University of Texas
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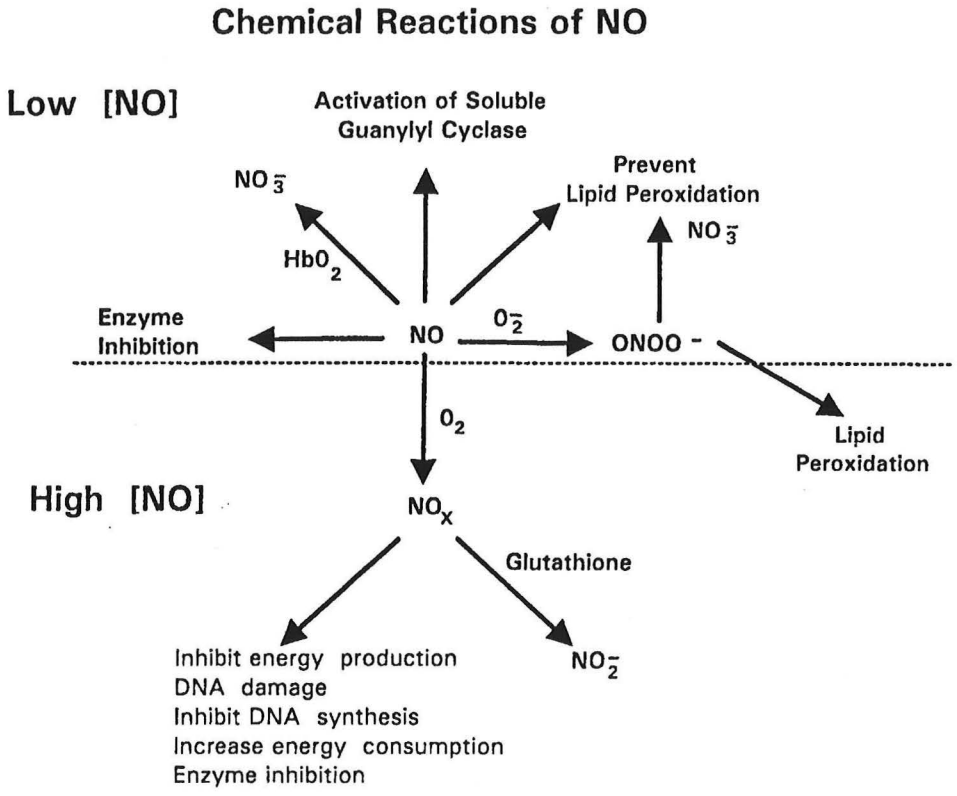
Nitric Oxide: Friend and Foe



Dr. Robert Alan Star
Medical Grand Rounds
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INTRODUCTION

Over the past 15 years, the gas nitric oxide (NO) has risen above its initial reputation as an environmental pollutant found in cigarette smoke and smog to a critically important molecule that has a myriad of regulatory actions throughout the body. By virtue of its complicated chemistry and biological activity, NO is a pleotrophic molecule that can kill cells, yet also protects cells from other toxins. Perhaps the most surprising aspect of NO is that it remained undiscovered for so long. This has been corrected. Research on NO initially increased at an exponential rate but has now stabilized at the rate of approximately 1400 articles per year (**Figure 1**). Since my Grand Rounds on NO in human vascular disease two years ago, the world's literature on NO has more than doubled. I will focus on 4 new areas of NO research: 1) evolving uses of NO, 2) trials of NO synthase inhibitors in inflammation, 3) why NO is both friend and foe, and 4) a new strategy to inhibit NO and inflammation which may be more successful than current NO synthase inhibitors.

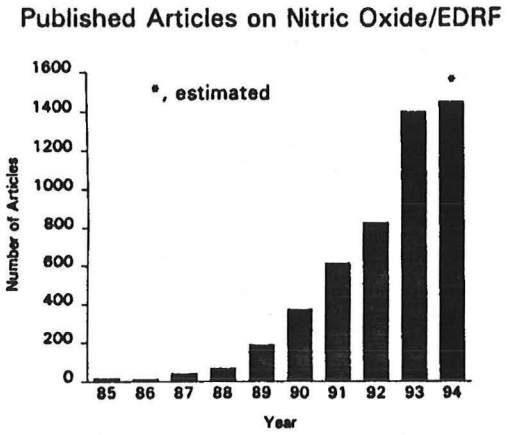


Figure 1

HISTORICAL OVERVIEW.

It has been known since 1916 that mammals produce nitrite. This observation was largely ignored until the 1980's when Tannenbaum found that urinary nitrate excretion was increased during infection, and Furchgott and Zawadzki reported the existence of an endothelium derived relaxation factor (EDRF) (1). In 1987, Hibbs found that macrophage cytotoxicity is dependent on converting arginine to nitrate or nitrite. In a completely separate line of investigation, Palmer, and then Ignarro, discovered that EDRF was NO or a closely related molecule (2-5). This served to join the inflammatory and vascular aspects of NO/EDRF research. In the early years it was discovered that NO has a multitude of functions including vasodilation, inflammation, neurotransmission and intracellular signalling. It was immediately obvious that either NO or NO inhibitors might be useful for treating disease, particularly inflammatory states. In 1991-3 the first three NO synthase (NOS) isoforms were cloned (6-10). The widespread distribution of NO and its importance in multiple signalling pathways led to this simple molecule being named "Molecule of the Year" by Science magazine in 1992. However, as is true in many areas of science, the NO field rapidly became filled with controversy and unanswered questions. For example: Is EDRF really NO? How does NO act? What is the chemistry of NO?

By 1991, studies began to suggest that NO also is cytoprotective, particularly for superoxide-induced injury. By 1992-3, the initial enthusiasm for using NO synthase inhibitors to treat stroke and inflammatory diseases was rapidly dampened when it became apparent that NO inhibitors could worsen disease. In 1994, several more NO synthase isoforms were cloned (11,12), and several drug companies gave up searching for NO synthase inhibitors. The reasons for this discouragement will be discussed.

The field is at a crossroad in 1995. Clever molecular biology techniques are being exploited to examine the structure, function, and regulation of the NO synthases (13-17). The chemical reactions of NO are better understood, but getting more complicated. However, pharmacologic options for manipulating the NO pathway and treating clinical disease seem to have stalled. Or have they? I will end in a hopeful note showing some of our present work on an alternative strategy to inhibit the synthesis of NO which may prove clinically useful.

PHYSIOLOGY OF NITRIC OXIDE.

Signalling cascade. NO carries out more important functions than virtually any other known messenger molecule. Because it possesses an extra electron, it is highly chemically reactive and has a short half life (< 5 seconds). The short half life indicates that the effects of NO are limited to a local site of action of approximately 200-600 microns. **Figure 2** shows a simplified schematic pathway of NO signalling system. NO is synthesized from L-arginine by NO synthase. NO diffuses within the cell or to an adjacent cell where it stimulates soluble guanylyl cyclase. The resultant increase in cyclic GMP in the target cell produces many of the physiologic effects of NO. NO also binds to the heme group of hemoglobin; NO is converted to nitrate. This serves to limit the biological effectiveness of vascular NO to cells in close proximity to the endothelial cell. NO can also interact directly with iron and heme-containing enzymes such as aconitase and ribonucleotide reductase. At higher concentrations, NO combines with molecular oxygen to form nitrogen oxide radicals (NOx) which chemically modifies cellular proteins, DNA, and cell membranes (see below).

