The Discovery of Nitric Oxide as the Endogenous Nitrovasodilator

SALVADOR MONCADA, RICHARD M. J. PALMER, AND E. ANNIE HIGGS

SUMMARY Endothelium-derived relaxing factor (EDRF) is a labile humoral agent released by vascular endothelium that mediates the relaxation induced by some vasodilators, including acetylcholine and bradykinin. EDRF also inhibits platelet aggregation, induces disaggregation of aggregated platelets, and inhibits platelet adhesion to vascular endothelium. These actions of EDRF are mediated through stimulation of the soluble guanylate cyclase and the consequent elevation of cyclic guanosine 3',5'-monophosphate. EDRF has been identified as nitric oxide (NO). The pharmacology of NO and EDRF is indistinguishable; furthermore, sufficient NO is released from endothelial cells to account for the biological activities of EDRF. Organic nitrates exert their vasodilator activity following conversion to NO in vascular smooth muscle cells. Thus, NO may be considered the endogenous nitrovasodilator. NO is synthesized by vascular endothelium from the terminal guanido nitrogen atom(s) of the amino acid L-arginine. This indicates the existence of an enzymic pathway in which L-arginine is the endogenous precursor for the synthesis of NO. The discovery of the release of NO by vascular endothelial cells, the biosynthetic pathway leading to its generation, and its interaction with other vasoactive substances opens up new avenues for research into the physiology and pathophysiology of the vessel wall. (Hypertension 12: 365–372, 1988)

KEY WORDS • hypertension • endothelium-derived relaxing factor • nitric oxide • nitrovasodilators

THE tone of the vascular smooth muscle is regulated by a multitude of vasoactive agents that may reach the vessel wall through the bloodstream, as do epinephrine, angiotensin, or vasopressin, or may be released by adrenergic, cholinergic, or other nerve terminals in the vessel wall. All these agents interact to maintain the blood pressure within very predictable values. Failure to do so leads to changes considered abnormal, the most common of which is hypertension.

What long remained unsuspected is that factors generated by cells within the vessel wall are also important regulators of smooth muscle tone and, more significantly, that the vascular endothelium is responsible for the generation of these vasoactive substances. Moncada et al., while studying the metabolism of arachidonic acid in the vessel wall, discovered prostacyclin, a powerful vasodilator generated preferentially by the vascular endothelium. Endothelium-derived relaxing factor (EDRF) was subsequently added by Furchgott and Zawadzki to the list of mediators that regulate vascular reactivity.

In the last 2 years it has been demonstrated that EDRF is nitric oxide and that its biosynthetic precursor is L-arginine. These discoveries have not only revealed the existence of an endogenous nitrovasodilator implicated in the regulation of vascular tone, but also identified its enzymatic generation through a pathway amenable to biochemical and pharmacological manipulation. The relevance of these findings to the understanding of the physiology or pathophysiology of the vessel wall as well as their possible therapeutic implications will be discussed in this review.

History of EDRF

Acetylcholine (ACh), a potent hypotensive agent, was known on occasion either to have no effect or to produce a slight contraction of vascular tissues in vitro. The cause of this discrepancy was revealed in 1980 when Furchgott and Zawadzki showed that the relaxation induced by ACh was dependent on the presence of the endothelium and provided evidence for the release of a labile humoral factor that causes relaxation. This substance, which was later called EDRF, was shown to be distinct from prostaglandins.
cyclic guanosine 3',5'-monophosphate (cGMP), consequent to stimulation of the soluble guanylate cyclase, is associated with endothelium-dependent relaxation, EDRF-induced vascular relaxation, and inhibition of platelet aggregation. Moreover, endothelium-dependent relaxation and the actions of EDRF on smooth muscle and platelets are potentiated by M&B 22948 and MY 5445, two selective inhibitors of cGMP phosphodiesterase.

We and others have found that superoxide dismutase protects EDRF from breakdown and reverses the action of a diverse group of compounds described as inhibitors of EDRF. These and other results led us to suggest that EDRF is destroyed by superoxide anions (O_2^-), but not by other oxygen-derived radicals, and that some inhibitors of EDRF act by generating O_2^-.

