Endothelium-Derived Vascular Relaxing Factor
MICHAEL J. PEACH, ALEX L. LOEB, HAROLD A. SINGER, AND JOANNE SAYE

SUMMARY A large number and variety of compounds (acetylcholine, adenosine diphosphate, adenosine triphosphate, arachidonic acid, bradykinin, Ca2+ ionophores, calcitonin gene-related peptide, histamine, hydralazine, substance P, thrombin, and vasoactive intestinal polypeptide) have been shown to relax arterial smooth muscle indirectly. The endothelium in muscular arteries from several species appears to have receptors for these vasodilators. Binding of one of these compounds to its endothelial receptors results in the release (and presumably synthesis) of substance(s) that act on arterial smooth muscle to cause relaxation. The name endothelium-derived relaxing factor (EDRF) has been proposed for the substance or substances responsible for inhibition of contraction. Studies to determine additivity of endothelium-dependent relaxing agents and sensitivity of EDRF-mediated responses to a variety of inhibitors suggest that a single factor or a single common mechanism induces relaxation of vascular smooth muscle. Pharmacological studies have been equivocal with regard to the postulated involvement of phospholipases or arachidonic acid and to the suggestion that EDRF is an oxidative, noncycloxygenase product of arachidonate. Experiments on transfer of EDRF and reversal of endothelium-dependent relaxation consistently indicate that EDRF is quite labile. There is convincing evidence that EDRF activates smooth muscle guanylate cyclase, which results in an increase in intracellular cyclic guanosine 3',5'-monophosphate levels. The stimulation of guanylate cyclase by EDRF provides a valuable and sensitive parameter for studies with arteries as well as cells in culture. At present, the identity of EDRF and its role in cardiovascular homeostasis are unknown. (Hypertension 7 [Suppl I]: I-94-I-100, 1985)

KEY WORDS • eicosanoid • cell culture • arachidonic acid • phospholipase • antioxidants • vascular smooth muscle • guanylate cyclase • cyclic GMP

ONE of the first reports of endothelium-dependent vasodilation was published in 1980 and addressed the vascular responses to acetylcholine (Ach). In preparations with intact endothelium, low concentrations of Ach (10^-8-10^-6 M) were found to relax precontracted rabbit aorta, while higher concentrations (>10^-4 M) exerted direct contractile effects independent of the presence of endothelium.1 It was also postulated that the endothelium released a factor that acted on arterial smooth muscle to promote relaxation of muscle tone. Evidence for transfer of a relaxing factor from endothelium to muscle was obtained by placing the intima of an intact piece of aorta against the lumenal surface of a bioassay aortic strip with no endothelium, which demonstrated that dilation in response to Ach necessitated the donor endothelium. Prostacyclin was an immediate candidate for the factor; however, endothelium-dependent vasodilation was obtained in arteries (i.e., rabbit thoracic aorta) that do not respond to prostacyclin and the response was not altered by treatment with cyclooxygenase inhibitors (nonsteroidal antiinflammatory agents).1 The term endothelium-derived relaxing factor (EDRF) was proposed for the substance that induces smooth muscle relaxation.2

Since the initial reports, numerous investigators have confirmed that the endothelium is requisite for cholinergic-induced vasodilation in a number of arterial preparations and an extensive list of other endothelium-dependent vasoactive substances has been generated (for reviews, see refs. 2, 3, and Table 1). The list of vasodilators includes potential physiological dilators such as Ach, adenosine diphosphate, adenosine triphosphate (ATP), bradykinin, substance P, thrombin, calcitonin gene-related peptide and histamine, as well as pharmacological vasodilators such as high concentrations of arachidonic acid, other saturated and unsaturated fatty acids, hydralazine, A23187, and melitinin (see Table 1 for references). The capacity to produce EDRF has been demonstrated in numerous species including humans (Table 1). Production and release appears to be a property primarily of arterial endothelium, although it has been documented in some venous preparations.11 Most of the arterial preparations studied have been conduit arteries, though some work.
has been done in vascular beds, which suggests that resistance vessels also may produce or respond to EDRF. Despite the seemingly universal nature of the factor, a given agent does not necessarily relax all arterial preparations (see Table 1). In the absence of any direct evidence, this finding probably reflects the endothelium receptor complement for various vasodilators. In addition, not all cholinergic vasodilation may be endothelium dependent; Brayden and Bevan have reported the existence of direct muscarinic neurogenic vasodilation in the postauricular artery in the cat.

