Abstract: Oxidative stress plays a pivotal role in the development of diabetes complications, both microvascular and cardiovascular. The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium. This increased superoxide production causes the activation of 5 major pathways involved in the pathogenesis of complications: polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isofoms, and overactivity of the hexosamine pathway. It also directly inactivates 2 critical antiatherosclerotic enzymes, endothelial nitric oxide synthase and prostacyclin synthase. Through these pathways, increased intracellular reactive oxygen species (ROS) cause defective angiogenesis in response to ischemia, activate a number of proinflammatory pathways, and cause long-lasting epigenetic changes that drive persistent expression of proinflammatory genes after glycemia is normalized (“hyperglycemic memory”). Atherosclerosis and cardiomyopathy in type 2 diabetes are caused in part by pathway-selective insulin resistance, which increases mitochondrial ROS production from free fatty acids and by inactivation of antiatherosclerosis enzymes by ROS. Overexpression of superoxide dismutase in transgenic diabetic mice prevents diabetic retinopathy, nephropathy, and cardiomyopathy. The aim of this review is to highlight advances in understanding the role of metabolite-generated ROS in the development of diabetic complications. (Circ Res. 2010;107:1058-1070.)

Key Words: hyperglycemia ▪ mitochondria ▪ metabolic memory ▪ epigenetic modifications ▪ insulin resistance
(CVD) risk 3- to 8-fold. Thus, more than 30% of patients hospitalized with acute myocardial infarction have diabetes, and 35% have impaired glucose tolerance. Finally, new blood vessel growth in response to ischemia is impaired in diabetes, resulting in decreased collateral vessel formation in ischemic hearts and in nonhealing foot ulcers. Hyperglycemia causes tissue damage through 5 major mechanisms: (1) increased flux of glucose and other sugars through the polyol pathway; (2) increased intracellular formation of AGEs (advanced glycation end products); (3) increased expression of the receptor for AGEs and its activating ligands; (4) activation of protein kinase (PKC) isoforms; and (5) overactivity of the hexosamine pathway.

Several lines of evidence indicate that all 5 mechanisms are activated by a single upstream event: mitochondrial overproduction of reactive oxygen species (ROS). In the diabetic microvasculature, this is a consequence of intracellular hyperglycemia. In the diabetic macrovascular and in the heart, in contrast, this appears to be a consequence of increased oxidation of fatty acids, resulting in part from pathway-specific insulin resistance.

**Role of Hyperglycemia in Microvascular Complications**

Overall, diabetic microvascular complications are caused by prolonged exposure to high glucose levels (Figure 1). The extent of diabetic tissue damage is also determined by genetic determinants of individual susceptibility and, as with atherosclerosis, by the presence of such independent accelerating factors as hypertension and dyslipidemia. This role of hyperglycemia has been established by large-scale prospective studies for both type 1 and type 2 diabetes, the DCCT/EDIC (Diabetes Control and Complications Trial), and the UKPDS (UK Prospective Diabetes Study). Similar data have been reported by the Steno-2 study. However, further analysis of the DCCT data shows that although intensive therapy reduced the risk of sustained retinopathy progression by 73% compared to standard treatment, hemoglobin (Hb)A1c and duration of diabetes (glycemic exposure) explained only ~11% of the variation in retinopathy risk for the entire study population, suggesting that the remaining 89% of the variation in risk is presumably explained by aspects of glycemia not captured by HbA1c.

Because every cell in the body of people with diabetes is exposed to abnormally high glucose concentrations, why does hyperglycemia selectively damage some cell types and not others? The targeting of specific cell types by generalized hyperglycemia reflects the failure of those cells to downregulate their uptake of glucose when extracellular glucose concentrations are elevated. Cells that are not directly susceptible to direct hyperglycemic damage show an inverse relationship between extracellular glucose concentrations and glucose transport. In contrast, vascular endothelial cells, major targets of hyperglycemic damage, show no significant change in glucose transport rate when glucose concentration is elevated, resulting in intracellular hyperglycemia.

**Mechanisms of Hyperglycemia-Induced Damage**

The majority of publications regarding the mechanisms underlying hyperglycemia-induced diabetic vascular damage focus on the 5 major mechanisms indicated above. However, the results of clinical studies in which only 1 of these pathways is blocked have been disappointing. This led to...
the hypothesis in 2000 that all 5 mechanisms are activated by a single upstream event: mitochondrial overproduction of the ROS.

**Increased Polyol Pathway Flux**

The polyol pathway is based on a family of aldo-keto reductase enzymes that can use as substrates a wide variety of carbonyl compounds and reduce these by NADPH to their respective sugar alcohols (polyols). It was first thought that glucose is converted to sorbitol by the enzyme aldose reductase, with sorbitol then oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH), with NAD$^+$ as a cofactor.