History of the Nitrovasodilators

Amyl nitrite was synthesized by Balard in 1844, and the inhalation of its vapors was observed by Guthrie to cause flushing of the face, throbbing of the carotid arteries, and acceleration of the pulse rate. The first report of its successful use in the treatment of angina pectoris was made in 1867 by Brunton, who, after observing that bleeding of the patient was beneficial in the relief of pain, concluded that a substance that reduced the arterial blood pressure should have the same effect. He observed that "On pouring from five to ten drops of the nitrite on a cloth and giving it to the patient to inhale, the physiological action took place in from thirty to sixty seconds; and simultaneously with the flushing of the face the pain completely disappeared, and generally did not return till its wonted time next night." Nitroglycerin (GTN) was synthesized by Sobrero shortly after Balard's synthesis of amyl nitrite but was only tested in the treatment of angina pectoris in 1879. Murrell, while describing what was a study on comparative clinical pharmacology, stated, "From a consideration of the physiological action of the drug, and more especially from the similarity existing between its general action and that of nitrite of amyl, I concluded that it would probably prove of service in the treatment of angina pectoris, and I am happy to say that this anticipation has been realised." The use of this class of compound for the treatment of hypertension also goes back a hundred years. Nitrates in general are still widely used in a number of conditions, including angina pectoris, congestive heart failure, blood pressure control during hypertensive emergencies, pulmonary hypertension, fibrinolytic therapy, percutaneous coronary angioplasty, and complications after cardiac catheterization.

For many years it was believed that the vasodilator action of GTN was due to its conversion in the bloodstream of an effective nitrite (NO_2^-) to explain the observed vasodilatation. They...
therefore concluded that the observed effect must be an action of the intact GTN molecule.

In 1977, two groups demonstrated independently that organic nitrates induced a dose-dependent increase in the levels of cGMP in smooth muscle. Subsequently, biochemical experiments showed that all the nitrovasodilators and nitric oxide (NO) activate the soluble guanylate cyclase. The precise way in which nitrovasodilators activate this enzyme has been the subject of much debate. Nitrovasodilators have recently been shown to generate NO in a nonenzymic reaction with cysteine. Furthermore, the concentrations of the nitrovasodilators that induced half-maximal stimulation of guanylate cyclase induced uniform release of NO in this reaction. Although this work does not provide evidence for such a process in the intact cell, the results strongly indicate that NO generation is the essential feature of the mechanism of action of nitrovasodilators.

The Two Tales Come Together

Widespread speculation about the chemical nature of EDRF developed soon after its discovery (see References 39, 40). In 1986, however, Furchgott and Ignarro independently suggested that EDRF may be NO or a closely related compound. We decided to investigate whether EDRF was indeed NO by comparing the pharmacological profile of EDRF and authentic NO on vascular strips and on platelets and by measuring directly the release of NO from porcine aortic endothelial cells in culture.

Both EDRF and NO caused a relaxation of the vascular strips that declined at the same rate during passage down the bioassay cascade. Furthermore, the rate of decay during transit in polypropylene tubes was slower, but similar, for both compounds. Both compounds also inhibited platelet aggregation and inhibited the disaggregation of aggregated platelets, and inhibited platelet adhesion. Moreover, their biological half-life as inhibitors of platelet aggregation was similar.

The actions of EDRF and NO on vascular strips and on platelets were similarly potentiated by superoxide dismutase and cytochrome c and inhibited by Fe\(^{2+}\) and some redox compounds. Furthermore, the potency of redox compounds as inhibitors of EDRF-induced and NO-induced vascular relaxation was attenuated by superoxide dismutase to a similar extent. Ignarro et al. have recently published data in agreement with these results.

Hemoglobin also inhibited the effect of EDRF and NO through a mechanism not involving O\(_2^-\). Finally, both compounds act on vascular smooth muscle and platelets through the stimulation of soluble guanylate cyclase and elevation of cGMP.

NO may be measured directly as the chemiluminescent product of its reaction with ozone. Using this method we have shown that the concentrations of bradykinin that induce the release of EDRF from endothelial cells in culture also cause a concentration-dependent release of NO. Moreover, the amounts of NO released by the cells are sufficient to account for both the relaxations of vascular strips and for the antiaggregating and antiadhesive activity of EDRF. We have also observed that the vascular relaxing activity released from fresh, perfused arteries of the rabbit by a number of agents, including ACh, substance P, and the calcium ionophore A23187, is accounted for by the amounts of NO released (unpublished observation, 1987).

