7 Microvascular Complications in Diabetes

35 Pathogenesis of Microvascular Complications

Ferdinando Giacco & Michael Brownlee

Diabetes Research Center, Departments of Medicine and Endocrinology, Albert Einstein College of Medicine, NY, USA

Keypoints

- Microvascular complications are caused by prolonged exposure to hyperglycemia.
- Hyperglycemia damages cell types that cannot downregulate glucose uptake, causing intracellular hyperglycemia.
- Intracellular hyperglycemia damages tissues by five major mechanisms: increased flux of glucose and other sugars through the polyol pathway; increased intracellular formation of advanced glycation end-products (AGEs); increased expression of the receptor for AGEs and its activating ligands; activation of protein kinase C isoforms; and overactivity of the hexosamine pathway.
- A single process – increased mitochondrial production of oxygen free radicals – activates each of these mechanisms.
- Persistent consequences of hyperglycemia-induced mitochondrial superoxide production may also explain the continuing progression of tissue damage after improvement of glycemic levels ("hyperglycemic memory").
- Different individual susceptibility to microvascular complications have been linked to polymorphisms in the superoxide dismutase 1 gene.
- Hyperglycemia-induced mitochondrial reactive oxygen species production impairs the neovascular response to ischemia by blunting hypoxia-inducible factor 1 transactivation.
- Hypertension accelerates microvascular damage by increasing intracellular hyperglycemia through upregulation of the glucose transporter 1.
- Potential mechanism-based therapeutic agents for diabetic microvascular complications include transketolase activators, poly(ADP-ribose) polymerase inhibitors and catalytic antioxidants.

Overview of diabetic complications

All forms of diabetes are characterized by hyperglycemia, a relative or absolute lack of insulin action, and the development of diabetes-specific pathology in the retina, renal glomerulus and peripheral nerve. Diabetes is also associated with accelerated atherosclerotic disease affecting arteries that supply the heart, brain and lower extremities. As a consequence of its disease-specific pathology, diabetes mellitus is now the leading cause of new blindness in people 20–74 years of age and the leading cause of end-stage renal disease (ESRD) in the developed world. Survival of patients with diabetic ESRD on dialysis is half that of those without diabetes. More than 60% of patients with diabetes are affected by neuropathy, which includes distal symmetrical polyneuropathy, mononeuropathies and a variety of autonomic neuropathies causing erectile dysfunction, urinary incontinence, gastroparesis and nocturnal diarrhoea. Diabetic accelerated lower extremity arterial disease in conjunction with neuropathy accounts for 50% of all non-traumatic amputations in the USA. Diabetes and impaired glucose tolerance increase cardiovascular disease (CVD) risk three- to eightfold. Thus, over 40% of patients hospitalized with acute myocardial infarction (MI) 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 non-healing foot ulcers. The focus of this chapter is on the microvascular complications comprising retinopathy, nephropathy and peripheral neuropathy.

Much of the impact of chronic diabetes falls on the microcirculation [1,2]. With long-standing disease, there is progressive narrowing and eventual occlusion of vascular lumina, resulting in impaired perfusion, ischemia and dysfunction of the affected tissues. Several processes contribute to microvascular occlusion. One of the earliest is increased vascular permeability, allowing extravasation of plasma proteins that accumulate as periodic acid–Schiff-positive deposits in the vessel walls. In addition, the extracellular matrix elaborated by perivascular cells such as pericytes (retina) and mesangial cells (glomerulus) is increased, brought about by changes in synthesis and turnover of its component proteins and glycosaminoglycans. As a result, the basement membrane is thickened in many tissues, including retinal capillaries and the vasa nervorum, while mesangial matrix is

Textbook of Diabetes, 4th edition. Edited by R. Holt, C. Cockram, A. Flyvbjerg and B. Goldstein.

expanded in the renal glomerulus. Hypertrophy and hyperplasia of endothelial, mesangial and arteriolar smooth muscle cells also contribute to vessel wall thickening. Finally, increased coagulability of the blood and adhesion of platelets and leukocytes to the endothelial surface lead to microthrombus formation and luminal occlusion.

The progressive narrowing and blockage of diabetic microvascular lumina are accompanied by loss of microvascular cells. In the retina, diabetes induces apoptosis of Müller cells and ganglion cells [3], pericytes and endothelial cells [4]. In the glomerulus, widespread capillary occlusion and declining renal function are associated with podocyte loss. In the vasa nervorum of diabetic nerves, endothelial cell and pericyte degeneration occur [5] and appear to precede functional abnormalities of peripheral nerves [6]. Increased apoptosis of cells in the retina, renal glomerulus and peripheral neurons is a prominent feature of diabetic microvascular tissue damage [7–11] and may also cause damage to adjacent cells.

Role of hyperglycemia in microvascular complications

Overall, diabetic microvascular complications are caused by prolonged exposure to high glucose levels. This has been established by large-scale prospective studies for both type 1 diabetes (T1DM) by the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study [DCCT/EDIC] [12] and for type 2 diabetes (T2DM) by the UK Prospective Diabetes Study [UKPDS] [13]). Similar data have been reported by the Steno-2 study [14].

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 such as vascular smooth muscle show an inverse relationship between extracellular glucose concentrations and glucose transport. In contrast, vascular endothelial cells, a major target of hyperglycemic damage, show no significant change in glucose transport rate when glucose concentration is elevated, resulting in intracellular hyperglycemia (Figure 35.1). These differences are caused in part by tissue-specific differences in expression and function of different glucose transporter (GLUT) proteins [15].

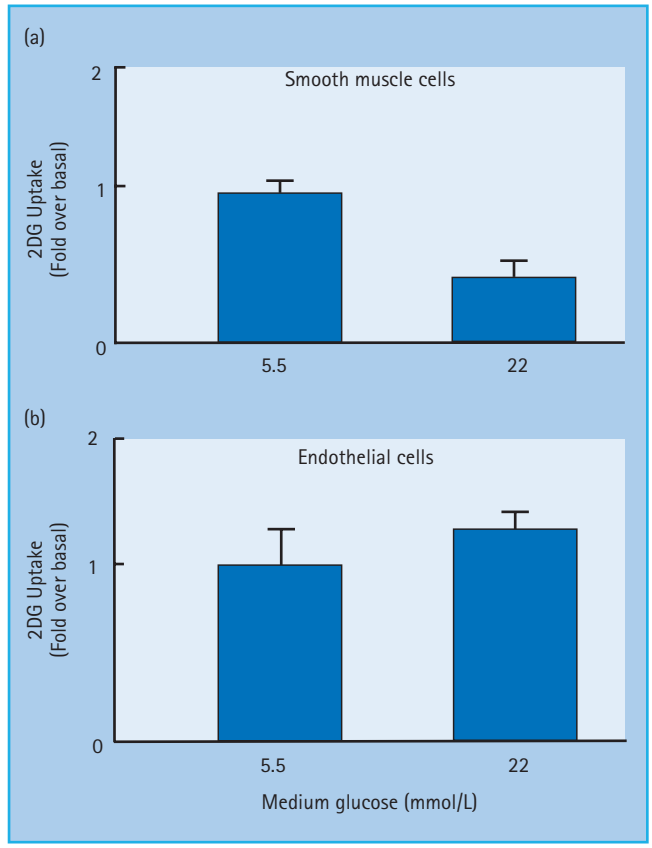

Figure 35.1 Lack of downregulation of glucose transport by hyperglycemia in cells affected by diabetic complications. (a) 2-Deoxyglucose uptake in vascular smooth muscle cells pre-exposed to 5.5 or 22 mmol/L glucose. (b) 2-Deoxyglucose uptake in aortic endothelial cells pre-exposed to 5.5 or 22 mmol/L glucose. Data from Kaiser N, Sasson S, Feener EP, Boukobza-Vardi N, Higashi S, Moller DE, *et al.* Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes* 1993; **42**:80–89.

Mechanisms of hyperglycemia-induced damage

There are nearly 2000 publications supporting five major mechanisms by which hyperglycemia causes diabetic complications:

1 Increased flux of glucose and other sugars through the polyol pathway;
2 Increased intracellular formation of advanced glycation end-products (AGEs);
3 Increased expression of the receptor for AGEs (RAGE) and its activating ligands;
4 Activation of protein kinase C (PKC) isoforms; and
5 Overactivity of the hexosamine pathway.

Despite this, the results of clinical studies in which one of these pathways is blocked have been disappointing. This led to the hypothesis in 2000 that all five mechanisms are activated by a single upstream event: mitochondrial overproduction of the reactive oxygen species (ROS) superoxide as a result of intracellular hyperglycemia. This provides a unifying hypothesis for the pathogenesis of diabetic complications.

