Nitric oxide: discovery and impact on clinical medicine

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Between 1987 and 1988 our research group made a couple of interesting findings. First, we discovered that vascular endothelial cells are able to generate nitric oxide gas (NO)[1], thus explaining the actions of the 'endothelium-derived relaxing factor' that had been described some seven years earlier[2]. And subsequently we found that NO was synthesized directly from the aminoacid L-arginine[3]. Here I present an overview of the new understanding that NO has brought to physiology and pathophysiology, with particular emphasis on the therapeutic possibilities that have arisen as a consequence.

We started to call the biochemical pathway the L-arginine:NO pathway, and the enzyme responsible for this conversion NO synthase[3]. To demonstrate the formation of NO from L-arginine we cultured vascular endothelial cells on microcarrier beads and perfused them *in vitro* with two types of arginine, either uniformly labelled, i.e. with ^{15}N-labelled nitrogen in all the nitrogen atoms of the molecule, or guanidino labelled, i.e. labelled in two guanidino nitrogen atoms. The effluent was connected directly to a mass spectrometer and we measured the formation of ^{15}N-labelled NO. Every time we stimulated the cells with bradykinin, we could see the release of ^{15}N-labelled NO and, since the amounts that were generated from both types of arginine were very similar, we concluded that NO was specifically made from the guanidino nitrogen atoms of the aminoacid[4]. Shortly after this it was discovered that, in the process of making NO, molecular oxygen was introduced in the formation of N-hydroxy L-arginine, which is an intermediate in the synthesis and later on in the formation of L-citrulline, which is the co-product of the generation of NO. The NO produced in this way is usually transferred from a generator cell to an effector cell where it exerts a biological function. In the case of the vasculature, it goes from the endothelial to the smooth muscle cell to activate the soluble guanylate cyclase and by doing so it produces vascular relaxation[5].

We also identified an inhibitor of the synthesis of NO. This compound, N^G-monomethyl L-arginine (L-NMMA), is a mono-methylated version of L-arginine and structurally is the same as L-arginine except that it has a methyl group on one of the guanidino nitrogen terminals. This compound blocks the generation of NO at two specific points in the pathway, one before and one after the generation of the intermediate N-hydroxy L-arginine. Although many inhibitors have been described subsequently, this compound remains the most important biochemical and pharmacological tool for the study of the roles of NO in biological systems[3,6].

Over the past ten years the molecular biology of the pathway has been elucidated[7]. We now know that there are three distinct enzyme isoforms that synthesize NO: one is the endothelial NO synthase (eNOS), which was the first to be identified in the vascular endothelium; later on an enzyme in the neuronal tissue, mainly in the brain, was identified and is known as neuronal NO synthase (nNOS); and a third isoform, an inducible NO synthase (iNOS) that was identified first in macrophages and is not present in non-activated cells, can be generated *de novo* when white cells are incubated either with lipopolysaccharide or with certain cytokines. The isoforms of the enzyme are very similar, with an overall homology of about 50% between them. They are highly complex structures, with an oxygenase domain and a reductase domain, and they have consensus sites for the binding of different biochemical co-factors, including flavin mononucleotide, flavin adenine dinucleotide, nicotinamide adenine dinucleotide phosphate and calmodulin. There is an overall homology between the NO synthases and the cytochrome P450 reductase, but the latter enzyme lacks the binding site for L-arginine. The three isoforms of NO synthase are encoded by three different genes present in different chromosomes in humans. The nNOS is encoded by a gene present in chromosome 12, the eNOS is encoded by a gene present in chromosome 7 and the iNOS is encoded by a gene in chromosome 17. The work on these three isoforms has given rise to large areas of research concerning the biological roles of the L-arginine:NO pathway. The first one relates to the physiology and pathophysiology of the pathway in the cardiovascular system; the second to the role of NO in the central and peripheral nervous tissue and the third is the role of NO in immunology and inflammation. About twenty thousand papers have already been published on the subject of NO and it is impossible here even to mention all aspects of this work.

