Molecular Insights and Therapeutic Targets for Diabetic Endothelial Dysfunction

Jian Xu, PhD; Ming-Hui Zou, MD, PhD

Diabetes mellitus and its associated complications are major health problems in the developed world. Diabetes mellitus is associated with an increased risk of cardiovascular disease (CVD) even in the presence of intensive glycemic control. Indeed, 75% of diabetic patients will die of CVD. Patients with type 1 and type 2 diabetes mellitus have an increased risk for the 3 major types of macrovascular disease (coronary heart disease, peripheral vascular disease, and stroke). A striking feature of diabetic cardiovascular complications is the appearance of accelerated atherosclerosis, which anatomically resembles atherosclerosis in nondiabetic individuals but is more extensive and occurs at an earlier age. Substantial clinical and experimental evidence suggests that endothelial dysfunction is a crucial early step in the development of atherosclerosis. Evidence also suggests that it participates in plaque progression and the clinical emergence of cardiovascular events. Endothelial dysfunction is characterized by impaired endothelium-dependent vasodilation and “endothelial activation,” which is associated with a proinflammatory, proliferative, and procoagulatory milieu that promotes initiation and complications of atherogenesis. Endothelial dysfunction associated with insulin resistance appears to precede the development of overt hyperglycemia in patients with type 2 diabetes mellitus. Therefore, endothelial dysfunction may be a critical early target for the prevention of atherosclerosis and CVD in patients with diabetes mellitus or insulin resistance. A synergistic cross talk exists among the conventional cardiovascular risk factors associated with diabetes mellitus, and such cross talk contributes to disruption of endothelial integrity and acceleration of atherosclerosis. However, the biochemical and cellular links between elevated blood glucose levels and endothelial dysfunction remain incompletely understood. This review will focus on the multifactorial nature of endothelial dysfunction in diabetes mellitus, the relationship between endothelial dysfunction and conventional cardiovascular risk factors, and the translational potential of molecular targets such as the AMP-activated protein kinase (AMPK) for treating endothelial dysfunction in diabetes mellitus.

Endothelial Function and Cardiovascular Health

Endothelial Function and Vascular Homeostasis

Originally considered to be simply a physical barrier between the blood and vascular wall, the endothelium is now recognized as the most important component of normal vascular homeostasis, because it serves to maintain the anticoagulant, antiplatelet, and fibrinolytic phenotypes of vascular cells. The maintenance of vascular homeostasis is accomplished through the release of numerous dilator and constrictor substances (the endothelium is regarded as a complex endocrine and paracrine organ; Figure 1). The most important factor released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor. Other endothelium-derived vasodilators include prostacyclin, bradykinin, and an uncharacterized endothelium-derived hyperpolarizing factor. Prostacyclin acts synergistically with NO to inhibit platelet aggregation. Bradykinin stimulates release of NO, prostacyclin, and endothelium-derived hyperpolarizing factor to inhibit platelet aggregation. Bradykinin also stimulates production of tissue plasminogen activator and thus may play an important role in fibrinolysis. The endothelium also produces vasoconstrictor substances such as endothelin (the most potent endogenous vasoconstrictor identified to date), reactive oxygen species (ROS), prostaglandin H2 (PGH2), thromboxane A2 (TXA2), and angiotensin II. Angiotensin II is not only a vasoconstrictor but also a pro-oxidant that stimulates production of endothelin. Endothelin and angiotensin II promote proliferation of smooth muscle cells and in this way contribute to plaque formation. Large amounts of endothelin are produced by activated macrophages and vascular smooth muscle cells, the cellular components of atherosclerotic plaques. Because the endothelium plays a key role in vascular homeostasis, damage to this cellular layer will upset the balance between vasoconstriction and vasodilation, initiating a number of events/processes that promote or exacerbate atherosclerosis. These include endothelial permeabilization, platelet aggregation, leukocyte adhesion, and cytokine production.

Assays of Endothelial Function in the Clinic

An improved understanding of the vascular biology of the endothelium and appreciation of the central role of the
endothelium in vascular disease has enabled the development of clinical tests to evaluate the functional properties of normal and activated endothelium. Ideally, such tests should be safe, noninvasive, reproducible, inexpensive, and standardized between laboratories. The results should also reflect the dynamic biology of the endothelium throughout the natural history of atherosclerotic disease, define subclinical disease processes, and provide prognostic information for risk stratification in the later clinical phase. No single test currently fulfills these requirements, which necessitates the use of several tests to characterize the multiple aspects of endothelial biology. Because endothelial function cannot be measured directly in humans, estimates may be obtained indirectly by measuring the endothelium-dependent vasodilation (vasoregulation), plasma levels of endothelium-derived regulatory proteins (NO, endothelium-derived hyperpolarizing factor, prostacyclin [PGI₂], TXA₂, and endothelin-1 [ET-1]), and possibly microalbuminuria. Additional aspects of endothelial function that can be measured are coagulation, fibrinolysis, inflammation, and angiogenesis. Other vascular properties such as arterial stiffness and intima/media thickness of the carotid artery are likely only endothelium dependent in part.

Endothelial function was first measured in humans in 1986 by assessing responses of the epicardial arteries to infused acetylcholine, an invasive approach. The first noninvasive approach was described in 1992. In this test, the diameter of an artery was measured by noninvasive ultrasound before and after an increase in shear stress (provided by reactive hyperemia), with the degree of dilation reflecting (in large part) arterial endothelial NO release. Measurement of ultrasound-based flow-mediated dilation in the brachial artery has intrinsic appeal, because it approaches certain criteria for endothelial function assessment. Nevertheless, it is not yet ready for widespread use in risk stratification (see Deanfield et al and Schalkwijk et al for the advantages and disadvantages of the available methods). Other noninvasive vascular testing approaches have been proposed in recent years and include pulse-wave analysis, pulse-wave velocity measurement, and pulse amplitude tonometry, such as reactive hyperemia pulse amplitude tonometry measured in the fingertips. Overall, clinical assessments of endothelial function not only have provided novel insights into pathophysiology but also have enabled detection of early disease, quantification of risk, judgment of response to interventions designed to prevent progression of early disease, and reduction of later adverse events in patients.

**Figure 1.** Endothelium, as a complex endocrine and paracrine organ, plays a crucial role in the maintenance of vascular homeostasis. Far beyond being simply a physical barrier between the blood and vascular wall, the endothelium is now recognized as the most important component of normal vascular homeostasis, through which the anticoagulant, antiplatelet, and fibrinolytic phenotypes of vascular cells can be maintained. Essentially, the maintenance of vascular homeostasis by endothelium is accomplished through the release of vasoprotective factors (eg, NO/endothelium-derived relaxing factor, prostacyclin, bradykinin, and endothelium-derived hyperpolarizing factor [EDHF]) and harmful substances (eg, endothelin [ET], ROS, endothelium-derived COX-dependent vasoconstricting factor [EDCF], and angiotensin II [Ang II]). Damage to endothelium will disrupt the balance between vasoprotective factors and harmful substances, initiating a number of events/processes that promote or exacerbate atherosclerosis via increased endothelial permeabilization, platelet aggregation, leukocyte adhesion, and cytokine production.

**Endothelial Dysfunction and CVD Prognosis**

The term “endothelial dysfunction” encompasses several pathological conditions, including altered anticoagulant and antiinflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. However, in much of the literature, this term...
often refers to reduced bioavailability of endothelium-derived relaxation factors (such as NO, prostacyclin, or endothelium-derived hyperpolarizing factor) and, consequently, impairment in the vasodilatory effects of these relaxation factors. The concept of endothelial dysfunction has attracted considerable attention, because the identification of a measurable parameter for endothelial dysfunction may link complex molecular phenomena to vascular pathologies such as atherosclerosis.

Numerous experimental studies have demonstrated that maintenance of the functional integrity of the endothelium exerts potent antiatherosclerotic and antithrombotic effects.

Endothelial injury and dysfunction are associated with a number of known vascular diseases. For example, traditional CVD risk factors such as advanced age, hypertension, smoking, and low HDL cholesterol are associated with endothelial dysfunction. Importantly, these risk factors typically produce endothelial dysfunction before they generate true atherosclerosis, which suggests that a unifying mechanism links endothelial dysfunction to atherosclerosis and vascular disease. Although endothelial dysfunction is clearly a feature of atherosclerosis, it has been difficult to prove that it is required for the development and clinical manifestation of vascular disease. Nevertheless, several studies have proven the prognostic value of endothelial function, which is most often clinically assessed as a vasodilator response to pharmacological or mechanical stimuli. For example, impaired vasodilation in either coronary or peripheral vessels identifies those individuals at increased risk for future cardiovascular events. In addition, impaired brachial endothelial function predicts both short-term and long-term outcome in patients undergoing vascular surgery. Thus, prospective studies clearly indicate that impaired endothelial function identifies patients at increased vascular risk, providing a “barometer” for vascular health.

