

## ENDOTHELIUM-DERIVED RELAXING FACTOR: DISCOVERY, EARLY STUDIES, AND IDENTIFICATION AS NITRIC OXIDE

Nobel Lecture, December 8, 1998

by

ROBERT F. FURCHGOTT

Department of Pharmacology, State University of New York, Health Science Center at Brooklyn, Brooklyn, NY 11203, USA

### AN ACCIDENTAL FINDING

In a paper published forty-five years ago, in which I introduced the helical strip of the rabbit aorta as a useful preparation for quantitative pharmacological studies on vasoactive drugs and drug-receptor interactions, I reported on the responses of this preparation to a number of catecholamines, sodium nitrite, histamine and acetylcholine (ACh). One somewhat surprising finding (Furchgott and Bhadrakom, 1953) was that ACh, well recognized as a very potent vasodilator in whole animal and perfused organ studies, elicited no relaxation but only contraction of the aortic strip, whether or not the strip was tested at rest or precontracted with a vasoconstrictor like norepinephrine. Somewhat later, we used ACh as the contracting agent when determining the relative potencies of norepinephrine, epinephrine and isoproterenol as relaxing agents acting via  $\beta$ -adrenoceptors on helical strips in which  $\alpha$ -adrenoceptors had been irreversibly blocked by pretreatment with dibenamine (Furchgott, 1954). The relative potencies of these catecholamines as relaxants (approximately 1:50:200) indicated a  $\beta_2$ -type adrenoceptor.

In the mid-1970s, in pharmacological studies on isolated strips of guinea-pig tracheal smooth muscle, we obtained evidence that the  $\beta$ -adrenoceptors of that muscle, which according to dogma were pure  $\beta_2$ -type, frequently had a fairly high admixture of the  $\beta_1$  type with the  $\beta_2$ -type. In view of this unexpected finding, I decided that it would be well to reexamine the type of  $\beta$ -receptor in the smooth muscle of rabbit thoracic aorta. The plan was first to determine anew in a series of experiments the relative potencies of norepinephrine, epinephrine and isoproterenol in producing relaxation of preparations of rabbit aorta after dibenamine pretreatment. To produce contraction after the pretreatment, we planned to use a muscarinic agonist like carbachol or ACh, as in our earlier studies. However, in the very first experiment of this planned series in May 1978, my technician did not follow my directions correctly: early in the experiment before blocking the  $\alpha$ -adrenergic receptors with dibenamine, he tested carbachol for its contracting activity before rather than after (as prescribed in the directions) washout of a previous test dose of norepinephrine. The response to carbachol was not a contrac-

tion, but was a partial relaxation of the norepinephrine-induced contraction. ACh was then tested. It too produced relaxation of the aortic preparation precontracted with norepinephrine (Fig. 1). This was the first time that I had ever observed relaxation of rabbit aorta in response to muscarinic agonists over the many years that I had been using this blood vessel for *in vitro* studies. The unexpected relaxation of rabbit aorta *in vitro* by muscarinic agonists was very exciting, for it was an effect that was in accord with the potent vasodilating action of these agonists *in vivo*.

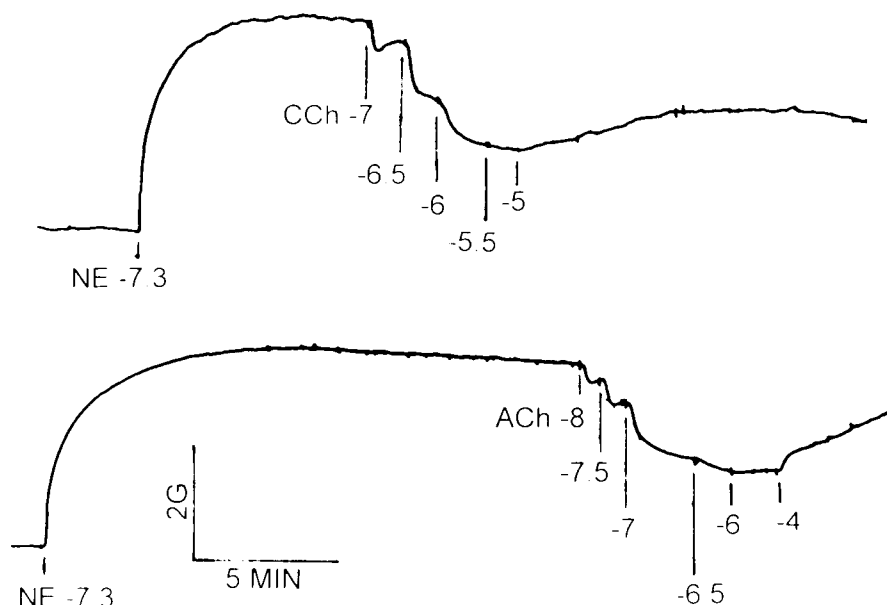


Figure 1. Polygraph tracings from the first experiment in the author's laboratory in which the muscarinic agonists, carbachol (CCh) and acetylcholine (ACh), unexpectedly produced relaxation of transverse rings of rabbit thoracic aorta. In this and all other experiments, unless otherwise noted, aortic preparations (rings or strips) were mounted in 20-ml all-glass organ chambers containing Krebs-bicarbonate solutions aerated with 95%O<sub>2</sub>-5%CO<sub>2</sub>, and kept at 37°. Tracings show changes in tension under isometric conditions. (For details of experimental procedures, see Furchgott and Zawadzki, 1980.) NE is norepinephrine. In this and all other figures unless otherwise noted, cumulative concentrations of drugs in organ chambers are expressed as log molar concentrations.

## DISCOVERY AND EARLY STUDIES ON EDRF

Why had we failed to observe ACh-induced relaxation of aortic preparations in the past, and why did it now become manifest? A detailed account of our investigation of this matter has been published (Furchgott, 1993; 1996), so it will suffice here to outline briefly the sequence of findings. First, we were aware that the kind of preparation of rabbit thoracic aorta that we were now using was the transverse ring, rather than the helical strip which we had used in all of the earlier experiments with muscarinic agonists.

Several experiments were therefore carried out in which we compared the responses to cumulative concentrations of carbachol or ACh on helical strips

and transverse rings prepared from the same aorta. Only the rings responded with relaxation to these muscarinic agonists, while the strips responded with contraction (Fig. 2). After several weeks of work, we recognized that in any

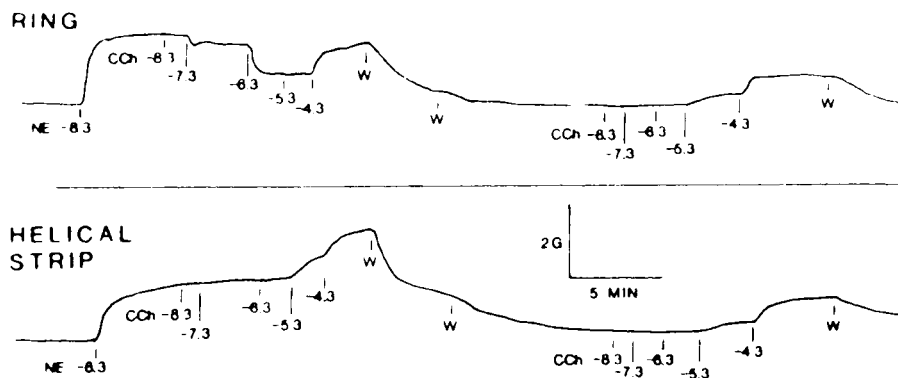


Figure 2. Comparison of responses to carbachol (CCh) of a transverse ring and a helical strip prepared from the same rabbit thoracic aorta. The ring (2.5-mm wide) was mounted on a pair of L-shaped hooks for attachment to the base of the organ chamber and to the force transducer. The strip, prepared by our standard method, had thread ties at each end for attachment. Only the ring, when precontracted with norepinephrine (NE), relaxed in response to low concentrations of CCh. It was subsequently recognized that in our standard method of preparing strips, the endothelial cells were unintentionally rubbed off the intimal surface. (There is no relaxation of the ring in response to CCh added after washout (W) of NE because such preparations exhibit practically no active contractile tension in the absence of vasoconstrictor agents.) In this figure, concentrations of drugs are expressed as log of concentrations in g per ml. Reproduced with permission from Furchgott (1993).

