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Endothelial regulation of vascular tone: From basic biology to clinical application

PETER GANZ, MD

At the cardiac catheterization laboratory at Brigham and Women's Hospital, investigations of endothelial function were initiated nearly two decades ago in order to provide an explanation why atherosclerotic arteries paradoxically constrict in response to the same stimuli that lead to vasodilation of healthy arteries. For example, with exercise performed at the time of cardiac catheterization, epicardial arteries of healthy subjects were found to dilate.[1] In patients with classical stable angina, however, paradoxical vasoconstriction typically occurred at sites of coronary stenoses or even at mildly irregular arterial segments (Figure 1).[1] A similar pattern of dilation of normal human coronary arteries and paradoxical constriction of atherosclerotic coronary arteries is found in response to other stimuli that cause ischemia in daily life, including mental stress,[2] exposure to cold,[3] or with simple increases in heart rate.[4] When superimposed on a coronary stenosis, this vasoconstriction markedly augments stenosis resistance and lowers the rate pressure product threshold for the development of myocardial ischemia.

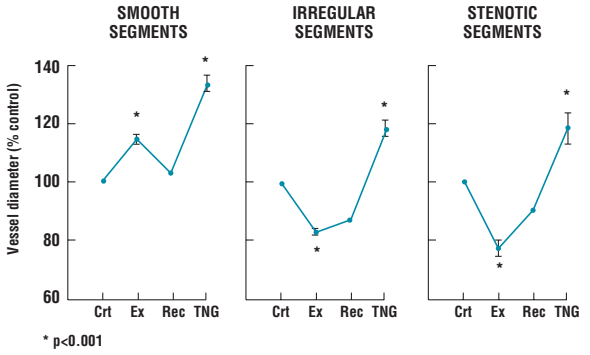
When endothelium was first reported to produce a vasodilator factor (endothelium-derived relaxing factor),[5,6] it was thought that possibly endothelial damage, a characteristic of atherosclerotic arteries, impaired endothelium-dependent vasodilation, 'tipping the balance' of vasomotor tone toward vasoconstriction.

Endothelium-derived relaxing factor (nitric oxide)

Perhaps the most important vasodilator substance produced by the endothelial cells is endothelium-derived relaxing factor (EDRF).[7,8] The discovery of EDRF in 1980 by Furchgott resulted from the observation that intact endothelium was required for acetylcholine-induced vasodilation of rabbit aorta rings.[5] In the presence of endothelium, acetylcholine produced dose-dependent vasodilation. When the endothelium was removed, only constriction was induced by acetylcholine (Figure 2). Accordingly, it became apparent that acetylcholine has two distinct and opposite actions on vasomotor tone: an endothelium-dependent dilation and a smooth muscle-mediated constriction. In most healthy arteries, endothelium-dependent vasodilation predominates over direct vasoconstriction so that the net effect of acetylcholine is vasodilation.

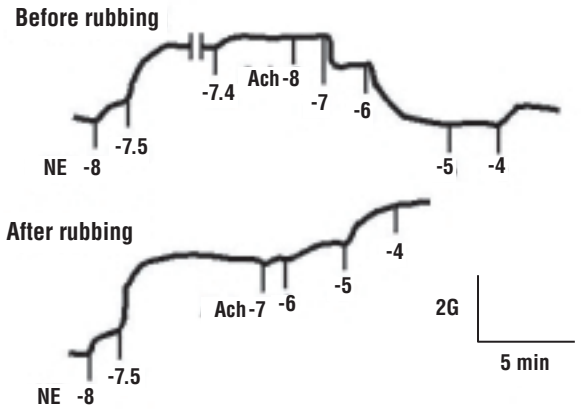
Subsequently, EDRF has been identified as the nitric oxide (NO) radical.[9,10] NO is formed in endothelial cells from L-arginine by the action of enzyme NO synthase (eNOS) (Figure 3). In this reaction, the terminal nitrogen from the guanidine group of L-arginine gives rise to NO. This reaction also requires molecular oxygen and several cofactors including calmodulin, tetrahydrobiopterin (THB_4), NADPH, flavin adenine dinucleotide and flavin mononucleotide and also produces L-citrulline as a byproduct (Figure 3).[9,10] Within the endothelium, eNOS is localized preferentially to invaginations in cell membranes called caveoli and a protein caveolin helps to maintain eNOS in an inactive state by binding to calmodulin in the eNOS complex.[11-13] The eNOS enzyme is activated by binding calcium to calmodulin, displacing the inhibitor caveolin. Once NO is formed, its relaxing effect is mediated by diffusion from the endothelium to smooth