CARDIOVASCULAR SYSTEM

In 1989 we found that if we injected L-NMMA, the NO synthase inhibitor, intravenously into an anaesthetized

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animal there was a long-lasting increase in blood pressure[3]. This increase in blood pressure is accompanied by inhibition of NO synthesis in the vessel wall and can be reversed immediately by giving L-arginine. This experiment suggested that NO was probably generated constantly in the interior of the vessel wall and was responsible for the maintenance of a vasodilator tone. We went on to show that L-NMMA caused a constriction in every single vascular bed that we tested, including the brachial circulation of man[8]. In an experiment with Patrick Vallance at St George's Hospital we cannulated the brachial artery of volunteers and measured forearm blood flow by plethysmography. When L-NMMA was given intravenously there was a vasoconstriction in the circulation of the forearm. We also found that if we gave L-NMMA orally to animals it produced a sustained hypertension for as long as it was ingested[9]. These were significant observations because L-NMMA, unlike noradrenaline or angiotensin, is devoid of any vasoconstrictor activity in the vasculature. L-NMMA produces vasoconstriction *in vivo* by taking away a normal physiological NO-dependent vasodilator tone, significant in the control and regulation of blood flow and blood pressure. So we proposed that there is an active vasodilator system in the vasculature, represented by the generation of NO, which is constantly opposed by vasoconstrictor influences in the vessel wall. These experiments have been repeated in many different laboratories around the world and probably the most important recent development in this area is that mutant mice, in which the gene that encodes eNOS has been deleted, have been shown to have higher blood pressure than the wild type controls[10]. We have recently carried out some studies in these eNOS knockout animals and have found that, while in wild-type (control) mice the difference in blood pressure between female and male animals is not significant, in the eNOS knock-out animals the males have a much greater increase in blood pressure than the females (Rees DD, Monkhouse JE, Davies N, Huang P, and Moncada S, unpublished) (Figure 1). Such animals will be very important from now on for identification of the phenotype which represents the lack of NO synthase in the vasculature. Since NO is also an inhibitor of platelet aggregation and vascular smooth muscle cell proliferation, playing a general homoeostatic regulatory role in the vessel wall, studies in these animals will throw light on the relevance of NO not only in relation to the homoeostatic functioning of the vascular wall but also in relation to its response to injury.

Before the discovery of NO, the general view was that hypertension was most often due to excessive vasoconstrictor activity in the vasculature. However, our studies indicated that a lack of vasodilator tone might have exactly the same result. We conducted some studies in collaboration with Patrick Vallance in which we measured

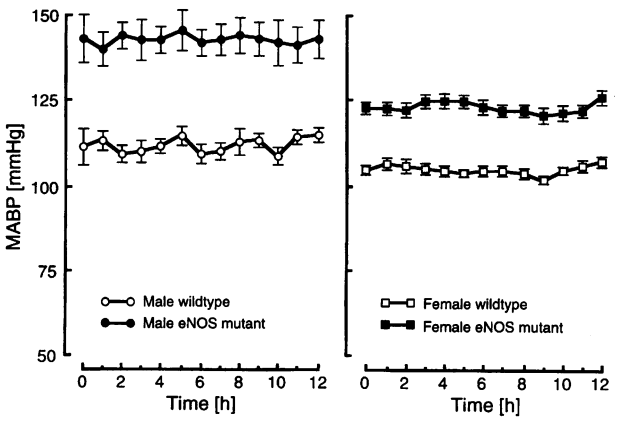

Figure 1 **Mean arterial blood pressure (MAPB) in conscious mice, wild-type and eNOS mutant mice.** [Data from Rees DD, Monkhouse JE, Davies N, Huang P and Moncada S, unpublished]

forearm blood flow in normotensive patients and in hypertensive patients[11]. We compared the responses to noradrenaline, which directly constricts the vasculature, with those to L-NMMA, which constricts by taking away the dilator tone. In the normotensive subjects we could construct dose–response curves that were superimposable. In the hypertensive patients, however, the vasoconstriction induced by L-NMMA was reduced, suggesting that there is a decrease in NO production in the vasculature of hypertensive patients. Since then, much circumstantial and direct evidence has been published pointing to a reduction in NO generation in the vasculature of patients with essential hypertension.

