REVIEW

Nitric oxide

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Nitric oxide is a gas generally known by its chemical formula NO, or NO[•]. The dot denotes an unpaired electron, which is the definition of a free radical; possessing this, NO is highly reactive and quite different from the anaesthetic agent nitrous oxide (N₂O), which is extremely stable. Until recently, NO was best known as a constituent of car exhaust fumes, contributing to the photochemical smog of cities such as Los Angeles. In contrast, the work which crucially altered this perception was performed in a leafy Kentish suburb at the Wellcome Foundation in Beckenham, UK. In 1987, it was shown that NO was the long sought endothelium derived relaxing factor (EDRF).¹ This was a crucial discovery for cardiovascular biology, and it soon became evident that NO was produced by many cell types and performed diverse functions, including inhibition of platelet aggregation and mediation of the cytotoxic action of activated macrophages, and had a role in central and peripheral neurotransmission.

NO is enzymically synthesised from L-arginine by oxidation of one of the terminal guanidino nitrogen atoms of L-arginine,²—a process inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (LNMMA). Figure 1 illustrates the mechanism by which NO mediates vasodilatation. NO is synthesised within the endothelial cell and released in response to vasodilator stimuli such as acetylcholine and bradykinin. Released NO diffuses to adjacent vascular smooth muscle cells (VSMC), where it activates guanylate cyclase (GCase) so increasing cyclic GMP (cGMP) concentrations. Increased intracellular cGMP concentrations cause relaxation of VSMC,

resulting in vasodilatation. However, the key to understanding the diverse roles of NO is that NO synthases (NOS) generate NO by both constitutive and cytokine/endotoxin induced pathways.

Enzymology—constitutive and inducible NO synthases

The original description of endothelial cell NO generation involved the *constitutive* enzyme (cNOS),^{1,2} which is also present in the adrenal gland,³ platelets,⁴ fibroblasts,⁵ polymorphonuclear leucocytes (PMN),⁶ brain,^{7,8} retina,⁹ and some non-adrenergic non-cholinergic nerve terminals.¹⁰ The *inducible* enzyme (iNOS) is present in many cells including endothelial cells,^{11,12} VSMC,¹³ macrophages,^{14,15} PMN,¹⁶ lymphocytes,¹⁷ fibroblasts,⁵ hepatocytes,¹⁸ mast cells,¹⁹ renal mesangial cells,²⁰ rabbit articular chondrocytes,^{21,22} and rabbit synovial fibroblasts.²³ NO synthesis by cells in which iNOS has been induced is *several orders of magnitude greater* than in cells producing NO via cNOS.

The NO synthases require NADPH, tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and haem as cofactors, but the constitutive enzyme is Ca²⁺/calmodulin dependent and the inducible enzyme is Ca²⁺/calmodulin independent. The cerebellar cNOS was the first to be isolated^{24,25} and cloned,²⁶ revealing a 1433 amino acid 160 kDa protein. The cell membrane located endothelial cNOS (both bovine^{27–29} and human^{30,31}) are about 130 kDa and exhibit only 58% sequence homology with rat cerebellar cNOS,²⁷ compared with the 51% homology between rat cerebellar cNOS and mouse macrophage iNOS³² which, like other cellular iNOS, is a cytoplasmic protein of about 130 kDa.^{32–36} The notations 'eNOS' and 'nNOS' have been used for the cNOS of endothelial and neuronal cells, respectively, though neither is confined to its respective cell type. The genes for the human eNOS, nNOS, and iNOS are located on chromosomes 7, 12, and 17 respectively.^{37–40} Cloning²⁶ and other studies⁴¹ also suggest that NOS are cytochrome P-450-type haemoproteins. Essentially, these function by transferring electrons via flavin moieties to haem, and it is likely that FAD/FMN fulfil a similar role in relation to NOS during the oxidation of L-arginine to NO. It is also clear that the tight binding of calmodulin to iNOS explains its Ca²⁺/calmodulin independence,⁴² that cNOS phosphorylation via several protein kinases may be an important influence on NOS

Figure 1 Mechanism of nitric oxide (NO) mediated vasodilatation in response to various stimuli such as bradykinin or acetylcholine. NO diffuses into adjacent vascular smooth muscle cells (VSMC), activating guanylate cyclase (GCase), increasing cGMP levels, and producing VSMC relaxation and vasodilatation. In contrast, NO inhibits platelet adhesion and aggregation. cNOS = Constitutive NO synthase; L-Arg = L-arginine; L-Cit = L-citrulline.

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activity,⁴³ and that the similarity between the NOS haem moiety and that of GCase represents a mechanism by which NO might achieve feedback inhibition of NOS.⁴⁴

Cytokine induction

The best recognised inducers of iNOS are interferon gamma (IFN γ), tumour necrosis factor α (TNF α), interleukin-1 (IL-1), and lipopolysaccharide (LPS)/endotoxin.^{12 13 20-23} Induction of iNOS is suppressed by TGF β , IL-4 and IL-10 alone,^{45 46} and synergistically in macrophages,⁴⁷ whilst IL-8 inhibits iNOS induction in PMN.⁴⁸ The inducer and suppressor cytokines noted above correspond to those secreted by the Th1 and Th2 subsets of CD4 positive T cells (see below: Immune system). Glucocorticoids also inhibit the induction, but not the activity, of the inducible enzyme.¹¹ The cytotoxic potential of NO demands strict regulation of iNOS expression, thus generation of significant quantities of NO requires a specific order of stimulation by at least two agents.⁴⁹ This is illustrated by the synergy between IFN γ and LPS which, respectively, were recognised as primer and trigger of various macrophage functions, including cytotoxicity, before NO was shown to mediate the latter. Mouse macrophages produce similar quantities of NO when costimulated with IFN γ and LPS or IFN γ followed by LPS, but LPS followed by IFN γ yields much less NO⁴⁹ in proportion to the length of preincubation with LPS.⁵⁰ NO production is also sustained by continued stimulation with LPS, but not IFN γ .⁴⁹ Regulation of iNOS expression may occur at many levels, though the synergistic effect of IFN γ and LPS is principally transcriptional.⁵¹ Recent studies of the promoter region of the mouse iNOS gene have identified putative binding sites for several transcription factors,⁵² including that of an IFN γ responsive transcription factor critical to the synergy between IFN γ and LPS.⁵³