Aldose reductase is found in tissues such as nerve, retina, lens, glomerulus, and vascular cells. In many of these tissues, glucose uptake is mediated by insulin-independent GLUTs; intracellular glucose concentrations, therefore, rise in parallel with hyperglycemia. Several mechanisms have been proposed to explain how hyperglycemia-induced increases in polyol pathway flux could damage the tissues involved. The most cited is an increase in redox stress caused by the consumption of NADPH. Because NADPH is a cofactor required to regenerate reduced glutathione (GSH), and GSH is an important scavenger of ROS, this could induce or exacerbate intracellular oxidative stress. Indeed, overexpression of human aldose reductase increased atherosclerosis in diabetic mice and reduced the expression of genes that regulate regeneration of glutathione. Reduced GSH is also depleted in the lens of transgenic mice that overexpress aldose reductase and in diabetic rat lens compared to nondiabetic lens. It has also been demonstrated that decreased glutathiolation of cellular proteins is related to decreased NO availability in diabetic rats, which would decrease GSN0. Restoring the NO levels in diabetic animals increases glutathiolation of cellular proteins, inhibits aldose reductase activity, and prevents sorbitol accumulation.

In diabetic vascular cells, glucose does not appear to be the substrate for aldose reductase, because the $K_m$ of aldose reductase for glucose in 100 mmol/L, whereas the intracellular concentration of glucose in diabetic endothelial cells is 30 mmol/mg protein.

Although the aldehyde form of glucose is a much better substrate for aldose reductase than are the ring forms, with $K_m$ of 0.66 μmol/L for the aldehyde form of glucose, the aldehyde form of glucose represents only 0.002% of the total glucose. Thus, a substrate concentration required for effective catalysis to occur may not be common. Glycolytic metabolites of glucose such as glyceraldehyde 3-phosphate, for which aldose reductase has a much higher affinity, may be the physiologically relevant substrate.

**Increased Intracellular AGE Formation**

AGEs are formed by the nonenzymatic reaction of glucose and other glyating compounds derived both from glucose and from increased fatty acid oxidation in arterial endothelial cells and most likely heart (eg, dicarboxyls such as 3-deoxyglucosone, methylglyoxal, and glyoxal) with proteins. Intracellular production of AGE precursors can damage cells by 3 general mechanisms. Firstly, intracellular proteins modified by AGEs have altered function. Secondly, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with matrix receptors (integrins) that are expressed on the surface of cells. Finally, plasma proteins modified by AGE precursors bind to AGE receptors on cells such as macrophages, vascular endothelial cells, and vascular smooth muscle cells. Receptor for AGE (RAGE) binding induces the production of ROS, which in turn activates the pleiotropic transcription factor nuclear factor (NF)-κB, causing multiple pathological changes in gene expression. In addition, increased metabolite-induced methylglyoxal in arterial endothelial cells and in mouse kidney endothelial cells increases methylglyoxal modification of the corepressor mSin3A. Methylglyoxal modification of mSin3A results in increased recruitment of O-linked N-acetylglycosamine (O-GlcNAc) transferase, with consequent increased modification of Sp3 by O-GlcNAc.

This modification of Sp3 causes decreased binding to a glucose-responsive GC-box in the angiopoietin (Ang)-2 promoter, resulting in increased Ang-2 expression. Increased Ang-2 expression induced by high glucose in renal endothelial cells increased expression of intracellular adhesion molecule-1 and vascular cell adhesion molecule (VCAM)-1. In cells and in kidneys from diabetic mice, increased expression of Ang-2 sensitized microvascular endothelial cells to the proinflammatory effects of tumor necrosis factor α. It has been also recently demonstrated that methylglyoxal covalently modifies the 20S proteasome, decreasing its activity in the diabetic kidney, and reduces the polyubiquitin precursor 19S-85a, indicating a new link between hyperglycemia and impairment of cell function.

Clinically, diabetes is associated with poor outcomes following acute vascular occlusive events. Diabetic animals have a decreased vascular density following hindlimb ischemia and impaired wound healing. Human angiograms demonstrate fewer collateral vessels in diabetic patients compared with nondiabetic controls. Clinically, this contributes to increased rates of lower limb amputation, heart failure, and increased mortality after ischemic events. These defects result in part from a failure to form adequate compensatory vasculogenesis in response to ischemia. AGEs appear to play a central role in this failure. High glucose induces a decrease in transactivation by the transcription factor hypoxia-inducible factor (HIF)-1α, which mediates hypoxia-stimulated chemokine and vascular endothelial growth factor (VEGF) production by hypoxic tissue, as well as chemokine receptor and endothelial nitric oxide synthase (eNOS) expression in endothelial precursor cells in the bone marrow. Decreasing superoxide in diabetic mice by either transgenic expression of manganese superoxide dismutase (MnSOD) or by administration of a superoxide dismutase (SOD) mimetic corrected posts ischemic defects in neovascularization, oxygen delivery, and chemokine expression and normalized tissue survival. Decreased HIF-1α functional activity was specifically caused by impaired HIF-1α binding to the coactivator p300. Hyperglycemia-induced covalent modification of p300 by the dicarboxyl metabolite methylglyoxal is responsible for this decreased association (Figure 2). In diabetic mouse models of
impaired angiogenesis and wound healing, decreasing mitochondria ROS formation normalizes both ischemia-induced new vessel formation and wound healing. 34,35