**Endothelial Dysfunction in Diabetes Mellitus**

Vascular injury is the principal complication of all forms of diabetes mellitus. One of the main events underlying vascular injury is the development of endothelial dysfunction, which predisposes diabetic patients to cardiovascular complications and microthrombus formation. Multiple studies in patients and in vitro have revealed that hyperglycemia alters endothelial metabolism and function in such a way that could lead to vascular disease. In diabetic patients and animals, endothelial dysfunction precedes clinically significant atherosclerotic vascular disease and may play a role in its pathogenesis. An overwhelming mass of data demonstrates that endothelial dysfunction occurs in animal models of diabetes and in human blood vessels from diabetic patients.

Abnormalities in endothelium-dependent relaxation have been described in patients with diabetes, and impaired glucose tolerance has been described in young normoglycemic relatives of patients with type 2 diabetes mellitus. Similar abnormalities have been detected in individuals with acutely increased blood glucose and insulin levels resulting from glucose infusion.

**Clinical Relevance of Endothelial Dysfunction in Diabetic Patients**

Accumulating evidence suggests that endothelial dysfunction is an early marker for atherosclerosis and can be detected before angiographic or ultrasound-assisted detection of structural changes to the vessel wall. Multiple risk factors have been found to predict endothelial dysfunction, and many of these risk factors are also associated with a predisposition to atherosclerosis.

**Accelerated Atherosclerosis**

The most common and devastating complications of diabetes mellitus are cardiovascular complications, with these events being the major cause of hospital admissions. A striking feature of diabetic cardiovascular complications is accelerated atherosclerosis, which is associated with metabolic syndrome, insulin resistance, and oxidative stress. The Diabetes Control and Complications Trial for type 1 diabetes mellitus and the United Kingdom Prospective Diabetes Study for type 2 diabetes mellitus have established the importance of intensive glycemic control in dramatically reducing the devastating complications that result from poorly controlled diabetes mellitus. Both trials demonstrated the efficacy of intensive glucose control in reducing the risk of microvascular complications such as retinopathy, neuropathy, and nephropathy. Recent findings from the long-term follow-up of participants in the Diabetes Control and Complications Trial reveal that study participants who intensively controlled blood glucose had a decreased incidence of atherosclerosis, which suggests that hyperglycemia is important in the development of macrovascular diseases in diabetes mellitus. Accordingly, endothelium-dependent relaxation is impaired in patients with type 2 diabetes mellitus and individuals with an acute elevation of blood glucose and insulin levels due to glucose infusion.

**Dysfunctional Endothelial Progenitor Cells**

The presence of endothelial progenitor cells (EPCs) was first demonstrated by showing that CD34+ hematopoietic progenitor cells purified from adults can differentiate, acquire an endothelial phenotype ex vivo, and be incorporated into neovessels at sites of ischemia. Circulating bone marrow–derived endothelial progenitor cells were then identified in the adult. Most convincingly, bone marrow–transplanted genetically tagged cells were found to colonize implanted Dacron grafts. Regardless of the origin of circulating EPCs, this pool of circulating endothelial cells may function in an endogenous repair mechanism that serves to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery. Increasing evidence supports the notion that maintenance of the endothelial monolayer may prevent thrombotic complications and atherosclerotic lesion development. Transplantation of apolipoprotein E–deficient mice with wild-type bone marrow reduces atherosclerotic lesion formation. Various risk factors for coronary artery disease, such as diabetes mellitus, hypercholesterolemia, hypertension, and smoking, affect the number and functional activity of EPCs in healthy volunteers and in patients with coronary artery disease. Likewise, diabetic mice and patients exhibit...
reduced functional activity of EPCs.\textsuperscript{46–48} In addition, factors that reduce cardiovascular risk, such as statins\textsuperscript{49–52} or exercise,\textsuperscript{53} elevate the number of EPCs, which enhances endothelial repair. Thus, the balance of atheroprotective and proatherosclerotic factors may influence EPC numbers and subsequently reendothelialization capacity.\textsuperscript{53} A few studies have shown that injected EPCs home to sites of ischemia, incorporate into the newly formed capillaries, and augment neovascularization.\textsuperscript{54} A strong inverse correlation exists between EPC number and cardiovascular risk, as assessed by the combined Framingham risk factor score.\textsuperscript{44} Consistent with this finding, analysis of flow-mediated brachial artery reactivity has revealed a significant relation between endothelial function and EPC number. Together, these findings suggest that EPCs are critical to the maintenance of endothelial integrity, and EPC dysfunction contributes to the pathogenesis of vascular disease.\textsuperscript{47}

\textbf{Wound Healing}

More than 100 known physiological factors contribute to wound-healing deficiencies in individuals with diabetes mellitus. These factors include growth factor production, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue, keratinocyte and fibroblast migration and proliferation, number of epidermal nerves, bone healing, and the balance between extracellular matrix production and remodeling by matrix metalloproteinases (see Brem and Tomic-Canic\textsuperscript{55} for a detailed discussion). Optimum healing of a cutaneous wound requires orchestration of complex biological and molecular events such as cell migration, cell proliferation, and extracellular matrix deposition and remodeling.\textsuperscript{56} Wound healing is a cellular response that must be appropriate and precise to the injury. This response involves subsequent activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. However, this orderly progression of the healing process is impaired in chronic wounds, including those that result from diabetes mellitus. Several pathogenic abnormalities ranging from disease-specific intrinsic flaws (blood supply, angiogenesis, and matrix turnover) to extrinsic factors (infection and continued trauma) contribute to the failed healing. Impaired healing of diabetic ulcers is caused by several intrinsic factors (neuropathy, vascular problems, and other complicating diabetes-induced systemic effects) and extrinsic factors (wound infection, callus formation, and excessive pressure to the site). Recently, identification of key factors that participate in EPC-mediated diabetic wound healing has gained increasing attention. Gallagher and colleagues\textsuperscript{57} demonstrated that in diabetic mice, hyperoxia enhances mobilization of circulating EPCs from the bone marrow to the peripheral circulation. Local injection of the chemokine stromal cell-derived factor-1α then recruits these EPCs to the cutaneous wound site to accelerate wound healing.

\textbf{Causes of Endothelial Dysfunction in Diabetes Mellitus}

\textbf{Hyperglycemia}

Prolonged exposure to hyperglycemia is now recognized as a major factor in the pathogenesis of diabetic complications, including atherosclerosis. Hyperglycemia induces a large number of cellular alterations\textsuperscript{58} in vascular tissue that potentially accelerate the atherosclerotic process. Animal and human studies have identified at least 5 major mechanisms that account for most of the pathological alterations observed in the diabetic vasculature\textsuperscript{25,58}: (1) Nonenzymatic glycosylation of proteins and lipids interferes with normal protein function by disrupting molecular conformation, altering enzymatic activity, reducing degradative capacity, and interfering with receptor recognition. In addition, glycosylated proteins interact with a specific receptor present on all cells relevant to the atherosclerotic process, including monocyte-derived macrophages, endothelial cells, and smooth muscle cells. The interaction of glycosylated proteins with their receptor induces oxidative stress and proinflammatory responses. (2) Protein kinase C activation alters growth factor expression. (3) Shunting of excess intracellular glucose into the hexosamine pathway leads to O-linked glycosylation of various enzymes and perturbs normal enzyme function. (4) Hyperglycemia increases oxidative stress through several pathways. A major mechanism appears to be superoxide (O\textsuperscript{2}−) overproduction by the mitochondrial electron transport chain. (5) Hyperglycemia promotes inflammation through induction of cytokine secretion by several cell types, including monocytes and adipocytes. Importantly, hyperglycemia-induced oxidative stress is tightly linked with other hyperglycemia-dependent mechanisms of vascular damage described above, namely, formation of advanced glycation end products, protein kinase C activation, and increased flux through the hexosamine pathway (Figure 2).