Cellular effects and mechanisms

The function of nitric oxide as an intercellular messenger is enhanced by its low molecular weight, high diffusibility, and lipid solubility, whilst the cellular effects of NO owe much to its reactivity as a free radical. The best described interactions are those with iron containing proteins and the superoxide anion (O $_2^-$). The principal action of constitutive NO is activation of GCase by binding to the haem group of the enzyme; this is believed to result from breakage of the haem-protein bond induced by NO-haem binding. The result is an increase in intracellular cGMP, achieving smooth muscle cell relaxation principally by decreasing intracellular Ca $^{2+}$. Conversely, cytokine induced NO production mediates cytotoxicity in the target cells of macrophages by inhibiting non-haem iron-sulphur centred enzymes and releasing iron.⁵⁴ Key iron-sulphur (Fe-S) enzymes inhibited by NO synthesis include aconitase, the rate limiting enzyme of

the citric acid cycle, complexes I and II in the mitochondrial electron transport chain, and ribonucleotide reductase which synthesizes the deoxynucleosides required for cell replication.^{14 54} In contrast, NO also inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase by stimulating its ADP-ribosylation.⁵⁵

The above information suggests that substantial NO induced increases in intracellular free iron concentrations occur directly as a result of NO releasing iron from both iron containing enzymes⁵⁶ and ferritin.⁵⁷ However, this may be mediated by NO via an Fe-S containing protein (iron regulatory factor (IRF)) involved in cellular iron homeostasis. IRF binds to iron responsive elements in the mRNAs of ferritin and the transferrin receptor. In iron replete cells, iron responsive element binding by IRF is low, whereas in iron poor cells such binding by IRF increases, impairing iron storage via ferritin. NO increases iron responsive element binding by IRF,^{58 59} decreasing the biosynthesis of ferritin, which should lead to the observed increases in intracellular free iron levels. The latter would also enhance the generation of reactive oxygen species via the Fenton and Haber-Weiss reactions.

The interaction of NO with O $_2^-$ is quite different from that with iron, and is one of several possible pathways for NO breakdown in vivo. The result is the elimination of both radicals.⁶⁰ In the vasculature, this would abrogate the vasodilator and antithrombotic properties of NO, but also remove O $_2^-$, which is implicated in ischaemic reperfusion injury of both the myocardium⁶¹ and the synovium.⁶² Under some circumstances the above mechanism would appear homeostatic were it not that the reaction product, the peroxynitrite anion (ONOO $^-$), is a powerful oxidising agent that may also yield the hydroxyl radical.^{63 64} Whilst contributing to the cytotoxicity of activated macrophages and neutrophils, formation of peroxynitrite and the hydroxyl radical may also mediate tissue injury in hypoxic-reperfusion injury, acute inflammation and other situations generating NO or O $_2^-$.

In aqueous solution, NO is spontaneously oxidised by oxygen to nitrite (NO $_2^-$), which in ex vivo studies is the major metabolite of NO in the coronary circulation.⁶⁵ However, in vivo NO $_2^-$ may be further oxidised by erythrocyte haemoglobin to nitrate (NO $_3^-$) which is also the end product of the interaction of NO and O $_2^-$. The half life of NO is between three and 50 seconds, but may be much longer depending on ambient NO, oxygen and O $_2^-$ concentrations.¹ The half life may also be extended physiologically by NO forming nitrosoproteins with vasorelaxant properties attributable to NO release,⁶⁶ enabling NO to exert effects at distant sites.

Below, we briefly consider actions of NO in the cardiovascular, nervous and immune systems that help to illuminate the pathophysiological relevance of NO to patients with rheumatic diseases including multisystem disorders.

The cardiovascular system

The simplified representation of endothelium dependent vasodilatation by NO (fig 1) shows that NO is released by endothelial cells in response to several agonists. NO is the major influence on basal vascular tone in vitro and in vivo,⁶⁷ including that of the coronary circulation.⁶⁸ NOS inhibitors such as LNMMA produce vasoconstriction in both the arterial and venous systems including the micro-circulation, with the most marked effects evident in large arterial resistance vessels,⁶⁹ though these effects do vary between species. In man, NO mediates both basal and stimulated arterial vasodilatation and stimulated venodilatation.^{70 71} Conversion to NO or direct release of NO is also the mechanism by which organic nitrates exert their beneficial cardiovascular effects.

Thus NO is anticipated to have a central role in the endothelial dysfunction and pathophysiology of atherosclerosis and essential hypertension. The former is a complex process that may have multiple effects on coronary endothelial NO synthesis-release and vice versa. Vasorelaxation is impaired in early atherosclerosis,⁷² and recent studies of coronary artery explants show that basal and stimulated NO release are impaired in established disease, not just at sites of atheroma but throughout the vessel, implying generalised endothelial dysfunction.⁷³ Cholesterol, and especially its low density lipoprotein fraction (LDL), may be responsible for such dysfunction, as both are known to impair endothelium dependent vasodilatation and NO release,⁷⁴ whilst L-arginine (the precursor of NO) reverses lipid induced endothelial dysfunction in both animals⁷⁵ and man.⁷⁶ Lipids may exert this effect via impairment of endothelial cell NO synthesis, though inactivation of NO by oxidised LDL⁷⁷ and LDL interactions with guanylate cyclase⁷⁸ may also be important. However, impaired vasodilatation is only one consequence of the presence of decreased amounts of NO in the endothelial milieu. NO has the potential to inactivate O₂⁻ which would otherwise oxidise LDL⁷⁹—a modification proposed as crucial in atherosclerosis as it promotes the uptake of LDL by macrophages.⁸⁰ Generation of reactive oxygen species is also presumed to be a principal factor in NO mediated hypoxic-reperfusion injury.^{81 82}

