*Biochemical Pharmacology*, Vol. 37, No. 13, pp. 2495–2501, 1988.
Printed in Great Britain.

0006–2952/88 $3.00 + 0.00


# COMMENTARY

# ENDOTHELIUM-DERIVED RELAXING FACTOR

## IDENTIFICATION AS NITRIC OXIDE AND ROLE IN THE CONTROL OF VASCULAR TONE AND PLATELET FUNCTION

SALVADOR MONCADA,* MAREK W. RADOMSKI and RICHARD M. J. PALMER
Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, U.K.

The vascular endothelium is now widely recognized as an organ with a variety of functions important in the regulation of blood vessel tone and permeability, the coagulation of blood, the activity of leukocytes, and the reactivity of platelets. The endothelium contributes to the regulation of vascular tone by releasing powerful vasodilators such as prostacyclin [1] and endothelium-derived relaxing factor (EDRF; [2]) and by metabolizing vasoactive substances such as catecholamines, angiotensin, bradykinin and prostaglandins [3].

The role of the vascular endothelium as a modulator of platelet and leukocyte interactions with the vessel wall has been the subject of much attention, first as a result of the discovery that prostacyclin is an inhibitor of platelet aggregation and leukocyte activation [4] and, second, as a consequence of the finding that EDRF is not only an inhibitor of platelet aggregation [5] but also of platelet adhesion [6, 7].

In this review we will discuss the evidence for the identification of EDRF as nitric oxide (NO) and for its role as a regulator of vascular tone and platelet–vessel wall interactions.

### *What is EDRF?*

The discovery of the obligatory role of the endothelium in the vascular relaxation induced by acetylcholine and the demonstration that a humoral factor was involved in this action led to the identification of many stimuli which induce endothelium-dependent relaxation, the description of a number of inhibitors of this mechanism, and considerable speculation about the nature of this extremely labile vasoactive substance named "endothelium-derived relaxing factor" [5, 8].

Whether endothelium-dependent relaxation is always mediated by the release of EDRF is not yet known; however, developments in this field of research are such that a set of well-defined criteria can now be used to identify EDRF as a distinct entity.

Endothelium-derived relaxing factor is a non-prostanoid, labile (half-life 3–50 sec) humoral agent released from the vascular endothelium by a variety of substances. Endothelium-derived relaxing factor relaxes vascular smooth muscle and inhibits platelet aggregation and platelet adhesion via the elevation of cGMP levels [5]. It is destroyed by oxygen [9] and superoxide anions ($O_2^-$; [9, 10]) but not by other reactive oxygen species, and its action both on vascular strips and on platelets is inhibited by hemoglobin (Hb; [11–13]) and by some redox compounds [13, 14]. We suggest that, from now on, studies on the mechanisms involved in endothelium-dependent relaxation, the identification of other humoral factors presumed to be involved in this action, or the effect of inhibitors should be rigorously analyzed in terms of these criteria.

The adoption of methods of bioassay in which the active material is transferred from a donor to a detector tissue has resulted in most of the information known about EDRF. The perfusion of vascular endothelial cells cultured on microcarriers and the direct bioassay of the perfusate on a series of de-endothelialized vascular strips [12, 15] or on platelet suspensions [13] created the conditions for important advances in this field. This arrangement increases the number of endothelial cells that can be studied at one time and thus the amount of EDRF that can be generated. Moreover, by separating the generation of EDRF from the biological or chemical detector systems, this method allows the study of the effect of physical or chemical intervention on the generation, stability and action of EDRF. Finally, it allows the simultaneous detection of EDRF and other substances generated or metabolized by the endothelium.

### *Inhibition and release of EDRF*

Using our bioassay system [12], we re-examined the mechanism of action of some compounds identified as inhibitors of EDRF [16]. We confirmed that compounds such as phenidone, BW755C, dithiothreitol and hydroquinone inhibit the action of EDRF released from the cells. Furthermore, the inhibitory action of these compounds was attenuated by concomitant infusion of superoxide dismutase (SOD), leading us to suggest that these compounds inactivate EDRF by generating $O_2^-$. This hypothesis was confirmed by demonstrating that another generator of $O_2^-$, pyrogallol, inhibits the action of EDRF and that cytochrome *c*, an $O_2^-$ scavenger, potentiates the action of EDRF [14].

* Address correspondence to: Dr. S. Moncada, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, England.

