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NITRIC OXIDE – THE SECRET SYMPATHY*

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> It is the secret sympathy,
> The silver link, the silken tie,
> Which heart to heart and mind to mind,
> In body and in soul can bind.
>
> *Sir Walter Scott, The Lay of the Last Minstrel*

Nitric oxide, an inorganic radical gas, is also one of the most important modulators of cellular function in biology; only since the 1970s has this importance become apparent. The purpose of this paper is to review the discovery of this ubiquitous mediator, and to discuss how nitric oxide activity is central to the action of many systems. Its particular importance in cardiovascular biology will be highlighted, especially the emerging role of nitric oxide in normal myocardial function and heart disease will be discussed.

The existence of nitric oxide (NO) has long been known to chemists - a simple molecule. Its structure, N=O, with an unpaired electron makes it a highly reactive species, rapidly metabolized within a few seconds in mammalian cells.

DISCOVERY OF THE BIOLOGICAL IMPORTANCE OF NITRIC OXIDE

Four independent cornerstones of investigation led to the discovery of the importance of nitric oxide in biological systems;[1] these are illustrated in Figure 1.

(i) Mammalian cells generate a reactive nitrogen oxide from the guanidino end of the amino acid, L-arginine.

(ii) These reactive nitrogen oxides mediate a physiological response in macrophages. Chemically modified L-arginine analogues block the synthesis of nitrogen oxides.

(iii) The endothelium also produces nitric oxide. Its action within the vasculature had previously been the subject of intensive research by vascular biologists, who had identified some of its functions, and ascribed them to Endothelial-Derived Relaxing Factor (EDRF).

(iv) Nitric oxide is produced by neural tissue and has properties of a neurotransmitter.

The background to the discovery of each of these pathways will be described individually: all involve a common substrate (L-arginine).

Macrophages as a source of nitric oxide

Interest in biological nitrates developed following the establishment of an association of nitrosamines with carcinoma of the stomach; attempts to study this association in animal models and human subjects showed that mammals synthesize nitrates.[2,3] At first it was considered that intestinal bacteria were the source of these nitrates, but studies with germ-free rats showed that these animals also produced nitrogen oxides. An important observation was made when a human subject became ill with intercurrent

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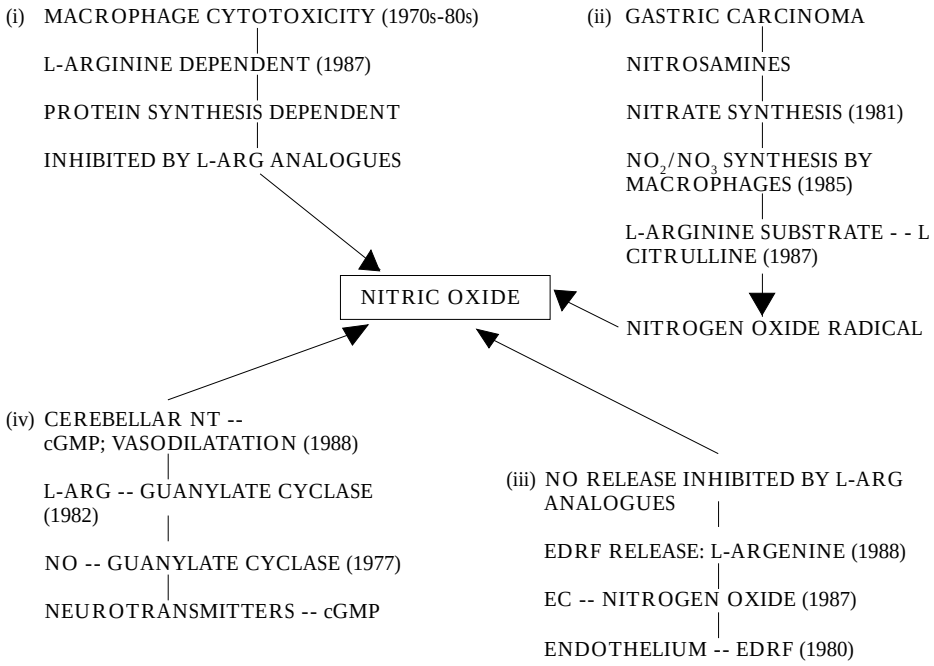