Increased polyol pathway flux

The polyol pathway is based on a family of aldoketo reductase enzymes which can utilize as substrates a wide variety of sugar-derived carbonyl compounds and reduce these by nicotinic acid

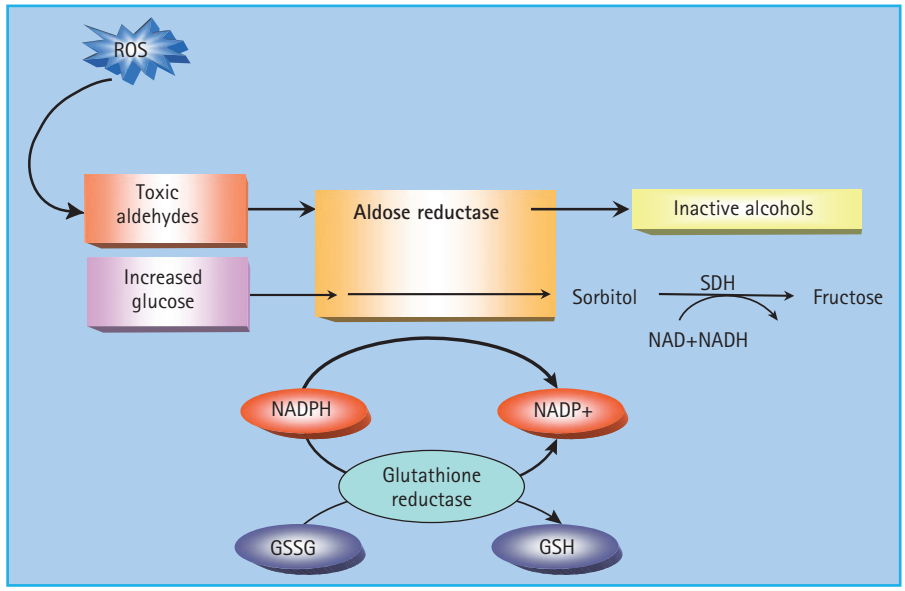

Figure 35.2 The polyol pathway. When glucose concentration is normal, aldose reductase reduces toxic aldehydes generated by reactive oxygen species (ROS) to inactive alcohols. With intracellular hyperglycemia, it can also reduce glucose to sorbitol. Both reactions use nicotinic acid adenine dinucleotide phosphate (NADPH) as a co-factor. When aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented. Sorbitol-dehydrogenase (SDH) oxidizes sorbitol to fructose using NAD^+ as a co-factor. GSSG, glutathione disulfide (oxidized glutathione).

adenine dinucleotide phosphate (NADPH) to their respective sugar alcohols (polyols). The classic representation holds that glucose is converted to sorbitol, and galactose to galactitol. Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH), with NAD^+ being reduced to NADH (Figure 35.2).

The first and rate-limiting step of the polyol pathway is governed by aldose reductase, which is found in tissues such as nerve, retina, lens, glomerulus and blood vessel wall. In these tissues, glucose uptake is mediated by GLUT proteins other than GLUT-4 and so does not require insulin; 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. These include sorbitol-induced osmotic stress, decreased cytosolic Na/K^+-ATPase activity, increased cytosolic $NADH/NAD^+$, and decreased cytosolic NADPH. It was originally suggested that intracellular accumulation of sorbitol, which does not diffuse easily across cell membranes, could result in osmotic damage, but it is now clear that sorbitol levels in diabetic vessels and nerves are far too low to do this. Another early suggestion was that increased flux through the polyol pathway led to decreased phosphatidylinositol synthesis, and that this inhibited Na/K^+-ATPase activity. The latter abnormality does occur in diabetes, but has recently been shown to result from hyperglycemia-induced activation of PKC which increases the production of two inhibitors of Na/K^+-ATPase, arachidonate and prostaglandin E_2 [16].

It has also been suggested that the reduction of glucose to sorbitol by NADPH (Figure 35.2) consumes the latter. NADPH is a co-factor required to regenerate reduced glutathione (GSH); as 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 GSH [17]. Reduced GSH is depleted in the lens of transgenic mice that overexpress aldose reductase and in diabetic rat lens compared with non-diabetic lens [18,19]. It has also been recently demonstrated that decreased glutathiolation of cellular proteins is related to decreased nitric oxide (NO) availability in diabetic rats which would decrease S-nitrosoglutathione (GSNO). Restoring the NO levels in diabetic animals increases glutathiolation of cellular proteins, inhibits aldose reductase activity and prevents sorbitol accumulation.

Moreover, hyperglycemia can also inhibit glucose-6-phosphate dehydrogenase, the major source of NADPH regeneration, which may further reduce NADPH concentration in some vascular cells and neurons [20].

In diabetic vascular cells, however, glucose does not appear to be the substrate for aldose reductase, because the Michaelis constant (Km) of aldose reductase for glucose is 100 mmol/L, while the intracellular concentration of glucose in diabetic retina is 0.15 mmol/L [21,22]. Glycolytic metabolites of glucose such as glyceraldehyde-3-phosphate, for which aldose reductase has much higher affinity, may be the physiologically relevant substrate.

Increased intracellular AGE formation

AGEs are formed by the reaction of glucose and other glycating compounds (e.g. dicarbonyls such as 3-deoxyglucosone, methylglyoxal and glyoxal) with proteins and, to a lesser extent, nucleic acids. The reactions proceed through a series of stages that are initially reversible and yield early glycation products, but eventually undergo irreversible changes that markedly impair the structural, enzymatic or signaling functions of the glycated proteins (Figure 35.3). A familiar example of this process yields glycated hemoglobin (HbA_{1c}). AGEs are found in increased amounts in

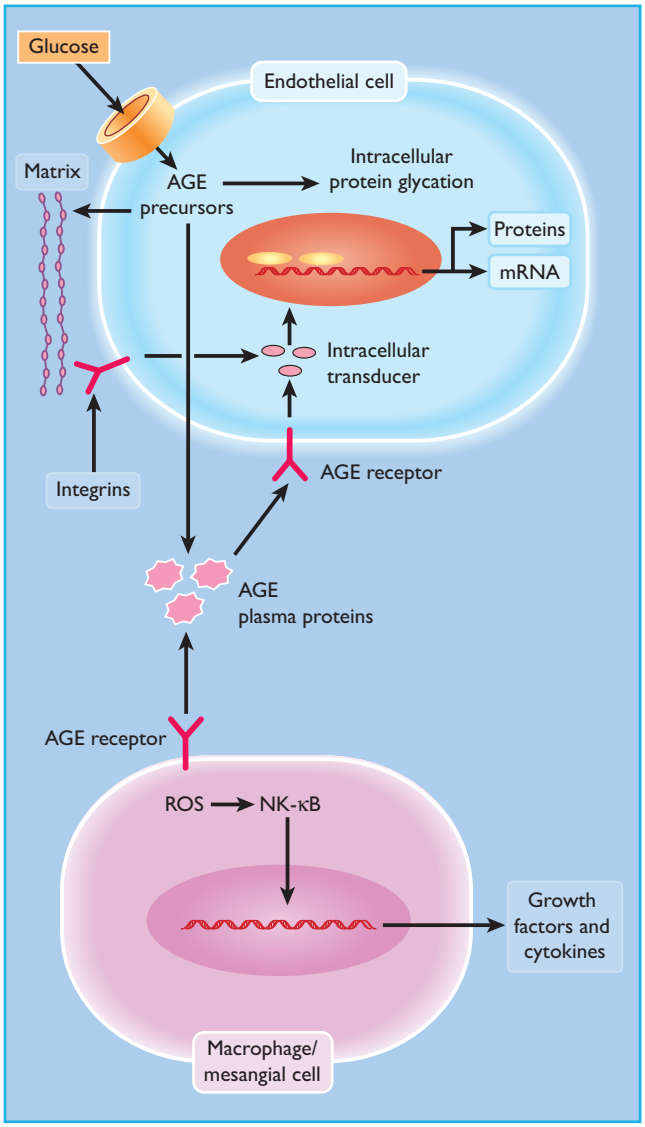

Figure 35.3 Increased production of intracellular advanced glycation end-products (AGE) precursors damages cells by three mechanisms: modification of intracellular proteins; modification of the extracellular matrix (upper panel); and interactions with AGE receptors such as RAGE in endothelial cells and macrophages. NFκB, nuclear factor κB; ROS, reactive oxygen species.

extracellular structures of diabetic retinal vessels [23–25] and renal glomeruli [26–28], where they can cause damage through the mechanisms described below.

These AGEs were originally thought to arise from non-enzymatic reactions between extracellular proteins and glucose; however, the rate of AGE formation from glucose is orders of magnitude slower than that induced by glucose-derived dicarbonyl precursors generated intracellularly, and it now seems likely that raised intracellular glucose is the primary initiating event in the formation of both intracellular and extracellular AGEs [29]. AGEs can arise intracellularly from the autooxidation of glucose to glyoxal [30], the decomposition of an Amadori product to the 3-deoxyglucosone, or the fragmentation of glyceraldehyde-3-phosphate to yield methylglyoxal [31]. All these reactive intracellular dicarbonyls react readily with uncharged amino groups of intracellular and extracellular proteins to form AGEs. Methylglyoxsal is the major intracellular AGE precursor [32,33].

Intracellular effects of AGEs

Intracellular production of AGE precursors can damage cells by three general mechanisms. First, 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) which are expressed on the surface of cells. Finally, plasma proteins modified by AGE precursors bind to AGE receptors on cells such as macrophages; binding induces the production of ROS, which in turn activates the pleiotropic transcription factor, nuclear factor κB (NFκB), causing multiple pathologic changes in gene expression [34].

It has been recently demonstrated that AGE modification of intracellular protein can be involved in diabetic retinopathy. In diabetes, retinal capillary formation is regulated by complex context-dependent interactions among pro- and anti-angiogenic factors [35,36], including angiopoietin-2 (Ang-2). When vascular endothelial growth factor (VEGF) levels are insufficient, Ang-2 causes endothelial cell death and vessel regression. Diabetes induces a significant increase in retinal expression of Ang-2 in rat [37], and diabetic Ang-2 +/– mice have both decreased pericyte loss and reduced acellular capillary formation [38].

Moreover, in mouse kidney endothelial cells, high glucose causes increased methylglyoxal modification of the corepressor mSin3A. Methylglyoxal modification of mSin3A results in increased recruitment of O-GlcNAc-transferase, with consequent increased modification of Sp3 by *O*-linked *N*-acetylglucosamine. This modification of Sp3 causes decreased binding to a glucose-responsive GC-box in the 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 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in cells and in kidneys from diabetic mice and sensitized microvascular endothelial cells to the pro-inflammatory effects of tumor necrosis factor α (TNF-α) [39].