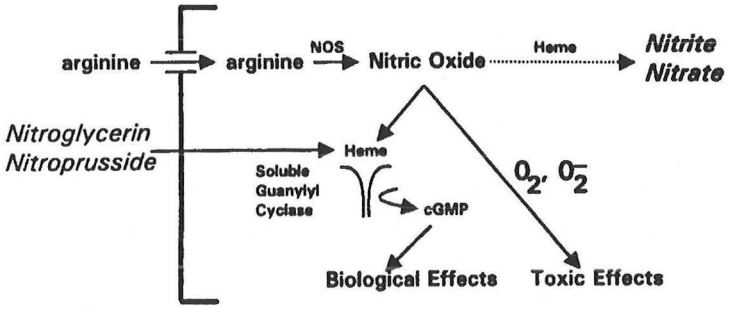


Figure 2

NO synthases. Currently, there are 5 isoforms of NO synthase named for the locations from which they were cloned. It is traditional to classify these isoforms as constitutive (unchanging enzyme abundance) or inducible. However, recent studies have shown that all the isoforms can be chronically regulated, so that the term constitutive is incorrect. **Figure 3** shows the differences in acute and chronic regulation of these isoforms. The so-called brain or neuronal isoform is found in neurons and in many cells throughout the body is acutely regulated primarily by cell calcium (6,18). There are now 3 highly homologous inducible isoforms (macrophage, vascular smooth muscle cell, and hepatic) whose synthesis is controlled primarily at the level of gene transcription by cytokines (7,9,12,19,20). These 3 isoforms are primarily responsible for NO produced during inflammation (see below). In collaboration with R. Tyler Miller in Nephrology, we found that inducible NOS can also be increased independent of cytokines in cells transfected with $G\alpha_{13}$ or activated $G\alpha_q$ (21). Activity of the hepatic isoform is also acutely controlled by cell calcium (10). The endothelial isoform is found in endothelial cells, and is primarily responsible for causing vasodilatation (22). It is acutely regulated by cell calcium, and chronically by alterations in blood vessel flow. Increases in flow increase the amount of NOS enzyme, leading to vasodilatation.

Regulation of Nitric Oxide Synthase Isoforms

#	Isoform	Acute	Chronic Regulation
I	Brain	Ca^{2+}	Hypoxia
IIm	Macrophage		Cytokines
IIs	Smooth Muscle		Cytokines
IIh	Hepatic	Ca^{2+}	Cytokines
III	Endothelial	Ca^{2+}	Flow, TNF

Figure 3

NO promotes organ blood flow. NO has several different modes of action. Its major mode of action is to promote organ blood flow (**Figure 4**). NO produced in endothelial cells diffuses to underlying vascular smooth muscle cells and causes vasodilatation. NO also inhibits platelet aggregation, preventing microthrombi (23,24). The NO is formed either within platelets or by nearby endothelial cells. NO acts locally to prevent platelet aggregation near the vascular wall; an effect on platelets in the center of the vessel is unlikely since NO is destroyed by hemoglobin. NO also inhibits neutrophil adhesion and neutrophil plugging (25). Finally, long term NO synthesis prevents intimal hyperplasia (26).

NO as messenger. NO also acts as a messenger both between cells and within cells. NO communicates between cells in its roles as vasodilator, neurotransmitter in the central nervous system and myenteric plexus of the gastrointestinal tract, and

modulator of tubular glomerular feedback (27-29). NO also acts as a second messenger inside platelets (24). We recently found that NO functions as an intracellular second messenger to prolong hormone action (**Figure 5**) in pancreatic, endothelial, renal epithelial and interstitial cells (21,30).