AGE-modified proteins in the circulation can affect a range of cells and tissues. A specific RAGE has been shown to mediate signal transduction via generation of ROS, activation of NF-κB, and p21 ras. 36–39 AGE signaling can be blocked in cells by expression of RAGE antisense cDNA 38 or anti-RAGE ribozyme. 39 It has been also recently demonstrated that the RAGE–NF-κB axis operates in diabetic neuropathy, mediating functional sensory deficits. 40 In endothelial cells, AGE binding to its receptor alters the expression of several genes, including thrombomodulin, tissue factor and VCAM-1. 41–44 These effects induce procoagulatory changes on the endothelial cell surface and increase the adhesion of inflammatory cells to the endothelium. In addition, endothelial AGE receptor binding appears to mediate, in part, the increased vascular permeability induced by diabetes, probably through the induction of VEGF. 45–48 RAGE deficiency attenuates the development of atherosclerosis in the diabetic apoE model of accelerated atherosclerosis. Diabetic RAGE−/−/apoE−/− mice had significantly reduced atherosclerotic plaque area. These beneficial effects on the vasculature were associated with attenuation of leukocyte recruitment; decreased expression of proinflammatory mediators, including the NF-κB subunit p65, VCAM-1, and MCP-1; and reduced oxidative stress. 49

It is important to note that more recent studies indicate that AGEs at the concentrations found in diabetic sera may not be the major ligand for RAGE. Rather, several endogenously produced proinflammatory protein ligands have been identified, which activate RAGE at low concentrations. These include several members of the S100 calgranulin family and HMGB1 (high-mobility group box 1). Each of these is increased by diabetic hyperglycemia. 50 Ligation of these ligands with RAGE causes cooperative interaction with the innate immune system signaling molecule TLR-4 (toll-like receptor 4). 51,52 Expression of RAGE, S100A8, S100A12, and HMGB1 are all increased by high glucose in cell culture and in diabetic animals. This hyperglycemia-induced overexpression is mediated by ROS-induced methylglyoxal, which increases binding of the transcription factors NF-κB and AP-1 to the promoters of RAGE and RAGE ligands, respectively. 48

Increased Protein Kinase C Activation

PKCs are a family of at least 11 isoforms that are widely distributed in mammalian tissues. The enzyme phosphorylates various target proteins. The activity of the classic isoforms is dependent on both Ca2+ ions and phosphatidylserine and is greatly enhanced by diacylglycerol (DAG). 10 Persistent and excessive activation of several PKC isoforms operates as a third common pathway mediating tissue injury induced by diabetes-induced ROS. This results primarily from enhanced de novo synthesis of DAG from glucose via triose phosphate, whose availability is increased because increased ROS inhibit activity of the glycolytic enzyme GAPDH, raising intracellular levels of the DAG precursor triose phosphate. 53–56 Evidence suggests that the enhanced activity of PKC isoforms could also result from the interaction between AGEs and their cell-surface receptors. 57 Hyperglycemia primarily activates the β and δ isoforms of PKC in cultured vascular cells. 58–60 In the diabetic retina, hyperglycemia persistently activates protein kinase C and p38α mitogen-activated protein kinase (MAPK) to increase the expression of a previously unknown target of PKC signaling, SHP-1 (Src homology-2 domain–containing phosphatase-1), a protein tyrosine phosphatase. This signaling cascade leads to platelet-derived growth factor (PDGF) receptor-β dephosphorylation and a reduction in downstream signaling from

Figure 2. Model of ischemia-induced neovascularization in normal and high glucose. A, In the presence of normal glucose concentration, ischemia-stabilized HIF-1α forms heterodimers with ARNT which bind the coactivator p300. This complex binds to the hypoxia response element (HRE) and activates expression of genes required for neovascularization. B, High glucose–induced methylglyoxal (MG) modifies HIF-1α and p300, inhibiting complex binding to the HREs of genes required for neovascularization. Data are from Thangarajah et al 34,35 and Ceradini et al. 35 (Illustration Credit: Ben Smith/Cosmocyte).
this receptor, resulting in pericyte apoptosis. The same pathway, activated by increased fatty acid oxidation in insulin-resistant arterial endothelial cells and heart, may play an equally important role in diabetic atherosclerosis and cardiomyopathy. Overactivity of PKC has been implicated in the decreased NO production in smooth muscle cells, and has been shown to inhibit insulin-stimulated expression of eNOS in cultured endothelial cells. Activation of PKC by high glucose also induces expression of the permeability-enhancing factor VEFG in vascular smooth muscle cells.

