function, and alterations in the properties of surface mucus are thought to enhance colonization by Gram negative bacteria.[9;13;14]

Pasteurella haemolytica has several virulence factors that contribute to clinical disease. These include adherence factors, a polysaccharide capsule that inhibits phagocytosis, a lipopolysaccharide that induces leukocyte emigration and activation, and a secreted leukotoxin (Lkt) that activates and kills bovine cells.[15]

Many bacteria utilize fimbriae to mediate adherence. Fimbriae are composed of proteins that act as lectins, allowing recognition and binding of bacteria to cell surface receptors. Two structurally distinct types of fimbriae have been identified on *P. haemolytica* A1 but the cell surface receptor they bind and their importance as virulence factors remain undescribed.[16] *P. haemolytica* A1 also produces a neutral protease and neuraminidase that may aid adhesion to host cells by cleaving fibronectin and surface sialic acid residues exposing potential binding sites on the cell surface.[15;17;18] Capsular polysaccharides appear to play a role in adherence of bacteria to host cells and also decrease phagocytosis and killing of *P. haemolytica* by bovine neutrophils.[18;19]

Sequential studies of the morphology of pneumonia caused by P. *haemolytica* A1 indicate that there is an early and rapid emigration of neutrophils into the alveolus, followed by platelet aggregation and serofibrinous exudation involving the alveolus, interlobular septa and pleural surface. Hemorrhage, thrombosis and necrosis occur later.[7;12] Most morphologic aspects of the natural disease can be reproduced by airway instillation of LPS and leukotoxin. In these lesions, LPS, Lkt and capsular polysaccharide are physically associated with neutrophils, pulmonary intravascular and alveolar macrophages, endothelial cells and epithelial cells.[20] Additionally these cells display morphologic features of activation and leukocytotoxicity. The relative contributions of LPS and Lkt virulence factors are difficult to differentiate because they form tight complexes, in vitro.[21;22]

Pasteurella haemolytica leukotoxin is a heat labile, 104 kDa protein that is a member of the RTX (repeats in toxin) family of pore forming toxins.[23;24] RTX toxins are characterized by a repeat domain consisting of multiple copies of a conserved nine amino acid cassette that is critical for cytotoxic action.[25] Leukotoxin exhibits cytocidal activity against bovine granulocytes, mononuclear phagocytes, and platelets.[9] RTX mediated cytotoxic mechanisms are incompletely understood. At high concentrations leukocytotoxicity appears to be due to membrane damage with pore formation and osmotic cytolysis.[23;26;27] At lower concentrations Lkt causes changes typical of apoptosis including internucleosomal DNA fragmentation, Poly(ADP ribose) polymerase cleavage, and translocation of phosphatidylserine groups to the outer membrane

leaflet.[23;28;29] Antibodies to β2 integrins block association of Lkt with bovine cell membranes and apoptosis.[23;30] This evidence suggests β2 integrins are the cell surface receptor for Lkt and provides an explanation for the cell- and species-specific activity of leukotoxin.

Leukotoxin causes degranulation, elaboration of arachidonic acid mediators and initiation of the respiratory burst in neutrophils, platelet activation and aggregation, and cytokine secretion by alveolar macrophages.[31-36] Leukotoxin is thought to contribute to pulmonary injury by release of hydrolytic enzymes, arachidonic acid metabolites and other mediators.[37-39] Significant pneumonic lesions are produced experimentally by a Lkt deletion mutant of *Pasteurella haemolytica*, but are considerably less necrotizing and severe than that caused by the isogenic parent strain.[40] This indicates Lkt contributes significantly to virulence, but is not the sole virulence factor.

Lipopolysaccharide comprises 12-25 % of the dry weight of *P. haemolytica* A1 and, like other Gram negative organisms, is composed of a core oligosaccharide, a biologically active lipid A component and a polysaccharide O antigen.[15] LPS acts on macrophages, endothelial and respiratory epithelial cells to initiate the generation or release of myriad inflammatory mediators, including, oxygen radicals, hydrolytic enzymes, arachidonic acid metabolites, and cytokines, as well as initiation of kinin generation and clotting and complement cascades.[9] LPS derived from *P. haemolytica* A1 exhibits direct cytotoxic activity on bovine endothelial cells, induces tissue factor expression on endothelial cells, and cytokine synthesis in bovine alveolar macrophages.[41-43]

Neutrophil emigration with subsequent activation and necrosis are prominent morphologic characteristics of BRD and neutrophil depletion decreases pulmonary injury in experimental pasteurellosis.[37;44] The importance of neutrophils in causing pulmonary injury is well described in models of endotoxemia with cytokine overproduction. These observations suggest that the signals and mechanisms that result in neutrophil infiltration in pasteurellosis are key to an understanding of pathogenesis. Il-1β, TNF-α, and Il-8 are expressed in experimentally induced pasteurellosis, in lung tissue sections cultured with *P. haemolytica* A1, and by alveolar macrophages exposed to *P. haemolytica* A1, or Lkt and LPS derived from this bacteria.[36;43;45-47] Elaboration of these mediators is thought to produce pulmonary injury by acting as chemotaxins, inducing the elaboration of other mediators that are chemotactic, inducing expression of adhesion molecules, and enhancing neutrophil-mediated injury.

Alveolar macrophages show the morphologic and functional features of activation upon exposure to *P. haemolytica* , and via cytokine secretion appear to play a central role in initiation of the inflammatory response to this agent. A hallmark of murine macrophage activation is iNOS expression[48] and nitric oxide-dependent cytostasis is recognized as essential for control of some infectious agents in rodents.[49;50] The inducible form of NOS is also readily expressed in bovine alveolar macrophages, however the efficacy of this antimicrobial effector pathway in killing *P. haemolytica* A1 has not been evaluated. The goals of this work are to: i) establish the effectiveness of *Pasteurella haemolytica* as an inducer of NO· production in macrophages, ii) evaluate the susceptibility of *P.*

haemolytica A1 to NO· and the secondary reactive nitrogen oxide, peroxynitrite, and iii) determine if macrophages are able to control growth of *Pasteurella haemolytica* in a NO· dependent fashion.

Material and Methods

Isolation of Alveolar Macrophages

Bovine alveolar macrophages were collected from fresh lungs obtained at an abattoir. Lungs from slaughter cattle were inspected and those free of gross pathology were chosen for lavage. Lungs were lavaged with 1-3 liters of Hanks balanced salt solution (HBSS) without calcium, pH 7.25 (GIBCO, Grand Island,NY). Lavage fluids were filtered through sterile gauze and spun at 200 *x*g. for 8 minutes at 4 C°. Cell pellets were gently resuspended and washed twice with 50 ml ice cold HBSS, resuspended in Dulbecco's Minimal Essential Medium (DMEM) (Whittaker Bioproducts, Walkersville,MD) and enumerated with a hemocytometer. Lavage isolates yielding greater than 90 % macrophages (Wright-Giemsa stained cytocentrifuge preparations) with greater than 90% viability (trypan blue dye exclusion) were used for studies.

Determination of Nitric Oxide Production by Macrophages Exposed to *Pasteurella haemolytica* A1

Bovine alveolar macrophages were cultured at $1x10^6$ cells ml^{-1} ($5x10^5$ cells/well) in 48 well flat bottom polystyrene plates or $1x10^5$ cells well in 96 well plates (Costar, Cambridge, MA). Culture media was DMEM with 20 mM HEPES, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2.5 μg ml^{-1} fungizone, and 2mM L-

glutamine (all from Whittaker Bioproducts). Bovine alveolar macrophages were cultured with the following as single or combined stimuli: 1, 10, 100 or 1000 ng ml^{-1} LPS from *E. coli* (O55:B5, Sigma, St. Louis, MO) or leukotoxin-free LPS purified from *P. haemolytica* A1 12996 (kindly provided by Dr. S. Maheswaran, University of Minnesota); 100 U ml^{-1} rbIFN-γ (Ciba-Geigy, Basel, Switzerland); 100 U ml^{-1} rbIl-1β (American Cyanamid, Princeton, NJ); and 2% FBS (Hyclone,Logan, UT) as single or combined stimuli. The NOS inhibitors used were either 2 mM N^G-monomethyl-L-arginine (N^GMMA; Calbiochem, San Diego, CA), or 2mM Aminoguanidine (AG; Sigma). In some experiments, macrophages were exposed to heat-killed log phase leukotoxin-deficient *P. haemolytica* A1 D153 with or without rbIFN-γ costimulus. Macrophage cultures were incubated at 37^0C in the presence of 5% CO_2 for 24 and 48 hours. Cell free supernatants were collected after centrifugation (10 minutes at 300 xg), and were assayed for nitrite immediately or frozen at -20°C until assay.

Nitrite Assay

Nitrogen oxide production was estimated by measuring nitrite in cell free supernatants by the Griess reaction.[51] In this assay, nitrite reacts with the Griess reagent to form a purple dye.[51] Briefly, 100 µl Griess reagent (aqueous solution of: 0.5% sulfanilamide, 0.05% naphthylethylene-diamine dihydrochloride, & 2.5% phosphoric acid; Sigma) was added to an equal volume of supernatant in 96 well flat bottom microtiter plates (Costar, Cambridge, MA). Plates were incubated at room temperature for five minutes. Absorbance at 550 nm was measured with a microplate reader (Bio-Tek; Winooski, VT). A standard curve for each assay was

generated using dilutions of sodium nitrite (Sigma) in DMEM in the concentration range 0-100 μM.

Bacterial Culture and Enumeration

P. haemolytica A1 D153 and the isogenic leukotoxin-deficient mutant, D153 lkt$^-$ (kindly provided by Dr. Briggs, National Animal Disease Laboratory, Ames, IA) as well as *Escherichia coli* were maintained on blood agar plates at room temperature. For experiments, colonies from a freshly streaked plate incubated overnight at 37°C were picked and grown to log phase in brain heart infusion broth at 37°C in an orbital shaker. Bacteria were pelleted and washed twice with PBS. Bacteria were resuspended in DMEM without antibiotics and adjusted to the desired concentration by comparing transmittance at 650nm with a standard curve. In experiments with macrophages, bacteria were opsonized by a 10 minute incubation with pooled sera (5 % in DMEM) from adult beef cows in which complement had been deactivated by heat treatment (56°C for 30 minutes). Stock suspensions used to set up experiments and bacteria remaining at the termination of studies were enumerated by duplicate colony counts of 5 μl aliquots of serial dilutions (10^0 to 10^{-6}) following overnight incubation at 37°C on blood agar plates. Only those dilutions yielding between 3 and 40 visibly separate and distinct colonies were counted.

Killing of *Pasteurella haemolytica* by Compounds that Spontaneously Release Reactive Nitrogen Oxides

The susceptibility of *P. haemolytica* A1 to killing by reactive nitrogen oxides was determined by incubation of bacterial suspensions with solutions of chemical compounds that spontaneously generate reactive nitrogen oxides. The compounds used were SNAP (S-Nitroso-N-acetyl-D,L-penicillamine) which spontaneously generates NO· in aqueous solution in the presence of oxygen, and SIN-1 (3-morpholinosydnonimine) which is stable in acidic solutions, but generates NO· and $O_2^{\cdot -}$ which form peroxynitrite at physiologic pH in the presence of oxygen. Stock solutions of SNAP and SIN-1 were prepared immediately before each experiment. SNAP was dissolved in 5mM KPO_4 containing 10% DMSO (pH 7.4) and SIN-1 was dissolved in 5mM KPO_4 (pH 5.6). N-acetyl D,L-penicillamine (NAP; an inactive counterpart of SNAP) and $NaNO_2$ were used as controls and were also dissolved in 5mM KPO_4 (pH 7.4). SNAP and SIN-1 were purchased from Alexis, San Diego, CA . NAP and $NaNO_2$ were purchased from Sigma.

Log-phase *P. haemolytica* A1 D153 suspensions were adjusted to 10^8 cfu ml^{-1} in DMEM containing 20 mM HEPES, 2 mM L-glutamine, pH 7.25 and 9 ml of the bacterial suspension was aliquoted into 125 ml Erlenmeyer flasks. Each flask also received 1 ml of SNAP, SIN-1, NAP or $NaNO_2$ diluted in DMEM to produce final concentrations of 0.05, 0.1, 0.25, 0.5 and 1 mM for SNAP; 1 mM for NAP; 0, 0.5, 1, 1.5 and 2 mM for SIN-1; and 0, 25, 50, 100, 250, and 500 μM for $NaNO_2$. Flasks were incubated for 2 hours at 37°C on a rotating incubator.