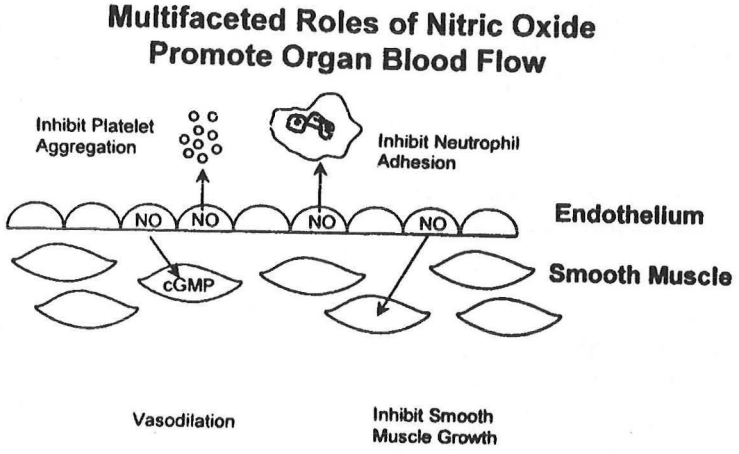


Figure 4

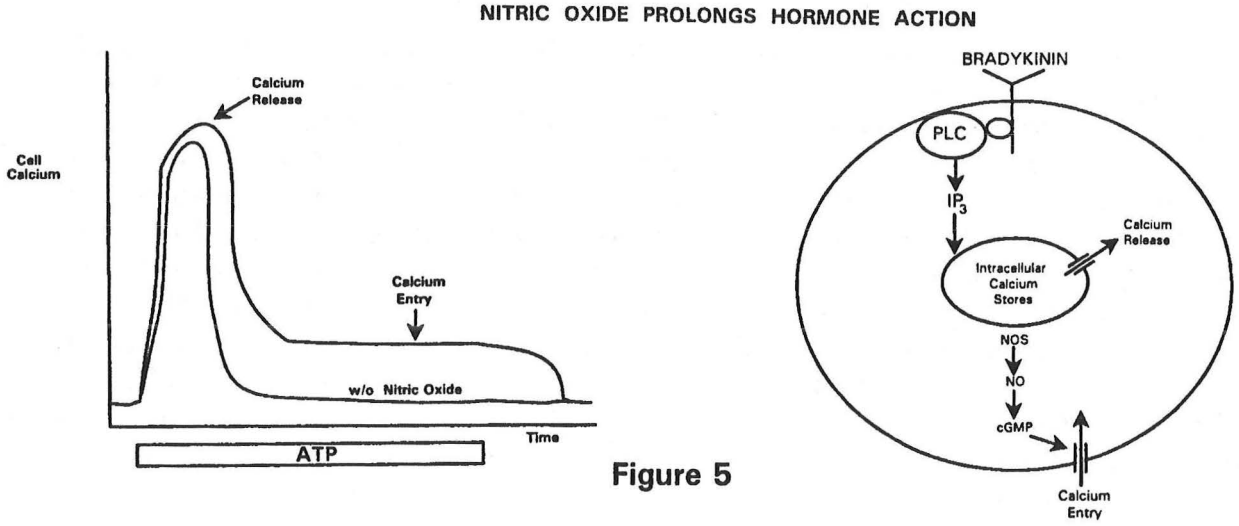


Figure 5

Stimulation with calcium mobilizing agonist causes a transient increase in cell calcium due to IP_3 mediated calcium release from intracellular stores. The higher cell calcium then leads to down stream physiologic events. Were calcium only to come from intracellular stores, depletion of the intracellular stores would cause a rapid loss in physiologic action. However, emptying of the intracellular calcium pools activates NO synthase, generating cyclic GMP, which then increases calcium entry in the plasma membrane. This increased calcium entry elevates cell calcium, allowing physiologic processes to proceed. When the agonist is withdrawn, NO remains elevated, allowing continued calcium entry to refill the intracellular stores in preparation for the next hormonal stimulation. Once the intracellular stores are full, NO synthesis shuts off.

NEWER USES OF NO

Organic nitrates (nitroglycerin) and nitroprusside are biotransformed to NO inside cells. These agents have been used to treat angina, myocardial infarction, and

some forms of congestive heart failure. This topic has been the subject of a recent Grand Rounds (31). The discussion will be limited to one established usage (inhaled NO) that is currently being evaluated in a large scale clinical trial, and two exciting new usages which are under evaluation in experimental animals.

Pulmonary hypertension / ARDS. NO is being used to treat pulmonary hypertension and ARDS in adults (32-35). Inhaled NO (5-20 parts per million) produces selective relaxation of the pulmonary vascular smooth muscle without dilating the systemic vasculature. A systemic effect is prevented because NO has a short half life, NO is rapidly inactivated by hemoglobin, and because the adventitial tissue of the lung is relatively impermeable to NO. **Figure 6** shows how NO can selectively vasodilate areas of the lung that are ventilated and thus improve the matching of ventilation to perfusion. Inhaled NO rapidly reverses pulmonary vasoconstriction due to hypoxia, congenital heart disease and persistent pulmonary hypertension in the newborn. Inhaled NO may be beneficial even in nonresponders because of its anti-inflammatory actions (see below).

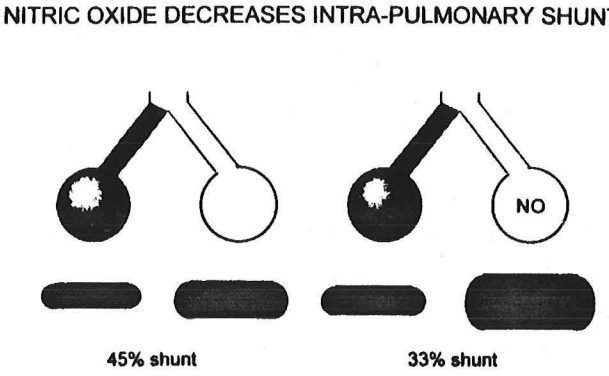


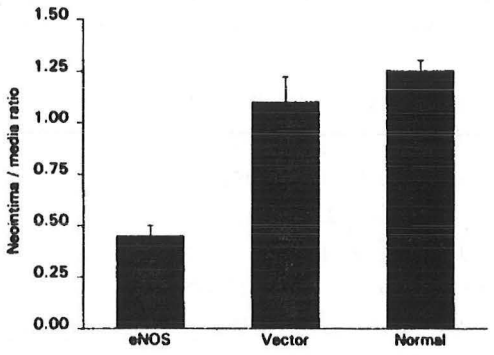
Figure 6

NO to prevent vascular restenosis. Restenosis following angioplasty is a major clinical problem, occurring in 12-43% of patients, and thus serves as the 'Achilles heal' of angioplasty (36,37). Restenosis and vascular injury have been reviewed in two recent Grand Rounds by Drs. Richard Lange and Robert Meidell (38,39); only a summary will be presented here. Restenosis is a complex process involving injury to the endothelium, exposure of structural components of the arterial wall, and release of thrombogenic, vasoactive, and mitogenic factors. The end result is an abnormal migration and proliferation of vascular smooth muscle cells in the intima (neointimal proliferation) which impedes blood flow through the vessel. In addition to the obvious structural alterations, there are functional alterations including endothelial cell dysfunction with decreased NO release and action. NO might inhibit vascular injury since NO inhibits platelet aggregation, leukocyte adhesion and vascular smooth muscle proliferation and migration *in vitro*. Supplementation of arginine to hypercholesterolemic rabbits inhibited atherosclerosis and endothelial cell function, whereas NO inhibitors accelerate atherosclerosis (40,41).

Dzau and coworkers tested whether NO is an effective inhibitor of neointimal hyperplasia *in vivo* using a rat carotid artery balloon injury model, which replicates

some but not all of the features of human restenosis (42). The vascular endothelium was denuded by balloon injury, and endothelial NOS expression was restored using a highly efficient Sendai virus/liposome *in vivo* gene transfer technique into vascular smooth muscle cells. Vessel NO production was restored to that of normal untreated vessels. Neointimal formation at 14 days after balloon injury was inhibited by 70% (**Figure 7**). Similar results have been obtained by perfusing the artery with a long-acting NO agonist at the time of balloon injury (J. Stamler, Duke University). This study shows that NO inhibits vascular lesions *in vivo*, and shows the feasibility of using gene therapy to treat vascular disorders. Unfortunately, success in rat and rabbit models does not predict success in preventing human restenosis. Nevertheless, this suggests that either long acting NO or a gene transfer technique might be a therapeutically useful approach to prevent restenosis in humans.

Gene Therapy with Endothelial NOS Reduces Neointimal Formation after Balloon Injury



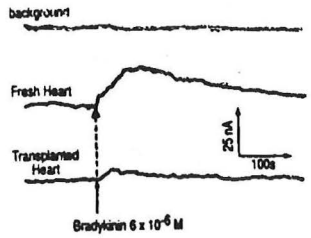
von der Leyen, et al. PNAS, 1995

Figure 7

NO to improve organ transplantation. Successful vascular organ transplantation depends on surmounting the hurdles of survival outside the body (cold ischemia time) and prevention of subsequent immunologic destruction. The heart and lungs are particularly susceptible to ischemia/reoxygenation injury. Cold ischemia times longer than 3-4 hrs cause organ dysfunction which is manifested by atrial arrhythmias and right atrial dysfunction (heart) or elevated vascular resistance and poor gas exchange (lung). Since prospective immunologic crossmatching can not be performed during this window, there is an increased rate of immunologic rejection and accelerated coronary artery atherosclerosis. The short cold ischemia time also prevents dissemination of available organs on a more equitable basis. Ischemia/reperfusion injury may also be important cause of long-term organ dysfunction mediated by alterations in cytokines, adhesion molecules, etc. Current preservation strategies have involved use of organ preservation solutions, and methods to prevent oxygen free radical injury to the blood vessels and organ parenchyma. Therefore, transplantation could be improved by lengthening the perfusion time and limiting perfusion damage.