kind of preparation (ring or strip), gentle rubbing of the intimal surface, whether intentionally or unintentionally (as had occurred, unbeknown to us, in our standard method of preparing helical strips), eliminated the relaxing response to ACh and other muscarinic agonists. Strips as well as rings gave excellent relaxation when care was taken not to rub the intimal surface. That the loss of the relaxation response to ACh after intimal rubbing was the result of mechanical removal of the endothelial cells was clearly demonstrated with the use of a silver staining procedure to allow *en face* microscopy of the intimal surfaces. It was also demonstrated that complete removal of endothelial cells of the aorta by pre-incubation of the intimal surface with collagenase also resulted in a complete loss of the relaxation response to ACh.

Figure 3 is a slightly modified figure from the first full paper on the obligatory role of endothelial cells in the relaxation of arterial smooth muscle by ACh (Furchgott and Zawadzki, 1980). In that paper, we also reported that in addition to the thoracic aorta, a number of other arteries from the rabbit and various arteries from other species of laboratory animals also exhibited relaxation in response to ACh only if endothelial cells were present. Cyclooxygenase inhibitors did not interfere with the endothelium-dependent relaxation. One hypothesis to explain the obligatory role of endothelial cells was that ACh acting on a muscarinic receptor of these cells stimulates them to release a non-prostanoid substance that diffuses to and activates relaxation of

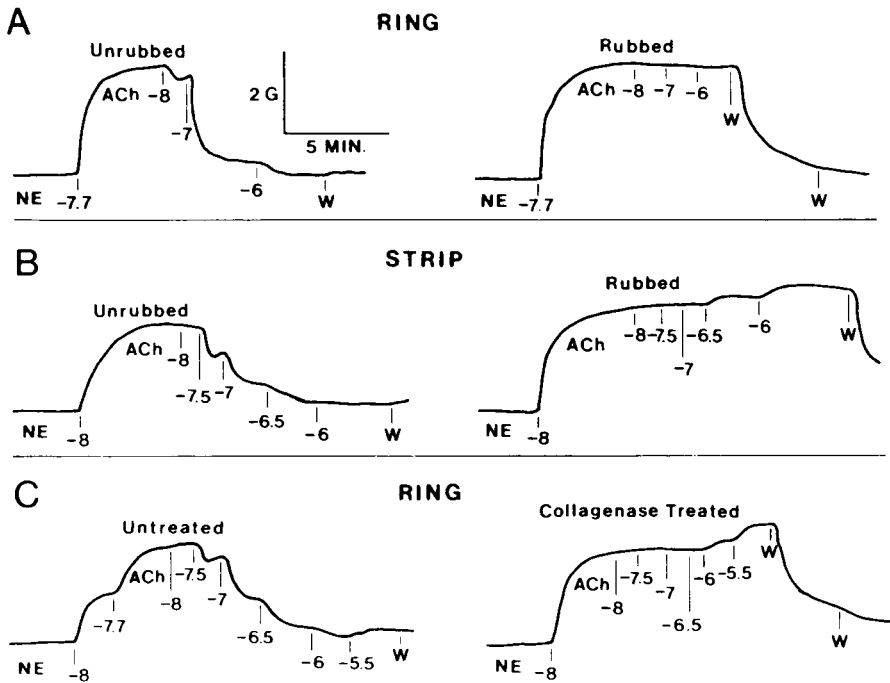


Figure 3. Recordings showing loss of relaxing response of rabbit aorta to ACh after complete removal of endothelial cells. In each panel, the recording on the left was made before removal and that on the right after removal of endothelial cells. The cells were removed from the ring in panel A by rubbing its intimal surface with a small wooden stick, from the transverse strip in panel B by rubbing its intimal surface on moistened filter paper, and from the ring in panel C by exposing its intimal surface to collagenase. After the procedure to remove endothelial cells, each preparation was allowed to re-equilibrate in its organ chamber for about one hour before being retested. Reproduced with permission from Furchgott *et al.* (1981).

the subjacent smooth muscle cells. Direct evidence for this hypothesis was obtained with the so-called "sandwich" procedure in which it was shown that a transverse strip of aorta freed of endothelial cells and therefore unable to relax on exposure to ACh, could be made to relax in response to ACh if it were remounted for recording with its endothelium-free intimal surface placed against the endothelium-containing intimal surface of a longitudinal strip of aorta (Fig. 4). The ACh-induced relaxation of the transverse strip in the "sandwich" demonstrated that ACh stimulated the release of a diffusible relaxing substance (or substances) from the endothelial cells of the longitudinal strip (Furchgott and Zawadzki, 1980). The relaxing substance was later referred to as EDRF (for endothelium-derived relaxing factor) (Cherry *et al.*, 1982).

It should be noted here that although we had never observed relaxation of rabbit aortic preparations by ACh in my laboratory prior to our accidental finding in 1978, there had been occasional reports from other investigators of relaxation of isolated preparations of arteries in response to that agent. For example, Jelliffe (1962) had reported that a chain of thread-tied rings of rabbit aorta precontracted with serotonin, relaxed in response to low concentra-

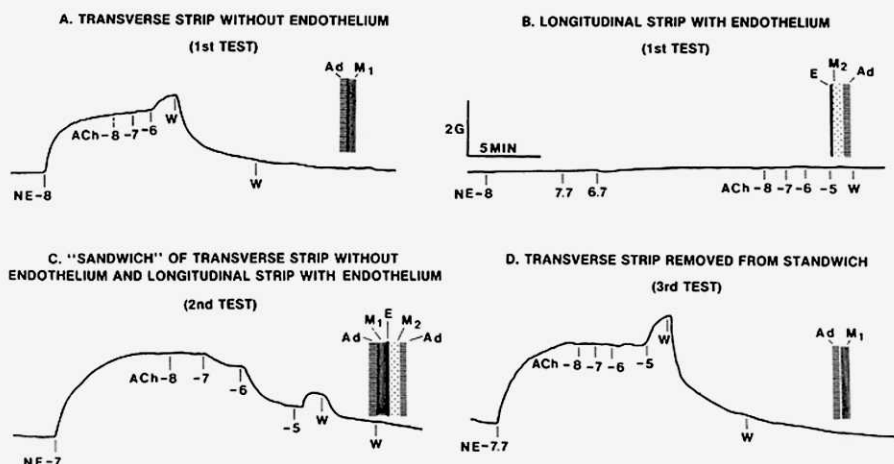


Figure 4. "Sandwich" experiment demonstrating release of a relaxing substance from endothelial cells in response to ACh. **A:** On a transverse strip of rabbit aorta with endothelial cells removed by rubbing, ACh failed to relax the NE-induced tone. **B:** On a longitudinal strip from the same aorta with endothelium present, NE and ACh produced only trivial changes in tension because of the orientation of the smooth muscle cells. **C:** On a "sandwich" made from the transverse strip without endothelium and the longitudinal strip with endothelium mounted together in clips with their entire intimal surfaces apposed, and allowed to re-equilibrate, ACh now produced relaxation of the NE-induced tone. **D:** After removing the longitudinal strip with endothelium from the endothelium-denuded transverse strip, the latter after re-equilibration, again gave no relaxation in response to ACh. In the diagrams of the vertical cross-sections of the preparations, Ad is adventitia; E is endothelial cell layer; M<sub>1</sub> and M<sub>2</sub>, respectively, are medial layers of smooth muscle cells oriented vertically and horizontally. Reproduced with permission from Furchgott *et al.* (1981).