Following incubation, aliquots of each flask were serial diluted for enumeration of bacteria and measurement of nitrite concentration.

Assessment of Nitric Oxide- Mediated Killing of *P. haemolytica* A1 and *E. coli* by Macrophages

These experiments utilized opsonized log-phase *E. coli* , *P. haemolytica* D153 and the isogenic lkt⁻ deletion mutant. Bactericidal assays were performed after an initial 24 hour culture to induce iNOS expression in macrophages. Treatments included: control (no stimulus or inhibitor); stimulus (100 U ml^{-1} rbIFN-γ and 10 ng ml^{-1} LPS (E. coli, O55:B5), stimulus plus NOS inhibitor (2 mM AG); or inhibitor alone. Two experimental approaches were utilized.

The first was a microtiter plate based system conducted accorded to the published protocol.[52] In this assay, bacterial survival was assessed before and after a 90 minute incubation period by measuring the ability of dehydrogenases in viable bacteria to reduce the yellow tetrazolium salt [3(4,5-dimethyl thiazole-2-yl-2,5 diphenyltetrazolium bromide] (MTT) to a purple formazan which is measured with an automated microplate reader at 570 nm. Briefly, bacterial suspensions are aliquoted into 96 well plates containing macrophages. Contact between bacteria and macrophages is enhanced by a 15 minute centrifuge spin at 250 xg at 4°C. Plates are incubated for 10 minutes at 37°C to allow phagocytosis and residual bacteria are removed by washing wells twice with DMEM. Initial bacterial numbers in the T=0 plates are assessed at this time point. Bacteria surviving after the killing assay were measured after re-establishment of the appropriate volume and content of culture media in each treatment and an additional 90 minute incubation period.

At each of these time points, macrophages are lysed by a 5 minute incubation with 0.5% saponin, after which 100 μl tryptose phosphate broth is added to each well. Plates are then incubated for 4 hours at 37°C. After this incubation, 10 μl of MTT (5 mg ml^{-1}) is added to each well and plates are incubated an additional 20 minutes and absorbance is measured.

In the second experimental approach, macrophages ($1x10^6$ ml^{-1}) were cultured in 12 x 75 mm polypropylene tubes, with bacteria enumerated after a phagocytosis step (T=0) and following an incubation to allow killing (T=90 minutes). After the initial 24 hour culture, log phase opsonized bacteria were added to each tube resulting in a bacteria to macrophage ratio of 4:1 to 15:1. Tubes were capped and rotated end over end for 15 minutes at 37°C to allow phagocytosis to occur. Phagocytosis was stopped and free bacteria were then removed by 2 five minute centrifuge washes at 250 xg at 4°C. The original volume and concentration of stimulus or inhibitor used in the initial 24 hour incubation was then re-established in each tube and 100 μl aliquots were removed from each tube to verify phagocytosis (Wright-Giemsa stained cytocentrifuge preparations) and to establish an initial count of viable bacteria in each treatment. Tubes were then incubated another 90 minutes at 37°C with end over end rotation to allow killing. Bacteria at initiation and termination of the experiment were enumerated by limiting dilutional analysis as described, except dilutions were performed with distillled H_2O to lyse macrophages. Additionally, the proportion of viable macrophages remaining in each treatment at the end of each experiment was assessed by trypan blue dye exclusion.

Cytotoxicity Assay

Cytotoxic effect of selected treatments on cultured macrophages was assessed using the MTT assay as described.[53] In this assay, mitochondrial dehydrogenases in viable cells reduce the tetrazolium salt, [3(4,5-dimethyl thiazole-2-yl)2,5 diphenyltetrazolium bromide] (MTT) to a purple formazan, which can be measured by absorbance at 570 nm. Briefly, MTT solution (5mg ml^{-1} in PBS, Sigma) is added to cell wells and incubated at 37 C° for 4-6 hours. Ten and 25 μl of MTT solution is used for 96 and 48 well plates respectively. Following incubation, the supernatant is aspirated and 100 μl of acidified 2-propanol (0.04N HCl in isopropyl alcohol) and 20 μl of sodium dodecyl sulfate (3% w/v in water) are added to each well to disrupt cells and solubilize the colored product. The contents of each well are mixed by aspirating up and down with a micropipette and the plate contents mixed on an orbital shaker for 5-10 minutes. Absorbance is measured on a microplate reader (Bio-Tek; Winooski, VT). at 570 nm. Determinations were performed on triplicate wells.

Results

Endotoxin from *E. coli* and *P. haemolytica* A1 are equipotent inducers of NO· production by macrophages, exhibiting a dose response effect as single stimuli (data not shown) and enhancement of the response to rbIL-1β (data not shown) and rbIFN-γ (Fig. 13). Heat-killed leukotoxin deficient bacteria induced NO· production by macrophages as a single stimulus at a bacterial particle to

macrophage ratio of 10:1(Fig. 14). Higher bacterial particle to macrophage ratios or the addition of rbIFN-γ failed to significantly augment this response (Fig. 14).

The nitrogen oxide generating compounds SNAP and SIN-1killed *P. haemolytica* in a dose-dependent fashion. Neither the inactive SNAP analog NAP (Fig. 15) nor nitrite had an effect on bacterial viability (data not shown). Bacterial killing by SNAP was curvilinear with 0.5 mM killing 2 logs of bacteria while producing 200 μM nitrite (Fig. 15). No further reduction in bacterial numbers was apparent with higher concentrations of SNAP although increasing concentrations resulted in the generation of more nitrite. No appreciable bacterial killing was apparent with 0.5 mM SIN-1, while a linear relationship was present between bacterial killing and nitrite accumulation for SIN-1 concentrations between 0.5 and 2 mM (Fig. 16). The linearity of this relationship was not investigated at higher SIN-1concentrations. Killing of 2 logs of bacteria by SIN-1 was present at a final nitrite concentration of nearly 500 μM (Fig. 16).

In tube based assays, killing of leukotoxin deficient *P. haemolytica* A1 was apparent in macrophages which were either unstimulated or cultured with NOS inhibitor alone, while there was net proliferation of bacteria in macrophages stimulated to generate NO· (Fig. 17). There was no apparent effect of treatment on the ability of macrophages to phagocytose bacteria (data not shown). In tube based killing assays, *E. coli* proliferated in all treatments, however there was a statistically insignificant trend toward lesser proliferation in treatments containing either unstimulated macrophages or macrophages cultured with AG alone (Fig. 18).

Wild type *P. haemolytica* A1 D153 readily killed macrophages, such that few viable macrophages remained at the end of killing assays (data not shown). There was also remarkable loss of viability of macrophages in tube based killing assays (Fig. 19). There was net proliferation of leukotoxin deficient *P. haemolytica* in microtiter plate based assays without any significant differences among treatments (Fig. 20). Macrophages had only modest loss of viability in sham-inoculated microtiter plates processed in parallel with killing assays (Fig. 20).

Discussion

The purpose of this work was to address whether or not macrophage derived NO· may play a role in defense of the respiratory tract against *P. haemolytica* A1.

Specific goals were; i) to measure the ability of macrophages to elaborate NO· in response to *P. haemolytica* and LPS derived from this bacterium, ii) determine if *P. haemolytica* is susceptible to killing by reactive nitrogen oxides, and iii) determine if NO· production by macrophages exerts a bacteriostatic or microbicidal effect on *P. haemolytica, in vitro*.

Significant findings included: i) killed *P. haemolytica* and LPS derived from *P. haemolytica* effectively stimulate NO· production by macrophages, ii) *P. haemolytica* is killed by chemically generated reactive nitrogen oxides, and iii) macrophages are able to kill lkt- *P. haemolytica* A1, but stimulation of macrophages for NO· production abrogated this effect, *in vitro*.

The ability of bovine macrophages to express iNOS and elaborate NO· in response to cytokines, bacteria and their products is firmly established.[54-56] The data presented here reveal that macrophages respond to killed *P. haemolytica* A1 and LPS derived from *P. haemolytica* A1 by generating NO· in a dose and time dependent manner similar to that previously observed for other microbial stimuli and their products.[54-56] Additionally this work confirms an earlier study demonstrating NO· production by macrophages exposed to *P. haemolytica* A1 or LPS derived from this bacterium.[57] Cytokines known to enhance LPS-induced NO· production by macrophages are present in the lung tissue of cattle suffering from pneumonic pasteurellosis and lavage fluid from the lungs of cattle with experimental pasteurellosis contain increased nitrite levels.[45;57] Additionally, iNOS expression by macrophages has recently been demonstrated by immunohistochemistry in the lungs of young cattle with spontaneous pneumonia due to *P. haemolytica* A1 and *Arcanobacterium pyogenes*.[58] Whether or not iNOS expression in pneumonic pasteurellosis plays a role in microbial killing remains unknown.

The results of this study reveal that *P. haemolytica* A1 are readily killed by chemically generated reactive nitrogen oxides, but that reactive nitrogen oxide species vary in their ability to kill *P. haemolytica* A1, and the quantity of reactive nitrogen species necessary, as reflected in nitrite accumulation, far exceeds the concentrations macrophages can be demonstrated to produce *in vitro*.

Compounds that spontaneously donate NO· have microbicidal effects against a range of pathogens including bacteria, several eukaryotic infectious agents, and viruses.[59] Bacteria vary by species in susceptibility to RNS-mediated

cytostatic or microbicidal effects and also vary in susceptibility to different RNS. There are few explanations for this variation. S-nitrosothiols are bacteriostatic and peroxynitrite is microbicidal for *Salmonella typhimurium* and *Escherichia coli*, while these species are resistant to NO·.[60;61] In contrast, peroxynitrite is ineffective, while NO· and S-nitrosothiols kill *Staphylococcus aureus.*[62]

The mechanisms for reactive nitrogen oxide-mediated bacterial cytostasis and killing and mechanisms of bacterial resistance are largely speculative. Reactive nitrogen oxides are known to interact with a variety of molecular targets including transition metals, iron sulfur clusters, heme, thiols, tyrosine residues and nucleic acids, but much of this work derives from work using eukaryotic target cells. In bacterial species, NO· mediated actions are known to include inhibition of iron sulfur or sulfhydryl containing enzymes involved in energy metabolism, membrane sulfhydryls and DNA.[60;63-67]

Some bacteria resist RNS mediated damage via NO· inducible detoxifying nitric oxide reductases or nitric oxide dioxygenases.[63;68] Inhibition of expression of the latter enzyme enhances susceptibility of *E. coli* to NO· mediated cytotoxic actions.[63] Access of RNS to the interior of the cell influences its cytotoxic potential. *Salmonella typhimurium* imports small peptides, including S-nitrosoglutathione, via a specialized dipeptide permease and NO· mediated growth inhibition of this bacterium is dependent on the presence of this transporter.[60] In general, bacterial resistance to RNS mediated cytostasis and killing appear to rely heavily on antioxidant cellular defenses, particularly low molecular weight thiol containing compounds. Mutant *Salmonella* which cannot

synthesize glutathione or homocysteine are exquisitely sensitive to killing by RNS, including NO·.[67;69] Supplementation with homocysteine restores resistance to RNS.[69] Additionally, these bacteria are of low virulence in wild type mice, but are pathogenic following NOS inhibition, *in vivo*.[67] The presence of high concentrations of low molecular weight thiols in enteric bacteria probably accounts for their observed resistance to NO·.[67] Gram positive bacteria, such as Staphylococci contain low levels of glutathione[70] and are susceptible to killing by NO·.[62]

Other microbial antioxidant defense systems that appear to mitigate RNS-induced damage include, Glucose-6-phosphate dehydrogenase, which provides NADPH reducing equivalents for regeneration of thiols[71], Cu/Zn superoxide dismutase, by limiting peroxynitrite generation[67;72], and the RecBCD exonuclease which is required for DNA repair[60]. The SoxRS regulon in *E. coli* is induced by NO· and encodes genes for glucose-6-phosphate dehydrogenase and manganese superoxide dismutase.[73] *E. coli* deficient in these genes are susceptible to RNS-dependent killing by macrophages.[71] The oxyR regulon in *E. coli* mediates resistance to S-nitrosothiols and encodes catalase and glutathione reductase.[74] Despite these findings, *Salmonella typhimurium* defective in both soxS and oxyR regulons, although sensitive to RNS *in vitro*, retain virulence in mice.[67] This illustrates our lack of a comprehensive understanding of the importance and relationship between RNS resistance mechanisms and RNS-mediated host defenses in infectious disease.