In addition to mediating hyperglycemia-induced abnormalities of blood flow and permeability, activation of PKC may contribute to the accumulation of microvascular matrix protein by inducing expression of transforming growth factor (TGF)-β1, fibronectin, and type IV collagen in both cultured mesangial cells and in glomeruli of diabetic rats. This effect also appears to be mediated by inhibition of NO production by PKC. Hyperglycemia-induced activation of PKC has also been implicated in the overexpression of the fibrinolytic inhibitor, plasminogen activator inhibitor (PAI)-1.

Increased Hexosamine Pathway Flux

Hyperglycemia and insulin resistance–induced excess fatty acid oxidation also appear to contribute to the pathogenesis of diabetic complications by increasing the flux of fructose 6-phosphate into the hexosamine pathway. In this pathway, fructose 6-phosphate is diverted from glycolysis to provide substrate for the rate-limiting enzyme of this pathway, glutamine:fructose 6-phosphate amidotransferase (GFAT). GFAT converts fructose 6-phosphate to glucosamine 6-phosphate, which is then converted to UDP-N-acetylglucosamine. Specific O-GlcNac transferases use this substrate to posttranslationally modify specific serine and threonine residues on cytoplasmic and nuclear proteins by O-GlcNAc. Inhibition of GFAT blocks hyperglycemia-induced increases in the transcription of both TGF-α and TGF-β1.

Although it is not entirely clear how increased flux through the hexosamine pathway mediates hyperglycemia-induced increases in the gene transcription of key genes such as TGF-α, TGF-β1, and PAI-1, it has been shown that hyperglycemia causes a 4-fold increase in O-GlcNAcylation of the transcription factor Sp1, which mediates hyperglycemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells and of TGF-β1 and PAI-1 in arterial endothelial cells.

Of particular relevance to diabetic vascular complications is the inhibition of eNOS activity in arterial endothelial cells by O-GlcNAcylation at the Akt activation site of eNOS protein. Hyperglycemia also increases GFAT activity in aortic smooth muscle cells, which increases O-GlcNAc-modification of several proteins in these cells.

Finally, diabetic hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation, which reduced sarcoplasmic reticulum Ca(2+)-ATPase 2a (SERCA2a) mRNA and protein expression, and decreased SERCA2a promoter activity. In isolated perfused rat hearts, increased GlcNAcylation inhibited phenylephrine-induced inotropy by impairing capacitative Ca(2+)-entry (CCE), the influx of Ca(2+) through plasma membrane channels activated in response to depletion of endoplasmic or sarcoplasmic reticulum Ca(2+) stores.

A Single Process Underlies Different Hyperglycemia-Induced Pathogenic Mechanisms: Mitochondrial Superoxide Production

Specific inhibitors of aldose reductase activity, AGE formation, RAGE ligand binding, PKC activation, and hexosamine pathway flux each ameliorate various diabetes-induced abnormalities in cell culture or animal models, but it has not been clear whether these processes are interconnected or might have a common cause. Moreover, all of the above abnormalities are rapidly corrected when euglycemia is restored, which makes the phenomenon of hyperglycemic memory conceptually difficult to explain (see below). It has now been established that all of the different pathogenic mechanisms described above stem from a single hyperglycemia-induced process, namely overproduction of superoxide by the mitochondrial electron-transport chain. Superoxide is the initial oxygen free radical formed by the mitochondria, which is then converted to other more reactive species that can damage cells in numerous ways.

Normally, electron transfer through complexes I, III, and IV extrudes protons outward into the intermembrane space, generating a proton gradient that drives ATP synthase (complex V) as protons pass back through the inner membrane into the matrix. In contrast, in diabetic cells with high intracellular glucose concentration, there is more glucose-derived pyruvate being oxidized in the TCA cycle, increasing the flux of electron donors (NADH and FADH2) into the electron transport chain. As a result, the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked, causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide (Figure 3). The mitochondrial isoform of the enzyme SOD degrades this oxygen free radical to hydrogen peroxide, which is then converted to H2O and O2 by other enzymes. In primary arterial endothelial cells in culture, intracellular hyperglycemia increases the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation and, subsequently, increases production of ROS. It has been also demonstrated that dynamic changes in mitochondrial morphology are associated with high glucose-induced overproduction of ROS. Inhibition of mitochondrial fission prevented periodic fluctuation of ROS production during high glucose exposure. Neither hyperglycemia nor increased fatty acid oxidation in vascular endothelium increases ROS nor activates any of the pathways when either the voltage gradient across the mitochondrial membrane is collapsed by uncoupling protein 1 (UCP-1) or when the superoxide produced is degraded by MnSOD. Moreover hyperglycemia does not induce ROS formation in so-called rho zero endothelial cells, where the mitochondrial electron transport chain has been inhibited. Although it thus appears that the mitochondria are required for the initiation of hyperglycemia-induced superoxi-
ide production, much evidence indicates that this, in turn, can activate a number of other superoxide production pathways that may amplify the original damaging effect of hyperglycemia. These include redox changes, NADPH oxidases, and uncoupled eNOS.