FIGURE 1
Discovery of nitric oxide biology.

infection during one study. Excretion of nitrates (NO_{3^-}) became markedly elevated and this finding was confirmed in animal experiments.[4,5] It appeared that macrophages accounted for, at least in part, the excess production of nitrogen oxides. Explanted macrophages were able to secrete these substances,[5] generated from oxidation of a guanidino nitrogen from the amino acid, L-arginine.[6] This oxidation yielded the amino acid citrulline, and a reactive compound, more reactive than either nitrite or nitrate, which could nitrosate amines to toxic nitrosamines.[7]

Independently, other researchers were studying the oxidative injury that macrophages cause to tumour cells and fungi. Hibbs had been studying this field since the early 1970s[8] and, in 1987, showed that macrophage cytotoxicity required L-arginine, and that modification of the L-arginine molecule blocked both nitrite production and cytotoxicity of macrophages.[9] The production of nitrogen compounds also required protein synthesis, implying induction of an enzyme within the macrophages.[10] The development of analogues of L-arginine which inhibit synthesis of nitrogen oxides has led to a virtual explosion in nitric oxide research.

Endothelium as a source of nitric oxide

Furchgott and Zawadski showed in their landmark study in 1980[11] that endothelium is not merely the inert lining of a hollow tube, but that it can influence adjacent smooth muscle in the vascular wall via a mediator they termed EDRF. The identification of EDRF as nitric oxide happened during the subsequent eight years, although as early as 1977 Arnold had shown nitric oxide capable of activating guanylate cyclase in different tissues, the enzyme now known to be the target for nitric oxide in smooth muscle.[12] In 1986 Ignarro *et al* independently suggested that EDRF might be

nitric oxide.[13] In 1987, two groups showed that stimulated endothelium released a nitrogen oxide which was neither nitrite or nitrate; a chemiluminescent assay failed to identify which nitrogen oxide it was.[14,15] In 1988, evidence from Moncada's group, amongst others, showed that EDRF release from cultured endothelial cells required L-arginine,[16,17] and that release of nitric oxide from endothelium was inhibited by analogues of L-arginine.[18]

Neural tissue as a source of nitric oxide

Neurobiologists had known since the early 1970s that some neurotransmitters elevated cyclic guanosine monophosphate (GMP) concentrations in neural tissue.[19] In 1977, Miki showed that nitric oxide stimulated the guanylate cyclase enzyme in mouse cerebral cortex.[20] In 1982 L-arginine was shown to activate guanylate cyclase in neuroblastoma cells,[21] and in 1988 cerebellar tissue was shown to release a mediator with properties like nitric oxide, which both elevated cyclic GMP and relaxed vascular smooth muscle.[22] Release of nitric oxide and citrulline by neural tissue was inhibited by an analogue of L-arginine, evidence that synaptic cells contained the nitric oxide synthase enzyme.[23]

Biosynthesis of nitric oxide

Nitric oxide is synthesized from the amino acid, L-arginine. It was initially believed that cells from different tissues had different synthetic pathways, but recent work has refuted this. Figure 2 shows the pathway of nitric oxide production from L-arginine. Both oxidation steps are catalysed by the enzyme, nitric oxide synthase; NADPH donates three electrons, molecular oxygen two more.

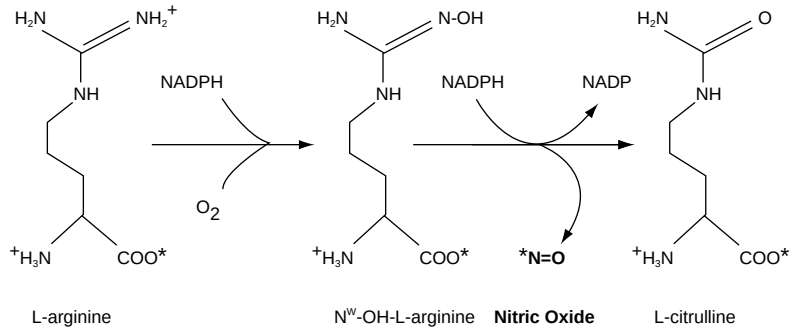

FIGURE 2
Synthesis of nitric oxide from L-arginine.