P. haemolytica A1 express superoxide dismutase which may mediate resistance to NO·, but the presence or absence of other resistance factors is unknown. The very high concentrations of reactive nitrogen oxides required to kill *P. haemolytica* in cell free systems suggest that macrophages are unlikely to produce sufficient NO· to significantly impact *P. haemolytica*, although bacteria within a phagosome are exposed to additional reactive species which may act synergistically with NO·.[61;75] *P. haemolytica* A1 reduce nitrate to nitrite in log phase growth and remain viable in culture for up to 70 hours with nitrite levels as high as 400 μm.[76] Because acidification of these cultures would reasonably be expected to occur within this time frame and because acidification of nitrite containing solutions results in generation of species such as peroxynitrous acid and peroxynitrite,[77-79] it seems likely that *P. haemolytica* A1 has evolved protective mechanisms to deal with reactive nitrogen oxides. The same reasoning predicts *P. haemolytica* A1 would also likely be resistant to RNS generated within an acidic phagolysosome.

In the experimental protocols used here, control macrophages exhibited microbicidal activity against *P. haemolytica* , but stimulation of macrophages for NO· production abrogated this effect. Findings with *E. coli* displayed a similar trend. Microbicidal activity of bovine alveolar macrophages against *E. coli* has been demonstrated,[80] however the multiple anti-oxidant defensive mechanisms possessed by *E. coli* likely explain the inability to demonstrate a NO· mediated microbicidal action of macrophages in this study. The failure of macrophages stimulated by IFN-γ and LPS to kill *P. haemolytica* A1 while control macrophages

demonstrated readily measurable microbicidal activity may be due to differential control of the production of reactive nitrogen and oxygen species in macrophages and perhaps by treatment differences in macrophage cytotoxicity which was demonstrated following termination of killing assays.

P. haemolytica A1 is susceptible to killing by $O2^{\cdot -}$ (despite the presence of periplasmic superoxide dismutase)[81] and bovine macrophages are known to produce $O_2^{\cdot -}$ when stimulated by *P. haemolytica* A1 as a phagocytic stimulus.[82] Additionally, generation of oxygen radicals by bovine phagocytes is temporally related to bacterial killing.[80] In rodent macrophages, NO· and $O_2^{\cdot -}$ production are differentially regulated. IFN-γ priming followed by a phagocytic signal activates oxygen radical generation, while IFN-γ with LPS as a secondary signal results in iNOS expression with concurrent depression in oxygen radical production.[83;84] This signal specific differential regulation likely serves to restrict oxidative damage to the host, but also limits the variety of radical based microbicidal effector molecules the macrophage can bring to bear on an infectious agent.[85] Whether or not oxygen radical and NO· production by bovine macrophages are differentially regulated has not been studied. If induction of iNOS by IFN-γ and LPS in bovine macrophages depresses oxygen radical production in response to a phagocytic stimulus, then this activation signal may explain the lack of microbicidal activity against *P. haemolytica* A1 noted in stimulated macrophages in this study. Conversely, if both oxygen and nitrogen based radicals are co-produced then quenching of reactive oxygen species by NO· may explain the noted differences.[86] The latter explanation seems less likely

however, because stimulated macrophages co-cultured with NOS inhibitor do not generate significant quantities of NO· yet displayed a lack of microbicidal activity similar to that of stimulated macrophages cultured without a NOS inhibitor.

Induction for iNOS expression and subsequent exposure to leukotoxin-deficient *P. haemolytica* A1 was associated with significant cytotoxicity of macrophages present at the termination of killing assays. Macrophage cytotoxicity may underlie the treatment differences noted in killing assays, but the cause of this cytotoxicity is unknown. In some studies, high output NO· production is associated with initiation of apoptosis in macrophages, while in others no relationship is apparent.[78] In bovine macrophages, LPS induced cytoxicity appears unrelated to NO· generation, but the mechanisms resulting in loss of viability in this study were undetermined.[87]

Induction of iNOS in macrophages causes activation, revealed morphologically by angular profiles in cell culture which reflect changes in cytoskeletal organization and expression of adhesive molecules. Centrifugation and resuspension of these cells may have resulted in cellular damage and explain the cytotoxic effect noted in tube based assays. This hypothesis was evaluated by conducting killing assays using a microtiter plate based system in which centrifugal shear forces experienced by activated macrophages would be lessened by adhesion to the flat bottom of the well. These assays yielded similar results for cytotoxicity to macrophages but no apparent NO· mediated microbicidal activity against *P. haemolytica* A1. It is possible that addition of another signal (bacteria) to macrophages previously activated for iNOS

expression may have initiated apoptosis or resulted in necrosis. Difficulties in ensuring removal of non-phagocytosed bacteria in the microtiter plate likely underlie the lack of demonstrable treatment differences in killing assays.

Bovine alveolar macrophages are known to kill wild type *P. haemolytica* A1 at low particle to macrophage ratios, with leukocytotoxicity predominating at ratios of 20:1 or greater.[80;88] The cytotoxic effect of wild type *P. haemolytica* A1 on macrophages noted in this study suggests that in the natural disease with the characteristic acute onset and fulminate course, the ability of macrophages to induce iNOS and elaborate NO· may not be a significant factor in host resistance. The relative importance of iNOS expression and NO· production by macrophages in field cases of bovine pasteurellosis remains unproven.

Summary

In this and the preceding chapters, evidence has been presented that demonstrates iNOS is readily inducible in bovine alveolar macrophages exposed to bacterial products and cytokines known to be present in pneumonic tissue. Although *P. haemolytica* A1 are susceptible to killing by chemically-generated reactive nitrogen oxides *in vitro*, killing occurs at much higher concentrations than that produced by stimulated macrophages, *in vitro*. It appears doubtful that bovine macrophage-generated NO· has a significant microbicidal effect *in vivo*. In the experimental protocols used here, killing of leukotoxin-deficient *P. haemolytica* A1 by unstimulated macrophages was demonstrated, but pretreatment for macrophage stimulation and NO· production resulted in

considerable loss of macrophage viability, which may have abrogated measurement of a NO· mediated effect. Thus, evidence of the possible relevance of iNOS expression by bovine alveolar macrophages in pneumonic pasteurellosis remains elusive.

Viral pathogens are often identified concurrently with *P. haemolytica* A1 in the bovine respiratory disease complex. The following chapter describes experiments designed to address the effects of these viruses on macrophage NO· production and the effect of NO· production on virus replication in macrophages.

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Appendix

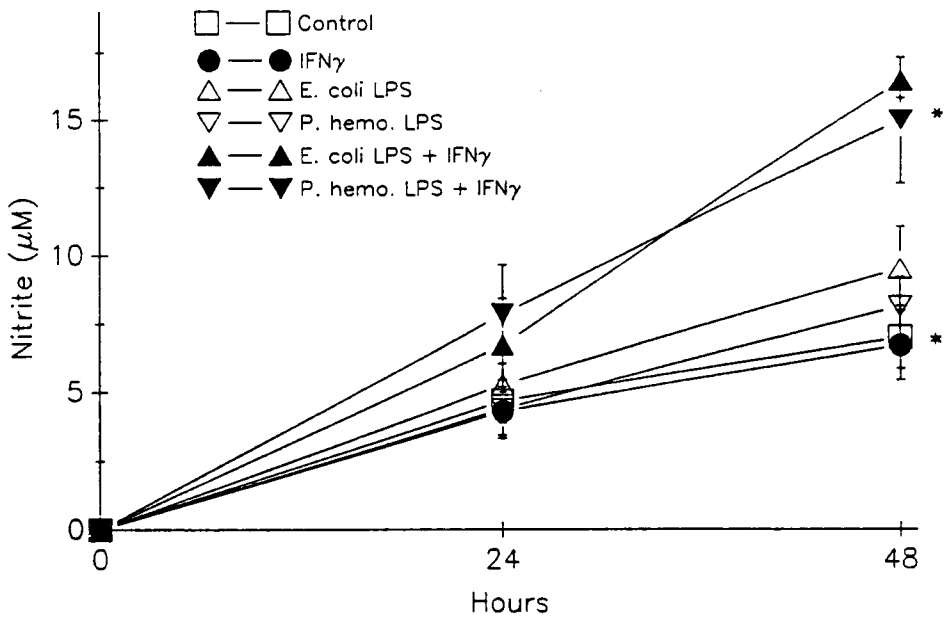

Figure 13. Comparative efficacy of LPS from *E. coli* and *P. haemolytica* A1 as stimuli for nitric oxide production by bovine alveolar macrophages. Stimuli included; rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 or leukotoxin deficient *P. haemolytica* A1 D153 (10 ng ml^{-1}). Data are presented as the means and standard deviations from 3 experiments. * ($p<0.01$)

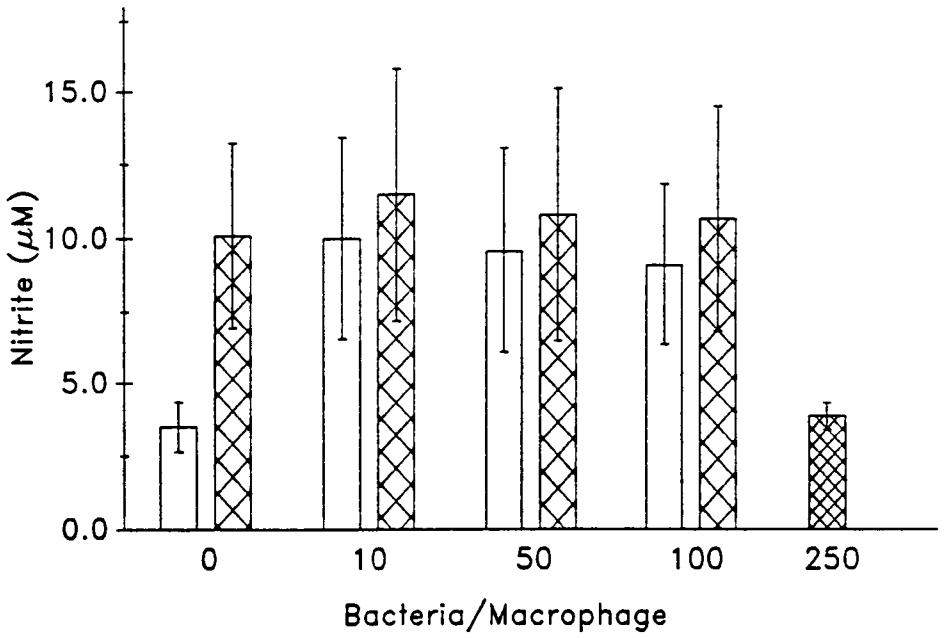

Figure 14. Nitric oxide production by bovine alveolar macrophages exposed to heat-killed leukotoxin-deficient *P. haemolytica* D153. Macrophages were treated for 48 hours prior to nitrite determination in culture supernatants. Treatments included: open bars, no co-stimulus; wide crosshatch, rbIFN-γ (100 U ml^{-1}); and narrow crosshatch, rbIFN-γ (100 U ml^{-1}) and aminoguanidine (2 mM). Data are presented as the means and standard deviations from 3 experiments.