Hyperglycemia might not be the sole factor responsible for endothelial dysfunction in diabetes mellitus. Other factors such as dyslipidemia, elevated free fatty acids (FFAs), inflammation, and insulin resistance can cause endothelial dysfunction. For example, endothelial dysfunction might precede the development of overt hyperglycemia in type 2 diabetes mellitus and prediabetic states (family history of type 2 diabetes mellitus and prior gestational diabetes, as well as in obese but nondiabetic individuals).\textsuperscript{59} Consistent with these observations, inflammation and endothelial activation are evident at birth in offspring of women with type 1 diabetes mellitus.\textsuperscript{60} In the nonobese diabetic (NOD) mouse, the preferred and accepted animal model of spontaneous autoimmune type 1 diabetes mellitus, there is endothelial dysfunction characterized by a paradoxical vasoconstriction that precedes the hyperglycemia, which suggests a hyperglycemia-independent effect on endothelial function in type 1 diabetes mellitus. This could explain the clinical observation that certain people are very prone to complications (despite good glycemic control), whereas others are not, regardless of glycemic status.

\textbf{Fatty Acids}

Both type 1 and type 2 diabetes mellitus are associated with an increase in circulating FFAs.\textsuperscript{61} Elevation of plasma FFA levels through infusion of a lipid emulsion and heparin has been shown to impair endothelium-mediated vasodilation (and NO production) in human volunteers undergoing a hyperinsulinemic-euglycemic clamp.\textsuperscript{62} Interestingly, such el-
Hyperglycemia

- NAD(P)H oxidase / Mitochondrial O$_2^-$
- eNOS uncoupling
- EDCF
- AGE
- PKC
- Hexosamine flux
- Endothelin
- LDL oxidation
- ONOO$^-$ (with other ROS/RNS)
- 6-10 x 10$^{-9}$ M$^{-1}$S$^{-1}$

Inflammation

- Insulin resistance
- PGIS nitration
- NO bioactivity
- Stress signaling
- Inflammation
- Sorbitol

Diabetic Endothelial Dysfunction

**Figure 2.** Origin of endothelial dysfunction in diabetes mellitus. Endothelial dysfunction in diabetes can be induced solely by or by a combination of (1) hyperglycemia, (2) fatty acids, (3) inflammation, and (4) insulin resistance. Prolonged exposure to hyperglycemia is now recognized as a major factor in the pathogenesis of diabetic complications, including atherosclerosis, mechanistically involving enhanced enzymatic and nonenzymatic protein/lipid glycosylation, protein kinase C activation, inflammation, and ROS production. Other factors including dyslipidemia, elevated FFAs, inflammation, and insulin resistance, can cause endothelial dysfunction. ROS indicates reactive nitrogen species; EDCF, endothelium-derived COX-dependent vasocostriciting factor; AGE, advanced glycation end products; and PKC, protein kinase C.

FFAs could account for abnormalities in the vascular reactivity of both arterial and venous vascular beds observed in diabetes mellitus and obesity.$^{68,69}$

**Insulin Resistance**

Endothelial cells express the cognate insulin receptor (IR), which belongs to a family of membrane-bound receptors with intrinsic tyrosine kinase activity, whose ligands include growth factors such as insulin-like growth factor-1, vascular endothelial growth factor, platelet-derived growth factor, and epidermal growth factor. In addition to crucial metabolic actions, insulin plays a critical role in the maintenance of physiological endothelial function through its ability to stimulate NO release via a cascade of signaling that involves activation of the PI3K-Akt axis and the downstream serine phosphorylation of endothelial NO synthase (eNOS). In addition to NO-dependent vasodilatory actions, insulin stimulates endothelial release of the vasodilator ET-1, as suggested by increased insulin vasodilatory effects in patients with insulin resistance and endothelial dysfunction.$^{3,73,74}$

Inflammation

The discovery that metabolic conditions such as obesity and type 2 diabetes mellitus (insulin resistance) are associated with inflammation was first described in a seminal publication by Hotamisligil et al in 1993.$^{70}$ This group demonstrated that adipocytes constitutively express the proinflammatory cytokine tumor necrosis factor-α (TNF-α), and adipocyte TNF-α expression is markedly increased in obese animals. They also showed that neutralization of TNF-α with soluble TNF-α receptor decreases insulin resistance in these animals.$^{70}$ These observations provided the first link between increased expression and plasma concentrations of a proinflammatory cytokine and insulin resistance.$^{71}$ Further work in humans has confirmed that obesity, a major risk factor for type 2 diabetes mellitus, and diabetes itself are inflammatory conditions, as indicated by increased plasma concentrations of C-reactive protein, interleukin-6, and plasminogen activator-inhibitor-1 (see Dandona et al$^{71,72}$ for details). At least 2 mechanisms might promote inflammation. First, glucose and macronutrient intake increase both oxidative stress and inflammatory changes. Thus, chronic overnutrition (obesity) might induce a proinflammatory state with oxidative stress. Second, increases in TNF-α and interleukin-6 associated with obesity and type 2 diabetes mellitus might interfere with insulin action by suppressing insulin signal transduction and thus the antinflammatory effect of insulin. This, in turn, may promote inflammation.$^{72}$ Hyperglycemia exacerbates the inflammation associated with type 2 diabetes mellitus. Notably, atherosclerosis links inflammation, obesity, insulin resistance, and type 2 diabetes mellitus. Moreover, it is responsible for the major cause of death (acute myocardial infarction) in this patient population and is itself an inflammatory process.$^{72}$ Therefore, inflammation is an effector of not only endothelial dysfunction$^{3,73,74}$ but also insulin resistance and atherosclerosis.
insulin-signaling branches, including Ras/mitogen-activated protein kinase–dependent pathways, are unaffected (Figure 3). In addition, metabolic insulin resistance is usually paralleled by a compensatory hyperinsulinemia to maintain euglycemia. Thus, consequent hyperinsulinemia in insulin-resistant states will overdrive unaffected mitogen-activated protein kinase–dependent pathways. In endothelium, decreased PI3K signaling and increased mitogen-activated protein kinase signaling in response to insulin may lead to decreased production of NO and increased secretion of ET-1, a characteristic of endothelial dysfunction. Indeed, insulin-resistant patients have elevated plasma ET-1 levels, and hyperinsulinemia increases ET-1 secretion in humans. Pharmacological blockade of ET-1 receptors (ET-A isoform) improves endothelial function in obese and diabetic patients but not in lean, insulin-sensitive subjects.

Endothelial dysfunction might also play a causal role in the development of insulin resistance. Insulin can relax resistance vessels and increase blood flow to skeletal muscle. Insulin acts on the vasculature in 3 discrete steps to enhance its own delivery to muscle/fat tissues: (1) Relaxation of resistance vessels to increase total blood flow; (2) relaxation of precapillary arterioles to increase the microvascular exchange surface perfused within skeletal muscle (microvascular recruitment); and (3) the transendothelial transport of insulin. Indeed, insulin resistance is associated with functional disturbances of the coronary circulation. Conversely, insulin infusion improves coronary flow, even in the setting of type 2 diabetes mellitus and coronary artery disease. Thus, such an imbalance between production of NO and secretion of ET-1 leads to decreased blood flow, which worsens insulin resistance. The reciprocal relationship of insulin resistance and endothelial dysfunction has been a subject of several excellent reviews.75

Mediators of Vascular Endothelial Dysfunction in Diabetes Mellitus

NO is the single most important factor for maintaining vascular endothelial function. NO is a gaseous free radical molecule and is synthesized by the action of the enzyme NO synthase (NOS). In endothelial cells, NO is quickly quenched and release of NO by cells and tissues has been the subject of intense investigation. A number of studies suggest that decreased NO bioactivity associated with these conditions is due to either quenching of normally released NO or impairment of NOS activity.17,76 In the glomeruli of diabetic rats, TxA2 and protein kinase C mediate the impairment of NO-dependent cGMP generation and are thought to do so by decreasing NO production.77 In vitro studies of human umbilical vein endothelial cells show that elevated glucose inhibits NO production.78
Loss of endothelium-dependent relaxation has been described in various animal models of diabetes and in patients. Several mechanisms, including activation of the protein kinase pathway,\textsuperscript{79} the posttranslational modification of eNOS through the hexosamine pathway,\textsuperscript{67} downregulation of the expression of eNOS (as opposed to inhibition of its catalytic activity),\textsuperscript{90} and \textit{S}-nitrosylation of eNOS,\textsuperscript{81} have also been ascribed to diabetic endothelial dysfunction, respectively.