tions of ACh; and Vanhoutte (1974) and Toda (1974) had found that on a number of canine arteries prepared as strips, ACh elicited significant relaxation of spontaneous or drug-induced contractions. After our findings on the obligatory role of endothelial cells in ACh-induced relaxation of arteries, it became apparent that these earlier examples of ACh-induced relaxation were dependent on the presence of endothelial cells in the preparations of the arteries being studied.

It is of some interest that an unintentional removal of endothelial cells in another vascular preparation used in my laboratory may have prevented the discovery of endothelium-dependent vasodilation at an earlier date. That preparation was the perfused rabbit central ear artery which Odd Steinsland, while a graduate student in my laboratory in the early 1970s, used for investigating the inhibition by ACh and other muscarinic agonists of adrenergic neurotransmission by their action on prejunctional muscarinic inhibitory receptors. He readily demonstrated that ACh was a potent inhibitor of neurotransmission, as evidenced by its inhibition of both the release of norepinephrine and the vasoconstriction in response to nerve stimulation (Steinsland *et al.*, 1973). To make sure that the inhibition of the vasoconstriction was only due to inhibition of transmitter release, he tested whether infused ACh could inhibit vasoconstriction produced by infused norepinephrine, and

found that it did not (Steinsland *et al.*, 1973). About ten years later, after endothelium-dependent relaxation by ACh had been found in a large variety of arterial preparations, we were puzzled by the earlier finding that the perfused ear artery constricted with infused norepinephrine, had failed to show any vasodilation in response to ACh. Dr. Steinsland provided us with the answer. In his earlier work, he had not taken precautions to prevent air bubbles from entering the perfusion line and passing through the ear artery. When care was now taken to avoid the occurrence of bubbles in the perfusion stream through the artery, ACh elicited good vasodilation (relaxation) of norepinephrine-induced vasoconstriction (Furchgott *et al.*, 1990b). Apparently, in the earlier work, the bubbles had mechanically removed the endothelial cells and we had been unaware of this action.

### EARLY STUDIES ON AGENTS INDUCING AND INHIBITING ENDOTHELIUM-DEPENDENT RELAXATION

Within a few years after the discovery that ACh-induced relaxation of isolated arteries was endothelium-dependent, a number of other endothelium-dependent relaxing agents were identified in organ chamber experiments using rings or strips of blood vessels from a variety of species, including man. Among these were the calcium ionophore A23187, ATP and ADP, substance P, bradykinin, histamine, thrombin, serotonin and vasopressin. [For references to first reports on endothelium-dependent relaxation by these and other agents, see Furchgott and Vanhoutte (1989) and Furchgott (1990)]. The early finding that the calcium ionophore A23187 was a very potent endothelium-dependent relaxing agent on all vessels tested suggested a central role for calcium in the release, or synthesis and release, of EDRF by endothelium-dependent relaxing agents (Furchgott 1981).

It was soon recognized in this early work that, depending on the agent, endothelium-dependent relaxation might be limited to certain species, or even to specific arteries from a given species. For example, whereas ACh relaxed by this mechanism practically all systemic arteries from all mammalian species tested, histamine, another potent vasodilator, produced endothelium-dependent relaxation of rat (van de Voorde and Leusen, 1983) but not of rabbit arteries (Furchgott, 1983). Another example was bradykinin, which gave endothelium-dependent relaxation of isolated canine and human arteries but not of rabbit or cat arteries (Cherry *et al.*, 1982). As examples of vessel specificity, it was found that noradrenaline activated endothelium-dependent relaxation in isolated canine coronary arteries by acting on  $\alpha_2$ -adrenoceptors of the endothelial cells, but failed to activate this kind of relaxation in canine systemic arteries (Cocks and Angus, 1983), and that serotonin activated endothelium-dependent relaxation in canine coronary arteries but not in canine systemic arteries (Cocks and Angus, 1983; Cohen *et al.*, 1983).

Among the conditions and agents which were found to inhibit endothelium-dependent relaxation of arteries in the early organ chamber experiments were anoxia and some agents that were recognized as inhibitors in the

metabolic pathway of arachidonic acid (AA) from phospholipids through lipoxygenase (Furchgott and Zawadzki, 1980; Furchgott *et al.*, 1981). Because it had already been reported that in certain smooth muscles there was a positive relationship between an increase in cyclic guanosine monophosphate (cGMP) and relaxation (Katsuki and Murad, 1977; Böhme *et al.*, 1978; Murad *et al.*, 1978, 1979) and that guanylate cyclase (G-cyclase) was markedly stimulated by hydroperoxides of AA (Hidaka and Asano, 1977; Goldberg *et al.*, 1978), I speculated that (1) EDRF is either a short-lived hydroperoxide or free radical arising as an intermediate product in the oxidation of liberated AA by the lipoxygenase pathway, and (2) that EDRF stimulates the G-cyclase of the vascular smooth muscle, causing an increase in cGMP which then somehow activates relaxation (Furchgott *et al.*, 1981). As it turned out, this early speculation about the nature of EDRF was wrong, but that about the role of cGMP proved to be right.

#### CYCLIC GMP AS A MEDIATOR FOR EDRF AND NITROVASODILATORS

The speculation that EDRF released by ACh would stimulate an increase in cGMP was soon proven correct in a number of independent studies – e.g., Rapoport and Murad (1983) using rat aorta, Diamond and Chu (1983) and Furchgott *et al.* (1984) using rabbit aorta, Holzmann (1982) using bovine coronary artery, and Ignarro *et al.* (1984) using bovine pulmonary artery. All of these investigators showed that a rise in cGMP accompanied the ACh-induced relaxation of the blood vessel, consistent with the proposal that the increase in cGMP in the muscle has a causal role in the relaxation (Fig. 5).

