Exploring vascular nitric oxide in health and disease

The Goulstonian Lecture 1996

Several clinical pharmacologists have delivered the Goulstonian Lecture, but one in particular is relevant to the nitric oxide story. Thomas Lauder Brunton, a physician at St Bartholomew’s Hospital, spoke in 1867 to the title of ‘Clinical pharmacology and therapeutics: or medicine past and present’. In an astonishingly wide ranging lecture he appeared to anticipate receptor theory: ‘Before therapeutics can become a science, the physician must know the action of his drugs, just as the locksmith does that of his keys, and since pharmacology is still so young, it is little wonder that medicine is yet only an art’. He expounded his belief that by understanding drug action it is possible to probe physiology and pathophysiology and that such an understanding underpins rational and effective therapeutics. To illustrate his point, he described how he anticipated that the cardiovascular effects of amyl nitrite would be of benefit in the treatment of angina pectoris. Of course he was correct, and this observation led to the introduction of nitrosodilators into clinical practice, with glyceryl trinitrate appearing in 1879. Not content with introducing a new class of drug, he wished to understand the mechanism of action, and although unable to undertake direct experiments to explore the possibility, he mused ‘whether in nitrite of amyl there is some other nitrous compound, more potent yet still more unstable, must be determined by future research’. Over 100 years later the unstable nitrous compound was identified as nitric oxide.

Endothelium-dependent relaxation and EDRF

The modern nitric oxide story started in 1980 in the laboratory of Professor Robert Furchgott where he was studying the effects of acetylcholine on rabbit aorta. In a simple and elegant series of studies Furchgott and Zawadzki demonstrated that the ability of acetylcholine to relax the aorta was absolutely dependent on the presence of an intact endothelium (Fig 1). If this monolayer of cells was removed, the vessel would no longer relax to acetylcholine although it would still respond to glyceryl trinitrate. They coined the term ‘endothelium-dependent relaxation’ and went on to show that the relaxation was mediated by the release of a labile substance which they named endothelium-derived relaxing factor, or EDRF. EDRF exerted its relaxant effects by activating guanylate cyclase and increasing cGMP levels. That the endothelium should exert such a profound effect on the tone of smooth muscle was a surprise. But greater surprises were in store and few could have predicted the protean biological and clinical implications of the discovery.

Fig 1. A, Endothelium-dependent relaxation. Rings of rabbit aorta were contracted with noradrenaline (NA) and relaxed with increasing concentrations (log M) of acetylcholine (ACh). The left hand trace is from a vessel with an intact endothelium. The right hand trace is from a vessel in which the endothelium was removed by passing a pipe cleaner through the lumen. (Reproduced from Furchgott and Zawadzki with the permission of the authors and the publisher). B, Response to acetylcholine in a hand vein. Venodilatation to acetylcholine was studied in the hand veins of healthy volunteers. Acetylcholine (1 nmol/min) caused vasodilatation (C) that was abolished by removal of endothelium (-E). In a separate study, the vasodilatation in an endothelium intact vessel was abolished by infusion of nitric oxide synthase inhibitor L-NMMA. The studies demonstrate that nitric oxide mediates endothelium-dependent dilatation in a human vessel in vivo.

This article is based on the Goulstonian Lecture given at the Royal College of Physicians in November 1996 by Professor Patrick Vallance, Professor of Clinical Pharmacology and Therapeutics at University College London.
Endothelium-dependent relaxation in the human vasculature

In the early 1980s endothelium-dependent relaxation was demonstrated in a variety of blood vessels isolated from species ranging from birds and reptiles to rats, rabbits, dogs and humans. In 1987, together with Dr Joe Collier, we attempted to determine whether it also occurred in a human vessel in vivo. We tried to reproduce Furchgott and Zawadzki's experiment on a vein on the back of the hand. These vessels were chosen for three reasons. First, we wanted to use a vessel in which endothelial damage (which might initiate thrombosis) would not produce serious clinical sequelae. Second, a method was available that allowed us to assess contraction and relaxation of these vessels in situ. Third, it was possible to infuse vasoactive agents locally into the study vessel and construct full dose response curves whilst administering a total dose of drug orders of magnitude lower than a systemically effective dose.