On the other hand, a number of studies have demonstrated that NO release is increased under hyperglycemic and diabetic conditions. Similarly, NOS activity in heart endothelium is increased in diabetic rats.\textsuperscript{82} Increased NO production due to inducible NOS (NOS-II) upregulation has been reported in pancreatic islet cells,\textsuperscript{83,84} murine macrophages,\textsuperscript{85} glomerular mesangial cells,\textsuperscript{85,86} and placental tissues of diabetic individuals.\textsuperscript{87} This paradoxical phenomenon might be best explained by a phenomenon called “eNOS uncoupling.” Increasing evidence suggests that in diabetes mellitus, the function of eNOS (or NOS-III) is altered, which leads to production of O$_2^-$ by this enzyme rather than NO (eNOS uncoupling). This transformation of eNOS from a protective enzyme to a contributor of oxidative stress has been observed in several in vitro models, in animal models of CVDs, and in patients with cardiovascular risk factors (reviewed in Cai et al\textsuperscript{100} and Forstermann and Munzel\textsuperscript{101}). Several studies from us\textsuperscript{89,91} and others\textsuperscript{92} suggest that 2 interrelated mechanisms, including oxidation of the zinc-thiolate center of eNOS and tetrahydrobiopterin (BH$_4$), an essential cofactor for eNOS, might play a particularly important role in regulating NO and O$_2^-$ production by eNOS in diabetes mellitus (see below).

\textbf{Reactive Oxygen Species}

Recent studies indicate that the endothelial dysfunction associated with diabetes mellitus results from the formation of oxidants and free radicals within and near the vascular endothelium.\textsuperscript{93,94} A number of functional studies have demonstrated that diabetic blood vessels respond with an improved endothelium-dependent relaxant response when treated with various antioxidant agents, including superoxide dismutase (SOD).\textsuperscript{95–99} The role of O$_2^-$ in the development of diabetic endothelial dysfunction is supported by the fact that many biochemical pathways strictly associated with hyperglycemia (glucose auto-oxidation, the polyol pathway, prostanoid synthesis, and protein glycation) can increase the production of free radicals and oxidants. Both hyperglycemia and FFAs have also been shown to alter cellular oxidative stress.\textsuperscript{36,61,99} Exposure of endothelial cells to high glucose or to FFAs augments O$_2^-$ production. In vivo studies of diabetic BB rats show that endothelium-mediated relaxation is diminished in these animals, possibly owing to NO quenching by increased O$_2^-$ production.\textsuperscript{99} Intense investigation has identified several sources of O$_2^-$ in vascular endothelial cells. Studies performed by Inoguchi et al\textsuperscript{100} suggest that oxidative stress is secondary to activation of NAD(P)H oxidase and is protein kinase C-$\beta$-dependent, whereas work by Brownlee\textsuperscript{58} suggests that hyperglycemia-induced oxidative stress from mitochondria is the earliest event in the development of cardiovascular complications in diabetes mellitus.

\textbf{Reactive Nitrogen Species}

The half-life of NO and therefore its biological activity are decisively determined by oxygen-derived free radicals such as O$_2^-$, O$_2^-$ rapidly reacts with NO to form the highly reactive intermediate ONOO$^-$\textsuperscript{100,101} The rapid bimolecular reaction between NO and O$_2^-$ that yields ONOO$^-$ (ONOO$^-$, rate constant equal to 5 to $10 \times 10^9$ mol $\cdot$ L$^{-1}$ $\cdot$ s$^{-1}$) is 3 to 4 times faster than the dismutation of superoxide by SOD. Therefore, ONOO$^-$ formation represents a major potential pathway of NO reactivity that relies on the rates of tissue O$_2^-$ generation (reviewed in Zou et al\textsuperscript{102,103}), and many of the biological effects attributed to NO are in fact mediated by ONOO$^-$. The production of O$_2^-$ has been long known to play a causal role in the development of endothelial dysfunction and cardiovascular complications in diabetes mellitus. From a chemical point of view, neither NO nor O$_2^-$ alone is considered a strong oxidant of most types of biological molecules.\textsuperscript{100,101} Recent evidence indicates that reaction of NO with O$_2^-$, which was initially viewed as a route for NO inactivation, yields the potent oxidizing species ONOO$^-$. ONOO$^-$ exhibits direct oxidative reactivity and becomes protonated to form peroxynitrous acid (ONOOH) at physiological pH (pK$_\text{a}$=6.8). Direct bimolecular reaction of NO with O$_2^-$ produces ONOO$^-$ at nearly diffusion-limited rates (5 to $10 \times 10^9$ mol $\cdot$ L$^{-1}$ $\cdot$ s$^{-1}$).\textsuperscript{100,101} This rate is at least 3 times faster than the rate of enzymatic dismutation of O$_2^-$ catalyzed by SOD at neutral pH (K$_\text{cat}$=2$\times$10$^9$ mol $\cdot$ L$^{-1}$ $\cdot$ s$^{-1}$).\textsuperscript{100} Thus, ONOO$^-$ formation, which is potentially a major pathway governing NO reactivity, depends on local rates of NO and O$_2^-$ production. ONOO$^-$ is a potent oxidant capable of directly oxidizing thiol groups and others. It also
nitrites and hydroxylates aromatic groups, including those of tyrosine, tryptophan, and guanine. This process is mediated by both 1- and 2-electron transfer reactions. Finally, ONOO• is capable of reaction with metal centers to yield a species that has the reactivity of nitronium (NO2+), an oxidizing and nitrating intermediate. All of these reactions occur when ONOO• encounters biological molecules such as enzymes and lipids, and they result in altered functions of these molecules.100,101

A characteristic reaction of ONOO• is the nitration of protein-bound tyrosine residues. Antibodies against nitrated tyrosine can be used to detect the ONOO• “footprint.”104 ONOO• reaction products have been detected in many conditions, including diabetes mellitus. Moreover, decomposition of ONOO• or pharmacological inhibition of ONOO• formation has been shown to be of benefit in these conditions. Recent clinical data reveal that vascular tissues from diabetic patients and animals exhibit significantly more intense 3-nitrotyrosine staining than tissues from normal controls.1,101,103,105 There are multiple lines of evidence demonstrating the formation of ONOO• in the diabetic vasculature, both in experimental models and in humans (reviewed in Zou et al.102,103). The tissues and species in which ONOO• has been identified in experimental animals (rodent and nonrodent species) and in humans include plasma, kidney, blood vessels (especially endothelium), retina, heart, and peripheral nerves and have been reviewed recently.105

Increased 3-nitrotyrosine levels have also been described in plasma from humans infused with glucose.106 Importantly, Frustaci et al.107 analyzed ventricular myocardial biopsy samples from diabetic patients and found that apoptosis was increased 61-fold in endothelial cells and 85-fold in cardiomyocytes. In both cell types, apoptosis was strongly associated with positive 3-nitrotyrosine staining, which suggests that apoptosis is likely caused by ONOO• generation. Accumulating evidence suggests that ONOO• might play a central role in the initiation of vascular endothelial dysfunction by altering the balance of endothelial cell−derived dilators/constrictors.

**Prostacyclin Deficiency**

Prostanoids are produced when arachidonic acid is released from the plasma membrane by phospholipases and metabolized by cyclooxygenases (COXs) and specific isomerases. The profile of the prostanoids that are produced can vary dramatically during the course of a response. Prostanoid production depends on the activity of the 2 COX isoenzymes within cells. PGH2 is produced by both COX isoforms (ie, COX-1 and COX-2) and is the common substrate for a series of specific synthase enzymes that produce prostaglandin (PG) D1 (PGD2), PGE2, PGF2α, PGH2, and TXA2. Importantly, the differential expression of these enzymes within the cells situated at inflammation sites determines the profile of prostanoid production.

Like other prostaglandins and TXA2, PGH2 is produced by the 2 rate-limiting cyclooxygenases, COX-1 and COX-2. These COX enzymes form the prostaglandin endoperoxide, PGH2, which is then enzymatically transformed into PGI2 by prostacyclin synthase (PGIS) in blood vessels or into TXA2 by TXA2 synthase in platelets. In blood vessels, particularly larger arteries, the predominant prostaglandin is PGI2.108 The ability of blood vessels to generate PGI2 is essential to the integrity of the endothelium.108,109 Vascular PGI2 synthesis and release are reduced in diabetes mellitus.108–115 Blood vessels obtained from animals with streptozotocin- or alloxan-induced diabetes show a reduced production of PGI2, whereas platelets from these animals release more TXA2 than normal platelets. In diabetic patients, PGI2 production by blood vessels is depressed. In patients with proliferative retinopathy and pregnancy, urinary and circulating levels of 6-keto-PGF1α are reduced.116–119 Decreased PGI2 has been linked to platelet hyperaggregability, increased platelet adhesiveness, and increased release of PGH2/TXA2 in diabetes mellitus.108–115 Moreover, a reduction in PGI2 production has been proposed to accelerate atherosclerosis during the pathogenesis of macroangiopathy and microangiopathy in diabetic patients.109,111 In mice, PGI2 deficiency elicited by PGIS gene depletion results in the development of vascular disorders and thickening of vascular walls,119 which suggests that PGI2 is important in the homeostasis of blood vessels.