NO inhibits platelet adhesion,⁸³ the key step in thrombosis in addition to platelet aggregation which results in the release of potent vasoconstrictors and mitogens. In vivo NO is crucial in opposing platelet induced vasoconstriction⁸⁴ caused by agents such as thromboxane; however, other platelet products such as serotonin and ADP/ATP also stimulate endothelial cell NO release, opposing vasoconstriction, and inhibit platelet function in tandem with platelet derived NO⁴ and prostacyclin.⁸⁵ Platelet derived mitogens are also the presumed cause of VSMC proliferation characteristic of atheroma, which NO antagonises directly via an antiproliferative effect on VSMC.⁸⁶ The significance of negative platelet regulation by organic nitrates in

clinical usage is debatable. Mediated via NO, nitrates are, of course, potent vasodilators, though their long term efficacy is limited by the development of tolerance, the mechanism of which remains controversial. Explanations include depletion of intracellular thiols essential for the conversion of organic nitrates to NO in addition to the regulation of GCase⁸⁷ and desensitisation of GCase to NO⁸⁸ making VSMC less responsive to nitrates.

Impaired NO dependent vasorelaxation is evident in both animal models of hypertension and hypertensive patients.⁸⁹ Moreover, orally administered NOS inhibitors produced sustained hypertension for the duration of studies lasting five to eight weeks.^{90 91} Potential mechanisms include a central nervous system (CNS) action⁹² or alterations in renal homeostasis, as there is increasing evidence that NO has important actions in the kidney. NOS inhibitors cause renal vasoconstriction, a decrease in GFR, and an increase in glomerular capillary pressure,⁹³ whilst increased urinary nitrite (NO₂⁻) and nitrate (NO₃⁻) in response to a high salt intake implicate NO in the renal mechanisms concerned with regulating blood pressure.⁹⁴ This is supported by data that NO influences perfusion pressure control of renin secretion⁹⁴ and that low doses of NOS inhibitors cause volume dependent hypertension.⁹⁵ Impaired NO synthesis is also implicated in the hypertensive response of salt sensitive Dahl/Rapp rats fed a high salt diet—an effect both prevented and reversed by feeding L-arginine.⁹⁶

The nervous system

Research to date has focused on the constitutive nNOS present in both the central and peripheral nervous systems, although iNOS has been described.⁹⁷ NO synthesis, originally described in the forebrain and cerebellum, is evident throughout the brain,⁹⁸ including the cerebral cortex, corpus striatum, mid-brain, hippocampus, and pituitary. Moreover, immunological and in situ RNA hybridisation studies have shown that nNOS is responsible for the activity of neuronal NADPH diaphorase—a well established histochemical stain.^{99 100} This is important, because NADPH diaphorase activity (and thus nNOS) is present in only a small percentage of neurones that are particularly resistant to degeneration in Huntington's chorea,¹⁰¹ hypoxia,¹⁰² and neurotoxicity caused by excitatory amino acid neurotransmitters.¹⁰³ Principal among the last of these is glutamate, which, acting via stimulation of the postsynaptic N-methyl-D-aspartate receptor (NMDA), is a proven mediator of neurotoxicity in models of stroke and a candidate mediator in neurodegenerative disorders. NMDA receptor activation may achieve different cellular effects via receptor diversity, but the most prominent effect is a Ca²⁺ influx, which would be expected to increase neuronal Ca²⁺ concentrations sufficiently to activate the Ca²⁺ dependent nNOS. Experimental evidence from several groups has confirmed that NO mediates glutamate neurotoxicity in vitro¹⁰⁴

and in vivo.^{79 105 106} The neuroprotective effect of superoxide dismutase implicates peroxynitrite or its decomposition products.¹⁰⁷ However, some NO releasing compounds are neuroprotective,¹⁰⁷ supporting the proposal that the redox state of NO determines toxicity.¹⁰⁸ It is suggested that while NO[•] is neurotoxic, NO⁺, the nitrosonium ion, is neuroprotective. A plausible explanation is that NO⁺, by reacting with thiol groups, nitrosylates the NMDA receptor, thus blocking NMDA receptor neurotoxicity mediated by NO[•]. Clinical trials are currently evaluating NMDA receptor antagonists in acute stroke, but inhibition of NOS in a mouse model of acute stroke proved more effective in limiting neuronal damage than did NMDA receptor antagonism.¹⁰⁹ The evidence above suggests nNOS inhibition may have a therapeutic role in stroke and neurodegenerative disorders.

CNS neurotoxicity is clearly an aberrant pathological consequence of NOS activity that also has important physiological functions in the brain. This is particularly true in the 'molecular' layer of the cerebellum, where granule and basket cells produce NO in response to NMDA receptor activation by mossy fibres and parallel fibres, respectively.¹¹⁰ Probable targets of NO are adjacent cells such as astrocytes and other glia, in addition to neurones and the GCa of the presynaptic terminal¹¹¹ (fig 2). This last possibility suggests the potential of NO to act as a *retrograde synaptic messenger*,¹¹² presumably modulating synaptic transmission, and stimulated the hypothesis that NO has a role in the plasticity of the CNS.¹¹³ This is sustained by evidence that, in the cerebellum, NO mediates long term synaptic depression,¹¹⁴ believed to be the cellular mechanism of cerebellar learning and analogous to the long term potentiation in the hippocampus that is implicated in learning and memory.¹¹² NO has been shown to enhance and NOS inhibitors to impair¹¹⁵ long term potentiation in the hippocampus, whilst in vivo NO inhibition blocks spatial learning.¹¹⁶ It has

been proposed that NO modulates synaptic efficacy by acting as a local temporo-spatial signal, strengthening synapses that fire in a co-ordinated manner and weakening those that do not, thus linking local neuronal activity.¹¹³ The same authors also proposed a role for NO in the development of the nervous system and the coupling of neural activity to local increases in cerebral blood flow, demonstrated by positron emission tomography (PET). Indeed, nNOS does mediate vasodilatation,¹¹⁷ and NOS inhibition uncouples the increased cortical blood flow that occurs with peripheral nerve stimulation.^{118 119}

