History of Discovery

How We Learned to Say NO

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What seest thou else
In the dark backward and abysm of time
—William Shakespeare

First, there was Robert Furchgott. He simply and brilliantly demonstrated that endothelial cells play a pivotal role in relaxations evoked by acetylcholine in isolated arteries, and do so by activating muscarinic receptors of these cells. Actually, the quest for the understanding of the mechanism(s) underlying the vasodilator effect of acetylcholine in vivo (in contrast with the vasoconstrictor effect that the cholinergic transmitter almost always had in vitro) had started many years before that and led to the earlier interpretation that this could be explained best by a prejunctional (presynaptic) muscarinic effect resulting in the local inhibition of adrenergic neurotransmission in the blood vessel wall. To be honest, I am still convinced that if acetylcholine released from cholinergic nerves (the most likely, if not the only, source of acetylcholine in our body) contributes to the control of vasomotor tone, it does so by inhibiting the release of norepinephrine in the adventitia rather than by diffusing through the media to the endothelial cells. When asked the role is of muscarinic receptors on endothelial cells, I usually respond by saying that they have been placed there to make sure that Robert Furchgott, who already had contributed immensely to pharmacology before discovering endothelium-dependent relaxations, would make the trip to Stockholm! Yet his simple pharmacological experiments had opened a totally new avenue not only in vascular pharmacology and physiology but in science in general as they set the stage for the discovery of the role of nitric oxide (NO) in biology.

EDRF

In his seminal paper, Robert Furchgott elegantly demonstrated (using what he called “sandwich” preparations: a layering of strips of arteries with and without endothelium whereby the contractile responses are measured only in the strip without endothelium) that the endothelium-dependence of the response to acetylcholine was attributable to the diffusion of a powerful vasodilator substance from the endothelial cells to the vascular smooth muscle cells. He immediately ruled out prostacyclin, known to be produced by endothelial cells, as the mediator responsible for the phenomenon. Faced with the unknown nature of the mediator, he coined the term “endothelium-derived relaxing factor,” soon transformed into the reassuring abbreviation “EDRF” which hid our lack of knowledge in a comforting scientific jargon (Figure 1). Originally, and not so surprisingly (Quote 1), the proposal of the essential role of endothelial cells in local vasomotor control met with little interest, not to say disbelief. Only a few laboratories (in Antwerp, Belgium; Birmingham, Ala; Cardiff, UK; Freiburg, Germany; and later Rochester, Minn) went on to confirm the existence of endothelium-dependent responses and initiated the search for the identity of the mysterious EDRF. More sophisticated superfusion-bioassay systems were designed permitting to apply pharmacological inhibitors to either the endothelial cells or the vascular smooth muscle (eg, 5). The first conclusion of these superfusion-bioassay studies was that the biological half-life of EDRF was disappointingly brief (in the order of seconds), which made identification by conventional chemical techniques impossible. The early pharmacological studies yielded heated, sometimes amusing, debates as to the nature of EDRF, confounded as they were by the conclusion that besides prostacyclin and the Furchgott EDRF endothelial cells could generate other signals leading to endothelium-dependent relaxations. The latter multiple signals (Figure 2) eventually became known as endothelium-derived hyperpolarizing factors (EDHF) and play a prominent role in smaller arteries and resistance vessels. In addition, it soon became obvious that in veins, and under certain conditions in arteries as well, the endothelial cells can generate endothelium-derived contracting factors (EDCF), which considerably adds to the complexity of the analysis of endothelium-dependent responses. Irrespective of that complexity, the quest was started to find more physiological stimuli than acetylcholine able to evoke endothelium-dependent relaxations. Over the years that followed, we were amazed to realize that physical forces (increases in shear stress), circulating hormones (catecholamines, vasopressin), platelet products (serotonin, adenosine diphosphate), autacoids (histamine, bradykinin), prostaglandin E4, and thrombin share with the cholinergic transmitter the ability to elicit endothelium-dependent changes in the tone of the underlying smooth muscle. Of those more physiological stimuli, increases in shear stress merit a special mention as they explain the endothelium-dependency of flow-
mediated vasodilatation, a response which to date probably allows the most accurate estimation of endothelial function in humans. In those early years, the first demonstrations were made also that endothelium-dependent responsiveness can be blunted by pathological conditions, including myocardial infarction, paving the way for the current concept that endothelial dysfunction precedes or at least accompanies vascular disease and indeed is a predictor of cardiovascular events.

Nitric Oxide

Two sets of observations were seminal in the thought process that led to the proposal that the Furchgott EDRF is NO. First, it soon appeared convincingly that EDRF, whatever it may be, was stimulating soluble guanylyl cyclase in the vascular smooth muscle cells. That enzyme catalyzes the formation of the cyclic nucleotide, cGMP (cyclic GMP), which is well known to initiate relaxation of vascular smooth muscle. Second, in early 1985 the author’s laboratory reported that under superfusion-bioassay conditions superoxide anions were scavenging EDRF. After a long battle with reviewers and editors, the full description of these observations finally was published at the same time as a similar conclusion reached by Salvador Moncada and colleagues. Ferid Murad had demonstrated earlier that NO activates soluble guanylyl cyclase and is scavenged by superoxide anions. Based on those findings and his own work with acidified nitrite, Robert Furchgott...
proposed during a session of the symposium “Mechanisms of Vasodilatation,” held in Rochester (Minn) in 1986, that his EDRF was nothing else but NO.22 In the same session, Louis Ignarro developed his arguments to reach the same conclusion.23 The participants of the meeting will never forget the electrifying atmosphere of that session (Quote 2). The biology of NO was born and, eventually, 12 years later Robert Furchgott, Louis Ignarro, and Ferid Murad shared the Nobel Prize.