Effects of AGEs on extracellular matrix

AGE formation alters the functional properties of several important matrix molecules. Collagen was the first matrix protein in which glucose-derived AGEs were shown to form covalent intermolecular bonds. This process is partly mediated by H_2O_2 production [40,41]. With type I collagen, this cross-linking causes expansion of molecular packing [42], while AGE formation on type IV collagen from basement membrane inhibits the normal lateral association of these molecules into a network-like structure by interfering with binding of the non-collagenous NC1 domain to the helix-rich domain [43]. AGE formation on laminin

prevents the molecules from self-assembling into a polymer and also decreases binding with type IV collagen and heparan sulfate proteoglycan [44].

These AGE-induced cross-links alter tissue function, notably in blood vessels. AGEs decrease elasticity in arteries from diabetic rats, even after vascular tone is abolished, and increase fluid filtration across the carotid artery [45]. *In vitro*, AGE formation on intact glomerular basement membrane increases its permeability to albumin in a manner that resembles the abnormal permeability of diabetic nephropathy [46,47].

AGE formation on extracellular matrix also interferes with the ways in which cells interact with the matrix. For example, methylglyoxal modification of type IV collagen's cell-binding domains decreases endothelial cell adhesion and inhibits angiogenesis [48].

AGE formation on a 6-amino acid, growth-promoting sequence in the A chain of the laminin molecule markedly reduces neurite outgrowth [49], while AGE modification of vitronectin reduces its ability to promote cell attachment [50]. In addition, matrix glycation impairs agonist-induced Ca^{2+} increases which might adversely affect the regulatory functions of endothelium [51].

Receptor-mediated biologic effects of AGEs

AGE-modified proteins in the circulation can affect a range of cells and tissues. Specific receptors for AGEs were first identified on monocytes and macrophages. Two AGE-binding proteins isolated from rat liver, identified as OTS-48 (60 kDa) and 80K-H (90 kDa) [52], are both present on monocytes and macrophages; antisera against either protein block AGE binding [53]. AGE protein binding to this receptor stimulates macrophages to produce cytokines, including interleukin-1, TNF-α, transforming growth factor β (TGF-β), macrophage colony-stimulating factor and granulocyte–macrophage colony-stimulating factor, as well as insulin-like growth factor I (IGF-I). These factors appear to be produced at concentrations that can increase glomerular synthesis of type IV collagen and induce chemotaxis and proliferation of both arterial smooth muscle cells and macrophages [54–62]. The macrophage scavenger receptor type II (class A), galectin-3 and CD36 (a member of the class B macrophage scavenger receptor family) have also been shown to recognize AGEs [63–67].

AGE receptors have also been identified on glomerular mesangial cells. *In vitro*, AGE protein binding to its receptor on mesangial cells stimulates secretion of platelet-derived growth factor which in turn mediates mesangial cells to produce type IV collagen, laminin and heparan sulfate proteoglycan [68,69].

Vascular endothelial cells and other cell types also express specific AGE receptors (RAGEs), notably 35-kDa and 46-kDa AGE-binding proteins that have been purified to homogeneity [70–72]. The N-terminal sequence of the 35-kDa protein is identical to lactoferrin, whereas the 46-kDa AGE-binding protein is a novel member of the immunoglobulin superfamily, containing three disulfide-bonded immunoglobulin homology units. RAGE have been shown to mediate signal transduction via generation of ROS, activation of NFκB, and p21 *ras* [73–75]. AGE signaling can be blocked in cells by expression of RAGE antisense cDNA [76] or anti-RAGE ribozyme [77]. It has been also recently demonstrated that a RAGE–NFκB axis operates in diabetic neuropathy by mediating functional sensory deficits [78].

In endothelial cells, AGE binding to its receptor alters the expression of several genes, including thrombomodulin, tissue factor and VCAM-1 [79–81]. 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 [82–85]. 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 pro-inflammatory mediators, including the NFκB subunit p65, VCAM-1, and monocyte chemotactic protein 1 (MCP-1) and reduced oxidative stress [86].

It is important to note that more recent studies indicate that AGEs at the concentrations found in diabetic sera are not the major ligand for RAGE. Rather, several pro-inflammatory protein ligands have been identified, which activate RAGE at low concentrations. These include several members of the S100 calgranulin family and high mobility group box 1 (HMGB1), all of which are increased by diabetic hyperglycemia. Binding of these ligands with RAGE causes cooperative interaction with the innate immune system signaling molecule toll-like receptor 4 (TLR-4) [87,88].

Increased protein kinase C activation

The group of PKCs consist of at least 11 isoforms that are widely distributed in mammalian tissues. The activity of the classic isoforms is dependent on both Ca^{2+} ions and phosphatidylserine and is greatly enhanced by diacylglycerol (DAG). Persistent and excessive activation of several PKC isoforms might also operate as a third common pathway mediating tissue injury induced by hyperglycemia and associated biochemical and metabolic abnormalities. This results primarily from enhanced *de novo* synthesis of DAG from glucose via triose phosphates, whose availability is increased because raised intracellular glucose levels enhance glucose flux through the glycolytic pathway [89–92]. Finally, recent evidence suggests that the enhanced activity of PKC isoforms could also result from the interaction between AGEs and their cell-surface receptors [93]. Hyperglycemia primarily activates the β and δ isoforms of PKC, both in cultured vascular cells [94–96] and in the retina and glomeruli of diabetic animals [91,92,93], but increases in other isoforms have also been found, such as PKC-α and PKC-ε isoforms in the retina [89] and PKC-α and PKC-δ in the glomerulus of diabetic rats [97,98] (Figure 35.4).

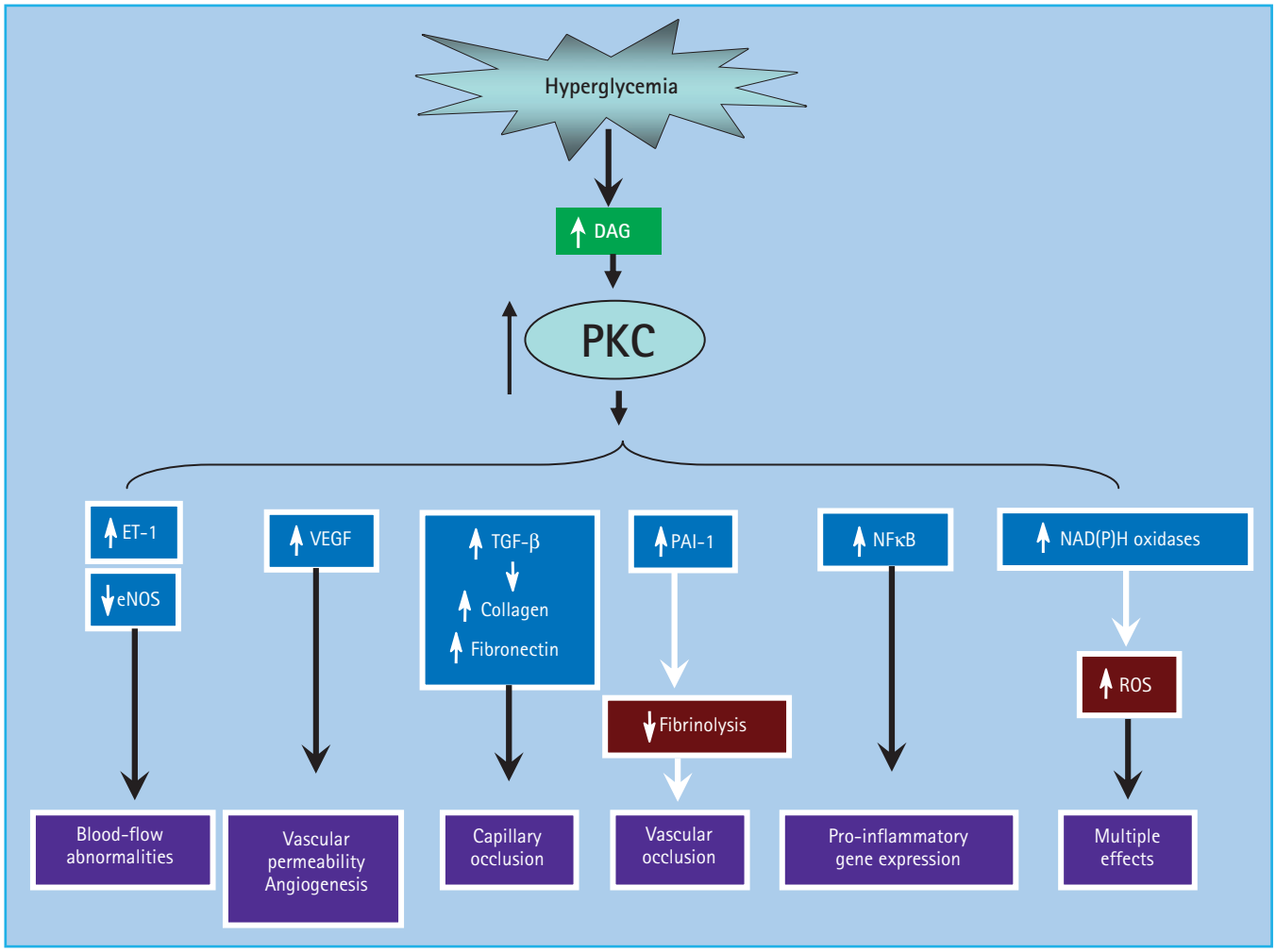

Figure 35.4 Activation of protein kinase C (PKC) by *de novo* synthesis of diacylglycerol (DAG) and some of its pathologic consequences. eNOS, endothelial NO synthase; ET-1, endothelin-1; NADPH, nicotinic acid adenine dinucleotide phosphate; NFκB, nuclear factor κB; PAI-1, plasminogen activator inhibitor 1; ROS, reactive oxygen species; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

In early experimental diabetes, activation of PKC-β isoforms has been shown to mediate the diabetes-related decreases in retinal and renal blood flow [99], perhaps by depressing the production of the vasodilator NO and/or increasing endothelin-1, a potent vasoconstrictor. Overactivity of PKC has been implicated in the decreased NO production by the glomerulus in experimental diabetes [100] and by smooth muscle cells in the presence of high glucose levels [101], and has been shown to inhibit insulin-stimulated expression of endothelial NO synthase (eNOS) in cultured endothelial cells [102]. Hyperglycemia increases the ability of endothelin-1 to stimulate mitogen activated protein kinase (MAPK) activity in glomerular mesangial cells, and this occurs by activating PKC isoforms [103]. The increased endothelial cell permeability induced by high glucose in cultured cells is mediated by activation of PKC-α [104]; activation of PKC by high glucose also induces expression of the permeability-enhancing factor VEGF in smooth muscle cells [105].