Three different nitric oxide synthase (NOS) enzymes are currently recognised in man: a neuronal synthase (nNOS or NOS1) exists in many neuronal tissues; a constitutive, endothelial cell enzyme (eNOS or NOS3) is present in vascular endothelial cells and many other cell types; and a third is induced under certain conditions, for example tissue injury or inflammation. This inducible enzyme (iNOS or NOS2) appears to be particularly important in disease states.

The action of NO can be blocked experimentally by modifying the L-arginine amino acid substrate, a technique used widely in experimental models. Induction of inducible nitric oxide synthase can be effectively prevented by corticosteroids,[24] and by some cytokines, for example transforming growth factor-ß (TGF-ß), and some interleukins.[25]

Moncada has summarised the importance in terms of phylogenetics and evolution of nitric oxide biology (invited lecture to the American Heart Association, November 1991).[26] Not only do all mammals examined employ nitric oxide as an intercellular mediator, but even primitive invertebrates use nitric oxide in their circulatory 'blood-like' cells as a chemical messenger, and probably as a cytotoxic agent too.

INTRACELLULAR SITE OF ACTION OF NITRIC OXIDE

In many tissues the principal target for nitric oxide is the soluble guanylate cyclase enzyme.[27] Guanylate cyclase is activated by nitrosation of its haem moiety by nitric oxide. Elevation of intracellular cGMP concentrations in vascular smooth muscle brings about vasorelaxation by mechanisms which are, as yet, only partially characterised, but which must ultimately involve cytosolic calcium. Cyclic GMP probably enhances phosphorylation of key proteins involved in handling Ca^{2+}, perhaps along with direct inhibition of Ca^{2+} binding to contractile proteins. The overall result is a reduction in free calcium, perhaps together with reduced sensitivity of contractile proteins to Ca^{2+}.

Within tumour cells macrophages exert their cytotoxic action by releasing nitric oxide which inhibits Fe-S proteins such as NADH.[1] Nitric oxide also inhibits tumour cell DNA synthesis[28] and also reacts with superoxide anions in a reaction that can either detoxify both molecules, or generates the powerful oxidants, nitrogen dioxide and hydroxyl radical.[29]

NITRIC OXIDE AND BLOOD FLOW

The most important stimulus to nitric oxide release may be shear stress on the endothelial luminal surface, secondary to flow of blood. Flow-induced dilatation, long known but unexplained by physiologists, is substantially accounted for by mechanisms involving release of nitric oxide from endothelium. It has been considered that vasodilatation of blood vessels supplying ischaemic tissue is caused by accumulation of metabolites, but recent studies have shown that vasoactive substances from endothelium also contribute to vascular changes brought about by hypoxia. For example, NO and the prostaglandins PGI_2 and PGE_2 are all released from hypoxic cultured endothelial cells suggesting that oxygen sensors are located on endothelium.[30] Park has also recently shown that NO, adenosine and vasodilatory prostaglandins are all involved in hypoxic coronary vasodilatation.[31]

Within the microvasculature there also appears to be a tonic release of nitric oxide, where it has been shown to act as a vasodilator in several different preparations.[32] Infusion of inhibitory analogues of L-arginine elevate blood pressure substantially, by inhibiting this basal release of nitric oxide from resistance vessels. The implication is, therefore, that nitric oxide is involved in the maintenance of normal vascular tone.

NITRIC OXIDE, HYPERTENSION AND ATHEROSCLEROSIS

Infusion of inhibitors of nitric oxide synthase raises blood pressure in animals, making it an attractive hypothesis that disturbances in endothelial function may contribute to hypertension. Endothelial responses are impaired in animal models of hypertension, and in some patients with high blood pressure.[33,34,35] Some possible theories include: impairment of nitric oxide release from arteriolar endothelium; abnormalities of nitric oxide agonist receptors; altered nitric oxide diffusion because of smooth muscle hypertrophy; and alterations in cellular second messengers/cellular breakdown of cAMP

and cGMP. However, abnormalities of nitric oxide biology do not account for all the abnormalities of blood pressure and blood flow seen in hypertensive subjects.