In the diabetic heart, overexpression of MnSOD or catalase protects cardiac mitochondria from oxidative damage, improves respiration, and normalizes mass in diabetic mitochondria. MnSOD also prevents the morphological changes in diabetic hearts and completely normalizes contractility in diabetic cardiomyocytes.91,92 In endothelial cells increased MnSOD or UCP-2 expression inhibits both hyperglycemia- and fatty acid–induced inactivation of the antiatherosclerosis endothelial enzyme prostacyclin synthase by nitration in diabetes.93–95 Overexpression of either MnSOD and UCP-1 also prevents inhibition of eNOS activity by these metabolites.90

In humans, skin fibroblast gene expression profiles from 2 groups of type 1 diabetic patients (20-year duration with a very fast [fast track] versus 20-year duration with a very slow [slow track] rate of development of diabetic nephropathy lesions) showed that the fast-track group had increased expression of oxidative phosphorylation genes and electron transport system complex II and TCA cycle genes compared to that of the slow-track group. This association is consistent with a central role for mitochondrial ROS production in the pathogenesis of diabetic complications.96

**Hyperglycemia-Induced Mitochondrial Superoxide Production Activates the Five Damaging Pathways by Inhibiting GAPDH**

Diabetes in animals and patients, and hyperglycemia in cells, all decrease the activity of the key glycolytic enzyme GAPDH in cell types that develop intracellular hyperglycemia. Inhibition of GAPDH activity by hyperglycemia does not occur when mitochondrial overproduction of superoxide is prevented by either UCP-1 or MnSOD.99 When GAPDH activity is inhibited, the levels of all the glycolytic intermediates that are upstream of GAPDH increase. This then increases the flux into the 5 pathways described earlier. Increased levels of the upstream glycolytic metabolite fructose 6-phosphate increase, which then increases flux through the hexosamine pathway, where fructose 6-phosphate is converted by the enzyme GFAT to UDP–N-acetylglucosamine (UDP-GlcNAc). Finally, inhibition of GAPDH increases intracellular levels of the first glycolytic metabolite, glucose. This increases flux through the polyol pathway, where the enzyme aldose reductase reduces it (or glyceralddehyde 3-phosphate), consuming NADPH in the process. Inhibition of GAPDH activity in 5 mmol/L glucose using antisense DNA elevates the activity of each of the major pathways of hyperglycemic damage to the same extent as that induced by hyperglycemia (Figure 4).97

Hyperglycemia-induced superoxide inhibits GAPDH activity in vivo by modifying the enzyme with polymers of ADP-ribose.97 By inhibiting mitochondrial superoxide production with either UCP-1 or MnSOD, both modification of GAPDH by poly(ADP-ribose) and reduction of its activity by hyperglycemia were prevented. Most importantly, both modification of GAPDH by poly(ADP-ribose) and reduction of its activity by hyperglycemia were also prevented by a specific
inhibitor of poly(ADP-ribose) polymerase (PARP), the enzyme that makes these polymers of ADP-ribose. Normally, PARP resides in the nucleus in an inactive form, waiting for DNA damage to activate it. When increased intracellular glucose generates increased ROS in the mitochondria, free radicals induce DNA strand breaks, thereby activating PARP. Both hyperglycemia-induced processes are prevented by either UCP-1 or MnSOD. Once activated, PARP splits the NAD⁺ molecule into its 2 component parts: nicotinic acid and ADP-ribose. PARP then proceeds to make polymers of ADP-ribose, which accumulate on GAPDH and other nuclear proteins. GAPDH is commonly thought to reside exclusively in the cytosol. However, it normally shuttles in and out of the nucleus, where it plays a critical role in DNA repair (Figure 4).  