The elucidation of 'vascular evoked responses' using PET has advanced our understanding of pain and, specifically, its cortical appreciation. NO is a candidate mediator of such phenomena and is implicated in pain. NO exhibits a peripheral analgesic effect via stimulation of GCa and increased cGMP concentrations¹²⁰⁻¹²² and, when administered systemically, L-arginine also has a central antinociceptive action via opioidergic pathways.^{123 124} In contrast NO also mediates hyperalgesia at the spinal or supraspinal level¹²⁵⁻¹²⁷ and is implicated in both the development of tolerance to morphine,¹²⁸ and thalamocortical function.¹²⁹ The above suggests not only an important role for NO in pain mechanisms, but that its actions may be pro- or antinociceptive at different sites.

Key sites of NO synthesis within the autonomic nervous system are non-adrenergic, non-cholinergic (NANC) nerve terminals that generally serve to relax smooth muscle. NO appears to be the principal NANC neurotransmitter, and NO mediated NANC function has been identified in the gastrointestinal tract,^{130 131} corpus cavernosum,¹³² the respiratory system,¹³³ and arterial vessels.¹³⁴ Evidence suggests that NO is the physiological mediator of penile erection,¹³⁵ modulates the effects of bronchoconstrictors,¹³⁶ and has a key role in gut sphincter control:¹³⁷ mice with an nNOS gene knockout develop normally without histological abnormalities of the CNS, but with dilation of the stomach and pyloric muscle hypertrophy similar to that of pyloric stenosis in human infants (see below).¹³⁸

Figure 2 Model for the role of nitric oxide (NO) as a CNS neurotransmitter. Glutamate (glu) is an excitatory neurotransmitter acting on the N-methyl-D-aspartate receptor (NMDAr). NMDAr stimulation produces a postsynaptic calcium influx (Ca) resulting in NO synthesis by the calcium dependent constitutive NO synthase (NOS). NO may act as a retrograde neurotransmitter acting on the guanylate cyclase (GCa) of the presynaptic terminal to enhance neurotransmission. Diffusing to adjacent cells, it may also link and enhance coordinated local neuronal activity. There is also strong evidence that NO mediates NMDA neurotoxicity.

The immune system

IMMUNOREGULATION, INFLAMMATION AND RHEUMATIC DISEASES

The discovery that NO was EDRF, was soon followed by the realisation that NO was also responsible for the cytotoxicity of activated macrophages¹²⁻¹⁴ and recognition of constitutive and cytokine inducible NOS. However, NO has a broader and more complex role in immune functions and inflammation, in which both cNOS and iNOS are relevant. Important in this respect has been the recent focus on the role of the endothelium in inflammation. At the endothelial level, the principal actions of constitutive NO synthesis are vasodilatation and inhibition of platelet aggregation. These functions are particularly relevant to both the abnormal vascular tone

associated with many inflammatory rheumatic diseases, evinced by Raynaud's phenomenon and the presence in chronic inflammatory diseases of microvascular abnormalities including microthrombi.¹³⁹ Indeed, a primary vascular pathogenesis has been proposed for both Crohn's disease¹⁴⁰ and rheumatoid arthritis,¹³⁹ whilst in the latter there is also evidence that exercising joints causes synovial hypoxia-reperfusion leading to further microvascular injury.^{141 142}

In the circumstance of hypoxia-reperfusion, NO has the potential to mediate or modulate free radical induced injury, though another important consequence of hypoxia-reperfusion is an increase in leucocyte adherence to endothelium.^{142a} In a cat model of myocardial ischaemia-reperfusion, impaired basal NO release during reperfusion accounted for the increased leucocyte adhesion observed.¹⁴³ Perfusion of normal cat mesenteric venules with NOS inhibitors produced a 15-fold increase in leucocyte adherence, but lesser increases in leucocyte emigration.¹⁴⁴ Further studies showed a marked increase in leucocyte adhesion, emigration, and vascular permeability 30 minutes after perfusion of *N*^G-nitro-L-arginine methyl ester (L-NAME), which was reduced by monoclonal antibodies to CD18, intercellular adhesion molecule-1, or P-selectin.¹⁴⁵ Subsequent evidence suggests that the L-NAME induced increases in leucocyte adhesion are the result of endothelial O₂⁻ and mast cell mediators unopposed by endothelial NO,¹⁴⁶ which is consistent with in vitro evidence that NO blocks histamine and platelet activating factor release by mast cells.^{147 148} This is further persuasive evidence that the cNOS has an important role in stabilising the endothelium and opposing early inflammatory changes. Conversely, in rat models of immune complex mediated alveolitis and dermal vasculitis, NOS inhibitors greatly reduced indices of tissue injury.^{149 150} Confirmation of NO as a principal effector mechanism of immune mediated vascular injury has therapeutic implications. Moreover, the protective effect of inhibiting iNOS in this circumstance is entirely compatible with the expected beneficial effects of constitutive NO release outlined above.