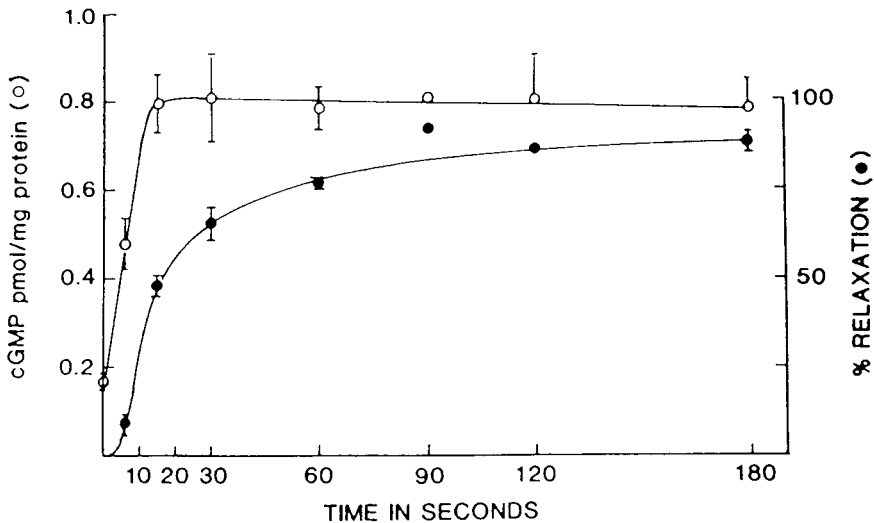


Figure 5. Time course of changes of cyclic GMP (cGMP) and of relaxation of endothelium-containing rings of rabbit thoracic aorta after addition of ACh. Norepinephrine ( $0.1 \mu\text{M}$ ) was first added to produce a steady contraction. ACh ( $1 \mu\text{M}$ ) was then added, and the tissue was instantly frozen in liquid nitrogen after the desired period of exposure to ACh. The level of cyclic AMP (not shown) did not change significantly during the endothelium-dependent relaxation. Reproduced with permission from Furchgott *et al.* (1984).

Endothelium-dependent relaxation of rat aorta by A23187 and by histamine (Rapoport and Murad, 1983) and of rabbit aorta by A23187 (Furchgott *et al.*, 1984) was also accompanied by a rise in cGMP.

It is well to stress at this point that Murad and coworkers (see Murad *et al.*, 1978 and 1979 for reviews) had previously found that nitric oxide (NO) is a very potent stimulus of G-cyclase, and had proposed that a number of agents (e.g. glyceryl trinitrate, nitroprusside, azide) which they and others had found to increase cGMP and produce relaxation of certain non-vascular smooth muscle preparations, do so by generating NO as the proximal activator of G-cyclase. Also, Ignarro and his coworkers (Gruetter *et al.*, 1979) had found that the relaxation of a vascular preparation (bovine coronary artery) by NO as well as by nitroprusside was associated with an increase in cGMP. However, despite these early findings with NO, none of the investigators who several years later showed that endothelium-dependent relaxation of arteries in organ chamber experiments was also correlated with an increase in cGMP (see above) hypothesized that EDRF might be the free radical NO. Certain key findings had yet to be made to foster this novel proposal.

#### FURTHER STUDIES ON EDRF, INCLUDING THOSE WITH PERFUSION-BIOASSAY PROCEDURES

##### *Hemoglobin and Methylene Blue as Inhibitors*

Two agents that became widely used as inhibitors of endothelium-dependent relaxation were hemoglobin (Hb) and methylene blue (MB). Hb had been found by Murad *et al.* (1978) to inhibit guanylate cyclase in cellular extracts, and by workers at Glasgow University (Bowman *et al.*, 1982) to inhibit the relaxation of strips of bovine retractor penis (BRP) muscle in response either to nonadrenergic, noncholinergic (NANC) nerve stimulation or to an acid-activated extract of that tissue. Gruetter *et al.* (1981) had reported that methemoglobin (methHb) interfered with NO-induced increases in vascular cGMP. In our laboratory, work with Hb began in 1983 when William Martin, who had studied the properties of extracts of the BRP muscle while a graduate student at Glasgow University (Gillespie *et al.*, 1981; Bowman *et al.*, 1981), came to my laboratory as a postdoctoral fellow (see below). It was soon found that Hb was an extremely potent and fast acting inhibitor of endothelium-dependent relaxation and of the associated rise in cGMP induced by ACh, A23187 and other agents on rabbit and rat aorta (Furchgott *et al.*, 1984; Martin *et al.*, 1985a, 1986a). Myoglobin was as effective as Hb, but methHb only inhibited very weakly (Martin *et al.*, 1985b) (Fig. 6).

The degree of potentiation by Hb of contractions of arterial rings by vasoconstrictors such as phenylephrine also provided a measure of the relaxing activity of basally released EDRF (Martin *et al.*, 1986a). Using this measure, the relaxing activity of basally released EDRF in the case of rings of rat aorta appeared to be considerably greater than in the case of rings of rabbit aorta. With rings from both species, addition of phosphodiesterase inhibitors (such as zaprinast and IBMX) produced relaxation associated with increases in



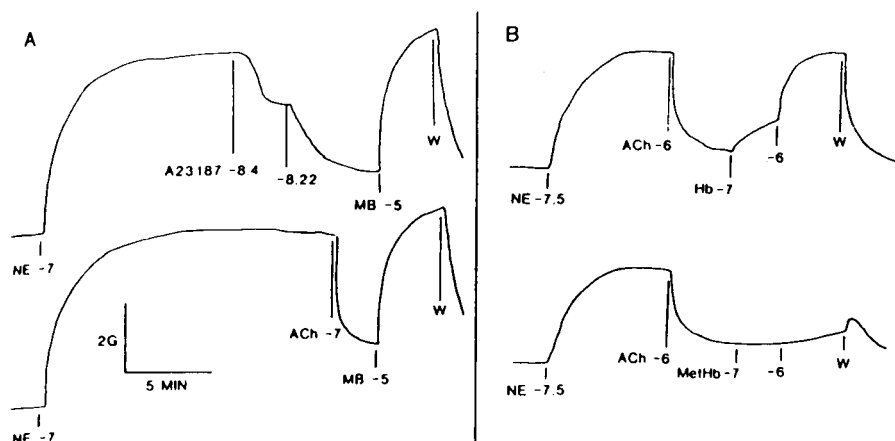


Figure 6. Examples of inhibition by methylene blue (MB) and by hemoglobin (Hb) of endothelium-dependent relaxation in rings of rabbit aorta. **A.** Addition of MB ( $10 \mu\text{M}$ ) rapidly and completely inhibited the ongoing relaxation produced by the addition of ACh and that produced by addition of the calcium ionophore A23187. **B.** Addition of Hb at  $1 \mu\text{M}$  rapidly and almost completely inhibited the relaxation produced by addition of ACh; however, methemoglobin (MetHb) at the same concentration inhibited ACh-induced relaxation only to a very small extent.

cGMP (Martin *et al.*, 1986b). The responses to these inhibitors of the enzymes which hydrolyze cGMP to GMP were more pronounced in the case of rat aorta than in rabbit aorta, consistent with a higher basal release of EDRF and higher basal stimulation of G-cyclase in the former. The relaxation of endothelium-containing rings of both species produced by phosphodiesterase inhibitors could be completely blocked by Hb.

It was originally proposed that Hb inhibits relaxation and the rise in cGMP by binding EDRF as it diffuses from the endothelial cells, thus preventing it from reaching and activating the G-cyclase in the muscle cells (Martin *et al.*, 1985a). However, after the identification of EDRF as NO, it became apparent that the scavenging of EDRF by Hb under the usual aerobic conditions was the result of the very rapid reaction of  $\text{HbO}_2$  with NO to form  $\text{NO}_3^-$  and metHb, as first reported by Doyle and Hoekstra (1981).