Figure 1: Responses of epicardial coronary arteries to supine bicycle exercise in patients with normal coronary arteries (left panel), irregular coronary arteries (middle panel), or coronary arteries with stenoses (right panel). Irregular arteries and stenoses exhibit a paradoxical constrictor response to exercise with a preserved dilator response to nitroglycerin.[1]



Ctr = control, Ex = exercise, Rec = recovery, TNG =nitroglycerin.

Figure 2: Relaxation by acetylcholine (Ach) of rings of rabbit aorta preconstricted by norepinephrine (NE). Aortic rings were exposed to increasing concentrations of Ach with endothelium intact or removed by rubbing with a wooden stick. This representative tracing shows loss of relaxation in response to Ach with removal of endothelium and appearance of constriction.[103]



muscle cells where it causes an activation of intracellular guanylate cyclase, a rise in cyclic guanosine monophosphate (cGMP), and a consequent fall in intracellular calcium.[14,15] NO released from endothelial cells has a short half-life, limited by interaction with oxygen-derived free radicals in tissues, principally superoxide.[16]

Most vasodilators, acetylcholine being only one example, essentially require the endothelium for their action. For example, serotonin and adenosine diphosphate (ADP) (substances derived from aggregating platelets), thrombin (product of thrombosis), other chemical stimuli (bradykinin, histamine), and increases in shear stress related to increases in blood flow (so-called flow-mediated dilation), all release NO from the endothelium.[17] Endothelium-derived NO also attenuates the action of many vasoconstrictors, including alpha-adrenergic agonists.[18] Only a few vasodilators act independently of the endothelium by relaxing vascular smooth muscle directly. These include nitroglycerin and other nitrates that are transformed to NO. The discovery of an endogenous NO vasodilator pathway solved the long-lasting mystery as to why exogenously administered nitrates are capable of eliciting vasodilation in atherosclerotic, as well as normal vessels. However, while exogenous nitrates are plagued by tolerance,[19] this is not a significant problem for the endogenous endothelial NO pathway.

Endothelium-dependent dilation is impaired with atherosclerosis

Endothelium-dependent vasodilation was first discovered in the aorta of healthy rabbits. Clinical cardiologists wanted to understand the importance of this pathway in healthy human epicardial coronary arteries and whether it was impaired in patients with coronary atherosclerosis. Accordingly, patients with normal coronary arteries who were undergoing cardiac catheterization were administered acetylcholine directly into the coronary arteries at the same concentrations used in the experimental studies by Furchgott; vasodilation was observed (Figure 4).[20] This acetylcholine-induced vasodilation can be inhibited by blocking NO synthesis, eg, with N^G-monomethyl-L-arginine (L-NMMA), confirming that this is a NO-dependent response.[21,22] Other endothelium-dependent agents shown to dilate healthy human coronary arteries include flow-mediated dilation,[23,24] serotonin, histamine, bradykinin, and substance P[17].

In contrast to its effect in normal human coronary arteries, acetylcholine constricts human atherosclerotic coronary arteries, reflecting the loss of NO and the unopposed constrictor action of acetylcholine on vascular smooth muscle.[20] The finding of endothelial vasodilator dysfunction in atherosclerotic human coronary arteries has been reinforced by similar observations for other stimuli that release NO, including flow-mediated dilation, serotonin, and ADP.[17]

Endothelium-dependent dilation is impaired with atherogenic risk factors

The loss of endothelium-dependent dilation occurs in the early stages of atherosclerosis, even before it can be detected by angiography[25,26] or by ultrasound.[27,28] Studies have suggested that this loss of NO bioavailability is related to the presence of risk factors for atherosclerosis.[25] Essentially, all risk factors that lead to atherosclerosis also impair endothelial vasodilator function in human arteries, including: dyslipidemia; hypertension; type I and type II diabetes mellitus; active and passive cigarette smoking; menopause; hyperhomocystinemia; aging; family history of atherosclerosis and certain infections.[29,30] In addition to risk factors, sites of disturbed hemodynamic shear stress, such as coronary bifurcations, have a predilection toward atherosclerosis and show impaired endothelium-dependent vasodilation before atherosclerosis can be detected.[31]

