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### Hindsight

As a young medical resident, I encountered a patient suffering from spontaneous coronary vasospasm and was puzzled by these dramatic alterations in vasomotion. This encounter piqued my interest in understanding the drivers of vascular reactivity. In a paper published in the *JCI*, my colleagues and I revealed a role for superoxide production in the vascular dysfunction associated with hypercholesterolemia. Subsequent work by our group and others has unveiled complex associations between ROS generation and vascular disease.

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# From ST segments to endothelial pathophysiology: hypercholesterolemia and endothelial superoxide production

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**As a young medical resident, I encountered a patient suffering from spontaneous coronary vasospasm and was puzzled by these dramatic alterations in vasomotion. This encounter piqued my interest in understanding the drivers of vascular reactivity. In a paper published in the *JCI*, my colleagues and I revealed a role for superoxide production in the vascular dysfunction associated with hypercholesterolemia. Subsequent work by our group and others has unveiled complex associations between ROS generation and vascular disease.**

While a second-year internal medicine resident on the Duke coronary care unit in the 1970s, I admitted a young lady who was having repeated episodes of chest pain and profound ST-segment elevation on her electrocardiogram. During several of these episodes, she developed ventricular fibrillation that required cardioversion. An emergency cardiac catheterization revealed that her coronary arteries were overtly normal, but her left anterior descending coronary artery spontaneously developed spasm to the point of closure. Thankfully, these episodes eventually resolved. She obviously had variant angina, and I was impressed with the dramatic nature of her illness and the idea that diseases could alter vasomotion in such a striking fashion.

## An experimental model and search for mechanisms

After my residency and cardiology fellowship, I accepted a postdoctoral research fellowship under the tutelage of Melvin Marcus, Allyn Mark, and Frank Abboud at the University of Iowa. There, I met Donald Heistad and Mark Armstrong, who were studying the effects of cholesterol and atherosclerosis on vascular function. Don had made the exciting discovery that hypercholesterolemia altered vascular reactivity. Specifically, he demonstrated that administration of serotonin, normally a vasodilator, paradoxically evokes severe vascular spasm in cholesterol-fed monkeys

(1). Other groups made similar observations in cholesterol-fed rabbits (2). These findings were reminiscent of my patient described above. Shortly before Don's report, Robert Furchgott discovered that agonists, including acetylcholine, bradykinin, serotonin, and vasopressin, promote vasodilation by stimulating endothelium-derived relaxing factor (EDRF) release (3, 4); however, these same agonists evoked vasoconstriction following removal of the endothelium by mechanical abrasion. In discussions with Don and Mark, we hypothesized that the endothelium of cholesterol-fed monkeys was deficient in EDRF production. During a stint in Paul Vanhoutte's laboratory at the Mayo Clinic, I learned to perform isometric tension studies of isolated vessels and established this method at the University of Iowa. Don and Mark provided iliac arteries from cholesterol-fed and control animals, and we demonstrated that atherosclerotic vessels had severely blunted endothelium-dependent vasodilation and sporadically demonstrated paradoxical vasoconstriction, much like the coronary artery of my patient at Duke (5). Others confirmed that hypercholesterolemia alters endothelium-dependent vasodilation (6), which was a fascinating result with clinical implications; however, the mechanisms responsible for hypercholesterolemia-associated changes in endothelium-dependent vasodilation were unclear.

## Identifying a role of ROS

Our understanding of disease-dependent changes in EDRF production was advanced by the nearly concurrent observations that the EDRF was NO (7) and that the EDRF

could be inactivated by superoxide (8). Because NO and superoxide react with one another at a near-diffusion-limited rate (9), we hypothesized that the hypercholesterolemia-associated defect in endothelium-dependent vasodilation was due to oxidative inactivation of NO by superoxide. At the time (late 1980s), apolipoprotein E-null mice, which develop severe hypercholesterolemia, were not available; therefore, we used cholesterol-fed rabbits as a model of hypercholesterolemia and early atherosclerosis. Fortunately, around this time I met Jim Bates, a faculty member in the Department of Anesthesia at the University of Iowa, who contributed substantially to these experiments (Figure 1). We used ozone-linked chemiluminescence to measure NO release from isolated perfused vascular segments and observed that vessels from cholesterol-fed rabbits produced NO, but it was largely released in the oxidized form of nitrite ( $\text{NO}_2^-$ ) (10). Furthermore, cholesterol-fed rabbits treated with the superoxide scavenger polyethylene-glycolated superoxide dismutase exhibited improved endothelium-dependent vasodilation (11). Together, our results strongly suggested superoxide involvement in hypercholesterolemia-associated vascular dysfunction and in other molecular events in atherosclerosis.