MB had first been shown by Gruetter *et al.* (1981) to inhibit stimulation of G-cyclase by nitrovasodilators in cell-free systems and to inhibit both the relaxation and increase in cGMP elicited by these agents in vascular smooth muscle. Subsequently, MB was found to inhibit both the endothelium-dependent relaxation and accompanying increase in cGMP produced by ACh in bovine arteries (Holzman, 1982; Ignarro *et al.*, 1984) and by ACh and A23187 in rabbit aorta (Furchgott *et al.*, 1984; Martin *et al.*, 1985a). Holzman (1982) and Ignarro *et al.* (1984) concluded that the inhibition of relaxation was the result of the inhibition of G-cyclase by the MB. On the other hand, the present author and his coworkers, although agreeing that such enzyme inhibition accounted for the sustained inhibition by MB developed during prolonged exposure of the rabbit aorta, proposed that the extremely fast inhibition of endothelium-dependent relaxation immediately after addition

of MB to an organ chamber (as in Fig. 6) resulted from some more direct inactivation of EDRF by the MB (Furchgott *et al.*, 1984; Martin *et al.*, 1985a). It now appears that this more direct inactivation is due to superoxide anions, generated by reactions of the MB in the tissue (also see Wolin *et al.*, 1990).

#### *Results with Perfusion-Bioassay Procedures*

For more direct studies on the properties of EDRF than were possible in organ chamber experiments, perfusion-bioassay procedures were developed. These procedures, although first developed in laboratories other than my own, should be considered here, because they produced results which contributed much to the identification of EDRF. In such procedures, endothelial cells are upstream in the perfusion (or superfusion) cascade and an endothelium-denuded arterial preparation (usually a ring or one or more strips) whose contractile state is monitored, is downstream to bioassay EDRF in the perfusion fluid. In some procedures, the endothelial cells upstream were native cells on the luminal surface of a perfused artery (e.g. Forstermann *et al.*, 1984, Griffith *et al.*, 1984, Rubanyi *et al.*, 1985, Khan and Furchgott, 1987); in other procedures, they were cultured cells (usually from bovine or porcine aortas) on the surface of microcarrier beads contained in a perfused column (e.g. Cocks *et al.*, 1985, Gryglewski *et al.*, 1986).

Perfusion-bioassay procedures allowed one to determine clearly whether an inhibitor of endothelium-dependent relaxation interfered with the synthesis/release of EDRF or inactivated the EDRF after its release. In addition, by altering the transit time of the perfusate between the endothelial cells and the bioassay preparation, it was possible to study the rate of decay of EDRF. Surprisingly, estimated half-life values for EDRF ranged from as low as 4-6 s to as high as 50 s depending on the biological preparations and the experimental conditions. The likely explanation of these discrepant values came with the important finding that superoxide dismutase (SOD) markedly stabilizes EDRF released from either endothelial cells on arteries (Rubanyi and Vanhoutte 1986) or cultured endothelial cells on microcarrier beads (Gryglewski *et al.*, 1986), clearly showing that the superoxide anion ( $O_2^-$ ) very rapidly inactivates EDRF. Thus, the marked differences in half-life of EDRF reported from different laboratories could largely be attributed to differences in concentrations of  $O_2^-$  in the perfusion fluid leaving the endothelial cells under the different experimental conditions used. Subsequently, Moncada *et al.* (1986) were able to show with their perfusion-bioassay procedure that inhibition of endothelium-dependent relaxation by certain reducing agents (e.g. hydroquinone, phenidone and pyrogallol) originally demonstrated in organ chamber experiments, was principally the result of inactivation of EDRF by  $O_2^-$  formed in a single-electron oxidation of the reducing agent by  $O_2$ .

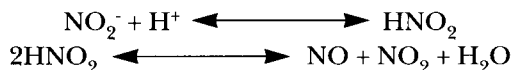
Development of perfusion-bioassay procedures also led to the important finding that increases in shear stress on endothelial cells of the perfused artery with increases in rate of flow of the perfusion fluid resulted in increases in the rate of release of EDRF (e.g. Holtz *et al.*, 1984; Rubanyi *et al.*, 1986). Increase in flow through arteries both *in situ* (Holtz *et al.* 1984; Kaiser

and Sparks 1986; Pohl *et al.*, 1986) and *in vitro* produced vasodilation that could not be blocked by inhibitors of cyclooxygenase but was abolished by removal of the endothelium. These findings concerning increased EDRF release with increased shear stress were important for they provided evidence that EDRF has a continuous physiological role in the control of regional blood flow and regulation of blood pressure.

#### FINDINGS THAT LED TO THE IDENTIFICATION OF EDRF AS NO

While Billy Martin was working in my laboratory (1983-1985), I became interested in the work that he and others had done in John Gillespie's laboratory in Glasgow in trying to identify the neurotransmitter released from NANC (non-adrenergic, non-cholinergic) nerves that elicit relaxation of bovine retractor penis (BRP) muscle and certain other smooth muscle preparations. I learned that both relaxation produced by NANC nerve stimulation and relaxation produced by a partly purified extract made by Martin from the BRP muscle (referred to as BRP inhibitory factor or BRPIF) were both inhibited by Hb (Bowman *et al.*, 1982) and were both associated with increases in cGMP in the muscle (Bowman and Drummond, 1984). One important aspect of Martin's work with BRPIF extract was that it had to be acidified for a short period prior to testing in order for it to have a relaxing effect on smooth muscle preparations of retractor penis and arteries in organ baths (Gillespie *et al.*, 1981). Once activated by acidification, the BRPIF extract gave good relaxations of the smooth muscle preparations, but the relaxations were transient, lasting no more than a few minutes.

As discussed in the paper that I presented at a symposium in Rochester, MN, in July, 1986 (Furchgott, 1988), I was struck by the similarities of the transient relaxations produced by the acid-activated BRPIF extract of Martin and the transient relaxations of rabbit aorta produced by acidified solutions of sodium nitrite ( $\text{NaNO}_2$ ). That acidified, but not neutral solutions of  $\text{NaNO}_2$ , produce strong transient relaxations of rabbit aorta had been found about fifteen years earlier in my laboratory when a new postdoctoral fellow, assigned to obtain cumulative dose-response curves for  $\text{NaNO}_2$ , accidentally made his standard solutions of that salt with acidified diluting fluid (used in my laboratory for making stable standard solutions of catecholamines) rather than with neutral diluting fluid (Fig. 7). At that time, I became aware that the  $\text{HNO}_2$  ( $\text{pK}_a$  of 3.2) produced by acidification of  $\text{NaNO}_2$  solutions immediately generates low concentrations of NO and  $\text{NO}_2$  as a result of a reversible dismutation.



However, when a solution of acidified nitrite is added to the oxygenated, well-buffered physiological salt solution used in our organ chambers, any NO present will be removed very rapidly – thus accounting for the transiency of the relaxation of the aortic rings receiving acidified nitrite.