It is unlikely, however, that all inhibitors of EDRF act via this mechanism. Hemoglobin may have a complex action involving binding of EDRF [17], inhibition of guanylate cyclase [18] and, under some circumstances, generation of $O_2^-$ during auto-oxidation [19]. We have observed that, while low concentrations of Hb (<100 nM) inhibit EDRF-induced relaxations of vascular strips, much larger concentrations are needed to block the vascular relaxation induced by nitroglycerin (GTN) and nitroprusside, two compounds that also induce vasodilatation via the elevation of cGMP. Furthermore, the effect of Hb on EDRF-induced relaxation is not attenuated by SOD [20]. These observations indicate that binding of EDRF is likely to be the principal mechanism by which Hb inhibits the action of EDRF.

Methylene blue, which has redox properties and may also inhibit guanylate cyclase, inhibits endothelium-dependent relaxation [11] and EDRF-induced relaxations [12]. Like Hb, this compound may have a complex mechanism of action for we have observed that, although methylene blue inhibits the vascular relaxations induced by EDRF and GTN, it potentiates those induced by sodium nitroprusside, suggesting that inhibition of guanylate cyclase does not contribute to its action.

Several lines of evidence implicate fatty acids, mobilized by phospholipases, or their metabolites in the generation of EDRF. The phospholipase $A_2$ inhibitors, mepacrine and *p*-bromophenacyl bromide, and the cytochrome P-450 inhibitor, SKF-525A, inhibit endothelium-dependent relaxation [8, 21], and melittin, a phospholipase $A_2$ activator, causes endothelium-dependent relaxation [22]. The specificities of these compounds are questionable, particularly at high concentrations. We have observed that, while the release of both EDRF and prostacyclin is inhibited when the endothelial cells are incubated with dexamethasone for 24 hr, only the release of prostacyclin is inhibited by a short incubation with lipocortin, indicating a lack of involvement of phospholipase $A_2$ in the release of EDRF (our unpublished observations). Exogenous phospholipase C causes endothelium-dependent relaxation [22], whereas phorbol myristate acetate, a protein kinase C activator which imitates the consequences of phospholipase C activation, is an inhibitor of endothelium-dependent relaxation [23]. Caution should be exercised in the interpretation of these results since, because of their physical properties, these compounds may have an action similar to that of saturated and unsaturated fatty acids [24], consisting of a non-specific perturbation of the membrane, rather than a specific chemical interaction.

We have also recently re-examined the action of SKF-525A and have observed that it does not inhibit EDRF release from endothelial cells at concentrations that inhibit ACh-induced endothelium-dependent relaxation [25]. We have also observed that high concentrations of SKF-525A induce the release of EDRF and cause endothelium-dependent relaxation of rabbit aortic rings. Thimerosal, a putative inhibitor of acyl CoA : lysolecithin acyl transferase, also induces EDRF release. This has been suggested to occur as a result of a rise in free arachidonic acid leading to the generation of EDRF [26].

SKF-525A and thimerosal, like many other compounds, induce the release of both prostacyclin and EDRF [25, 26]. The precise connection between these two processes remains to be established. In this context it is interesting that activation of phospholipase C, leading to arachidonic acid release, induces elevated cGMP levels in some cells [27]. This phenomenon, which has been attributed to the formation of lipid peroxides known to stimulate guanylate cyclase [28], has also been observed in endothelial cells when stimulated with a variety of agents that release prostacyclin [29]. It is tempting to speculate that in endothelial cells the increases in cGMP are due to the simultaneous release of EDRF and prostacyclin.

*Identification of EDRF*

A widespread debate about the identity of EDRF preceded the suggestion by both Furchgott [30] and Ignarro *et al.* [31] that EDRF might be NO or a NO-like species. We decided to investigate whether EDRF was NO using two approaches: first, we compared the pharmacological profile of EDRF and authentic NO on vascular strips and on platelets and, second, we measured directly the release of NO from porcine aortic endothelial cells in culture [32].

*Comparative pharmacology.* Both EDRF and NO caused a transient relaxation of the vascular strips which declined at the same rate during passage down the cascade. Furthermore, the rate of decay was slower, but similar for both compounds, during transit in polypropylene tubes (Fig. 1). Both compounds also inhibited platelet aggregation [13], induced the disaggregation of aggregated platelets [33] and inhibited platelet adhesion [6, 7]. Moreover, their biological half-lives as inhibitors of platelet aggregation were similar [13].