Figure 3: Endothelial cell production of NO by the action of NO synthase (eNOS) on L-arginine. This reaction requires a number of cofactors, including tetrahydrobiopterin [THB_4] and NADPH. A rise in intracellular Ca^{++} in response to vasodilator agonists or shear stress displaces the inhibitor caveolin from calmodulin activating eNOS. NO diffuses to vascular smooth muscle and causes relaxation by activating guanylate cyclase [GC], thereby increasing intracellular cyclic guanosine monophosphate [cGMP].

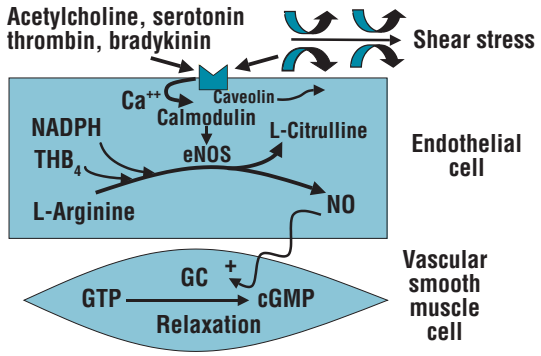
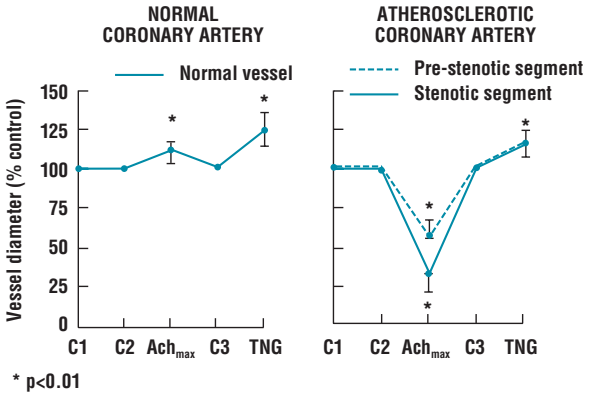


Figure 4: Responses of coronary arteries to intracoronary administration of an endothelium-dependent vasodilator (acetylcholine) and a direct smooth muscle vasodilator (TNG) in patients with normal coronary arteries (left panel) and atherosclerotic coronary arteries (right panel). Atherosclerotic arteries exhibit a paradoxical constrictor response to acetylcholine with a preserved dilator response to nitroglycerin.[20]



C1 = control; C2 = vehicle control; Ach_{max} = response to maximal dose of acetylcholine; C3 =repeated control; TNG = nitroglycerin.

Atherogenic risk factors can rapidly modulate and impair endothelial function. For example, the release of NO from endothelial cells in culture is diminished within 30 minutes of adding low-density lipoprotein (LDL) or oxidized LDL particles.[32] Risk factors can be introduced experimentally (and temporarily) into healthy human volunteers and their effect on endothelial function assessed. Administering a load of methionine to raise homocysteine concentration,[33] a high fat meal to generate atherogenic lipoprotein remnant particles,[34] or infusing high concentrations of glucose to mimic diabetes mellitus,[35] all reduce the availability of NO in minutes to hours, and each can result in reduced endothelium-dependent dilation. Surgical ovariectomy in women free of cardiovascular disease, a procedure that results in premature menopause, is also followed by a rapid loss of endothelium-derived NO.[36] The results of investigations that have introduced risk factors into subjects free of cardiovascular disease also helped to establish a causal link between risk factors and endothelial injury in humans.

Recent data suggest that certain infections can promote the development of atherosclerosis, but the underlying mechanisms for this association are incompletely understood. Vaccination of healthy volunteers with *Salmonella typhi* has been used as a model of infection. It elicits an inflammatory response, followed by a marked depression in endothelial function (assessed as a response to several vasodilators in the forearm circulation),[37] providing a plausible, though speculative mechanistic link between infection, endothelial function, and atherosclerosis (Figure 5).