Two recent studies demonstrated that nitric oxide levels in the affluent of preserved heterotopically transplanted hearts and lungs fall dramatically as the cold perfusion time increases (43,44) (**Figure 8**). This is associated with coronary artery endothelial dysfunction and less vasodilator-induced NO release. Surprisingly, NO

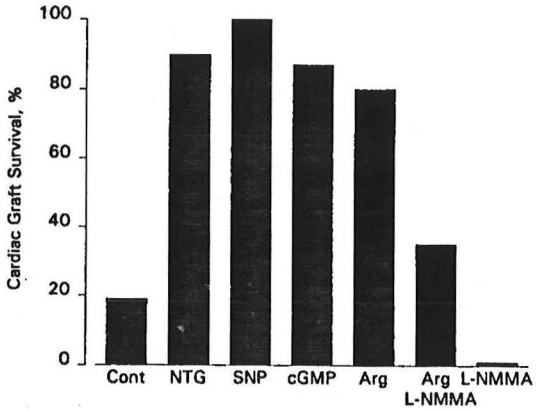
NO Response to Bradykinin is Decreased in Preserved / Transplanted Hearts



Pinsky, et. al. J.Clin. Invest., 1994

Figure 8

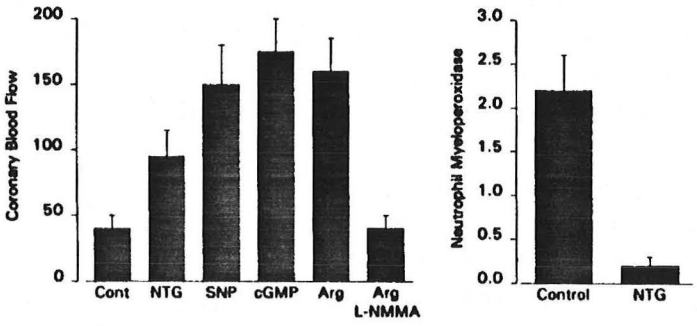
Role of NO Pathway in Enhancing Cardiac Graft Survival



data from Pinsky, et al, JCI, 1994

Figure 9

Effect of NO Pathway on Graft Blood Flow and Leukostasis



data from Pinsky, et al, JCI, 1994

Figure 10

production is enhanced, but NO concentrations remain low because of increased superoxide formation which destroys NO. Supplementation of the NO pathway with either nitroglycerin, sodium nitroprusside, arginine or cyclic GMP dramatically increased graft survival from 20% to 90%, whereas inhibition of NO synthase worsened graft survival (**Figure 9**). Addition of nitroglycerin to the University of Wisconsin solution, the standard clinical perfusion solution, enhanced survival of transplanted organs. Baboon hearts were preserved successfully for an unprecedented 24 hr using several cold storage and supplemented with nitroprusside.

The mechanism of enhanced survival may involve NO stimulation of blood flow and decreases in neutrophil infiltration (**Figure 10**); neutrophil plugging may contribute to the no-reflow phenomenon. Augmentation of the NO pathway at either level of NO of cyclic GMP may provide a clinically useful approach to normalize organ function at the critical early stages following organ transplantation, and hopefully will also prevent longer term damage to transplanted organs.

OVERPRODUCTION OF NO IN INFLAMMATION

Evidence for increased NO production. Figure 11 lists the clinical states in which overproduction of NO has been thought to be pathogenic in causing disease (45). Because of the wide range and large numbers of disorders, discussion will be limited to arthritis, glomerulonephritis, and sepsis. Sepsis increases blood and urine nitrite and nitrate, two stable end products of NO metabolism (46-49). In localized inflammations such as arthritis and glomerulonephritis, nitrate/nitrite have been detected in joint fluid and glomerular tissues (50-52). In all of these states, molecular techniques have shown increases in inducible NOS mRNA and protein (53-55). In addition, nitrated proteins have been detected by anti-nitrotyrosine antibodies in blood, joint fluid, and inflammatory tissues (51). NO is also increased in ischemic stroke, and is thought to enhance neurotoxicity (56-58).

Overproduction of Nitric Oxide

- Systemic inflammation
  endotoxemia, IL-2 chemotherapy
- Local inflammation
  arthritis, glomerulonephritis,
  organ rejection
  ? aortic aneurysms, atherosclerosis
- CNS
  stroke, neurodegenerative diseases

Figure 11

Cellular mechanisms of NO and NOx toxicity. NO by itself is not toxic to most cells; however, NO reacts with oxygen to form unidentified reactive nitrogen oxide free radicals (NOx) which are cytotoxic (59) (**Figure 12**). NO and NOx inhibit heme and iron containing enzymes that are responsible for mitochondrial respiration (aconitase, etc.) and DNA synthesis (ribonucleotide reductase). NO also decreases protein synthesis. High level of NO induce strand breaks by deadenation of double stranded DNA and also by a direct chemical tract on single stranded DNA. Repair of these strand breaks stimulates poly-ADP ribose synthase (PARS) (60,61). This enzyme requires 4 ATP molecules per base repaired and causes energy depletion in cultured cells. NO also chemically modifies the cysteine and tyrosine amino acids in proteins,

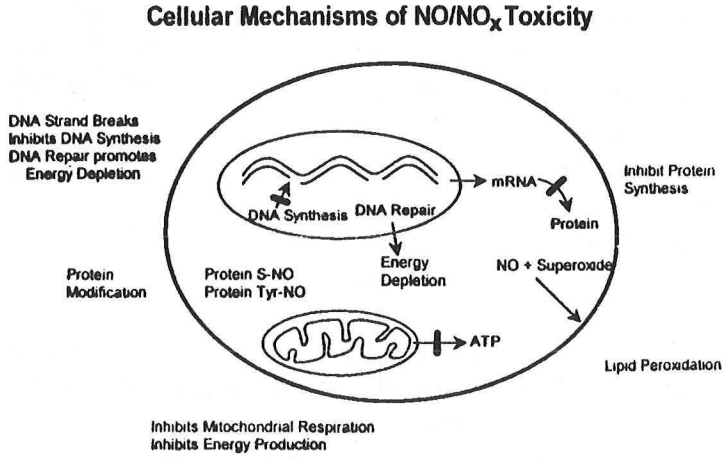


Figure 12

forming S-nitrosylated proteins and nitrated proteins, respectively (62,63). S-nitrosylation can inactivate proteins (for example, NMDA receptor, GAPDH, protein kinase C, phosphotyrosine protein phosphatase (64)). Peroxynitrite, the product of NO and superoxide metabolism, can cause lipid peroxidation (65). Thus, NO and its metabolites can cause cell death by a variety of mechanisms.

Since NO is cytotoxic and mediates some of the pathophysiological events associated with inflammation, then inflammation should be suppressed by NO antagonists. But nature is not so simple. We will focus on the effects of NO synthesis inhibition in arthritis, glomerulonephritis, and sepsis.