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 TGF-β1, fibronectin and type IV collagen in both cultured mesangial cells [106,107] and in glomeruli of diabetic rats [97]. This effect also appears to be mediated through the inhibition of NO production by PKC [108]. Hyperglycemia-induced activation of PKC has also been implicated in the overexpression of the fibrinolytic inhibitor, plasminogen activator inhibitor 1 (PAI-1) [109], and in the activation of NFκB in cultured endothelial cells and vascular smooth muscle cells [110,111].

Increased hexosamine pathway flux

Several data suggest that hyperglycemia could cause diabetic complications by shunting glucose into the hexosamine pathway [112–115]. Here, fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions that utilize UDP-*N*-

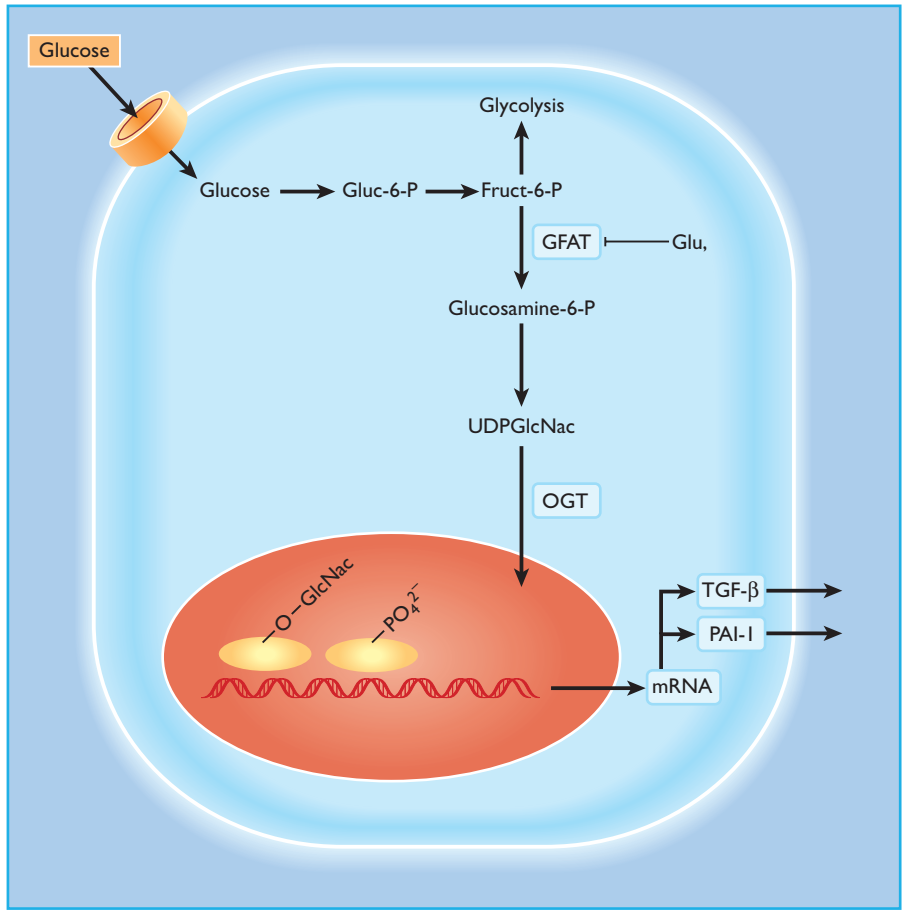

Figure 35.5 The glucosamine pathway. Glucosamine-6-phosphate is generated from fructose-6-phosphate and glutamine, by glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of this pathway. Glucosamine-6-phosphate is converted to UDP-*N*-acetylglucosamine (UDP-GlcNac), which can glycosylate transcription factors and thus enhance transcription of genes including plasminogen activator inhibitor 1 (PAI-1) and transforming growth factor β1 (TGF-β1). OGT, O-linked N-acetylglucosamine (GLcNac) transferase.

acetylglucosamine, particularly the formation of *O*-linked *N*-acetylglucosamine. This pathway has been shown to have an important role in hyperglycemia-induced and fat-induced insulin resistance [116–118]. The rate-limiting step in the conversion of glucose to glucosamine is regulated by glutamine:fructose-6-phosphate amidotransferase (GFAT), and inhibition of this enzyme blocks hyperglycemia-induced increases in the transcription of both TGF-α [112] and TGF-β1 [113].

It is not entirely clear how increased glucose 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, however, it has been shown that the transcription factor Sp1 regulates hyperglycemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells [119], raising the possibility that covalent glycation of Sp1 by *N*-acetylglucosamine to form its *O*-GlcNacylated derivative could explain how hexosamine pathway activation might operate. Virtually every RNA polymerase II transcription factor examined is *O*-GlcNacylated [120], and this glycosylated form of Sp1 appears to be more transcriptionally active than its non-glycosylated counterpart [121]. A fourfold increase in Sp1 *O*-GlcNacylation (caused by inhibition of the enzyme *O*-GlcNac-β-*N*-acetylglucosaminidase) resulted in a reciprocal 30% decrease in the level of serine/threonine phosphorylation of Sp1; thus, *O*-GlcNacylation and phosphorylation may compete to modify the same sites on Sp1 (Figure 35.5) [122].

GlcNac modification of Sp1 may regulate other glucose-responsive genes in addition to TGF-β1 and PAI-1. Glucose-responsive transcription of the acetylcoenzyme A carboxylase gene (the rate-limiting enzyme for fatty acid synthesis) is regulated by Sp1 sites, and post-transcriptional modification of Sp1 may similarly be responsible [123,124]. Because so many RNA polymerase II transcription factors are *O*-GlcNacylated [120], others in addition to Sp1 may be regulated by reciprocal modification (glycosylation vs phosphorylation) at key serine and threonine residues and so cause gene transcription to be glucose-responsive. In addition to transcription factors, many other nuclear and cytoplasmic proteins can be modified by *O*-GlcNac moities perturbing their normal function and regulation. One example relevant to diabetes is the inhibition of eNOS activity by *O*-GlcNacylation at the Akt site of eNOS protein [125–127]. Hyperglycemia also increases GFAT activity in aortic smooth muscle cells which increases *O*-GlcNac modification of several proteins in these cells [128]. Overall, activation of the hexosamine pathway by hyperglycemia may result in many changes in both gene expression and in protein function that

together contribute to the pathogenesis of diabetic complications. Recently, increased modification of key signaling molecules by *O*-GlcNAc was shown to cause reduced insulin signal transduction [129]. Pathway selective insulin resistance in vascular cells and resultant overactivation of the MAPK pathway by hyperinsulinemia could contribute further to diabetic microvascular damage [130].

A single process underlying 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 [99,131–134]. Moreover, all the above abnormalities are rapidly corrected when euglycemia is restored, which makes the phenomenon of hyperglycemic memory conceptually difficult to explain.

It has now been established that all of the different pathogenic mechanisms described above stem from a single hyperglycemia-induced process, overproduction of superoxide by the mitochondrial electron-transport chain [135,136]. 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. [137]. To understand how this occurs, mitochondrial glucose metabolism is briefly reviewed (Figure 35.6).

Intracellular glucose oxidation begins with glycolysis in the cytoplasm, which generates NADH and pyruvate. Cytoplasmic NADH can donate reducing equivalents to the mitochondrial electron-transport chain via two shuttle systems, or it can reduce pyruvate to lactate, which leaves the cell to act as a substrate for hepatic gluconeogenesis. Pyruvate can also be transported into the mitochondria where it is oxidized by the tricarboxylic acid (TCA) cycle to produce CO_2, H_2O, four molecules of NADH and one molecule of reduced flavine adenine dinucleotide ($FADH_2$). Mitochondrial NADH and $FADH_2$ provide energy for ATP production via oxidative phosphorylation by the electron transport chain. Electron flow through the mitochondrial electron transport chain is effected by four enzyme complexes, plus cytochrome *c* and the mobile carrier ubiquinone, all of which lie in the inner mitochondrial membrane [137]. NADH derived from both cytosolic glucose oxidation and mitochondrial TCA cycle activity donates electrons to NADH:ubiquinone oxidoreductase (Complex I) which ultimately transfers its electrons to ubiquinone. Ubiquinone can also be reduced by electrons donated from several $FADH_2$-containing dehydrogenases, including succinate:ubiquinone oxidoreductase (Complex II) and glycerol-3-phosphate dehydrogenase. Electrons from reduced ubiquinone are then transferred to ubiquinol:cytochrome *c* oxidoreductase (Complex III) by the Q cycle which generates ubisemiquinone radicals [138]. Electron transport then proceeds through cytochrome *c*, cytochrome *c* oxidase (Complex IV) and, finally, molecular oxygen.