A variety of techniques has been used to remove the endothelium, with success verified by silver staining followed by light microscopy or by scanning transmission electron microscopy. These techniques encompass 1) mechanical or physical (e.g., rubbing, scraping, insertion of suture or swab through the lumen, or balloon catheterization), 2) enzymatic, and 3) chemical (perfusion with hypertonic medium or exposure to p-bromophenacyl bromide methods. When harsh measures are used to remove the endothelium, several layers of smooth muscle cells or much of the medial layer may also be damaged and alter responses to vasoconstrictors as well as endothelium-independent vasodilators.

Involvement of Arachidonic Acid

Among the first observations regarding endothelium-dependent vasodilation, relaxation by i.c.osatsrayenoic acid (ETYA), an inhibitor of cyclooxygenase and lipoxygenase, and quinacrine, a phospholipase inhibitor. However, there was no inhibitory effect with cyclooxygenase inhibitors such as indomethacin. These basic observations have been confirmed several times in rabbit aorta and similar observations have been made in other species and preparations (see Table 1). The apparent lability of EDRF and the reversal of the relaxation response by anoxia, directed considerable attention toward arachidonic acid and non-cyclooxygenase oxidative products of arachidonate as potential mediators of endothelium-dependent relaxation. Another phospholipase inhibitor, p-bromophenacyl bromide, and several reported lipoxygenase inhibitors such as BW755C, nordihydroguararetic acid (NDGA), nafazatrom, and phenidone have also been shown to inhibit the endothelium-dependent relaxation in rabbit aorta. Conversely, others have reported that BW755C and p-bromophenacyl bromide are ineffective inhibitors.

Other indirect evidence consistent with the hypothesis of phospholipase activation with subsequent release and metabolism of arachidonate to yield an active EDRF relates to the Ca2+ dependence of the relaxation response. The calcium ionophore A23187 has been shown to be the most efficacious of the endothelium-dependent agents on rabbit aorta. Endothelium-dependent relaxation by methacholine (Mch) or A23187 is markedly attenuated by removing Ca2+ in the bathing medium or adding calcium channel blockers (verapamil, nifedipine, SKF525A). In addition to A23187 and muscarinic agonists, other endothelium-dependent vasodilators such as adenosine nucleotides, bradykinin, norepinephrine, and histamine have been shown to be coupled to Ca2+ in other systems. Bradykinin, A23187 and thrombin have been shown to stimulate phospholipase and arachidonic acid release from cultured fibroblasts in a Ca2+-dependent manner. Thus, receptor-mediated increases in intracellular Ca2+ are most likely the initial step in the synthesis and release of EDRF.

As with other tissues, there is indirect evidence that the Ca2+ signal may be coupled to phospholipase activation in endothelium. Although prostacyclin is not EDRF, there is evidence that prostacyclin and other cyclooxygenase products of arachidonic acid are produced and released in several arterial preparations in response to endothelium-dependent agonists, which indicates the activation of phospholipases and release of the substrate arachidonic acid. Recently, the phospholipase activator melittin has been shown to stimulate prostacyclin-independent, endothelium-dependent relaxation in rabbit aorta (Peach MJ, Loeb AL, unpublished observations, 1985) and prostacyclin release from cultured endothelium. Melittin-induced relaxation in rabbit aorta was blocked by treatment with NDGA (Peach MJ, Loeb AL, unpublished observations, 1985).

Exogenous arachidonate can induce endothelium-dependent vasodilation. Obviously, the arachidonate may be oxidized to prostacyclin and cause relaxation in any artery that responds to this eicosanoid. However, formation of cyclooxygenase products can be prevented by pretreatment with indomethacin or aspirin. In rabbit and rat aorta, arachidonic acid induces endothelium-dependent dilation and indomethacin potentiates the relaxation response, probably by blocking the generation of contractile prostanoids. Pretreatment with, but not acute administration of, ETYA or NDGA or both blocks arachidonate-induced, endothelium-dependent relaxation. The arachidonate response was not altered by treatment with "Ca2+" buffer, nifedipine, or quinacrine, compounds whose blocking action presumably occurs before phospholipase activation, which would prevent release of EDRF by arachidonate. (Singer HA, Peach MJ, unpublished observations, 1982) These findings suggest that oxidation of arachidonate to a non-cyclooxygenase vasodilator product occurs in endothelium.