We next established methods to measure superoxide production by intact vessels. This was challenging, because many commonly used assays to study isolated enzymes or chemical reactions were either insensitive or subject to interference. After exploring several techniques, we settled on chemiluminescence-based methods to study intact vessels, and Dr. Yuichi Ohara, a postdoctoral fellow in our laboratory at the time, was instrumental in adapting this technique. We were surprised to discover that vessels from cholesterol-fed rabbits produced three times more superoxide than did normal vessels (12). Strikingly, removal of the vessel endothelium com-

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**Figure 1**

Collaboration. David Harrison (left), David Ku (center), and James Bates (right) at a Keystone Conference, circa 1990.

pletely normalized superoxide production. Thus, hypercholesterolemia stimulated superoxide production in the very cells that were synthesizing NO, leading to NO oxidation prior to leaving the endothelium. A consequence of this reaction is formation of the strong oxidant peroxynitrite ( $\text{OONO}^-$ ), which oxidizes LDL, depletes antioxidants, and leads to the formation of isoprostanes. The isoprostanes are particularly interesting. These compounds, discovered in the early 1990s by my current colleague Jack Roberts (13), are potent vasoconstrictors and could therefore contribute to abnormal vasomotion in conditions of excessive oxidation. ROS are also implicated in many aspects of vascular disease, including lipid oxidation (14), adhesion molecule expression (15), plate-

let aggregation (16), thrombosis, matrix remodeling, and plaque rupture (17). Thus, superoxide production by the endothelium not only affects vasomotor tone, but also contributes to lesion formation.

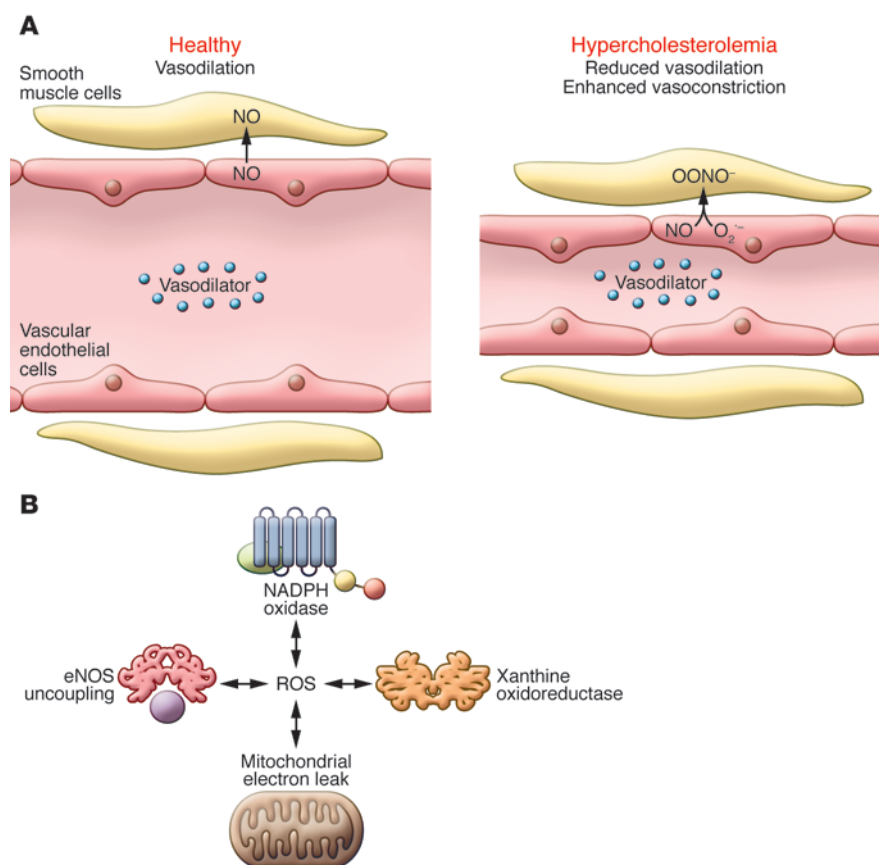
### Subsequent studies and complexity

Our discoveries led to a simple concept (Figure 2A): hypercholesterolemia stimulates an increase in superoxide, which oxidizes NO, preventing endothelium-dependent vasodilation and in some cases unmasking vasocon-