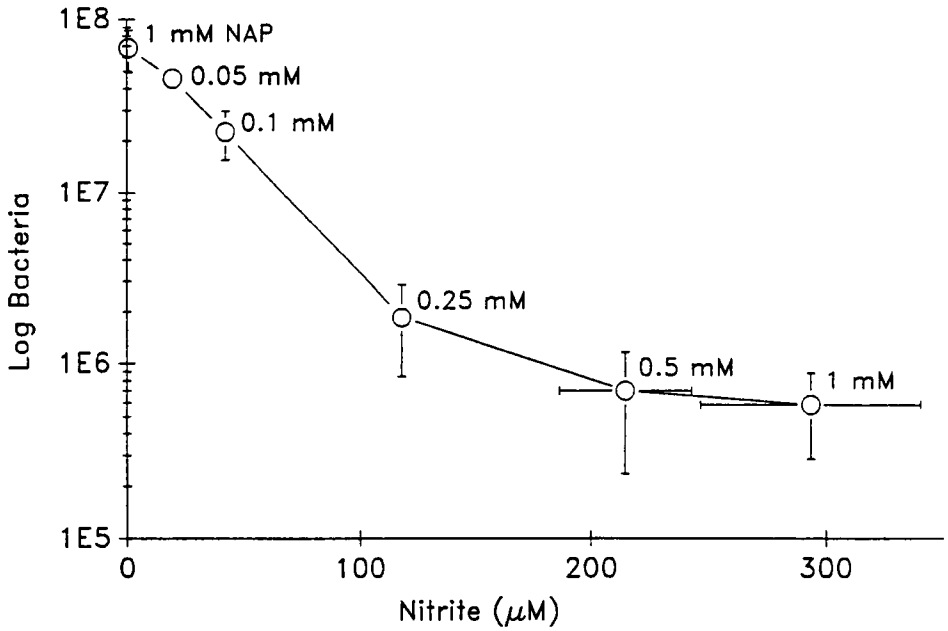

Figure 15. Killing of *P. haemolytica* A1 by reactive nitrogen oxide species derived from SNAP. Log viable bacteria and nitrite determinations were performed after 2 hours of incubation with 1 mM NAP or the concentrations of SNAP indicated. Data are presented as the means and standard deviations from 3 experiments.

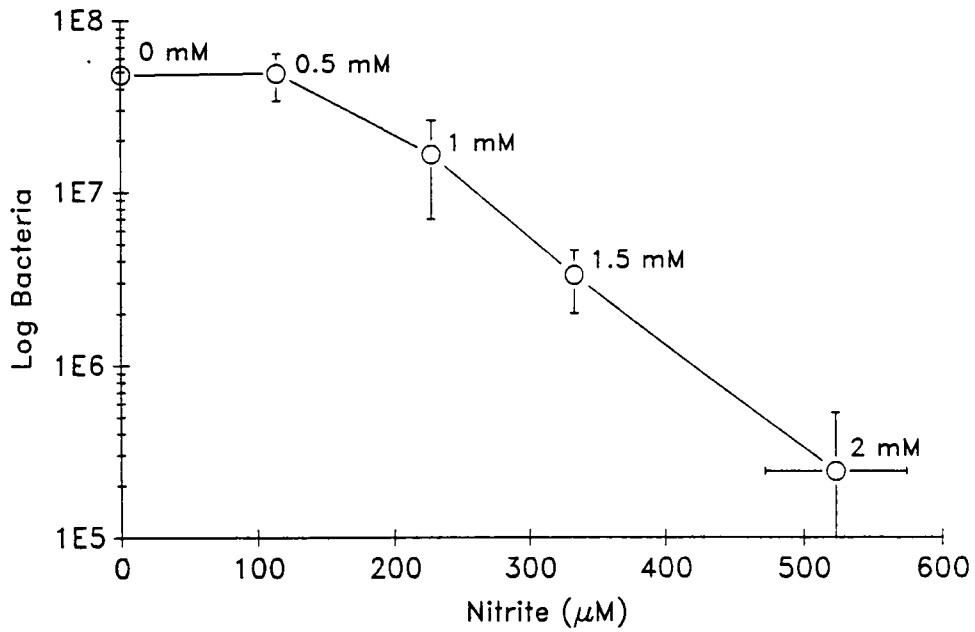

Figure 16. Killing of *P. haemolytica* A1 by reactive nitrogen oxide species derived from SIN-1. Log viable bacteria and nitrite determinations were performed after 2 hours of incubation with the concentrations of SIN-1indicated. Data are presented as the means and standard deviations of 3 experiments.

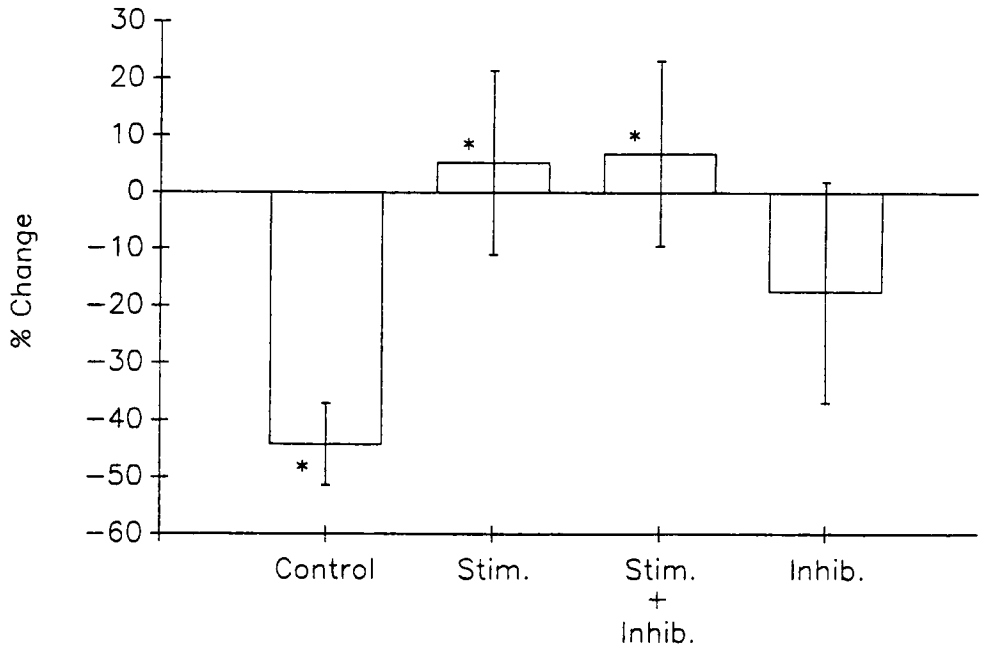

Figure 17. Tube-based macrophage microbicidal assay against leukotoxin-deficient *P. haemolytica* A1. Percent change in bacterial numbers at T=0 and T=90 minutes is indicated on the y axis Treatments include: Control, DMEM alone; Stim.; rbIFN-γ (100 U ml^{-1}) and LPS (10 ng ml^{-1}); Stim. + Inhib., rbIFN-γ and LPS and aminoguanidine (2 mM); and Inhib., aminoguanidine. Data are presented as the means and standard deviations from 6 experiments. * ($p<0.05$, versus control)

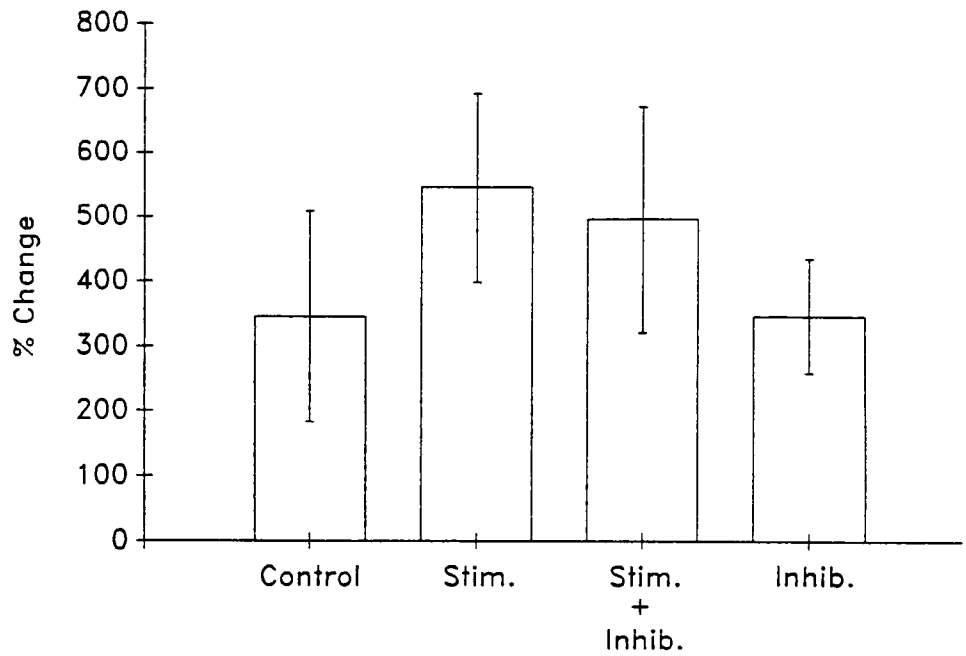

Figure 18. Tube-based macrophage microbicidal assay against *E. coli*. Percent change in bacterial numbers at T=0 and T=90 minutes is indicated on the y axis. Treatments include: Control, DMEM alone; Stim.; rbIFN-γ (100 U ml^{-1}) and LPS (10 ng ml^{-1}); Stim. + Inhib., rbIFN-γ and LPS and aminoguanidine(2 mM); and Inhib., aminoguanidine. Data are presented as the means and standard deviations from 4 experiments.

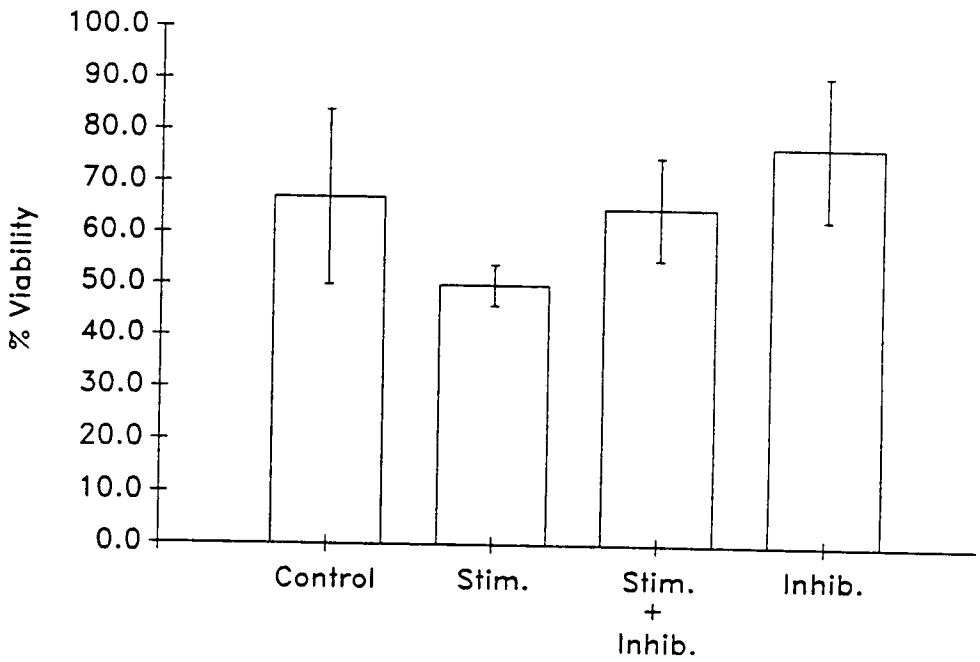

Figure 19. Macrophage viability following tube-based microbicidal assay against leukotoxin-deficient *P. haemolytica*. Treatments include: Control, DMEM alone; Stim.; rbIFN-γ (100 U ml^{-1}) and LPS (10 ng ml^{-1}); Stim. + Inhib., rbIFN-γ and LPS and aminoguanidine (2 mM); and Inhib., aminoguanidine (2mM). Data are presented as the means and standard deviations from 4 experiments.