Limited studies on the vasoactivity of lipoxygenase products have indicated that in general they are contractile agonists. Recently, it was reported that several hydroxylated eicosatetraenoic acids and C-6-sulfidopeptide leukotrienes, which are also lipoxygenase products, could not mimic the actions of EDRF in rabbit aorta. This evidence does not, of course, rule out the existence of as yet undiscovered labile lipoxygenase metabolites with vasodilator activity.

Arachidonic acid can also be oxidized by cytochrome P-450 monooxygenases in endothelium to epoxides, as has been shown in liver and kidney. There is evidence that cytochrome P-450 may...
Table 1  Characterization of Endothelium-Dependent Vasodilators

<table>
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<tr>
<th>Vasodilator</th>
<th>Species</th>
<th>Vessel</th>
<th>Endo requirement</th>
<th>Indomethacin</th>
<th>ETYA</th>
<th>Quinacrine</th>
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- 8
- 10
- 11
- 15
- 16
- 17
- 18
- 19
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- 21
- 22
- 23
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- 25
- 26
Other saturated and unsaturated fatty acids

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<th>Vasodilator</th>
<th>Species</th>
<th>Vessel</th>
<th>Endo requirement</th>
<th>Indomethacin</th>
<th>ETYA</th>
<th>Quinacrine</th>
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Blanks indicate data unknown

Endo = endothelium, ETYA = eicosatetrayenoic acid

be present in vascular endothelium,\textsuperscript{11,13} and, using cytochrome P-450 inhibitors, we have provided indirect evidence that such a pathway could be active in the production of an EDRF.\textsuperscript{19} Validation of the pathway requires more direct evidence regarding the activity of cytochrome P-450 and its ability to metabolize arachidonic in preparations shown to have endothelium-dependent relaxation responses.

Despite the evidence summarized above, the role of phospholipase activation and arachidonic acid release in the production and release of EDRF is difficult to assess with certainty. Activation of phospholipase with melittin results in endothelium-dependent relaxation in rabbit thoracic aorta (Peach MJ, Loeb AL, unpublished results, 1985).\textsuperscript{26} Phospholipase inhibitors such as quinacrine and p-bromophenacylbromide block or reverse vasodilation in response to melittin\textsuperscript{26} and Ach\textsuperscript{13,26} however, quinacrine does not antagonize the response to ATP, Bradykinin, or A23187 in rabbit aorta or to ATP and thrombin in canine femoral artery.\textsuperscript{13}

Glucocorticoid pretreatment, which has been shown to inhibit phospholipase in other cellular systems,\textsuperscript{13} has not been reported to alter responses to endothelium-dependent vasodilators. In rats treated with dexamethasone for 7 days, we found normal vasodilator response to Mch (Peach MJ, Singer HA, unpublished observation). In addition to arachidonic acid, several other unsaturated and saturated fatty acids (but not ETYA) also promote endothelium-dependent responses,\textsuperscript{13} and it has been suggested that these lipids (including arachidone) are not precursors but rather promote the synthesis (or release) of EDRF.\textsuperscript{1} It is possible that multiple pathways for EDRF formation exist or that multiple EDRFs exist,\textsuperscript{1} and this could explain the contradictory evidence regarding involvement of phospholipases and arachidonic acid in the endothelium-dependent response. It is also possible that the pharmacological evidence is misleading and that arachidonic acid is not directly involved in the relaxation responses. Strictly speaking, the endothelium is known to release the vasodilators prostacyclin\textsuperscript{1,21,32} and platelet-activating factor,\textsuperscript{56} both of which should be considered known EDRFs.

Maximal doses of the various endothelium-dependent dilators are not additive to each other or to the Ca\textsuperscript{2+} ionophore A23187,\textsuperscript{57} which has the greatest maximum efficacy of all the compounds that induce EDRF release.\textsuperscript{22,55} The response to A23187 is additive to that obtained with a maximum concentration of Ach or ATP, but only to the level of maximal relaxation induced by A23187 alone.\textsuperscript{55} These addition studies are consistent with release of either a common factor or multiple factors with a common mechanism of action. The varied maximum efficacies for relaxation among the dilator substances that act through the endothelium probably reflect the magnitude of receptor-mediated Ca\textsuperscript{2+} influx and therefore the amount of EDRF produced (or released). The efficacy of A23187 probably is determined by the maximum rate at which EDRF can be released or reflects the ultimate capacity of EDRF to induce smooth muscle relaxation.