NO-Mediated Oxidative Damage in Arthritis

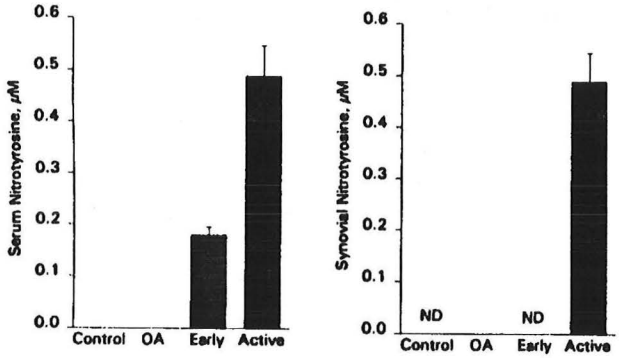
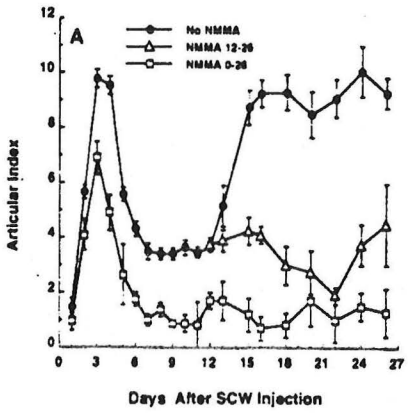


Figure 13

NOS inhibition in arthritis. Because NO has a short half life, any NO involved in arthritis joint injury must be synthesized locally (50). NO can be synthesized by capillaries, infiltrating leukocytes, resident mesenchymal cells, chondrocytes, synovial fibroblasts and other cells. Nearly all of these cells can be induced to produce high levels of NO by inflammatory cytokines. NO is synthesized in arthritic joints as evidenced by accumulation of nitrite/nitrate in human synovial fluid. The 'footprint' of NO, nitrotyrosine, is increased in inflammatory arthritides (51) (**Figure 13**). NO is clearly involved in arthritic inflammation.

Suppression of Arthritis by L-NMMA



McCartney-Francis, et al., J. Expt. Med., 1993

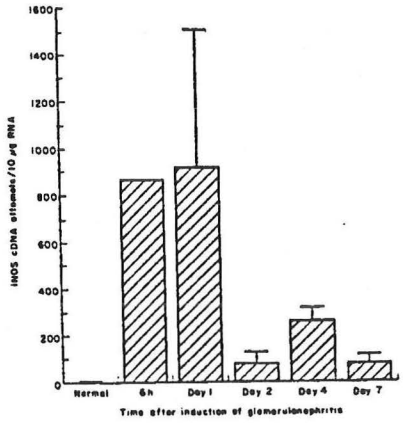
Figure 14

NOS inhibitors given systemically inhibit arthritis in several animal models of inflammation including immune complex arthritis, carrageenan dermatitis, and arthritis, and an arthritis caused by injection of streptococcal cell wall fragments (54,66-68). The later model is characterized by a biphasic pattern of tissue destruction caused by acute inflammation of the joints which is followed by a chronic, T cell- and monocyte- meditated erosive arthritis (**Figure 14**). The arginine analogue L-NMMA reduced inflammation and tissue damage even when its administration was delayed until after the onset of symptoms. This is one of the few success stories of NOS inhibitors; showing that NO promotes

inflammation in inflammatory arthritis.

NOS inhibition in glomerulonephritis. Glomerulonephritis is the third most common cause of end stage kidney disease leading to dialysis or transplantation, in part, because it is not easily treated. Several forms of glomerulonephritis are caused by infiltration of the kidney with inflammatory cells, including post-infectious glomerulonephritis, Goodpasture's disease, and crescentic glomerulonephritis. Glomerulonephritis is an immune-mediated disease in which neutrophils and macrophages cause glomerular injury. The precipitating event is usually the intraglomerular deposition of immunoglobulin, which triggers the elaboration of an array of chemoattractants.

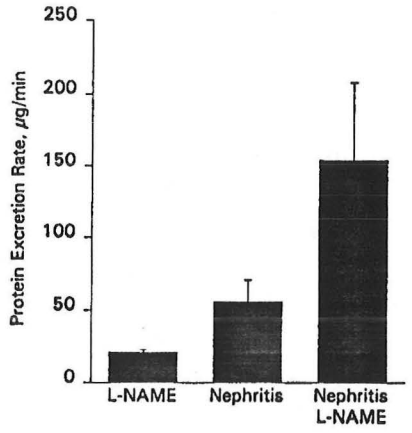
Levels of Indicible Nitric Oxide Synthase in Rat Anti-GBM Glomerulonephritis



Cook et al., Clin. Expt. Immunol, 1994

Figure 15

NO Inhibition Worsens Proteinuria During Anti-GBM Nephritis in Rats



Munger, et al. JASN, 1994

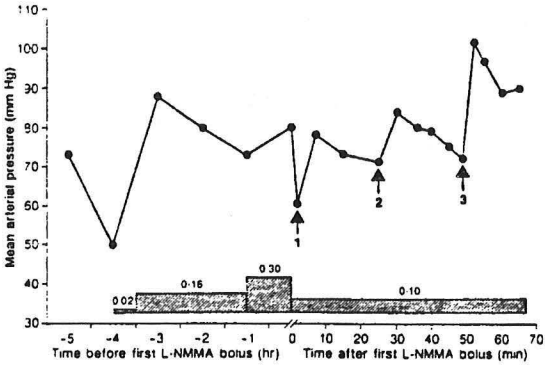
Figure 16

Most of our current knowledge about glomerular injury has been gained through study of immune mediated experimental models which reproduce the histologic features of human disease (69). The initial phase consists of infiltration by neutrophils. After 4-6 hrs, the glomeruli are infiltrated by macrophages, which produce most of the glomerular injury (70). NO synthesis and expression of the macrophage isoform of NO synthase is increased in nephritic glomeruli (52,55,71) (**Figure 15**). The role of NO in mediating glomerular damage during glomerulonephritis is unknown. L-NMMA decreases proteinuria in lupus nephritis (66). However, recent studies have shown that acute NO inhibition increases proteinuria in normal and nephritic rats (72,73) presumably by altering renal hemodynamics, although neutrophil infiltration is enhanced (74) (**Figure 16**). Chronic NO inhibition causes proteinuria, histologic damage, hypertension, mononuclear cell infiltration, and decreases survival

in rats (75). Thus, inhibition of NO synthase in glomerulonephritis promotes renal injury, probably by both immune mediated pathways and by increasing blood pressure.