Electron transfer through Complexes I, III and IV extrudes protons outwards into the intermembrane space, generating a proton gradient that drives ATP synthase (Complex V) as protons

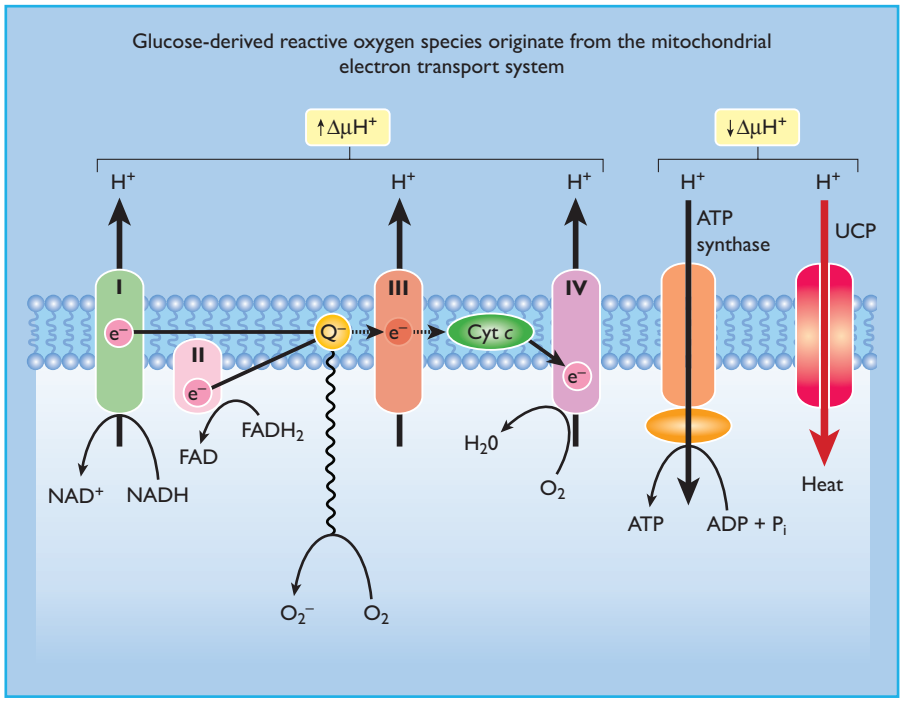

Figure 35.6 Mitochondrial metabolism. Flow of electrons (e^-) through the electron transport chain in the inner mitochondrial membrane pumps H^+ ions into the intermembrane space; superoxide is generated as a consequence of one electron leak. H^+ ions can pass back across the inner membrane along their concentration gradient, either via ATP synthase (to produce ATP) or via uncoupling proteins (UCP). When intracellular hyperglycemia increases electron flux by generating more NADH and $FADH_2$, more superoxide is produced. Cyt c, cytochrome *c*; Q, ubiquinone.

pass back through the inner membrane into the matrix. That is what happens in normal cells. In contrast, in diabetic cells with high glucose inside, there is more glucose being oxidized in the TCA cycle which in effect pushes more electron donors (NADH and $FADH_2$) into the electron transport chain. As a result of this, the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside Complex III is blocked [139], causing the electrons to back up to co-enzyme Q which donates the electrons one at a time to molecular oxygen and thereby generating superoxide. The mitochondrial isoform of the enzyme superoxide dismutase degrades this oxygen free radical to hydrogen peroxide which is then converted to H_2O and O_2 by other enzymes. Intracellular hyperglycemia did, indeed, increase the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation [140] and, subsequently, increase in production of ROS.

It has been also recently demonstrated that dynamic changes in mitochondrial morphology are associated with high glucose-induced overproduction of ROS and inhibition of mitochondrial fission prevented periodic fluctuation of ROS production during high glucose exposure [141]. Hyperglycemia does not increase ROS and does not activate 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 manganese superoxide dismutase (MnSOD) [142]. Overexpression of either MnSOD and UCP-1 also prevents inhibition of eNOS activity by hyperglycemia [125]. Importantly, inhibition of hyperglycemia-induced superoxide overproduction using a transgenic approach (superoxide dismutase [SOD]) also prevents long-term experimental diabetic nephropathy and retinopathy [143]. In humans, skin fibroblast gene expression profiles from two groups of patients with T1DM – 20 with very fast (fast track) versus 20 with very slow (slow track) rate of development of diabetic nephropathy lesions – showed that the fast-track group has increased expression of oxidative phosporylation genes, 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 [144].

Hyperglycemia-induced mitochondrial superoxide production activates the five damaging pathways by inhibiting GAPDH

Diabetes in animals and humans and hyperglycemia in cells decrease the activity of the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (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 [145]. When GAPDH activity is inhibited, the level of all the glycolytic intermediates that are upstream of GAPDH increase. An increased level of the upstream glycolytic metabolite glyceraldehyde-3-phosphate activates two major pathways. It activates the AGE pathway because the major intracellular AGE precursor methylglyoxal is formed non-enzymatically from glyceraldehyde-3 phosphate. Hyperglycemia-induced methylglyoxal formation has recently been shown to cause both increased expression of RAGE and its activating ligands S100 calgranulins and HMGB1 (M. Brownlee, unpublished).

Increased glyceraldehyde-3-phosphate also activates the classic PKC pathway, because the activator of PKC, DAG, is also formed from glyceraldehyde-3 phosphate. Further upstream, levels of the glycolytic metabolite fructose-6 phosphate increase, which 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 glyceraldehyde-3-phosphate), consuming NADPH in the process (Figure 35.7). Inhibition of GAPDH in 5 mmol/L activity using antisense DNA elevates the activity of each of the major pathways of hyperglycemic damage to the same extent as that induced by hyperglycemia [146].

Hyperglycemia-induced mitochondrial superoxide production inhibits GAPDH by activating poly(ADP-ribose) polymerase

Hyperglycemia-induced superoxide inhibits GAPDH activity *in vivo* by modifying the enzyme with polymers of ADP-ribose [146]. By inhibiting mitochondrial superoxide production with either UCP-1 or MnSOD, both modification of GAPDH by ADP-ribose and reduction of its activity by hyperglycemia are prevented. Most importantly, both modification of GAPDH by ADP-ribose and reduction of its activity by hyperglycemia are 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 [146]. Once activated, PARP splits the NAD^+ molecule into its two 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. In fact, it normally shuttles in and out of the nucleus, where it has a critical role in DNA repair (a summary of the integrated mechanism is shown in Figure 35.8) [147,148].

Glycemic memory

In 1993, the results of the landmark DCCT study showed that, in people with short-duration T1DM, intensive glycemic

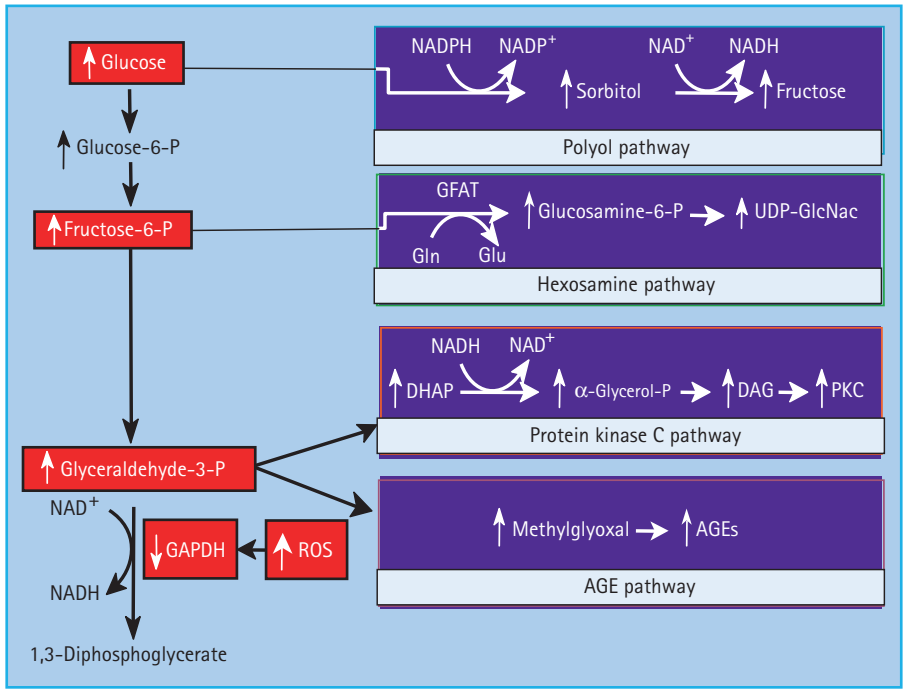

Figure 35.7 Mitochondrial overproduction of superoxide activates the major pathways of hyperglycemic damage by inhibiting glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