Endothelium-dependent vasodilation is impaired in resistance vessels with atherogenic risk factors

While vascular blood flow can be limited by atherosclerotic stenoses in large arteries, second-to-second regulation of blood flow is generally accomplished in resistance arterioles. Endothelium-dependent vasodilation occurs not only in large conduit arteries such as epicardial arteries, it is also a mechanism that controls dilation of small (resistance) vessels.[38] In addition to NO, other endothelium-derived factors may contribute to vasodilation of coronary resistance vessels, including prostacyclin and endothelium-derived hyperpolarizing factors.[24,39-41] Although atherosclerosis is typically absent from resistance vessels, atherogenic risk factors markedly impair the responses of resistance vessels to endothelium-dependent vasodilator stimuli.[38,42,43] This is not altogether surprising since most atherogenic risk factors are transmitted in circulating blood and are in contact with endothelium throughout the systemic vasculature.[44]

The failure of blood flow to increase in response to metabolic stimuli in patients with atherosclerosis can be attributed in part to a reduced endothelium-dependent metabolic vasodilation in resistance vessels, and is a mechanism that contributes to myocardial ischemia.[38]

Figure 5: Forearm blood flow responses to incremental doses of bradykinin, an endothelium-dependent vasodilator, in subjects vaccinated against *S. typhi.* (n = 6). The marked depression in the vasodilator response to bradykinin present at 8 hours post-vaccination coincides with an inflammatory response.[37]

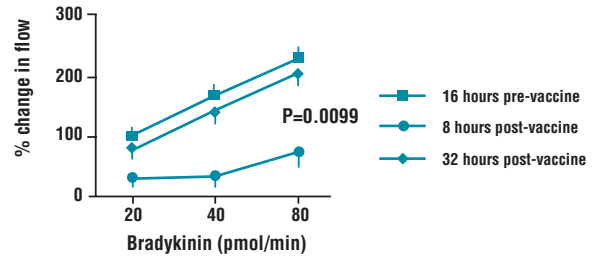
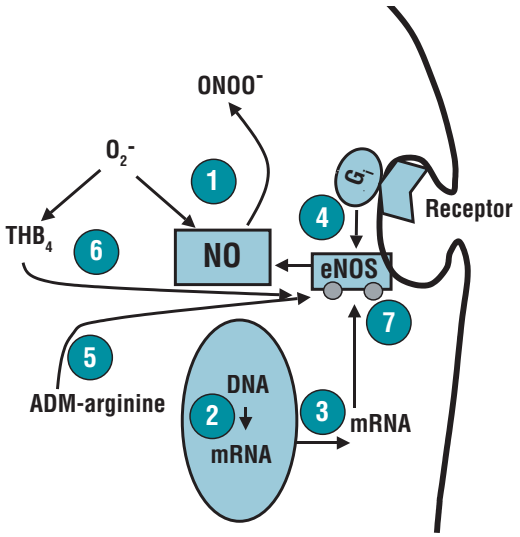


Figure 6: Mechanisms of endothelial dysfunction in hypercholesterolemia.



Hypercholesterolemia, and other cardiovascular risk factors, reduce the bioavailability of NO by:

1. increasing the generation of superoxide [O_2^-], which combines with NO to generate a vasoinactive, potentially toxic, product $ONOO^-$
2. reducing the transcription and
3. stability of messenger RNA encoding for NO synthese
4. interfering with coupling of endothelial receptors to associated G-proteins required to signal transduction
5. favoring accumulation of asymmetric dimethyl arginine [ADM-arginine], a competitive antagonist to L-arginine, the substrate for NO synthesis
6. causing a loss of tetrahydrobiopterin [THB_4], a cofactor for eNOS – in the absence of THB_4, eNOS generates superoxide rather than NO, exacerbating NO inactivation
7. favoring the accumulation of caveolin, an inhibitor of eNOS activity.