NOS inhibition in endotoxemic shock. NO production in macrophages is tightly regulated, requiring two different signals to activate NO production. Macrophages require both a global stimulus (endotoxin) and a local stimulus (usually TNF) before they will produce large amounts of NO. LPS injection induces the synthesis of an inducible macrophage isoform of NO synthase (53). The vascular smooth muscle isoform is induced throughout the body including cardiac and vascular smooth muscle, renal tubules, and renal interstitial cells (12,21,76-78). Its induction in cardiac myocytes and smooth muscle cells attenuates the contraction of these cells to agonists, and may contribute to the myocardial depression seen in sepsis (76). NO is thought to form a basis for a primitive immune system which kills intracellular bacteria, parasites and fungi, and prevents viral invasion or viral replication (28,79,80). Septic patients have higher plasma and urine nitrate/nitrite levels which correlate with decreased systemic vascular resistance and hypotension, suggesting that overproduction of NO causes detrimental systemic effects. This idea was supported by initial studies that showed that inhibition of NO synthesis by infusion of NO synthase inhibitors raised blood pressure in experimental animals and humans with gram-negative sepsis (48,49,81,82) (**Figure 17**). This led many researchers to hypothesize that NO production was unconstrained during sepsis, and suggesting that NOS inhibitors would be useful in the treatment of septic shock.

Effect of L-NMMA on Hypotension in a Patient with Septic Shock

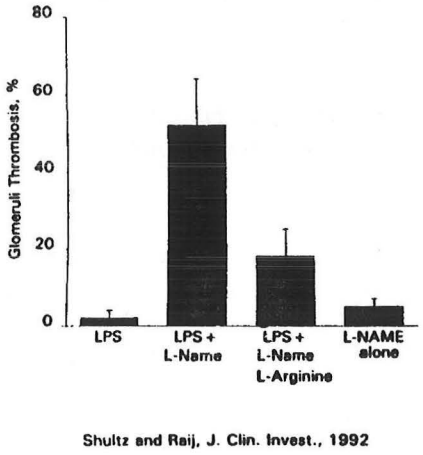


Petros, et.al., Lancet, 1991

Figure 17

However, more recent studies found that NO synthase inhibitors increase the death rate in dogs with endotoxin sepsis, despite raising vascular resistance (83). NOS inhibitors also increase mortality in staphylococcal enterotoxin B-induced shock in mice (84). NOS inhibition prolongs the increase in TNF and IFN-γ seen in this model. Antibodies directed against either TNF or IFN-γ protect mice from NOS inhibitor-induced death, suggesting that NO down-regulates IFN-γ and TNF production. Indeed, many of the systemic hemodynamic consequences of endotoxemia are in part mediated by TNF (85). NOS inhibition during sepsis causes organ ischemia or focal infarction. This has been best demonstrated in the kidney in which there is a dramatic decrease in glomerular blood flow, with platelet aggregation and capillary thrombosis (86,87) (**Figure 18**). These changes were reversed by arginine or nitroprusside infusions (86,88), indicating that a critical amount of locally produced NO is necessary

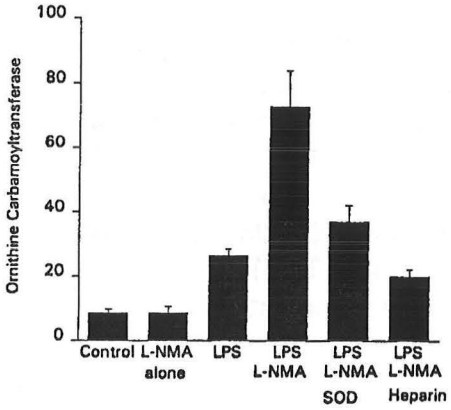
L-NAME Causes Glomerular Thrombosis during Endotoxemia



Shultz and Raij, J. Clin. Invest., 1992

Figure 18

NOS Inhibition Causes Intra-Hepatic Thrombosis during Endotoxemia



Harbrecht, et al., J. Leukocyte Biol., 1992

Figure 19

to maintain organ perfusion and to prevent vascular thrombosis. Similar changes are seen in the liver, with increased transaminases, intrahepatic thrombosis, and focal infarction in several different liver injury models (89,90) (**Figure 19**).

But why does NO inhibition increase organ damage during sepsis, and in some models of glomerulonephritis? How does NO protect cells and organs from damage during inflammation?

NO IS FRIEND AND FOE.

Biology of NO. How is it that a single, potentially toxic, molecule is involved in many important physiological processes and cytoprotective as well? The answers depend on the complicated biological and chemical actions of NO (**Figure 20**). The biological effects of NO are probably most critical in determining whether NO promotes or prevents tissue damage. NO protects organs from ischemia by regulating organ blood flow as a vasodilator and an anti-thrombotic agent. Removing vascular NO production causes organ ischemia or focal infarction. Replacing endogenous NO with either excess arginine or nitroprusside reverses the harmful effects of L-NAME on glomerular

Biological Actions of NO

Cytoprotective	Cytotoxic
· Maintain organ perfusion	· Increase TNF
· vasodilatation	· Increase COX-2
· inhibit micro-thrombosis	
· inhibit neutrophil adhesion	
· Inflammatory cytokines	
· decrease TNF *in vivo*	

Figure 20

filtration rate and capillary thrombosis seen during sepsis (88). This indicates that a critical amount of locally produced NO is necessary to maintain organ perfusion and to prevent vascular thrombosis. NO also inhibits neutrophil adhesion which prevents neutrophil plugging and migration into sites of inflammation (25).

NO can likewise initiate biological events that promote organ/cell injury. NO can directly stimulate the constitutive and inducible forms of cyclo-oxygenase (COX), causing synthesis of pro-inflammatory prostaglandins (91,92). NO can increase TNFα production in mononuclear cells (93,94).

N O C h e m i s t r y . Whereas NO can promote tissue injury (**Figure 12**), it can also prevent tissue injury caused by other toxic molecules. The chemistry of NO depends on the NO concentration (**Figure 21**). At low concentrations of NO (i.e., < 10 μM) that might be generated by brain or endothelial NOS, NO directly reacts with 1) heme proteins such as soluble guanylyl cyclase, 2) oxyhemoglobin and be degraded to nitrate, 3) free radical lipid intermediates, resulting in the chain termination of lipid peroxidation, 4) superoxide, forming peroxynitrite or $ONOO^-$, and 5) directly inhibits some enzymes (ribonucleotide reductase, P450). The relative toxicities of superoxide and peroxynitrite are controversial and likely depend on the particular cell. However, low concentrations of peroxynitrite can be rapidly transformed to nitrite, thus forming a pathway for detoxifying superoxide, and preventing the damage normally associated with reactive superoxide species.