Whether NO is released as such, or as an unstable precursor, has not been established. The comparative pharmacology of NO and EDRF, especially the studies on the stability of EDRF, argue against the latter possibility unless the lifetime of the intermediate is in the order of fractions of a second rather than seconds.

All this pharmacological and biochemical evidence clearly demonstrates that EDRF is NO (Table 1) and that it fulfills all the criteria necessary to be classified as a biological mediator. It is remarkable that in 1987, 120 years after the first clinical use of the organic nitrates, we saw the clarification of the mechanism of action of this group of compounds and the identification of the endogenous mediator that they imitate.

NO is a highly unstable substance that is rapidly converted to NO\(_2^-\) and NO\(_3^-\) in oxygenated solutions. Furthermore, hemoglobin has a greater affinity for NO than for O\(_2^-\). Thus, NO may act only as a result of direct transfer from cell to cell, the memhranes of which it crosses rapidly. NO may therefore be a local hormone in the absolute sense, never circulating, nor even reaching the extracellular space under normal circumstances.

Endothelium-dependent relaxation in perfused vascular beds in vitro has been reported in several preparations, yet there are no reports of the release of EDRF or NO from these preparations. We have studied the release of NO from isolated segments of rabbit aorta (unpublished observations, 1987) and from the rabbit perfused heart. We have found that, while stimulation with ACh leads to the release of NO into the perfusate of a segment of rabbit aorta (unpublished observations, 1987), no biological activity can be detected in the cardiac effluent. Nitrite, however, which is devoid of biological activity at low concentrations, is released into the effluent of the heart, suggesting that NO is released by ACh but that it is rapidly oxidized, presumably in the microcirculation.

All this evidence demonstrating that EDRF is NO does not, however, exclude the possibility that mechanisms other than the release of NO play a role in endothelium-dependent vascular relaxation. Their existence would not be surprising, since mechanisms subserving a biological function are usually multiple. Hyperpolarization of smooth muscle or direct cell to cell communication should be thoroughly investigated to establish their relevance in the regulation of vascular smooth muscle tone.
TABLE 1. EDRF Is Nitric Oxide

<table>
<thead>
<tr>
<th>Property</th>
<th>EDRF</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Released by endothelial cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Relaxes vascular smooth muscle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibits platelet aggregation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Induces platelet disaggregation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibits platelet adhesion</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stability (t1/2, in sec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down cascade</td>
<td>3.6±0.1</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>Through polypropylene tubes</td>
<td>30.9±1.9</td>
<td>30.4±2.2</td>
</tr>
<tr>
<td>Receptor</td>
<td>Soluble</td>
<td>Soluble</td>
</tr>
<tr>
<td>2nd messenger</td>
<td>guanylate cyclase</td>
<td>guanylate cyclase</td>
</tr>
<tr>
<td>Inhibited directly by hemoglobin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibited indirectly by redox compounds</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Potentiated by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD, cytochrome c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M&amp;B 22948, MY 5445</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Not affected by methemoglobin, HL 725*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reacts with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide anions (O2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Binds to ion exchange columns</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

Half-life (t1/2) values are means ± SE. NO = nitric oxide; SOD = superoxide dismutase. + = positive; ± = positive in some and negative in others.

Biosynthesis of Nitric Oxide

Where does NO come from? This has been one of the most intriguing questions arising from the discovery of its release from endothelial cells. We have recently demonstrated that endothelial cells in culture synthesize NO from the terminal guanido nitrogen atom(s) of the amino acid L-arginine.4 This reaction is specific, for other close analogues of L-arginine, including its d-isomer, are not substrates. Furthermore, one of them, L-N°-monomethyl arginine, inhibits this process in endothelial cells.5 We have also demonstrated that L-arginine produces a small but significant endothelium-dependent relaxation in vascular rings. In contrast, L-N°-monomethyl arginine produces a small endothelium-dependent contraction, and each compound inhibits the actions of the other.5 Most strikingly, L-N°-monomethyl arginine inhibits relaxation induced by endothelium-dependent vasodilators, and this effect is reversed by L-arginine. These data suggest that there is a specific enzymic route to NO that may be amenable to pharmacological and biochemical manipulation. Further work will elucidate the consequences of such manipulation in vivo.