Antioxidants and nonspecific radical scavengers (e.g., dithiothreitol, cysteine, phenidone, tetrahydroborate, phenylhydrazine, hydroquinone, resorcinol, butylated hydroxytoluene, α-tocopherols) inhibit endothelium-dependent relaxation.\textsuperscript{6,8,18-22,25,26} Coupled with the ETYA and NDGA data, this finding constituted the basis for the postulate that EDRF is a hydroperoxy fatty acid or a hydroxy fatty acid.\textsuperscript{14} Recently, Griffith et al.\textsuperscript{26} reported data from a series of superfusion transfer experiments that bring into question the actual mechanism for the inhibitory action of NDGA and other lipoygenase inhibitors with antioxidant activity. These investigators found that several antioxidant or free radical scavenger compounds (cysteine, dithiothreitol, phenylhydrazine, hydroquinone, phenidone, and NDGA) inactivated EDRF in solution and did not need to act on the endothelium to inhibit endothelium-dependent relaxation. Their studies involved perfusion of a large (7-8 cm) segment of intact rabbit aorta that was connected by a cannula to a bioassay coronary artery denuded of endothelium. By varying the distance for transfer between donor endothelium and bioassay ring, they estimated a half-life for EDRF in solution of 6.3 ± 0.6 seconds. Drugs were introduced into the perfusate before or after the aortic segment. Agents such as NDGA and phenidone blocked the EDRF response even when added after the endothelial source (aorta) The donor endothelium, therefore, was not exposed to these compounds. The series of effective blockers of EDRF identified in this study suggests that EDRF contains a highly reactive carbonyl group (ketone, aldehyde, or lactone). Griffith et al.\textsuperscript{5} proposed that the blocking agents interfered with this chemical group and enhanced the lability of EDRF in solution. These agents do inactivate EDRF, but it is unlikely that carbonyl function is involved. Although ascorbate\textsuperscript{4} and metabsulfitite (Peach MJ, unpublished observation, 1984) have not been shown to inhibit EDRF,
these antioxidants have been found to impair the inhibitory actions of hydroquinone on EDRF-induced relaxation (Peach MJ, unpublished observation, 1984). Therefore, ascorbate and metabisulfite may impair an oxidative step that must occur before any reaction between EDRF and compounds such as hydroquinone or resorcinol takes place. Such a requisite oxidative step(s) would argue against a straightforward quinone or resorcinol takes place. Such a requisite oxidative step(s) would argue against a straightforward reaction.

Hemoglobin also has been shown to attenuate EDRF-dependent responses. Furchgott et al. suggested that because of its size this heme-containing protein can sequester or inactivate EDRF in solution. Several of these antioxidants and radical scavengers also may inhibit guanylate cyclase activity, which would result in attenuation of relaxation induced by EDRF.

### Cyclic GMP and Endothelium-Derived Relaxing Factor Induced Vascular Smooth Muscle Relaxation

In 1983, Rapoport and Murad reported that the accumulation of cyclic guanosine 3',5'-monophosphate (cGMP) in rat aorta in response to ACh, histamine, and A23187 was dependent on the endothelium. The increase in aortic cyclic GMP levels with sodium nitroprusside or nitroglycerin had no endothelial dependence. Other laboratories have confirmed that a variety of endothelium-dependent dilators in other species and arteries stimulate guanylate cyclase in smooth muscle. Thus, the EDRF released in an intact vessel acts on muscle and cyclic GMP levels increase.

Regardless of the controversy over the precise role of cyclic GMP in smooth muscle relaxation, the exposure of smooth muscle to EDRF is reflected by the change in cyclic GMP concentration. Cyclic GMP accumulation therefore represents an important biochemical parameter that reflects the interaction of EDRF with muscle and can be readily exploited in tissue and cell culture studies.