The actions of EDRF and NO on platelets and on vascular strips were similarly potentiated by SOD and cytochrome *c* and inhibited by $Fe^{2+}$ and some redox compounds [13, 20]. Hemoglobin also inhibited the effects of EDRF and NO through a mechanism not involving $O_2^-$. Furthermore, the potencies of redox compounds as inhibitors of EDRF- and NO-induced vascular relaxation were similarly attenuated by SOD. Finally, direct measurements of cGMP, or studies with selective inhibitors of its specific phosphodiesterase, have demonstrated that both compounds act on vascular smooth muscle and platelets via the stimulation of guanylate cyclase and elevation of cGMP.

*Chemical detection of NO.* Nitric oxide is a component of cigarette smoke and of automobile engine exhaust emissions contributing to the formation of smog. Methods have been developed, therefore, to measure NO, either indirectly through colorimetric determination of its breakdown product, $NO_2^-$, or directly as the chemiluminescent product of its reaction with ozone.

Using the latter method we have shown that the concentrations of bradykinin which induce the release of EDRF from endothelial cells in culture also cause a concentration-dependent release of NO [32]. Furthermore, we have established that the amounts of NO released by the cells are sufficient to account both for relaxations of the vascular strips

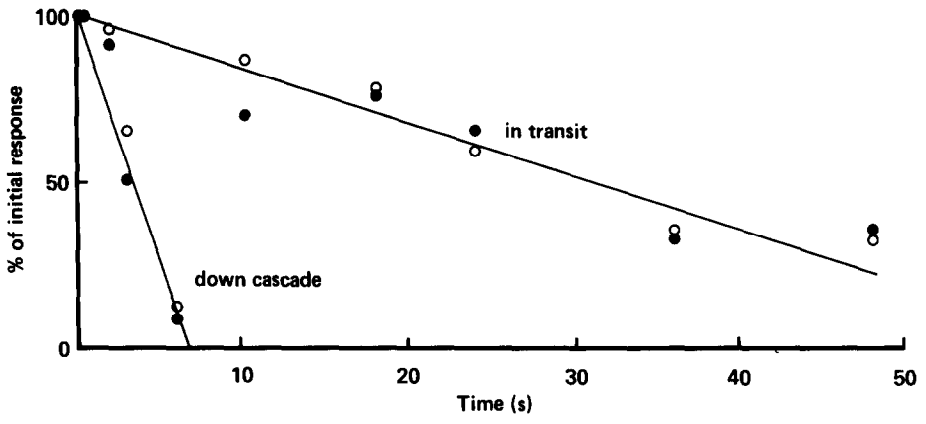


Fig. 1. Decay of EDRF and NO during passage down the bioassay cascade and in transit through a polypropylene tube. Key: (●) EDRF, and (○) NO. Data are from Ref. 32.

and for the anti-aggregating and anti-adhesive activities of EDRF [7, 32, 33]. We have also observed that the vascular relaxing activity released from fresh perfused rabbit, cat and dog arteries by a number of agents, including ACh, substance P and bradykinin, is also accounted for by the amounts of NO released (our unpublished observations).

The evidence for the release of NO is not based solely on the chemical measurement of NO but on the correspondence between the chemical and the biological assays. In most of our studies the fluid containing the biologically active material is transferred rapidly to the bioassay system or the chemiluminescence detector, allowing little time for the breakdown of NO. In these experiments the correspondence between EDRF measured by bioassay and the NO detected by chemiluminescence is complete. When longer times elapse between these two events, as for example when NO is determined after decay to $NO_2^-$ in stored cell supernatants, caution should be exercised in extrapolating data back to the amounts of NO released, as the breakdown of NO needs to be examined under the conditions of the experiment.

In summary, we have provided pharmacological and biochemical evidence to demonstrate that EDRF is NO and that NO satisfies the criteria described by Dale [34] for identification of a biological mediator.

Table 1. Comparison of EDRF and nitric oxide

| | EDRF | NO |
|---|---|---|
| Released by endothelial cells | + | + |
| Relaxes vascular smooth muscle | + | + |
| Inhibits platelet aggregation | + | + |
| Induces platelet disaggregation | + | + |
| Inhibits platelet adhesion | + | + |
| Stability ($T_{1/2}$): | | |
| Down cascade | 3.6 ± 0.1 sec | 4.1 ± 0.2 sec |
| Through polypropylene tubes | 30.9 ± 1.9 sec | 30.4 ± 2.2 sec |
| "Receptor" | Soluble guanylate cyclase | Soluble guanylate cyclase |
| 2nd Messenger | cGMP | cGMP |
| Inhibited directly by Hb | + | + |
| Inhibited indirectly by redox compounds | + | + |
| Potentiated by: | | |
| SOD, cytochrome *c* | + | + |
| M&B 22948, MY-5445 | + | + |
| Not affected by: | | |
| MetHb, HL 725 | + | + |
| Reacts with: | | |
| Superoxide | + | + |
| and $O_2$ | + | + |
| 'Binds' to "Bond Elut" columns | ± | ± |

