Endothelium

Lead Article

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The endothelium: a modulator of cardiovascular health and disease

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The endothelial cells lining the luminal surface of blood vessels are involved in numerous regulatory functions, such as control of vascular smooth muscle cell proliferation, adhesion of leukocytes and platelets, permeability, and inflammatory responses.
The endothelium also has thrombolytic and fibrinolytic properties. Its metabolic activity contributes to the regulation of the oxidation of plasma lipids, angiotensin II formation, and the degradation of circulating catecholamines and kinins. In addition, the endothelium plays an important role in the regulation of vascular smooth muscle tone by releasing both relaxing and contracting factors. Endothelium-dependent relaxations are mediated primarily by nitric oxide, but also by endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin. The contracting factors are endothelin-1, metabolites from the cyclooxygenase pathway, and superoxide anions.
Under physiological conditions, a precise and balanced release of relaxing and contracting factors ensures adequate organ perfusion. However, this balance is altered in disease states such as atherosclerosis, diabetes, chronic heart failure, coronary artery disease, or hypertension, thereby contributing to the further development of vascular diseases.

Keywords: nitric oxide; endothelium-dependent hyperpolarization; endothelin; thromboxane; hypertension; atherosclerosis; endothelial dysfunction

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The endothelial cells that line the luminal surface of all blood vessels constitute an important organ. The endothelium is involved in numerous regulatory functions, such as the control of vascular smooth muscle proliferation, leukocyte and platelet adhesion, vascular permeability, and inflammation. It also has thrombolytic and fibrinolytic properties. In addition, its metabolic activity regulates oxidation of plasma lipids, angiotensin II formation, and the degradation of circulating catecholamines and kinins. This paper updates previous reviews and only cites a limited number of references. Readers are invited to look at the Bibliography of 100 Key References at the end of this issue for a more comprehensive selection of papers.

SELECTED ABBREVIATIONS AND ACRONYMS

ADMA	asymmetrical dimethyl-L-arginine
CNP	C-type natriuretic peptide
EDCF	endothelium-derived contracting factor
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
FAD	flavin adenine dinucleotide
ICAM-1	intercellular cell adhesion molecule–1
L-NAME	L-nitro-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MCP-1	monocyte chemoattractant protein–1
NANC	nonadrenergic noncholinergic (nerves)
NF-κB	nuclear factor kappa B
NO	nitric oxide
PDGF	platelet-derived growth factor
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule–1

In 1980, Robert Furchgott demonstrated that endothelial cells generate vasoactive subtances.[1] This seminal observation was crucial to the understanding of the regulation of vascular smooth muscle tone. Furchgott's simple pharmacological experiment initiated countless studies on a wide variety of blood vessels, shedding new light on the physiological role of nitric oxide.

Although nitric oxide appears to be the major vasodilator released by endothelial cells in the vast majority of blood vessels, other substances, some of them still unknown, may play a role as well. Soon after Furchgott's discovery, it became clear that endothelial cells not only release relaxing factors, but also produce contracting substances.[2] This concept led to the discovery of the endothelin peptides in the late 80s.

The release of endothelium-derived vasoactive subtances is not only triggered by acetylcholine, but also controlled by a host of neuromediators and by shear forces exerted by the blood flowing through the blood vessel.[2-4] Under physiological conditions, a precise and balanced release of relaxing and contracting factors ensures adequate organ perfusion. However, this balance is altered in disease states such as atherosclerosis, diabetes, chronic heart failure, coronary artery disease, or hypertension. Although the role of endothelial vasoactive factors as primary initiators of vascular diseases is still under debate, their dysfunctional release or their inactivation can have serious consequences for the vascular wall.

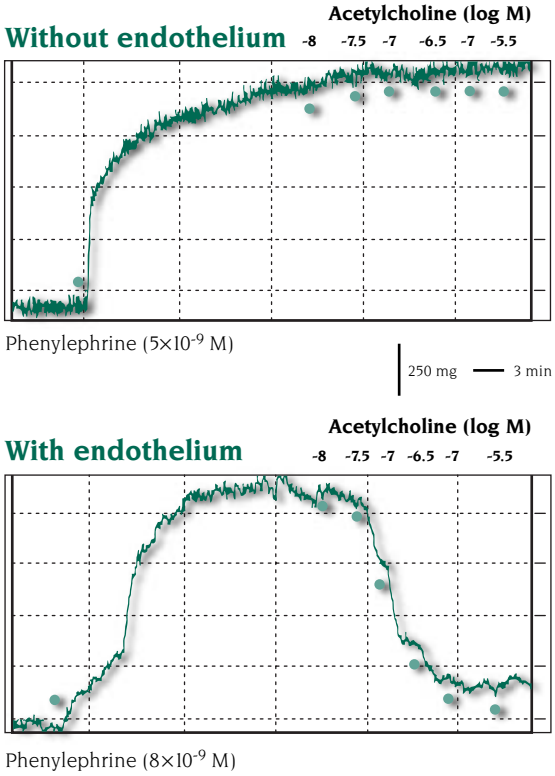

Figure 1. *Typical recording showing the role of the endothelium in the regulation of vascular smooth muscle tone. The relaxation to acetylcholine (shown in the bottom trace) is lost after mechanically removing endothelial cells from the aorta of a mouse (top trace).*
The recording shows the points at which each agonist was added (in molar concentration). The preparations were first contracted with phenylephrine before exposure to increasing concentrations of acetylcholine.
C.M. Boulanger, unpublished data.

ENDOTHELIUM-DERIVED RELAXING FACTORS

Acetylcholine and other mediators release endothelium-derived relaxing factor(s) (EDRF) in many arteries and veins from different species, including human blood vessels *(Figure 1)*.[1] Endothelium-dependent relaxation appeared early in evolution, even in species with a primitive cardiovascular system, which implies that these endothelial mediators fulfill a primordial role. Identification of the factors mediating endothelial-dependent relaxation was made difficult because of their short half-lives under in vitro experimental conditions. Indeed, bioassay experiments demonstrating that EDRF could diffuse from the endothelium to the smooth muscle also showed that the relaxing activity of the perfusate from a blood vessel was quickly lost when the transit time between the donor segment and the detector (an artery without endothelium) exceeded a few seconds. These bioassay experiments permitted the identification of the crucial role of superoxide anions in the inactivation of EDRF.

Nitric oxide

In 1986, Furchgott and Ignarro independently proposed nitric oxide, or a related nitroso compound, as the primary mediator of endothelium-dependent relaxation. This was based on pharmacological similarities between EDRF and nitric oxide, the active component of the nitrovasodilators, which have been used for therapy. Moncada and colleagues then identified nitric oxide (NO) as EDRF, which is synthesized following the conversion of endothelial L-arginine into L-citrulline.[2,5,6]

Endothelium-dependent relaxation can be inhibited by analogs of L-arginine such as N^G-monomethyl-L-arginine (L-NMMA) or L-nitro-arginine methyl ester (L-NAME), which compete with the natural precursor

L-arginine at the catalytic site of the enzyme.[6]
When infused intravenously, L-NMMA induces a long-lasting increase in blood pressure. This suggests that the continuous basal release of NO by the endothelium contributes to the regulation of peripheral resistance. Indeed, mice lacking the gene for the endothelial isoform of NO synthase (type III) have a slightly higher arterial blood pressure than wild-type animals.

Mechanisms of NO release from the endothelium

The endothelial enzyme that produces NO (type III NO synthase) is a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxygenase whose mechanism of action involves the oxidation of one of the guanidine nitrogen atoms of L-arginine.[5,6] This NO synthase is located in the caveolae of the plasma membrane of endothelial cells *(Figure 2)*.

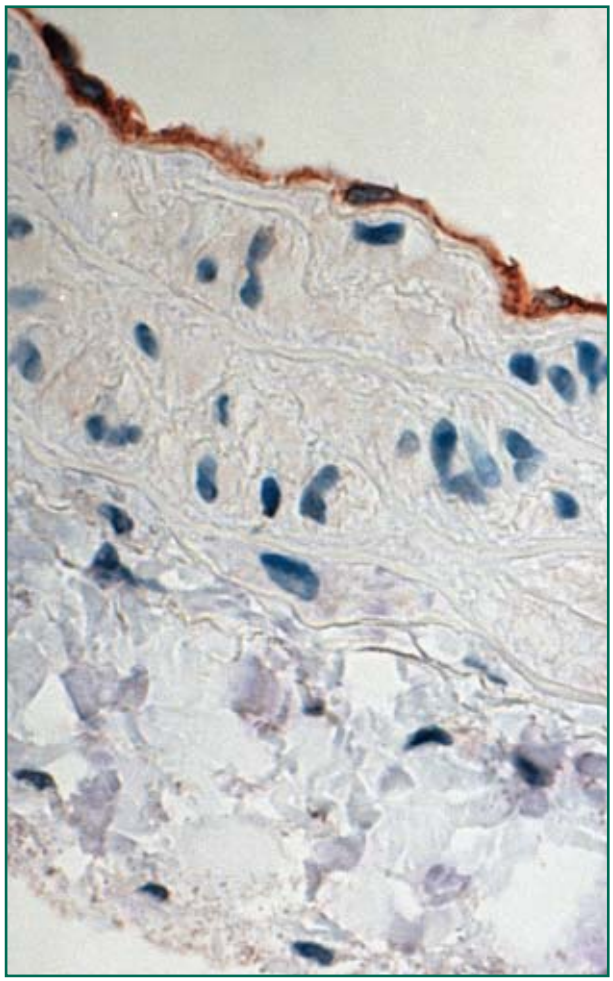

Figure 2. *Immunohistochemistry showing the expression of endothelial type III NO synthase restricted to the endothelium in a rat carotid artery. Courtesy of Dr R.S. Geske, Baylor College of Medicine, Houston, Tex, USA.*

The production of NO by the endothelium requires the enzyme to be membrane-bound as well as the presence of cofactors (such as tetrahydrobiopterin, flavin adenine dinucleotide (FAD), and flavin mononucleotide), and substrate (L-arginine).[7,8] The enzyme binds calmodulin in a calcium-dependent manner and can therefore be activated by stimuli that increase the concentration of intracellular free calcium (such as acetylcholine or bradykinin). However, the release of endothelial NO can also occur in the absence of increase in intracellular free calcium, as observed in endothelial cells exposed to shear stress.[9]

Under normal conditions, the availability of the semi-essential amino acid L-arginine in the plasma (about 100 to 200 µmol/L) does not appear to be a limiting factor for the production of NO by type III NO synthase (K_m^{arg}: 5 to 10 µmol/L). Endothelial cells not only take up circulating L-arginine by means of the y^+ transporter but can also recycle L-citrulline into L-arginine. Endogenous inhibitors of NO synthase such as asymmetrical dimethyl-L-arginine (ADMA) or L-NMMA may interfere with L-arginine uptake and activation of the NO synthase(s), but the degree of NO synthase activation will depend upon the L-arginine: (ADMA + L-NMMA) ratio. The circulating levels of these endogenous inhibitors appear augmented in renal failure and in animal models of atherosclerosis.

Although type III NO synthase is also called "constitutive" NO synthase, the level of its messenger RNA (mRNA) can be regulated.[6,7,10] Indeed, type III NO synthase mRNA levels appear to be augmented by shear stress and by exposure to vascular endothelial cell growth factor. Estrogens may also regulate the expression of this endothelial isoform, although this has not been observed consistently. In addition, type III NO synthase mRNA is decreased by oxidized LDL, while tumor necrosis factor α (TNF-α) shortens its half-life. This could contribute to pathophysiological changes in NO production by the blood vessel wall. Furthermore, the subsequent production of endothelial NO may be affected by changes in membrane localization of the enzyme (which is regulated by posttranslational palmytoylation and myristoylation) or its interaction with caveolin-1, as well as by alterations in the availability of L-arginine or the unavailability of cofactors.[7,10,11] Finally, the amount of active NO released by endothelial cells depends on the level of superoxide anions that are able to degrade the mediator. Therefore, alteration of these different steps involved in the activation of endothelial NO release may contribute to the development of vascular disease.

Other NO synthase isoforms in the vessel wall

Another calcium-dependent NO synthase (type I) is expressed in nitrergic neurons innervating the adventitia of some cerebral arteries. Its role in the regulation of these blood vessels is not fully elucidated. NO released following activation of type I NO synthase is the mediator of the nonadrenergic noncholinergic innervation (NANC nerves).

Exposure to cytokines or lipopolysaccharides induces the expression of a calcium-independent NO synthase (type II) in the vessel wall.[7,10] Once induced, this isoform spontaneously releases large and sustained amounts of the mediator, which may in part explain the hyporeactivity to contractile agents observed in septic shock. However, this type II isoform is "constitutively" expressed in the renal medulla, where it may participate in the control of arterial blood pressure.

Effects of endothelial NO

Relaxation of vascular smooth muscle

Endothelial NO causes the relaxation of vascular smooth muscle and can be regarded as a physiological antagonist of endogenous vasoconstrictors such as catecholamines, angiotensin II, or endothelin-1. NO stimulates soluble guanylate cyclase and augments the levels of cyclic guanosine monophosphate (GMP) in vascular smooth muscle cells *(Figure 3)*.[12] This increase in cyclic GMP could contribute to the relaxation of the smooth muscle by decreasing Ca^{2+} influx, increasing calcium uptake by Ca^{2+} ATPases, or by a direct interaction at the level of contractile proteins. In some blood vessels, cyclic GMP specifically inhibits phosphodiesterase activity and may prevent the degradation of cyclic AMP, thus augmenting cyclic AMP–mediated relaxations. In addition, NO may cause relaxation by interacting with K^+ channels either directly or through an increase in cyclic GMP, as observed in the rabbit and rat aorta.[13,14]

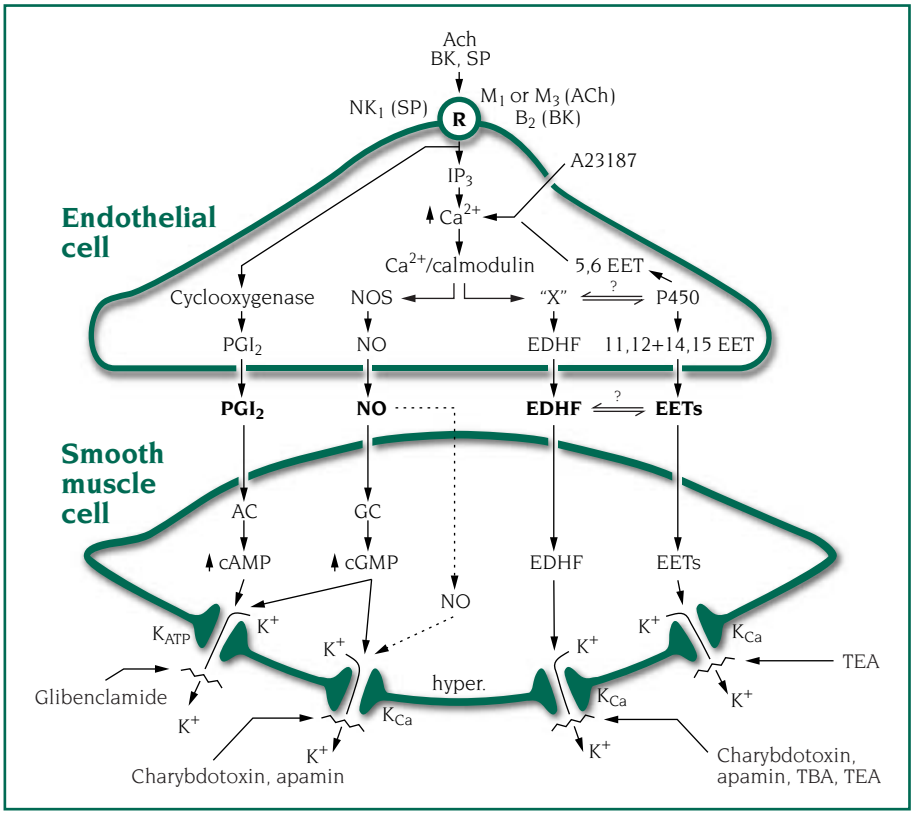

Figure 3. *Schematic representation of the release of endothelium-derived relaxing factors from endothelial cells and their effect on vascular smooth muscle cells. AC, adenylate cyclase; Ach, acetylcholine; A23187, calcium ionophore A23187; BK, bradykinin; B_2, bradykinin B_2 receptor; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; EET, epoxyeicosatrienoic acid; GC, guanylate cyclase; IP_3, inositol triphosphate; K_{ATP}, K_{Ca} potassium channels; M_1, M_3, muscarinic M_1 or M_3 receptor subtype; NK_1, neurokinin receptor; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; P450, cytochrome P450; SP, substance P; TBA, tetrabutylammonium; TEA, tetraethylammonium. The broken line indicates the action of an inhibitor or an antagonist. Reproduced from ref 12: Vanhoutte PM. Endothelial dysfunction and inhibition of converting enzyme.* Eur Heart J. *1998;19(suppl J): J7-J15. Copyright © 1998, The European Society of Cardiology. With permission.*

In addition to directly relaxing vascular smooth muscle, NO also regulates the production of the potent vasoconstrictor endothelin-1 in endothelial cells *(Figure 4)*.[15] This effect is mediated by an increase in cyclic GMP and is also observed with atrial natriuretic factor.

NO can also regulate the tone of vascular smooth muscle following a complex interaction with hemoglobin from red blood cells, glutathione, and small erythrocytic thiols. Release of oxygen to tissue is accompanied by the transfer of NO, bound to a cysteine group in hemoglobin, to small erythrocytic thiols where it can be released to cause relaxation of blood vessels. These data suggest that NO may be more than a purely local regulator of vascular smooth muscle tone, as previously thought.

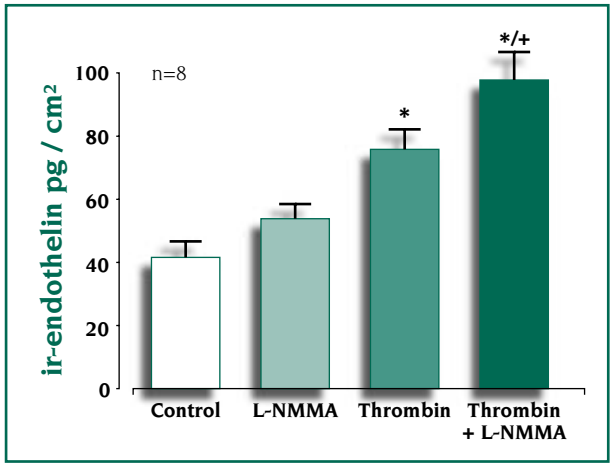

Figure 4. *Inhibition by nitric oxide (NO) of the release of endothelin-1 from the porcine aorta. The NO synthase inhibitor L-NMMA (2×10^{-4} M) augments the release of endothelin following stimulation with thrombin (4 U/mL), demonstrating that NO exerts a negative regulatory feedback on the release of the peptide. The amount of peptide produced is expressed as picograms immunoreactive (ir) endothelin released per square cm intimal surface after 4 h incubation (n=8). * indicates a significant difference as compared with control; + indicates a significant difference of preparations stimulated with thrombin in the presence and absence of L-NMMA (P<0.05). Reproduced from ref 15: Boulanger C, Lüscher TF. Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide.* J Clin Invest. *1990;85:587-590. Copyright © 1990, The American Society for Clinical Investigation, Inc. With permission.*

Antiproliferative effects?