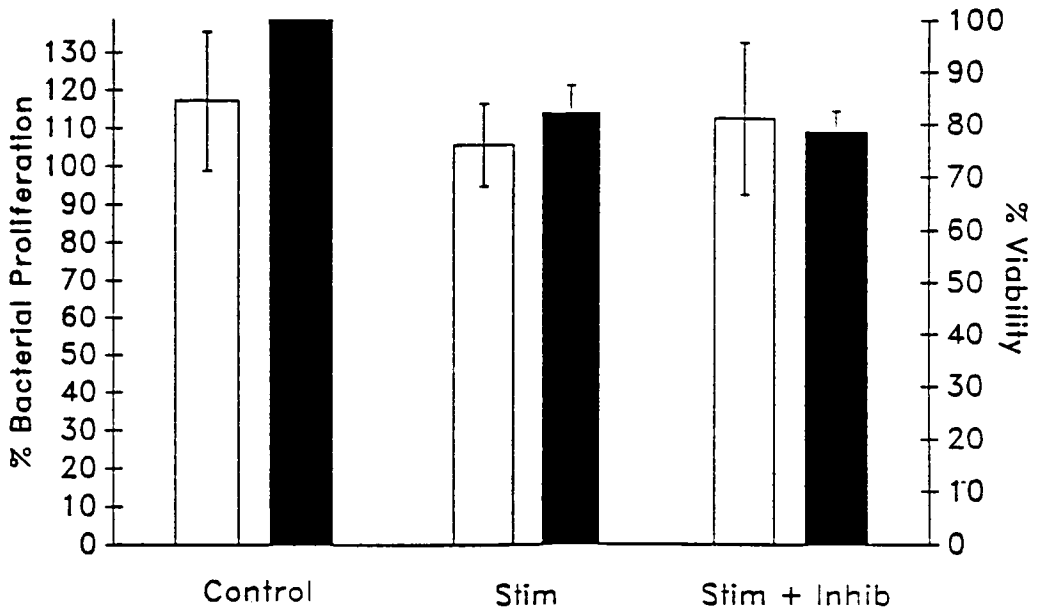

Figure 20. Microbicidal activity against leukotoxin deficient *P. haemolytica* and viability of macrophages in microtiter plate assay. Open bars, percent change in bacterial numbers between T=0 and T=90 minutes. Closed bars, percent viable macrophages at T=90 versus control. Treatments include: Control, DMEM alone; Stim.; rbIFN-γ (100 U ml^{-1}) and LPS (10 ng ml^{-1}); Stim. + Inhib., rbIFN-γ and LPS and aminoguanidine (2 mM). Data are presented as the means and standard deviations from 3 experiments.

PART 4

Effect of Virus Infection on Nitric Oxide Production by Bovine Alveolar Macrophages and Effect of Nitric Oxide Production on Macrophage Permissiveness for Virus Replication

Abstract

Viral pneumonias are a common cause of economic loss to the cattle industry. Viral pneumonia often predisposes to bacterial super-infection of the lung further compounding morbidity and economic loss. Alveolar macrophages play a central role in defense of the lower respiratory tract and iNOS expression and NO· production are considered key components of macrophage activation. The work presented here was designed to assess the potential effect of infection of macrophages by common bovine viral pathogens on the ability of macrophages to generate NO·; and to determine if prior stimulation of macrophages for iNOS expression alters their permissiveness for viral replication. The data indicates that Bovine herpes virus (BHV), Bovine virus diarrhea virus (BVD) and Parainfluenza type 3 (PI3) infection of bovine alveolar macrophages depresses NO· production. This effect is due to loss of viability of macrophages infected with BHV and BVD, but is due to an alteration of cell function in PI3 infected macrophages. BVD and PI3 readily replicated in macrophages, but there was only minimal replication of BHV in these cells. Prior stimulation of macrophages for NO· production did not significantly effect the replicative ability of any of these viruses in macrophages.

Introduction

Viral pneumonias are a common cause of economic loss to the cattle industry.[1] Viral agents cause clinically significant disease as single agents but more importantly, viral respiratory pathogens predispose to bacterial super-

infection of the lung resulting in significant morbidity, mortality and economic loss.[2;3] Viral infection predisposes the lung to bacterial superinfection by disrupting mucociliary escalator function, altering the properties of mucus, increasing cell surface fibronectin expression, altering cell surface receptor expression and alveolar macrophage function.[3-6] Collectively, these changes reduce bacterial clearance and enhance bacterial adhesion and colonization of the lung.

Viral agents implicated in the bovine respiratory disease complex (BRD) that commonly infect cattle in North America include Bovine herpes virus type 1(BHV), Bovine virus diarrhea virus (BVD), Parainfluenza type 3 (PI3), and Bovine respiratory syncytial virus (BRSV). There is limited evidence suggesting infection with pneumotropic bovine coronavirus occasionally causes clinically significant disease in fed cattle.[7] BHV, BVD, PI3 and BRSV all cause significant alterations in macrophage function but only BHV, BVD and PI3 readily replicate within macrophages.[8;9]

Bovine herpes virus 1, an alpha herpes virus, is the cause of infectious bovine rhinotracheitis. The virus produces a fibrinonecrotic upper respiratory infection with a mortality rate that ranges up to 10% in severe outbreaks.[7] Disease outbreaks occur most frequently when cattle from diverse sources are congregated at sale barns and feedlots.[7] Viral latency occurs in paravertebral ganglia and stress-associated recrudescense with subsequent exposure of immunologically naïve animals likely plays a major epidemiological role.[7]

Productive lytic infection of alveolar macrophages occurs early in infection, although the degree to which macrophages are permissive for BHV infection is controversial.[7;8;10] BHV infected macrophages secrete interferons,[11] display increased procoagulant activity,[12] MHC-II expression, and prostaglandin E_2 production, and secrete increased quantities of lysosomal enzymes[13] but display depressed TNF-α[14] and Il-1β secretion,[13] markedly diminished Fc and complement mediated phagocytosis,[15;16] and depressed antibody dependent cellular cytotoxicity.[16] Ironically, despite these changes documented *in vitro*, the ability of BHV-infected macrophages to kill *Staphylococcus epidermis* was unaffected, *in vitro*.[15]

Parainfluenza virus type 3, a paramyxovirus, causes clinically insignificant or mild disease as a single agent, but is an important and common co-pathogen in outbreaks of the bovine respiratory disease complex.[7] Natural disease is characterized by fever with cough and spontaneous resolution within a few days, if uncomplicated by bacterial superinfection.[7] PI3 infects many cell types in the upper respiratory tract, resulting in mucopurulent rhinitis, bronchitis and bronchiolitis.[7] Lytic infection of the respiratory epithelium alters mucociliary clearance. PI3 infection also alters the function of macrophages and lymphocytes. Macrophages support productive infection of PI3 resulting in altered function and cellular lysis.[8;17] PI3 infected macrophages display enhanced procoagulant activity[12] and prostaglandin E_2 secretion,[18] decreased TNF-α secretion,[14] depressed oxygen radical generation,[19] depressed

phagocytosis[15;17;20] and microbial killing activity,[18] and decreased ability to serve as accessory cells in mitogen-stimulated lymphocyte proliferation.[21]

Bovine viral diarrhea virus (BVD) is a member of the *Pestivirus* genus of the family Flaviviridae. BVD infection is common in North American cattle. There are two biotypes, designated cytopathic and non-cytopathic based on their effects on host cells, *in vitro*.[22] Infection by cytopathic BVD in a naïve immunocompetent animal typically results in a low-grade diarrheic syndrome, but occasionally produces a severe disease characterized by thrombocytopenia, hemorrhage and high mortality.[23;24] Many infections with the noncytopathic type are acquired during gestation. Intrauterine infection results in abortion, fetal abnormalities, an immune response with clearance of the virus, or persistent infection with tolerance, depending on the fetal age at infection.[22;24] However, many noncytopathic infections are clinically silent and persistently infected animals serve as a source for spread of the virus in a herd. These individuals may experience a severe disease characterized by mucosal ulceration if subsequently infected with an antigenically-related cytopathic virus type; this is termed "mucosal disease".[22;24] Mucosal disease may also be produced in persistently infected individuals by genetic recombination of noncytopathic virus with expression of a p80 protein that encodes a protease virulence factor.[25]

Cattle suffering BRD are often co-infected with BVD.[24;26] There is only limited evidence that BVD can function as a primary pulmonary pathogen, however disease caused by BHV, BRSV and *P. haemolytica* are more severe

when there is concurrent BVD infection.[27-31] The mechanisms responsible for this observation are incompletely understood. Research in this area reveal that bovine viral diarrhea virus infection alters the number, proportion and MHC expression of circulating T and B cells.[32] BVD infection of alveolar macrophages depresses Fc and complement receptor expression, phagocytosis, microbicidal activity, and decreases production of neutrophil chemotactic factors.[33] BVD infection decreases production of TNF-α by macrophages,[34] but enhances procoagulant activity[12] and prostaglandin E_2 secretion.[35] Of note to the work presented here, it has been shown that infection of bovine macrophages with non-cytopathic BVD primes them for NO· production.[36] Additionally, infection of macrophages with the cytopathic type results in secretion of IFN-α[37] that decreases LPS-induced NO· production and promotes LPS-induced apoptosis.[38;39]

Bovine alveolar macrophages are known to express iNOS and generate NO· in response to stimuli present in pneumonic tissue. NO· production is known to be a feature of macrophage activation in murine macrophages and macrophage-generated NO· is important in the control or pathogenesis of viral infections in rodents.[40-42] Viruses susceptible to replication inhibition by NO· *in vitro* or *in vivo*, include taxonomically diverse viruses represented by genera that are enveloped, encapsidated, and contain both DNA and RNA genomes.[43] Among these, are agents similar to those associated with respiratory disease in

cattle, including the flavivirus, Japanese Encephalitis Virus,[44] the alpha herpes virus, Herpes simplex,[45;46] and Influenza virus.[41]

It is clear that many of the functions of bovine alveolar macrophages may be markedly altered by viral infection, however apart from the few experiments with BVD-infected macrophages just outlined, no work has addressed the potential effects of respiratory viruses on production of NO· by bovine macrophages. Additionally, the effect of stimulation of macrophages for iNOS expression on the permissiveness of these cells for viral replication has not been determined. The goals of the present study were to determine if: i.) viral infection of macrophages alters their ability to express NO·, and ii.) activation of macrophages for NO· synthesis alters their permissiveness for productive viral infection.

Materials and Methods

Isolation of Alveolar Macrophages

Bovine alveolar macrophages were collected from fresh lungs obtained at an abattoir. Lungs from slaughter cattle were inspected and those free of gross pathology were chosen for lavage. Lungs were lavaged with 1-3 liters of Hanks balanced salt solution (HBSS) without calcium, pH 7.25 (GIBCO, Grand Island,NY). Lavage fluids were filtered through sterile gauze and spun at 200 *x*g. for 8 minutes at 4 C°. Cell pellets were gently resuspended and washed twice with 50 ml ice cold HBSS, resuspended in Dulbecco's Minimal Essential Medium (DMEM) (Whittaker

Bioproducts, Walkersville,MD) and enumerated with a hemocytometer. Lavage isolates yielding greater than 90 % macrophages (Wright-Giemsa stained cytocentrifuge preparations) with greater than 90% viability (trypan blue dye exclusion) were used for studies.

Culture and Virus Infection of Alveolar Macrophages

Bovine alveolar macrophages were cultured at $1x10^6$ cells/ml ($5x10^5$ cells/well) in 48 well flat bottom polystyrene plates (Costar, Cambridge, MA). Culture media was DMEM with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml fungizone, and 2mM L-glutamine (all from Whittaker Bioproducts). Stimuli for NO· production were 10 ng ml^{-1} LPS (E. coli O55:B5, Sigma, St. Louis, MO); and 100 U ml^{-1} rbIFNγ (Ciba-Geigy, Basel, Switzerland). The NOS inhibitor used was 2mM Aminoguanidine (AG; Sigma).

Macrophages were infected with virus by inoculation of duplicate wells with virus at multiplicities of infection ranging from 1 viral particle per macrophage (10^0 moi) to 1 viral particle per 10^6 macrophages (10^{-6} moi). Virus inoculation was performed by aspirating supernatant from each well, followed by addition of 40 μl aliquots of viral suspension. Plates were rocked for 10 minutes, viral suspension was aspirated and the appropriate volume and composition of cell culture media and stimuli were re-established in each well. Macrophage cultures were incubated at 37^0C in the presence of 5% CO_2. Cell free supernatants were collected after centrifugation (10 minutes at 300 xg), and were assayed for nitrite immediately or frozen at -20°C until assay. Virus infection of macrophages was confirmed by

staining with virus specific fluorescent antibody (VMRD, Pullman, WA) and fluorescent microscopy.