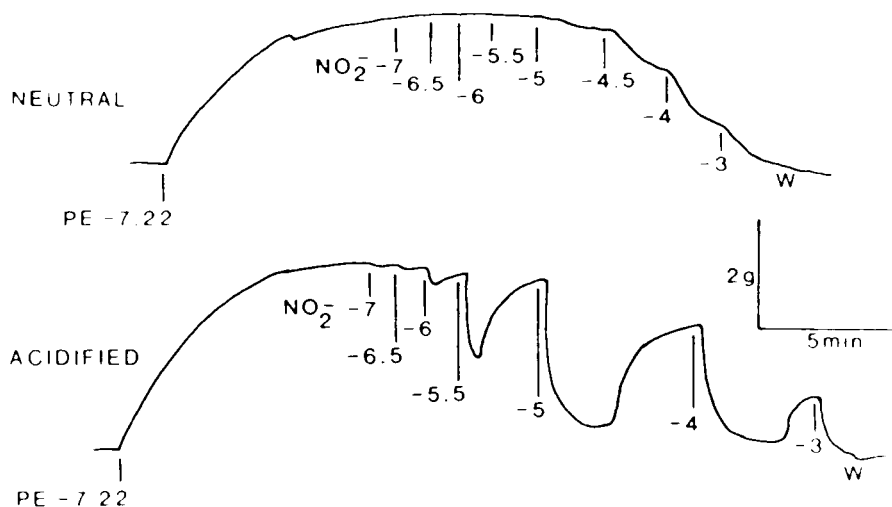


Figure 7. Comparison of the relaxing effects of cumulative additions of neutral and acidified solutions of sodium nitrite on rings of rabbit aorta. In this typical experiment, two rings from the same aorta after contraction with phenylephrine (PE), were exposed, respectively, to additions of  $\text{NaNO}_2$  diluted with neutral physiological saline and to additions of  $\text{NaNO}_2$  diluted with the saline acidified with 11 mM HCl. The concentrations shown are the final nitrite concentrations (log molar) in the organ chambers. Note the striking transient relaxations in the case of the acidified nitrite solutions. The rings in this experiment were denuded of endothelial cells, but results were essentially the same when endothelial cells were present. Reproduced with permission from Furchgott (1988).

In the spring of 1986, recalling the much earlier accidental finding in my laboratory with acidified sodium nitrite solutions, I began to consider the possibility that Martin's acid-activatable BRPIF was inorganic nitrite. An encouraging early finding was that the degree of transient relaxation as a function of pH of the added  $\text{NaNO}_2$  solution in the case of rabbit aorta was similar to that found by Martin for his added extract in the case of the BRP – maximal transient relaxation at a pH of 2 or lower, and approximately half-maximal at a pH of 3.

By the time I had begun to use acidified nitrite solutions as a source of NO in experiments with rabbit aorta in 1986, I had also become aware of the recent findings by Gryglewski, Palmer and Moncada (1986) and by Rubanyi and Vanhoutte (1986) demonstrating that the superoxide anion ( $\text{O}_2^-$ ) rapidly inactivates EDRF and that superoxide dismutase (SOD) protects against this inactivation. Assuming that the free radical NO might rapidly react with the free radical  $\text{O}_2^-$ , to form inactive  $\text{NO}_3^-$ , I began to speculate on the possibility that EDRF is NO. [Later, it was pointed out by Beckman *et al.* (1990) that the immediate product of the reaction between NO and  $\text{O}_2^-$  is peroxynitrite ( $\text{ONOO}^-$ ), which is protonated to  $\text{HONOO}$  ( $\text{pK}_a = 6.8$ ), which decays within seconds at physiological pH.]

My speculation in the spring of 1986 that EDRF might be NO led to a large number of experiments on rabbit aorta, carried out with the assistance of D. Jothianandan and M.T. Khan, comparing the characteristics of relaxation by

NO (present in acidified solutions of  $\text{NaNO}_2$ ) with the characteristics of relaxation by EDRF (released by ACh). It was found that relaxation by NO like that produced by EDRF was strongly inhibited by Hb, strongly inhibited by MB, strongly inhibited by superoxide generators (e.g., hydroquinone, phenidone, ferrous sulfate), and potentiated by SOD when superoxide was limiting the degree of relaxation (Fig. 8). It was already known that relaxation of

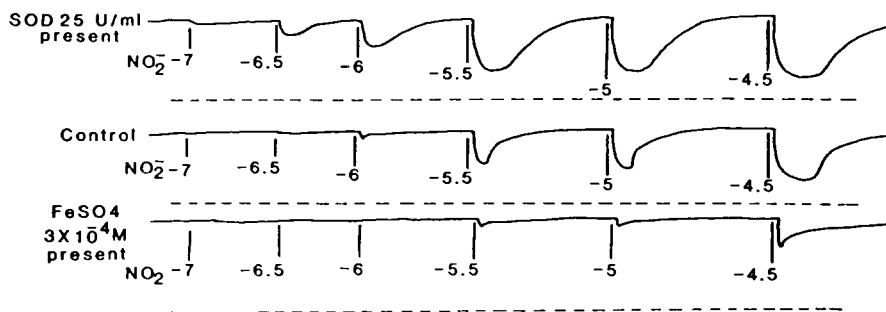


Figure 8. Transient relaxations of endothelium-free rings of rabbit aorta by nitric oxide present in acidified solutions (pH 2) of  $\text{NaNO}_2$ , potentiation of the relaxation by superoxide dismutase (SOD), and inhibition by  $\text{FeSO}_4$  (a superoxide generator). Three matched rings from one aorta were contracted above basal tone (dashed line) with phenylephrine. SOD and  $\text{FeSO}_4$  were added about five min before first additions of the acidified nitrite. Reproduced with permission from Furchgott (1988).

vascular smooth muscle by NO, like that by EDRF, is accompanied by an increase in cGMP. On finding the characteristics of NO and EDRF so similar, I had no hesitancy in proposing in the paper which I delivered at the July, 1986, symposium that EDRF is NO (Furchgott, 1988). [Shortly after the symposium, we began using solutions of NO gas rather than acidified nitrite as a source of NO in our experiments and obtained identical results (Fig. 9).]

In my symposium presentation, I also proposed that the acid-activatable inhibitory factor in the BRP extract of Martin and Gillespie is inorganic nitrite, and that the possibility had to be considered that the neurotransmitter of NANC nerves might also be NO (Furchgott, 1988).

At the same symposium at which I proposed that EDRF is NO, Ignarro, on the basis of studies in his laboratory on isolated bovine pulmonary arteries, independently made the same proposal (Ignarro *et al.*, 1988). (Unfortunately, the papers presented at that symposium were delayed in publication until 1988.) Soon after the 1986 symposium, three laboratories utilized perfusion-bioassay procedures to compare more accurately the biological and chemical characteristics of EDRF and NO. Ignarro and coworkers using bovine pulmonary vessels (Ignarro *et al.* 1987), Moncada and coworkers using cultured porcine aortic endothelial cells (Palmer *et al.*, 1987), and Khan and myself using rabbit aorta (Khan and Furchgott, 1987) as a source of EDRF, all found EDRF released upstream and NO infused upstream to have similar rates of decay, similar susceptibility to inhibitors like Hb and superoxide generators, similar stabilization by SOD, etc. In addition, Ignarro's laboratory presented