Endothelial NO may contribute to inhibition of the proliferation of vascular smooth muscle cells, as suggested by coculture experiments. However, the antiproliferative effect of NO and NO donors observed in cultured vascular smooth muscle cells is most convincing at high concentrations, which appear to be more compatible with the release of NO from an inducible NO synthase. Moreover, the antiproliferative effect of NO may depend upon the growth factors present in the medium, since culture experiments suggest that NO in the presence of fibroblast growth factor may actually stimulate vascular smooth muscle proliferation.

NO may have indirect antiproliferative effects, as it also downregulates the production of growth factors by endothelial cells such as endothelin-1 and the platelet-derived growth factor (PDGF) B-chain. This effect appears to be mediated by an increase in cyclic GMP.

Protective role and antiatherogenic effect

Soon after the demonstration that NO could mediate endothelium-dependent relaxation, it was observed that NO, in synergy with prostacyclin, prevents platelet adhesion and aggregation at the endothelial surface. By inhibiting the degranulation of platelets, NO prevents the release of vasoconstrictors and growth factors such as thromboxane A_2, serotonin, and PDGF.

By impairing the activation of nuclear factor kappa B (NF-κB), NO negatively regulates the expression of adhesion proteins (such as vascular cell adhesion molecule–1 [VCAM-1], intercellular adhesion molecule–1 [ICAM-1], and monocyte chemoattractant protein–1 [MCP-1], the latter being a chemokine mediator of inflammation involved in the recruitment of circulating monocytes. NO, therefore, has a protective role against the initial events involved in cellular adhesion, which precedes the formation of macrophages/foam cells and the development of subsequent atherosclerotic lesions. In addition, NO prevents the oxidation of low-density lipoproteins, another crucial event in the development of atherosclerosis.

Endothelium-derived hyperpolarizing factor(s)

Endothelium-dependent hyperpolarization has been observed in a large variety of blood vessels, including human coronary arteries. Although endothelium-derived NO itself can cause hyperpolarization of vascular smooth muscle cells, endothelium-dependent hyperpolarization is mediated mainly by a distinct diffusible substance termed endothelium-derived hyperpolarizing factor (EDHF) *(Figure 3)*. The identity of EDHF is unknown, although several candidates have been proposed, such as ammonium anions, cytochrome P450 metabolites, hydrogen peroxide, cannabinoids, and potassium ions (see also the article by M. Félétou, in this issue).

The intracellular pathway leading to EDHF synthesis is also unclear, but is likely to involve an increase in intracellular calcium, since the calcium ionophore A23187 causes endothelium-dependent hyperpolarization and the latter is absent in calcium-free medium. Since, depending on the species, endothelium-dependent hyperpolarization is mediated by the activation of either K_{Ca} or K_{ATP} channels in vascular smooth muscle, several endothelium-dependent hyperpolarizing factors may exist.

Prostacyclin

Prostacyclin is formed from arachidonic acid following the activation, in turn, of phospholipase A_2, cyclooxygenase, and prostacyclin synthase. Its release,

which depends mainly upon calcium release from intracellular stores, is observed during activation of endothelial cells by shear stress or different agonists, which also cause the release of NO. In certain cases, endothelium-derived prostacyclin causes endothelium-dependent relaxation of vascular smooth muscle cells by stimulating adenylate cyclase and increasing the level of cyclic AMP, but in most cases prostacyclin has rather weak vasodilator properties *(Figure 3)*.

Other relaxing factors

Carbon monoxide

Carbon monoxide (CO) is synthesized during activation of hemoxygenase-1 and -2 in the blood vessel wall. It may contribute to endothelium-dependent relaxation in blood vessels that are insensitive to NO synthase inhibitors. Since CO augments cyclic GMP levels, the relaxation it causes is sensitive to inhibitors of soluble guanylate cyclase. CO also downregulates the expression of endothelin-1 and growth factors in endothelial cells.

C-type natriuretic peptide

Endothelial cells produce C-type natriuretic peptide (CNP). Exogenous CNP causes relaxation of isolated arteries and veins by activation of particulate guanylate cyclase, and, in some blood vessels, by activation of soluble guanylate cyclase and calcium-dependent K^+ channels. However, evidence for endothelial CNP-mediated endothelium-dependent relaxation in vivo is still awaited. Experiments on cocultures of endothelial and smooth muscle cells suggest that endothelial cells may release sufficient amounts of CNP to negatively control the proliferation of vascular smooth muscle cells.

Parathyroid hormone–related peptide

Parathyroid hormone–related peptide may be synthesized both by endothelial cells and by vascular smooth muscle cells. This peptide induces relaxation of isolated vessels and causes hypotension when infused in vivo. As with CNP, a possible role of this peptide in endothelium-dependent regulation of vascular smooth muscle tone has yet to be demonstrated.

RELEASE OF RELAXING FACTORS

Besides acetylcholine, there are a number of physiological stimuli or mechanisms that can cause endothelium-dependent relaxation.

Shear stress

The shear stress exerted by the blood flowing on the arterial wall is one of the main factors in the release of relaxing mediators.[2,4,16] This conclusion was reached from studies showing that an increase in flow rate through an isolated artery substantially increased the relaxation. A similar result was obtained if a stable flow rate was replaced by a pulsatile one *(Figure 5)*.[17] This effect explains why flow-induced dilatation is endothelium-dependent in the intact organism. Thus, if resistance vessels in a peripheral organ (heart or skeletal muscle) suddenly dilate, the resulting surge of blood causes dilatation of large arteries irrigating that organ. This dilatation is not observed in arteries without endothelium. These flow-induced changes in vessel diameter tend to normalize wall shear stress, making it return to baseline values. The augmented release of endothelium-derived relaxing factors induced by shear stress results—at least in part—from the activation of the kinin-kallikrein system and the local formation of bradykinin in the vessel wall.

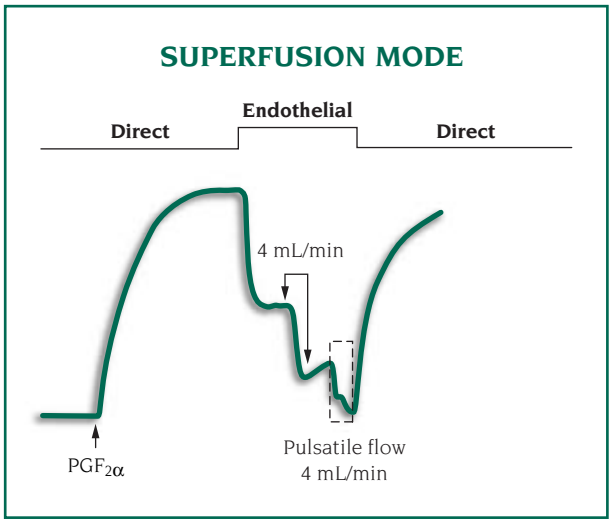

Figure 5. *Effect of increase in flow rate under bioassay conditions. A bioassay ring without endothelium (canine coronary artery) is superfused either with the effluent of a stainless tube (direct superfusion) or with the perfusate through a segment of femoral artery with endothelium (endothelial superfusion). This bioassay ring is used to detect the relaxant activity of the perfusate at an initial flow rate of 2 mL/min. The bioassay ring is first contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and then moved under the endothelial superfusion. A partial relaxation is observed in the bioassay ring, due to the production of relaxing substances by the endothelium of the perfused segment. The relaxation is augmented by increasing the flow rate from 2 to 4 mL/min, and pulsatile flow induces a full relaxation of the bioassay ring. The increased release of relaxing factors is best explained by the effect of shear stress on the endothelial cells. Adapted from ref 17: Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor.* Am J Physiol. *1986;250(6, pt 2):H1145-H1149. Copyright © 1986, American Society for Investigative Pathology. With permission.*

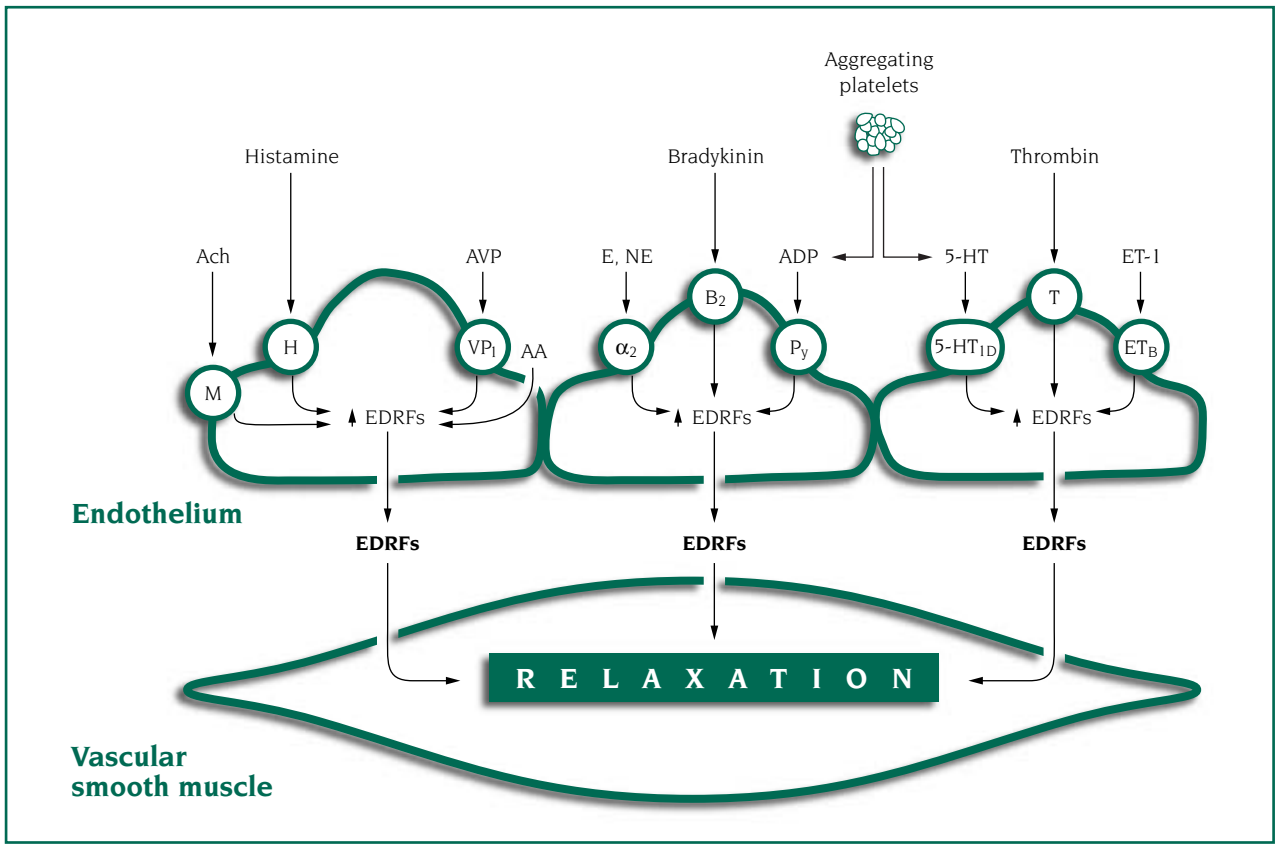

Figure 6. *Activation of endothelial receptors and release of endothelium-derived relaxing factors (EDRF), which cause the relaxation of the underlying smooth muscle. AA, arachidonic acid; α_2, α_2-adrenoceptor; Ach, acetylcholine; ADP, adenosine diphosphate; AVP, arginine vasopressin; B_2, bradykinin-2 receptor; E, epinephrine; ET_B, endothelin-B receptor subtype; H, histamine receptor; 5-HT, serotonin, 5-hydroxytryptamine; 5-HT_{1D}, 5-HT_{1D} serotonergic receptor; M, muscarinic receptor; NE, norepinephrine; Py, purinergic receptor; T, thrombin receptor; VP_1, vasopressinergic-1 receptor. Reproduced from ref 12: Vanhoutte PM. Endothelial dysfunction and inhibition of converting enzyme.* Eur Heart J. *1998;19(suppl J):J7-J15. Copyright © 1998, The European Society of Cardiology. With persmission.*

Endothelium-dependent relaxation mediated by shear stress involves integrins (transmembrane glycoproteins involved in cell–matrix interactions) and the endothelial cytoskeleton. Indeed, both inhibition of integrin binding to the extracellular matrix and disruption of the endothelial cytoskeleton specifically inhibit flow-induced dilation without affecting endothelium-dependent relaxation to agonists. Shear stress on the endothelial cells can stimulate the immediate release of NO, prostacyclin, and EDHF. In addition, long-term exposure to shear stress upregulates the expression of type III NO synthase in cultured endothelial cells. Therefore, the continuous stimulation of endothelial NO release by flowing blood probably contributes to the protective role of the endothelium against platelet, neutrophil, and monocyte adhesion in the intact organism. In addition, when blood flow is augmented for a sustained period of time, NO participates in the long-term increase in diameter of the vessel.

Activation of endothelial receptors

The endogenous substances stimulating the release of relaxing factors are either circulating hormones, autacoids formed by the blood vessel wall, or substances released during coagulation of the blood *(Figure 6)*.[2,12,16]

Hormones

Epinephrine (adrenaline), norepinephrine (noradrenaline), and synthetic α_2-adrenergic agonists can cause endothelium-dependent relaxations, which are prevented by α_2-adrenergic antagonists, thus demonstrating the presence of α_2-adrenoreceptors on these endothelial cells. It is likely that endothelium-dependent relaxations participate in the vasodilator effect of catecholamines in some blood vessels (like the coronary arteries). In rat cerebral arterioles, α_2-adrenergic agonists cause relaxation by augmenting the direct relaxing effect of basally released NO on vascular smooth

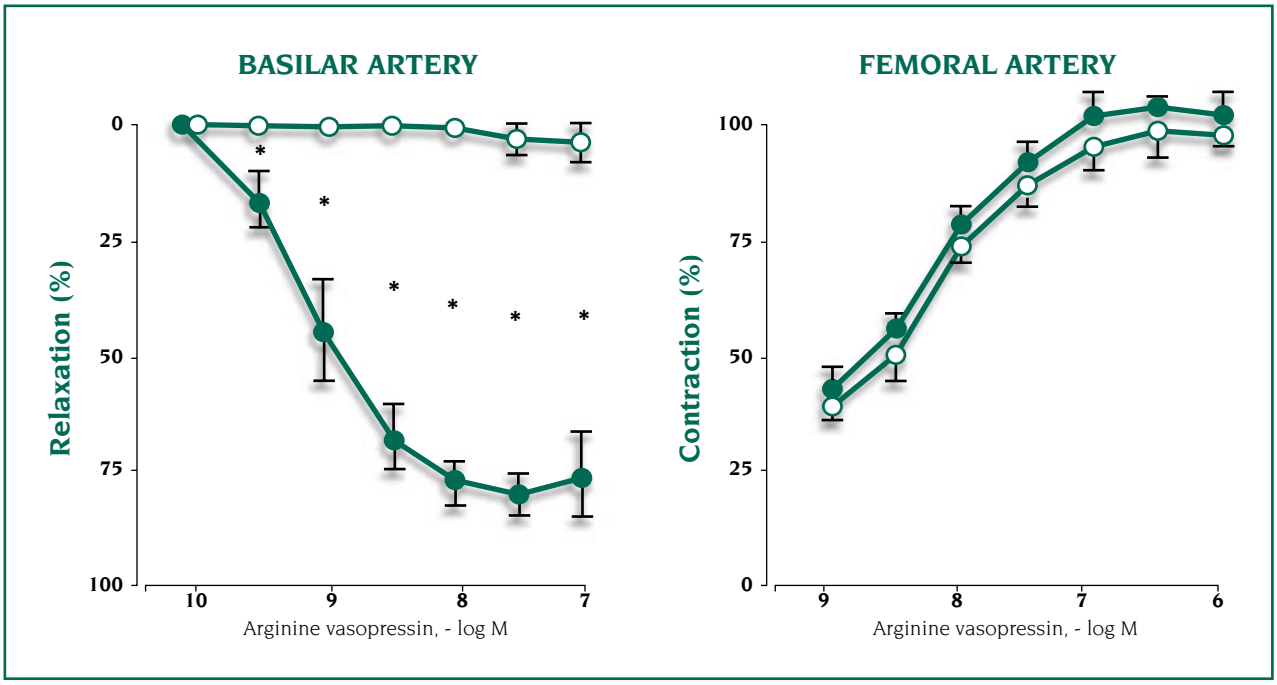

Figure 7. *Differential effect of arginine-vasopressin on vascular reactivity in canine cerebral and peripheral arteries.* ***Left:*** *arginine-vasopressin induces profound relaxation in basilar arteries with endothelium (closed circles), but not in those without endothelium (open circles).* ***Right:*** *in contrast, arginine vasopressin directly contracts the smooth muscle of the femoral artery without causing the release of endothelial vasoactive factors. *The difference between the two types of preparations is statistically significant (P<0.05). Adapted from ref 18: Katusic ZS, Shepherd JT, Vanhoutte PM. Vasopressin causes endothelin-dependent relaxations of the canine basilar artery.* Circ Res. *1984;55:575-579. Copyright © 1984, American Heart Association. With permission.*

muscle cells and not by augmenting the release of NO from the endothelium. The exact mechanism of the permissive role of NO in these vessels is unknown.

Vasopressin and oxytocin cause endothelium-dependent responses by acting on endothelial V_1 vasopressinergic receptors in certain arteries. This effect is particularly striking in cerebral arteries where vasopressin causes potent endothelium-dependent relaxation, while it does not have this effect in the peripheral circulation *(Figure 7)*.[18] The endothelium-dependent dilatation induced by vasopressin may contribute to the preferential redistribution of blood flow to the brain when the hormone is secreted, eg, during hemorrhage.

Autacoids

The autacoids that release EDRF include histamine, bradykinin, and various neuropeptides.

Histamine causes potent endothelium-dependent relaxation in many blood vessels. The endothelial action of histamine in arterioles likely accounts for the local vasodilatation and hence for the rubor characteristic of the histamine response.

Various neuropeptides, in particular substance P, cause endothelium-dependent relaxation. The endothelial effect of substance P may be involved in the local vasodilatation characteristic of antidromal (axon reflex) stimulation of sensory nerves.

Bradykinin is a potent stimulator of the release of EDRF and causes major precapillary vasodilatation, possibly by its action on endothelial B_2 kinin receptors. Bradykinin releases both NO and, at higher concentrations, EDHF, as observed in human coronary arteries *(Figure 3)*. Since the precursors of bradykinin (kininogens) are found throughout the body and particularly in platelets, and given that the vessel wall contains the enzymes required to transform these kininogens into bradykinin, locally generated bradykinin may have a more important role in the regulation of vasomotor tone than was originally thought. Indeed, several observations suggest that the local production of bradykinin mediates endothelium-dependent relaxations to increases in flow. Inhibition of local bradykinin degradation by angiotensin-converting enzyme inhibitors may contribute to the beneficial effect of these compounds, in addition to inhibiting the formation of angiotensin II.

Platelet products and thrombin

The endothelial action of thrombin and of the platelet products 5-hydroxytryptamine (serotonin) and adenosine diphosphate (ADP) is of crucial importance in the protective role of the endothelium against undesired coagulation. Thus, local platelet aggregation, with the inevitable release of ADP and serotonin, as well as the local formation of thrombin, leads to massive endothelium-dependent vasodilatation *(Figure 6)*. This helps to eliminate platelet microaggregates. In addition, NO, in synergy with prostacyclin, also inhibits further platelet adhesion and aggregation, thereby eliminating the danger of vascular occlusion. Conversely, if the endothelial lining is damaged, eg, as a result of trauma, aggregation proceeds, resulting in continuous release of serotonin and thromboxane A_2, which have unrestricted access to the smooth muscle. Hence, the smooth muscle contracts and the blood vessel closes, giving rise to the vascular phase of hemostasis.