**Glycemic Memory**

In 1993, the results of the landmark Diabetes Control and Complications Trial (DCCT) showed that in people with short-duration type 1 diabetes, intensive glycemic control dramatically reduced the occurrence and severity of diabetic microvascular complications. After the announcement of the DCCT results, many patients who had been in the standard therapy group adopted more intensive therapeutic regimens, and their level of glycemic control improved, as measured by the HbA1c test. At the same time, the mean level of HbA1c worsened for patients who had been in the intensive therapy group. The post-DCCT HbA1c values for both groups have become statistically identical during the approximate 14 years of follow-up in the ongoing Epidemiology of Diabetes Interventions and Complications Study (EDIC).  

Surprisingly and provocatively, however, the effects of a 6.5-year difference in HbA1c during the DCCT on the incidence of retinopathy and nephropathy persisted, and have become even greater after the subsequent 14 years of follow-up. People in the standard therapy group continue to have a higher incidence of complications, even with an improvement in glycemic control during the 14 years of EDIC, whereas people in the intensive therapy group continue to have a lower incidence of both microvascular complications and CVD, even with a deterioration in glycemic control during the EDIC years. Intensive treatment reduced the risk of any CVD event by 42% and the risk of nonfatal myocardial infarction, stroke, or death from any cause, despite an early loss of glycemic differences (also termed the “legacy effect”). A continued benefit was evident during the 10-year posttrial follow-up among overweight patients. Glycemic memory has several important clinical implications: (1) early tight control is very important; (2) cure of diabetes may not prevent subsequent development of complications; and (3) novel therapies that reverse hyperglycemic memory may be needed. The continuing progression of tissue damage after the correction of hyperglycemia (“hyperglycemic memory”) may be explained in part by persistent epigenetic changes caused by hyperglycemia-induced mitochondrial superoxide production.  

Posttranslational modifications of histones cause chromatin remodeling and changes in levels of gene expression. Because these modifications do not involve differences in DNA sequence, they are called “epigenetic.” Transient hyperglycemia, at a level sufficient to increase mitochondrial ROS production, induces long-lasting activating epigenetic changes in the proximal promoter of the NF-κB subunit p65 in human aortic endothelial cells (16 hours exposure) and in aortic cells in vivo nondiabetic mice (6 hours exposure). These epigenetic changes (recruitment of Set 7 and H3K4 monomethylation) cause sustained increases in p65 gene expression and in the expression of p65-dependent proinflammatory genes. Both the epigenetic changes and the gene expression changes persist for at least 6 days of subsequent normal glycemia in cultured cells, and for months in previously diabetic mice whose β-cell function recovered. Hyperglycemia-induced epigenetic changes and increased p65 expression are prevented by normalizing mitochondrial superoxide production or superoxide-induced methylglyoxal. These results highlight the dramatic and long-lasting effects that short-term hyperglycemic spikes can have on vascular cells and suggest that transient spikes of hyperglycemia may be an HbA1c-independent risk factor for diabetic complications. Demethylation of another histone lysine residue in the proximal p65 promoter, H3K9, is also induced by hyperglycemia-induced overproduction of ROS. This reduces inhibition of p65 gene expression, and thus acts synergistically with the activating methylation of histone 3 lysine 4 (Figure 5). Consistent with these observations, others have shown similar epigenetic changes in lymphocytes from patients with type 1 diabetes and in vascular smooth muscle cells derived from db/db mice.  

**Insulin Resistance, ROS, and Atherosclerosis**

Insulin resistance (IR) occurs in the majority of patients with type 2 diabetes, and in two-thirds of subjects with impaired glucose tolerance. Both of these groups have a significantly higher risk of developing CVD. To isolate the effects of IR from those of hyperglycemia and diabetes, several studies have evaluated subjects with normal glucose tolerance. In nonobese subjects without diabetes, IR predicted the development of CVD independently of other known risk factors. In another group of subjects without diabetes or impaired glucose tolerance, those in the highest quintile of IR had a 2.0-fold increase in CVD risk compared with those in the lowest quintile after adjustment for 11 known cardiovascular risk factors, including LDL, triglycerides, HDL, systolic blood pressure, and smoking. These data indicate that IR itself promotes atherogenesis in the absence of hyperglycemia. IR in adipocytes increases release of free fatty acids (FFAs) from stored triglyceride, and increased oxidation of FFAs in aortic endothelial cells due to lack of insulin stimulation of malonyl CoA production causes increased production of superoxide by the mitochondrial electron transport chain. By activating the same mechanisms as hyperglycemia-induced ROS, FFA-induced overproduction
of superoxide activates a variety of proinflammatory signals and inactivates 2 important antiatherogenic enzymes: prostacyclin synthase and eNOS. In 2 nondiabetic rodent models of insulin resistance, inactivation of prostacyclin synthase and eNOS was prevented by inhibition of FFA release from adipose tissue, by inhibition of the rate-limiting enzyme for fatty acid oxidation in mitochondria (carnitine palmitoyl-transferase I), and by reduction of superoxide levels (Figure 6).116 Human atherosclerotic samples obtained during vascular surgery show greater mitochondrial DNA damage than nonatherosclerotic samples obtained from age-matched transplant donors, consistent with increased ROS production. Mitochondrial damage precedes the development of atherosclerosis and tracks with lesion extent in apoE-null mice, and mitochondrial dysfunction caused by heterozygous deficiency of a superoxide dismutase (SOD2) increases atherosclerosis and vascular mitochondrial damage in the same model.117,118