Assessment of endothelial function by brachial ultrasound

The recognition of the 'systemic nature' of endothelial dysfunction[44] has facilitated the development of approaches that noninvasively assess endothelial function in the brachial artery using high-resolution external ultrasound.[28,45-47] The brachial artery dilation in response to reactive hyperemia is indeed mediated by NO[48] and correlates reasonably well with coronary responses to acetylcholine.[27] This ultrasound approach has permitted studies of endothelial function in populations of asymptomatic subjects in whom cardiac catheterization is not indicated, and has been useful in studies of the reversibility of early arterial damage, using various antiatherogenic strategies.[28]

Molecular basis of NO deficiency in atherosclerosis

The loss of NO bioavailability in the setting of atherogenic risk factors or in established atherosclerosis is caused by reduced biosynthesis, as well as accelerated breakdown of NO (Figure 6).[11-13,16,49-55] Factors that decrease NO synthesis include:

- reduced transcription and/or stability of messenger RNA encoding for NO synthase resulting in diminished eNOS enzyme;
- defective coupling of endothelial receptors to associated G-proteins that are involved in downstream signaling;
- accumulation of asymmetric dimethyl arginine (ADM-arginine) that acts as a competitive antagonist to the substrate of L-arginine used by eNOS;
- a rise in the concentration of caveolin, an inhibitor of eNOS activation.

Accelerated breakdown of NO is caused by an excess production of superoxide free radicals (O_2^-) that inactivate NO, and in the process, generate a potentially toxic product peroxynitrite ($ONOO^-$).[16,56] Superoxide free radicals in atherosclerotic arteries are byproducts of several enzymatic pathways including xanthine oxidase and NADH/NADPH oxidase.[16] Excess superoxide also leads to a degradation of tetrahydrobiopterin, a cofactor for eNOS enzyme.[49,54,57] In the absence of this cofactor, eNOS produces superoxide rather than NO, further augmenting oxidant stress and NO breakdown (Figure 6).[54]

Nitric oxide is a potent antiatherogenic molecule

NO is a multipotent molecule with antiatherogenic properties. NO inhibits the recruitment and differentiation of inflammatory cells in the arterial intima by reducing the production of chemotactic cytokines, leukocyte adhesion molecules, and factors that encourage the differentiation of monocytes into macrophages.[58-61] NO also inhibits the production of tissue factor, a highly thrombogenic molecule.[62,63] Lastly, NO inhibits the proliferation of vascular smooth muscle cells.[64]

Accordingly, as well as being a vasodilator, NO has antiatherogenic and plaque-stabilizing actions that have been substantiated by experimental and clinical investigations. For example, eNOS deficiency introduced experimentally by a genetic mutation, markedly accelerates coronary arteriosclerosis in hypercholesterolemic mice, in association with myocardial ischemia, infarction, and heart failure.[65] In humans, naturally occurring mutations in eNOS that reduce its activity are associated with an increased incidence of myocardial infarction (MI), as well as vasospasm and hypertension.[66-68] In cardiac transplant recipients, vasoconstriction to acetylcholine indicative of endothelial dysfunction is associated with rapid development of coronary arteriosclerosis.[69] Furthermore, patients undergoing cardiac catheterization found to have evidence of coronary endothelial dysfunction by acetylcholine testing, have a far greater incidence of adverse coronary events in subsequent follow-up compared to patients with preserved endothelial function.[70,71]

The role of endothelial dysfunction in clinical ischemic syndromes

Coronary endothelial dysfunction in epicardial or resistance vessels is typically accompanied by myocardial perfusion defects, suggestive of ischemia.[72-74] Furthermore,

daily activities including exercise, mental stress, or exposure to cold paradoxically constrict atherosclerotic arteries, increasing stenosis resistance.[1-3] The responses during these stimuli parallel closely the responses to acetylcholine suggesting that they are determined by the health of the endothelium.[1-3] These stimuli are accompanied by activation of the sympathetic nervous system, by an increase in circulating catecholamines, and by increases in coronary blood flow secondary to a rise in myocardial oxygen demand. In patients with dysfunctional endothelium, the loss of flow-mediated and catecholamine-stimulated NO release permits unopposed constriction to catecholamines.[18] Thus, the loss of NO may contribute to impaired dilation or exaggerated constriction of epicardial and resistance vessels and thereby to myocardial ischemia. Conversely, improvement in endothelial function, achieved by cholesterol lowering therapy, is paralleled by a reduction in myocardial ischemia[75] and improved myocardial perfusion.[76-80]