NO is also an effector and regulator of white cell function, as lymphocytes,¹⁷ PMN¹⁶ and macrophages¹⁴ all possess NOS activity. As with the endothelium, the effect of NO is likely to be determined by the concentration of NO in the local milieu and thus reflect local iNOS activity, illustrated by the effect of NO on PMN and monocyte chemotaxis.^{151 152} The key cytotoxic effector in PMN is O₂⁻ and though NO has an analogous role in macrophages, it was once thought of little relevance to PMN. In fact, phorbol myristate acetate (PMA) stimulation of the neutrophil respiratory burst generates both NO and O₂⁻, though increases in NO synthesis occur at lower PMA concentrations, plateau earlier, and are generally less than those of O₂⁻.¹⁵³ Purification of the iNOS in human PMN reveals an associated 22 kDa protein that generates O₂⁻, whilst stimulation of the whole fraction by PMA increases O₂⁻ and

decreases NO generation.¹⁵⁴ NO has also been shown to inhibit PMN O₂⁻ production via a direct effect on the membrane NADPH oxidase,¹⁵⁵ and to inactivate the O₂⁻ generating enzyme xanthine dehydrogenase/oxidase in macrophages,¹⁵⁶ suggesting negative feedback regulation of O₂⁻ generation by NO and vice versa. The above suggests a close regulatory relationship between NO and O₂⁻ and the reactive oxygen species generating potential of this interaction.

Low levels of NO have been implicated in lymphocyte activation and proliferation.¹⁵⁹ NO donors such as sodium nitroprusside, and to a lesser degree gaseous NO, increase lymphocyte uptake of glucose (an early event during lymphocyte activation), stimulate TNF α production and nuclear transcription factor κ B (NF- κ B) binding activity and enhance activity of the tyrosine kinase, p56^{lck}, which is implicated in lymphocyte signalling events.¹⁵⁷ All the above are effected via a cGMP independent mechanism, though guanylate cyclase activation by NO may be necessary for optimal lymphocyte proliferation.¹⁵⁸ L-Arginine depletion and NOS inhibitors also impair phytohaemagglutinin (PHA) stimulated proliferation,¹⁵⁸ whilst dietary L-arginine supplementation in man increases lymphocyte mitogenic responses to concanavilin A (con A) and PHA.¹⁵⁹ L-Arginine has also been shown both in vitro and in vivo to enhance natural killer and lymphokine activated killer activity.¹⁶⁰

Paradoxically, high concentrations of NO which occur following macrophage activation suppress antigen presenting cell activity¹⁶¹ and T cell proliferation. NOS inhibition produced a striking increase in PHA induced proliferation in rat spleen cell cultures, and greatly enhanced both the allogeneic mixed lymphocyte reaction and the development of lymphocyte cytotoxicity in both rats and mice.¹⁶² Mills confirmed the observation that NOS inhibitors enhanced mitogen induced proliferative responses, that this was true for a variety of mitogens that activate different lymphocyte subsets, that macrophages were the 'suppressor' cells producing NO, and that differences between rat and mouse responses were probably quantitative.¹⁶³ Concurrent studies reinforced these findings and showed that T cell IFN γ was required to induce the NO mediated suppressive effects of mouse peritoneal macrophages.¹⁶⁴

These findings are of very direct relevance to organ transplantation. In vivo studies of a sponge matrix allograft model revealed much greater concentrations of NO breakdown products (NO₂/NO₃⁻) in allograft fluid compared with syngeneic graft fluid, whilst in vitro allogeneic graft infiltrating cells produced more NO than corresponding syngeneic cells, and only developed cytotoxicity when treated with the NOS inhibitor LNMMA.¹⁶⁵ In rat studies, LNMMA also promoted allospecific cytotoxicity¹⁶⁶ and restored depressed mitogenic responses to con A.¹⁶⁷ Thus macrophage NO synthesis, induced by cytokines derived from activated T cells, may effect negative feedback by suppressing T cell proliferation.

Direct evidence of NO production during allograft rejection *in vivo* has been provided by electron paramagnetic resonance (EPR). In rat heart allografts, rejection corresponds with the appearance of an NO signal on EPR in both blood and graft tissue, which is prevented by the immunosuppressive, FK506.¹⁶⁸ After small bowel transplantation, both rejection and graft versus host disease (GVHD) were preceded by increased serum $\text{NO}_2^-/\text{NO}_3^-$ concentrations, but were prevented clinically and histologically (as was the increase in serum $\text{NO}_2^-/\text{NO}_3^-$ levels) by treating transplant recipients with FK506.¹⁶⁹ Widening the range of allogeneic grafts undertaken yielded similar findings,¹⁷⁰ confirming $\text{NO}_2^-/\text{NO}_3^-$ concentrations as an early marker of acute rejection or GVHD that merit further investigation in clinical practice. However, this proposal raises the questions whether NO synthesis in this context is a specific indicator of immune activation, and whether its net effect is to inhibit or promote rejection or GVHD. Whilst NO is cytostatic, limiting lymphocyte proliferation, increased NO synthesis may reflect macrophage and cytotoxic lymphocyte activity that is responsible for rejection and GVHD—a notion supported by the protective effect of LNMMA in GVHD.¹⁷¹

More evidence is also emerging in respect of the differential effects of cytokines on iNOS activity, and vice versa. As noted above, $\text{IFN}\gamma$ and TNF are key inducers, and IL-4 and IL-10 key suppressors, of iNOS activity, produced by the Th1 and Th2 subsets of CD4 T cells, respectively. Activated Th1 cells produce large quantities of NO in comparison to undetectable levels in Th2 cells; however, NO synthesis by Th1 cells exerts negative feedback by suppressing their production of $\text{IFN}\gamma$ and IL-2.¹⁷² NO does not appear to have any significant effect in respect of Th2 cytokine production. Given the important proliferative effects of IL-2 on Th1 and CD8 cells, it is argued that NO prevents the over expansion of the T cell subsets that mediate immunopathology.¹⁷² Also important in this context is that NO exerts negative feedback by inhibiting both iNOS and cNOS via the haem moiety of NOS.¹⁷³

Inhibiting NO also modulates carrageenin and dextran induced acute inflammation in the rat¹⁷⁴ and ADP/bradykinin induced changes in microvascular permeability in the hamster cheek pouch model.¹⁷⁵ In addition, NO mediates neurogenic plasma exudation in the lungs of guinea pigs,¹⁷⁶ oedema formation caused by substance P¹⁷⁷ and, together with sensory nerves, skin inflammation caused by bradykinin.¹⁷⁸ NO inhibition also reduced neurogenic inflammation in adult rats, which was prevented by treatment with capsaicin in the neonatal period.¹⁷⁹ These findings suggest a central role for NO in neurogenic inflammation.