ENDOTHELIUM-DEPENDENT CONTRACTION

Endothelial cells also can initiate contraction of the underlying smooth muscle by releasing vasoconstrictor substances. Endothelium-derived contracting factors (EDCFs) include the peptide endothelin, vasoconstrictor prostanoids such as thromboxane A_2 and prostaglandin H_2, as well as superoxide anions and components of the renin-angiotensin system. Whereas the release of NO and other relaxing factors appears to be decreased in many vascular diseases, the release of EDCFs is, by contrast, augmented.[2,16]

Contractions blocked by cyclooxygenase inhibitors

A group of EDCFs is generated by the metabolism of arachidonic acid through the cyclooxygenase pathway.[2] As shown in *Figure 8,* particularly in peripheral veins, but also in the cerebral circulation and in some

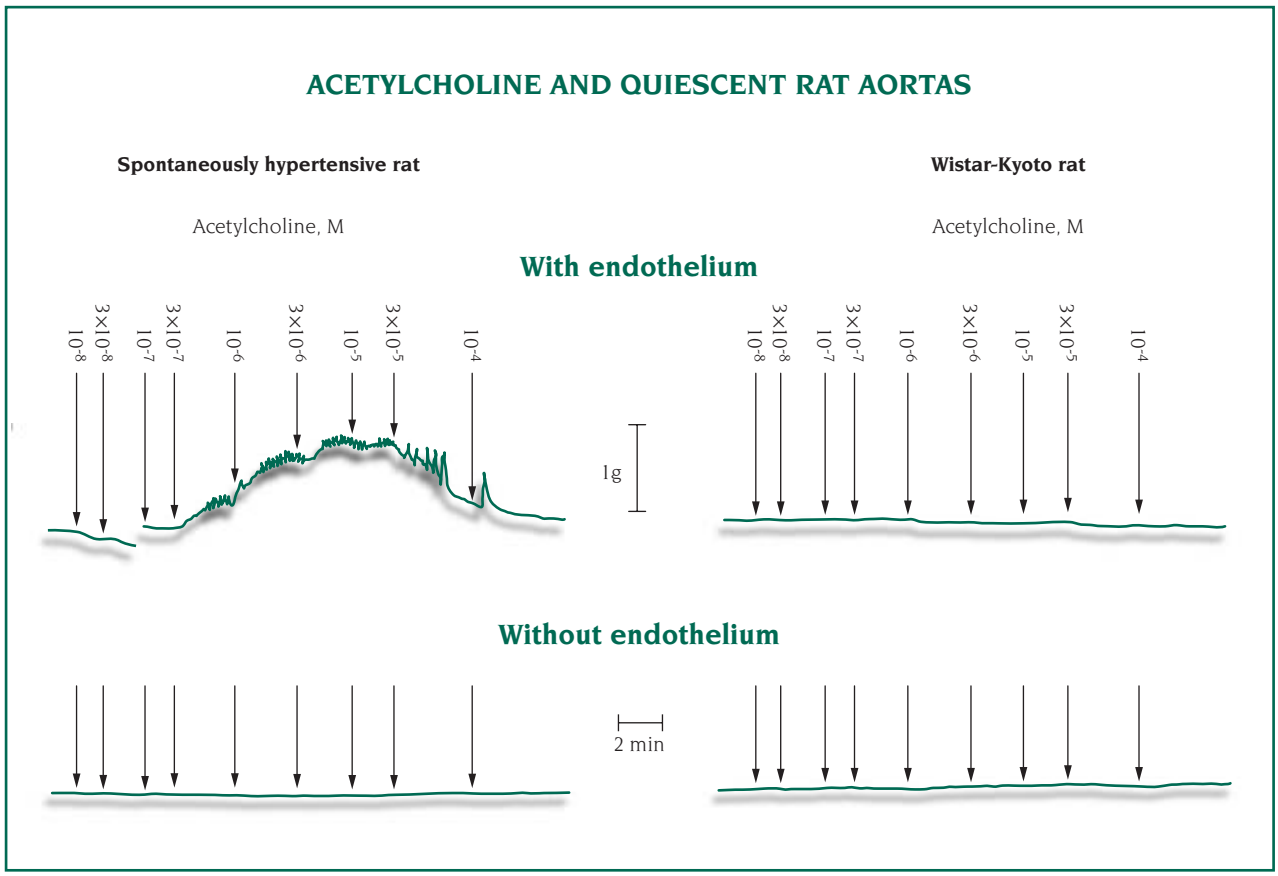

Figure 8. *Isometric tension changes in quiescent rings of rat aorta with (above) and without (below) endothelium, from normotensive and spontaneously hypertensive animals (right and left, respectively). Acetylcholine induces endothelium-dependent contractions only in aortas from the spontaneously hypertensive rats. Based on data from ref 20.*

arteries from hypertensive animals, endothelium-dependent contractions are mediated by thromboxane A_2 or prostaglandin H_2, which activate the same thromboxane-endoperoxide receptor.[16,19,20] These mediators may be involved in the autoregulation of the cerebral circulation. Few physiological stimuli cause endothelium-dependent contractions that are sensitive to inhibitors of cyclooxygenase. An important response is probably that to stretch *(Figure 9)*.[21] Thus, endothelium-dependent contraction of a cerebral artery in response to stretch closely resembles the autoregulatory response. A possible explanation for the autoregulation of the cerebral circulation initiated by a sudden stretch of the vessel wall in response to an increase in blood pressure is the release of endothelium-derived contracting factors, which would activate the underlying smooth muscle to restore a normal flow rate.

In addition, the cyclooxygenase pathway is a source of superoxide anions, which can cause contraction directly or indirectly by inactivating EDRF-NO. Endothelial cells also produce reactive oxygen species

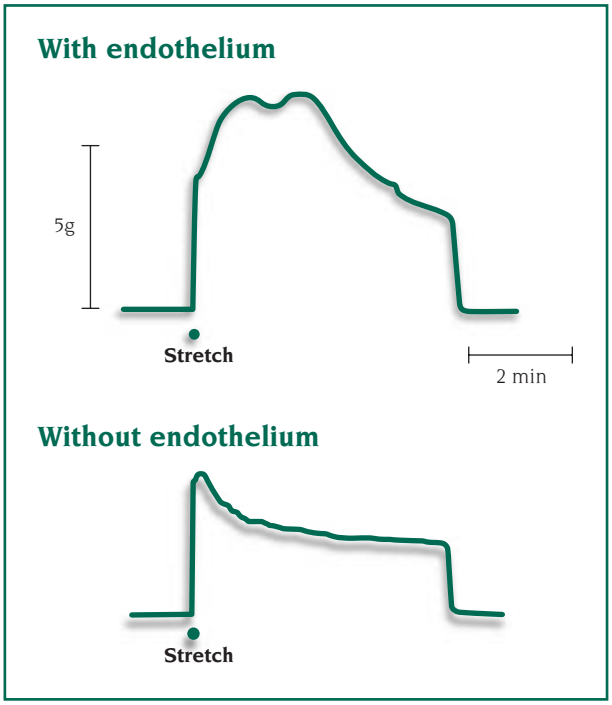

Figure 9. *Isometric tension response of canine basilar artery rings with (above) and without (below) endothelium to sudden stretch. In the rings without endothelium, the rapid increase in tension in response to stretch is followed by a slow decrease in tension (hysteresis). In the rings with endothelium, on the other hand, stretch induces a contraction which is stable for approximately 2 minutes. This experiment shows that stretch causes endothelium-dependent contractions in the canine basilar artery with a time course comparable to that of the autoregulatory response. Based on data from ref 21.*

either through the xanthine oxidase and NADPH-oxidase systems, or the NO synthases when available concentrations of tetrahydrobiopterin or L-arginine are too low.

Hypoxia-induced contraction

Some blood vessels, particularly coronary, cerebral, and pulmonary arteries, rapidly contract when exposed to sudden hypoxia. This endothelium-dependent contraction is caused by the transfer of a diffusible substance, which is still unknown. It is not dependent on the cyclooxygenase pathway nor on the release of endothelin. The phenomenon is exacerbated by a reduced release of NO.

Endothelin

Although endothelin exists in three isoforms (endothelin-1, -2, and -3), endothelial cells exclusively produce endothelin-1.[22] Translation of the mRNA generates preproendothelin, which is converted to big endothelin; its conversion to the mature peptide endothelin-1 by endothelin-converting enzymes is necessary for the development of its vascular activity. The expression of mRNA and the release of the peptide from cultured endothelial cells is stimulated by thrombin, transforming growth factor β1, interleukin-1, epinephrine, angiotensin II, arginine vasopressin, calcium ionophore, and phorbol ester. Endothelin-1 causes vasodilation at lower concentrations by activating endothelial ET_B receptors coupled to the release of NO, prostacyclin, and EDHF, while at higher concentrations it causes marked and sustained contractions by activation of ET_A, and, in some blood vessels, of ET_B receptors on vascular smooth muscle.[22,23] Circulating levels of endothelin-1 are low, suggesting either discrete endogenous production under physiological conditions, the presence of potent inhibitory mechanisms (such as the negative control induced by NO), or preferential abluminal release of the peptide towards vascular smooth muscle cells.

ENDOTHELIUM-DERIVED FACTORS AND PATHOLOGY

Regenerated endothelium

In a healthy adult body, under normal conditions (and in the absence of physiological local angiogenesis as observed in women), endothelial cells proliferate at a very low rate. This rate of proliferation increases with aging, after angioplasty, or under pathological conditions

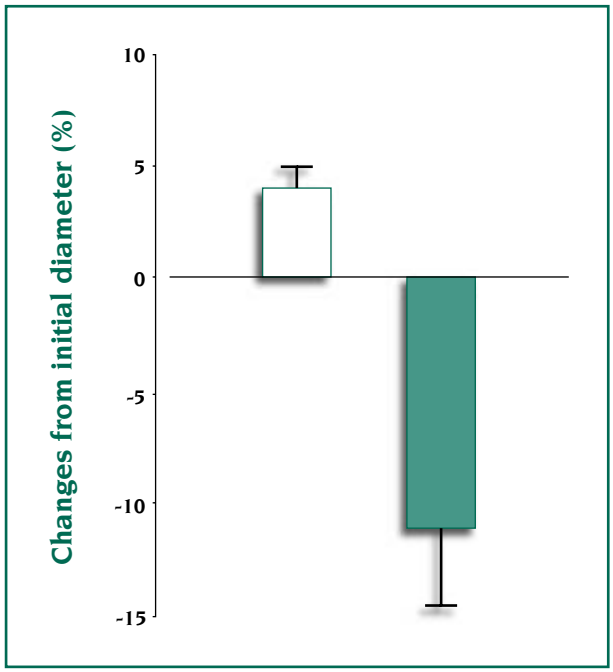

Figure 10. *Changes in porcine coronary artery diameter following intracoronary infusion of serotonin (10 mg/kg). In control coronary arteries (open bar), serotonin causes a slight increase in diameter, while in coronary arteries with regenerated endothelium (hatched bar), serotonin decreases the diameter. C.M. Boulanger, unpublished data.*

such as hypertension. However, the newly generated endothelial cells appear to be dysfunctional.[24] In the model of regenerated endothelium following balloon abrasion of the porcine coronary artery, a selective dysfunctional release of relaxing factors is observed in response to agonists activating receptors linked to pertussis toxin–sensitive Gi proteins (such as serotonin 5-HT_{1D}, endothelin ET_B, and α_2-adrenergic receptors). However, the response of regenerated endothelial cells to agonists activating receptors not coupled to pertussis toxin–sensitive Gi proteins is maintained (eg, response to bradykinin or ADP).[16] These dysfunctional endothelial cells can promote the occurrence of coronary vasospasm in response to ergonovine or platelet-derived products such as serotonin, by leaving unopposed the direct contractile effect of the serotonergic agonist on vascular smooth muscle cells *(Figure 10)*. This vasospastic event is associated with signs of severe cardiac ischemia. In porcine coronary arteries, this selective dysfunction of regenerated endothelial cells is still observed 5 to 6 months after the abrasion. Administration of growth factors (such as fibroblast growth factor or vascular endothelial cell growth factor) may accelerate the regrowth of the endothelium, but no information is available regarding a possible beneficial effect of these growth factors on endothelial dysfunction. It has been also shown that vessels growing de novo in the intact organism, as in collateral formation or tumor angiogenesis, are hypersensitive to the vasoconstrictor effect of serotonin; this can also be explained by the absence of an appropriate endothelium-dependent response to serotonin.

The fact that the response to all endothelial receptors coupled to pertussis toxin–sensitive Gi proteins is selectively altered has suggested that these Gi proteins may be dysfunctional in regenerated endothelial cells. Regenerated endothelial cells express a normal amount of α-subunits of Gi proteins as compared with normal endothelial cells, but their function may be altered. Interestingly, Gi proteins are preferentially expressed in endothelial cells from large human epicardial coronary arteries in contrast to those from small coronary arterioles, which are less prone to the occurrence of atherosclerosis.

Reperfusion injury

A situation similar to that of the dysfunction of regenerated endothelial cells occurs during abrupt reperfusion. When the blood flow is restored following temporary occlusion of a coronary artery, the reperfusion injury involves not only the myocardium, but also the blood vessel wall. In canine coronary arteries, 1 hour after reperfusion, almost all endothelium-dependent responses have disappeared or are severely inhibited. This is not surprising, as reperfusion involves profound metabolic changes due to the reintroduction of oxygen and massive free radical formation. A greater cause for concern is that 3 months after reperfusion injury, the reactivity of the reperfused artery is still abnormal, particularly with regard to the response of platelet aggregation and thrombin. As a result, hyperconstriction is observed and platelets and other circulating cells adhere to the reperfused segment. This abnormal adhesion can lead to the release of vasoconstrictors or growth factors from adherent cells, or favor the transmigration of monocytes to the subendothelium, initiating a local inflammatory response. If the reperfusion is carried out slowly, the subsequent chronic endothelial dysfunction may not be so dramatic.

Atherosclerosis

A significant decrease in endothelium-dependent relaxation has been observed in various models of atherosclerosis as well as in human atherosclerotic coronary arteries. As with regenerated endothelial cells,

the first endothelium-dependent response to be lost is that to serotonin.[16,24] The most important mechanism in the decrease in endothelium-dependent relaxation appears to be a reduced release of NO.[13] As the disease progresses over the years and the artery thickens and stiffens, it becomes increasingly difficult for NO to reach the vascular smooth muscle before it is inactivated. Animal models of atherosclerosis have evidenced the presence of an inducible NO synthase expressed in the vessel wall, although the amount of active NO produced is significantly impaired due to an increased formation of superoxide anions.[13,25] The beneficial effect of chronic treatment with L-arginine in some animal models of atherosclerosis may be explained by an increased availability of the substrate of both isoforms, resulting in a greater generation of NO, although a decreased formation of superoxide anions certainly plays a role as well. Inhibition of superoxide anions in the atherosclerotic wall may also explain the beneficial effect of chronic treatment with antioxidants. An augmented expression of endothelin-1 has been observed in the human atherosclerotic plaque, but is unlikely to directly contribute to the impaired endothelium-dependent vasodilation, since endothelin receptor antagonists do not improve endothelium-dependent responses.

It is tempting to hypothesize that endothelial dysfunction is one of the initial steps involved in the development of atherosclerosis. As endothelial cells regenerate with aging or following exposure to risk factors such as hypertension, smoking, or hyperlipidemia, their protective role against platelet, monocyte, or neutrophil adhesion diminishes. This may set the stage for the development of an inflammatory response, which, together with the presence of oxidized low-density lipoprotein in the vessel wall, further worsens the initial endothelial dysfunction.

Hypertension

In most animal models of hypertension, the endothelium-dependent relaxation to acetylcholine (and other agonists) is impaired.[16,19] However, this endothelial dysfunction presents different characteristics depending on the model studied. The impairment of endothelial function is either associated with a decreased production of NO and/or a concomitant release of endothelial contracting factors that impair the effects of NO. This may explain the apparent discrepancy between the results from studies evaluating endothelial function and the contribution of NO in hypertensive subjects. Indeed, endothelium-dependent dilatation is either decreased or unchanged in patients with high blood pressure. However, most studies suggest a dysfunctional release or effect of EDRF in hypertensive vessels.

In salt-sensitive hypertensive Dahl rats, endothelium-dependent relaxation is decreased as a result of impaired release of NO associated with decreased activity of the calcium-dependent NO synthase in blood vessels and increased levels of the endogenous inhibitor ADMA. In this model, exposure to L-arginine restores endothelium-dependent relaxation. In conduit arteries of spontaneously hypertensive rats (genetic hypertension), endothelium-dependent relaxation to acetylcholine, ADP, or serotonin is impaired due to the release of endoperoxides, in particular prostaglandin H_2, which activate thromboxane-endoperoxide receptors.[19] Endothelial function is normalized following inhibition of cyclooxygenase activity or blockade of thromboxane-endoperoxide receptors. The endothelial dysfunction associated with activation of thromboxane-endoperoxide receptors may result from impaired NO action on smooth muscle cells or altered endothelial NO release. Calcium-dependent NO activity is increased in homogenates from spontaneously hypertensive rat (SHR) blood vessels, suggesting either an increased expression of endothelial NO synthase or the presence of another isoform. The presence of a cyclooxygenase-dependent EDCF in hypertension is supported by the fact that a cyclooxygenase inhibitor (indomethacin) improves the endothelium-dependent response to acetylcholine in patients with essential hypertension. In the microcirculation of the SHR, endothelium-dependent relaxation to acetylcholine appears to be decreased as a result of the concomitant release of superoxide anions.

Endothelial dysfunction in hypertension appears to be a consequence of high blood pressure, since the majority of antihypertensive treatments normalize these responses. However, it may also amplify the increase in vascular resistance, since the inhibition of NO release (as in type III NO synthase knockouts or after enzyme inhibition by L-arginine analogs) causes an increase in blood pressure.

Heart failure

Heart failure is a clinical syndrome that occurs late in the course of coronary artery disease and other vascular diseases. It is accompanied by a generalized increase in peripheral vascular resistance, which may result from compensatory mechanisms involving neural, hormonal, or local factors. Endothelial cell dysfunction may also contribute to the peripheral vasoconstriction.[26] Indeed, decreased endothelium-dependent relaxation has been reported in several experi-

mental models of heart failure. Likewise, in patients with heart failure, the vasodilator response to acetylcholine is impaired. Endothelial dysfunction may be specific to certain responses (eg, acetylcholine), but has not been observed with other endothelium-dependent vasodilators (eg, substance P, the calcium ionophore A23187). In addition, plasma levels of endothelin-1 increase in proportion to the severity of cardiac failure. Chronic treatment with a combined ET_A-ET_B endothelin antagonist significantly improves long-term survival in rat models of heart failure.

Cerebral vasospasm

Experimental cerebral vasospasm is associated with a disappearence or dramatic decrease in endothelium-dependent relaxation. However, the release of endothelium-derived NO appears to be normal as evidenced from bioassay experiments and measurement of cyclic GMP. The abnormality in endothelium-dependent relaxation involves a decreased response of vascular smooth muscle to endothelial NO. This appears logical, since the cerebral vasospasm that normally follows subarachnoid hemorrhage is associated with the presence in the tissues of hemoglobin, a scavenger of NO. In addition, the potent vasconstrictor endothelin-1 may play a role in the spastic response, since endothelin-1 levels are increased in cerebrospinal fluid and endothelin antagonists normalize the response in subarachnoid hemorrhage.