Atherosclerosis is characterised by the development of plaques. These lesions develop within the intima, replacing healthy endothelium with a hyperplastic collection of lipid-rich material, macrophages, smooth muscle cells and fibrous tissue, usually covered by endothelium. Endothelium overlying stable plaques is abnormal, both in function and in ultrastructural appearance. In animals made hyperlipidaemic, endothelial responses to agents which stimulate nitric oxide release are diminished, while responses to endothelium-independent (e.g. sodium nitroprusside) vasoactive agents are intact.[36]

Endothelial dysfunction has also been demonstrated in experimental models of diabetes,[32] and patients with diabetes (both IDDM and NIDDM) may have abnormal vasodilator responses because of dysfunctional endothelium. Insulin itself is an NO-dependent vasodilator, but in diabetics its actions as a potential stimulus to atheroma may outweigh this beneficial action.

In patients with atheromatous coronary artery disease, impaired responses to endothelium-dependent vasodilators also occur. Loss of tonic nitric oxide release at sites of atheroma might predispose towards focal coronary spasm. Moreover, nitric oxide activity in the peripheral vasculature is impaired in models of chronic heart failure, and in the coronary arteries of patients with dilated cardiomyopathy.[32] Thus, there is much evidence indicating the importance of nitric oxide in atherosclerotic disease. However, within the coronary circulation, nitric oxide is only one of a number of mediators that control blood flow. Indeed, NO inhibition by modified L-arginine (L-NMMA) in patients with normal coronary arteries has shown only modest effects on blood flow.[37]

RELEASE OF NITRIC OXIDE FROM NITROVASODILATOR DRUGS

Organic nitrates have been used to treat angina pectoris for over a century, but only recently has their mechanism of action been established.[32] Nitro-vasodilator drugs act within blood vessel walls by being metabolized to release nitric oxide. These drugs are thus a convenient source of nitric oxide for experimental purposes. In the past it was generally assumed that nitrates did not have much effect on myocardial contractility. However, the proof that this was indeed the case was never well substantiated. The isolated, functioning cardiac myocyte, suspended in an experimental cell bath is a suitable model to study the effects of nitrates and nitric oxide on the heart in the absence of haemodynamic changes, and this experimental system is used in many of the studies described below.

ACTIONS OF NITRIC OXIDE IN THE HEART

Endocardial endothelium and its role in the modulation of myocardial contraction

Until recently endocardium was considered, like vascular endothelium, not to be of much importance other than as a structural element of the heart, but with an important role in preventing thrombus formation within cardiac chambers. However, in a classic paper, Brutsaert showed that damage to the endocardial surface of isolated papillary muscle caused a reduction in isometric tension,[38] thus suggesting tonic release of a positive inotropic substance by endocardial endothelium. A reduction in contraction was also detected in endocardium-intact preparations with administration of lipid-soluble analogues of cGMP, or of sodium nitroprusside.[39]

Evidence from a number of laboratories, but principally from Shah's group in Cardiff, UK,[40] showed that using endocardial cells on beads in a cascade system, endocardial

endothelium appears to release both nitric oxide as a modulatory, negatively inotropic agent, and a positive inotropic agent, now is considered to be the vasoconstrictor peptide, endothelin.

Nitric oxide and myocardial contractility

The heart is very vascular and thus full of vascular endothelium, providing the contracting cells, the myocytes, with a rich blood supply. Each cardiac myocyte is within a few millimetres of an adjacent capillary, well within the diffusing distance of nitric oxide. By 1990 it was established that, within the peripheral vasculature, NO controls vascular smooth muscle tone, and experiments were begun to determine whether heart muscle also was similarly influenced by this novel mediator.

In 1991 our group in London provided early cellular evidence that NO could modify cardiac myocyte contractility.[41] The nitrates, glyceryl trinitrate and isosorbide dinitrate had no effect, but sodium nitroprusside attenuated myocyte contraction (Figure 3).