striction. The phrase, “reduced NO bioavailability” became commonly used in reference to conditions in which NO is produced but biologically unavailable through oxidation. Subsequent work from many groups expanded this concept, and it is now clear that as atherosclerosis progresses, plaque-associated and vascular smooth muscle cells produce ROS (18, 19). We initially found that oxypurinol reduced vascular superoxide production and endothelium-dependent vasodilation, suggesting a role for xanthine oxidase in early atherosclerosis (12). Subsequently, we learned that additional sources of ROS are important in vascular diseases, including the NADPH oxidases, uncoupled NOSs, and mitochondria. Our laboratory and others have shown that these various sources are interdependent (Figure 2B), and ROS from one source can activate ROS production by others in a feed-forward fashion (20–22). The NADPH oxidases appear to lead these interactions. For example, NADPH oxidase-pro-

**Figure 2**

Evolving concepts of vascular ROS production. (A) Healthy vascular ECs respond to the presence of vasodilators by producing NO, which diffuses into smooth muscles and promotes vasodilation. Under hypercholesterolemia conditions, vascular ECs produce superoxide in addition to NO in response to vasodilators. Superoxide and NO interact within vascular ECs to produce  $\text{OONO}^-$ , which is a strong oxidant that contributes to vascular dysfunction in individuals with hypercholesterolemia. (B) Interplay between various sources of ROS in vascular cells. NADPH oxidase-produced ROS can oxidize the eNOS cofactor tetrahydrobiopterin (purple), which uncouples these enzymes, resulting in ROS production. NADPH oxidase-associated ROS also disrupt mitochondrial electron transfer, leading to ROS production. Furthermore, ROS from other sources promote the formation of ROS by xanthine oxidoreductase. This feed-forward nature of ROS production promotes several pathophysiological states.





duced ROS can oxidize tetrahydrobiopterin, an NOS cofactor, and this event uncouples these enzymes, resulting in the production of superoxide rather than NO (23). My colleague, Hua Cai, revealed that hydrogen peroxide inhibits the expression of dihydrofolate reductase, an enzyme involved in maintaining cellular levels of tetrahydrobiopterin, leading to eNOS uncoupling (24). Furthermore, NADPH oxidase-associated ROS disrupt mitochondrial electron transfer, leading to ROS production (25). The feed-forward nature of ROS production is recapitulated in several pathophysiological states (20).

It should be noted that all methods for measuring superoxide and related ROS are imperfect. Dr. Sergey Dikalov joined our group and helped us adapt additional methods for ROS detection and quantification, including electron spin resonance, HPLC to monitor oxidation of dihydroethidium, and cytochrome c reduction, as well as hydrogen peroxide-detecting methods. Use of these methods confirmed our initial chemiluminescence-based measurements and proved extremely useful.

### Clinical hurdles and therapeutic implications

Several large clinical trials analyzing the use of antioxidant vitamins in cardiovascular disease have failed to show benefit. In fact, high doses of vitamin E seem to be harmful (26). We now understand that ROS act as signaling molecules and are essential for cell growth and survival. Hydrogen peroxide released from the endothelial mitochondria acts as a hyperpolarizing factor to mediate vasodilation (27). Because ROS production can be highly localized and have different roles in various subcellular compartments, treatment with nonspecific antioxidants has proven problematic. An alternative approach has been to develop specific NADPH oxidase (NOX) inhibitors. Recently, Gray and colleagues demonstrated that hyperglycemia activates NOX1 (28), and deletion of *Nox1* in mice prevented the diabetes-associated acceleration of atherosclerosis. Excitingly, Gray et al. determined that pharmacological inhibition of NOX1 attenuates lesion formation in hypercholesterolemic mice (28). Mitochondria-targeted antioxidants have proven beneficial in experimental hypertension and type 2 diabetes, perhaps by specifically targeting pathological ROS in mitochondria, without disrupting ROS signaling at other cellular sites (29).

In the years since our initial observations, it has become clear that the etiology of coronary spasm is complicated and involves perturbed endothelial NO production, oxidative injury, inflammation, and enhanced vascular smooth muscle constriction. Other causes of endothelial dysfunction in addition to NO oxidation have been described. Nevertheless, the experience of caring for a patient with coronary spasm left a lasting impression and attracted me to this area of research.

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