Virus Propagation and Enumeration

Viruses used for these studies were kindly provided by Dr. L.N.D. Potgieter of the University of Tennessee and included cytopathic Bovine viral diarrhea virus (Singer strain), Bovine herpes virus type 1(Cooper strain) and Parainfluenza type 3 (a field strain). Viruses were propagated and enumerated on Madin Darby Bovine Kidney cells (MDBK) as previously described.[47] MDBK cells were grown and maintained in DMEM containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml fungizone, and 2mM L-glutamine (all from Whittaker Bioproducts) and 2% gamma irradiated, certified virus free, FBS (Hyclone, Logan,UT). Viral propagation and enumeration were performed using established methods.[47] Briefly, freshly thawed aliquots of virus in a small volume of DMEM were placed on MDBK cells grown to approximately 75% confluence. Flasks were rocked at room temperature for 15 minutes, after which the flasks were rinsed twice with DMEM and incubated at 37^0C in the presence of 5% CO_2. Flasks were visually monitored and harvested when cytopathic effect was well established. Virus was recovered by 3 freeze thaw cycles followed by a 15 minute centrifugation at 1000 xg to remove cell fragments. Virus was then pelleted by centrifugation for 2 hours at 19,000 rpm at 4^0C, and the resulting pellet was re-suspended in DMEM containing 10%FBS, aliquoted, and frozen at -70°C.

Virus was titered by limiting dilutional analysis as previously described.[47] Briefly, stock suspensions were serially diluted (10^{-1} to 10^{-12}) and 100 μl inoculated into duplicate wells containing MDBK cells grown to approximately 75% confluence in 48 well plates. Plates were visually monitored for cytopathic effect daily for 7 days. The tissue culture infective dose was determined to be the average of the highest dilutions that resulted in cytopathic effect.

Effect of Virus Infection on Nitric Oxide Production by Bovine Alveolar Macrophages

In these experiments, macrophages were infected with virus for 24 hours prior to stimulation for NO· production. Viral inoculum ranged from 1 viral particle per macrophage (multiplicity of infection of 10^0; moi 10^0) to 1 particle per 10^6 macrophages (moi 10^{-6}). After an additional 48 hours of culture, cell viability and nitrite accumulation in culture supernatant, as an indicator of NO· generation, were measured. Controls included both virus-infected and sham-infected macrophages, which were unstimulated, activated and co-cultured with the NOS inhibitor aminoguanidine, or cultured with aminoguanidine alone.

Effect of Nitric Oxide Production on Viral Proliferation in Bovine Alveolar Macrophages

In these experiments, macrophages were cultured for 24 hours with stimuli known to cause activation and induce NO· production. Controls included unstimulated macrophages and activated macrophages cocultured with aminoguanidine. Following the initial incubation, macrophages were infected

with virus at a moi of 10^{-1} and viral titers determined after an additional 48 hours in culture.

Nitrite Assays

Nitric oxide production was estimated by measuring nitrite in cell free supernatants by the Griess reaction.[48] In this assay, nitrite reacts with the Griess reagent to form a purple dye.[48] Briefly, 100 μl Griess reagent (aqueous solution of: 0.5% sulfanilamide, 0.05% naphthylethylene-diamine dihydrochloride, & 2.5% phosphoric acid; Sigma) was added to an equal volume of supernatant in 96 well flat bottom microtiter plates (Costar, Cambridge, MA). Plates were incubated at room temperature for five minutes. Absorbance at 550 nm was measured with a microplate reader (Bio-Tek; Winooski, VT). A standard curve for each assay was generated using dilutions of sodium nitrite (Sigma) in DMEM in the concentration range 0-100 μM.

Cytotoxicity Assay

Cytotoxic effect of treatments on macrophages was assessed using the MTT assay as described.[49] In this assay, mitochondrial dehydrogenases in viable cells reduce the tetrazolium salt, [3(4,5-dimethyl thiazole-2-yl)2,5 diphenyltetrazolium bromide] (MTT) to a purple formazan, which can be measured by absorbance at 570 nm. Briefly, 25 μl of MTT solution (5mg/ml in PBS, Sigma) is added to cell wells and incubated at 37 C° for 4-6 hours. Following incubation, the supernatant is aspirated and 100 μl of acidified 2-propanol (0.04N HCl in isopropyl alcohol) and 20 μl of sodium dodecyl sulfate (3% w/v in water) are added to each

well to disrupt cells and solubilize the colored product. The contents of each well are mixed by aspirating up and down with a micropipette and the plate contents mixed on an orbital shaker for 5-10 minutes. Absorbance is measured on a microplate reader (Bio-Tek; Winooski, VT) at 570nm. Determinations were performed on duplicate wells.

Statistical Analysis

Where applicable, differences between treatments were compared by repeated measures ANOVA and student's t-test. Linear relationship between selected data pairs was assessed by regression analysis. A p value of <0.05 was considered significant.

Results

Exposure of uninfected macrophages to IFN-γ and LPS resulted in a significant increase in nitrite accumulation in the media (Figs. 21-23). Nitrite concentration was decreased to basal levels by inclusion of the NOS inhibitor, aminoguanidine (Fig. 21). Infection of macrophages by all three viruses in the absence of stimulus for NO· production resulted in a small, but statistically insignificant increase in nitrite (Figs.21-23). Infection by all three viruses at moi of 10^0 significantly decreased nitrite accumulation in response to IFN-γ and LPS (Figs. 21-23). Nitrite accumulation in virus infected macrophages increased as the moi decreased (Figs. 21-23). Nitrite accumulation reached levels not significantly

different than that in uninfected, stimulated macrophages at a moi of 10^{-4} for BHV (Fig. 21) and 10^{-1} for BVD (Fig. 22) and PI3 (Fig. 23).

Treatment of macrophages for NO· production resulted in a statistically insignificant decrease in viability (Figs. 21-23). Viability was not affected by co-treatment with a NOS inhibitor (data not shown). Infection of macrophages by BHV and BVD at high infective doses resulted in significant loss of viability when compared to uninfected macrophages, regardless of whether or not they were stimulated for NO· production or exposed to a NOS inhibitor (Figs. 21 & 22). No significant effect of infection by PI3 on viability was identified in any treatment (Fig. 23). Changes in nitrite production by infective dose was linearly related to loss of viability in macrophages infected with BVD (r=.9959)(Fig. 22). A similar, but statistically insignificant, trend was apparent in BHV infected macrophages (r=.7975) (Fig. 21).

Infection of macrophages by all three viruses in these experiments was confirmed by positive immunostaining with virus specific fluorescent antibody. Viral antigen and related fluorescence was most prominent in macrophages infected with high doses of BHV and BVD. Very few cells displayed positive immunostaining at low moi with any of the viruses (data not shown).

In experiments designed to assess the effect of prior stimulation of macrophages for iNOS induction prior to viral infection, i.e., whether or not stimulation alters viral infectivity or replication, no statistically significant treatment differences in viral titers were documented. However, in all experiments there was

a trend for the highest viral titers in control macrophages, intermediate titers in cells co-treated with NOS inducing stimuli and NOS inhibitor, and lowest titers in macrophages receiving stimuli for iNOS induction alone (Figs. 24-26). The data in figure 25 (BVD) appeared provocative, but was nevertheless statistically insignificant. Of the three viruses, BHV produced the lowest titers. Viable BHV was not recovered in one or more treatments in some experiments and viral titers were never higher than 10^2 (Fig. 24). In contrast, BVD and PI3 were readily recovered from macrophages cultured for 48 hours in all treatments, with titers in the range $10^{3.7}$ to $10^{4.7}$ viable viral particles per milliliter (Figs. 25 & 26).

Discussion

Nitric oxide production has been shown to limit replication of taxonomically diverse viruses both *in vivo* and *in vitro*.[43] As might be expected with actions on diverse agents, NO· limits viral replication by diverse mechanisms including inhibition of protein,[44;50] RNA,[44] and DNA[50] synthesis and nitrosylation of viral structural proteins.[43] Production of NO· is associated with tissue injury in some viral respiratory infections.[41;42] Because NO· production is one of the key activation pathways in macrophages, virus infection of macrophages is known to produce defects in macrophage function, and alveolar macrophages play a central role in defense of the lower respiratory tract, this study was designed to assess: i) the effects of virus infection on the ability of bovine alveolar macrophages to elaborate

NO·, and ii) determine if induction of iNOS influences viral replication in bovine alveolar macrophages.

Key findings include: i) BHV, BVD and PI3 infection of macrophages restricts their ability to elaborate NO·, ii) the decrement in NO· production in BHV and BVD infected macrophages is associated with a loss of macrophage viability, iii) PI3 infection decreases NO· production without affecting macrophage viability, and iv) induction of NO· does result in a statistically significant restriction of the ability of these viruses to replicate in bovine alveolar macrophages.

The restriction of NO· production by BHV and BVD infected macrophages appears to be due to loss of macrophage viability. Cytolytic infection of bovine macrophages by these viruses has been demonstrated previously,[8;51] and in the case of BVD infection is known to be due to initiation of apoptosis.[39] Loss of viability in BHV infected macrophages may be due to viral cytolysis, however initiation of apoptosis may have contributed to cell death and stimulation for NO· production may have promoted this pathway.[48] Herpes virus infection of macrophages is known to induce production of interferons.[11;52] IFN-α is one of the interferons produced and both IFN-α and culture supernatants from BVD-infected macrophages prime uninfected macrophages for activation-induced apoptosis.[37;38] Additionally, apoptosis has recently been demonstrated as the method of cell death in Herpes simplex infected murine macrophages.[53] Although not statistically significant, loss of viability of BHV infected macrophages stimulated for iNOS expression was higher than that of unstimulated macrophages infected with the

same viral inoculum (moi 10^0), suggesting that both activation signals and viral infection contribute to loss of viability in this model system.

PI3 is also known to cause cytolytic infection of macrophages, but cytolysis develops after 3 or more days in culture at high infective doses.[8] The modest cytotoxicity noted after 72 hours of infection in the work reported here is consistent with that time course. The nature of the alteration leading to decreased NO· production by PI3 infected macrophages is unknown. Assessment of iNOS mRNA and protein expression in PI3 infected cells at various time points would be useful future experiments in describing the functional defect induced by virus infection, and investigation of signal transduction pathways may also yield mechanistic insight. Virus infection of macrophages is known to alter the activation signals required and signal transduction pathways associated with defensive responses, including expression of iNOS.[54-56] Currently, only a single study of this type is reported using bovine macrophages. In this work, NO· production was enhanced in bovine macrophages infected with noncytopathic BVD, but the mechanistic basis was not investigated.[34] PI3 infection of bovine macrophages is known to cause depletion of a calcium-independent Protein Kinase C pathway which is associated with depressed oxygen radical generation.[19] This mechanism is thought to underlie the decrement in bactericidal activity demonstrated in PI3 infected bovine macrophages.[18] Although there is a growing body of knowledge addressing signaling pathways leading to iNOS expression in a variety of cell types,[57;58] relatively little work has been done in bovine macrophages.[59] Studies addressing the signal transduction pathways utilized by bovine macrophages in the expression

of iNOS and alterations of these pathways by viral infection present an opportune target for advancing our understanding of the pathogenesis of the bovine respiratory disease complex.

The inability of prior stimulation for NO· production to limit viral replication in macrophages suggests that NO· may not be an important infection-resistance auto-effector for macrophages. Although there was a trend toward decreased replication in macrophages stimulated for NO· production, this was not statistically significant. An experimental approach utilizing permissive, virus-infected MDBK cells and a dose-range of NO· spontaneously generated by chemical compounds accompanied by parallel experiments using infected MDBK cells co-cultured with macrophages stimulated for NO· production may prove useful in future studies for determining the potential role of NO· production in limiting viral replication.

The inability to demonstrate altered permissiveness in macrophages activated by LPS and IFN-γ is problematic because the antiviral function of interferons, including IFN-γ added as an activation stimulus in this experiment, are well known.[60] A critical assessment of the experimental protocol suggests experiments using a longer time course are indicated. BVD and PI3 infected macrophages generate rising titers within 2-7 days of infection, *in vitro*.[8] PI3 and BVD were readily recovered in the work reported here, although titers suggested only minimal replication had occurred during the incubation period. An incubation period longer than the 48 hours used in this study may be necessary to detect differences between treatments.