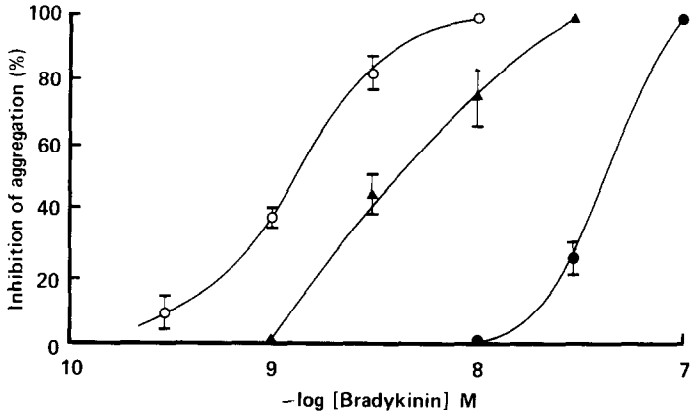


Fig. 2. Inhibition of platelet aggregation by aliquots (0.1 ml) from incubates of 1.5 ml endothelial cells stimulated with bradykinin (0.3–100 nM). Key: (○) control; (▲) cells treated with hemoglobin (100 nM), and (●) cells treated with indomethacin (10 μM). Data are from Ref. 33.

*Is NO the whole story?*

Is all the chemical and pharmacological evidence to date consistent with the conclusion that EDRF is NO? The answer is probably yes (Table 1). The wide variation in the half-life reported for EDRF can probably be explained in terms of the relative contributions of $O_2$ and $O_2^-$ to the inactivation of NO. In addition, some NO may diffuse into the air in cascade bioassay experiments, contributing to the shorter half-lives of NO and EDRF on cascades compared to those in polypropylene tubes. Oxygen is known to react rapidly with NO to form $NO_2$ which in solution forms $NO_2^-$ and $NO_3^-$, both of which are almost inactive on platelets and vascular strips [32, 33]. Nitric oxide has also been shown to react with $O_2^-$ to form $NO_3^-$ [35]. Interestingly, Borland *et al.* [36] have shown recently that the half-life of NO is shorter in air than in cigarette smoke, but not in smoke filtered to remove particulate matter, suggesting that particulate material can modify the decay of NO. Thus, different "half-lives" are likely to be reported as the experimental conditions vary from laboratory to laboratory, making these studies of limited value.

It has been known for over 100 years that NO binds to Hb. Inhibition of endothelium-dependent relaxation and inactivation of EDRF is consistent with its binding to Hb. This has been confirmed experimentally using Hb-agarose columns to which EDRF binds [17].

Some recent reports, however, seem to be at variance with the evidence in support of EDRF being NO. EDRF has been reported to be stabilized by acidification [37], a condition that would not be expected to stabilize NO. However, this can be explained in terms of the transformation of NO into $NO_2^-$, which can generate NO when acidified. In support of this we have shown recently that $NO_2^-$ has the same chromatographic mobility as that reported by these authors for the "stable form" of EDRF.

Some authors have reported that EDRF only relaxes vascular smooth muscle, whereas NO relaxes vascular, tracheal and *Taenia coli* smooth muscle [38, 39]. These results are difficult to analyze since neither group presents evidence showing that equivalent amounts of EDRF and NO were compared. We have observed that the guinea pig tracheal strip is approximately 30 times less sensitive to infusions of NO than the rabbit aortic strip, suggesting that the amount of EDRF required for its relaxation is in excess of that released by our porcine cells in culture.

There are other aspects to consider when analyzing the differential bioassay of EDRF and NO: first, the kinetics of the interaction between NO and $O_2$ or $O_2^-$ may differ according to the conditions of the experiment and, second, the responses of the bioassay tissues to a bolus or to an infusion of an agent are quantitatively different. The release of NO after stimulation is likely to occur at variable rates for periods in excess of 1 min. Because of this, the comparison between NO released from cells or tissues and bolus injections of NO may be misleading. This also explains why slightly lower concentrations of Hb are required to inhibit EDRF-induced relaxations than those required to inhibit similar relaxations induced by authentic NO given as a short infusion [20].