THREE KEY QUESTIONS

In the following section of this issue, three experts will focus on some of the points raised in this paper. Michel Félétou and Paul Vanhoutte reply to the question of which factors are involved in endothelium-dependent relaxation: **"is nitric oxide the only answer?"** and clearly show that NO should not be viewed as eclipsing the role of other candidates. Endothelin is known to contribute to the pathogenesis of a number of cardiovascular diseases, and so Thomas Lüscher and Francesco Cosentino ask **"does endothelin play a role in hypertension?"** and the corollary, what is the potential for endothelin receptor antagonists to become part of the therapeutic arsenal against hypertension? But however exciting future drugs may appear, those that seem familiar to us may still hold a few surprises: Helmut Drexler, in his contribution entitled **"does bradykinin play a role in the regulation of vascular tone?"** illustrates this point by showing that part of the beneficial effect of ACE inhibition may be attributable to an improvement in endothelial function via an increase in bradykinin availability.

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Endothelium

Expert Answers to Three Key Questions

1

Is nitric oxide the only answer?

M. Félétou, P.M. Vanhoutte

2

Does endothelin play a role in hypertension?

T.F. Lüscher, F. Cosentino

3

Does bradykinin play a role in the regulation of vascular tone in humans?

H. Drexler

Is nitric oxide the only answer?

Michel Félétou, PhD*; Paul M. Vanhoutte, MD, PhD†

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Endothelium-dependent relaxations cannot be explained only by the release of nitric oxide and/or prostacyclin. Another still unidentified substance—endothelium-derived hyperpolarizing factor (EDHF)—which hyperpolarizes the underlying vascular smooth muscle cells, may also contribute to endothelium-dependent relaxations. In human, endothelium-dependent hyperpolarizations are observed in blood vessels that exhibit endothelium-dependent relaxations partially or totally resistant to inhibitors of nitric oxide synthase and cyclooxygenase. The contribution of the EDHF response is more important in smaller than in larger arteries. The suggestion that EDHF could be a metabolite of arachidonic acid formed through cytochrome P450 is still controversial. The identification of EDHF and/or the discovery of specific inhibitors of its synthesis action will permit a better understanding of its physiological and pathophysiological role(s).

Keywords: EDHF; endothelium; hyperpolarization; hyperpolarizing factor; membrane potential; potassium channel; prostacyclin; smooth muscle; vasodilatation

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Nitric oxide (NO) produced from L-arginine by endothelial nitric oxide synthase (NOS) and prostacyclin produced from arachidonic acid by cyclooxygenase have been identified as endothelium-derived vasodilators[1] (see also article by Boulanger and Vanhoutte on page 3). However, in various blood vessels from different species, endothelium-dependent relaxations are partially or totally resistant to inhibitors of cyclooxygenase and NOS *(Figure 1)*. In these blood vessels, muscarinic agonists, bradykinin, or substance P elicit endothelium-dependent hyperpolarizations of vascular smooth muscle cells that are also partially or totally resistant to inhibitors of cyclooxygenase and NOS, suggesting the existence of an additional mechanism for endothelial control of vasomotor tone *(Figure 2)*. These endothelium-dependent responses are observed without an

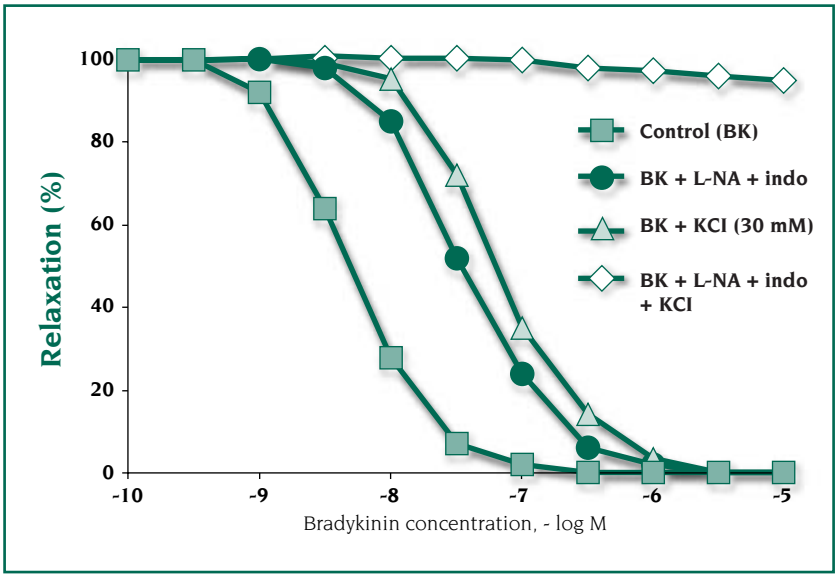

Figure 1. *Concentration-relaxation curves to cumulative addition of bradykinin (BK) in porcine coronary artery. Bradykinin induces an endothelium-dependent relaxation that is minimally affected by the presence of L-nitro-arginine (L-NA: 10^{-4} M) and indomethacin (indo: 5×10^{-6} M), inhibitors of nitric oxide synthase (NOS) and cyclooxygenase, respectively. The component of the relaxation that subsists in the presence of these inhibitors is attributed to the release of a factor inducing hyperpolarization of the vascular smooth muscle cells (endothelium-derived hyperpolarizing factor, EDHF). The hyperpolarization is blocked by raising the potassium concentration (KCl: 30 mM) of the extracellular medium. This also produces an inhibition of the endothelium-dependent relaxation to bradykinin. The combination of inhibitors of NOS and cyclooxygenase and the elevated potassium concentration abolishes the relaxation to bradykinin. These experiments suggest that the activation of three pathways (NOS, cyclooxygenase, and the release of unidentified EDHF) contributes to endothelium-dependent relaxations in the porcine coronary artery. Similar observations have been reported for various animal and human isolated arteries.*

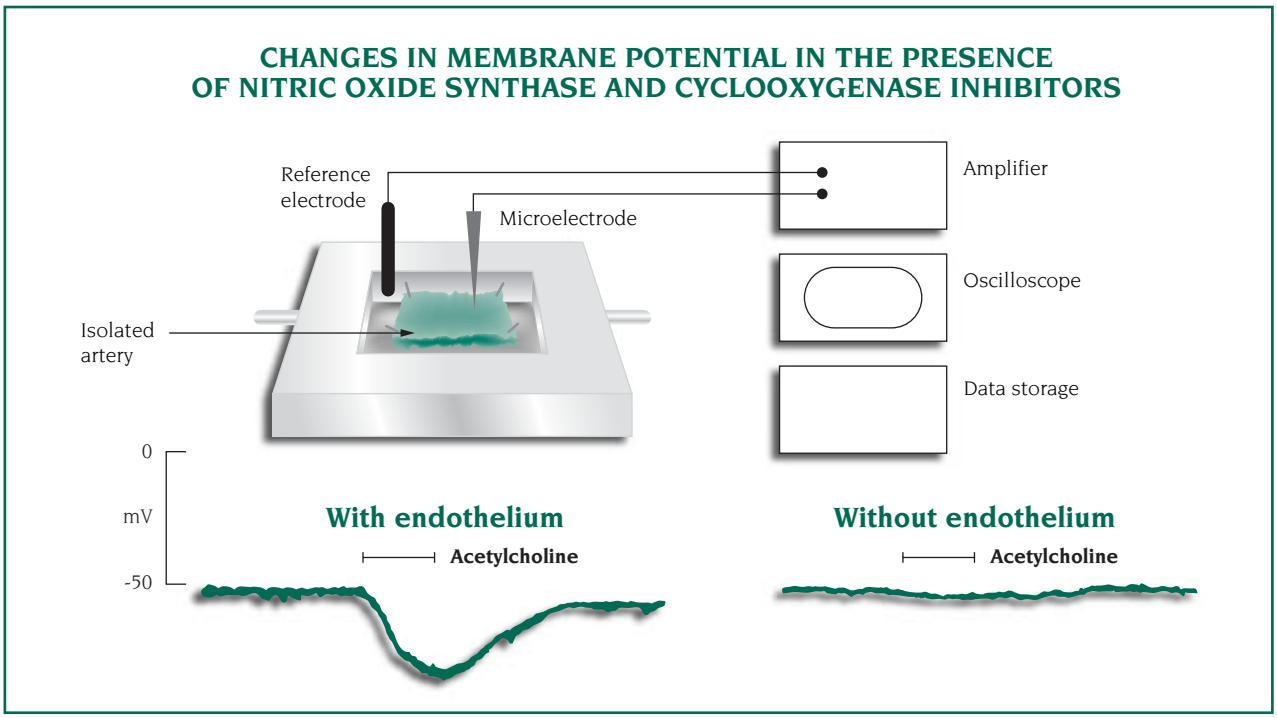

Figure 2. *Recording of endothelium-dependent hyperpolarization. The membrane potential of vascular smooth muscle cells is measured with a microelectrode inserted into a single cell impaled at random from the media of a strip of an isolated artery. Drugs such as acetylcholine produce hyperpolarization of the vascular smooth muscle cells only in the presence of the endothelial cells. This hyperpolarization is not affected by the presence of inhibitors of nitric oxide synthase and cyclooxygenase. This again suggests that EDHF is responsible for the endothelium-dependent hyperpolarization.*

increase in intracellular levels of cyclic nucleotides (cyclic GMP and cyclic AMP) in the smooth muscle cells, indicating that neither NO nor prostacyclin are responsible. Substances that produce endothelium-dependent hyperpolarization of vascular smooth muscle cells also hyperpolarize endothelial cells, with the same time course. Thus, cell-to-cell conduction could explain endothelium-dependent hyperpolarization. Indeed, direct electrical coupling between endothelial and vascular smooth muscle cells may be relevant at the microcirculatory level where myoendothelial junctions are numerous. However, this is unlikely to play an important role in the larger blood vessels in which endothelium-dependent hyperpolarizations have been reported so far *(Figure 3)*. Thus, endothelium-dependent hyperpolarizations resistant to inhibitors of NOS and cyclooxygenase have been attributed to the release of an as yet unidentified diffusible substance, termed endothelium-derived hyperpolarizing factor (EDHF), and may contribute to endothelium-dependent relaxations.[2,3]

DOES EDHF EXIST IN HUMAN BLOOD VESSELS?

Endothelium-dependent hyperpolarizations associated with endothelium-dependent relaxations resistant to inhibitors of NOS and cyclooxygenase have been recorded in human coronary, pial, and gastroepiploic arteries.[4-6] Endothelium-dependent relaxations resistant to inhibitors of NOS and cyclooxygenase, which are generally attributed to EDHF release, have also been observed in subcutaneous, omental, renal, and radial arteries. By contrast, EDHF-dependent responses are minimal in the internal thoracic artery and in the basilar artery. As in animal arteries, the contribution of the EDHF response is significantly greater in smaller than in larger human arteries.[6,7] Therefore, EDHF may play a significant role in the local regulation of peripheral vascular resistance and thus of the distribution of blood flow.

It is difficult to evaluate, in the intact human, the involvement of EDHF in the vasodilator responses to various stimuli, as specific inhibitors of its synthesis or action are not available. An EDHF mechanism is often suggested to explain vasodilatations in the intact organism that are resistant to inhibitors of NOS. However, other interpretations are possible. First of all, most of the human studies do not involve the administration of an inhibitor

of cyclooxygenase. Although in normal subjects inhibitors of cyclooxygenase do not appear to affect vasodilatation in response to mediators such as acetylcholine, the continuous release of vasodilator prostanoids contributes to the regulation of resting forearm blood flow in humans.[8] Furthermore, complete blockade of NOS is difficult to obtain, and/or the nonendothelial effect of the vasodilators, such as a direct effect on the smooth muscle cells or an inhibitory effect on the sympathetic nerve endings, cannot be excluded easily. Therefore, the exact role of EDHF in the control of human blood vessel tone is still unknown.[7]

HOW ARE HYPERPOLARIZATION AND RELAXATION RELATED?

The hyperpolarization of the cell membrane of vascular smooth muscle cells and the resulting reduction in Ca^{2+} entry explain the endothelium-dependent relaxations caused by EDHF. Indeed, hyperpolarization of smooth muscle cells reduces the open-state probability of voltage-dependent calcium channels, thereby decreasing calcium influx and lowering intracellular calcium levels. In addition, the hyperpolarization may reduce the increase in intracellular phosphatidylinositol turnover caused by agonist-induced receptor activation and therefore decrease the release of calcium from intracellular stores.

MECHANISM UNDERLYING ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION

The amplitude of the endothelium-dependent hyperpolarization is inversely related to the extracellular

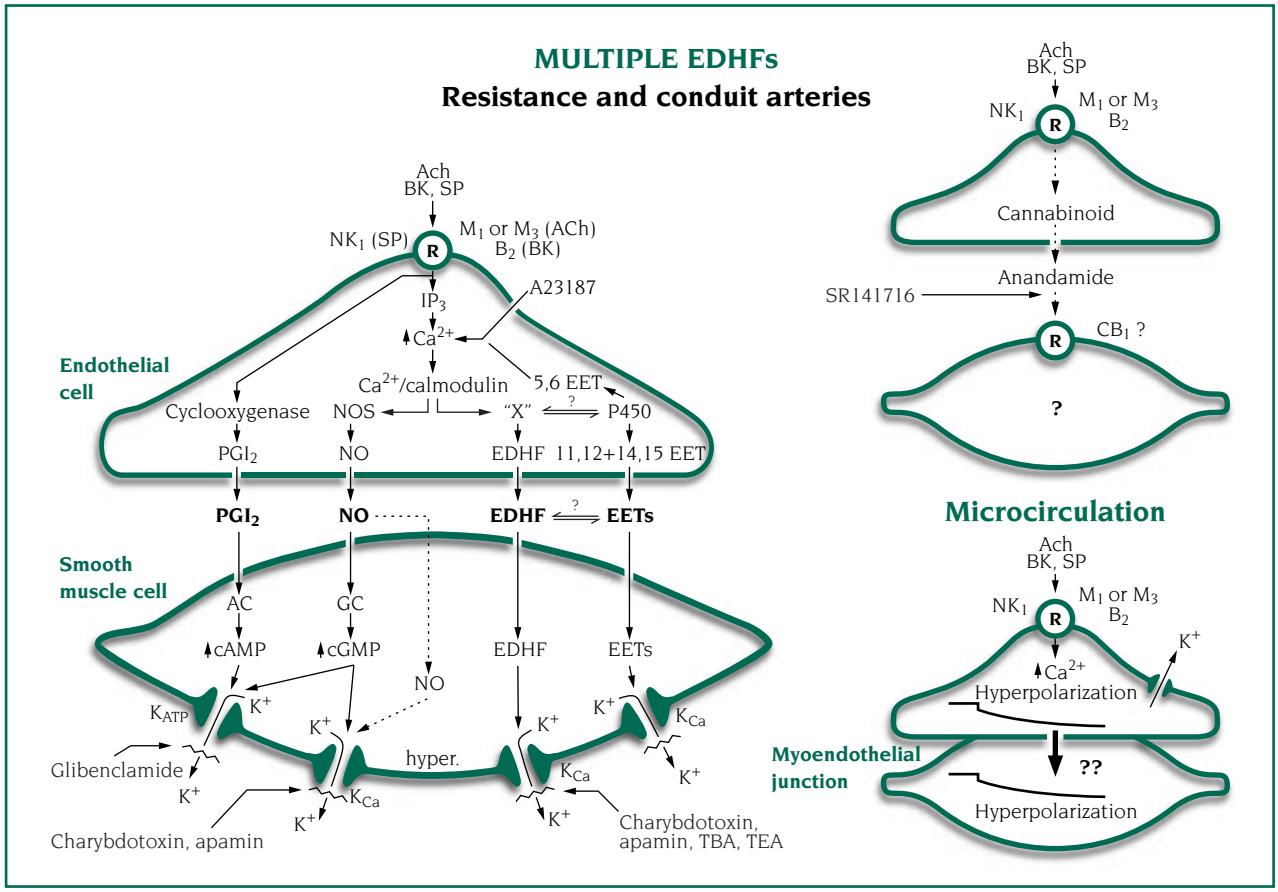

***Figure 3.** Possible mechanisms leading to endothelium-dependent hyperpolarizations. A23187, calcium ionophore; AC, adenylate cyclase; Ach, acetylcholine; ATP, adenosine triphosphate; B_2, B_2 bradykinin receptor subtype; BK, bradykinin; cAMP, cyclic adenosine monophosphate; CB_1, cannabinoid receptor; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; 5,6 EET, 5,6-epoxyeicosatrienoic acid; 11,12 EET, 11,12-epoxyeicosatrienoic acid; 14,15 EET, 14,15-epoxyeicosatrienoic acid, GC, guanylate cyclase; IP_3, inositol triphosphate; K_{ATP}, K_{Ca}, potassium channels; M_1, M_3, muscarinic receptor subtype M_1 or M_3; NK_1, NK_1 neurokinin receptor subtype; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; R, receptor; SR141716, an antagonist of CB_1 receptors; SP, substance P; TBA, tetrabutylammonium; TEA, tetraethylammonium. TBA and TEA are nonspecific inhibitors of potassium channels when used at high concentrations (>5 mM). However, at lower concentrations (1-3 mM) these drugs act selectively on calcium-activated potassium channels (K_{Ca}).*

concentration of K^+ ions, and it disappears at K^+ concentrations higher than 25 mM. Endothelium-dependent hyperpolarizations are associated with an increase in rubidium flux confirming the importance of potassium movement in the response. Nonselective potassium channel inhibitors, such as tetraethylammonium or tetrabutylammonium, prevent the hyperpolarization in the cell membrane of vascular smooth muscle cells. These observations concur, suggesting that endothelium-dependent hyperpolarization involves the opening of a potassium channel. In animal as well as human blood vessels, endothelium-dependent hyperpolarizations are insensitive to glibenclamide, indicating that they are independent of the activation of ATP-sensitive potassium channels. In most blood vessels, they are minimally or not at all affected by toxins that block either large conductance calcium-activated potassium channels (charybdotoxin, iberiotoxin) or small conductance calcium-activated potassium channels (apamin, scillatoxin) when given individually. However, hyperpolarizations can be prevented by the combination of charybdotoxin plus apamin *(Figure 3)*. Although the potassium conductance involved in the endothelium-dependent hyperpolarization in human blood vessels has not been identified with precision, the limited data available are consistent with observations already made in other species.[2,7]

IS EDHF A CYTOCHROME P450 DERIVATIVE?

In blood vessels from species such as the rat, guinea pig, and rabbit, endothelium-derived or exogenously added NO as well as prostacyclin (and its stable analog iloprost) hyperpolarize the vascular smooth muscle cells. Responses attributed to EDHF are observed in the presence of inhibitors of NOS and cyclooxygenase. Nevertheless, a residual release of NO and/or prostacyclin, due to an uncomplete inhibition of NOS and/or cyclooxygenase, is conceivable, and this could explain endothelium-dependent hyperpolarizations. However, hyperpolarization in response to NO, depending on the species, involves either ATP-sensitive potassium channels (sensitive to glibenclamide), or large conductance calcium-activated potassium channels (sensitive to iberiotoxin, charybdotoxin, or low concentrations of tetraethylammonium or tetrabutylammonium), while that evoked by prostacyclin involves ATP-sensitive potassium channels exclusively *(Figure 3)*. Therefore, the mechanism underlying these hyperpolarizations clearly differs from that attributed to EDHF.[2,7]

Molecules such as anandamide, carbon monoxide, hydroxyl radicals, and hydrogen peroxide are all putative EDHFs, as they are produced by the endothelial cells and induce hyperpolarization of the smooth muscle cells. However, the evidence confirming the role of these molecules as EDHF is either weak or inexistent.[3,7]

EDHF may be a short-lived metabolite of arachidonic acid, possibly produced through the cytochrome P450 monooxygenase pathway.[9] Inhibitors of this pathway inhibit endothelium-dependent relaxations resistant to inhibitors of NOS and cyclooxygenase in the perfused heart and kidney of the rat and in the isolated porcine and bovine coronary arteries. Some metabolites of arachidonic acid, formed through cytochrome P450, activate potassium channels in vascular smooth muscle cells.