### Endothelium-Derived Relaxing Factor in Cultured Cells

With cultured endothelium, no specific binding of the muscarinic radioligand [3H]QNB occurs and no detectable vasodilator substance is released into the medium during exposure to Ach; however, rabbit aortic endothelium, either freshly obtained by adhesion to glass coverslips or removed after exposure of intact aortic rings to [3H]QNB, demonstrates atropine sensitive (specific), saturable binding of the radioligand (Peach MJ, unpublished observation, 1984). Therefore, the muscarinic receptor appears to be extremely labile during isolation of endothelial cells, and this receptor is not phenotypically expressed under conditions of endothelial cell culture. In contrast, other receptor types (bradykinin, ATP, adenosine diphosphate) are expressed by cultured endothelium from bovine and porcine aorta. These receptors are coupled to induce Ca2+ flux (Ca2+ influx), K+ efflux, and quin 2 fluorescence, and to activate phospholipase A2 (release of arachidonate and prostacyclin). In spite of the presence of functional receptors, transfer experiments from cultured endothelium to cultured smooth muscle or to an arterial ring (strip) without endothelium have failed to demonstrate release of EDRF in response to bradykinin, ATP, and Ach. However, large concentrations of A23187 have been shown to release substances from cultured cells. When endothelial cells are grown on carrier beads, large numbers of cells can be readily placed in a column and superfused. In response to A23187, column superfused cells appear to release prostacyclin, ATP, and possibly other compounds, one of which may be EDRF.

Recently, we have begun these types of studies using cultured pig aortic endothelium and vascular smooth muscle. In monolayer cultures of porcine aortic endothelium or aortic smooth muscle, Ach did not stimulate cyclic GMP accumulation. However, the cultured muscle cells responded to 10^{-6} M sodium nitroprusside with a fivefold increase in cyclic GMP. The cultured porcine aortic smooth muscle cells do have receptors for Ach ([3H]QNB binding) and undergo a shape change (contraction) in response to hormones and neurotransmitters; however, cyclic GMP levels did not change in response to muscarinic agonists. When the two cell types were cultured together and then exposed to either ATP or methacholine, we observed an increase in cyclic GMP levels similar to that seen in aortic ring preparations. Thus, it appears that the phenotypic expression of EDRF synthesis and release — certainly the presence of muscarinic receptor on endothelium and of the EDRF receptor on muscle cells — requires the presence of both cell types in the culture. The addition of endothelial cells to a monolayer culture of smooth muscle for 3 days resulted in a culture that also responded to a cholinergic agonist with an increase in cyclic GMP levels. The rise in cyclic GMP induced by a muscarinic compound could be completely blocked by the presence of ETYA, which suggests EDRF stimulation of guanylate cyclase (Figure 1). These recent successes with cultured endothelial cells and EDRF release hold great promise for production of large quantities of the factor for isolation and chemical characterization. With the cultured cells, sustained production by large amounts of endothelium should enhance attempts to trap EDRF with columns (C_{18}, ion exchange, hemoglobin Sepharose) or solvents.

Although the identity of EDRF remains unknown, it is clear that the endothelium modulates the ability of arterial smooth muscle to generate and sustain tone by releasing a relaxing factor and that the endothelial cell has receptors for a large number of vasoactive substances. Changes in any of these endothelial receptors or in the capacity of the endothelium to produce and release EDRF or in smooth muscle responsiveness to EDRF could play a major role in diabetic vascular disease, hypertension, vascular spasms, atherosclerosis, or other types of vascular pathophysiology.
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Figure 1 Evidence for endothelium-derived relaxing factor mediated cyclic GMP (cGMP) elevation in cultured cells. Homogeneous aortic smooth muscle cultures at confluence had homogeneous endothelial cells plated on top. After 5 days of coculture, some dishes were pretreated with eicosatrayenoic acid (ETYA, I x 10^-4 M) for 10 minutes. Thirty seconds after the addition of methacholine (Mch) (I x 10^-5 M) the medium was removed and replaced by 0.1 N HCl. After extraction in HCI, cyclic GMP was acetylated and quantified by radioimmunoassay (New England Nuclear, Boston, MA, USA). Protein concentration was determined by the protein dye binding method (Bio-Rad Laboratories, Richmond, CA, USA). The cyclic GMP levels with ETYA alone were not different from those of the control (data not shown). When endothelium and muscle were combined, Mch induced an increase in cyclic GMP concentration that was prevented by pretreatment with ETYA.
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