**Endothelium-Derived COX-Dependent Vasooconstriction Factor**

Increased release of the endothelium-derived COX-dependent vasoconstriction factor in high glucose−exposed aortas was first described by Tesfamariam and colleagues.120,121 Endothelium-derived COX-dependent vasoconstrictor factor was subsequently identified as prostaglandin endoperoxide, PGD2, or PGH2 (reviewed in Zou et al.). Both TXA2 and PGH2 stimulate contraction of vascular smooth muscle via activation of the TXA2/PGI2 receptor (TPr).122–124 Activation of TPr in vascular cells also induces apoptosis,125,126 abnormal expression of adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial-leukocyte adhesion molecule-1),127,128 and mitogenic or hypertrophic activities.129–132 The actions of PGH2/TXA2 are opposed by prostacyclin. The major sources of TXA2 are platelets, polymorphonuclear leukocytes, and monocytes.

One of the hallmarks of diabetic endothelium is its propensity to produce and release vasoconstrictors, as manifested by the presence of endothelium-dependent contractions. These findings suggest that alterations in arachidonic acid metabolism via the COX pathway are important for the synergism between vascular disease and diabetes mellitus. Aortic release of PGH2/TXA2-like vasoconstrictors is increased in hyperglycemic animals with alloxan- or streptozotocin-induced diabetes,97,120,121,133–142 Furthermore, in the same models, the impaired relaxation is restored by COX inhibitors such as indomethacin or TPr blockers but not by TXA2 synthase inhibitors.97,120,121,133–142 This suggests that constrictor responses are mediated by PGH2 rather than by TXA2. In contrast, in blood vessels of normal animals, PGH2 is metabolized primarily by PGIS to yield PGI2, which minimizes PGH2-mediated responses in the aorta. Decreased activity of PGIS or increased COX activity likely causes PGH2-dependent vasoconstriction that contributes to impaired relaxation in diabetes mellitus. Generation of PGH2...
appears to be related to hyperglycemia rather than elevated lipids. Both in vitro and in vivo, elevated glucose reduces the production of 6-keto-PGF₁α and promotes generation of PGH₁/THA, which results in PGI₁/THA imbalance.97,120,121,133–142 Interestingly, a number of free radical scavengers, such as SOD, prevent and restore impaired endothelium-dependent relaxation, which suggests that free radicals play a major role in the generation of the vasoconstrictor PGH₁ in diabetes mellitus.97,120,121,133–142 Furthermore, endothelium-independent vasodilation in response to sodium nitroprusside does not differ between aortic rings exposed to control and elevated glucose levels, which suggests that hyperglycemia-associated endothelial dysfunction is related to endothelium-derived NO.

More recent data reveal that S18886, an orally active TXA₂ receptor antagonist in clinical development for use in secondary prevention of thrombotic events in CVD, inhibits inflammation and the accelerated atherogenesis caused by diabetes mellitus, most likely by counteracting the effects on endothelial function and adhesion molecule expression of eicosanoids stimulated by the diabetic milieu.143 Although potential endogenous ligands for TPr remain to be identified, the study by Zuccollo et al143 provides important evidence for TPr-dependent atherogenesis in diabetic cardiovascular complications. How TPr enhanced atherogenesis remains unknown. Interestingly, we and others have observed a TPr-dependent ROS production in several cell types144 (M. Zhang, MD, PhD, M.H. Zou, MD, PhD, unpublished observations, 2009) and in vivo.145,146 Thus, it is highly likely that TPr and ROS might mutually promote their effects in vascular cells to worsen endothelial dysfunction and atherosclerosis. Thus, the way in which TPr triggers ROS warrants further investigation.

**Increased Production of Endothelins**

ET-1 is primarily synthesized by vascular endothelial cells and acts on the vascular smooth muscle. This factor participates in the development of vascular diseases through its vasoconstrictor and mitogenic effects.147 In addition, ET-1 is reported to exert its pathological effects by increasing the formation of ROS and reactive nitrogen species in vascular endothelial cells.148 Interestingly, ROS are reported to increase endothelin.149 Thus, ONOO⁻ and endothelin might form a feed-forward loop to worsen endothelial dysfunction in diabetes mellitus.

Clinical evidence has demonstrated that alterations in plasma ET-1 concentrations are associated with various physiological abnormalities. In a cross-sectional study, ET-1 was associated with urinary albumin excretion, with plasma ET-1 levels progressively increasing in patients with normoalbuminuria, microalbuminuria, and macroalbuminuria.150 ET-1 levels are also higher in patients with type 2 diabetes mellitus than in healthy subjects, and this increase in ET-1 levels is accompanied by increased levels of oxidative stress markers, proinflammatory markers, and (downstream) adhesion molecules.151 ET-1 is a marker of endothelial dysfunction and is responsible in part for the endothelial dysfunction associated with diabetes mellitus. Therefore, it may also play an important role in the development of diabetic arterial disease.147 This vascular peptide is released from human adipose tissue and provides a link between fat accumulation and insulin resistance.152 Increasing evidence suggests that chronic activation of the ET-1 system can lead to heterologous desensitization of the glucose-regulatory and mitogenic actions of insulin, with subsequent development of glucose intolerance, hyperinsulinemia, impaired endothelial function, and exacerbation of CVD. However, prospective trials are needed to assess whether ET-1 antagonists are better than conventional therapies in preventing the development of insulin resistance and the progression of diabetes mellitus.153

**Peroxynitrite as the Unifying Mechanism for Endothelial Dysfunction in Diabetes Mellitus**

ONOO⁻ Increases Tyrosine Nitration of Prostacyclin Synthase

Our recently published work86,154,155 has provided new insights into how hyperglycemia increases reactive nitrogen species and affects cell function. Exposure of cultured human aortic endothelial cells to clinically relevant concentrations of high glucose increases the production of both NO and O₂⁻ and consequently decreases the bioactivity of NO, as seen by decreased levels of cGMP. This suggests that NO is inactivated through its reaction with O₂⁻. Simultaneously, high glucose increases PGIS nitration and decreases PGIS activity. We have also shown that activation of TPr in human aortic endothelial cells modulates both adhesion molecule expression and apoptosis, with each being significantly attenuated by a TPr antagonist or COX inhibition. Thus, TPr may be activated by PGH₁ as a result of PGIS nitration and inactivation. Consistent with this observation, S18886, a potent TPr antagonist, markedly attenuates diabetes-enhanced aortic lesions in vivo.143 We also found that blocking TPr prevents the dramatic enhancement in atherogenesis caused by diabetes mellitus in the Apo-E−/− deficient mouse with a concomitant endothelium-dependent constriction sensitive to the TPr antagonist SQ29548 (Zou et al, unpublished observations). This observation may explain not only why diabetes mellitus decreases PGls levels but also why increases occur in its precursor, PGH₂, which activates TPr.

Thus, diabetes mellitus likely acts via hyperglycemia/hyperlipidemia to increase O₂⁻ and then ONOO⁻, resulting in PGIS nitration and consequent TPr stimulation. This, in turn, contributes to the initiation and progression of vascular complications in diabetes mellitus through downregulation of the protective actions of NO and PGls and accumulation of nonmetabolized PGH₂, which tips the balance toward platelet aggregation, atheroma accumulation, and thrombus formation (Figure 5).

**ONOO⁻ Causes eNOS Uncoupling**

When we studied diabetes-enhanced PGls nitration, we found that both diabetic eNOS⁻/− mice and mice overexpressing human SOD (hsSOD−/− mice) exhibited less PGls nitration and released less O₂⁻ than their diabetic littermates.156 Conversely, PGIs activity was significantly preserved in both eNOS⁻/− and hsSOD−/− mice.156 Thus, tyrosine nitration of PGls is most likely mediated by ONOO⁻ formed endoge-
nously from NO and $O_2^-$ in diabetes mellitus, because both eNOS$^{+/+}$ or hSOD$^{+/+}$ mice had significantly attenuated diabetes-enhanced PGIS nitration and inhibition. Although we cannot totally exclude the possibility of peroxidase-catalyzed PGIS nitration, our data strongly suggest that ONOO$^-$ derived from eNOS is likely to be responsible for the increased PGIS nitration caused by diabetes mellitus in vivo.