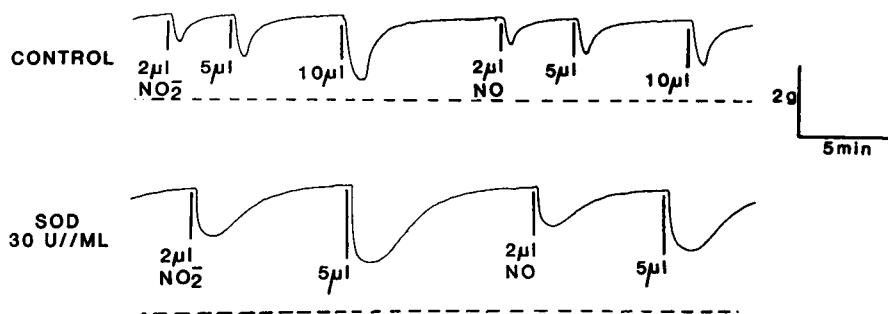


Figure 9. Similarity of the relaxing effects of a solution of acidified sodium nitrite and a solution of nitric oxide gas on rings of rabbit aorta in the absence and presence of superoxide dismutase (SOD). The acidified nitrite was 2.5 mM at pH 2.0. The NO solution was 15  $\mu$ M. (See Furchgott *et al.* (1990a) for method for making solutions of NO.) Additions were to 20 ml of Krebs solution in each organ chamber, so that 2  $\mu$ l of the NO solution gave an initial concentration of 1.5 nM. Both rings were precontracted with phenylephrine. The potentiation by SOD of the transient relaxations produced by the NO in the acidified nitrite and in the NO solution indicates that there was a considerable endogenous production of the superoxide anion  $O_2^-$  in the organ chambers.

spectroscopic evidence that the product of the reaction of released EDRF and Hb was the same as that of NO and Hb, and Moncada's laboratory showed that the amount of NO released by bradykinin (as determined by a chemiluminescence assay) could fully account for the relaxation of the bioassay strip by the EDRF released by the peptide. A year later, Moncada and coworkers made the major finding that the source of endothelial NO was a guanidinium nitrogen of L-arginine and that the enzyme responsible for its formation was an oxygenase (now called endothelial nitric oxide synthase or eNOS) (Palmer *et al.*, 1988). These findings marked the beginning of a major worldwide expansion of research on the role of NO in vascular physiology and pathophysiology.

Despite the evidence for the identity of EDRF and NO, some results obtained in various laboratories in the late 1980s did not appear to be completely consistent with that conclusion. [For a review of these apparent or real inconsistencies, see Furchgott *et al.*, 1990a.] Nevertheless, as of now, it has become generally accepted that EDRF is either NO, or some adduct that readily releases NO, or perhaps a mixture of NO and some adduct of NO. This does not preclude a contribution to endothelium-dependent relaxation in some blood vessels from the release of a non-nitric oxide endothelium-derived hyperpolarizing factor (EDHF) along with EDRF (for review see Garland *et al.*, 1995).

#### ACKNOWLEDGEMENT

The research in the author's laboratory referred to in this article was supported by grants from the National Heart, Lung, and Blood Institute Of the National Institutes of Health.

## REFERENCES

- Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620–1624.
- Böhme, E., Graf, H. and Schultz, G. (1978) Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. *Adv. Cyclic Nucleotide Res.* **9**, 131–143.
- Bowman, A., and Drummond, A.H. (1984) Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle. *Br. J. Pharmacol.*, **81**, 665–674.
- Bowman, A., Gillespie, J.S., and Martin, W. (1981) Actions on the cardiovascular system of an inhibitory material extracted from the bovine retractor penis. *Br. J. Pharmacol.* **72**, 365–372.
- Bowman, A., Gillespie, J.S., and Pollock, D. (1982) Oxyhaemoglobin blocks nonadrenergic inhibition in the bovine retractor penis muscle. *Eur. J. Pharmacol.* **85**, 221–224.
- Cherry, P.D., Furchgott, R.F., Zawadzki, J.V., and Jothianandan, D. (1982) The role of endothelial cells in the relaxation of isolated arteries by bradykinin. *Proc. Nat. Acad. Sci.* **79**, 2106–2110.
- Cocks, T.M. and Angus, J.A. (1983) Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* **305**, 627–630.
- Cocks, T.M., Angus, J.A., Campbell, J.H. and Campbell, G.R. (1985) Release and properties of endothelium-derived relaxing factor (EDRF) from endothelial cells in culture. *J. Cell Physiol.* **123**, 310–320.
- Cohen, R.A., Shepherd, J.T. and Vanhoutte, P.M. (1983) 5-Hydroxytryptamine can mediate endothelium-dependent relaxation of coronary arteries. *Am. J. Physiol.* **245**, H1077–1080.
- Diamond, J. and Chu, E.B. (1983) Possible role for cyclic GMP in endothelium-dependent relaxation of rabbit aorta by ACh. Comparison with nitroglycerin. *Res. Comm. Chem. Pathol. Pharmacol.* **41**, 369–381.
- Doyle, M.P., and Hoekstra, J.W. (1981) Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **14**, 351–358.
- Forstermann, U., Trogisch, G. and Busse, R. (1984) Species-dependent differences in the value of endothelium-derived vascular relaxing factor. *Eur. J. Pharmacol.* **106**, 639–643.
- Furchgott, R.F. (1954) Dibenamine blockade in strips of rabbit aorta and its use in differentiating receptors. *J. Pharmacol. Exp. Ther.* **111**, 265–284.
- Furchgott, R.F. (1981) The requirement for endothelial cells in the relaxation of arteries by ACh and some other vasodilators. *Trends Pharmacol. Sci.* **2**, 173–176.
- Furchgott, R.F. (1983) Role of endothelium in the responses of vascular smooth muscle. *Circ. Res.* **53**, 557–573.
- Furchgott, R.F. (1988) Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: *Vasodilatation: Vascular Smooth Muscle, Peptides, and Endothelium*, edited by P.M. Vanhoutte, pp. 401–414, Raven Press, New York.
- Furchgott, R.F. (1990) The 1989 Ulf von Euler Lecture: Studies on endothelium-dependent vasodilation and the endothelium-derived relaxing factor. *Acta Physiol. Scand.* **139**, 257–270.
- Furchgott, R.F. (1993) The discovery of endothelium-dependent relaxation. *Circulation* **87**(Suppl V) V3–V8.
- Furchgott, R.F. (1996) The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *J.A.M.A.* **276**, 1186–1188.
- Furchgott, R.F. and Bhadrakom, S. (1953) Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J. Pharmacol. Exp. Therap.*, **108**, 129–143.