In animal studies there are models of hypertension in which production of NO is high and other models in which production of NO is low; this has created some confusion in the published work[12]. We have suggested recently that, in relation to NO, there might actually be two types of hypertension. In a normal situation vasoconstrictor influences are opposed by the production of NO; however, in one type of hypertension there may be an increase in vasoconstrictor factors which could lead to an increased production of NO to act as a protective mechanism. That type of hypertension would be associated with an enhanced production of NO. In another situation there may be a decrease in NO production and here the normal vasoconstrictor activity in the vessel wall would be unopposed, leading to abnormal constriction. We started to look for an animal model in which this latter situation—reduced generation of NO—occurred. We used the Sabra rat, of which there is a hypertension-prone subset, and in those animals we found a reduction in circulating oxidation products of NO such as nitrite and nitrate[13]. Furthermore,

if we gave L-NMMA to inhibit production of NO, the resultant vasoconstriction was greater in the normotensive controls than in the hypertensives. Thus we have identified an animal model of hypertension in which there is clearly a decrease in production of NO. This animal model also has lower sodium excretion than the normotensive controls. Since NO is involved in the excretion of sodium in the kidney we have suggested that a decrease in production of NO might lead to two accompanying defects—namely, an increase in vascular reactivity and a decrease in sodium excretion.

In addition to its vasodilator actions, NO inhibits platelet aggregation and vascular smooth muscle cell proliferation[5]. Thus it plays a homoeostatic role in the vasculature. A reduction in synthesis of NO in the vasculature may result in conditions such as atherosclerosis[12]. If synthesis of NO in an animal is inhibited and vascular damage is produced, neointimal proliferation accelerates and an atherosclerotic plaque is formed. This process can be prevented by administration of drugs like nitroglycerin that donate NO, and there are already some indications that the same process occurs in man[12]. Thus orally administered L-arginine seems to improve endothelial dysfunction and reduce monocyte endothelial cell adhesion in young men with advanced arterial disease. This potential anti-atherosclerotic action of L-arginine requires further investigation. Interestingly, oestrogens increase the production of NO in the vasculature and this effect may have implications in relation to the protection of women against vascular disease. Animals treated with 17-beta oestradiol for several days have above-normal NO synthase activity in heart, kidney, skeletal muscle and cerebellum[14]. Oestradiol increases not only the activity of eNOS and nNOS, but also the production of enzyme itself. The observation that this enzyme induction also occurs in pregnancy has led to the suggestion that it may account for the decrease in vascular tone and contractility of the vasculature as well as the increase in gastrointestinal transit time that occur in pregnancy. In addition, the effect of oestrogens on the generation of NO may account at least in part for the relative freedom of premenopausal women from heart disease.

Nitric oxide is therefore an endogenous nitrovasodilator substance the action of which is imitated by drugs such as nitroglycerin and sodium nitroprusside that have been in clinical use for over a hundred years. These compounds can be converted chemically into NO and thus mimic its actions and produce mainly vascular relaxation. It may now be possible to start designing drugs that will exploit the other actions of NO—for example, inhibition of platelet aggregation. We have been using a compound called S-nitrosoglutathione (GSNO), which is a more potent inhibitor of platelet aggregation than a vasodilator. This differs from nitroglycerin, which causes vasodilatation but little or no inhibition of platelet aggregation. Administration of GSNO into the brachial circulation of humans causes 100% inhibition of platelet aggregation at very low doses which do not increase forearm blood flow[15]. Administration of nitroglycerin results in the opposite pharmacological profile. These observations led us to look at GSNO in patients subjected to angioplasty, in whom we measured the expression of p-selectin or of IIb/IIIa as indicators of activation of platelets before and during the procedure[16]. These patients, who are already treated with heparin, nitroglycerin and aspirin, still show some degree of platelet activation across the coronary circulation. However, addition of GSNO to the normal cocktail of drugs inhibited the expression of p-selectin and the expression of IIb/IIIa. If the aggregation of platelets during the process of angioplasty results in vessel occlusion because of cell proliferation, GSNO may become a useful drug to inhibit platelet aggregation and prevent the restenosis which occurs in about 30% of cases.