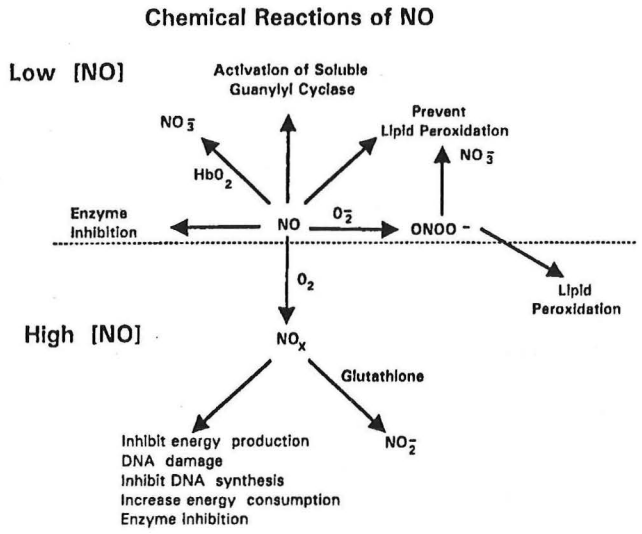


Figure 21

At higher NO concentrations, NO reacts with oxygen to form NOx. This reaction possesses a third order rate equation, so that the half life of NO in aqueous solutions is proportional to its concentration. Under biological conditions, as NO diffuses from its cellular source, the concentration drops and the lifetime increases. This allows NO to find its biological target without being destroyed by O_2. However, at high concentrations within or near a cell membrane, the autoxidation of NO generates large amounts of NOx, which mediate many of NO's cytotoxic effects. The NOx is rapidly hydrolyzed in aqueous solutions to nitrite. However, NOx is also able to attack proteins and glutathione, forming S-nitrosothiol adducts. Some of these adducts can then slowly release NO into solution, and thus may represent biological

forms of EDRF. However, they can irreversibly inhibit enzymes (aconitase, P450, GADPH, PKC, alkyltransferases, DNA repair enzymes), release Fe from Fe and hemeproteins, degrade zinc finger motifs, and damage DNA (deadenation causing single strand breaks, and double strand breaks).

At low concentrations, NO is more likely to be involved in physiological signalling and cytoprotective functions, whereas, at high concentrations, NO is more likely to be toxic. Unfortunately, whether NO will be cytoprotective or cytotoxic is difficult to determine on strictly chemical or biochemical bases. The most important factors include [NO], [superoxide], [O_2], superoxide dismutase, the site of NO synthesis (inside or outside cells), and ill defined cellular factors. Complete inhibition of NOS is counterproductive (the 'dark side' of NOS inhibitors), because it prevents the helpful chemical and biological actions of NO. Indeed, it may be necessary to modulate NO synthesis, and not completely inhibit it.

***In vivo* evidence for dual biological and chemical effects.** The relative importance of biological or chemical effects can only be studied in whole animals since cultured cells do not require blood flow. The increased liver damage following NOS inhibition in sepsis can be reduced by superoxide dismutase/deferoxamine or heparin (**Figure 19**), indicating that NO prevents chemical damage from superoxide and hydroxyl radicals, and that NO prevents biological damage from intravascular thrombosis (90). Since heparin prevented most of the injury, it is likely that NO inhibition causes intrahepatic thrombosis, followed by ischemic hepatic injury. Studies employing in vivo microscopy found that NOS inhibitors increased neutrophil adherence, and reduced sinusoidal blood flow (95). These studies indicate that NO plays a significant role in stabilizing the hepatic microcirculation during endotoxemia by direct biological effects on blood vessels, platelets, neutrophils, and chemical actions to prevent superoxide and oxygen free radical injury. Taken together, the studies show that NOS inhibition during sepsis causes both kidney and liver injury by removal of biological (vasodilation, platelets, neutrophils) and chemical (free radical scavenger) cytoprotective effects of NO.

NO THERAPY: DESIGN OF SUPERSELECTIVE NOS INHIBITORS

The above examples suggest that selective inducible NOS inhibitors might be useful for treating sepsis and local inflammation; other studies have suggested and shown that selective brain NOS inhibitors would be useful for treating stroke. NOS has a multitude of co-factors and potential regulatory sites, more than any other enzyme, that could be exploited for designing inhibitors (**Figure 22**). The classic NO synthase inhibitors block arginine entry/binding at the arginine binding site. **Unfortunately, the current NO synthase inhibitors are not specific enough to inhibit an individual isoform.** The best inhibitors only show about a 60-fold selectivity for inducible NOS; it is estimated that 1,000-fold specificity will be required to be useful clinically.

Since arginine-based NOS inhibitors are not clinically useful, perhaps a new approach is needed. NO synthase might also be inhibiting by interfering with other active sites such as BH4, NADPH or flavins; however with the exception of BH4 analogies which are not specific, these are presently not available. Cyclosporine and FK506 are known to phosphorylate and inactivate brain NO synthase (96).

Potential Sites of Regulation of Inducible NOS

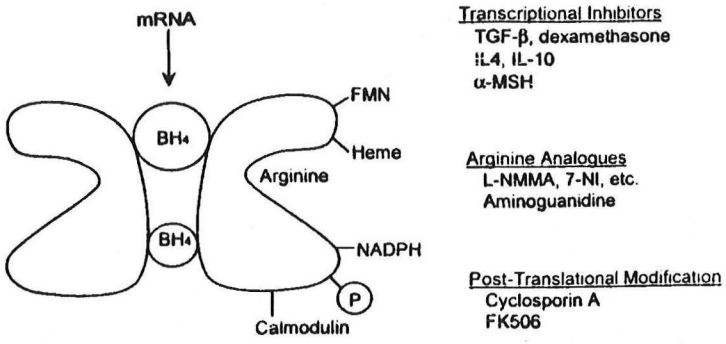


Figure 22

Indeed, cyclosporin A has been shown to decrease the volume of infarcted brain tissue during ligation of middle cerebral artery. In addition to direct inhibition of NO synthase catabolic activity, it is possible to prevent the formation of NO synthase enzyme by interfering at the level of transcription or altering mRNA stability. Transcriptional inhibitors include dexamethasone, TGFβ, IL-4, IL-10, and the neurotransmitter α-melanocyte stimulating hormone (α-MSH).

A NEW CLASS OF NO SYNTHESIS INHIBITORS

Clearly what is needed is a 'magic bullet' that will inhibit NO in a tissue specific manner. However, current arginine-based inhibitors are not specific enough to use in inflammation. Agents which can prevent the formation of NO synthases at the level of gene transcription might be more useful. Corticosteroids work at the level of gene transcription, but their clinical use is limited by side effects. We have focused on α-MSH because of its ability to inhibit inflammation in a wide variety of anti-inflammatory models.

Anti-inflammatory actions of α-MSH. α-MSH is a 13 amino acid peptide produced by the pituitary which we have found is also produced by macrophages (97,98). While this hormone is best known for changing skin color in amphibia, its effects on human melanocyte pigmentation is very minimal. Instead α-MSH is an extremely potent

Effect of α-MSH and Prednisolone in Adjuvant-Induced Arthritis

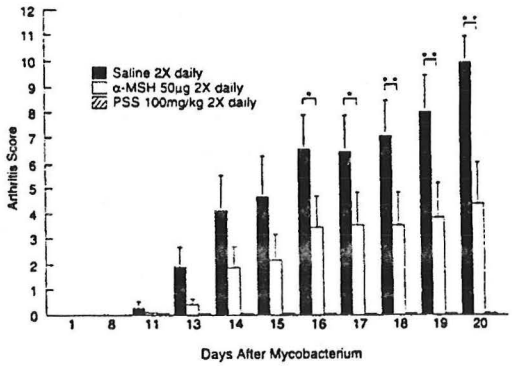


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Figure 23

antipyretic and anti-inflammatory agent [reviewed in (97)]. α-MSH is antipyretic in laboratory animals at 10,000-fold lower molar concentration than acetaminophen. α-MSH is anti-inflammatory in many models of inflammation including both systemic inflammation and localized forms of inflammation. **Figure 23** shows the effect of α-MSH in experimental arthritis (99). MSH decreased the inflammatory pannus, joint space destruction, and inflammatory score in this model. α-MSH is increased in synovial fluid in patients with rheumatoid arthritis (100).