Alveolar macrophages support replicative infection of BHV, however titers in the work reported here and in previous investigations were low.[8;10] The vast majority of BHV infected macrophages were positive for viral antigen by fluorescent antibody in this study, but earlier studies reveal that although virus infection is widespread, only 4-5% of infected macrophages produce infectious centers at a moi of 10^{-1}.[10] Similarly, mouse macrophages infected by Herpes simplex display widespread infection which is accompanied by significant cytolysis without generation of significant titers.[53] Thermal decay of BHV in culture[8] at 37°C as well as exposure to lysosomal enzymes from devitalized macrophages may also have limited virus recovery from macrophages. An experimental protocol similar to the one used in this work has been successful in demonstrating NO· mediated restriction of herpes virus replication in the murine macrophage cell line, Raw 264.7,[45] however, an approach using a permissive cell line as outlined above is suggested as a more promising approach to the question of whether or not NO· can restrict replication of bovine respiratory viruses, *in vitro*.

Summary

This work adds restriction of NO· production to the list of functional defects produced by infection of bovine macrophages with respiratory viruses. The question regarding the ability of macrophage-generated NO· to restrict virus replication remains unresolved. The potential consequences of a virus-mediated decrement of macrophage NO· production in the bovine respiratory disease

complex are conjectural because, to date, an antibacterial function of bovine macrophage generated NO· has not been demonstrated.[61;62] In addition, NO· mediated pulmonary damage is increased in viral pneumonias caused by influenza and herpes virus,[41;42] and both positive and negative effects of NO· production are documented in models of experimental lung injury induced by endotoxin.[63;64]

In the following chapter an attempt will be made to place the role of NO· production by bovine macrophages in perspective and future studies will be suggested.

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Appendix

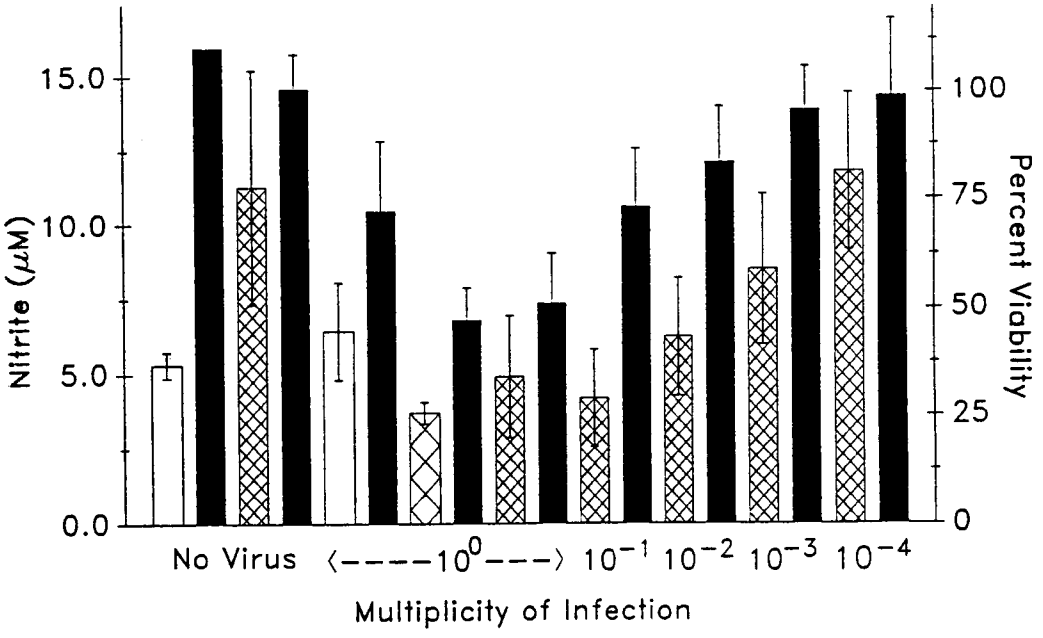

Figure 21. Effect of BHV infection on NO· production and viability of bovine alveolar macrophages. Open bars indicate macrophages received no stimulus for NO· production. Narrow crosshatch indicates treatment with rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}). Wide crosshatch indicates stimuli include IFN-γ, LPS and Aminoguanidine (2 mM). Macrophages were infected with virus at the multiplicities of infection (moi) indicated. Viability of macrophages exposed to each experimental condition is indicated by the closed bar to the right. Data are presented as the means and standard deviations from five experiments.

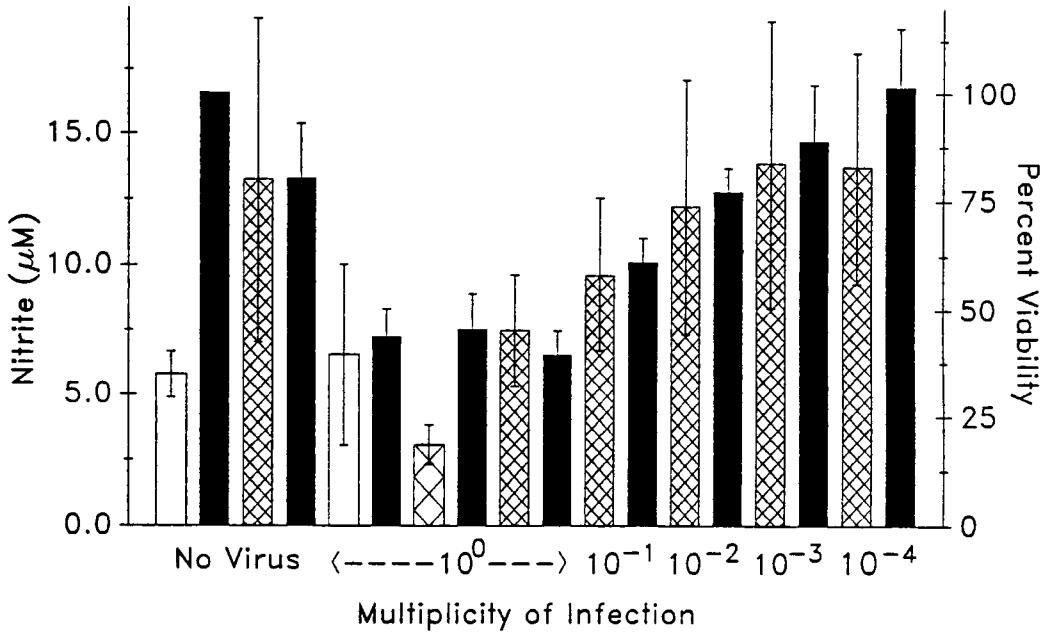

Figure 22. Effect of BVD infection on NO· production and viability of bovine alveolar macrophages. Open bars indicate macrophages received no stimulus for NO· production. Narrow crosshatch indicates treatment with rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}). Wide crosshatch indicates stimuli include IFN-γ, LPS and Aminoguanidine (2 mM). Macrophages were infected with virus at the multiplicities of infection (moi) indicated. Viability of macrophages exposed to each experimental condition is indicated by the closed bar to the right. Data are presented as the means and standard deviations from four experiments.

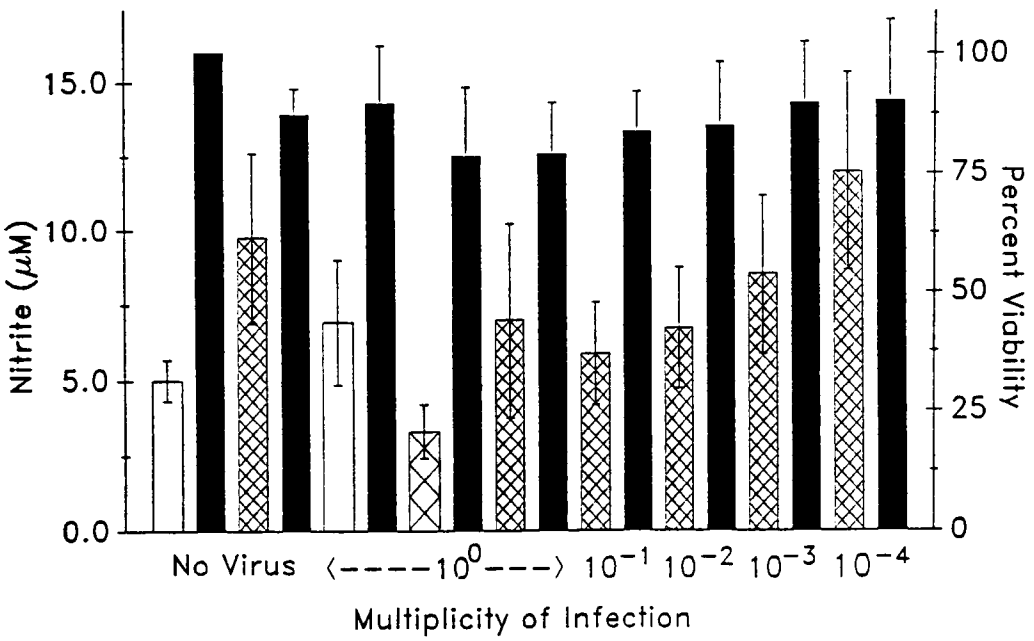

Figure 23. Effect of PI3 infection on NO· production and viability of bovine alveolar macrophages. Open bars indicate macrophages received no stimulus for NO· production. Narrow crosshatch indicates treatment with rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}). Wide crosshatch indicates stimuli include IFN-γ, LPS and Aminoguanidine (2 mM). Macrophages were infected with virus at the multiplicities of infection (moi) indicated. Viability of macrophages exposed to each experimental condition is indicated by the closed bar to the right. Data are presented as the means and standard deviations from five experiments.

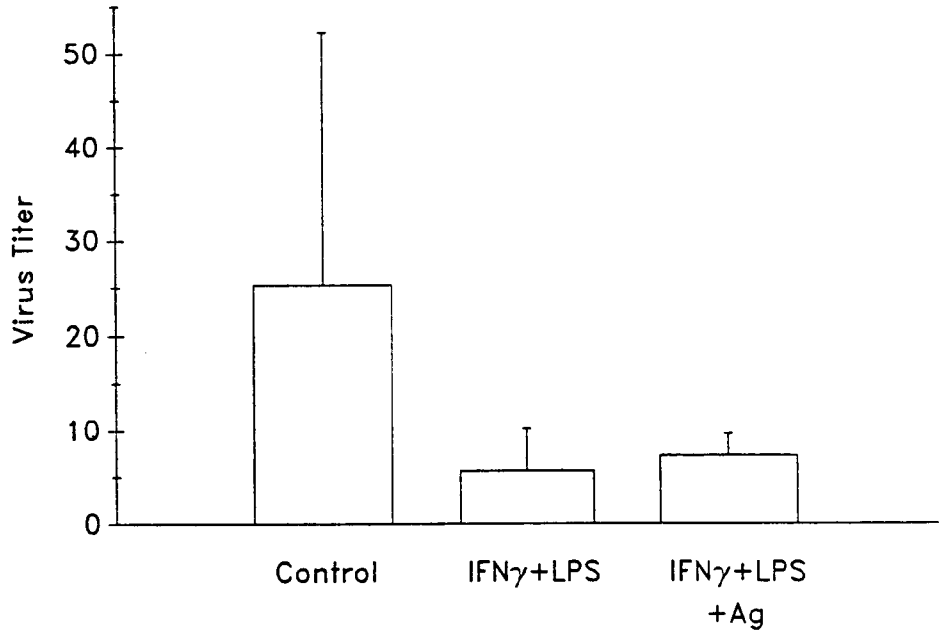

Figure 24. Titer of BHV recovered from macrophages stimulated for NO· production. Treatments include: Control, DMEM alone; rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}); rbIFN-γ, LPS and Ag (aminoguanidine,2 mM). Data are presented as the means and standard deviations of five experiments.