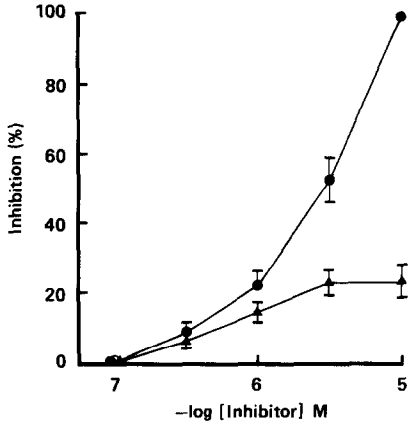


Fig. 3. Inhibition of human platelet adhesion to fibrillar collagen by NO (●) and prostacyclin (▲). Data are from Ref. 7.

EDRF has been reported to bind to anion exchange columns [15, 40], while NO does not [40]. We also have observed binding of EDRF to anion exchange columns (our unpublished results). Amounts of NO that induce comparable relaxation of the bioassay tissues also bind to the columns, although to a lesser extent. NO is not anionic, but it is known to react with amines, and therefore it is possible that the interaction of EDRF and NO with these columns is a chemical reaction.

What cannot be excluded at present is the existence of mechanisms other than the release of NO that play roles in endothelium-dependent relaxation. Their existence would not be surprising since it is well known that mechanisms subserving a biological function are usually multiple. It is important to stress, however, that endothelium-dependent relaxations that are susceptible to inhibition by Hb and methylene blue have been demonstrated in many vascular preparations, indicating the involvement of NO and cGMP, whereas evidence for other mechanisms is scanty. Grace *et al.* [41] have shown that calcitonin gene-related peptide (CGRP) causes endothelium-dependent relaxation of rat aortic rings associated with a rise in cAMP rather than cGMP. These authors suggested that there is a synergism between cAMP elevated by CGRP and the basal level of cGMP, which is higher in tissues with endothelium.

Other mechanisms have been suggested including mediators such as $NH_3$ [42], or the involvement of ion channels which may regulate the release of EDRF and/or propagate signals from the endothelium via gap junctions [43, 44]. An endothelium-dependent hyperpolarization of vascular smooth muscle cells, probably independent of NO, has also been suggested [42]. All of these observations require confirmation before the existence of endothelium-dependent vasodilator mechanisms other than NO can be firmly established.

### *Interactions with prostacyclin*

The supernatants of endothelial cells stimulated with low concentrations of bradykinin contain undetectable amounts of NO and concentrations of prostacyclin too low to explain the anti-aggregating activity observed when these supernatants are added to platelets. This is the result of a synergistic interaction between NO and prostacyclin (Fig. 2; [33]). As a result, we have suggested that the very low concentrations of prostacyclin found in plasma may, after all, have a physiological effect in regulating platelet aggregability if acting on a background of NO release.

Like prostacyclin, therefore, NO is a powerful inhibitor of platelet aggregation and an inducer of platelet disaggregation. Interestingly, NO differs from prostacyclin in that it is also an effective inhibitor of platelet adhesion (Fig. 3). The fact that we did not observe a synergistic interaction between these two compounds on platelet adhesion 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 anti-thrombotic action.

Prostacyclin and NO are powerful vasodilators. The interaction between NO and prostacyclin as vasodilators remains to be studied. Preliminary evidence from our laboratory has not shown synergy between NO and prostacyclin in the rabbit mesenteric artery strip (our unpublished observations).

The anti-aggregating and disaggregating activity of NO and prostacyclin are mediated by cGMP and cAMP respectively. Interestingly, the inhibition of platelet adhesion by both NO and prostacyclin is potentiated by M&B 22948 and by MY 5445, two selective cGMP phosphodiesterase inhibitors, but not by the specific cAMP phosphodiesterase inhibitor HL 725. Forskolin also inhibits platelet adhesion, and its effect is potentiated by M&B 22498 but not by HL 725. These data suggest that the cGMP system in platelets selectively controls those membrane properties associated with adhesion. The fact that both prostacyclin and forskolin elevate platelet cGMP [45, 46] is consistent with our hypothesis. If such a mechanism also regulates the adhesive properties of neutrophils, monocytes or other cells, then our observation may have important physiological and pathological implications.