Insulin dependent diabetes illustrates the multifunctional role of NO in an autoimmune disease that involves T cell dependent and cytokine mediated destruction of the pancreatic islet β cell.¹⁸⁰ NO production by human islets is greatly enhanced by combinations of IL-1 β ,

TNF, or $\text{IFN}\gamma$, while several studies suggest inhibition of insulin secretion by IL-1 β and other cytokines is mediated by NO.^{181 182} Both islet cells and macrophages have been shown to kill islet cells via NO *in vitro*.¹⁸³ Interestingly, cyclosporin A protects islet cells from NO cytotoxicity *in vitro*, but NOS inhibitors are far more effective,¹⁸⁴ and partially suppress the development of streptozotocin induced diabetes,¹⁸⁵ suggesting a therapeutic potential for NOS inhibitors such as aminoguanidine. The latter reduces diabetic vascular dysfunction *in vivo*,¹⁸⁶ though its effectiveness could in part result from direct inhibition of the formation of advanced glycosylation products that are known to inactivate NO *in vitro* and *in vivo*.

Despite the wealth of evidence above, there are few published studies measuring NO synthesis in human diseases, inflammatory or otherwise. Our studies implied increased NO synthesis in both rheumatoid and osteoarthritis,¹⁸⁷ whilst others have demonstrated similar findings in response to sepsis^{188 189} or cytokine chemotherapy^{190 191} and in biopsy material from patients with ulcerative colitis.^{192 193} Using NO_2^- as an index of NO synthesis, we showed increased NO_2^- concentrations in the serum of patients with rheumatoid arthritis (RA) compared with controls, and in synovial fluid compared with serum from RA patients, implying NO synthesis within the joint.¹⁸⁷ This was to be expected, as NO generating cells such as endothelial cells, PMN, macrophages, and synoviocytes are found within inflamed synovium. There is, however, accumulating evidence that NOS inhibitors modulate inflammation in animal models, endorsing a role for NO in inflammatory rheumatic disorders including vasculitis. NO_2^- production by synovial tissue and peripheral blood mononuclear cells was increased in streptococcal cell wall induced arthritis and suppressed by LNMMA.¹⁹⁴ Importantly, this was accompanied not only by a marked reduction in the articular index during the acute inflammatory phase (days 1–6), but also by a dramatic reduction during the chronic T cell-macrophage phase of inflammation that was also evident but less marked if LNMMA was started on day 12 after induction of the arthritis. Synovial histology confirmed a reduction in the cellular infiltrate and erosions in the LNMMA treated animals. In rat adjuvant arthritis, L-NAME decreased, whilst L-arginine (the precursor of NO) increased paw swelling throughout the course of arthritis.¹⁷⁴ T cell proliferative responses and macrophage production of NO_2^- and acid phosphatase were decreased and increased in cells harvested from L-NAME and L-arginine treated animals, respectively. The inbred MRL-lpr/lpr mouse is a recognised model for human systemic lupus erythematosus that spontaneously develops an autoimmune disorder characterised by DNA antibodies, arthritis, nephritis, and vasculitis. Urinary $\text{NO}_2^-/\text{NO}_3^-$ concentrations were demonstrated to be greater in MRL-lpr/lpr mice compared with other strains, and increased with the onset

of nephritis.¹⁹⁵ Administration of LNMMA reduced not only urinary $\text{NO}_2^-/\text{NO}_3^-$ levels, but also histological indices of both arthritis and renal disease, including vasculitis and proteinuria. These findings are consistent with studies of rat models of immune complex mediated alveolitis and dermal vasculitis in which NOS inhibitors greatly reduced indices of tissue injury.^{149–150} The above observations provide strong evidence for a major role of NO as a mediator of inflammatory joint disease and connective tissue disorders.

Because of its role as the regulator of basal vascular tone and principal vasodilator, one would anticipate an important role for NO in the regulation of blood flow to the joint. Indeed, inhibition of NOS by intra-arterial infusion of L-NAME substantially reduced basal blood flow and increased sympathetic vasoconstriction in both normal and inflamed rabbit knees.¹⁹⁶ Moreover, the reaction of NO with O_2^- to form peroxynitrite makes NO very pertinent to synovial hypoxic reperfusion injury. Such injury occurs because, in the presence of even a modest effusion, joint exercise leads to intra-articular pressure increases that exceed synovial capillary perfusion pressure, impairing synovial blood flow.¹⁴¹ We investigated this in vivo by measuring synovial fluid NO_2^- levels before and after exercise (10 minutes walking) or rest (controls) in 18 patients with active RA, and found a significant decrease of 30% in mean synovial fluid NO_2^- concentrations after exercise, compared with a non-significant increase of 13% noted for control patients who were rested (unpublished data). The decreases in synovial fluid NO_2^- were more striking and persistent in patients with clinically and biochemically very active disease. These findings suggest synovial NOS activity may be impaired during joint exercise. Probable explanations include hypoxia or transmural pressure increases during exercise that inhibit endothelial NO production in some vessels,¹⁴² and decreased shear stress as a result of impaired flow; shear stress generally stimulates endothelial NO production. However, these factors may only partially account for exercise induced decreases in synovial fluid NO_2^- , as new evidence suggests an alternative explanation with quite different implications. Zweier *et al* used an oxygen stable ferrous iron spin trap to estimate NO concentrations in an ex vivo model of cardiac ischaemia-reperfusion.¹⁹⁷ They found that, during ischaemia, NO may be generated non-enzymatically by the reduction of NO_2^- to NO, and that the formation of NO correlates with increasing duration of ischaemia and decreasing tissue pH, and is responsible for impaired myocardial contractility. It is probable that a similar phenomenon occurs in the synovium during joint exercise and may be responsible for the observed exercise induced decreases in synovial fluid NO_2^- . Clearly, this last possibility requires confirmation, preferably by direct measurement of NO in vivo. The implications for the pathogenesis and persistence of rheumatoid synovitis are profound, as increased NO formation could have

many adverse consequences, either directly or as a result of greater NO concentrations increasing radical generation via the reaction with O_2^- .