Interactions of Nitric Oxide with Prostacyclin

Prostacyclin and NO synergize with each other as inhibitors of platelet aggregation and inducers of platelet disaggregation.13 As a result, we have suggested that the very low concentrations of prostacyclin found in plasma may have a physiological effect in regulating platelet aggregability if it is acting on a background of NO release.10 Interestingly, NO differs from prostacyclin in that it is also an effective inhibitor of platelet adhesion. The fact that we did not observe a synergistic interaction between these two compounds on platelet adhesion17 suggests that the physiological process of platelet adhesion and repair of the vessel wall may proceed under circumstances in which both substances, acting in concert, are exerting a powerful antithrombotic action.

The subcellular mechanisms underlying the actions of NO and prostacyclin are the cGMP and the cyclic adenosine 3',5'-monophosphate (cAMP) systems, respectively. It is interesting that in some situations, such as inhibition of platelet aggregation and induction of disaggregation, there is a synergy between the two, while in others, such as inhibition of platelet adhesion, there may even be some antagonism.17 The precise interaction of both systems in the control of vascular tone remains to be studied. Results obtained in our laboratory have not shown synergy between NO and prostacyclin as vasodilators in the rabbit mesenteric artery strip or the arterioles of the hamster cheek pouch (unpublished observations, 1987).

Physiological, Pathological, and Clinical Implications

There is circumstantial evidence indicating that endothelium-dependent vasodilatation occurs in vivo.
The alterations in vessel diameter that follow changes in blood flow are endothelium-dependent, and it has been suggested that endothelium-dependent relaxation coordinates increased flow responses through vascular beds. Treatment with methylene blue and endothelial damage with a laser abolish the response to vasodilators in vivo without affecting the response to sodium nitroprusside.

Morphological and biochemical changes occur in blood vessels during hypertension (for a review, see Reference 56). Whether alterations in the availability of L-arginine, in the synthesis of NO, or in the sensitivity to its vasodilator action contribute to the development of hypertension should be investigated.

A link between endogenous NO\textsuperscript{−} and hypertension was suggested many years ago as a result of interest in the mechanism of action of nitrovasodilators. Measurements of NO\textsuperscript{−} in blood revealed concentrations of 10 \mu g/dl. However, there was no correlation between blood NO\textsuperscript{−} levels and blood pressure, although a marked reduction in NO\textsuperscript{−} levels was observed in older subjects.

Decreased endothelium-dependent relaxation in vessels from hypertensive animals has been reported by some workers. Interestingly, the impaired endothelium-dependent responses of aortic tissue from hypertensive rats as well as the accompanying reduced levels of cGMP were reversed by restoring the blood pressure to normal. Other workers, however, have reported no change or an increase in endothelium-dependent relaxation in arteries from hypertensive animals. All these experiments are not strictly comparable, since different vascular preparations from different species stimulated with different agonists were used. Thus, there is a need for a systematic study of endothelium-dependent vasodilators in hypertension.

A reduction in the release of EDRF from the vascular endothelium or a decrease in the endothelium-dependent relaxation has been demonstrated in vascular tissue obtained from rabbits made atherosclerotic and in human atherosclerotic coronary arteries. Endothelial damage also induces an increase in basal tone and enhanced responses to vasoconstrictor substances in the coronary arteries of the atherosclerotic miniature swine. The reasons for these changes need to be elucidated; however, it is tempting to suggest that they may be related to the generation of O\textsubscript{2} and released by monocytes that accumulate during the atherosclerotic process. In this context, it is interesting that the enzyme that generates prostacyclin is inhibited by lipid peroxides and that oxygen-derived radicals and lipid peroxides have long been suspected to play a role in the development of atherosclerosis.