Muscarinic agonists induce not only endothelium-dependent relaxation and hyperpolarization, but also the release of epoxyeicosatrienoic acids from the endothelial cells. These responses are inhibited by reasonably selective inhibitors of P450 monooxygenase. The cytochrome P450 metabolites produced by the endothelial cells increase the open-state probability of calcium-activated potassium channels sensitive to tetraethylammonium or charybdotoxin, and induce hyperpolarization of coronary arterial smooth muscle cells. Taken together, these observations support the hypothesis that epoxyeicosatrienoic acids could be EDHFs at least in blood vessels such as the bovine coronary artery *(Figure 3)*.[10]

However, in human coronary and omental arteries, the involvement of metabolites of arachidonic acid formed through cytochrome P450 is unlikely, as several inhibitors of the enzyme have no effect on the responses to EDHF. This is in agreement with what is observed in blood vessels of rats, guinea pigs, dogs, and pigs, in which chemically unrelated inhibitors of cytochrome P450 do not inhibit the EDHF responses, or do so in a nonspecific way.[11] Indeed, at high concentrations, inhibitors of cytochrome P450 are unspecific and can inhibit hyperpolarizations induced by potassium-channel openers such as levcromakalim.

In human renal arteries, the inhibition of the relaxation attributed to EDHF by two anesthetic agents, etomidate and thiopental, may indicate the involvement of cytochrome P450. Cultured endothelial cells from the human umbilical vein synthesized a transferable β-naphtoflavone–inducible hyperpolarizing substance.[12] However, activation of cytochrome P450 in human

endothelial cells may be a more general requirement for increasing the intracellular calcium concentration and thus the release of endothelium-derived factors such as NO and EDHF *(Figure 3)*.
The fundamental endothelial function of products of cytochrome P450 may confuse the issue when interpreting results of studies investigating the effects of inhibitors of cytochrome P450 on EDHF-mediated responses.[2,7]

Although its existence has been established, the identity of EDHF is still elusive. The possibility of multiple EDHF(s) depending on the species or the size of the blood vessel has to be considered *(Figure 3)*.

DO CARDIOVASCULAR DISEASES ALTER EDHF-MEDIATED RESPONSES?

In aging animals and in various animal models of diseases including hypertension, diabetes, and endotoxemia, as well as in humans (although the number of observations is limited), endothelium-dependent hyperpolarizations are diminished. The absence of endothelium-dependent hyperpolarization may contribute to the abnormal vascular responses observed under these pathologic conditions.[4,6] Conversely, enhancement of EDHF-mediated responses contributes to the antihypertensive and cardioprotective action of drugs such as angiotensin-converting enzyme inhibitors,[4] estrogen, diets rich in ω_3-unsaturated fatty acids, and exercise training.

CONCLUSION

Besides the release of NO and prostacyclin, endothelial cells cause hyperpolarization of the underlying vascular smooth muscle cells and this contributes to the endothelium-dependent relaxations. Studies in animal blood vessels show that this phenomenon is due to a diffusible factor, termed endothelium-derived hyperpolarizing factor (EDHF), which activates potassium channels on the smooth muscle cells.
The identity of EDHF and the exact nature of the potassium channel involved remain to be determined. Only through the development of selective inhibitors of the synthesis or action of EDHF, will its role be able to be evaluated fully in humans.

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Does endothelin play a role in hypertension?

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It is generally accepted that endothelin-1 (ET-1) may contribute to the pathogenesis of a number of cardiovascular diseases. Although still controversial, ET-1 may also be involved in blood pressure regulation as well as functional and structural changes in arterial hypertension. Beneficial effects of ET-1 antagonism in experimental models of hypertension have set the stage for the clinical use of ET-1–receptor antagonists. However, it is still a matter of debate whether selective ET_A or nonselective ET_A/ET_B-receptor antagonists provide the best approach to blocking the effects of ET-1 in man. Future head-to-head comparative, large-scale studies with these pharmacological agents will provide us with further insight into the pathophysiological role of ET-1 and eventually prove whether they represent a therapeutic advance in the treatment of our patients.

Keywords: blood pressure; endothelial dysfunction; nitric oxide; angiotensin II; vascular remodeling; endothelin-1 receptor antagonists

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It is becoming increasingly clear that locally synthesized or activated mediators play a major role in determining vascular tone and structure and may therefore influence the elevated peripheral vascular resistance, which is the hallmark of arterial hypertension.[1] In this context, the vascular endothelium, strategically positioned between the blood compartment and vascular smooth muscle cells, is an important modulator of vascular function through the release of potent substances capable of modifying adjacent cell function.[1] The possibility that the vascular endothelium might be involved directly in the increased peripheral resistance, via an enhanced release of constricting and/or decreased release of relaxing factors, remains an attractive hypothesis. Endothelin (ET) is a member of the endothelium-derived vasoactive factors with constrictor and proliferative properties. It is not surprising that ever since its discovery in 1988[2] the potential involvement of this potent vasoconstrictor in hypertension has stimulated research on its biological actions, their modulation by the hypertensive state, as well as hastened the development of specific antagonists.

To imply a major role for ET in the development and maintenance of hypertension, one would have to determine if the response to ET is altered in hypertension and its production increased, and whether or not ET antagonists may prevent or reverse hypertension.

BACKGROUND

There are three structurally related ET isopeptides in human and other mammalian species, named ET-1, ET-2, and ET-3.[2,3] ET-1 is mainly produced by endothelial cells.[3] The production of ET-1 is well characterized and involves the final transformation of big ET-1 catalyzed by a putative endothelin-converting enzyme (ECE) *(Figure 1).*[3] ET-1 is released in response to different stimulating agents. Among the stimuli capable of eliciting ET-1 release are low shear stress, thrombin, angiotensin II, vasopressin, norepinephrine, and transforming growth factor β.[3] ET-1 is the most potent vasoconstrictor known to date and typically produces vasoconstriction of conduit and resistance arteries in the range of 0.1 to 1 nmol/L. Most of this effect is mediated by ET_A receptors, but in some vascular beds including of humans, ET_B receptors may also be involved *(Figure 1).*[3] These are membrane-bound receptors consisting of seven transmembrane domains and are coupled to G-proteins. ET-1 is predominantly secreted towards the vascular smooth muscle cells, where it binds to ET_A and ET_B receptors and causes vasoconstriction with a potency 100-fold higher than norepinephrine. However, ET_B receptors are also expressed on endothelial cells and linked to nitric oxide and prosta-

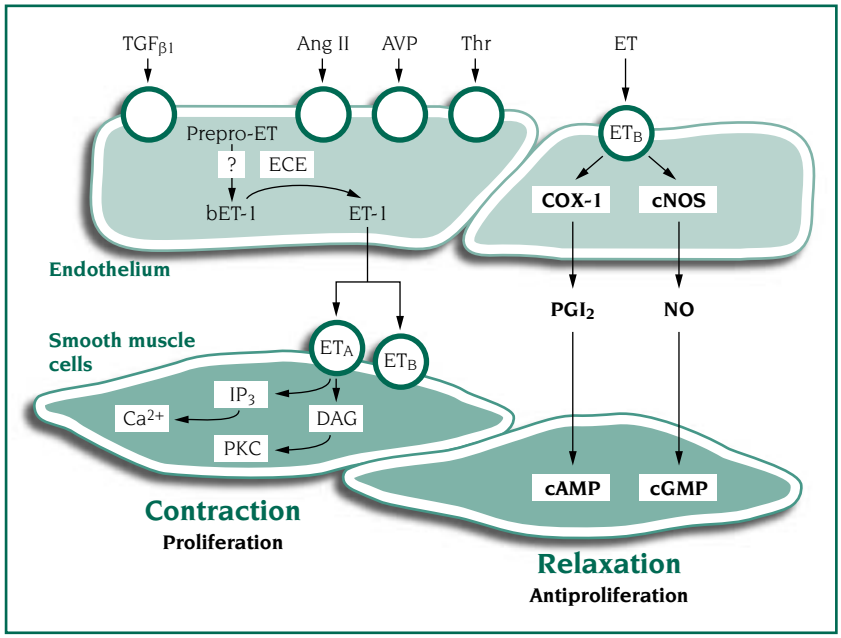

Figure 1. *The vascular endothelin system. Schematic diagram showing that production of ET-1 is stimulated by receptor-operated agonists (open circles). ET-1 can activate ET_A and ET_B receptors on vascular smooth muscle (mediating contraction and proliferation), and ET_B receptors on endothelial cells (mediating the release of NO and PGI_2). At the level of vascular smooth muscle, both NO and PGI_2 blunt or prevent ET-1–induced effects. Ang II, angiotensin II; AVP, arginine vasopressin; bET-1, big endothelin-1; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; cNOS, constitutive nitric oxide synthase; COX-1, cyclooxygenase-1; DAG, diacylglycerol; ECE, endothelin-converting enzyme; ET, endothelin; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; IP_3, inositol 1,4,5-triphosphate; NO, nitric oxide; PGI_2, prostacyclin; PKC, protein kinase C; Prepro-ET, preproendothelin; $TGF_{\beta 1}$, transforming growth factor β1; Thr, thrombin.*

cyclin formation *(Figure 1)*.[3,4] This explains why, in intact animals and in the human forearm circulation in vivo, ET-1 causes a transient vasodilatation at lower concentrations that precedes its pressor effect.[4]

Recently, an increasing number of ET-1–receptor antagonists have been synthesized. Certain compounds inhibit ET_A receptors only, while others interfere with both ET_A and ET_B receptors. The ET_A receptor has been implicated in the vasoconstrictive and mitogenic effects of ET-1, whereas ET_B-receptor activation has been shown to mediate either vasodilation or vasoconstriction. These observations have fueled the debate as to whether ET_A-receptor selective or combined $ET_{A/B}$-receptor antagonists would represent a more appropriate therapeutic strategy. Indeed, combined $ET_{A/B}$-receptor antagonists may prove to be better therapeutic agents, since they block both types of receptors. However, by antagonizing the ET_B receptor, these nonselective antagonists also inhibit ET-mediated endothelium-dependent vasorelaxation.[4]

BIOLOGICAL ACTIONS OF ET-1

In order to be a candidate for hypertension, a local vasoconstrictor is expected to exert a certain control on systemic arterial pressure. Both acute and chronic administration of exogenous ET-1 causes hypertension in rats and dogs.[3,4] Acute studies in man have also confirmed these findings. In healthy subjects, ET-1 infusion decreases, and receptor antagonism increases, forearm blood flow, indicating a role of ET-1 in the regulation of vascular tone.[4] In most forms of experimental hypertension, such as in spontaneously hypertensive rats (SHR),[3,4] N^G-nitro-L-arginine methyl ester (L-NAME),[5] deoxycorticosterone acetate (DOCA)-salt,[3] and angiotensin II,[6] as well as in essential hypertensive patients,[3] the maximal contraction to ET-1 is reduced in isolated vessels as compared to control arteries. However, these observations obtained either in conduit or resistance arteries of some vascular beds may not apply to all the vascular tree. Indeed, in the renal circulation, which is important for long-term arterial pressure regulation, the vasoconstrictor responses to ET-1 are maintained[7] or even increased[4] at least in the SHR. One study in cutaneous hand veins of hypertensive subjects suggested an enhanced response to ET-1.[3,4] In patients with essential hypertension, infusion of ET-1 induces a rise in blood pressure and systemic vascular resistance, while cardiac index and natriuresis are reduced.[8] Interestingly, normotensive offspring of hypertensive parents exhibit enhanced endothelin plasma responses to mental stress, indicating that genetically determined activation of the ET system is already present at this early stage of disease.[9] Furthermore, ET-1 gene expression is enhanced in small arteries of patients with moderate-to-severe hypertension, whereas the expression is similar in control subjects and patients with untreated mild hypertension.[10]

In addition to direct vasoconstriction, ET-1 may also facilitate the vascular contraction to other agonists. Indeed, very low concen-

trations of this peptide, which by themselves exert no significant contraction, can amplify contractions to norepinephrine and serotonin in rats as well as in humans.[3,4]

ET-1 has also been reported to be a mitogen and a trophic factor[3,4] and could therefore be involved in the modification of the structure of the cardiovascular system. These more permanent changes of the vasculature (vascular remodeling) may then contribute to the maintenance of hypertension by increasing peripheral resistance and enhancing the effect of other vasoconstrictors. In contrast to eutrophic remodeling, which seems to occur through a rearrangement of a similar amount of vascular material around a smaller lumen, hypertrophic remodeling involves an increase in mass of the vessel wall (increased cross-sectional area). The proliferative properties of ET-1 may be involved in the latter process and contribute to hypertension, independently of the direct and indirect vasoconstriction it produces.

Several studies have shown an association between the local content of ET-1 and hypertrophy of small arteries. In DOCA-salt hypertensive rats, hypertrophy is associated with an increased production of ET-1 in mesenteric arteries *(Figure 2)*, even if the circulating levels of the peptide are normal.[3] In arteries from L-NAME[11] and SHR[3] models of hypertension, characterized by eutrophic remodeling, there is no increase in local concentration. Treatment of SHR with DOCA and salt,[3] but not with L-NAME,[12] produces overexpression of local endothelial ET-1 and hypertrophic remodeling of resistance arteries. We have recently found that the chronic administration of angiotensin II increases the concentration of ET-1 in mesenteric arteries and

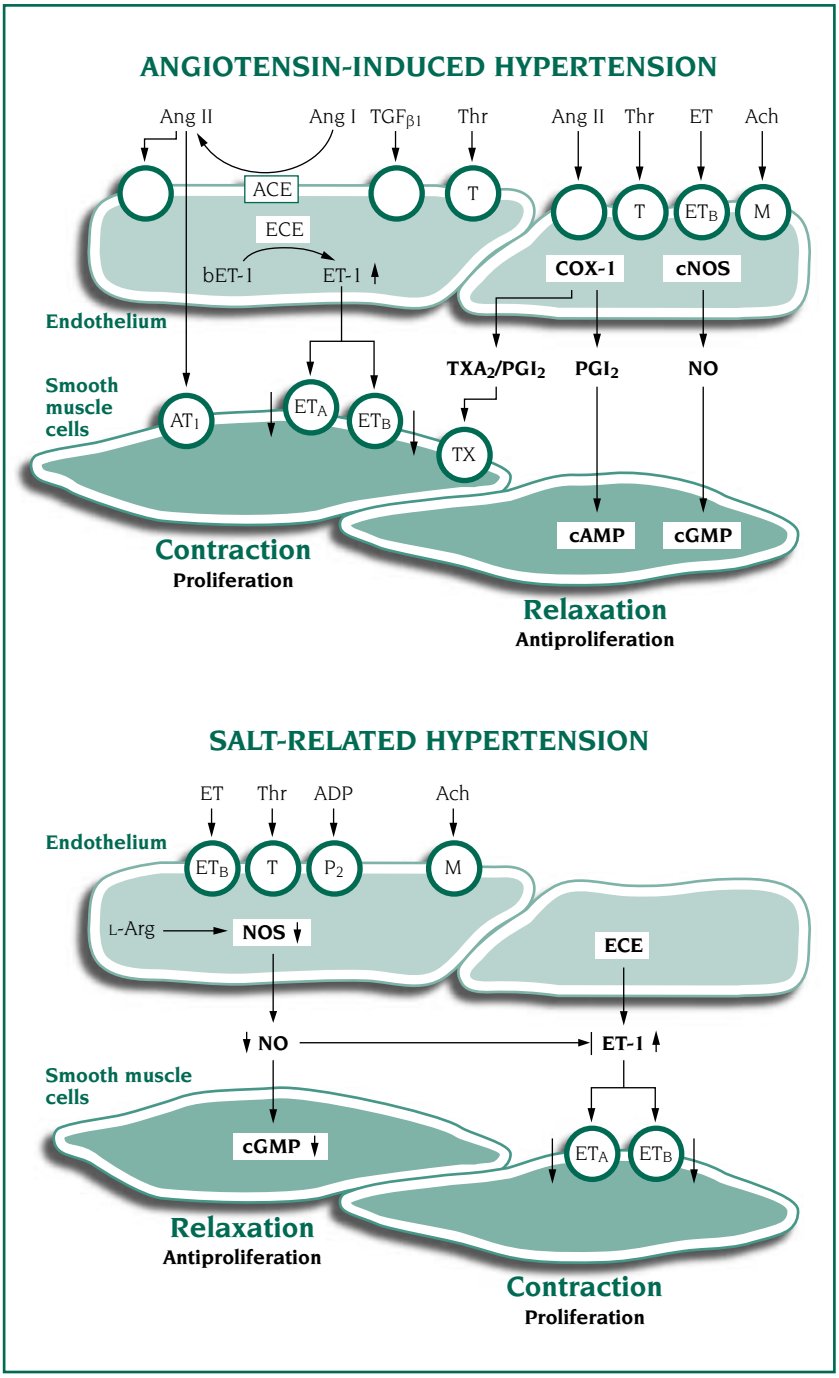

***Figure 2.** Endothelial function and hypertension. In angiotensin-induced hypertension, the concentration of ET-1 is increased and this may shift the balance among different endothelium-derived vasoactive factors in favor of vasoconstrictive and proliferative mediators. Indeed, local ET-1 levels in this model were related to endothelial dysfunction and vascular hypertrophy. In salt-related hypertension (Dahl rats, DOCA-salt hypertension), NO production is reduced, whereas production of ET-1 is significantly increased. Ach, acetylcholine; ACE, angiotensin-converting enzyme; ADP, adenosine diphosphate; Ang I, angiotensin I; Ang II, angiotensin II; bET-1, big endothelin-1 ; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; DOCA, desoxycorticosterone acetate; ECE, endothelin-converting enzyme; ET, endothelin; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; L-Arg, L-arginine; M, muscarinic receptor; NO, nitric oxide; NOS, nitric oxide synthase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; Thr, thrombin; TXA_2, thromboxane A_2.*

that this is associated with vascular hypertrophy *(Figure 2)*.[6,13] In contrast to these findings, renovascular models of hypertension seem to overexpress ET-1 when the renin-angiotensin system is not activated. Indeed, the normo-renin 1-kidney/1-clip hypertensive rats express ET-1 in an exaggerated fashion, while 2-kidney/1-clip hypertensive rats have normal expression and high renin activity.[14]

Taken together, these results suggest that the association between hypertrophic remodeling of resistance arteries and local ET-1 content is rather consistent and does not seem to depend on the level of arterial pressure. It is therefore of interest that in essential hypertensive patients, eutrophic remodeling prevails and there is no increase in the local concentration of ET-1. However, in patients suffering from severe hypertension, ET-1 is overexpressed in subcutaneous arteries and the vessels exhibit the characteristics of hypertophic remodeling.

PLASMA LEVELS OF ET-1

The circulating levels of ET-1 are not increased in most models of experimental or in human hypertension,[3,4] although a recent study suggests that there may be racial differences. Specific activation of ET in African-Americans, who often present with severe and salt-sensitive (low-renin) hypertension, points to severity and salt-sensitivity as important denominators of the activation of the ET system in hypertension.
It must be noted, however, that plasma levels may not represent a good index of local vascular production of ET-1, since more than twice as much ET-1 is released abluminally by endothelial cells.[3,4] This is in agreement with the increasing understanding of ET-1 as a hormone that acts in a paracrine fashion. Plasma levels of ET-1 were correlated with arterial pressure only in patients with ET-secreting hemangioendotheliomas.[3,4] Thus, in these rare cases, it appears that circulating ET-1 does indeed have a confirmed blood pressure–raising effect in humans.