Since NO also relaxes non-vascular smooth muscle, there may be other uses for nitrovasodilators. For example, NO is a powerful relaxant of uterine muscle, so NO donors may prove useful for prolongation of gestation. Thus, in addition to the development of new NO donors, new uses may be found for old ones.

NERVOUS SYSTEM

Nitric oxide is not just a vasodilator. It is also formed in the central nervous system where it activates the soluble guanylate cyclase. The wide but not uniform distribution of NO-synthesizing neurons in the central nervous system suggests a variety of functions[17,18]. The known physiological actions of NO in the brain include involvement in memory, the regulation of cerebral blood flow and the formation of cerebrospinal fluid. It is possible that NO is the substance that integrates neuronal function with blood flow, a phenomenon identified by Sherrington about a hundred years ago. In addition, NO may be the retrograde messenger that has been postulated in the process of 'long-term potentiation' which underlies the formation of memory. Glutamate is produced by a presynaptic terminal and this activates glutamate receptors, especially NMDA receptors, which in turn stimulate the L-arginine:NO pathway. Nitric oxide produced in this way travels to the presynaptic terminal and increases the production of glutamate, thus creating the state of long-term potentiation.

We are used to thinking of neurotransmitters as being synthesized in vesicles, stored there and released during nerve stimulation, after which they specifically activate certain receptors. Nitric oxide, however, seems to be synthesized on demand—i.e. after nerve stimulation—with

an action that spreads over a wide region; from a single point release in the brain, NO might influence a network of neurons over a distance of 100 μm. If this is the case then probably NO should be regarded more as a coordinator of neuronal function than as a simple neurotransmitter.

We now know that a large network of nerves in the periphery, which were classified when discovered about 25 years ago as non-adrenergic and non-cholinergic, can all be termed as nitrergic because they release NO[19–21]. These nerves are widely distributed in the blood vessels of the brain, in the airways and the pulmonary circulation, in the gastrointestinal tract and in the genitourinary tract. In the mammalian stomach NO produces the adaptive relaxation that occurs when the volume of the contents increases. Throughout the gastrointestinal system, especially in the sphincters, the nitrergic nerves produce a relaxant tone. In rats, administration of an NO synthase inhibitor has been shown to increase both intraluminal pressure and phasic movements[22]. So NO released by nitrergic nerves maintains a dilator tone throughout the gastrointestinal system. We looked at the possibility that there might be a human disease related to a decrease in NO generated by nNOS. We investigated the disease achalasia, in which there is a closure of the gastro-oesophageal sphincter, comparing biopsies of achalasia patients with those from patients who were to be operated on for other reasons. We found that in the patients with achalasia there was no immunostaining for NO synthase, while in the myenteric plexuses of patients who did not have achalasia there was clear staining for the enzyme[23]. At around the time we published this paper, there was a report also suggesting that children with pyloric stenosis had subnormal NO production in the area of the pyloric sphincter. Interestingly, if you 'knock out' the gene that encodes the nNOS, these mutant animals show a syndrome similar to hypertrophy of the pyloric sphincter[24].