We found that α-MSH also inhibits the development of proteinuria in a rat nephrotoxic serum glomerulonephritis which has all the features of human rapidly progressive glomerulonephritis. The rats injected with anti-glomerular basement membrane antibody develop macrophage infiltration and proteinuria. α-MSH reduced proteinuria by 80% and also dramatically decreased the degree of inflammation (**Figure 24**). Untreated animals had a diffuse proliferative glomerulonephritis whereas the animals treated with α-MSH had either mesangial proliferative glomerulonephritis or a segmental glomerular nephritis. This suggests that α-MSH can be used to treat glomerulonephritis, a model in which standard NO synthase inhibitor increase disease activity.

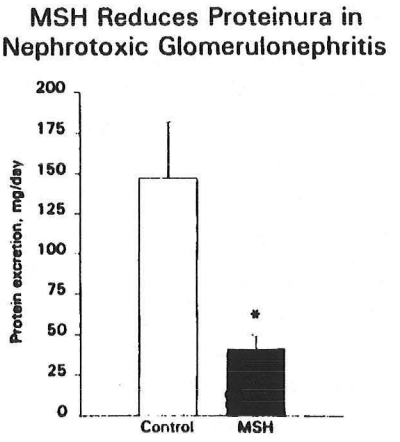

Figure 24

Mechanism of action. In collaboration with Dr. J.L. Lipton, in the Department of Physiology, we found that α-MSH inhibits NO synthesis in cultured macrophages with an EC_{50} of 1 nM which is 1,000-fold less than that of standard arginine analogs. α-MSH only inhibits NO production by about 50%. However, as described above, it may be advantageous to allow some NO production to sustain the cytoprotective functions of NO. α-MSH prevents cytokine induction of mRNA and protein for the macrophage form of NO synthase. Thus, α-MSH inhibits NO production at a different level than that of arginine analogues.

Other actions of α-MSH. One of the distinct advantages of α-MSH is that not only does it inhibit the synthesis of NO, but it also inhibits the inflammatory action of many cytokines including TNFα, IL-1, IL-6, IL-8, LTB_4, and platelet activating factor. In addition, α-MSH inhibits the synthesis of cytokines TNFα and IFN-γ by activated macrophages. **Figure 25** shows a summary of the cellular effects of α-MSH in both neutrophils and macrophages. α-MSH stimulates specific melanocortin receptors (MC1) in the cell membrane which act through cAMP to suppress induction of NO synthase. α-MSH decreases cytokine production which inhibits macrophage-induced tissue destruction, which decreases macrophage infiltration and activation. A surprising finding was that TNFα also increases macrophage production of α-MSH. Endogenous α-MSH produced by macrophages could therefore counterbalance the

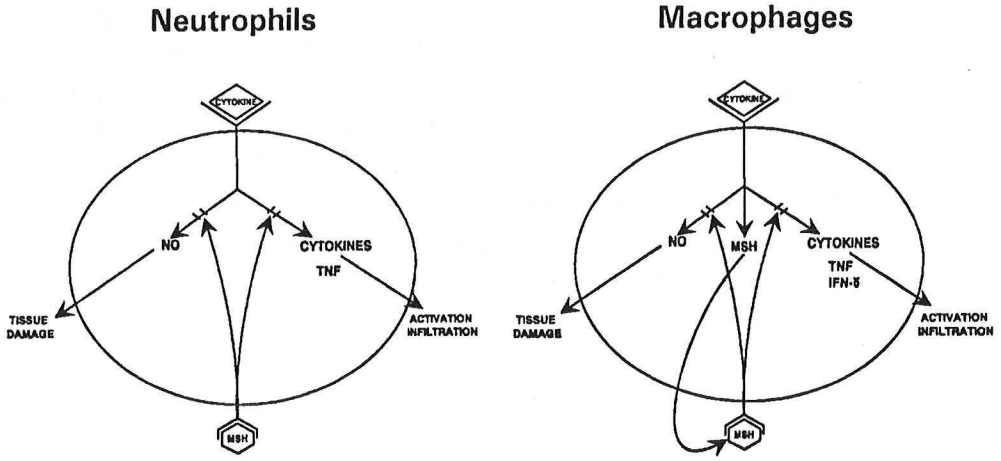

Figure 25

generation of chemoattractant cytokines. This would prevent full expression of a positive amplification loop, and serve to broke the inflammatory response by limiting recruitment and activation of circulating monocytes.

Advantages of α-MSH. α-MSH has several advantages over standard NO synthase. First of all it appears to be selective for the inducible isoform because of the cellular distribution of α-MSH receptors. α-MSH has no direct effect on blood pressure or heart rate in rats or humans (101-103). Second, α-MSH does not completely inhibit NO synthase so that small amount of NO production remaining may have cytoprotective actions. Thirdly, and perhaps most importantly, α-MSH inhibits inflammation at several points in the inflammatory cascade in addition to inhibition of NO synthase. In this regard, its anti-inflammatory spectrum is somewhat reminiscent of glucocorticoid.

Side Effects. α-MSH is well tolerated for up to 14-21 days in rats, with no obvious side effects. It takes 5000-fold excess above the antipyretic dose, injected into the brain, to kill 30% of rabbits (97). Human studies from the early 1960-1970's for use as a skin tanning agent showed only very minor side effects in reports involving about 50 human subjects (101-103). The reported side effects included mild skin darkening, transient increases in growth hormone, increases in LH/FSH in males but not females, increased verbal memory, and a mild sensation of hunger sensation. In summary, α-MSH is well tolerated, but extensive testing has not been performed.

CONCLUSION

I have focused on some of the clinical uses of NO and NO synthase inhibitors, with particular focus on the actions of NO during inflammation. Because NO has many effects throughout the body, one would guess that general NO agonists or NOS inhibitors might have many side effects. NO has been used in isolated organs to promote organ transplantation. Genetic manipulations have targeted endothelial NO synthase to vascular smooth muscle cells cells which inhibit intimal proliferation during restenosis. Unfortunately, the clinical utility of NOS inhibitors has been poor. Drug companies have tried to develop specific NO synthase inhibitors and thus far have been unsuccessful. However, I have shown that inhibiting inducible NO synthase at the level of gene transcription may be useful in the treatment of inflammatory diseases.

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