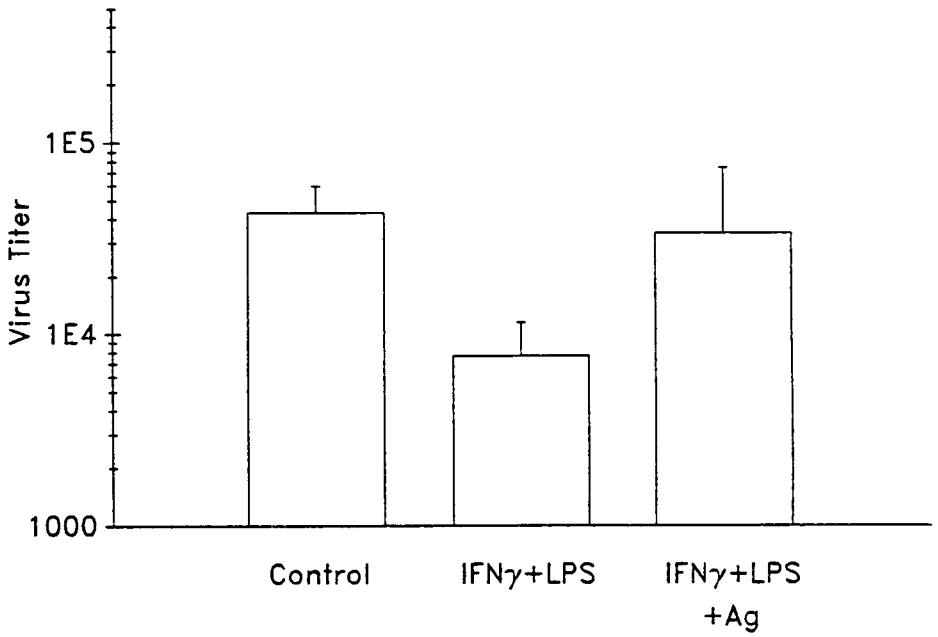

Figure 25. Titer of BVD recovered from macrophages stimulated for NO· production. Treatments include: Control, DMEM alone; rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}); rbIFN-γ, LPS and Ag (aminoguanidine,2 mM). Data are presented as the means and standard deviations of six experiments.

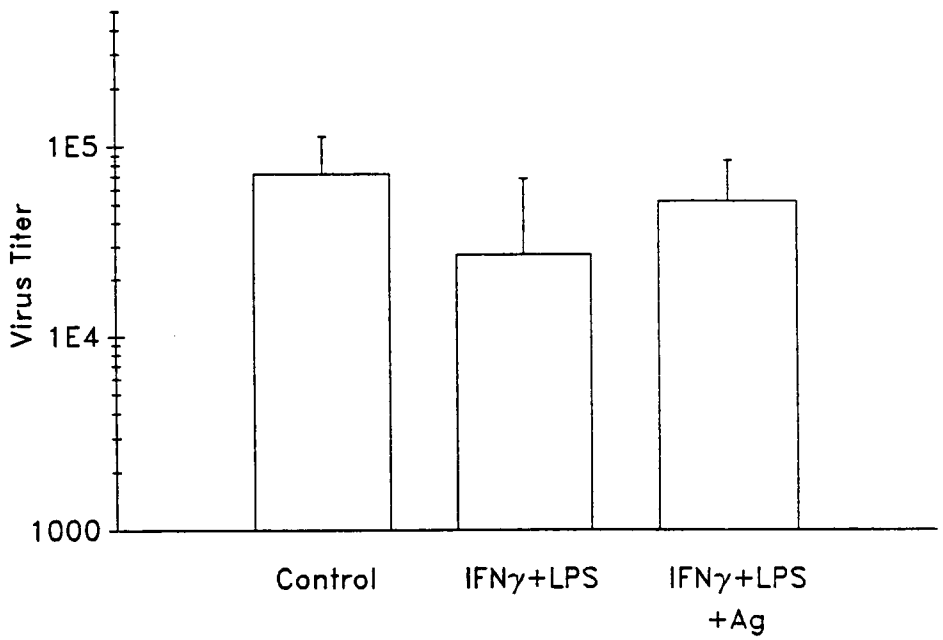

Figure 26. Titer of PI3 recovered from macrophages stimulated for NO· production. Treatments include: Control, DMEM alone; rbIFN-γ (100 U ml^{-1}) and LPS from *E. coli*, O55:B5 (10 ng ml^{-1}); rbIFN-γ, LPS and Ag (aminoguanidine,2 mM). Data are presented as the means and standard deviations of five experiments.

PART 5

Summary and Future Directions

Relevance of Macrophage Derived Nitric Oxide Production in Defense of the Bovine Lung

Inducible nitric oxide synthase is readily expressed in bovine alveolar macrophages exposed to stimuli known to exist in pneumonic tissue,[1] and iNOS expression has been demonstrated immunohistochemically in lung lesions from cattle with pneumonic pasteurellosis.[2] Clearly, iNOS expression by alveolar macrophages is part of the host response in pneumonic pasteurellosis, but the role and significance of NO· production in BRD remains conjectural.

An expanding literature describes the diverse targets and actions of NO· in infectious and inflammatory disease and documents numerous settings in which NO· production is found to be either critically important, irrelevant, or deleterious.[3] This information is derived from many different model systems and provides a broad and ever deepening database from which to extrapolate to the *in vivo* situation, however species-specific differences and *in vitro* versus *in vivo* modeling have resulted in numerous instances where data vary widely, serving as a reminder that the importance and role of NO· production in a specific setting cannot always be predicted. Central to this literature and of critical interest in regard to BRD are two basic issues. The first is whether or not NO· production serves to limit pathogen replication and the second is what role NO· production plays in the host inflammatory response.

Although there are many pathogens whose growth is restricted by RNS either *in vivo* or *in vitro*,[4;5] limitation of pathogen replication in a NO· dependent

fashion by bovine alveolar macrophages was not demonstrated in the work presented here. In concert with our results, neither has bovine macrophage derived NO· been shown to kill or limit replication of the bovine pulmonary pathogen *Haemophilus somnus.*[6] Moreover, the only other bovine pathogen studied so far in a similar model system, *Mycobacterium avium* subspecies *paratuberculosis*, was found to be susceptible to killing by chemically generated RNS at high concentrations, but bovine mononuclear phagocytes could not control replication of this agent in a NO· dependent fashion.[7] Once again, these results are in concert with our findings. These results and their implications are particularly disheartening because both are intracellular pathogens and, in general, NO· mediated microbicidal activity is believed to be greatest against intracellular organisms.[4] Thus, to date, a microbicidal or microbiostatic action of bovine macrophage-derived NO· has not been demonstrated against any bovine pathogen. This is in contrast to the numerous reports of potent NO· dependent microbicidal activity of murine macrophages against diverse infectious agents both *in vitro* and *in vivo*.

The lack of demonstrable antimicrobial activity against important respiratory pathogens in the bovine model systems examined to date does not mean that NO· production in bovine pneumonia is irrelevant; nitric oxide production may well have other positive and negative effects *in vivo*. For instance, vascular injury is a prominent feature of the lesions in BRD and is a central component in the pathogenesis of pulmonary injury in this disease complex.[8] It is possible that high output production of NO· by bovine alveolar macrophages may protect the vasculature by deactivating reactive oxygen species,[9] limiting vascular

permeability,[10] inhibiting leukocyte adhesion[11-13] and limiting platelet aggregation.[14] Additionally, NO· production could act to limit the severity of the systemic inflammatory reaction by inducing apoptosis in leukocytes and other cells that are "over-stimulated".[15] On the other hand, NO· production may lead to death by promoting excessive nitrosative and oxidative damage, resulting in enhanced inflammation in the lung.[15] Additionally, it is easy to envision that NO· production by the high output iNOS isoform may contribute to septic shock, multiple organ injury and mortality.[16] Cattle that die of BRD often retain sufficient structurally normal lung tissue to maintain pulmonary function but have evidence of sepsis, suggesting actions of NO· as part of the systemic inflammatory response may contribute to mortality.

These speculative roles of iNOS and NO· in infectious pneumonia emanate primarily from rodent studies. A comparative approach based on studies in species more closely related to cattle is hampered by insufficient information in which mechanistic explanations are often lacking. Ovine alveolar macrophages can be induced to generate NO·[17] in much the same fashion as bovine cells, while caprine macrophages produce little NO· in response to the same stimuli.[18] All three of these species suffer epidemiologically and morphologically similar respiratory diseases due to infection by *P. haemolytica* A1, PI3, and respiratory syncytial virus.[19] It is not known if stimuli present in the pneumonic lung result in similar iNOS expression and NO· production in these high (ovine, bovine) and low (caprine) iNOS responding species, but the presence of similar disease expression

in closely related species with differing *in vitro* NO· induction patterns suggests NO· may not be critical for host defense against pneumonia caused by these agents.

Future Directions

Many of the questions concerning specific effects of iNOS expression and NO· production in infectious pneumonia can be readily addressed with *in vitro* studies, but animal studies are required to place the function of this mediator in perspective. The time-proven approach of comparing experimentally infected animals with and without NOS inhibition could provide an indication of whether or not NO· contributes measurably to host defense or injury in BRD. Unfortunately these studies would be quite expensive; e.g. $135.00 per hour to infuse 2 mg/kg N^G-monomethyl-l-arginine into a 50 kg calf. Obviously, the alternative approach of a classic genetic knockout or transgenic animal that is used to such great effect in rodent models is not readily applicable to a large animal with a comparatively low and slow reproductive rate. Other experimental approaches, such as Gadolinium depletion of macrophages, cannot be adequately controlled. A better understanding of macrophage activation, signaling pathways, and the molecular biology of iNOS expression in bovine macrophages may make functional knockout of macrophage iNOS expression by gene therapy-like approaches practical in the future and allow pursuit of animal studies.

At the population level, field based studies may reveal useful information. Arginine supplementation is known to enhance immune responsiveness and can

enhance NO· production *in vitro*.[20;21] Outbreaks of respiratory disease often follow periods when dietary intake is altered or disrupted. Changes in rumen ecology secondary to stress, periods of forced inappetance, and changes in diet are known to be critical factors in other diseases of cattle such as polioencephalomalacia[22] and atypical interstitial pneumonia.[23] The blood levels and availability of arginine stores in cattle whose diets are disrupted are not known. Collection of plasma arginine data from shipped, fed, sick and healthy cattle and correlation with evidence of NO· production (urinary or blood nitrate) and occurrence of respiratory disease may provide data implicating altered ability to produce NO· as a host factor in the pathogenesis of BRD. Correlation of blood and urinary nitrates as well as exhaled NO· with clinicopathologic and outcome data may help develop diagnostic and prognostic criteria useful in economically managing outbreaks of BRD in feedlots.

Apart from respiratory disease, there are numerous bovine diseases where investigation of the role of iNOS expression appears warranted. A few are suggested below.

Mastitis is one of the most economically important diseases of dairy cattle.[24] Macrophages are important somatic cells that migrate into mammary secretion to monitor and maintain health of the gland.[25] Cattle suffering gram negative mastitis are often septic[26;27] and display clinical and pathologic evidence of septic shock,[28] a disease state in which NO· is known to be a major mediator.[16] Despite the potential importance of macrophage-derived NO· production in bovine mastitis there has been no significant work addressing the role of NO· in this disease.[29]

In addition, a more critical assessment of the role of iNOS expression in resistance to *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle is indicated, particularly in light of the controversial link between this agent and Crohn's disease in man.[30;31] A genetic approach, although time consuming, expensive, and technically difficult is suggested. Undoubtedly, many cattle exposed to this bacterium do not become carriers or develop clinical disease. Identification of these individuals and assessment of their innate immune response pattern, including NO· production, may lead to an understanding of the genetic control of resistance to this agent and allow genetic improvement. The long incubation period, lack of accurate ante mortem diagnostic tests, widespread occurrence, impracticality of treatment, and potential public health concerns suggest genetic progress would be a valuable tool in the control or eradication of this agent in a herd.[24;32] A similar approach is suggested for *Brucella abortus*, although in this case resistant cattle are already identified.[33]

Another common disease condition in which characterization of NO· production is likely to yield useful information is assessment of nitrates in bovine abortions. Elevated nitrates are commonly encountered in the ocular fluids of bovine abortuses with documented inflammatory or infectious disease.[34] Nitrate concentrations in these cases are often markedly increased, overlapping with those encountered in abortions due to true nitrate intoxication. This is widely recognized by veterinary diagnosticians but remains undocumented in the literature, which produces uncertainty about the diagnostic significance of elevated nitrates in an aborted fetus. Studies designed to assess the competency of fetal and placental

tissues to express iNOS as well as documentation of the effect of gestational age and effective stimuli would be useful. Additionally, a critical assessment of the mechanisms responsible for nitrate-induced abortion in large animals is lacking[35] such that the contribution of RNS and terminal oxidation products (i.e. nitrate) in the pathogenesis of infectious abortion remains unknown.

There are many diseases of cattle in which assessment of iNOS expression and NO· production would add to our understanding of the importance of this mediator and it's role in disease. Definition of the role of this enzyme and it's product in specific diseases will provide knowledge that may lead to improved management, diagnostic and therapeutic strategies that promote the health, welfare, and productivity of cattle.

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Vita

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