There is ample evidence showing that hemoglobin inhibits endothelium-dependent relaxation in different preparations. Hemoglobin also produces vasoconstriction in some of these preparations, suggesting an effect on the basal release of NO. However, part of this effect may be due to direct vasoconstriction, since methemoglobin, which has a lower affinity for NO than does hemoglobin, also induces vasoconstriction, at least in the isolated rabbit heart. Inhibition of NO by hemoglobin could play a role in the vasospasm that follows subarachnoid hemorrhage, which has long been suspected to be mediated by some product of lysed red blood cells. A recent study on the canine basilar artery in vitro shows that endothelium-dependent relaxation induced by A23187 is inhibited by hemoglobin and by cerebrospinal fluid obtained from patients with subarachnoid hemorrhage. Since NO is also an inhibitor of platelet activation, impairment of its formation in the vessel wall will not only predispose to vasoconstriction but also favor platelet adhesion, aggregation, and the consequent release of vasoconstrictor substances that will exacerbate the tendency to vasospasm. In this context, platelet products released during aggregation in vitro contract deendothelialized canine coronary artery rings whereas they induce relaxation when the endothelium is present.

A potent vasoconstrictor peptide, endothelin, has been shown to be released by endothelial cells in response to various chemical and physical stimuli. Whether endothelin is released with prostacyclin and NO or whether during pathological situations there is a shift from prostacyclin and NO to the release of endothelin or other vasoconstrictor factor(s) remains to be studied.

Conclusions and Perspectives

The discovery that NO is the endogenous nitrovasodilator and that synthetic nitrovasodilators act by releasing NO, to mimic the natural mediator, has important implications. Although organic nitrates have long been used clinically as vasodilators, their effect on other systems, notably platelets, either has not been understood or has been neglected, probably because GTN is almost inactive in platelets whereas sodium nitroprusside does inhibit aggregation. The present evidence suggests that only those nitrovasodilators that spontaneously release NO inhibit platelet aggregation, indicating that platelets lack the mechanisms required for the uptake or conversion (or both) of organic nitrates to NO.

Tolerance to GTN was originally observed in the late 19th century among workers handling this compound in munitions factories. The biochemical mechanism underlying this tolerance is thought to be the depletion in the cell of thiols responsible for the conversion of GTN to NO, since compounds such as sodium nitroprusside or the molsidomine metabolite SIN-1, which generate NO spontaneously, do not exhibit the same degree of tolerance, at least in vitro. Not all the data, however, agree with these results, and cross tolerance has been demonstrated not only between nitrovasodilators but also to endothelium-dependent vasodilatation.
In view of this finding, it is worth investigating whether prolonged exposure to organic nitrates inhibits the synthesis of NO by the vessel wall or whether these compounds desensitize the guanylate cyclase, not only to themselves but also to endogenous NO.

The release of NO has been suggested to occur predominantly at the abluminal surface of the endothelial cell.79 The characteristics of this release, as well as the precise way in which NO is transferred and later metabolized in the vascular smooth muscle, platelets, or other cells, needs to be studied.

The interactions between NO and other vasoactive substances such as prostacyclin, which acts by stimulating adenylate cyclase, and atrial natriuretic factor, which stimulates the particulate form of the guanylate cyclase, will be interesting to study, as will be its interactions with vasoconstrictor factors such as endothelin. All these vasoactive substances are generated or metabolized through pathways that are becoming clearly defined. Moreover, they act on receptors that in the future will be adequately classified. As a result of this, it is highly likely that new drugs that promote or interfere with these interactions will be developed.

Whether NO has other actions, such as modulating the behavior of leukocytes or controlling the replication of smooth muscle cells, is not known. A cytoprotective mechanism in platelets, similar to that of prostacyclin, has already been reported.80 NO could act as a cytoprotective agent either through an effect on cGMP, leading to intracellular calcium sequestration,81 or through its ability to react with and inactivate oxygen-derived radicals.82

Activated macrophages produce NO2 and NO3.83 This production, which probably proceeds through the formation of NO, contributes to their cytotoxic capacity.84 Furthermore, neutrophils have recently been shown to produce EDRF,85 and this EDRF is probably NO. Since macrophages and endothelial cells generate NO from the terminal guanido nitrogen atom(s) of L-arginine,4,46 this amino acid probably is also the precursor for NO generation in neutrophils. Thus, the biological consequences of pharmacological or biochemical manipulation of the pathway leading to NO generation are likely to be many and varied, both within and beyond the cardiovascular system.

The unraveling of the release of NO by the vascular endothelium, its interactions with prostacyclin, and their role as regulators of vascular tone and of platelet–vessel wall or leukocyte–vessel wall interactions is increasing our understanding of the physiology and pathophysiology of the cardiovascular system and will therefore enhance the possibilities of preventing and treating cardiovascular disease.

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