To remove the endothelium we isolated the vein from the circulation by means of two weighted occluding wedges and circulated distilled water through the isolated segment. This manoeuvre caused osmotic lysis of the fragile endothelial cells whilst leaving the more robust smooth muscle cells intact. In the healthy hand vein, acetylcholine produced a biphasic response comprising dilatation at low doses and constriction at higher doses. Once the endothelium was removed, the dilator response was lost (Fig 1) and the constrictor response enhanced. The dilatation to acetylcholine depended entirely upon the presence of an intact endothelium, whereas the constrictor actions of this hormone were due to a direct effect on smooth muscle. Thus, what Furchgott and Zawadzki had seen in the rabbit aorta in vitro occurred also in a human blood vessel in vivo.

Identification of EDRF as nitric oxide

On the basis of work undertaken in a number of laboratories, Furchgott and Ignarro independently suggested that EDRF might be an inorganic gas, nitric oxide. Hitherto, nitric oxide had been thought of as a pollutant, a toxic contaminant of cylinders of nitrous oxide or a product of electrochemical storms. However, within a year Moncada's group had clearly demonstrated that release of nitric oxide accounted for the action of EDRF, that endothelial cells synthesise nitric oxide from the amino acid L-arginine, and that the synthetic process could be inhibited by an analogue of L-arginine in which one of the guanidino nitrogen atoms is methylated (N\textsuperscript{6} monomethyl-L-arginine; L-NMMA; Fig 2). L-NMMA provided the pharmacological tool necessary to block the production of nitric oxide (EDRF) within the endothelium and determine the effect on smooth muscle.

Blocking nitric oxide synthesis in humans

In initial experiments, we infused L-NMMA into a hand vein and assessed the response to acetylcholine. Infusion of L-NMMA mimicked endothelial denudation; the dilator response to acetylcholine was blocked (Fig 1) and the constrictor response enhanced. L-NMMA did not alter the response to glyceryl trinitrate. The effect of L-NMMA seemed specific for the L-arginine:nitric oxide pathway, since the inactive stereoisomer (D-NMMA) was without effect, and the inhibitory actions of L-NMMA could be reversed by supplying excess substrate (L-arginine). Nitric oxide has a half-life in biological solutions of only a few seconds and initially it was not possible to confirm the specificity of the functional actions of L-NMMA at a biochemical level. However, more recently, electrochemical systems for detecting nitric oxide have been used.

---

**Fig 2. Nitric oxide is synthesised from the amino acid L-arginine in generator cells.** The process is inhibited by L-NMMA. In target cells, nitric oxide activates guanylate cyclase (GC) by interacting with the haem moiety of this enzyme. Commonly used nitrovasodilators mimic the endogenous pathway and (either spontaneously or due to active metabolism) break down to nitric oxide in cells.

---

**Diagram:**

- **Generator cell**
  - L-NMMA
  - O\textsubscript{2}
  - L-citrulline
  - L-arginine
  - NO synthase

- **Target cell**
  - GTP
  - cGMP
  - NO-haem GC
  - Glycerol trinitrate
  - Sodium nitroprusside
  - Amyl nitrite

---

**Journal of the Royal College of Physicians of London Vol. 31 No. 3 May/June 1997**
developed and it has been possible to record in the hand vein the burst of nitric oxide generated in response to acetylcholine, and demonstrate the inhibition of this process by L-NMMA. We concluded that EDRF is generated in a human vessel in vivo, that it mediates the dilator effects of acetylcholine and that nitric oxide generated from L-arginine accounts for the action of EDRF in these vessels.

**Basal generation of nitric oxide in the arterial system**

The hand vein system was useful to explore basic pharmacology in a controlled manner; but these vessels are unlikely to be of major physiological or pathophysiological significance, and we turned our attention to the forearm arterial bed. We infused L-NMMA directly into the brachial artery and assessed forearm blood flow by venous occlusion plethysmography.

