

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 research, 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 precontracted 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 precontract 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 marvelous 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

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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 precontracted 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 consen-

sus 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 precontracted rabbit aortic strips denuded of endothelium, and its NO content was measured by chemiluminescence (of NO and NO₂) and by mass spectrometry of ¹⁵NO following incubation with ¹⁵N-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 EC₅₀ 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 EC₅₀ (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 moiety 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 non-specific 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 tetrodotoxin-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 adventitiously. 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 be 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

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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 precontracted 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 supravenuous 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 $\mu\text{mol}/\text{min}$ (or D-NMMA, 4 $\mu\text{mol}/\text{min}$); and for 15 minutes thereafter, during the last 10 minutes of which 40 $\mu\text{mol}/\text{min}$ 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 precontracted 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 (THB₄), 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 THB₄ can be overcome by THB₄ 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 THB₄ exhibit cooperativity of binding to eNOS so that reduced bioavailability of THB₄ results in functional deficiency of L-arginine. The beneficial effect of folate may also relate to THB₄ 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

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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 precontracted 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 precontracted arteries, whereas the constrictor influence was not apparent when the artery was already precontracted. In venous preparations, by contrast, platelet products induced constriction whether the veins were quiescent or precontracted, 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-depend-

ent 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 adenylyl 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