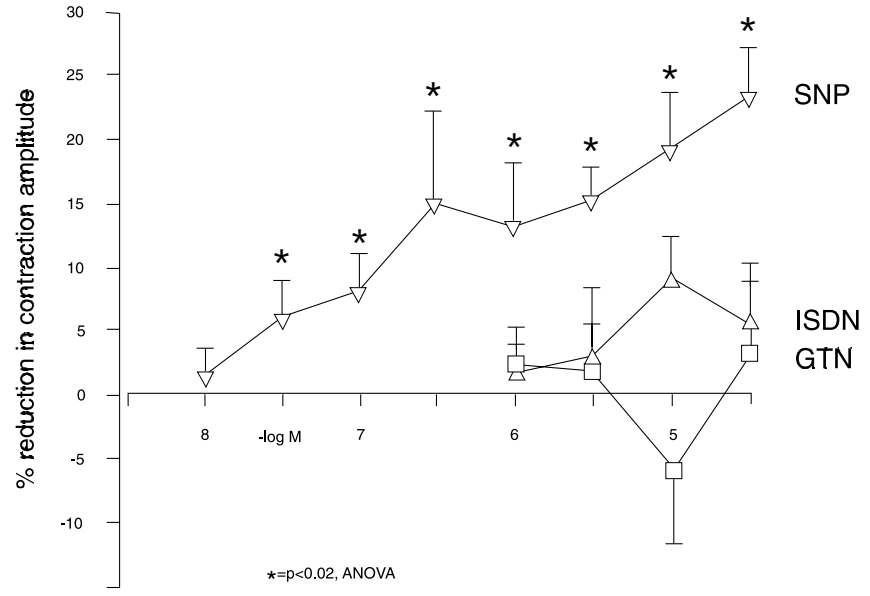

FIGURE 3

Effect of different nitro-vasodilators on myocyte contraction amplitude; differences are expressed as percentage reduction of control contractions; mean ±SEM of 6-10 experiments at each concentration of each drug. SNP=sodium nitroprusside; ISDN=isosorbide dinitrate; GTN=glyceryl trinitrate; (ANOVA=analysis of variance).

Studies over the next two years in this London laboratory and in other centres confirmed that exogenous NO, either from nitrate donor drugs or if applied in solution, had a negative inotropic effect. In a co-culture study of cardiac myocytes in close apposition to endothelium, modelling the microenvironment within the heart,

stimulation of endothelial cells to release NO, again reduced myocyte contraction.[42] At the same time experiments by Balligand, Ungureanu and Kelly identified similar findings, and carried the work much further:[43] they showed that cardiac myocytes themselves contain a small amount of eNOS, which appears important in modulating contractile responses to adrenergic and vagal influences.

Many laboratories around the world are currently studying NO biology in heart muscle[43] and many papers on this subject have been published over the last few years. The extent to which nitric oxide modulates normal cardiac function is not yet fully determined, but the involvement of NO in signalling pathways, within and between cells in the heart, is certain to be found crucial to cardiovascular regulation.

NITRIC OXIDE IN ENDOTOXIC SHOCK

While reduced NO activity may be important in atheroma and perhaps hypertension, there has been a great deal of interest in the overactivity of nitric oxide in endotoxic shock. Septicaemia and accompanying septic shock account for many hospital deaths, despite aggressive modern therapy. In the USA, an estimated 100,000 patients die from sepsis in hospital each year. One of the characteristic features of septic shock is profound hypotension caused by a decrease in peripheral vascular resistance, unusually resistant to both volume replacement and vasoconstrictor agents. As septic shock worsens, myocardial function deteriorates and ventricular dilatation develops with a reduction in ejection fraction. If the patient survives, the heart recovers, and ventricular size and function return to normal as the infection is controlled and circulatory function restored.[44]

Abnormalities of nitric oxide production in endotoxic shock

In endotoxic shock the presence of disseminated foreign antigen, together with the inflammatory response, causes induction of the inducible nitric oxide synthase (iNOS) in many cell types, including hepatocytes, fibroblasts and vascular smooth muscle. Subsequent production of large quantities of nitric oxide leads not only to haemodynamic instability, but also to widespread production of nitric oxide-based free radicals which have the potential to cause considerable damage to tissues. In endotoxic shock production of nitric oxide occurs within the muscle layer of the vessel wall, causing profound vasodilatation and a reduction in peripheral vascular resistance. Inhibitory analogues of L-arginine can reverse this hypotension, but cause a sustained increase in systemic vascular resistance and, at higher doses, a decrease in cardiac output.[44]