Flow through the forearm is predominantly through skeletal muscle and changes in flow at constant perfusion pressure represent changes in the contractile state of resistance vessels supplying skeletal muscle. Infusion of L-NMMA attenuated the dilator response to acetylcholine and produced an additional effect; basal (resting) blood flow fell (Fig 3). Indeed, blockade of nitric oxide synthesis in the forearm led to a halving of resting flow – a doubling of vascular resistance. It was clear that there was a continuous generation of nitric oxide in resistance vessels that provided a significant tonic dilator tone. The only other endogenous mediator known to exert such a profound and rapidly modulating local effect on vascular tone is noradrenaline released from sympathetic nerves (although the constrictor peptide endothelin also appears to provide a more slowly modulating influence). Blocking sympathetic neurotransmission leads to a two to threefold reduction in vascular resistance whereas blockade of endothelium-derived nitric oxide doubles it. These experiments with local delivery of L-NMMA confirmed that nitric oxide acts as a local mediator and does not have significant downstream or circulating effects; indeed, it is rapidly inactivated upon contact with haemoglobin.

**Probing disease states**

L-NMMA has been widely used to probe the L-arginine:nitric oxide pathway in animals and humans. Basal generation of nitric oxide occurs in virtually every arterial bed that has been studied (including cerebral, renal, mesenteric and cardiac) and systemic blockade of nitric oxide generation causes an immediate hypertension, indicating that normal arterial pressure is kept down by the continuous generation of nitric oxide. It was inevitable that alterations of nitric oxide-mediated dilatation would be implicated in disease states, and hypertension seemed an obvious starting point. Due to the difficulties in accurately measuring biochemical generation of nitric oxide, most researchers have adopted a functional approach, assessing changes in flow in response to endothelium-dependent dilators such as acetylcholine, or in response to blockade of basal nitric oxide generation with L-NMMA. In patients with essential hypertension, the constrictor response to L-NMMA is diminished, suggesting that basal nitric oxide-mediated dilatation is reduced. The change appears to be a reaction to the raised pressure rather than causal, since reduction of the pressure restores the response to L-NMMA towards normal. Functional changes in nitric oxide-mediated dilatation have also been detected in patients with type I or type II diabetes, hypercholesterolaemia, overt atheroma and even in smokers. What is the significance of these observations?

---

**Fig 3. The effects of L-NMMA on resting forearm blood flow.** L-NMMA was infused into the brachial artery of one arm and the other arm acted as a control. L-NMMA produced a dose-dependent fall in blood flow as endogenous nitric oxide synthesis was blocked.
Loss of nitric oxide would lead to increased vascular tone and reactivity, and might result in a 'stiffer' vascular system, manifest by a widening of pulse pressure, and impaired ability to accommodate increases in intravascular volume. But nitric oxide is also released luminally, where it inhibits platelet aggregation and adhesion\textsuperscript{15} and prevents the adhesion of white cells to the endothelium. In experimental animals, inhibition of nitric oxide synthesis promotes atherogenesis\textsuperscript{14,15} and it is tempting to speculate that changes in the generation or stability of nitric oxide, or alterations in the responsiveness of tissues to nitric oxide, might provide a mechanism to link apparently disparate risk factors to the common end point of atheroma formation. Consistent with this possibility, polymorphisms of the gene encoding endothelial nitric oxide, synthase appear to be associated with enhanced atherogenesis\textsuperscript{16}.

**Nitric oxide donors**

Studies with L-NMMA assess the overall functional effects of nitric oxide and cannot determine at which level any defect may lie. A reduced maximal response to L-NMMA might be due to less nitric oxide generated, more nitric oxide being destroyed before it reaches its target or a lower sensitivity of the target cells to nitric oxide. Different types of study will be needed to dissect the nature of any changes that occur with disease states. Nonetheless, delivery of extra nitric oxide to target cells seems an appropriate therapeutic goal. Here Brunton re-enters the story. Nitric oxide is the active drug formed when amyl nitrite, gyceryl trinitrate and other nitrovasodilators are broken down within the body. These drugs, which have been in clinical use for well over 100 years, are pro-drugs; donors of nitric oxide, that mimic the endogenous mediator\textsuperscript{17} (Fig 2). Is it possible to improve upon the existing nitric oxide donors? The challenge now is to design novel nitric oxide donors or activators of guanylate cyclase that show selectivity for individual cell types (platelets, white cells etc) or certain tissues. Already prototypes exist\textsuperscript{18} that exhibit platelet-selective effects. These are useful pharmacological tools and might be developed into novel therapies.