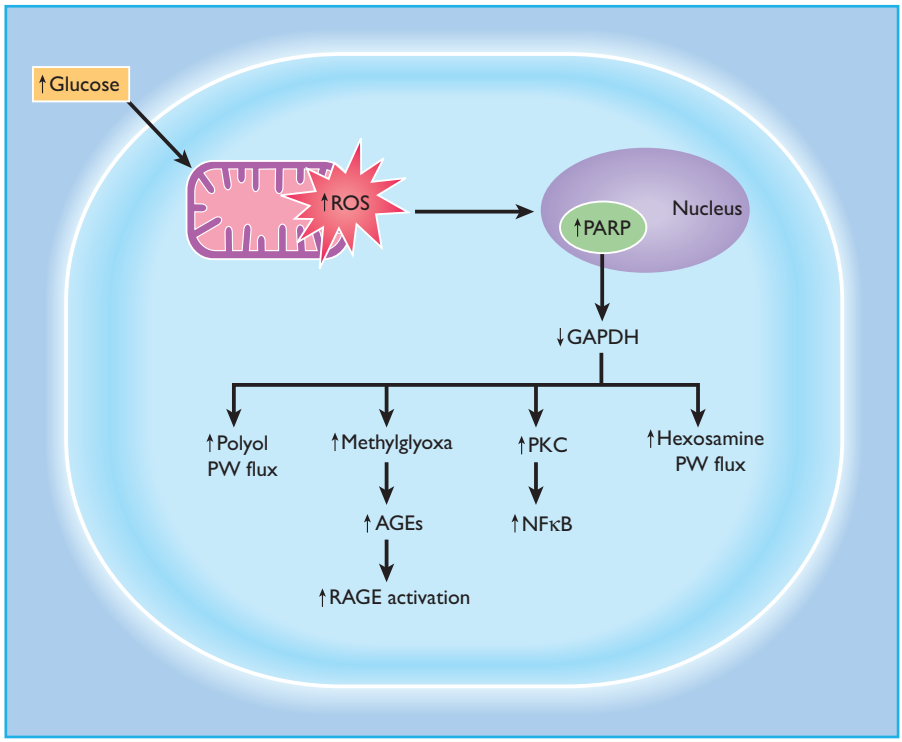

Figure 35.8 The unifying mechanism of hyperglycemia-induced cellular damage.

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 HbA_{1c}. At the same time, the mean level of HbA_{1c} worsened for patients who had been in the intensive therapy group. The post-DCCT HbA_{1c} values for both groups became statistically identical during the approximate 14 years of follow-up in the ongoing EDIC Study.

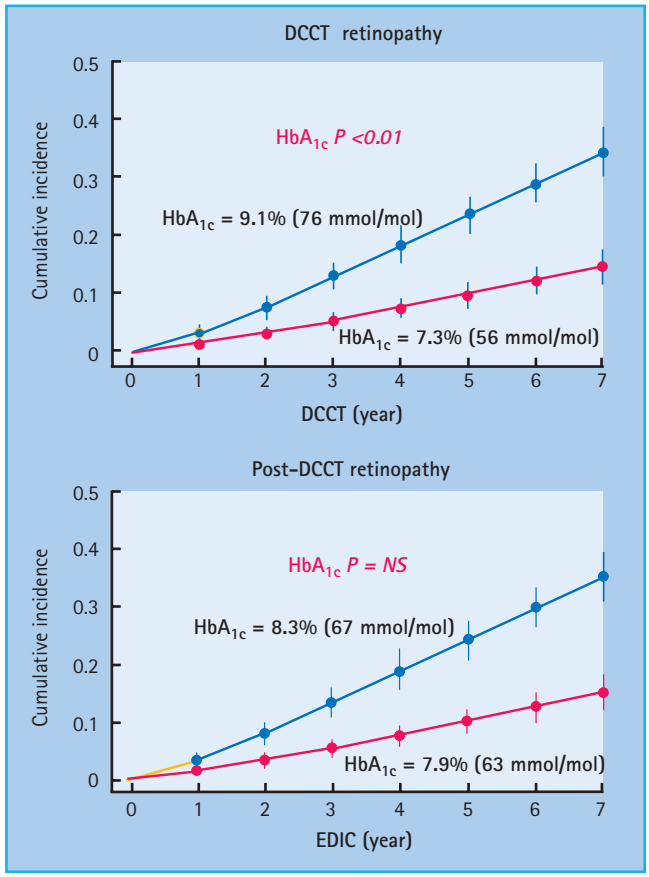

Figure 35.9 "Hyperglycemic memory": previous higher blood glucose levels make people with diabetes more susceptible to damage from subsequent lower blood glucose exposure. After the end of the Diabetes Control and Complications Trial (DCCT) study, the group that had been poorly controlled on conventional insulin therapy continued to have a higher incidence of diabetic retinopathy than the tightly controlled group given intensive therapy, even though post-trial HbA_{1c} levels were comparable in the two groups. EDIC, Epidemiology of Diabetes Interventions and Complications Study.

Surprisingly and provocatively, however, the effects of a 6.5-year difference in HbA_{1c} during the DCCT on the incidence of retinopathy and nephropathy have persisted and have even become greater over 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, while people in the intensive therapy group continue to have a lower incidence of complications, even with a deterioration in glycemic control during the EDIC years. This phenomenon has been given the name "glycemic memory" (Figure 35.9). More recent data indicate that glycemic memory also occurs in patients with T2DM. Indeed, the tight glucose control group from the UKPDS demonstrated a continued reduction in microvascular risk and emergent risk reductions for myocardial infarction and 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 post-trial follow-up among overweight patients [149–151]. 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.

Hyperglycemia-induced mitochondrial superoxide production may provide an explanation for the continuing progression of tissue damage after the correction of hyperglycemia ("hyperglycemic memory"). Post-translational modifications of histones cause chromatin remodeling and changes in levels of gene expression [152–154]. Because these modifications do not involve differences in DNA sequence, they are called "epigenetic" (Figure 35.10a). Transient hyperglycemia was recently shown to induce long-lasting activating epigenetic changes in the promoter of the NFκB-subunit p65 in human aortic endothelial cells (16 hours exposure) and in aortic cells *in vivo* in non-diabetic mice (6 hours exposure) which cause sustained increases in p65 gene expression (Figure 35.10b) and in the expression of p65-dependent pro-inflammatory genes. Both the epigenetic changes and the gene expression changes persist for at least 6 days of subsequent normal glycemia. Hyperglycemia-induced epigenetic changes and increased p65 expression are prevented by normalizing mitochondrial superoxide production or superoxide-induced methylglyoxal (Figure 35.10b,c) [155]. 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 HbA_{1c}-independent risk factor for diabetic complications.

Demethylation of another histone lysine residue, 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 [156]. Consistent with these observations, others have shown similar epigenetic changes in lymphocytes from patients with T1DM [157] and in vascular smooth muscle cells derived from *db/db* mice [158,159].

Determinants of individual susceptibility to hyperglycemia-induced damage

As with all complex diseases, the occurrence and progression of diabetic complications vary markedly among patients. Some patients have T1DM for over 50 years with minimal complications, while others manifest severe disease or death within 15 years after diagnosis. The control of blood glucose, as well as blood pressure and blood lipid profiles, are important factors in predicting the risk of complications, but they only partially explain the risk of complications for an individual patient. Therefore, genetic factors have been investigated for their influence on the risk of developing complications. An understanding of the genes involved in the susceptibility to or protection from