All 3 NOS isoforms are catalytically active only in dimeric form. In the NOS dimer, a tetra-coordinated zinc ion is held by 4 thiols (cysteine 94 and 99 in human eNOS), with 2 thiols being contributed by each 135-kDa monomer. Because it remains partially positively charged, the zinc-thiolate center is subject to attack by anionic oxidants such as ONOO$^-$. ONOO$^-$ reacts with zinc-thiolate clusters (5.2 x 10$^5$ mol $\cdot L^{-1} \cdot s^{-1}$) at least 1000 times faster than it reacts with cysteine thiols (6 x 10$^2$ mol $\cdot L^{-1} \cdot s^{-1}$) and 100 times faster than it reacts with BH4 (6 x 10$^3$ mol $\cdot L^{-1} \cdot s^{-1}$). Recently, we found that oxidation of the eNOS zinc-thiolate cluster by a small amount of ONOO$^-$ precipitates oxidative stress in cells exposed to elevated glucose. The catalytic activities of recombinant eNOS are exquisitely sensitive to ONOO$^-$-induced uncoupling, which decreases NO synthesis and increases $O_2^-$ production by the enzyme. An analysis of recombinant eNOS in endothelial cells revealed that exposure of these cells to elevated glucose induces loss of zinc from eNOS, disruption of SDS-resistant dimers, and uncoupling of eNOS activity. The same observations have been made in diabetic mouse tissues, which underscores the significance of this process under in vivo conditions.

Regulation of BH4 synthesis and bioavailability is a topic of great interest, because BH4 is essential in NOS function and vascular NO synthesis. BH4 is an essential cofactor for activity of all NOS enzymes. The exact role of BH4 in NOS catalysis is incompletely understood, but BH4 appears to facilitate electron transfer from the eNOS reductase domain and maintain the heme prosthetic group in its redox active form. BH4 also stabilizes the NOS dimer complex, increases dimerization of eNOS monomers, and lowers the $K_m$ for L-arginine binding. Recent studies suggest that in diabetes mellitus, reduced BH4 availability is an important contributor to reduced NO production and increased endothelial $O_2^-$ production. In vessel rings from diabetic or atherosclerotic animals and in mammary artery rings from diabetic patients, supplementation with high concentrations of BH4 improves some features of endothelial dysfunction. Accordingly, acute BH4 administration appears to augment NO-mediated effects on forearm blood flow in patients with diabetes mellitus or hypercholesterolemia. In addition, deficiency in BH4 has been reported in diabetes mellitus, and BH4 supplementation partially restores endothelial function.

The BH4 pool in endothelial cells is variable, and a continuous supply of BH4 is needed to maintain basal BH4 levels. BH4 is synthesized from GTP de novo. The first and rate-limiting step in this biosynthetic pathway is catalyzed by guanosine 5'-triphosphate cyclohydrolase I (GTPCH). Thus, GTPCH levels and activity are crucial for maintenance of BH4 bioavailability and endothelial function under both physiological and pathological conditions. Indeed, inhibition of GTPCH rapidly decreases BH4. GTPCH is constitutively expressed in endothelial cells, and endothelial GTPCH expression is markedly reduced in type 1 and type 2 diabetes mellitus rat models. In addition, aortic GTPCH activity is significantly lower in the insulin-resistance rat model than in control rats. Consistent with these results, GTPCH gene transfer or endothelial cell-specific GTPCH overexpression in mice increases BH4 bioavailability and improves endothelial function. In contrast, acute GTPCH knockdown by small interfering RNA technology uncouples eNOS and elevates blood pressure in mice. We have found that either ONOO$^-$ or high glucose significantly lowers BH4 levels by increasing 26S proteasome--dependent degradation of GTPCH. Furthermore, ONOO$^-$ releases zinc from the zinc-thiolate cluster of eNOS, which presumably leads to the formation of disulfide bonds between monomers. As a result, disruption of the SDS-resistant eNOS dimers occurs under reducing conditions. These exciting observations suggest that hyperglycemia might initially generate "kindling radicals" ($O_2^-$) and then a "bonfire" oxidant (ONOO$^-$) that uncouples eNOS through 26S proteasome--dependent degradation of GTPCH and oxidizes the zinc-thiolate center of eNOS, which is required for NO production (Figure 6).
ONOO$^-$ and Insulin Resistance

Insulin resistance is characterized by pathway-specific impairment in PI3K-dependent signaling in both metabolic and vascular insulin target tissues. Increasing evidence suggests that ROS and reactive nitrogen species play a causal role in the development of insulin resistance and consequent endothelial dysfunction. ONOO$^-$ inhibited Akt signaling in endothelial cells. Furthermore, ONOO$^-$ nitrates a critical tyrosine residue in the p85 regulatory subunit of PI3K, preventing its association with the catalytic p110 subunit and subsequent PI3K activation. ONOO$^-$ causes the nitration of tyrosine residues in rat insulin receptor substrate-1, including Tyr939, a critical site for the association of insulin receptor substrate-1 with the p85 subunit of PI3K. Thus, ONOO$^-$ reduces the protein levels of insulin receptor substrate-1 and associated PI3K activity, upstream of Akt/protein kinase B.165

Our recent work might have provided an alternative mechanism for ONOO$^-$-mediated insulin resistance and endothelial dysfunction in diabetes mellitus.166 In that study, we found that endogenous ONOO$^-$ generated during hyperglycemia exposure inhibits insulin-enhanced protein kinase B/Akt signaling in endothelial cells by increasing the phosphorylation and activity of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), elicited by the tumor suppressor LKB1, eventually leading to an inhibition of PI3K and apoptotic cell death. In vivo, the aortas of diabetic mice exhibited increased levels of nitrotyrosine together with increased phosphorylation of PTEN and LKB1 but reduced protein kinase B/Akt phosphorylation. These findings, therefore, suggest that hyperglycemia may promote insulin resistance and endothelial cell apoptosis in endothelial cells through protein kinase B/Akt downregulation via an ONOO$^-$-mediated, LKB1-dependent PTEN activation (Figure 7).

ONOO$^-$ and the Formation of Advanced Glycation End Products

$N^\omega$-(carboxymethyl)lysine (CML) is a major antigenic advanced glycation end-product structure. CML concentration, adjusted for age and duration of diabetes mellitus, is also increased in patients with diabetes mellitus. CML is also recognized by the receptor for advanced glycation end product (RAGE), and CML-RAGE interaction activates cell-signaling pathways such as nuclear factor-$\kappa$B (NF-$\kappa$B) and enhances the expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells. CML formation...
was increased with increasing ONOO\(^{-}\) concentrations when glycated human serum albumin (Amadori-modified protein) was incubated with ONOO\(^{-}\). CML was inhibited by amino-
guanidine, a trapping reagent for \(\alpha\)-oxoaldehydes.\(^{167}\) Thus, ONOO\(^{-}\) can induce protein modification by oxidative cleavage of the Amadori product and by generation of reactive 
\(\alpha\)-oxoaldehydes from glucose.

Pharmacological Therapies for Treating Endothelial Dysfunction in Diabetes Mellitus

If one accepts the premise that the endothelium is a "gate-
way" to vascular disease, then one would expect that ther-
apieties known to reduce cardiovascular risk would also improve 
endothelial function. This expectation has been fulfilled,
except in rare instances. Cholesterol lowering unequivocally 
reduces cardiovascular events,\(^{168,169}\) and multiple studies 
indicate this effect is accompanied by improved endothelial 
function,\(^{170,171}\) Angiotensin-converting enzyme inhibitors 
also reduce cardiovascular risk\(^{172}\) and improve endothelial 
function.\(^{173}\) Finally, patients with type 2 diabetes mellitus 
have abnormal endothelial function that is improved by 
metformin,\(^{174}\) a drug that reduces the myocardial infarction 
risk in overweight patients.\(^{175}\) Several pharmacological 
agents are thought to achieve vascular protection through 
mechanisms that go beyond their primary therapeutic actions 
(eg, hypotensive or hypocholesterolemic actions).