**Endogenous inhibitors of nitric oxide synthesis**

L-NMMA and arginine are closely related components (Fig 4). Might L-NMMA be formed endogenously and provide a mechanism to regulate nitric oxide generation? To address this question an assay was developed to extract synthetic L-NMMA from human urine and blood and the trace analysed to determine whether any endogenous compounds were also detected. Small amounts of endogenous L-NMMA were detected in plasma and urine but two other compounds with similar physicochemical properties were present in far greater quantities. From two litres of human urine it was possible to extract sufficient amounts of these compounds to identify them using mass spectrometry and nuclear magnetic resonance (NMR); they were asymmetric and symmetric dimethylarginine (ADMA and SDMA; Fig 4)\textsuperscript{19}.

One of the compounds we identified (ADMA) inhibited the activity of purified nitric oxide synthase, caused endothelium-dependent contractions of rat aorta (due to inhibition of basal nitric oxide), raised the blood pressure of anaesthetised guinea-pigs and reduced resting forearm blood flow in a manner similar to L-NMMA\textsuperscript{19}. In summary, in human plasma and in urine there is a compound, ADMA, that is a specific and selective inhibitor of nitric oxide synthase in vitro and in vivo, in animals and humans. It is not yet known whether ADMA acts physiologically to reduce nitric oxide generation, but circulating levels of dimethylarginines are increased in certain disease states (most notably in uraemia) and might contribute to some of the pathophysiology seen in those conditions. More recently, it has been demonstrated that endothelial cells have the metabolic machinery necessary to synthesise and degrade ADMA\textsuperscript{20} and attempts are being made to manipulate these pathways to determine the biological significance of this naturally occurring inhibitor of nitric oxide synthase (Fig 5).

**Overproduction of nitric oxide**

It is clear that underproduction of nitric oxide might contribute to disease, but what of overproduction? Now the story becomes more complex. Three isoforms of nitric oxide synthase have been identified; these are

---

**Fig 4. Structures of arginine (substrate for nitric oxide synthase) and two inhibitors, asymmetric dimethylarginine (ADMA) and N\textsuperscript{6} monomethyl-L-arginine (L-NMMA).** L-NMMA and ADMA are both naturally occurring compounds that compete with arginine and inhibit nitric oxide synthesis. Another endogenous compound, symmetric dimethylarginine (SDMA), in which methyl groups are present on each of the guanadino nitrogen atoms, does not act as an inhibitor of nitric oxide synthesis.
an endothelial isoform (eNOS; gene located on chromosome 7), a neuronal isoform (nNOS; gene located on chromosome 12) and a macrophage or inducible isoform (iNOS; gene located on chromosome 17). eNOS and nNOS are normal constituents of healthy cells (Fig 6), and both may contribute to physiological regulation of the cardiovascular system. It is assumed that the effects of l-NMMA in healthy individuals are due exclusively to inhibition of eNOS activity in endothelium. In the hand veins this is almost certainly the case, for we can detect no messenger RNA for any other isoform. However, it may not be true in the forearm or other vascular beds in which nitric oxide releasing (nitrergic) nerves may play a significant role. Certainly nitrergic nerves do control vascular tone in some situations; in the penis nitrergic nerves contribute to the process of erection and in the brain nitrergic nerves may help couple neuronal activation to vasodilatation to increase blood flow to metabolically active areas. To determine the role of nNOS in cardiovascular control in humans it will be necessary to develop selective inhibitors of this isoform. This is an exciting area of research in which clinical investigation will play an important part.