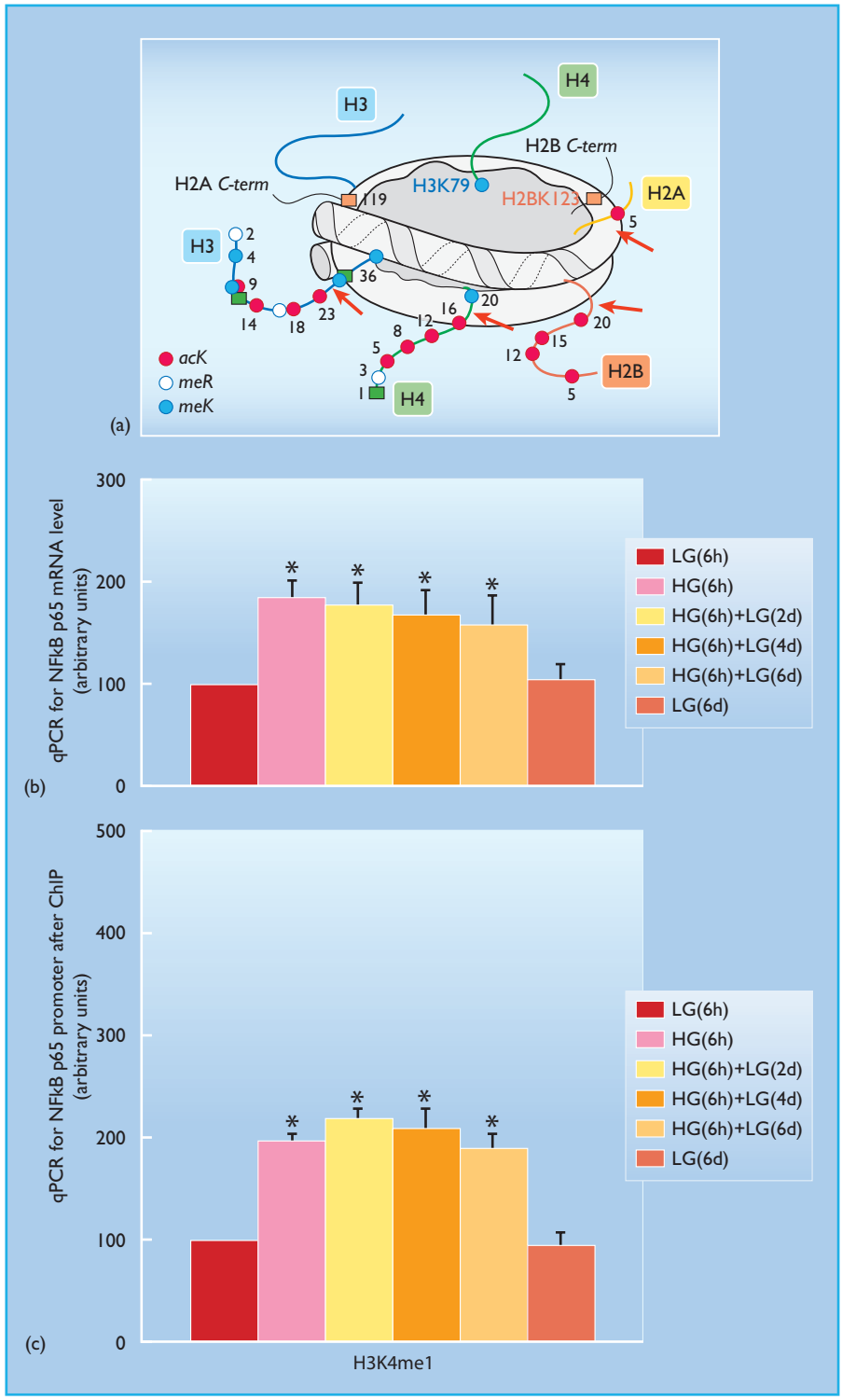

Figure 35.10 Transient hyperglycemia causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. (a) Schematic representation of several histone modifications (acK: acetylated lysine; meK, methylated lysine; meR: metylated arginine). (b and c) Transient hyperglycemia induces persistent increased expression of the NFκB-p65 subunit, caused by persistent epigenetic changes, including histone 3 lysine 4 monomethylationn (H3K4me1) in the proximal promoter of the NFκB-subunit p65. HG, high glucose; LG, low glucose. Data from El-Osta A, Brasacchio D, Yao D, Pocai A, Jones PL, Roeder RG, *et al*. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *J Exp Med* 2008; **205**:2409–2417.

diabetic complications can lead to both a better understanding of the pathophysiologic mechanisms, as well as new biomarkers and molecular targets for drug development. Familial clustering studies strongly support a role for genetic determinants of susceptibility to hyperglycemic damage.

In two studies of families that have two or more siblings with T1DM, if one sibling had advanced diabetic nephropathy, the other sibling with diabetes had a nephropathy risk of 83% and 72%, respectively. By contrast, the risk was only 17% or 22% if the index patient did not have diabetic nephropathy [160,161] or

retinopathy. Numerous associations have been made between various genetic polymorphisms and the risk of various diabetic complications. Those include the HLA-DQB10201/0302 alleles [162], polymorphisms of the aldose reductase gene [163], of the sorbitol dehydrogenase gene [164] and of the promoter of erythropoietin gene [165]. Study in European descent families of patients with T1DM showed a positive linkage and association with diabetic nephropathy of simple tandem repeat polymorphisms and single nucleotide polymorphisms in 20 genes. Five genes code for transcription factors and signaling molecules (*HNF1B1/TCF2*, *NRP1*, *PRKCB1*, *SMAD3* and *USF1*). Three genes code for components of the extracellular matrix (*COL4A1*, *LAMA4* and *LAMC1*), and two are involved in its degradation (*MMP9* and *TIMP3*). Three genes code for growth factors or growth factors receptors (*IGF-IR*, *TGFBR-2* and *TGFBR-3*). The others are genes likely to be important in kidney function (*AGRT1*, *AQP1*, *BCL2*, *CAT*, *GPX1*, *LPL* and *p22phox*) [166].

The DCCT/EDIC trial also reported familial clustering and association with gene polymorphisms. The odds ratio for risk of severe retinopathy in diabetic relatives of positive versus negative subjects from the conventional treatment group is 5.4; coronary artery calcification also showed familial clustering [167]. In the same cohort, an association of multiple superoxide dismutase 1 variants is associated with the development and progression of diabetic nephropathy [168].

In the future, the challenge will be to identify specific genes involved in the varying clinical severity of diabetic complications. Recent emphasis in human disease genetics has been on so-called modifying genes, i.e. genetic variants that are distinct from disease susceptibility genes and that modify the phenotypic and clinical expression of the disease genes. Studies show that genetic modifiers can be "tipping point" genes. This means that one gene changes the whole phenotype in an all-or-nothing fashion, in contrast with the incremental effects seen with changes in a large number of non-modifier genes. Many examples of modifier genes are known in model organisms, and several have been identified in humans [169,170].

Impaired neovascular response to ischemia

Tissue ischemia promotes vasculogenesis through chemokine-induced recruitment of bone marrow-derived endothelial progenitor cells (EPCs). Diabetes significantly impairs this process in tissues whose cells develop intracellular hyperglycemia. Many of the defects responsible for impaired vasculogenesis involve hypoxia-inducible factor 1 (HIF-1) regulated genes. Recently, HIF-1 function was shown to be impaired in diabetes because of ROS-induced modification of HIF-1α and its coactivator p300 by the glyoxalase 1 (GLO1) substrate methylglyoxal (Brownlee, unpublished data) [171]. Decreasing superoxide in diabetic mice by either transgenic expression of MnSOD or by administration of a small molecular weight SOD mimetic corrected post-ischemic defects in neovascularization, oxygen delivery and chemokine expression, and normalized tissue survival. In hypoxic fibroblasts cultured in high glucose, overexpression of GLO1 prevented reduced expression of both the EPC mobilizing chemokine stromal cell-derived factor 1 (SDF-1) and of VEGF which modulates growth and differentiation of recruited EPCs. In hypoxic EPCs cultured in high glucose, overexpression of GLO1 prevented reduced expression of both the SDF-1 receptor CXCR4, and endothelial nitric-oxide synthase, an enzyme essential for EPC mobilization. HIF-1α modification by methylglyoxal reduced heterodimer formation, and HIF-1α binding to all relevant promoters [171]. These results provide a basis for the rational design of new therapeutics to normalize impaired ischemia-induced vasculogenesis in patients with diabetes such as occurs in non-healing foot ulcers.

Hemodynamic factors

Hypertension is one of the most significant secondary risk factors for the development of microvascular vascular diabetic complications. In both retina and glomerulus, reduction of vascular surface area appears to occur first in microvessels with high perfusion pressure, and in patients with unilateral ophthalmic or renal artery stenosis there is a pronounced decrease in the severity of retinopathy or nephropathy on the affected side.

Tight control of blood pressure delays the progression of retinopathy and nephropathy, while elevated blood pressure accelerates the onset of nephropathy and its progression [172–174]. In the kidney, glomerular hypertension occurs with diabetes as a result of altered afferent and efferent arteriolar tone, increasing renal damage. This is one of the major targets of blockers of the angiotensin system (ARBs). How might hypertension connect to intracellular ROS generation, and activation of the five mechanisms of hyperglycemic damage? In hypertensive rats with glomerular hypertension, there is an 80% increase in glomerular GLUT-1 expression, associated with glomerulosclerosis and proteinuria. When mesangial cells, an important target for mechanically induced glomerular injury, were subjected to mechanical stretch, GLUT-1 protein expression was also upregulated, causing an increase in basal glucose transport. It has also been demonstrated that overexpression of GLUT-1 in mesangial cells grown in normal glucose concentration induces a diabetic cellular phenotype, with diabetic changes in gene expression [175,176]. Together, these data suggest that hypertension contributes to diabetic microvascular complications by further increasing intracellular hyperglycemia.

Potential mechanism-based therapeutic agents for diabetic complications

Transketolase activators

The first new class of potential therapeutic agents is transketolase activators. This concept originated from a feature of the unifying

mechanism. When increased superoxide inhibits GAPDH activity, the glycolytic intermediates above the enzyme accumulate and are then shunted into the four 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 another metabolic pathway, the pentose phosphate pathway [177]. Because in diabetes the concentration of these two glycolytic intermediates is high, activating transketolase could reduce their concentration by converting them to pentose phosphates. This would divert their flux away from three of the damaging pathways normally activated by hyperglycemia. This enzyme requires the vitamin thiamine as a co-factor. Although thiamine itself only activated transketolase by 25% in arterial endothelial cells, the thiamine derivative benfotiamine activated transketolase 250% in arterial endothelial cells.

Based on those findings, several groups have demonstrated the effectiveness of benfotiamine to counteract glucose-mediated toxicity in cultured endothelial cells, endothelial progenitor and endothelial cells and mouse models of diabetes [177–179]. Most importantly, it has been recently demonstrated that beneficial effects of benfotiamine on complication-causing pathways in rodent models of diabetic complications and in humans with T1DM [180]. Benfotiamine treatment prevented experimental diabetic retinopathy and nephropathy in mice, and treatment with high-dose thiamine reduced albuminuria in patients with T2DM. Diabetes has recently been implicated as a cause of intracellular thiamine depletion, which would impair normal transketolase conversion of hyperglycemia-induced elevation of glycolytic intermediates, further exacerbating activation of the five damaging pathways [181–183].

PARP inhibitors

The second new class of potential therapeutic agents based on the unified mechanism is PARP inhibitors. In cultured endothelial cells, a specific PARP inhibitor prevents hyperglycemia-induced activation of PKC, NFκB, intracellular AGE formation, and the hexosamine pathway [146]. In animal models of diabetes, PARP inhibition prevents podocyte apoptosis, ameliorates nephropathy and alleviates sensory neuropathy [184,185].

Catalytic antioxidants

Although increased superoxide activates the five damaging pathways implicated in the pathogenesis of microvascular complications, it is important to recognize that excess superoxide itself can also directly inhibit critical endothelial enzymes without any involvement of these four mechanisms. Two of these enzymes that are particularly important for vascular biology are eNOS and prostacyclin synthase. Both are dramatically inhibited in diabetic patients with diabetes and diabetic animals. To prevent direct oxidative inactivation of these key enzymes, it is necessary to directly reduce the amount of superoxide directly; however, conventional antioxidants are unlikely to do this effectively because conventional antioxidants neutralize reactive oxygen molecules on a one-for-one basis, while hyperglycemia-induced overproduction of superoxide is a continuous process. What is needed then is a new type of antioxidant, a catalytic antioxidant, such as an SOD/catalase mimetic [186], that works continuously. 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 anti-atherogenic enzyme. 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 five of the pathways implicated in hyperglycemic damage. 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 [143,187–192]. Together, these data strongly suggest that therapeutic correction of diabetes-induced superoxide overproduction may be a powerful approach for preventing diabetic microvascular complications.