Studies in primary knee osteoarthritis (OA) showed increased NO_2^- concentrations in OA synovial fluid compared with OA serum, and in OA serum compared with that of age and gender matched controls.¹⁸⁷ Explaining the latter observation challenges current concepts of OA, whilst the former suggests generation of NO within the osteoarthritic joint, but to a lesser degree than in RA. Although synovium may be responsible, chondrocytes are an alternative source of NO.^{21–22} The most effective inducers of NOS in rabbit articular chondrocytes were IL-1 and LPS, which also demonstrated pronounced synergy compared with the small increases in NOS activity attributable to IL-1 in combination with $\text{TNF}\alpha$ or $\text{IFN}\gamma$.²¹ Recent evidence that NO mediates suppression of proteoglycan synthesis by IL-1 suggests that NO is relevant to cartilage degradation in vivo.¹⁹⁸ The same study showed that cytokine-stimulated increases in NO production and decreased proteoglycan synthesis by rabbit articular slices in vitro were reversed by the NOS inhibitor, LNMMA. NOS activity has also recently been demonstrated in human chondrocytes in response to IL-1 β , $\text{TNF}\alpha$, or LPS alone,¹⁹⁹ which contrasts with the multiple cytokines required to induce NOS in other cells, including rabbit chondrocytes.

The potential for NO to modulate chondrocyte metabolism and perhaps cartilage degradation in RA and OA is complemented by the effect of NO on bone. Both mouse and human osteoblasts produce NO,^{200–202} with the human studies showing that osteoblast NOS expression is of the inducible, not the constitutive, type and that NO production greatly impairs osteoblast proliferation.²⁰² Some reports have suggested that NO inhibits osteoclast function and bone resorption,^{201–203} but other studies have shown that NO stimulates bone resorption.²⁰⁴ The latter is crucial, as it raises the possibility that NO mediates articular erosions in RA and offers the therapeutic promise that NOS inhibitors might prevent joint damage in RA. NO is clearly also relevant to other conditions exhibiting excessive osteoclastic bone resorption, such as Paget's disease and osteoporosis.

INFECTION AND CANCER

Cytotoxicity against microbes and tumour cells was one of the first recognised and most studied actions of NO. NO mediates the non-specific component of the T cell immune response and is particularly targeted against intracellular microbes and protozoa. NO mediated microbial killing can occur independently of T cells, as shown by the resistance of immunodeficient mice to *Listeria*.²⁰⁵ NO is also implicated in the response against or killing of leishmania, schistosoma, trypanosomes, *Plasmodium falciparum*, *Mycobacterium leprae* and *M. tuberculosis*, *Legionella pneumophila*, and several viruses.^{206–212} The

latter exclude some of the most important bacterial pathogens that are extracellular targets of the oxygen dependent cytotoxic mechanisms of PMN, though PMN can utilise NO in killing *Staphylococcus aureus*.²¹³

Candidate mechanisms by which NO effects cytostasis or cytotoxicity are via inhibition of iron centred enzymes and the formation of peroxynitrite, respectively. The former might explain the long recognised positive association between iron and infection. Indeed, excess iron reverses the cytostatic effects of NO on *Trypanosoma* sp.²¹⁴ However, another feature of trypanosomal infection, immunosuppression of the host, is also mediated by NO. In a manner analogous to the allogeneic mixed lymphocyte reaction, NO synthesis by host macrophages suppresses T cell proliferation.²¹⁵ Despite these findings, the evidence strongly favours a net antimicrobial effect for NO.

There are, however, circumstances in which NO may be detrimental. It has been hypothesised that excessive NO synthesis accounts for CNS dysfunction in cerebral malaria,²¹⁶ and there is definitive evidence that NO is the final common pathway leading to septic shock. The latter remains an important cause of mortality, especially in patients with underlying disease or the immunosuppressed—categories into which many rheumatic disease sufferers fall. Septic shock is initiated by endotoxins derived from bacterial cell walls which activate several humoral pathways and, most importantly, stimulate excessive cytokine release. Most interest has focused on TNF α as the principal mediator, though other cytokines are involved, as outcome correlates with the levels of IL-1, IFN γ and TNF α .²¹⁷ LPS, IL-1, IFN γ and TNF α are also the principal mediators of inducible NO synthesis in endothelial cells and VSMC, which are the probable cellular source of excessive intravascular NO release in septic shock.^{188 189} In man, increased NO production occurs in sepsis and greater concentrations are associated with decreased systemic vascular resistance and greater circulating concentrations of endotoxin.¹⁸⁸ In animal models of septic shock, hypotension is reversed by NOS inhibitors,^{218 219} however, high doses of the inhibitors may accelerate hypotension,²²⁰ resulting in underperfusion of vital organs and other potentially detrimental effects. Initial reports were that LNMMA reversed hypotension^{221 222} in patients with septic shock. A small randomised double blind placebo controlled study has shown that LNMMA increases blood pressure, systemic and pulmonary vascular resistance, and central venous pressure, but decreases cardiac output.²²³ Further studies are clearly required to assess its possible adverse effects and confirm its efficacy and influence on outcome. Selective inhibition of the inducible NOS of endothelial cells and VSMC may improve the therapeutic benefit/risk equation and be more successful than inhibition of either endotoxin or TNF α .