The L-arginine : NO pathway seems to be a primitive system that has developed along with the animal kingdom. There is now evidence that NO has very similar roles in primitive species to those it has in mammals. We have studied the relaxation of the stomach of the starfish. The stomach has a pyloric part and a cardiac part, and the animal everts its stomach in order to pick up food from the bottom of the ocean. Using an antibody that we had developed to identify mammalian nNOS, we found immunostaining of neurons and fibres in the starfish stomach[25]. Since the starfish is a primitive species, these observations indicate that the function of NO has been highly conserved throughout evolution. Another area in which nitrergic nerves play a role is the genitourinary tract. Penile erection in animals and human beings is totally dependent on the generation of NO by nerves that innervate structures in the corpus cavernosum. We have recently completed a study of the innervation of the human corpus cavernosum using immunostaining with a specific antibody for nNOS (Rodrigo J, Cellek S, Roile M, Martinez-Murillo R, Bentura M, Fernandez A, Serrano J, Martinez-Valasco J, Alonso D, Santacana M, Uttenthal L, Moncada S, unpublished). We found staining for nNOS around the blood vessels in the corpus cavernosum. There is evidence that the dysfunction of the gut and of penile erection that occurs in diabetes could be due to specific damage of the nitrergic innervation in these organs.

IMMUNOLOGY AND INFLAMMATION

The third isoform of NO synthase (iNOS) was originally identified in macrophages[26]. Circulating macrophages that are not activated do not produce NO, but if they are activated with certain cytokines or with lipopolysaccharide there is *de novo* induction of NO synthase, which generates NO in large quantities and for very long periods. This differs from eNOS and nNOS which release small quantities of NO in response to receptor stimulation. This iNOS in inflammatory cells uses NO as a cytostatic and cytotoxic agent[27,28]. Nitric oxide inhibits enzymes in the mitochondria, especially complex I and complex IV, as well as enzymes in the nucleus such as ribonucleotide reductase, responsible for the synthesis of DNA. Nitric oxide released by iNOS from murine macrophages is cytostatic and cytotoxic for protozoan parasites, fungal cells and bacteria. It has been shown that human macrophages can kill *Leishmania major* by the production of NO. Thus NO, either on its own or in combination with oxygen radicals, might be a more important cytotoxic or cytostatic agent than oxygen radicals themselves. We have found that topical application of an NO donor, S-nitrosopenicillamine, in patients with cutaneous leishmaniasis kills the parasites and heals the lesion[29] (Figure 2). Thus, it is possible that donors of NO might be used as cytostatic and cytotoxic agents in certain conditions.

Nitric oxide is now known to be released in large quantities in the vasculature in septic shock. Generation of excessive amounts of NO by the vasculature explains the hyperreactivity to vasoconstrictor agents and the hypotension of septic shock. This has led to the suggestion that NO synthase inhibitors might be used in the management of this condition. In human patients with septic shock, L-NMMA infusions have reversed the hypotension. Selective inhibitors of iNOS, which do not prevent the production of NO by eNOS, may prove more beneficial in treatment of septic shock[6,30].

Nitric oxide generated by iNOS is present in various inflammatory conditions of man including rheumatoid arthritis, Crohn's disease and asthma[31]. iNOS also contributes to conditions such as foreign body inflammation, specifically in septic loosening of joints. So in different