Cardiac failure in endotoxic shock

Until 1992, the cause of myocardial depression in endotoxaemia was considered to be a direct effect of endotoxin or an inflammatory mediator on myocardial tissue. The existence of a specific circulating myocardial depressant substance in endotoxic shock having been postulated, but not proven. While coronary perfusion abnormalities in patients with co-existing cardiac or coronary disease and endotoxic shock may account for segmental abnormalities of left ventricular function, in patients with global myocardial impairment and endotoxic shock the loss of function cannot be wholly explained by changes in coronary flow. As in the peripheral vasculature, multiple factors exist which depress cardiac function in endotoxic shock. However, there may be a common pathway for such mediators to impair myocardial contraction.

The hypothesis that overproduction of nitric oxide within cardiac muscle contributes to impaired function was addressed by four independent research groups during 1992. In health, cardiac myocytes do not produce much nitric oxide.[43] In experimental endotoxaemia, or following administration of inflammatory cytokines to isolated myocytes, iNOS is induced within cardiac myocytes. The subsequent generation of quantities of nitric oxide within the myocytes themselves is accompanied by a substantial loss of contractile function.[45] Both the generation of nitric oxide by myocytes and their depression of contractility can be reversed by specific inhibitors of the nitric oxide synthase enzyme (Figure 4). Pretreatment with high dose corticosteroids prevents the induction of this enzyme and blocks completely the impairment of contraction. Also, the cytokine TNFa and the interleukins IL-6 and IL-2 can cause marked depression of cardiac contraction in isolated papillary muscles, an effect mediated by nitric oxide generation within the cardiac muscle itself.[46]

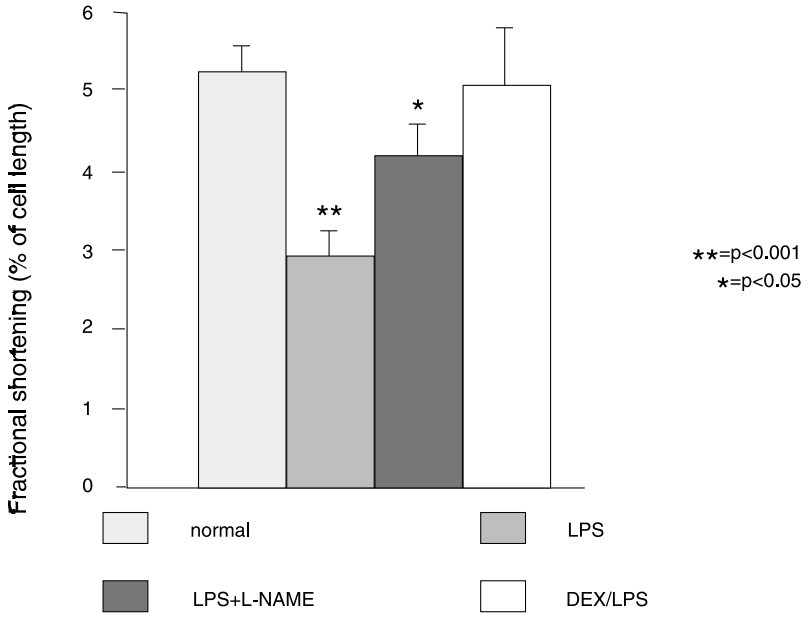

FIGURE 4
Effect of endotoxin (lipopolysaccharide, LPS) treatment on fractional shortening. Dex: dexamethasone. Mean ±SE of 32 (normal), 17 (LPS treated) and 7 (LPS + dexamethasone) experiments. (L-NAME=L-nitroarginine methyl ester.)

Furthermore, an overproduction of nitric oxide within the coronary microcirculation is found in experimental endotoxic shock.[47] This may not only perturb coronary blood flow in the microvasculature, but may also depress cardiac contraction. Hence,

several groups working independently arrived at the same conclusion that the heart failure in endotoxic shock is, at least in part, caused by nitric oxide over-production within the cardiac myocytes.