Cytokine induced hypotension is also crucial in deploying immunotherapy against cancer. Hypotension is a serious complication of IL-2 treatment which, in clinical trials, has shown

therapeutic benefit to patients with advanced melanoma and renal cell carcinoma.²²⁴ Two separate studies provided evidence that NO synthesis is dramatically increased after IL-2 treatment^{190 191} and that NO₂/NO₃⁻ concentrations correlate inversely with mean blood pressure.¹⁹² If planned clinical trials of NOS inhibitors prove successful, it may be possible to use larger doses of IL-2, with the prospect of improved clinical response.²²⁵

The tumouricidal effects of macrophage NO production are well recognised and amongst several candidate mechanisms is the inhibition of ribonucleotide reductase, probably via an essential tyrosyl radical.²²⁶ Moreover, conversion of L-arginine to ornithine/urea via arginase, and to NO via NOS are associated with tumour progression and rejection, respectively.²²⁷ Antitumour treatment strategies involving NO include stimulating macrophage iNOS²²⁸ and selectively impairing tumour blood flow with NOS inhibitors.²²⁹ However, NO also has carcinogenic potential, as it alters DNA in vitro and is mutagenic to bacteria²³⁰ and human cell lines,²³¹ furthermore, NO derived nitrosamines could explain the association between malignancy and inflammation. These effects are also highly relevant to inflammation, as NO attacks pancreatic islet cell DNA,²³² impairs enzymes that repair DNA,²³³ produces a pattern of DNA fragmentation typical of apoptosis,²³⁴ and directly induces apoptosis in tumour cells²³⁵ and macrophages.²³⁴ Recent evidence implicates p53, the tumour suppressor gene, in NO induced apoptosis, as NO and NO inhibitors respectively stimulate and prevent p53 expression and apoptosis.²³⁶

Other actions of NO

NO regulates basal pulmonary vascular tone,²³⁷ mediates vasodilatation, and attenuates vasoconstriction in vitro²³⁸ and ex vivo.²³⁹ Moreover, NO mediated responses are impaired in animals²⁴⁰ and humans with chronic hypoxia.^{241–243} Because of its local delivery and breakdown, inhaled NO is a *selective* pulmonary vasodilator that reverses acute hypoxic pulmonary vasoconstriction in vivo.²³⁷ In human studies, inhaled NO was beneficial in persistent pulmonary hypertension of the newborn^{244 245} and adult respiratory distress syndrome.²⁴⁶ NO mediated NANC nerve bronchodilatation is also the sole neural mechanism of airways dilatation in man.¹³⁵ Inhaled NO decreases methacholine induced bronchoconstriction in animals,¹³⁶ probably by directly relaxing bronchial smooth muscle, and has recently been shown to have a similar effect in humans.²⁴⁷ NO can also mediate neurogenic inflammation in the lung,¹⁷⁸ though histamine modulates mast cell degranulation by a negative feedback loop involving NO activation of GCase.²⁴⁸ Intriguingly, NO is also involved in the upregulation of ciliary beating,²⁴⁹ which is inhibited by pyocyanin from *Pseudomonas aeruginosa*.²⁵⁰

NO has been investigated most extensively in the gut, in respect of its role in NANC

neurotransmission. NO has been directly implicated in the control of sphincters of the lower oesophagus,¹³⁷ ileocolonic junction,¹³¹ and pylorus.²⁵¹ The last of these is of practical importance in infantile hypertrophic pyloric stenosis, in which defective pyloric relaxation may be aetiological. The absence of NADPH diaphorase activity in the hypertrophied circular musculature of affected infants suggests that a lack of NO synthase is the cause of pylorospasm, and perhaps the primary cause of this disorder;²⁵² this concept is supported by similar abnormalities in a mouse nNOS gene knockout model.¹³⁸ Increased NO synthesis is implicated in the haemodynamic consequences of cirrhosis leading to the hepatorenal syndrome.²⁵³ It contrasts with the contribution of decreased blood flow to acute non-steroidal gastric lesions in rats.²⁵⁴ NOS inhibitors reduce gastric mucosal blood flow²⁵⁵ and attenuate increased blood flow associated with gastric acid secretion,²⁵⁶ whilst organic nitrates reduce ethanol induced mucosal damage,²⁵⁷ but NOS inhibitors only cause mucosal damage in the presence of other factors such as the inhibition of vasodilatory prostaglandins and neuropeptides.²⁵⁸ Whilst animal studies suggest NO mediates the gastroprotective effects of several agents, NO is also implicated in gut inflammation—a paradox explained by the existence of constitutive and cytokine induced NOS. Increased NOS activity has been demonstrated in animal models of both ileal²⁵⁹ and colonic inflammation,²⁶⁰ whilst NOS inhibitors alleviate inflammation in a guinea pig model of ileitis.²⁶¹ In man there is both indirect¹⁹² and direct evidence¹⁹³ of increased NOS activity in ulcerative colitis, though not Crohn's disease.

The widespread actions of this simple organic molecule are astonishing, and clearly relevant to pathophysiological events in many tissues, circumstances and diverse medical fields. NO has many actions relevant to rheumatic diseases, being implicated in inflammation and immunoregulation, hypoxic-reperfusion injury and vasculitis, cartilage and bone physiology, peptic ulceration, and pain mechanisms. Understanding the role of NO in a wider context should also help clarify the influence of NO on rheumatic diseases. Studies are in progress in several clinical areas to establish the benefits and problems of manipulating NO synthesis and, together with current molecular studies of NOS expression and regulation, should improve the understanding of the multifarious roles of NO.

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