In terms of overproduction of nitric oxide it is iNOS that has received most attention. This isoform is considered part of the host-defence reaction and is expressed in a wide variety of cell types once they have been exposed to bacterial endotoxin and/or inflammatory cytokines. If rat blood vessels are exposed to endotoxin, they gradually lose tone and become hyporesponsive to vasoconstrictors. This is because iNOS is synthesised de novo and expressed throughout the vessel wall, in muscle layers as well as endothelium, and generates large quantities of nitric oxide. Might expression of iNOS and other production of nitric oxide be the reason that blood pressure falls during infection, and might it be the cause of vascular collapse in septic shock? Certainly there is evidence of increased generation of nitric oxide in sepsis; the circulatory levels of nitrate (a stable breakdown product of nitric oxide) are elevated, and injection of l-NMMA restores blood pressure to normal in patients who are resistant to conventional pressor agents (Fig 7). However, whilst these studies indicate that nitric oxide contributes to vasodilatation in septic shock, and suggest novel treatments, they do not clearly identify iNOS as the source of the nitric oxide since l-NMMA can inhibit all three isoforms of NOS. Human iNOS remains something of an enigma. It is easy to induce iNOS in rodents but difficult to do so in human cells and tissues. eNOS and nNOS are highly conserved between species, yet iNOS is not and appears to be evolving.

To explore the mechanisms by which sepsis induces nitric oxide generation in humans, the research turned again to the hand vein. A segment of hand vein was isolated from the circulation and into the isolated segment we instilled endotoxin or various pro-inflammatory cytokines. One hour later the endotoxin or cytokines were withdrawn and the vein was reconnected with the circulation. Endotoxin alone was insufficient to induce nitric oxide generation, but the pro-inflammatory interleukin-1 (IL-1β) was effective. A one-hour exposure to IL-1β produced a slowly developing hyporesponsiveness to constrictors that could be prevented by prior treatment with glucocorticoids and reversed by l-NMMA. This picture is consistent with induction of nitric oxide generation; by taking biopsies and using selective pharmacological probes it should now be possible to use this model to explore the molecular basis of inflammatory and infectious vasodilatation in a controlled and safe fashion in humans. In this model the generation of nitric oxide within the vessel wall acts as a powerful functional antagonist to dampen the sympathetic nervous system’s ability to cause vasoconstriction.
Conclusions

The discovery of nitric oxide has changed perception of vascular control. The basal nitric oxide-mediated dilator tone exerts a powerful influence on vascular behaviour and changes in its magnitude appear to contribute to disease states. There are many novel targets for drug treatments – for example, nitric oxide donors or stimulants to prevent or treat atheromatous, vasospastic or thrombotic disorders; and inhibitors of nitric oxide synthesis for septic shock or inflammatory vasodilatation. The studies I have undertaken with colleagues have been designed to use drugs and mersors to dissect the role of nitric oxide in the human vascular system. Drugs have been, and remain, a powerful way in which to dissect basic mechanisms of physiology and pathophysiology and explore genotype–phenotype relationships. In doing so, unexpected and exciting therapeutic opportunities may emerge.

Acknowledgements

The work described in the lecture was funded largely by the British Heart Foundation and Wellcome Trust. I owe a debt of gratitude to my teachers, Joe Collier and Salvador Moncada, and to the many colleagues and students who contributed to the research.