### *Concluding remarks*

All the findings described indicate that NO is the endogenous nitrovasodilator and that the soluble guanylate cyclase can be considered as the receptor for NO. The exact mechanism by which NO exerts its biological actions still remains to be determined. The extreme sensitivity of NO to inactivation by $O_2$ and Hb suggests that of all the "local hormones" NO is probably the one with the least possibility of acting as a circulating substance. Indeed, it is likely that NO is transferred directly to the vascular smooth muscle or to platelets which come in close contact with the endothelium. Whether NO also shares with prostacyclin the ability to modulate the behaviour of leukocytes or to control the replication of smooth muscle cells remains to be established. We have demonstrated recently that NO has a cytoprotective effect on platelets similar to that of prostacyclin [47].

Circumstantial evidence suggests a physiological role for EDRF. Endothelium-dependent vasodilatation has been demonstrated in a number of species *in vivo*, and removal of vascular endothelium enhances the responses to constrictor agents in several animal models. Endothelium-dependent relaxation is attenuated in animals with spontaneous or experimentally-induced hypertension [48, 49] or with dietary-induced atherosclerosis [50]. Human tissue from subjects with coronary disease or atherosclerosis also exhibits reduced endothelium-dependent vasodilatation [51, 52]. Low density lipoproteins, which are associated with the development of atherosclerosis, have been shown to inhibit endothelium-dependent relaxation [53]. The destruction of NO by oxygen radicals and/or the inhibition of prostacyclin biosynthesis by lipid peroxides [5] may, therefore, be a crucial biochemical step in the development of atherosclerosis. The measurement of NO *in vivo* will provide evidence for the biological significance of all the observations in this field. Such studies are not yet available. However, it is already clear that manipulation of the soluble guanylate

cyclase and/or the specific cGMP phosphodiesterase may offer possibilities for therapeutic intervention in different areas of disease ranging from hypertension to atherosclerosis.

One of the most intriguing remaining problems is the metabolic route to the formation of NO. Direct formation from $NO_2^-$ has been suggested [54]; however, experiments in our laboratory have shown that neither $NO_2^-$ nor $NO_3^-$ is able to enhance the release of NO by endothelial cells. In addition, we have shown that L-arginine, but not D-arginine, is a substrate for NO formation [55]. Macrophages have been shown to synthetize $NO_2^-$ and $NO_3^-$ from L-arginine [56] by arginine deiminase [57]. Whether this process is the same in endothelial cells is yet to be studied. Whether NO is released as such, or as an unstable precursor, is also not yet known. 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 precursor is in the order of fractions of seconds rather than seconds.

Endothelium-dependent relaxation in perfused vascular beds *in vitro* has been reported [58, 59], and yet there are no reports of the release of EDRF or NO from these preparations. It is likely that the release of EDRF is mostly abluminal [60] so that it never reaches the venous outflow of a circulatory bed. Alternatively, the fact that the release of EDRF or NO is easily demonstrable from fresh arterial but not from venous tissues suggests that the synthesis, release or metabolism of NO may be different in the venous side of the circulation. These possibilities should be investigated in connection with the whole problem of the release and fate of NO in the body.

What is becoming evident as our knowledge increases is that NO is an important mediator in the vessel wall, which may help to explain the pathophysiological bases of some diseases. This field of research is likely to produce more exciting discoveries in the near future.

*Acknowledgements*—The authors are indebted to Mrs. G. Henderson and Mrs. E. A. Higgs for assistance in the preparation of this manuscript.