Patients with recent MI or unstable angina show more pronounced endothelial dysfunction in the culprit artery compared to arteries with stable lesions of similar severity.[81,82] While intracoronary platelet aggregation and thrombosis is a hallmark in these acute ischemic syndromes, coronary constriction plays an important role as well. The products released from aggregating platelets or from thrombi, although dilating normal arteries, severely constrict atherosclerotic arteries, due to endothelial dysfunction.[83,84] The clinical significance of these findings is reinforced by observations that coronary concentrations of serotonin, a platelet release product, is markedly increased in patients with acute coronary syndromes.[85]

Endothelial dysfunction, and reduced NO in particular, may play an important role in destabilizing atherosclerotic plaques as well. An important feature of unstable plaques is inflammation. NO regulates multiple steps in the inflammatory cascade and NO reduction is accompanied by a rise in cytokines and chemoattractant factors (eg, interleukin-6, interleukin-8, monocyte chemotactive protein-1), leukocyte adhesion molecules, and factors that facilitate the differentiation of monocytes into macrophages (eg, macrophage colony-stimulating factor).[58-61]

Accordingly, endothelial dysfunction and deficiency of NO exacerbates myocardial ischemia in patients with stable angina or acute ischemic syndromes. In addition, endothelial dysfunction may predispose to a transition from stable to unstable ischemic syndromes.

Treatment of endothelial dysfunction in the clinical setting

Several strategies are effective at restoring endothelium-dependent dilation, augmenting myocardial perfusion, and in parallel, reducing myocardial ischemia or symptoms of angina. These include the use of lipid lowering agents, angiotensin-converting enzyme inhibitors, and therapeutic lifestyle changes.

The use of cholesterol-lowering agents (statins, cholestyramine, or LDL apheresis) has led to a significant improvement in endothelium-dependent dilation of coronary and peripheral arteries in patients with hypercholesterolemia.[17,86-90] This improvement is particularly rapid in resistance arterioles,[88] and takes longer in frankly atherosclerotic arteries where a significant amount of lipid has to be removed from the arterial intima before endothelial function returns to normal.[91,92]

Intensive reduction in cholesterol is associated with improved myocardial perfusion by positron-emission tomography (PET scan)[76-79] or thallium imaging[80] and reduced ischemia by ambulatory ECG monitoring.[75] Cholesterol lowering has been shown to markedly reduce evidence of myocardial ischemia on ambulatory ECG monitoring in hypercholesterolemic patients with stable angina over a 6-month period, a time course that parallels the improvement in endothelial function previously observed in similar patient populations (Figure 7).[75] Improvement in endothelial vasodilator function is also thought to be a mechanism that contributes to a reduction in recurrent ischemic episodes in patients with acute coronary syndromes after intensive cholesterol lowering.[93]

HMG-CoA reductase inhibitors (statins) restore endothelial function, in part by their ability to reduce serum cholesterol. Recent studies have suggested that several intermediates in the cholesterol synthetic pathway have a biological activity of their own (Figure 8). Inhibition of one such intermediate – geranyl-geranyl pyrophosphate – by statins results in increased synthesis of eNOS.[94-96] In animal studies, statins significantly reduce the size of ischemic cerebrovascular strokes by augmenting eNOS and thereby improving collateral blood flow.[97] The clinical importance of this 'non-lipid' effect of statins is under investigation. Drugs that selectively mimic this property of statins (eg, Rho kinase inhibitors) are undergoing clinical investigation.