References

1. Lauder Brunton T. *Medicine past and present*. London: Macmillan, 1980.
2. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373–6.
3. Collier J, Vallance P. EDRF is an endogenous vasodilator in man. *Br J Pharmacol* 1989;97:639–41.
4. Vallance P, Collier J. Biphasic response to acetylcholine in human veins: the role of the endothelium. *Clin Sci* 1990;78:101–4.
5. Palmer R, Ferrige A, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524–6.
6. Palmer R, Ashton D, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1986;320:664–6.
7. Vallance P, Collier J, Moncada S. Nitric oxide or nitric oxide synthase? *Nature* 1987;327:524–6.
8. Vallance P, Patton S, Bhagat K, MacAllister R, et al. Direct measurement of nitric oxide in humans. *Lancet* 1995;345:153–4.
9. Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arterial tone in man. *Lancet* 1989;i:997–1000.
10. Haynes W, Noon J, Walker B, Webb D. Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J Hypertens* 1993;11:1375–80.
11. Calver A, Collier J, Vallance P. Effects of local intra-arterial Nω-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears normal. *J Hypertens* 1992;10:1025–31.
12. Calver A, Collier J, Vallance P. Effects of l-NMMA in patients with treated hypertension. *Cardiovasc Res* 1994;28:1720–5.
13. Radomski M, Palmer R, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987;i:1057–8.
14. Cayatte A, Palacino J, Horton K, Cohen R. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler Thromb* 1994;14:753–9.
15. Naruse K, Shimizu K, Muramatsu M, Toki Y, et al. Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit aortic wall. *PGB2* does not contribute to impaired endothelium-dependent relaxation. *Arterioscler Thromb* 1994;14:746–52.
16. Wang X, Sim A, Badenhop R, McCredie R, Wilcken D. A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat Med* 1996;2:41–5.
17. Feelisch M, Noack E. Nitric oxide formation from nitrovasodilators occurs independently of haemoglobin or non-heme iron. *Eur J Pharmacol* 1987;142:465–9.
18. de Belder A, MacAllister R, Radomski M, Moncada S, Vallance P. Selective inhibition of platelet aggregation by nitrosoglutathione. *Cardiovasc Res* 1994;5:691–4.
19. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 1992;339:572–6.
20. MacAllister R, Whitley G, Parry H, Kimoto M, *et al*. Regulation of nitric oxide synthase by endogenous dimethylarginine. *Br J Pharmacol* 1996;119:1533–40.
21. Vallance P, Moncada S. The role of endogenous nitric oxide in human septic shock. *New Horizons* 1993;1:77–86.
22. Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypertension in patients with septic shock. *Lancet* 1991;338:1557–8.
23. Petros A, Leone A, Moncada S, Bennett D, Vallance P. Effect of a nitric oxide synthase inhibitor in patients with septic shock: a randomised study. *Cardiovasc Res* 1994;28:34–9.
24. Bhagat K, Vallance P. The local venous responses to endotoxin in humans. *Circulation* 1996;94:490–7.

Address for correspondence: Professor P Vallance, Professor of Clinical Pharmacology and Therapeutics, The Cruciform Project, University College London, The Rayne Institute, 5 University Street, London WC1 0JJ.

---

**FORTHCOMING PUBLICATION FROM THE ROYAL COLLEGE OF PHYSICIANS**

**CARDIAC REHABILITATION: GUIDELINES AND AUDIT STANDARDS**

Edited by David R Thompson, Gerald S Bowman, David de Bono and Anthony Hopkins

As the value of rehabilitation for patients with heart disease becomes more widely appreciated and accepted — both in terms of quality of life and cost effectiveness — clinicians, purchasers and patients are asking for more information, which research is providing.

The Royal Colleges of Physicians and Nursing and the British Cardiac Society have collaborated to produce this book which reviews evidence of the effectiveness of cardiac rehabilitation and includes guidelines and audit measures tailored to meet the needs of those running or setting up rehabilitation programmes. The success of cardiac rehabilitation may include medical and psychological care and social support — delivering them at an appropriate time and in an appropriate setting.

This book describes and evaluates the main components of cardiac rehabilitation programmes and will be a source of useful evidence and information on which purchasers and providers of specialist and primary health care can base clinical and service management decisions.

**Contents:**
- Foreword by Alistair Burt
- Introduction by D Thompson, G Bowman, D de Bono and A Hopkins
- Overview of cardiac rehabilitation by I Todd and L Cay
- The medical component of cardiac rehabilitation by A Mcleod
- The psychological component of cardiac rehabilitation by B Lewin
- The social component of cardiac rehabilitation by A Radley
- The exercise component of cardiac rehabilitation by A Hardman
- The vocational component of cardiac rehabilitation by M Joy and S Donald
- The economic component of cardiac rehabilitation by A Gray
- Guidelines and audit standards
- Audit proformas

Softcover, 141 pages ISBN 1 86016 048 4