## REFERENCES

1. Moncada S, Gryglewski R, Bunting S and Vane JR, An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263**: 663–665, 1976.
2. Furchgott RF and Zawadzki JV, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**: 373–376, 1980.
3. Vane JR, The release and fate of vasoactive hormones in the circulation. *Br J Pharmacol* **35**: 209–242, 1969.
4. Moncada S, Prostacyclin—discovery and biological importance. In: *Prostaglandins: Research and Clinical Update* (Eds. Longenecker GL and Schaffer SW), pp. 1–39. Raven Press, New York, 1985.
5. Moncada S, Palmer RMJ and Higgs EA, Prostacyclin and endothelium-derived relaxing factor: biological interactions and significance. In: *Thrombosis and Haemostasis* (Eds. Verstraete M, Vermylen J, Lijnen R and Arnout J), pp. 587–618. Leuven University Press, Leuven, 1987.
6. Radomski MW, Palmer RMJ and Moncada S, Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **ii**: 1057–1058, 1987.
7. Radomski MW, Palmer RMJ and Moncada S, The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun* **148**: 1482–1489, 1987.
8. Furchgott RF, The role of endothelium in the responses of vascular smooth muscle to drugs. *Annu Rev Pharmacol Toxicol* **24**: 175–197, 1984.
9. Rubanyi GM and Vanhoutte PM, Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am J Physiol* **250**: H-222–H-227, 1986.
10. Gryglewski RJ, Palmer RMJ and Moncada S, Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320**: 454–456, 1986.
11. Martin W, Villani GM, Jothianandan D and Furchgott RF, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* **232**: 708–716, 1985.
12. Gryglewski RJ, Moncada S and Palmer RMJ, Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br J Pharmacol* **87**: 685–694, 1986.
13. Radomski MW, Palmer RMJ and Moncada S, Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol* **92**: 181–187, 1987.
14. Moncada S, Palmer RMJ and Gryglewski RJ, Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* **83**: 9164–9168, 1986.
15. Cocks TM, Angus JA, Campbell JH and Campbell GR, Release and properties of endothelium-derived relaxing factor (EDRF) from endothelial cells in culture. *J Cell Physiol* **123**: 310–320, 1985.
16. Griffith TM, Edwards DH, Lewis MJ, Newby AC and Henderson AH, The nature of endothelium-derived vascular relaxant factor. *Nature* **308**: 645–647, 1984.
17. Martin W, Smith JA and White DG, The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide or bovine retractor penis inhibitory factor. *Br J Pharmacol* **89**: 563–571, 1986.
18. Murad F, Mittal CK, Arnold WP, Katsuki S and Kimura H, Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv Cyclic Nucleotide Res* **9**: 145–158, 1978.
19. Misra HP and Fridovich I, The generation of superoxide radical during the autooxidation of hemoglobin. *J Biol Chem* **247**: 6960–6962, 1972.
20. Hutchinson PJA, Palmer RMJ and Moncada S, Comparative pharmacology of EDRF and nitric oxide on vascular strips. *Eur J Pharmacol* **141**: 445–451, 1987.
21. Singer HA, Saye JA and Peach MJ, Effects of cytochrome P-450 inhibitors on endothelium-dependent relaxation in rabbit aorta. *Blood Vessels* **21**: 223–230, 1984.
22. Forstermann U and Neufang B, Endothelium-dependent vasodilation by melittin: are lipoxygenase products involved? *Am J Physiol* **249**: H14–H19, 1985.
23. Lewis MJ and Henderson AH, A phorbol ester inhibits the release of endothelium-derived relaxing factor. *Eur J Pharmacol* **137**: 167–171, 1987.
24. Furchgott RF, Cherry PD, Zawadzki JV and Jothianandan D, Endothelial cells as mediators of vasodilation of arteries. *J Cardiovasc Pharmacol* **6**: S336–S343, 1984.
25. Rees DD, Palmer RMJ and Moncada S, Effect of SKF