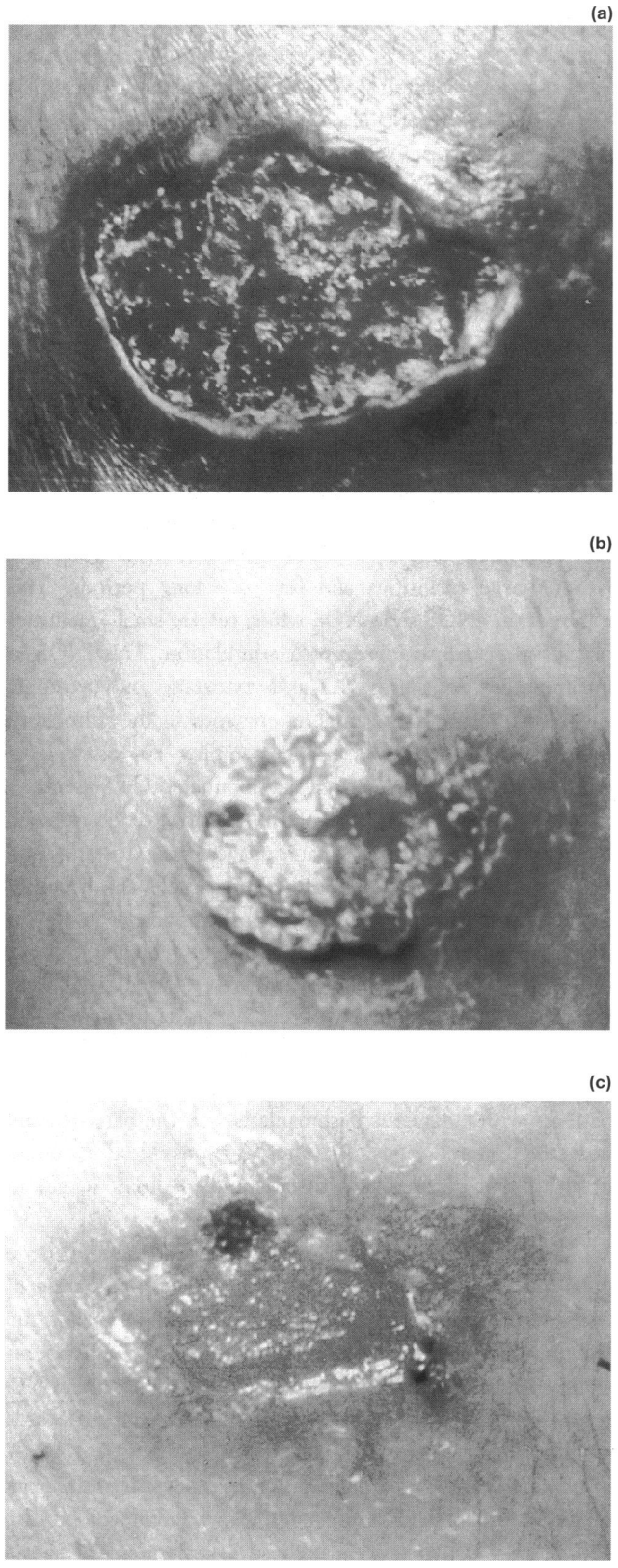

Figure 2 **Effect of treatment with S-nitrosopenicillamine on cutaneous leishmania ulcer.** (*a*) Typical leishmania ulcer (26 × 16 × 7 mm). (*b*) Topical application of a cream containing the NO donor for 5 days resulted in visible improvement (centre). (*c*) After 10 days of treatment the whole bed of the ulcer has become granulation tissue (see Reference 29)

forms of human and animal inflammation, NO is produced in large quantities and is probably an important mediator of the acute and chronic signs of inflammation. This has led to the idea that selective inhibitors of iNOS may in the future be useful in the treatment of inflammatory conditions.

One of the most interesting aspects of the biology of NO is the way in which it changes from being a physiological mediator to a cytostatic/cytotoxic agent. One possible mechanism is that NO interacts with superoxide, which is also generated in inflammatory conditions, to form peroxynitrite. This compound is a highly oxidant species that will produce tissue damage. We have been investigating this phenomenon in mitochondria and have found that NO selectively and at physiological concentrations reversibly inhibits the last enzyme in the respiratory cycle, cytochrome c oxidase (complex IV). In this way NO plays an important role as a regulator of cell respiration. If, however, NO is produced in large quantities for long periods it will inhibit cytochrome c oxidase in a way in which oxygen will not be able to displace it from the enzyme. In such a situation, superoxide may be generated in the mitochondria itself and interact with NO to form peroxynitrite, which we know will block complex I and complex III irreversibly. So from inhibition of complex IV, which might be a physiological way in which NO can regulate respiration, NO could have adverse effects through the irreversible inhibition of complex I[32].

In conclusion, NO is a ubiquitous molecule that not only has many different physiological functions, some of which remain to be unravelled, but also provides an immunological defence mechanism with implications for pathophysiology. Twelve years ago we were not even aware that NO was produced by the body; today we see it as a mediator with a very wide range of biological activities. Undoubtedly, understanding of the actions of NO will lead to new forms of therapy for human disease.

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