- Furchgott, R.F. and Vanhoutte, P.M. (1989) Endothelium-derived relaxing and contracting factors. *FASEB J.* **3**, 2007–2018.
- Furchgott, R.F., and Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by ACh. *Nature* **288**, 373–376.
- Furchgott, R.F., Zawadzki, J.V., and Cherry, P.D. (1981) Role of endothelium in the vasodilator response to ACh. In: *Vasodilatation*, edited by P. Vanhoutte and I. Leusen, pp. 49–66. Raven Press, New York.
- Furchgott, R.F., Cherry, P.D., Zawadzki, J.V., Jothianandan, D. (1984) Endothelial cells as mediators of vasodilation of arteries. *J. Cardiovasc. Pharmacol.* **6**, Suppl. 2, S336–S344.
- Furchgott, R.F., Jothianandan, D. and Freay, A.D. (1990b) Endothelium-derived relaxing factor: some old and new findings. In *Nitric Oxide from L-Arginine: A Bioregulatory System*, edited by S. Moncada and E.A. Higgs, pp. 5–17, Elsevier, Amsterdam.
- Furchgott, R.F., Khan, M.T. and Jothianandan, D. (1990a) Comparison of properties of nitric oxide and endothelium-derived relaxing factor: Some cautionary findings. In *Endothelium-derived Relaxing Factors*, edited by G.M. Rubanyi and P.M. Vanhoutte, pp. 8–21, Karger, Basel.
- Garland, C.J., Plane, F., Kemp, B.K., and Cocks, T.M. (1995) Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol. Sci.* **16**, 23–30.
- Gillespie, J.S., Hunter, J.C., and Martin, W. (1981) Some physical and chemical properties of the smooth muscle inhibitory factor in extracts of the bovine retractor penis muscle. *J. Physiol.* **315**, 111–125.
- Goldberg, N.D., Graff, G., Haddox, M.K., Stephenson, J.H., Glass, D.B., and Moser, M.E. (1978) Redox modulation of splenic cell soluble guanylate cyclase activity: Activation by hydrophilic and hydrophobic antioxidants represented by ascorbic and dehydroascorbic acids, fatty acid hydroperoxides, and prostaglandin endoperoxides. *Adv. Cyclic Nucleotide Res.* **9**, 101–130.
- Griffith, T.M., Edwards, D.H., Newby, A.C., Lewis, M.J., Newby, A.C. and Henderson, A.H. (1984) The nature of the endothelium-derived vascular relaxant factor. *Nature* **305**, 645–647.
- Gruetter, C.A., Barry, B.K., McNamara, D.B., Gruetter, D.Y., Kadowitz, P.J. and Ignarro, L.J. (1979) Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and carcinogenic nitrosoamine. *J. Cyclic Nucleotide Res.* **5**, 211–214.
- Gruetter, C.A., Gruetter, D.Y., Lyon, J.E., Kadowitz, P.J. and Ignarro, E.F. (1981) Relationship between cyclic guanosine 3',5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: Effects of methylene blue and methemoglobin. *J. Pharmacol. Exp. Ther.* **219**, 181–186.
- Gryglewski, P.J., Palmer, R.M. and Moncada, S.A. (1986) Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature* **320**, 454–456.
- Hidaka, H., and Asano, T. (1977) Stimulation of platelet guanylate cyclase by unsaturated fatty acid peroxides. *Proc. Natl. Acad. Sci. USA* **74**, 3657–3661.
- Holtz, J., Förstermann, U., Pohl, U., Giesler, M., and Bassenge, E. (1984) Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. *J. Cardiovasc. Pharmacol.* **6**, 1161–1169.
- Holzmann, S. (1982) Endothelium-induced relaxation by ACh associated with larger rises in cyclic GMP in coronary arterial strips. *J. Cyclic Nucleotide Res.* **8**, 409–419.
- Ignarro, L.J., Burke, T.M., Wood, K.S., Wolin, M.S., Kadowitz, P.J. (1984) Association between cyclic GMP accumulation and ACh-elicited relaxation of bovine intrapulmonary artery. *J. Pharmacol. Exp. Ther.* **228**, 682–690.
- Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9265–9269.
- Ignarro, L.J., Byrns, R.E. and Wood, K.S. (1988) Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radi-



- cal. In: *Vasodilatation: Vascular Smooth Muscle, Peptides, and Endothelium*, edited by P.M. Vanhoutte, pp. 427–435, Raven press, new York.
- Jelliffe, R.W. (1962) Dilator and constrictor effects of acetylcholine on isolated rabbit aortic chains. *J. Pharmacol. Exp. Ther.* **135**, 349–353.
- Kaiser, L., and Sparks, H.V., Jr. (1986) Mediation of flow-dependent arterial dilation by endothelial cells. *Circulatory Shock* **18**, 109–114.
- Katsuki, S., and Murad, F. (1977) Regulation of adenosine cyclic 3',5'-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol. Pharmacol.* **13**, 330–341.
- Khan, M.T. and Furchgott, R.F. (1987) Additional evidence that endothelium-derived relaxing factor is nitric oxide. In: *Pharmacology*, edited by M.J. Rand and C. Raper, pp. 341–344 Elsevier, Amsterdam.
- Martin, W., Villani, G.M., Jothianandan, D. and Furchgott, R.F. (1985a) 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.
- Martin, W., Villani, G.M., Jothianandan, D. and Furchgott, R.F. (1985b) Blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation of rabbit aorta by certain ferrous hemoproteins. *J. Pharmacol. Exp. Ther.* **233**, 679–685.
- Martin, W., Furchgott, R.F., Villani, G.M., and Jothianandan, D. (1986a) Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor (EDRF). *J. Pharmacol. Exp. Ther.* **237**, 529–538.
- Martin, W., Furchgott, R.F., Villani, G.M. and Jothianandan, D. (1986b) Phosphodiesterase inhibitors induce endothelium-dependent relaxation of rat and rabbit aorta by potentiating the effects of spontaneously released endothelium-derived relaxing factor (EDRF). *J. Pharmacol. Exp. Ther.* **237**, 539–547.
- Moncada, S., Palmer, R.M.J. and Gryglewski, R.J. (1986) Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9164–9168.
- Murad, F., Mittal, C.K., Arnold, W.P., Katsuki, S., Kimura, H. (1978) 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.
- Murad, F., Arnold, W.P., Mittal, C.K., Braughler, J.M. (1979) Properties and regulation of guanylate cyclase and some proposed functions of cyclic GMP. *Adv. Cyclic Nucleotide Res.* **11**: 175–204.
- Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature (Lond.)* **327**, 524–526.
- Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988a) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**, 664–666.
- Pohl, U., Holtz, J., Busse, R., Bassenge, E. (1986) Crucial role of endothelium in the vasodilator response to increased flow *in vivo*. *Hypertension* **8**, 37–44.
- Rapoport, R.M. and Murad, F. (1983) Agonist-induced endothelium-dependent relaxations in rat thoracic aorta may be mediated through cGMP. *Circ. Res.* **52**, 352–357.
- Rubanyi, G.M. and Vanhoutte, P.M. (1986) Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.* **250**, H822–H827.
- Rubanyi, G.M., Lorenz, R.R. and Vanhoutte, P.M. (1985) Bioassay of endothelium-derived relaxing factor. *Am. J. Physiol.* **251**, H1077–H1080.
- Rubanyi, G.M., Romero, J.C., Vanhoutte, P.M. (1986) Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.* **250**, H1145–H1149.
- Steinsland, O.S., Furchgott, R.F., Kirpekar, S.M. (1973) Inhibition of adrenergic neurotransmission by parasympathomimetics in the rabbit ear artery. *J. Pharmacol. Exp. Ther.* **184**, 346–356.
- Toda, N. (1974) The action of vasodilating drugs on isolated basilar, coronary and mesenteric arteries of the dog. *J. Pharmacol. Exp. Ther.* **193**, 376–384.
- Van de Voorde, J. and Leusen, I. (1983) Role of endothelium in the vasodilator responses of rat thoracic aorta to histamine. *Eur. J. Pharmacol.* **87**, 113–120.

- Vanhoutte, P.M. (1974) Inhibition by acetylcholine of adrenergic neurotransmission in vascular smooth muscle. *Circ. Res.* **34**, 317–326.
- Wolin, M.S., Cherry, P.D., Rodenburg, J.M., Messina, E.J., and Kaley, G. (1990) Methylene blue inhibits vasodilation of skeletal muscle arterioles to ACh and nitric oxide via the extracellular generation of superoxide anion. *J. Pharmacol. Exp. Ther.* **254**, 872–876.