525A on the release of nitric oxide and prostacyclin from endothelial cells. *Eur J Pharmacol* **150**: 149–154, 1988.
26. Forstermann U, Goppelt-Strube M, Frolich JC and Busse R, Inhibitors of acyl-coenzyme A: lysolecithin acyltransferase activate the production of endothelium-derived vascular relaxing factor. *J Pharmacol Exp Ther* **238**: 352–359, 1986.
27. Nishizuka Y, Turnover of inositol phospholipids and signal transduction. *Science* **225**: 1365–1370, 1984.
28. Graff G, Stephenson JH, Glass DB, Haddox MK and Goldberg ND, Activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides. *J Biol Chem* **253**: 7662–7676, 1978.
29. Brotherton AFA, Induction of prostacyclin biosynthesis is closely associated with increased guanosine 3′,5′-cyclic monophosphate accumulation in cultured human endothelium. *J Clin Invest* **78**: 1253–1260, 1986.
30. Furchgott RF, Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: *Mechanisms of Vasodilatation* (Ed. Vanhoutte PM), Vol. IV. Raven Press, New York, in press.
31. Ignarro LJ, Byrns RE and Wood KS, Biochemical and pharmacological properties of EDRF and its similarity to nitric oxide radical. In: *Mechanisms of Vasodilatation* (Ed. Vanhoutte PM), Vol. IV. Raven Press, New York, in press.
32. Palmer RMJ, Ferrige AG and Moncada S, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**: 524–526, 1987.
33. Radomski MW, Palmer RMJ and Moncada S, The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol* **92**: 639–646, 1987.
34. Dale HH, Progress in autopharmacology. A survey of present knowledge of the chemical regulation of certain functions by natural constituents of the tissues. *Bull Johns Hopk Hosp* **53**: 297–347, 1933.
35. Blough NV and Zafiriou OC, Reaction of superoxide with nitric oxide to form peroxonitrite in alkaline aqueous solution. *Inorg Chem* **24**: 3502–3504, 1985.
36. Borland C, Chamberlain A, Barber A, Thrush B and Higenbottam TW, A comparison between the rate of reaction of nitric oxide in the gas phase and in whole cigarette smoke. *Beitr Tabakforschung Int* **13**: 67–73, 1985.
37. Murray JJ, Fridovich I, Makhoul RG and Hagen PO. Stabilization and partial characterization of endothelium derived relaxing factor from cultured bovine aortic endothelial cells. *Biochem Biophys Res Commun* **141**: 689–696, 1986.
38. Dusting GJ, Read MA and Stewart AG, Endothelium-derived relaxing factor released from cultured cells; differentiation from nitric oxide. *Clin Exp Pharmacol Physiol* **15**: 83–92, 1988.
39. Shikano K and Berkowitz BA, Endothelium-derived relaxing factor is a selective relaxant of vascular smooth muscle. *J Pharmac Exp Ther* **243**: 55–60, 1987.
40. Long CJ, Shikano K and Berkowitz BA, Anion exchange resins discriminate between nitric oxide and EDRF. *Eur J Pharmacol* **142**: 317–318, 1987.
41. Grace GC, Dusting GJ, Kemp BE and Martin TJ, Endothelium and the vasodilator action of rat calcitonin gene-related peptide (CGRP). *Br J Pharmacol* **91**: 729–733, 1987.
42. Vanhoutte PM, The end of the quest? *Nature* **327**: 459–460, 1987.
43. Olesen S-P, Davies PF and Clapham DE, Muscarinic acetylcholine-activated $K^+$ current in bovine aortic endothelial cells. *Circ Res*, in press.
44. Lansman JB, Hallam TJ and Rink TJ, Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* **325**: 811–813, 1987.
45. Bruckdorfer KR, Buckley S and Hassall DG, The effect of low-density lipoproteins on the synthesis of cyclic nucleotides induced by prostacyclin in isolated platelets. *Biochem J* **223**: 189–196, 1984.
46. Siegl AM and Moroff G, Effect of forskolin on the maintenance of platelet properties during storage. *J Lab Clin Med* **108**: 354–359, 1986.
47. Radomski MW, Palmer RMJ, Read NG and Moncada S, Isolation and washing of human platelets with nitric oxide. *Thromb Res*, in press.
38. Van de Voorde J and Leusen I, Endothelium-dependent and independent relaxation of aortic rings from hypertensive rats. *Am J Physiol* **250**: H711–H717, 1986.
49. Lockette W, Otsuka Y and Carretero O, The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension* **8** [Suppl 11]: 11-61–66, 1986.
50. Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Coene M-C and Herman AG, Effect of hypercholesterolemia on vascular reactivity in the rabbit: 1: Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ Res* **58**: 552–564, 1986.
51. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW and Ganz P, Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* **315**: 1046–1051, 1986.
52. Forstermann U, Properties and mechanisms of production and action of endothelium-derived relaxing factor. *J Cardiovasc Pharmacol* **8** [Suppl 10]: S45–S51, 1986.
53. Andrews HE, Bruckdorfer KR, Dunn RC and Jacobs M, Low-density lipoproteins inhibit endothelium-dependent relaxation in rabbit aorta. *Nature* **327**: 237–239, 1987.
54. Martin W, Smith JA, Lewis MJ and Henderson AH, Evidence that the inactivated form of inhibitory factor extracted from bovine retractor penis is nitrite and the acid-activated form is nitric oxide. *Br J Pharmacol* **93**, 579–586, 1988.
55. Palmer RMJ, Ashton DS and Moncada S, Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, in press.
56. Stuehr DJ and Marletta MA, Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharaide. *Proc Natl Acad Sci USA* **82**: 7738–7742, 1985.
57. Hibbs JB Jr, Taintor RR and Vavrin Z, Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235**: 473–476, 1987.
58. Cherry PD and Gillis CN, Evidence for the role of endothelium-derived relaxing factor in acetycholine-induced vasodilatation in the intact lung. *J Pharmacol Exp Ther* **241**: 516–520, 1987.
59. Chen W-Z, Palmer RMJ and Moncada S, The effect of nitric oxide on the isolated perfused rabbit heart. *Br J Pharmacol* **92**: 643P, 1987.
60. Busse R, Trogisch G and Bassenge E, The role of endothelium in the control of vascular tone. *Basic Res Cardiol* **80**: 475–490, 1985.