Although the association of insulin resistance with CVD risk is clear, data concerning the relative role of hyperglycemia in promoting CVD in diabetes appear to be somewhat contradictory. In type 1 diabetes, where severe insulin resistance is not a major abnormality, lowering of HbA1c levels with more intensive insulin treatment during the Diabetes Control and Complications Trial reduced both atherosclerosis surrogates during the trial and actual CVD events years after the trial concluded. Intensive treatment reduced the risk of any CVD event by 42% and the risk of nonfatal myocardial infarction, stroke, or death from CVD by 57%.100 In contrast, intensive insulin treatment during the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial was associated with an unexpected excess of cardiovascular mortality in the intensive arm that caused the study to end early. Patients in this group were treated with a goal of lowering HbA1c to 6.0%. Overall, a higher average on-treatment A1C was a stronger predictor of mortality than the A1C for the last interval of follow-up or the decrease of A1C in the first year. Of note, the excess risk associated with intensive glycemic treatment occurred among those participants whose average A1C, contrary to the intent of the strategy, was >7%.119 How might these apparently conflicting results be explained? One possibility is that intensive therapy in extremely insulin resistant diabetic patients causes proatherogenic effects through overstimulation of insulin signaling pathways not affected by insulin resistance. In the liver, insulin exerts 2 predominant actions: it reduces glucose production (gluconeogenesis) and it increases the synthesis of fatty acids and triglycerides (lipogenesis). In the insulin-resistant state, only one of these actions is blocked in liver. Insulin loses its ability to reduce gluconeogenesis but it retains its ability to enhance lipogenesis.120,121

Thus, increasing insulin levels to overcome the hyperglycemia caused by pathway-selective insulin resistance likely overdrive nonresistant pathways of insulin signaling, including MAPK. In arterial endothelial cells, such selective overdrive of the MAPK pathway by insulin would stimulate cellular growth and migration and the production of prothrombotic and profibrotic factors. Insulin stimulates production of the vasoconstrictor endothelin-1, and increases cellular adhesion molecule expression. In arterial smooth muscle cells, overdrive of the MAPK pathway by high insulin levels would stimulate VSMC proliferation and migration and expression of angiotensinogen and AT1R.122,123

In rodent models of type 1 and type 2 diabetes, accumulation of triglyceride in the heart is associated with cardiac dysfunction and oxidative stress.124,125 Expression of antioxidant enzymes or treatment with antioxidant compounds can ameliorate both triglyceride-associated oxidative stress and diabetic cardiomyopathy.126,127 In vitro studies of cultured myocytes and endothelial cells have shown that excess saturated long chain fatty acids promote generation of ROS.128,129 Several mechanisms have been implicated including lipid activation of NADPH oxidase activity through the downstream production of ceramides, and signaling through pathways that converge on NF-κB.130,131 Oxidative stress may also be intimately linked to lipid-induced endoplasmic reticulum stress.132

Recently, a genetic screen for mutations that abrogate lipotoxic cell death led to the identification of a long
noncoding RNA, gadd7, which functions as a feed-forward amplifier of lipid-induced and generalized oxidative stress.\textsuperscript{133}

**Mechanism-Based Therapeutic Approaches**

Because existing methods of treating diabetes do not prevent diabetic complications, new mechanism-based therapeutic strategies are needed. One new class of potential therapeutic agents is transketolase activators. When increased superoxide inhibits GAPDH activity, the concentration of glycolytic intermediates above the enzyme accumulates which increases the flux into the 5 pathways of hyperglycemic damage. Two of these glycolytic intermediates, fructose 6-phosphate and glyceraldehyde 3-phosphate, are also the final products of the transketolase reaction, which is the rate-limiting enzyme in the nonoxidative part of the pentose phosphate pathway. Although the function of this enzyme is normally taught to be the conversion of pentose phosphates to glycolytic intermediates, it can also convert glycolytic intermediates to pentose phosphates. Because in diabetes the concentration of these 2 glycolytic intermediates is high, transketolase could reduce their concentration and thereby divert their flux away from 3 of the damaging pathways activated by hyperglycemia. This enzyme requires the vitamin thiamine as a cofactor. Although thiamine itself only activated transketolase \textasciitilde25\% in arterial endothelial cells, the thiamine derivative benfotiamine was found to activate transketolase 250\% in arterial endothelial cells. Based on those findings, several groups have demonstrated the ability of benfotiamine to counteract glucose-mediated toxicity in cultured endothelial cells, endothelial progenitor endothelial cells, and mouse models of diabetes.\textsuperscript{134–136} Most importantly, it has been recently demonstrated that beneficial effects of benfotiamine on complication-causing pathways in rodent models of diabetic complications also occur in humans with type 1 diabetes.\textsuperscript{137} Benfotiamine treatment prevented experimental diabetic retinopathy and nephropathy in mice, and treatment with high-dose thiamine reduced albuminuria in patients with Type 2 diabetes. Diabetes has recently been found to cause intracellular thiamine depletion, which would impair normal transketolase conversion of hyperglycemia-induced elevation of glycolytic intermediates to pentose phosphates, and further exacerbate activation of 3 of the damaging pathways.\textsuperscript{138–140}