Lipid-Lowering Drugs

Statin, a well-known cholesterol-lowering drug, has been 
shown to execute its vascular protective effect at least in part 
through AMPK activation.\(^{102,176–179}\) Berberine, an alkaloid 
isolated from a Chinese herb widely used as a drug to treat 
gastrointestinal infections, has recently been described as a 
new cholesterol-lowering drug.\(^{180}\) The cholesterol-lowering 
effect of berberine is distinct from that of the statin drugs in 
that it elevates expression of the LDL receptor through a 
posttranscriptional mechanism that stabilizes the mRNA.\(^{180}\)
Surprisingly, the increasingly emerging evidence links many 
of its beneficial effects on CVDs, such as suppression of 
proinflammatory responses in macrophage,\(^{181}\) improvement 
of glucose metabolism through induction of glycolysis,\(^{182}\) and 
prevention of hyperglycemia-induced endothelial injury and 
enhancement of vasodilation,\(^{183}\) to AMPK.

Antioxidants

Given the consistent and promising findings from experimen-
tal, clinical, and epidemiological investigations, the overall 
results of large-scale clinical studies investigating antioxidant 
(principally vitamin E and C) effects on CVD have been 
disappointing.\(^{184,185}\) At least several explanations may ac-
count for this outcome. First, the type of antioxidants used 
may be ineffective and nonspecific. For example, ONOO\(^{-}\) is 
a highly reactive oxidant with proteins, lipids, and DNA that 
causes tissue injury. The reaction of ONOO\(^{-}\) is at least 1000 
times faster with the zinc-thiolate complex (5.2\(\times\)10\(^{7}\) mol \cdot 
L\(^{-1}\)s\(^{-1}\)) than with cysteine thiols (6\(\times\)10\(^{3}\) mol \cdot 
L\(^{-1}\)s\(^{-1}\)) and 100 times faster than with BH\(_{4}\) (6\(\times\)10\(^{3}\) mol \cdot 
L\(^{-1}\)s\(^{-1}\)) or vitamin C or E. Thus, antioxidants such as vitamin E or C 
require extremely high concentrations to compete for 
ONOO\(^{-}\) with endogenous targets. Therefore, negative results 
with antioxidant vitamins cannot be generalized to all anti-
oxidants. Second, the dosing regimens and duration of ther-
apy may have been insufficient.\(^{186,187}\) Third, some antioxi-
dants, such as vitamin E and vitamin C, might possess a 
pro-oxidant property. Fourth, there is insufficient evidence to 
demonstrate that vitamin E reaches target cells. Fifth, the 
antioxidant potency of vitamins such as C and E is limited, 
because these antioxidants work as scavengers of existing 
except reactive species in a stoichiometric manner, and this 
approach represents a symptomatic approach to oxidative 
stress-associated clinical problems. Sixth, the trial design did 
not allow for recruitment of subjects based on evidence of 
elevated ROS formation.\(^{188}\) Indeed, the failed clinical trials of 
vitamin E may have not addressed possible benefits to 
subgroups with increased oxidative stress. Seventh, as has 
been eloquently argued elsewhere, treating the antioxidant 
vitamins as a single class of compounds with expected similar 
effects inappropriately disregards their wide range of chem-
ical properties and pharmacodynamics. Clinical trials to date 
have been conducted without any real understanding of the 
mechanisms of action or the concentrations of the various 
agents seen at different physiological sites. Eighth, in cohorts 
included in large trials, most subjects have significant CVD, 
in which damaging effects of oxidative stress may be irre-
versible. Finally, carefully designed clinical studies have 
revealed that vitamin E supplementation appears to reduce 
cardiovascular events in individuals with diabetes mellitus 
and the Hp 2-2 (haptoglobin, a major antioxidant protein) 
genotype.\(^{189}\)

On the basis of the new developments in our understanding 
of the pathophysiology of oxidative stress, it is clear that 
strategies to block the formation of reactive oxygen radicals 
will provide conclusive evidence on whether antioxidants 
should be part of the cardiovascular treatment plan in diabetes 
mellitus. Over the last decade, several classes of ONOO\(^{-}\) 
decomposition catalysts (eg, eboselen and the metalloporphy-
rin compounds FP-15, FeTPPS, and FeTMAPS) have been 
tested in a variety of experimental models of diabetic com-
lications in various animal models of diabetes (reviewed in 
Szabo\(^{190}\)). The results from these studies have demonstrated 
that the neutralization of ONOO\(^{-}\) significantly delays the 
development of endothelial dysfunction, cardiomyopathy, 
retinopathy, nephropathy, and neuropathy in diabetes mellitus 
(reviewed in Szabo\(^{190}\)).

Thiazolidinediones

Treatment with thiazolidinediones has been shown to im-
prove endothelium-dependent vasodilation in patients with 
diabetes mellitus and in individuals with metabolic syndrome 
but without diabetes after 8 weeks of rosiglitazone treatment. 
Moreover, in obese men with normal glucose tolerance, 12 
weeks of pioglitazone treatment reduced markers of endothe-
lial function, such as intercellular adhesion molecule-1, vas-
cular cell adhesion molecule-1, and E-selectin. Finally, in 
nondiabetic patients with major cardiovascular risk factors,
pioglitazone treatment improved both insulin sensitivity and endothelial vasodilator function. Consistently, thiazolidinediones have also been reported to reduce the levels of biomarkers (eg, C-reactive protein, TNF, and cytokines), intima-media thickness, restenosis, and plaque stability in diabetic and nondiabetic patients. The PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events) also reported that pioglitazone treatment led to a nonsignificant 10% reduction of the combined primary end point (mortality, nonfatal myocardial infarction, acute coronary syndrome, stroke, percutaneous or surgical coronary revascularization, peripheral revascularization, or leg amputation), although thiazolidinedione treatment significantly reduced the predefined, principal secondary end point of mortality, nonfatal myocardial infarction, and stroke by 16%. These data suggest that thiazolidinediones may have beneficial vascular properties independent of their effect on insulin sensitivity and inflammation. PROactive also showed an increase in hospitalization due to cardiac decompensation, which most likely stems from fluid retention in the kidney. Thus, the difference of improved endothelial function and increased cardiac incidence in thiazolidinedione-treated patients might be explained by the off-target effects of thiazolidinediones in other organs such as hearts and kidneys.

**Metformin**

The biguanide metformin is widely used in type 2 diabetes mellitus. Metformin is known to have beneficial vascular effects in animal studies and most importantly in clinical studies. Data from the United Kingdom Prospective Diabetes Study provided the first substantive prospective evidence that early and intensive intervention with metformin could reduce the incidence of myocardial infarction and increase survival in overweight patients with type 2 diabetes mellitus. Moreover, this apparently vasoprotective effect of metformin could not be explained by its blood glucose–lowering efficacy, because intensive therapy with a sulfonylurea or insulin produced similar glycemic control but afforded less protection against myocardial infarction and other macrovascular events. A large, randomized, double-blind trial with tranilast to prevent restenosis after percutaneous coronary intervention (the Presto trial) provided prospective data from patients with type 2 diabetes mellitus who were receiving metformin (n=887) versus other antidiabetic therapies that excluded a thiazolidinedione (n=1110). After 9 months, metformin reduced the incidence of myocardial infarctions by 69% and of all-cause mortality by 61%.

Metformin is known to improve sensitivity to the glycemic effects of insulin; however, metformin appears to have a direct protective effect on endothelial cells. Metformin is reported to reduce monocyte adhesion to human endothelial cells. Furthermore, several studies have shown that metformin therapy improves vascular reactivity. Metformin therapy for 3 months increased acetylcholine-induced endothelium-dependent vasodilation, whereas there was no significant increase in nitroprusside-induced endothelium-independent vasodilation in patients with type 2 diabetes mellitus. Other studies have further demonstrated that metformin-increased endothelium-dependent vasodilation appears to be independent of glycemia. Conversely, there are conflicting reports that metformin has no significant effects on endothelium-dependent vasodilation in patients with type 2 diabetes mellitus. The reasons for these discrepancies remain unknown, but they are likely related to the difference in the methods used for the assays of the endothelium-dependent relaxation and the patient populations studied.

**AMPK as an Emerging Therapy for Promoting Vascular Health in Diabetes Mellitus**

The pharmacological effects of metformin have recently been ascribed to its activation of AMPK. Certain beneficial effects of agents such as physical exercise, statins, thiazolidinediones, leptin, adiponectin, and rosiglitazone are mediated, at least in part, by activation of AMPK in endothelial cells. Although these effective therapies have distinct effects in improving endothelial function in diabetes mellitus, AMPK activation appears to be a shared molecular target in vascular tissues.