EFFECTS OF ENDOTHELIN ANTAGONISTS IN HYPERTENSION

Although we have suggested that angiotensin II is an in vivo stimulus for ET-1 production *(Figure 2)*,[6] the stimuli involved in other models of hypertension and in man have yet to be determined. In addition, the vascular structure may have an impact on the development and maintenance of hypertension, but the relationship between ET-1 and vascular hypertrophy, as well as the vascular effects of exogenous ET-1, represent only indirect evidence for a role of ET-1 in hypertension. Experiments with ET-1 receptor antagonists provide a more definitive assessment of its involvement.

Phosphoramidon, an inhibitor of endothelin-converting enzyme, reduces blood pressure in the stroke-prone SHR.[3,4] However, because this agent has a multiplicity of pharmacological effects, its blood pressure–lowering action cannot be attributed entirely to inhibition of the conversion of big-endothelin to ET-1. With the development of specific ET-1–receptor antagonists for ET_A and ET_B receptors,[3,4] research tools to better address this question have become available. Experimental findings suggest that ET-1 may be differently involved in different forms of hypertension. Indeed, in some animal models, such as the DOCA-salt hypertensive rat, ET-1–receptor blockade causes marked reduction in blood pressure, and this is also associated with regression of vascular hypertrophy.[3] Accordingly, ET-1 secretion is augmented in cultured endothelial cells from this model. However, the effects of ET-1 antagonism in other experimental models of hypertension, notably the SHR, are less clear. In the SHR, both circulating and vascular ET-1 as well as ET-1 tissue content of the renal medulla are reduced.[3] By contrast, in the stroke-prone SHR, the endothelin axis is activated, and ET-1 antagonism significantly reduces blood pressure and prevents cardiac and vascular hypertrophy.[15] In addition, in Dahl salt-sensitive rats, ET-1 levels are increased and ET-1 antagonists lower blood pressure, indicating that the ET system is particularly activated in severe, salt-sensitive hypertension *(Figure 2)*.[16]
Most interestingly, selective ET_A-receptor antagonism reduces blood pressure, vascular hypertrophy, and endothelial dysfunction in angiotensin II–induced hypertension.[6,13] These data strongly suggest that ET-1 antagonists may be of particular value in conditions of increased activity of the renin-angiotensin system. This is consistent with additional hypotensive effects of ET-1 receptor antagonism in hypertensive dogs already treated with an angiotensin-converting enzyme (ACE) inhibitor.[17] The discrepancy between the beneficial effects of selective ET_A-receptor blockade in angiotensin II–induced hypertension and the lack of effects in the 2-kidney, 1-clip model (high-renin model resembling renovascular hypertension in man) is difficult to reconcile. However, it is possible to speculate that there is a lower activation of the tissue renin-angiotensin system and/or enhanced activity of vasodilatory pathways preventing increased ET-1 production in this condition.

In nitric oxide–deficient hypertension induced by N^G-monomethyl-L-arginine (L-NMMA) or L-NAME, ET-1 production is enhanced, but the peptide is only involved in the increase in blood pressure under acute and not chronic conditions.[18] Interestingly, systemic hypertension induced by hepatic overexpression of human preproendothelin-1 in rats was reduced by an ET_A antagonist.[19]

GENE KNOCKOUT AND TRANSGENIC MODELS FOR ET-1

To further elucidate the role of ET-1, gene knockout and transgenic models have been developed. After targeted disruption of the ET-1 gene in mice, all homozygotes died, but surviving heterozygotes exhibited significant decrease in ET-1 levels, and, surprisingly, elevated blood pressure.[3] Since ET-1–deficient mice suffer from severe craniofacial malformations, they are therefore hypoxemic, which could contribute, via stimulation of the sympathetic nervous system, to the apparently paradoxical blood pressure increase independently of the changes in ET-1 production. ET-2 transgenic rats exhibit elevated ET plasma levels, but do not develop hypertension.[3] However, since the transgene is not expressed in the endothelium, these experiments do not contradict the hypothesis that in pathologic conditions overexpression of ET-1 in the blood vessel wall may play a pathogenic role in hypertension.

CONCLUSION

There is mounting evidence from animal and human studies that ET-1 is involved in the pathogenesis and maintenance of hypertension, at least in salt-sensitive and severe forms of the disease. First clinical trial evidence is now available demonstrating that combined $ET_{A/B}$-receptor antagonism effectively lowers blood pressure in patients with mild-to-moderate hypertension.[20] Indeed, the antihypertensive effect of bosentan was comparable with that of the ACE inhibitor enalapril.[20] There is an urgent need for intensive investigation with combined $ET_{A/B}$ and selective ET_A receptor antagonists to confirm these findings, determine the best approach to blocking the ET-1 axis in human hypertension, and characterize subsets of hypertensive patients who might benefit more from such pharmacological agents.

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Does bradykinin play a role in the regulation of vascular tone in humans?

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Recent experimental studies suggest that bradykinin, which induces endothelial release of nitric oxide (NO), prostacyclin, and/or endothelium-derived hyperpolarizing factor, plays an important role in the regulation of vascular tone at rest and during flow-stimulated conditions. In humans, endothelium-dependent vasodilation induced by bradykinin can be blocked by specific B_2-receptor blockers, and, in part, by NO synthase. Endogenous bradykinin contributes in an important way to the regulation of coronary vascular tone under resting and flow-stimulated conditions, in human peripheral and coronary arteries.
The beneficial effects exerted by the angiotensin-converting enzyme (ACE) inhibitors in heart failure and coronary artery disease could in part be explained by an increased availability of bradykinin, hence improved endothelial function, since ACE is identical to kininase II, which degrades bradykinin.

Keywords: endothelium; nitric oxide; flow-dependent vasodilation; endothelium-derived hyperpolarization factor; bradykinin; vascular tone; ACE inhibition

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The endothelium plays a fundamental and obligatory role in the regulation of vascular tone throughout the circulation as a result of the release of a variety of substances that modulate the contractile behavior of underlying vascular smooth muscle cells.[1] The phenomenon of endothelium-dependent relaxation has been demonstrated in human arteries[2] and has been attributed to the actions of the endogenous vasodilators such as endothelium-derived nitric oxide (NO) and prostacyclin.[1,3] Recent evidence has also emerged of an endothelium-derived hyperpolarizing factor (EDHF),[4] which also causes vasodilation and is distinct from NO and prostacyclin.[4,5] NO is released constantly in the basal state,[6] but the release of endothelium-derived vasodilators is also influenced by dynamic factors. The change in vascular tone in response to changes in flow,[7] which has been documented in the human coronary and peripheral arteries,[8] is also mediated by endothelium-dependent mechanism(s).[9] While the importance of the endothelium in regulating the aggregate hemodynamic properties of vascular networks is therefore beyond doubt,[10] the specific role of the different endogenous agents in mediating vasodilator responses in the human coronary circulation remain poorly understood.

Bradykinin is a vasoactive kinin that is liberated from its substrate kininogen by the action of kallikrein[11] and that is known to be involved in a wide range of biological processes. Bradykinin is a potent vasodilator which acts through endothelial bradykinin B_2 receptors to stimulate the release of endothelium-derived NO, prostacyclin,[12] and EDHF.[4] It was shown previously that bradykinin is released from endothelial cells[13,14] and that cultured human endothelial cells are able to generate vasoactive kinins in basal conditions.[15] In addition, there is evidence of basal bradykinin release in the heart[16] and of an endogenous kininogen/kinin system within the vascular wall.[17] The local concentration of bradykinin is affected by several enzymes, such as kininase II (angiotensin-converting enzyme) or the enzymes that degrade bradykinin to inactive peptides. Thus, there is evidence of an endogenous kininogen/kinin system within the vascular wall, raising the possibility that bradykinin plays an important role in mediating vasomotor responses in vivo. Numerous experimental studies have addressed the vascular effects of bradykinin and have reported physiological vascular effects of bradykinin in animal models. However, the role of bradykinin in modulating vascular tone in humans remains controversial. There is evidence that physiological doses of bradykinin exert vasodilator

effects in the human circulation, both in the forearm vasculature and the coronary circulation. This effect can be blocked either by specific bradykinin B_2 receptor blockers[18] or by the inhibition of NO synthesis.[19,20] Kuga et al showed that intracoronary infusion of bradykinin dilated human epicardial coronary arteries in vivo, and that the vasodilator effect of bradykinin was impaired in stenotic coronary arteries.[21] The degree of endothelium-dependent relaxations to bradykinin appears to be comparable between large vessels and microvessels. However, the contribtion of NO and EDHF is markedly dependent on vessel size: in large arteries, both NO and EDHF were shown to contribute equally to the vasorelaxation, whereas in microvessels most of the vasorelaxation was attributed to EDHF.[22] These in vitro observations have been confirmed, in part, by in vivo studies, which showed that the intracoronary infusion of bradykinin increased coronary diameter, but that the latter was significantly attenuated by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis.[23,24] Systemic plasma levels of bradykinin are very low, but can now be measured by sensitive techniques.[25] Systemic administration of bradykinin lowers blood pressure in a dose-related manner through marked reduction in peripheral vascular resistance.[26] The release of NO by bradykinin has been confirmed in vivo by direct measurement of NO in the hand circulation of healthy volunteers.[27] These studies in humans in vivo or in human vessels in vitro have clearly confirmed a large body of experimental data hat bradykinin is a vasodilating agent by release both prostaglandins, NO, and EDHF. However, these studies did not elucidate the role of endogenously produced bradykinin (or kinins) in the regulation of vascular tone in humans. The determination of the relative contribution of bradykinin to the regulation of vascular tone in humans in vivo has only recently been possible since the development of selective bradykinin antagonists, specifically those directed at the B_2 receptor through which bradykinin mediates its endogenous physiological actions. In this regard, the development of D-Arg[Hyp^3, Thi^5,D-Tic^7,Oic^8]bradykinin (HOE 140) has represented a major advance since it has been shown to be highly specific, to be 500 times more potent than the early bradykinin B_2 receptor antagonists,[28,29] and to abolish bradykinin-mediated cardiac effects in rats as well as inhibit the vasodilatory actions of exogenous bradykinin in man.[18]

A study carried out by us in 1995 represented the first time HOE 140 was used in the human coronary circulation, and was the first demonstration of the endogenous role of bradykinin in human coronary vasomotor control.[30] This study evidenced a consistent reduction in epicardial coronary area following bradykinin B_2 receptor blockade. Since flow is largely determined by changes in the caliber of resistance vessels (those less than 400 µ in diameter[31]), the reduction in coronary blood flow implies that endogenous bradykinin is also important in the regulation of normal basal vascular tone at the level of resistance vessels. These findings are consistent with the results of experimental studies that have also shown that bradykinin B_2 receptor blockade leads to a significant reduction in coronary blood flow in normotensive rats.[32] Similarly, Koller el al[33] showed that HOE 140 decreased the basal diameter of muscular arterioles, suggesting that bradykinin actively participated in the development of basal vascular tone in skeletal muscle microcirculation. In contrast, in isolated perfused human placenta, bradykinin was found to induce a thromboxane-mediated constriction,[34] suggesting that there were differences in the effects and mechanisms of action of bradykinin in different vascular regions. Our aforementioned study[30] also showed that, in addition to the influence on basal coronary tone, HOE 140 reduced the flow-dependent dilator response to papaverine. Flow-dependent dilation was thus shown to be an important mechanism in the regulation of the aggregate hemodynamic properties of vascular networks, and its presence in resistance as well as epicardial vessels was confirmed by Kuo et al.[35] The degree of flow-dependent dilation observed by us[30] (23.4±7.0%) was equivalent to that reported in our previous studies in patients with normal coronary arteries,[8] and the magnitude of the effects of HOE 140 on basal epicardial coronary area correlated significantly with those on flow-dependent dilation at the same site in the proximal vessel. These findings imply that the vasoconstrictive effects of the bradykinin B_2 receptor antagonist may be due to suppression of the effects of endogenous bradykinin released in response to increases in flow both at the level of conduit and resistance vessels. Indeed, Mombouli and Vanhoutte[36] showed that a bradykinin antagonist decreased the basal production (or release) of EDRF in perfused canine carotid arteries, suggesting the existence of local kinin-generating system(s) that would contribute to basal flow-dependent release of EDRF.

In our aforementioned study,[30] assessment of flow-dependent dilation before and after HOE 140 showed that there was a tendency for the papaverine-induced increase in blood flow to be smaller in the

presence than in the absence of the bradykinin B_2 receptor agonist. We had previously shown that repeated measurements of papaverine-induced flow-dependent dilation were highly reproducible in the same patient,[8] and experimental studies evidenced a linear relationship between the extent of increase in flow and the subsequent dilator response.[7] We observed[30] that the maximal blood flow increase in response to papaverine was similar before and after HOE 140 in half the patients studied and that in these, the degree of reduction in flow-dependent dilator response was comparable to that in the group as a whole. Therefore, the apparent reduction in flow-dependent dilation after HOE 140 was unlikely to merely reflect the change in papaverine-induced coronary flow reserve after bradykinin B_2 receptor blockade. Although the underlying mechanisms by which HOE 140 influences coronary vasomotor tone cannot be determined with certainty, the absence of any significant changes in blood pressure or heart rate during HOE 140 administration seems to indicate that the vasomotor response to HOE 140 is indeed attributable to the suppression of the local vasodilator actions of bradykinin rather than to compensatory hemodynamic mechanisms. Similarly, the presence of a normal dilator response to the endothelium-independent vasodilator nitroglycerin after HOE 140 suggests that its effects do not result from a change in vascular smooth muscle cell sensitivity. The vasodilator actions of bradykinin are largely mediated by the stimulated release of endothelium-derived NO, prostacyclin, and EDHF,[4] and it is therefore likely that HOE 140 acts by reducing the endogenous bradykinin-stimulated release of one or more of these endothelium-derived vasodilators. Human vessels obtained from the operating room have extended these findings by showing that the vasodilator effect of bradykinin is related, in part, to EDHF: previous observations have shown that the endothelium-dependent hyperpolarization response to bradykinin occurs in human coronary arteries from patients with different cardiac diseases including dilated and ischemic cardiomyopathy.[4] These data suggest that endogenous bradykinin has a role in modulating coronary tone in healthy human vessels as well as diseased arteries. Interestingly, while bradykinin appears to modulate both the basal and flow-mediated vasomotor tone in the coronary circulation, its role in the forearm vasculature was restricted to flow-mediated vascular response.[37]

Angiotensin-converting enzyme (ACE) inhibitors have undoubtedly become a cornerstone in the treatment of heart failure and are known to exert beneficial effects in patients with coronary artery disease.[38,39] So far, the beneficial effects of ACE inhibitors have been attributed to a reduction in angiotensin II and norepinephrine levels.[40] However, since ACE is identical to kininase II which inactivates bradykinin, ACE inhibition not only reduces angiotensin II, but is also associated with increased levels of bradykinin.[25] The contribution of kinins to the hypotensive effect of ACE inhibitors has been postulated,[41] but never established in humans for want of a bradykinin receptor antagonist suitable for use in humans. In this regard, the development of the bradykinin B_2–receptor antagonist HOE 140, now known as icatibant, has provided a valuable tool since it has been shown to be highly specific.[28] Furthermore, in humans, icatibant has been shown to inhibit bradykinin-induced vasodilation in the forearm resistance vessels.[18]

Experimental studies have shown that ACE inhibitors stimulate the endothelial release of NO and prostacyclin by a bradykinin-mediated mechanism,[42] thereby enhancing endothelial-dependent vasodilation. These experimental studies raised the question of whether or not ACE inhibition may improve endothelial function in humans and whether the potential beneficial effect of ACE inhibition is bradykinin-mediated. To answer these questions, we performed experiments in healthy volunteers and examined the effects of the ACE inhibitor quinaprilat, the selective bradykinin B_2–receptor antagonist icatibant, and their combination, on resting tone and flow-dependent, endothelium-mediated vasodilation of the radial artery in healthy volunteers. The major result was that ACE inhibition enhances flow-dependent, endothelium-mediated dilation in humans by a bradykinin-dependent mechanism *(Figure 1)*.[37] Icatibant attenuated flow-dependent, endothelium-mediated dilation of the radial artery consistent with our previous observations in coronary arteries.[30] Similarly, experimental studies have shown that a bradykinin antagonist decreases the production or release of endothelium-derived relaxing factors in isolated perfused arteries.[36] Conversely, quinaprilat, but not placebo, improved flow-dependent, endothelium-mediated dilation, an effect that was completely abolished during infusion of icatibant. Thus, our data support the concept that ACE inhibitors exert endothelium-dependent vascular effects related to increased local concentrations of endogenous kinins. In this respect, it is noteworthy that administration of ACE inhibitors causes an increase in plasma levels of bradykinin in humans.[25]

These observations may explain some of the beneficial effects of

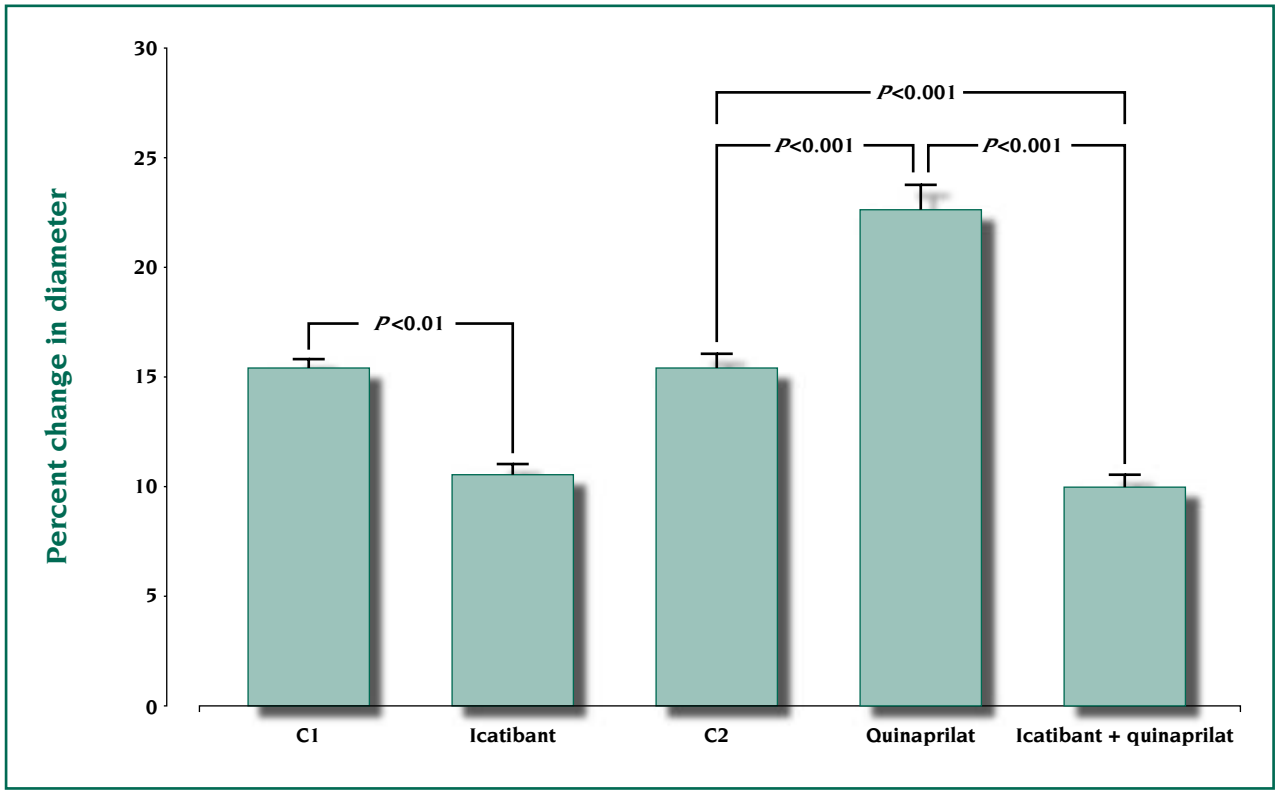

Figure 1. *Effect of bradykinin antagonism and ACE inhibition on flow-dependent dilation. Percentage change in radial artery diameter during reactive hyperemia (flow-dependent dilation) at control measurements 1 and 2 (C1 and C2) and during infusion of icatibant (HOE 140), quinaprilat, and both icatibant (HOE 140) and quinaprilat. Reproduced from ref 37: Hornig B, Kohler C, Drexler H. Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans.* Circulation. *1997;95:1115-1118. Copyright © 1997, American Heart Association. With permission.*

ACE inhibition, including those related to coronary artery disease. Two megatrials have shown that ACE inhibition reduces mortality and recurrence of angina after myocardial infarction.[38,39] Risk reduction in these trials was significant after 1 to 1.5 years of treatment, raising the question of whether or not ACE inhibition improves vascular function rather than the degree of coronary artery stenosis. In this respect, it is interesting to note that experimental[43] and clinical data[44] have shown that ACE inhibition restores coronary flow reserve, probably by a bradykinin-dependent mechanism, as suggested by the experimental study. It is conceivable that an improved endothelial function, related to accumulation of bradykinin, might provide vascular protection during long-term treatment with ACE inhibitors. The results of the Trial on Reversing ENdothelial Dysfunction (TREND)[45] support this concept. In this study, the effect of ACE inhibition on coronary artery endothelial function was determined in patients with established coronary atherosclerosis. In the quinapril-treated group, the initial vasoconstrictor response to intracoronary infusion of acetylcholine was dramatically reduced and, in part, normalized to a vasodilator response, whereas no change was observed in the placebo-treated group. These results suggest that quinapril, an ACE inhibitor with high tissue-binding affinity, attenuates impaired endothelial function in patients with coronary artery disease. This beneficial effect of long-term ACE inhibition could be due to reduction of angiotensin-II and/or an increase in bradykinin. Inhibition of angiotensin II generation may attenuate smooth muscle contraction and generation of superoxide anions through stimulation of NADH/NADPH oxidase systems of smooth muscle cells.[46] The latter would inactivate endothelium-derived NO and thereby cause endothelial dysfunction. In addition, bradykinin-induced augmentation of NO release by endothelial cells is promoted by ACE inhibition.