Therapeutic lifestyle changes can also improve coronary endothelial function. Exercise training restores endothelium-dependent vasodilatation both in epicardial

Figure 7: Effect of cholesterol lowering or placebo over 6 months on the number of episodes of ischemic ST-segment depression in patients with coronary disease. Two of 20 in the placebo group vs. 13 of 20 in the treatment group demonstrate complete resolution of ischemia.[75]

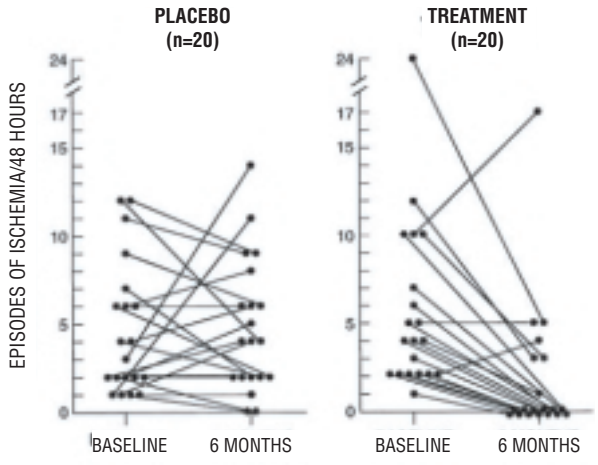
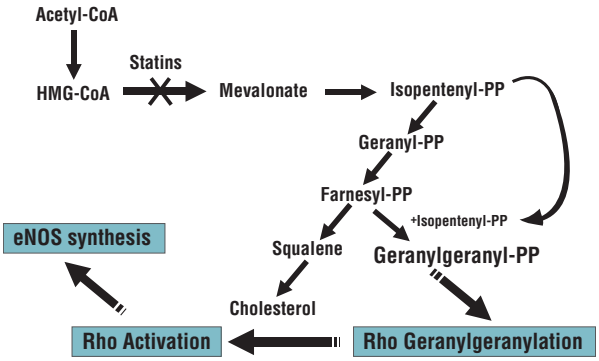


Figure 8: Inhibition of Rho by statins. Statins inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and block the synthesis of cholesterol and isoprenoids, including geranylgeranyl pyrophosphate. Modification of Rho by attachment of geranylgeranyl pyrophosphate permits the subsequent membrane translocation and activation of Rho. A reduced Rho activation by statins enhances the synthesis of endothelial NO synthase.[94]



coronary vessels and in resistance vessels in patients with coronary artery disease.[98] Cigarette smoking is associated with dose-related impairment of endothelium-dependent arterial dilation and this endothelial dysfunction appears to be reversed with smoking cessation.[99]

Several novel strategies that may improve endothelial function are under clinical investigation. There is a strong, inverse relationship between endothelial function and HDL cholesterol.[100,101] While high-density lipoprotein (HDL) might improve endothelial function by accelerating reverse cholesterol transport, it was recently discovered that HDL is also a direct, potent activator of the eNOS enzyme.[102] In preliminary clinical studies, infusion of reconstituted HDL improved endothelial function in hypercholesterolemic patients, a strategy that will be explored in future studies using more practical means of raising HDL.

Conclusion

Studies of the endothelial regulation of vascular tone have been particularly exhilarating for myself, and my closest senior collaborators, Dr A. Selwyn and Dr. M. Creager, (as well as many talented Fellows who have joined our laboratory) over the past two decades. The initial study of endothelial function in humans[20] has stimulated widespread interest in 'translational research' in endothelial function. Concepts taken from basic biology were tested in the clinical setting, and therapeutic approaches were developed to improve the lives of patients. At the same time, questions have been generated that will keep colleagues in basic science busy with research for many years to come. Endothelial function is a prime example of translational research that has come to clinical fruition.

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Peter Ganz, M.D. is an Associate Professor of Medicine at Harvard Medical School and the Director of Vascular Research in the Cardiac Catheterization Laboratory at the Brigham and Women's Hospital. Dr. Ganz received his M.D. from Harvard Medical School in 1976, and subsequently completed his residency at the Massachusetts General Hospital and cardiovascular fellowship at the Brigham and Women's Hospital. He has authored over 100 original articles and numerous review articles. He has served on the editorial board of several prominent journals.

Dr. Ganz's research interests have centered on endothelial function, as it pertains to the regulation of vascular tone or inflammation. He has been instrumental in advancing translational research in endothelial function, taking concepts derived from basic biology and applying them in the clinical setting. The National Heart, Lung and Blood Institute of the NIH has recognized the importance of Dr. Ganz's work by generous support throughout his career. Dr. Ganz can be reached at pganz@aol.com

The author serves as a consultant for Pfizer and Merck

Brigham and Women's Hospital,
Cardiovascular Division website:
www.heartdoc.org

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