A second new class of mechanism-based potential therapeutic agents are PARP inhibitors. In cultured arterial endothelial cells, a specific PARP inhibitor prevents hyperglycemia-induced activation of PKC, NF-\textit{k}B, intracellular AGE formation, and the hexosamine pathway. In animal models of diabetes, PARP inhibition prevents arterial endothelial cell injury and podocyte apoptosis, ameliorates nephropathy, and alleviates sensory neuropathy.\textsuperscript{141–143}

A third class of mechanism-based therapeutics are SOD/catalase mimetics. Excess superoxide itself directly inhibits critical antiatherosclerotic endothelial enzymes independent of activating the 5 damaging pathways implicated in metabolite-induced diabetic complications. Both of these enzymes (eNOS and prostacyclin synthase) are inhibited in diabetic patients and diabetic animals. To prevent oxidative inactivation of these key enzymes, in addition to preventing activation of the pathways discussed above, it is necessary to directly reduce the amount of superoxide. Conventional antioxidants are unlikely to do this effectively because conventional antioxidants neutralize reactive oxygen molecules on a one-for-one basis, whereas hyperglycemia-induced overproduction of superoxide is a continuous process. Based on observations of the beneficial effects of overexpression of antioxidant enzymes in mouse models, what is needed is a new type of antioxidant, a catalytic antioxidant, such as an SOD/catalase mimetic.\textsuperscript{144} Hyperglycemia-induced reactive oxygen overproduction directly reduces eNOS activity in diabetic aortas by 65\%. However, when these diabetic animals are treated with an SOD/catalase mimetic, there is no reduction in activity of this antiatherogenic enzyme.\textsuperscript{4} Similarly, but more dramatically, hyperglycemia-induced reactive oxygen overproduction directly reduces prostacyclin synthase activity in diabetic aortas by 95\%. Treatment of these diabetic animals with an SOD/catalase mimetic completely prevents diabetes-induced oxidative inactivation of aortic prostacyclin synthase, and also normalizes all 5 of the pathways implicated in hyperglycemic damage.\textsuperscript{4} Inhibition of hyperglycemia-induced ROS production in diabetic mice using either transgenic antioxidant enzyme expression or combinations of antioxidant compounds prevents the development of experimental diabetic retinopathy, nephropathy, neuropathy and cardiomyopathy.\textsuperscript{91,145–149} Together, these data strongly suggest that therapeutic correction of diabetes-induced superoxide overproduction may be a powerful approach for preventing diabetic complications.

**Summary**

Oxidative stress plays a pivotal role in the development of diabetes complications, both microvascular and cardiovascular. The experimental data discussed in this review demonstrate that the metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction. This increased superoxide production is the central and major mediator of diabetes tissue damage, causing the activation of 5 pathways involved in the pathogenesis of complications and direct inactivation of 2 antiatherosclerotic enzymes, eNOS and prostacyclin synthase. This model is strongly supported by in vitro and in vivo experiments. However, rodent models of diabetic atherosclerosis, cardiomyopathy, and, indeed, any diabetic microvascular complication do not recapitulate major aspects of the phenotypes of human diabetic complications. Large animal models such as pigs or nonhuman primates, in which diabetic CVD more closely resembles that in humans, have been generated,\textsuperscript{150,151} and future testing of ROS involvement in these models is the critical next step. Although conventional antioxidants such as vitamin E have not shown any benefit in human CVD, these conventional antioxidants neutralize reactive oxygen molecules on a one-for-one basis, whereas diabetes-induced overproduction of superoxide is a continuous process. Based on observations of the beneficial effects of overexpression of antioxidant enzymes in transgenic mouse models of CVD, catalytic antioxidants such as the family of SOD/catalase mimetic compounds are the most logical choice for reducing diabetes-induced ROS in the large animal models mentioned above.
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Disclosures
None.

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