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Endothelium

Summaries of Ten Seminal Papers

1

The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine

R.F. Furchgott, J.V. Zawadzki. *Nature*. 1980

2

Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide

L.J. Ignarro and others. *Proc Natl Acad Sci USA*. 1987

3

Vascular endothelial cells synthesize nitric oxide from L-arginine

R.M. Palmer and others. *Nature*. 1988

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A novel potent vasoconstrictor peptide produced by vascular endothelial cells

M. Yanagisawa and others. *Nature*. 1988

5

Crucial role of endothelium in the vasodilator response to increased flow in vivo

U. Pohl and others. *Hypertension*. 1986

6

Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries

M. Nakashima and others. *J Clin Invest*. 1993

7

Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man

P. Vallance and others. *Lancet*. 1989

8

Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries

P.L. Ludmer and others. *N Engl J Med*. 1986

9

Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta

R.L. Minor Jr and others. *J Clin Invest*.1990

10

Different interactions of platelets with arterial and venous coronary bypass vessels

Z.H. Yang and others. *Lancet*. 1991

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The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine

R.F. Furchgott, J.V. Zawadzki

Nature. 1980;288:373-376

This is where "EDRF" (endothelium-derived relaxing factor) began—though nowhere in the text does the term itself appear. The paper is a classic. It elucidated the paradox, long known to pharmacologists, that acetylcholine was a vasodilator in vivo, but only contracted isolated preparations in vitro. Not for the first time in reseach, it was a chance observation, followed up. If the story be true, Zawadzki, Furchgott's technician, obtained an unfamiliar relaxant response due, as it transpired, to unusually careful preparation of arterial strips and preservation of their delicate endothelial layer. Such folklore attaches perhaps more readily to milestone studies.

The simple series of pharmacological experiments showing that the explanation lay in the endothelium opened a major new chapter in cardiovascular physiology.
The study elegantly showed that the relaxant action of acetylcholine in preconstricted rabbit aorta strips with endothelium carefully preserved was abolished in strips with endothelium removed by gentle rubbing or collagenase. The relaxation was independent of the agent used to preconstrict the strip, it was related to the concentration of acetylcholine, and its loss was quantitatively related to the histologically measured loss of endothelial cells. "Sandwich" experiments were devised that showed that the relaxant effect was mediated by release of an extracellular agent. These neatly took advantage of muscle cell alignment whereby longitudinal strips contributed negligibly to force as measured. Longitudinal strips, with endothelium intact, were mounted intimal surface–to–intimal surface with transverse strips denuded of endothelium, and were able to restore endothelium-mediated relaxant responses to acetylcholine in the endothelium-denuded transverse strips. Further experiments excluded prostacyclin, cAMP, and cGMP as the agent responsible. Release of the agent was found to be oxygen-dependent. These findings were confirmed in a number of different mammalian species and artery types. The authors concluded that the potent vasodilator effect of acetylcholine in vivo was likely to be mediated by its action on the endothelium.

A relaxant effect of acetylcholine had been reported nearly 20 years earlier, but not recognized as endothelium-dependent. To those working on this phenomenon, this publication came as a "Eureka" moment. It defined a novel endogenous intercellular signaling system, whose further characterization and physiological and pathophysiological consequences were to grow exponentially over the years to come. The paper showed that acetylcholine was in effect a "double agent" in that it stimulated endothelium to release an agent that relaxed vascular smooth muscle and that it also stimulated vascular smooth muscle directly to contract: its net effect could thus be either vasodilatation or vasoconstriction, depending on whether endothelium was present and functionally normal or not. Acetylcholine came to be used as a standard laboratory and clinical investigation tool with which to test whether endothelium was functionally present or not (with due attention to dose-response, for at higher dose contraction tends to override dilatation).

With growing recognition of the physiological and pathophysiological importance of endothelium—"that marvellous factory" in Sir John Vane's words—the measurement of endothelial function is receiving increasing attention. Acetylcholine-induced responses remain a useful first approach, despite the fact that we now know that acetylcholine is but one of many agonists which can stimulate EDRF release, that physiologically relevant flow-related increase in EDRF involves different signaling pathways, that endothelial functions embrace more than the release of EDRF, and that acetylcholine-induced relaxation is only partly due to the factor we now know to be nitric oxide. This was the paper which introduced the saga of EDRF—and nitric oxide—and all that is following therefrom.

1980

TV addicts at last discover who shot "JR in "Dallas"; Mount St Helens (USA) erupts after being dormant for 120 years; and Jean-Paul Sartre, French philosopher and writer, dies, aged 74

Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide

L.J. Ignarro, G.M. Buga, K.S. Wood, R.E. Byrns, G. Chaudhuri

Proc Natl Acad Sci USA. 1987;84:9265-9269

Following the discovery of endothelium-derived relaxing factor (EDRF), the chase was on to identify it. Its further characterization led gradually to a growing realization that it had much in common with nitric oxide (NO), including its very short half-life, the association of its relaxant action with elevation of cGMP levels, and inhibition of its action by methylene blue or hemoglobin. This paper showed experimentally that the effects of authentic NO and EDRF were indistinguishable. It stands as a point of focus in the important discovery of NO as a biological agent, a discovery, as is often the case, towards which a number of different groups were converging.

A cascade bioassay was used to assay the effects of effluent from superfused segments of bovine coronary artery and vein, on preconstricted strips of endothelium-denuded artery or vein. These detector strips were arranged in series, thereby introducing 2-second delays between successive strips. The effects of endothelial effluent, basally and after stimulation by acetylcholine or the calcium ionophore A23187, and those of NO (prepared in oxygen-free solution) were directly compared. The half-lives of EDRF and NO were identical at 3.5 seconds, as previously reported by others. Their dilator effects were decreased by superoxide and increased by superoxide dismutase similarly. They were inhibited similarly by methylene blue (which inhibits soluble guanylate cyclase) and by hemoglobin (which competes with soluble guanylate cyclase for NO). They elevated tissue cyclic GMP levels similarly and in association with dilator responses that were similar. EDRF was also detected as NO by a spectrophotometric chemical method at quantitatively appropriate levels, and by reaction with hemoglobin to form nitrosylhemoglobin. The calcium ionophore A23187 stimulated endothelial cells, like vascular strips, to produce NO as thus measured. These data therefore provided direct evidence that EDRF was NO or possibly an unstable nitroso compound such as an S-nitrosothiol, between which the assay used could not distinguish. Proponents of the latter theoretical possibility have, with the passage of time, become less actively supportive, however, and the consensus now seems to have settled on the assumption that EDRF is indeed NO. NO does, however, bind reversibly to circulating thiols, such as those in albumin, which may thus serve as a buffer source of low-level NO, though the physiological relevance of this has not been explored.

That the simple molecule NO, a gas, could be a biological messenger was an important new concept. Not for the first time, the biological prototype of a pharmacological agent—in this case, the nitrovasodilator drugs—came to be discovered later. Much of the pharmacology of the nitrovasodilators was already known and could thus now be applied to EDRF, eg, its activation of soluble guanylate cyclase to elevate intracellular levels of cGMP and the consequences of this. It has become apparent that NO is of very primitive evolutionary origin. It is also now known to serve a multitude of physiological roles throughout mammalian cardiovascular systems, let alone in other systems and other organisms. It modulates vascular and myocardial contraction in novel and physiologically elegant ways. Its very short biological half-life, confirmed in this study, is central to its integrating role in coordinating changes in vascular diameter throughout the vascular bed in response to changes in flow. Indeed, it may be seen in many ways to contribute to cardiovascular "efficiency," measured in terms of tissue perfusion relative to cardiac work under differing hemodynamic conditions. It prevents adhesion of platelets and white blood cells to endothelium and its production in high concentration by activated leukocytes is part of their defense mechanism. The discovery of NO was indeed a landmark in biology. It has opened a new chapter in cardiovascular physiology and pathophysiology.

1987

General Motors' "Sunraycer" wins the first solar-powered car race in Australia;
"Platoon" wins the Best Picture Oscar;
and Rudolf Hess, Hitler's deputy from 1933 to 1941, dies, aged 93

Vascular endothelial cells synthesize nitric oxide from L-arginine

R.M. Palmer, D.S. Ashton, S. Moncada

Nature. 1988;333:664-666

Palmer et al took the endothelium-derived relaxing factor (EDRF) story an important step further by establishing the metabolic precursor for nitric oxide (NO) production as L-arginine and, on the basis of its strict structural and isomeric specificity, pointed to an enzymatic step in its production.

Two-week cultures of porcine aortic endothelial cells were incubated for a further 24 hours with or without L-arginine in the medium. The superfusion effluent was bioassayed for relaxant activity on preconstricted rabbit aortic strips denuded of endothelium, and its NO content was measured by chemiluminescence (of NO and NO_2^-) and by mass spectrometry of ^{15}NO following incubation with ^{15}N-labeled L-arginine. Effluent effects were compared with those induced directly by 50 nM glyceryl trinitrate (GTN) or by 44 nM NO. Prostacyclin was measured by radioimmunoassay of its stable breakdown product.

Addition of L-arginine (10 µmol) to the buffer increased its relaxant effect and its NO content (about 2-fold, with EC_{50} 3.5 µmol), but only if the cells had been deprived of L-arginine for 24 hours. D-Arginine was without effect. NO release from arginine-deprived cells stimulated by bradykinin was also enhanced by L-citrulline, but to a smaller extent (about 1.5-fold) and with 10-fold higher EC_{50} (35 µmol). Other amino acids were without effect. Control experiments showing that L-arginine infused directly onto detector strips was without effect confirmed that its enhancement of relaxant activity of endothelial cells was not due to enhancement of vascular smooth muscle responsiveness to released NO. Stimulation of the endothelial cells by the calcium ionophore A23187 resulted in more prolonged enhancement of NO release than with bradykinin, enabling the demonstration that it could be immediately and reversibly enhanced by coadministration of L-arginine, whereas L-citrulline resulted in only a slow and partially reversible response—evidence consistent with conversion to L-arginine as the directly contributory substrate. Prostacyclin release was uninfluenced by L-arginine. Radiolabeling of L-arginine showed that it was the terminal guanidino nitrogen atom that formed NO.

This study paved the way for the use of nonmetabolized analogs of L-arginine to block the production of NO. It provided a more specific inhibitor than hemoglobin, which competes with the heme moeity of soluble guanylate cyclase for NO and had been used previously, and enabled measurement of tonic NO activity. It identified the component of induced change, eg, in flow-mediated dilatation, attributable specifically to NO, which has become relevant as endothelium-mediated dilatation is now known not to be exclusively NO-dependent.

A naturally occurring nonmetabolized analog of L-arginine exists, at circulating levels that are increased in renal failure and may then reduce NO activity.

Supplementation with additional L-arginine improves endothelial function in most conditions where it is impaired, but not when it is normal. Although administration of L-arginine and certain other amino acids can induce nonspecific dilator effects, the effects at low dosage are stereospecific. The reason for this beneficial effect is still not clear. L-Arginine is available by interconversion from other amino acids, and it is normally present in circulating blood at levels that should ensure no substrate deficiency relative to the activity of the intracellular enzyme nitric oxide synthase (NOS), whose K_m is far exceeded by intracellular levels of L-arginine. There is some recent evidence that L-arginine binding to NOS may become functionally impaired when the availability of reduced tetrahydrobiopterin, a necessary cofactor for continuing NO production, is limited by increased oxidant stress within the cell. Competitive interaction with the endogenous inhibitor of L-arginine, dimethyl arginine, when its levels are raised (ie, in renal failure and hypercholesterolemia), has also been suggested.

1988

Carbon dating proves the Turin Shroud to be a fake;
Australian Peter Carey wins the Booker Prize for his novel "Oscar and Lucinda";
and Greek ship owner Christina Onassis, dies, aged 37

A novel potent vasoconstrictor peptide produced by vascular endothelial cells

M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, T. Masaki

Nature. 1988;332:411-415

Once endothelium had been recognized as capable of releasing vasoactive substances, it became apparent that it might also exert vasoconstrictor effects. Whether these resulted from the reduction of vasodilator influences or the release of directly active vasoconstrictor agents remained uncertain, however, for none of the latter had been identified. This paper from Japan therefore made a big impact by reporting not only the isolation of a novel peptide that was shown to be the most potent vasoconstrictor known, but also by describing the cloning of its precursor, with evidence that its production in endothelial cells was regulated at the transcriptional level.

From the supernatant of cultured porcine aortic endothelial cells, a vasoconstrictor substance was extracted. This substance, which the authors called endothelin, showed as a single peak on analytical anion-exchange and reversed phase high-performance liquid chromatography. Endothelin caused contraction of arterial strips prepared from various species including man, comparable in magnitude to potassium-induced contraction, but with an EC_{50} which was an order of magnitude lower than for any other constrictor agent. Given by IV bolus to rats it raised the blood pressure for more than 60 minutes. The constrictor response to endothelin of porcine coronary artery strips in vitro was not inhibited by any tested antagonist, suggesting that it exerted a direct constrictor action on vascular smooth muscle.

Preproendothelin cDNA was cloned and sequenced. Vascular endothelium was shown to contain mRNA encoding preproendothelin, indicating that endothelial cells produce endothelin by de novo synthesis. Mature endothelin was generated by previously unknown proteolytic processing involving an endopeptidase–endothelin-converting enzyme (ECE). The level of preproendothelin mRNA was increased by epinephrine, thrombin, and the calcium ionophore A23187—agents known to be capable of inducing vasoconstriction—but was reduced by shear stress, implying flow-dependent inhibition of endothelin production, which could contribute (synergistically with endothelium-derived relaxing factor [EDRF]) to flow-mediated vasodilatation.

The structure of endothelin was found to be similar to that of peptide toxins acting on membrane channels. Significant regional homologies were apparent between endothelin and α scorpion toxins binding to tetradotoxin-sensitive sodium channels. The fact that endothelin's action depended on extracellular calcium and that it was inhibited by nicardipine, which blocks L-type calcium channels, suggested that it might be an endogenous agonist of these channels. Although the active expression of endothelin mRNA in endothelial cells implied that endothelin might contribute tonically to vasomotor tone in vivo, the authors cautioned that these cultured endothelial cells may be more representative of dysfunctional endothelium than healthy endothelium in vivo. The prolonged induction and action of endothelin was contrasted with the relatively short time course of EDRF responses, as likely to be relevant to their respective physiological roles.

This study put endothelin on the map as a uniquely potent, endogenous, endothelium-derived "constricting factor." Contrary to the earlier supposition that endothelin may act predominantly as a protective vasoconstrictor in pathological states, it now appears that it exerts some tonic action normally. Circulating levels are increased in congestive heart failure where their therapeutic lowering could be beneficial, though this remains to be established in proper trials. Moreover, endothelin, as seems typical of vasoconstrictor agents, may also promote cell growth, with obvious implications in many cardiovascular disease states.

1988

Florence Griffith-Joyner (Flo-Jo) wins two gold medals at the Seoul Olympics;
"The Last Emperor" wins the Best Picture Oscar;
and Hannibal's trek across the Alps is recreated by UK cricketeer Ian Botham

Crucial role of endothelium in the vasodilator response to increased flow in vivo

U. Pohl, J. Holtz, R. Busse, E. Bassenge

Hypertension. 1986;8:37-44

The phenomenon of flow-related vasodilatation, described 50 years earlier and recently shown to be endothelium-dependent in vitro, is here confirmed as endothelium-dependent in vivo. This study is an object lesson in controlling for interdependent variables in interventional vascular studies. Flow, pressure, and external diameter were measured in femoral arteries of anesthetized dogs, with and without local endothelial removal. Endothelium-dependent dilatation was induced by proximal intra-arterial infusion of acetylcholine, keeping flow constant. Blood flow was increased by distal infusion of acetylcholine or opening an arteriovenous shunt. Endothelium-independent responses were induced by norepinephrine and glyceryl trinitrate (GTN) applied adventitially. An increase in flow resulted in dilatation after a brief (ca 2 min) time lag. The small associated fall in pressure caused a small initial passive reduction in artery diameter, which in control experiments was ingeniously reproduced by a Valsalva maneuver, thus excluding any contribution from myogenic relaxation (Bayliss effect). Flow-related dilatation was greater when resting diameter was not set at the extremes of vasodilatation or vasoconstriction by GTN or norepinephrine, implying that it modulated rather than overrode humoral vasomotor control. Flow-dependent dilatation was preserved after distal transection of the artery, excluding a peripherally conducted mechanism as previously proposed. Endothelial damage abolished flow-related dilatation and impaired acetylcholine-induced dilatation, while endothelium-independent responses remained unchanged. These experiments thus clearly demonstrated flow-related endothelium-dependent dilatation in vivo, without establishing the agent responsible, though previous studies had excluded prostaglandins, adrenergic mechanisms, and histamine.