Thus, there appears to be a common mechanism in endotoxic shock contributing to both cardiac and vascular dysfunction. Overproduction of nitric oxide within the peripheral vasculature and myocardium, at least in experimental models, contributes in a major way to the vasodilational loss of myocardial contractility seen in patients with endotoxic shock.

Therapeutic strategies for future therapy in endotoxic shock

Experimentally, pretreatment of animals with corticosteroids prevents the induction of the nitric oxide synthase enzyme within the heart and the vasculature.[44] In the clinical setting patients present with established infection, too late for steroids to be of such benefit. Neither specific monoclonal antibodies to endotoxin or to TNF (Tumour Necrosis Factor) have been successful in clinical trials. Although the mechanism of NO overproduction is understood, the existing experimental inhibitors of NOS are not specific for the inducible form of the enzyme which accounts for the overproduction of NO. In animal models of endotoxic shock, a small dose of non-specific NOS inhibitor blocks the excess production of nitric oxide, but higher doses cause inhibition of normal, endogenous nitric oxide production as well, resulting in intense vasoconstriction and cardiovascular collapse.[48]

Widespread inhibition of nitric oxide has potentially serious side-effects: for example, impairment of the normal anticoagulant status of endothelium and alteration of neurotransmission. Moreover, a degree of vasodilatation may in fact be valuable, allowing washout of toxic oxides of nitrogen from tissues.[48]

Encouraging results have been described in two clinical reports using non-specific NOS inhibitors. In two patients with septic shock, administration of L-arginine analogues caused short-term increases in blood pressure; cardiac output was increased in one patient and decreased in the other.[49] A further report from Edinburgh described short-lived improvement in a patient with hepatic failure following administration of methylene blue, an agent which inhibits the effects of nitric oxide on vascular tone.[50] The pharmaceutical industry is working hard on specific inhibitors of iNOS and these will likely be a powerful therapeutic tool for use on patients with severe sepsis.

NITRIC OXIDE AND OTHER HEART DISEASES

Although an excess of nitric oxide may explain heart failure in septic shock, the extent to which NO contributes to other heart conditions is less certain. It is tempting to assume that an overproduction of NO in more chronic conditions may likewise contribute to impairment of myocyte function, but the evidence for this is still fragmentary. It seems likely, particularly in inflammatory conditions with activation of inflammatory mediators, that NO production might be stimulated. For example, in a mouse model of acute myocarditis, iNOS activity could be detected in association with impaired cardiac function.[51] Dilated cardiomyopathy is also a condition associated with myocardial inflammation, and emerging data suggest that iNOS activity may be increased, at least in some cases. This evidence comes mostly from histological study of biopsy material from transplant or post-mortem material;[52,53] however, there is yet no sizeable study to substantiate these case reports.

Most cases of heart failure in the developed world are caused by ischaemic heart disease (IHD). There have been a few case reports of NO overactivity in biopsies of

myocardium from such patients, but there is currently less evidence of NO involvement than in IHD inflammatory conditions.

As part of the disease process of heart failure in ischaemic heart disease, like other forms of heart failure, death of individual cardiac myocytes occurs progressively as the condition worsens. It has recently been shown in an experimental model that this cell death, or apoptosis, may in some cases be triggered by nitric oxide.[54] Currently, a joint project between Edinburgh and Glasgow is addressing whether this occurs in patients with congestive heart failure.

SUMMARY AND CONCLUSIONS

Since the earliest evidence that unstable nitrogen oxides were involved in cell signalling 20 years ago, there has been an explosion of research into the actions of nitric oxide. Indeed there are over 11,000 references to nitric oxide listed by the US National Library of Medicine as of November 1997. The tremendous growth in the understanding of NO biology has been a period of great excitement in cardiovascular research. While much is known of the importance of nitric oxide in the vascular system, its role within the myocardium is still being pursued intensively and it is anticipated that a greater understanding of this action will help to explain some of the processes involved in heart diseases.

Nitric oxide may indeed be the silken tie that Sir Walter Scott perceived as the common thread linking the mechanisms of cardiac and cerebral function.

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