Of course, the first response of the biochemical establishment was disbelief, as the dogma was that mammalian cells are not supposed to produce NO (Quote 3). But Salvador Moncada had attended the symposium in Rochester and took the next giant leap forward! He and his colleagues cultured endothelial cells and demonstrated, using a chemiluminescence technique that when stimulated with bradykinin (a well known endothelium-dependent vasodilator) they indeed produced NO.24 Inspired by the work of John Hibbs on macrophages,25 they went on to prove that the endothelial cells are able to transform the amino acid L-arginine into NO and citrulline, isolated the endothelial enzyme fraction responsible, and provided the scientific community with inhibitors of the enzyme,26,27 of which L-NMMA is the prototype, still used for human research. Claude Bernard, one of the founders of physiology, said that one can only tell what a nerve does by cutting it. Likewise, the availability of inhibitors of NOS permitted the exploration of the physiological role of NO in isolated tissues and organs, and in the intact organism. Inhibitors of NOS augmented arterial blood pressure in vivo,28 implying a role for the endothelial mediator in cardiovascular homeostasis. However, it rapidly became clear that NO is more than an endothelial affair, and that indeed from memory to erection it affects almost every bodily function.

Then, the molecular biologists took over. Salomon Snyder and his group isolated nitric oxide syn(thesis)ase (NOS) from the brain (eg. 29). It soon turned out that there were 3 isoforms of the enzyme, numbered in the order of their cloning: neuronal NOS (nNOS, NOS 1), inducible NOS (iNOS, NOS 2), and finally endothelial NOS (eNOS, NOS 3). The author has always regretted that the endothelial isoform was given the last number, as it clearly was the first one identified biologically. The next major step was made by Paul Huang and colleagues, who genetically engineered mice with deletion of the eNOS gene.30 These animals have an increased arterial blood pressure, illustrating again a role of NO in the complex events that control cardiovascular homeostasis.

The rest is history. Armed with genetically modified animals and inhibitors of NOS, the systematic exploration of the role of NO in vascular health and disease became possible, leading to an explosion of knowledge concerning the production, its regulation, and its dysfunction (Figure 3).13 We have recognized that in a given blood vessel, the level of activity of eNOS, and thus the production of endothelium-derived NO, is not constant but can be upregulated by chronic increases in shear stress, estrogens, and the chronic intake of ω3-unsaturated fatty acids or polyphenols (in red wine, green tea, and dark chocolate), but also can be downregulated by high glucose and increased oxidative stress, to name but a few (see13,31). The primary function of NO as a major paracrine regulator of the tone of the underlying vascular smooth muscle was extended to those of inhibitor of platelet aggregation, in synergy with endothelium-derived prostacyclin,32 and of the growth of vascular smooth muscle cells.33 A number of autocrine regulatory roles of NO also emerged, including the reduced production by the endothelial cells of endothelin-134 and cyclooxygenase-derived EDCFS,35 inhibition of the expression of endothelial adhesion molecules and thus of the adhesion of platelets and white blood cells,13,32 and modulation of angiogenesis.31,36 We understand better the signaling cascade, in particular the role of Akt, in the phosphorylation that leads to activation of eNOS,31,37–39 The original concept that the activity of eNOS is strictly regulated by the intracellular Ca2+-concentration was revisited, as it appeared that in particular shear stress can cause Ca2+-independent increases in NO production by the enzyme (Fleming31,39). We have learned to appreciate the intricacies of the interactions...
between eNOS and caveolae.40,41 The extremely short half-life of the gaseous mediator turned out to be prolonged by the formation of NO-metabolites constituting a nonenzymatic source of the activator of soluble guanylyl cyclase.42 The scavenging of NO by superoxide anions, leading to the formation of peroxynitrite, has become a major player in genesis of endothelial dysfunction.43–46 Above all, the progressive inability of endothelial cells, prematurely aged by the exposure to risk factors, to generate sufficient NO is now believed (at least by the author) to be the initial step permitting the inflammatory response that leads to atherosclerosis (see11). We also have learned that the most widely used therapeutic agents for the treatment or prevention of cardiovascular disease share the pleiotropic action to enhance the ability of the endothelial cells to produce NO. This is the case for ACE-inhibitors, 47 statins,13 and third generation βadrenergic blockers. 78

Obviously, much remains to be learned about the precise regulation of the release of NO by endothelial cells, as well as about the consequences of the lack of endothelium-derived NO as a key factor in the complex chain of events that leads to vascular dysfunction in hypertension, diabetes, and atherosclerosis. We should not forget that this extraordinary scientific adventure started with a very simple pharmacological experiment in the rabbit aorta by Robert Furchgott 30 years ago.

**Quotes**

**Quote 1:** “A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die, and a new generation grows up that is familiar with it.” Max Planck.

**Quote 2:** “… The hunt for Furchgott’s endothelial factor came to an end during a scientific meeting at the Mayo Clinic…in the summer of 1986. At the meeting Furchgott concluded…that the factor was identical with NO. Ignarro supported this at the same meeting…[and] went one step further…. The hunt was over. The riddle concerning the endothelial factor was finally solved…. Sten Lindahl, Presentation Speech, Stockholm, 1998, The Nobel Prize in Physiology or Medicine 1998.

**Quote 3:** “The fact that an opinion has been widely held is no evidence whatever that it isn’t utterly absurd. Indeed, in view of the silliness of the majority of mankind, a widespread belief is more likely to be foolish than sensible.” Bertrand Russell.

**Disclosures**

None.

**References**

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