We now know that flow-related dilatation characterizes resistance as well as conduit arteries and that endothelium-derived relaxing factor (EDRF) is nitric oxide (NO), produced by endothelial NO synthase (eNOS), which is activated by shear stress or by agonists. Shear stress also influences the production of other endothelial vasomotor agents, eg, prostacyclin (which is increased) and endothelin (which is decreased).

Resistance vessels determine tissue perfusion and its distribution by local dilatation in response to "metabolic" signals (adenosine and opening of K_{ATP} channels) acting predominantly on arterioles. These are amplified by NO, which acts predominantly on small arteries. The very short half-life of NO enables it to integrate vascular behavior throughout the vascular bed in response to changes in flow. It is presumably this which underlies the experimental observation that maintenance of the same distribution of microvascular flow at different input flow rates is endothelial NO–dependent. Loss of flow-related dilatation is thereby likely to result in microvascular heterogeneity, limiting overall tissue perfusion when this can no longer adequately compensated by increased metabolic signals downstream. This could then prejudice flow-limited functions, despite normal (or compensatorily increased) flow into the bed—an intriguing hypothesis with potentially far-reaching consequences. In conduit arteries, flow-related dilatation contributes to increasing compliance at higher flow rates, reducing systolic pressure, the cardiac effects of reflected waves, and cardiac work—with correspondingly detrimental consequences when it is impaired. The phenomenon is relevant also to atherogenesis: NO has antiatherogenic activity through its protective effects against oxidant stress, cytokine transcription, and the inflammatory response, while atheroma develops preferentially at sites of low shear stress where NO production will be low.

Flow-related vasodilatation can now be measured clinically, providing a surrogate measure of generalized endothelial function.

1986

"Tigger" celebrates his 60th birthday;
Elie Wiesel is awarded the Nobel Peace Prize;
and pianist Vladimir Horowitz returns to Russia
for the first time in 61 years

Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries

M. Nakashima, J.V. Mombouli, A.A. Taylor, P.M. Vanhoutte

J Clin Invest. 1993;92:2867-2871

Vanhoutte and colleagues, who have contributed much to endothelial pharmacology, here investigate endothelium-dependent hyperpolarization in human arteries. The phenomenon was thought to be mediated by a diffusible substance known as endothelium-derived hyperpolarizing factor (EDHF), shown not to be nitric oxide (NO) or prostacyclin. The evidence indicated that it contributed to endothelium-dependent relaxation by opening K^+ channels in vascular smooth muscle. It is instructive to re-read this paper in the light of recent data which question the existence of EDHF as then envisaged: the paper stands up to the test.

Coronary artery rings were prepared from hearts of transplant patients, and studied with and without endothelium. Indomethacin and a nonmetabolized analog of L-arginine were added to prevent any contribution of cyclooxygenase products or NO. Resting membrane potential was the same whether endothelium was present or not. Hyperpolarization was transient with bradykinin and more prolonged with the calcium ionophore A23187, but only in preparations with intact endothelium. It was potentiated by the addition of an angiotensin-converting enzyme (ACE) inhibitor (inhibiting the breakdown of bradykinin). Similar hyperpolarization could be induced by a K_{ATP} channel opener (lemakalim) and blocked by the K_{ATP} channel antagonist glibenclamide. Glibenclamide, however, did not block bradykinin- or A23187-induced hyperpolarization, indicating that this was not mediated by opening of K_{ATP} channels. Bradykinin is known to stimulate endothelial NO production. However, the endothelium-dependent relaxation of preconstricted rings induced by bradykinin was not abolished by blocking NO production with a nonmetabolized L-arginine analog. Thus, both receptor-mediated and receptor-independent stimulants of endothelial NO release induced hyperpolarization that was not mediated by cyclooxygenase products or NO nor mediated by opening of K_{ATP} channels.

These in vitro findings broadly confirmed in human what had been shown in animal preparations. The identity of the putative EDHF remained, however, elusive. It was confirmed as not being NO or a cyclooxygenase product, but was shown to be dependent on intracellular calcium mobilization inhibitable by a calmodulin antagonist. There was considerable species variation in the type of potassium channel involved in the smooth muscle cell hyperpolarization. The consequence of hyperpolarization, however, would be to reduce voltage-dependent calcium influx, thereby contributing to a relaxant effect in addition to that induced by the multiple consequences of NO-induced elevation of intracellular cyclic guanosine monophosphate. The physiological implications remained uncertain.

It has become increasingly apparent, recently, that only part of the endothelium-dependent dilatation induced by acetylcholine could be attributed to NO and other agonists which stimulate NO production, and that "endothelium-derived relaxing factors" (EDRF) should perhaps embrace EDHF as well as NO. Very recent evidence indicates that endothelium-dependent hyperpolarization is due not to the release of a diffusible messenger between one cell and another, but to direct intercellular communication between endothelial and vascular smooth muscle cells through intact gap junctions. The phenomenon of endothelium-induced smooth muscle hyperpolarization independent of NO production is likely to be as important as NO in vasomotor control. It introduces the potential for new approaches to its control, experimentally and clinically, and for exploiting differences in the relative contributions of these two mechanisms in different arteries. Endothelium-dependent hyperpolarization is turning out to be important after all.

1993

Norwegian Erling Kaage completes
the first solo trek on foot to the South Pole;
the European Community is renamed
the European Union;
and UK pilot Barbara Harmer becomes
the first woman to fly Concorde

Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man

P. Vallance, J. Collier, S. Moncada

Lancet. 1989;2:997-1000

This paper is the outcome of a series of three studies by Moncada: the first was one of the papers showing that endothelium-derived relaxing factor (EDRF) was nitric oxide (NO); the second was a study showing that the amino acid L-arginine was the metabolic precursor of NO; and the third described the development of nonmetabolized analogs of L-arginine. These, by competitively blocking NO production, were to prove useful pharmacological tools with which to study the role of NO.

The experimental animal work had been done. Nonmetabolized analogs (eg, N^G-monomethyl-L-arginine [L-NMMA]) had been shown reversibly and stereospecifically to inhibit NO production by endothelial cells or arteries and to inhibit endothelium-dependent relaxation. In vivo, they increased blood pressure. It only remained for ethical approval to be granted for comparable studies to be carried out in man. Such approval was proving understandably difficult in the UK at that time. This particular study stands as probably the first demonstration of endothelial NO activity in man.

L-NMMA was infused into the brachial artery of 14 healthy young adults. Forearm blood flow was measured in both arms by mercury-in-rubber strain-gauge plethysmography, with upper arm cuffs inflated to supravenous pressure for 10 seconds in each 15-second cycle, the hands being excluded from the circulation by wrist cuffs inflated to supra-arterial pressure. Blood flow was measured in both arms three times during 30 minutes stabilization; and then at the end of 5-minute L-NMMA infusion periods at cumulative doses of 1, 2, and 4 µmol/min (or D-NMMA, 4 µmol/min); and for 15 minutes thereafter, during the last 10 minutes of which 40 µmol/min L-arginine (or D-arginine) was infused. L-NMMA dose-dependently reduced blood flow to plateau levels by 5 minutes, by up to 40% at the highest dose, with recovery to control levels some 60 minutes later. L-Arginine reversed the L-NMMA-induced reduction in blood flow. The effect was stereospecific, D-NMMA or D-arginine being without effect. Blood flow in the control arm remained constant and served both as a control to exclude systemic effects and as the reference against which to express blood flow readings in the experimental arm. Forearm blood flow was also increased dose-dependently by acetylcholine, an effect similarly reduced by L-NMMA without affecting glyceryl trinitrate (GTN)-induced increases in blood flow.

The study thus showed that endothelial NO contributes continuously to basal blood flow as well as to acetylcholine-induced vasodilatation in the forearm of healthy young subjects. It incidentally confirmed the stereospecificity of L-arginine as the substrate for endothelial production of NO. Of particular note was the unique magnitude of the NO contribution to basal flow, in marked contrast to the relatively negligible contributions of angiotensin II, prostaglandins, histamine, or serotonin, as tested by blockade of their respective actions. L-NMMA was observed to only partially inhibit the vasodilator effect of acetylcholine, suggesting that this is mediated only partly by NO. This is consistent with there also being a contribution from non–NO-mediated acetylcholine-induced hyperpolarization of vascular smooth muscle, which has been attributed to an endothelium-derived hyperpolarizing factor (EDHF). Recent experimental evidence indicates that this phenomenon is not due to a free agent acting as an intercellular messenger, but that it depends on direct heterocellular communication through gap junctions between endothelial and vascular smooth muscle cells.

1989

Pro-democracy activists are crushed in China's Tiananmen Square; Dustin Hoffman wins an Oscar as Best Actor for his performance in "Rain Man"; and Spanish artist Salvador Dali dies, aged 84

Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries

P.L. Ludmer, A.P. Selwyn, T.L. Shook, R.R. Wayne, G.H. Mudge, R.W. Alexander, P. Ganz

N Engl J Med. 1986;315:1046-1051

It was obvious from the time of Furchgott & Zawadski's classic paper *(see page 222)*, which showed that acetylcholine induced both endothelium-dependent dilatation in the presence of healthy endothelium and direct vascular smooth muscle contraction in the case of endothelial dysfunction or loss, that acetylcholine could be a useful pharmacological tool with which to test endothelial function. The concept of coronary spasm as a clinical entity in some patients had long existed, with coronary angiography later suggesting that this occurred preferentially at sites of minor atheroma. It was therefore unsurprising that acetylcholine, the useful "double agent," came to be tested in patients with coronary atheroma by relating vasomotor responses to angiographically demonstrable stenoses.

Responses to infusions of acetylcholine and glyceryl trinitrate (GTN) into the left anterior descending (LAD) coronary artery were measured by quantitative coronary angiography in 18 patients (12 M, 6 F): 4 "normal controls" with atypical chest pain; 6 patients with "minimal disease" (minimal LAD atheroma); 8 patients with "advanced disease" (significant LAD stenosis and inducible myocardial ischemia in the LAD territory). The control group showed modest (ca 10%) dose-dependent dilatation to acetylcholine. In the minimal disease group, acetylcholine constricted all diseased segments (by ca 35%) and some adjacent smooth segments (by ca 15%). In the severe disease group, acetylcholine dose-dependently constricted all stenotic as well as pre- and poststenotic segments (by ca 70%, with temporary occlusion in 5 patients) and other less severe stenotic sites. GTN induced dilatation in all groups. The controlled intracoronary infusion into the LAD was associated with no changes in diameter of the circumflex coronary artery and avoided the systemic consequences of methacholine or acetylcholine administration reported in previous studies. This study therefore provided good evidence that acetylcholine-induced vasodilatation was impaired at sites of angiographically demonstrable lesions in patients with coronary artery disease, consistent with endothelial dysfunction at these sites—without, as the authors acknowledge, either proving the presence of endothelial dysfunction or implying that acetylcholine mediates coronary spasm in vivo.

This study paved the way for clinical studies showing that constrictor responses to acetylcholine may be evidenced even in angiographically clean coronary arteries of subjects with known risk factors for atheroma, consistent with the presence of nonangiographically demonstrable atheromatous coronary lesions with vascular remodeling, and that these constrictor responses can revert to normal dilator responses with therapy aimed at improving endothelial function (eg, cholesterol-lowering agents or angiotensin-converting enzyme inhibitors). Impairment of acetylcholine-mediated dilator responses may not necessarily correlate with impairment of endothelial responses to agonists acting through other receptors or of flow-related responses, and loss of endothelium-mediated dilatation does not necessarily correlate with other components of endothelial dysfunction more directly relevant to atherogenesis and inflammation in the vascular wall. On the other hand, endothelial dysfunction seems to be generalized when present. Moreover it correlates with all known risk factors for atheroma and there are good grounds for attributing an antiatherogenic role to NO. Other more amenable arteries and less invasive methods of assessing their endothelial function are thus likely to give comparable information.

Practically applicable methods of measuring endothelial function should have a useful role in the better diagnosis and treatment of susceptibility to atherogenesis and its complications.

1986

High-temperature superconductors are discovered;
Riots break out in Haiti as the ruthless dictator
"Baby Doc" flees to France;
and Davina Thompson becomes the first recipient
of a triple heart, lung, and liver transplant

Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta

R.L. Minor Jr, P.R. Myers, R. Guerra Jr, J.N. Bates, D.G. Harrison

J Clin Invest. 1990;86:2109-2116

This was the first study to show that impaired nitric oxide (NO)-mediated endothelium-dependent dilatation might be due not to reduced production but to decreased availability of reactive NO. Indeed, this study set out to examine whether "impaired endothelium-dependent vasodilatation in atherosclerosis is associated with decreased synthesis of nitrogen oxides by the vascular endothelium."
The experimental findings must have come as a surprise.

The authors used the cholesterol-fed rabbit model, with rabbits fed a high-cholesterol diet for 2 to 5 weeks (resulting in hypercholesterolemia without atheroma) or 6 months (resulting in atheroma, but no endothelial loss), and control rabbits fed a normal diet. Aortic rings were prepared and superfused. Indomethacin was used to block cyclooxygenase products. Effluent from aortic rings from normal rabbits relaxed the detectors (de-endothelialized preconstricted pig artery rings), with additional relaxation when the aortic rings were stimulated by acetylcholine or the calcium ionophore A23187. With effluent from aortic rings of hypercholesterolemic or atheromatous rabbits, these relaxant responses were grossly impaired, whereas NO levels were paradoxically increased, and increased further following stimulation by acetylcholine or A23187. N^G-Monomethyl-L-arginine, a nonmetabolized analog of L-arginine, abolished the dilator responses, confirming that they were due to NO, whose relaxant action was somehow reduced when derived from hypercholesterolemic arteries. The loss of endothelium-dependent relaxant activity could not be attributed to impaired signal transduction or substrate deficiency as previously suggested, since NO production was increased. Accelerated oxidative degradation of NO was suggested. The explanation of the increased NO production remained unclear.

Subsequent work confirmed increased superoxide (O_2^-) in this model. A similar combination of decreased endothelium-dependent dilatation but increased NO and O_2^- was reported by the same workers in experimental nitrate tolerance, the O_2^- being endothelium– and angiotensin II–dependent, suggesting that its source could be endothelial NADH oxidase whose expression is increased by angiotensin II. Intriguingly, endothelial NO synthase (eNOS), which produces NO from L-arginine, can itself produce O_2^- when intracellular oxidant stress is increased, as in many conditions associated with endothelial dysfunction (hypercholesterolemia, atheroma, smoking, diabetes, hyperhomocysteinemia, and hypertension). eNOS production of NO rather than O_2^- depends on recycling of tetrahydrobiopterin (THB_4), a cofactor for eNOS, to its active reduced state. This can be impaired by increased oxidant stress, as may result from increased activity of endothelial NADH oxidase and xanthine oxidase. NO production is then switched to O_2^- production, further reducing the activity of such NO as is produced. Functional deficiency of reduced THB_4 can be overcome by THB_4 supplementation, as clinically confirmed in hypercholesterolemic patients. It may explain also the puzzling benefits of L-arginine supplementation if, as is suggested, L-arginine and THB_4 exhibit cooperativity of binding to eNOS so that reduced bioavailability of THB_4 results in functional deficiency of L-arginine. The beneficial effect of folate may also relate to THB_4 bioavailability, as well as to its lowering of homocysteine levels when these are raised.

These findings highlighted the importance of an abnormal intracellular redox state and of the balance between NO and O_2^- as key determinants not only of vasomotor tone but also of inflammatory processes and cell growth.
The emphasis of therapy is shifting towards normalizing oxidant status. This paper may be seen as having opened new avenues to understanding the pathogenesis of cardiovascular dysfunction and disease and to new therapeutic approaches.

1990

West Germany beats Argentina to win the World Cup; "Cinema Paradiso" wins the Best Foreign Film Oscar; and US entertainer Sammy Davis Jr dies, aged 74

Different interactions of platelets with arterial and venous coronary bypass vessels

Z.H. Yang, P. Stulz, L. von Segesser, E. Bauer, M. Turina, T.F. Lüscher

Lancet. 1991;337:939-943

Coronary artery bypass grafting has become a highly successful industry for relief of disabling angina, with benefit to life expectancy where this is reduced. Vein graft patency is, however, limited, due to technical surgical factors, thrombotic occlusion within weeks, and intimal hyperplasia some 10 years later. Internal mammary artery grafts have better long-term patency. The present paper is one of a series in which the authors explored possible differences in endothelial function between saphenous vein and internal mammary artery.

Ring preparations of internal mammary artery and saphenous vein obtained during coronary surgery were mounted in organ baths. Platelets from normal subjects were prepared in calcium-free buffer. Vasomotor concentration responses to the spontaneously aggregating platelets were measured in preparations with and without endothelium. Aggregating platelets release adenosine diphosphate (ADP), which stimulates endothelium to release nitric oxide (NO), and thromboxane and serotonin, which directly constrict smooth vessels, serotonin also stimulating endothelial NO production. Platelet products were found to relax preconstricted artery preparations—a response that was dependent on ADP, endothelium, and NO—but to constrict quiescent arteries, though to a smaller extent in the presence of endothelium. An endothelium-dependent relaxant influence was thus manifest in quiescent as well as preconstricted arteries, whereas the constrictor influence was not apparent when the artery was already preconstricted. In venous preparations, by contrast, platelet products induced constriction whether the veins were quiescent or preconstricted, and to a greater extent in the presence of endothelium, the constrictor response being mediated jointly by thromboxane and serotonin and enhanced in the presence of intact endothelium. Whether pharmacological blockade of the constrictor response in veins would have unmasked an endothelium-dependent dilator effect in the presence of intact endothelium was not tested. Morphological evidence confirming endothelial coverage was not reported. The study thus showed that platelet products induced both constrictor and endothelium-dependent relaxant responses in the arteries, the resultant response depending on conditions, but only thromboxane and serotonin mediated constrictor responses in the veins with an additional endothelium-related constrictor influence—consistent with the earlier investigations of single agents.

How these interesting, but ultimately empirical, findings relate to vascular responses under more physiological conditions in vivo and to the fate of the vessels after graft implantation is not so clear. Intimal hyperplasia may indeed be influenced by cGMP and cAMP levels in the vascular wall. The issue, however, is complex. Recent experimental studies indicate that saphenous vein guanylate and adenyl cyclase activities are decreased but NO production increased during adaptive changes to arterial conditions following graft implantation. The relevance of platelet aggregation following graft implantation is likely to be limited to the immediate postsurgical endothelial loss until re-coverage, complete within weeks, and to late neoatheromatous damage. Platelets may accordingly contribute to thrombotic occlusion within the first few weeks, whereas late graft occlusion is related to the consequences of intimal hyperplasia. The adaptive changes resulting from arterialization of these veins is likely to be the main contributory cause of this vascular pathology, with secondary changes in endothelial responses. Platelet endothelial interactions may not be the major influence in promoting intimal hyperplasia once thought. Growth-promoting cytokines, such as platelet-derived growth factor (PDGF), will then be derived predominantly from cells activated in the vascular wall, rather than directly from aggregating platelets.

1991

Sweden wins the Eurovision Song Contest in Rome;
Nigerian Ben Okri wins the Booker Prize
for his novel "The Famished Road";
and Freddie Mercury, lead singer of "Queen,"
dies of AIDS, aged 45

Endothelium

Bibliography of One Hundred Key Papers

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SEMINAL PAPER SUMMARIES

Seminal paper summaries take up one page of *Dialogues in Cardiovascular Medicine:* the length of each summary should **IMPERATIVELY be comprised between 500 and 600 words, ie, not exceed 3000 characters.** Summaries that are too short or too long will be returned to the author or edited by the Publisher. **No figures, tables or references should be included** in seminal paper summaries.