AMPK was initially identified as a sensor of cellular energy and is also likely a sensor of cellular redox status. AMPK is a heterotrimeric enzyme composed of a catalytic (α1 or α2) subunit and 2 regulatory (β1 or β2 and γ1, γ2, and γ3) subunits, all of which are encoded by separate genes, which makes it possible to form a total of 12 complexes. AMPKs are activated by 2 distinct signals: A Ca^{2+}-dependent pathway mediated by calcium calmodulin-dependent kinase-β (CaMKK-β) and an AMP-dependent pathway mediated by LKB1. These and other upstream kinases, including TGF-β–activated kinase-1 (Tak1), phosphorylate Thr172 on the α-subunit. Binding of AMP to the γ-subunit leads to allosteric activation of AMPK and protection of Thr172 from dephosphorylation, thereby maintaining the enzyme in the activated state. AMPK is a key regulator of carbohydrate and lipid metabolism, and activation of this protein kinase is expected to have multiple beneficial effects for diabetes mellitus, including suppression of lipogenesis and gluconeogenesis, as well as augmentation of fatty acid oxidation and glucose uptake. AMPK has also been shown to mediate the glucose-lowering efficacy of metformin in both experimental animals and humans. Thus, AMPK activation might indirectly improve vascular endothelial function via its improvement in metabolic profiles and insulin sensitivity. Because the effects of AMPK on other key metabolically relevant tissues such as liver, skeletal muscle, adipose, and hypothalamus have been a subject of several recent reviews, in the present report, we will focus the discussion on the beneficial effects of AMPK activation in endothelium and how AMPK activation could be used as a strategy to reverse endothelial dysfunction in diabetes mellitus and other CVDs (Figure 8). This hypothesis is strengthened by the findings from and others that AMPK is essential for maintaining endothelial homeostasis by increasing NO bioactivity and mitochondrial biogenesis and by suppressing inflammation and the production of ROS/ONOO− in endothelial cells.
AMPK-eNOS-NO Axis: AMPK Is Important for NO Bioactivity and Endothelial Function

The notion that AMPK contributes to eNOS bioactivity is supported by in vitro and in vivo work by our group and others. We were among the first to observe that blood vessels from α/AMPK-null mice exhibit defective eNOS-mediated NO production in response to estradiol. We also showed that AMPK promotes eNOS association with heat shock protein 90 (HSP90) and in this way is required for estradiol-induced eNOS activation. Similarly, in vivo activation of AMPK with the antidiabetic drug metformin stimulates NO synthesis, which is promoted by association of HSP90 with eNOS. The pharmacological AMPK activator 5-aminoimidazole-4-carboxamide ribose (AICAR) stimulates eNOS activity. Adiponectin, which is reduced in the plasma of patients with type 2 diabetes mellitus, protects against myocardial contractile dysfunction and limits infarct size after ischemia and reperfusion. The mechanism underlying this protective effect involves activation of AMPK and production of NO. Interestingly, adiponectin-mediated eNOS activation depends on AMPK-mediated eNOS association with HSP90. Ghrelin is a newly discovered gastric peptide that improves endothelial function and inhibits proatherogenic changes. A recent study of cultured endothelial cells and mice aortas revealed that AMPK-dependent eNOS activation underlies the vascular actions of ghrelin. Cilostazol, a selective inhibitor of phosphodiesterase 3, increases intracellular cAMP and activates protein kinase A, which leads to inhibition of platelet aggregation and induction of peripheral vasodilation. Administration of cilostazol to diabetic rats significantly restores endothelium-dependent vasodilation, an effect that is attributed at least in part to AMPK-dependent amelioration of eNOS activation and biotin metabolism in the aorta. Finally, AMPK is critical for eNOS activation by vascular endothelial growth factor, shear stress, and insulin. Thus, abundant data link AMPK to NO bioactivity.

AMPK Suppression of Inflammation

Vascular inflammation is a feature of endothelial dysfunction, and NF-κB activation is a major player in the development of vascular inflammation. AMPK activation via several mechanisms suppresses endothelial inflammation. Recent data suggest that AMPK is a master regulator of macrophage differentiation to an antiinflammatory phenotype. Such an antiinflammatory role has been suggested by data obtained with the AMPK activators in endothelial cells. Cacicedo and colleagues have reported that pharmacological or genetic activation of AMPK blunts palmitate- or TNF-induced NF-κB activation and consequent expression of adhesion molecules in endothelial cells. Furthermore, treatment of mice with AICAR reduces the severity of experimental autoimmune encephalomyelitis. AICAR has also been shown to reduce NOS-II synthesis by adipocytes, macrophages, myocytes, and glia. Similarly, metformin-induced AMPK activation inhibits cytokine-induced NF-κB activation in vascular endothelial cells and thus suppresses inflammation. Overall, AMPK appears to be a natural suppressor of NF-κB and vascular inflammation in endothelial cells.

AMPK Suppression of ROS/ONOO−

Although nontoxic levels of ROS/ONOO− modulate AMPK or AMPK-dependent pathways, activation of AMPK may suppress ROS/ONOO− generation. For example, rosiglitazone quenches oxidative stress elicited by high glucose by preventing NAD(P)H oxidase activation in an AMPK-dependent manner. Accumulating data also support the notion that AMPK activation exerts antiinflammatory responses, likely via suppression of the production of ROS such as ONOO−. Indeed, mice with deficient AMPKα2 exhibited increased ROS in parallel with impaired endothelium-dependent vasorelaxation in isolated aortas. Consistently, mice deficient in both the AMPKα2 and apolipoprotein E genes exhibit worse atherosclerosis that involves an alteration in NF-κB activation and NADP(H) oxidase expres-
AMPK activation also suppresses endothelial dysfunction by increasing cellular endogenous antioxidant potentials. Pharmacological activation of AMPK with AICAR markedly increased mitochondrial UPC-2 (uncoupling protein-2) expression and reduced both $O_2^-$ and PGIS nitration in diabetic wild-type mice but not in their AMPKα2-deficient counterparts in vivo. Similarly, Kukidome et al reported that AMPK activation by metformin normalizes hyperglycemia-induced mitochondria-derived ROS production by induction of Mn-SOD and promotion of mitochondrial biogenesis through activation of the AMPK-PGC-1α (peroxisome proliferator–activated receptor-γ coactivator-1α) pathway. This is important because mitochondria are a principal site for hyperglycemia-driven $O_2^-$ in endothelial cells.

**AMPK Increases Mitochondrial Biogenesis in Endothelial Cells**

AMPK activation has been shown to induce mitochondrial biogenesis via PGC-1α induction in the endothelium, consistent with another report. In parallel, AMPK increases cellular NAD$^+$ levels and enhances sirtuin 1 (SIRT1) activity, which results in the deacetylation and activation of PGC-1α. Indeed, chronic administration of AICAR in vivo prevented angiotensin II– or endotoxin-mediated JNK activation and endothelial injury, a process dependent on oxidative stress and vascular ROS production. This protective effect of AICAR was lost in mice lacking AMPK and PGC-1α. Thus, these data indicate that AMPK can direct adaptive changes in the mitochondria via PGC-1α, which enhances mitochondrial biogenesis and cellular resistance to stress.

**Concluding Remarks**

Macrovascular and microvascular disorders are the principal causes of morbidity and mortality in patients with diseases that involve the cardiovascular system (eg, atherosclerosis and diabetes mellitus). The endothelium has emerged as the key regulator of vascular homeostasis because it not only serves a barrier function but also acts as an active signal transducer that regulates vessel wall phenotype. Abnormal vasomotor responses and impaired endothelium-dependent vasodilation have been demonstrated in a number of vessels in a variety of animal models and in humans with CVDs. Endothelial dysfunction plays a key role in the development of these diseases, although the genesis of this endothelial dysfunction and its associated vasomotor abnormalities remains poorly understood. Hyperglycemia, fatty acids, inflammation, and insulin resistance are major inducers of endothelial dysfunction in diabetes mellitus and likely act through common mechanisms that involve reactive nitrogen species. Increasing evidence suggests that AMPK activation suppresses ROS but increases both NO release and mitochondrial biogenesis in endothelial cells. Thus, AMPK appears to be a therapeutic target for improving endothelial function in patients with diabetes mellitus and other forms of CVD.

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**Disclosures**

None.

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Endothelial Dysfunction in Diabetes Mellitus

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