References

1 Nathan D. Relationship between metabolic control and long term complications of diabetes. In: Kahn CR, Weir G, eds. *Joslin's Diabetes*. Philadelphia: Lea & Febiger, 1994: 620–30.

2 Skyler J. Diabetic complications: the importance of glucose control. *Endocrinol Metab Clin North Am* 1996; **25**:243–254.

3 Hammes HP, Federoff HJ, Brownlee M. Nerve growth factor prevents both neuroretinal programmed cell death and capillary pathology in experimental diabetes. *Mol Med* 1995; **1**:527–534.

4 Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 1996; **97**:2883–2890.

5 Giannini C, Dyck PJ. Ultrastructural morphometric features of human sural nerve endoneurial microvessels. *J Neuropathol Exp Neurol* 1993; **52**:361–369.

6 Giannini C, Dyck PJ. Basement membrane reduplication and pericyte degeneration precede development of diabetic polyneuropathy and are associated with its severity. *Ann Neurol* 1995; **37**:498–504.

7 Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 1996; **97**:2883–2890.

8 Hammes HP. Pericytes and the pathogenesis of diabetic retinopathy. *Horm Metab Res* 2005; **37**(Suppl 1):39–43.

9 Susztak K, Raff AC, Schiffer M, Böttinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocytedepletion at the onset of diabetic nephropathy. *Diabetes* 2006; **55**: 225–233.

10 Isermann B, Vinnikov IA, Madhusudhan T, Herzog S, Kashif M, Blautzik J, *et al*. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat Med* 2007; **13**:1349–1358.

11 Russell JW, Sullivan KA, Windebank AJ, Herrmann DN, Feldman EL. Neurons undergo apoptosis in animal and cell culture models of diabetes. *Neurobiol Dis* 1999; **6**:347–363.

12 Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**:977–986.

13 UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998; **352**:837–853.

14 Gaede P, Lund-Andersen H, Parving HH, Pedersen O. Effect of a multifactorial intervention on mortality in type 2 diabetes. *N Engl J Med* 2008; **358**:580–591.

15 Giardino I, Edelstein D, Brownlee M. BCL-2 expression or antioxidants prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells. *J Clin Invest* 1996; **97**:1422–1428.

16 Xia P, Kramer RM, King GL. Identification of the mechanism for the inhibition of Na/K-adenosine triphosphatase by hyperglycaemia involving activation of protein kinase C and cytosolic phospholipase A_2. *J Clin Invest* 1995; **96**:733.

17 Vikramadithyan RK, Hu Y, Noh HL, Liang CP, Hallam K, Tall AR, *et al.* Human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice. *J Clin Invest* 2005; **115**: 2434–2443.

18 Lee AY, Chung SS. Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J* 1999; **13**:23–30.

19 Chung SS, Ho EC, Lam KS, Chung SK. Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol* 2003; **14**(Suppl 3):S233–236. [Review]

20 Zhang Z, Apse K, Pang J, Stanton RC. High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. *J Biol Chem* 2000; **275**:40042–40047.

21 Bohren KM, Grimshaw CE, Gabbay KH. Catalytic effectiveness of human aldose reductase. Critical role of C-terminal domain. *J Biol Chem* 1992; **267**:20965–20970.

22 Zhang JZ, Gao L, Widness M, Xi X, Kern TS. Captopril inhibits glucose accumulation in retinal cells in diabetes. *Invest Ophthalmol Vis Sci* 2003; **44**:4001–4005.

23 Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci USA* 1991; **88**:11555–11558.

24 Stitt AW, Moore JE, Sharkey JA, Murphy G, Simpson DA, Bucala R, *et al.* Advanced glycation end products in vitreous: structural and functional implications for diabetic vitreopathy. *Invest Ophthalmol Vis Sci* 1998; **39**:2517–2523.

25 Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol* 1997; **150**:523–531.

26 Nishio T, Horii Y, Shiiki H, Yamamoto H, Makita Z, Bucala R, *et al.* Immunohistochemical detection of advanced glycosylation end products within the vascular lesions and glomeruli in diabetic nephropathy. *Hum Pathol* 1995; **26**:308–313.

27 Horie K, Miyata T, Maeda K, Miyata S, Sugiyama S, Sakai H, *et al.* Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions: implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. *J Clin Invest* 1997; **100**:2995–3004.

28 Niwa T, Katsuzaki T, Miyazaki S, Miyazaki T, Ishizaki Y, Hayase F, *et al.* Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. *J Clin Invest* 1997; **99**:1272–1280.

29 Degenhardt TP, Thorpe SR, Baynes JW. Chemical modification of proteins by methylglyoxal. *Cell Mol Biol* 1998; **44**:1139–1145.

30 Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* 1995; **34**:3702–3709.

31 Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J* 1990; **269**:1–11.

32 Ahmed N, Battah S, Karachalias N, Babaei-Jadidi R, Horanyi M, Baroti K, *et al.* Increased formation of methylglyoxal and protein glycation, oxidation and nitrosation in triosephosphate isomerase deficiency. *Biochim Biophys Acta* 2003; **1639**:121–132.

33 Takahashi M, Fujii J, Teshima T, Suzuki K, Shiba T, Taniguchi N. Identity of a major 3-deoxyglucosone-reducing enzyme with aldehyde reductase in rat liver established by amino acid sequencing and cDNA expression. *Gene* 1993; **127**:249–253.

34 Chang EY, Szallasi Z, Acs P, Raizada V, Wolfe PC, Fewtrell C, *et al.* Functional effects of overexpression of protein kinase C-alpha-beta-delta-epsilon, and -eta in the mast cell line RBL-2H3. *J Immunol* 1997; **159**:2624–2632.

35 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; **9**:653–660.

36 Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997; **277**:48–50.

37 Hammes HP, Lin J, Wagner P, Feng Y, Vom Hagen F, Krzizok T, *et al.* Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 2004; **53**:1104–1110.

38 Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, *et al.* Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 2002; **51**:3107–3112.

39 Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, *et al.* High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A. *J Biol Chem* 2007; **282**:31038–31045.

40 Elgawish A, Glomb M, Friedlander M, Monnier VM. Involvement of hydrogen peroxide in collagen cross-linking by high glucose *in vitro* and *in vivo*. *J Biol Chem* 1996; **271**:12964–12971.

41 Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 1986; **232**:1629–1632.

42 Tanaka S, Avigad G, Brodsky B, Eikenberry EF. Glycation induces expansion of the molecular packing of collagen. *J Mol Biol* 1988; **203**:495–505.

43 Tsilibary EC, Charonis AS, Reger LA, Wohlhueter RM, Furcht LT. The effect of non-enzymatic glycosylation on the binding of the main noncollagenous NC1 domain to type IV collagen. *J Biol Chem* 1988; **263**:4302–4308.

44 Charonis AS, Reger LA, Dege JE, Kouzi-Koliakos K, Furcht LT, Wohlhueter RM, *et al.* Laminin alterations after *in vitro* nonenzymatic glycosylation. *Diabetes* 1990; **39**:807–814.

45 Huijberts MS, Wolffenbuttel BH, Boudier HA, Crijns FR, Kruseman AC, Poitevin P, *et al.* Aminoguanidine treatment increases elasticity and decreases fluid filtration of large arteries from diabetic rats. *J Clin Invest* 1993; **92**:1407–1411.

46 Cochrane SM, Robinson GB. *In vitro* glycation of glomerular basement membrane alters its permeability: a possible mechanism in diabetic complications. *FEBS Lett* 1995; **375**:41–44.

47 Boyd-White J, Williams JC Jr. Effect of cross-linking on matrix permeability: a model for AGE-modified basement membranes. *Diabetes* 1996; **45**:348–353.

48 Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes* 2006; **55**:1961–1969.

49 Federoff HJ, Lawrence D, Brownlee M. Nonenzymatic glycosylation of laminin and the laminin peptide CIKVAVS inhibits neurite outgrowth. *Diabetes* 1993; **42**:509–513.

50 Hammes HP, Weiss A, Hess S, Araki N, Horiuchi S, Brownlee M, *et al.* Modification of vitronectin by advanced glycation alters functional properties *in vitro* and in the diabetic retina. *Lab Invest* 1996; **75**:325–338.

51 Bishara NB, Dunlop ME, Murphy TV, Darby IA, Sharmini Rajanayagam MA, Hill MA. Matrix protein glycation impairs agonist-induced intracellular Ca^{2+} signaling in endothelial cells. *J Cell Physiol* 2002; **193**:80–92.

52 Li YM, Mitsuhashi T, Wojciechowicz D, Shimizu N, Li J, Stitt A, *et al.* Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90–80K-H membrane proteins. *Proc Natl Acad Sci U S A* 1996; **93**:11047–11052.

53 Yang Z, Makita Z, Horii Y, Brunelle S, Cerami A, Sehajpal P, *et al.* Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose-modified proteins. *J Exp Med* 1991; **74**:515–524.

54 Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science* 1988; **240**:1546–1548.

55 Kirstein M, Aston C, Hintz R, Vlassara H. Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins. *J Clin Invest* 1992; **90**:439–446.

56 Yui S, Sasaki T, Horiuchi S, Yamazaki M. Induction of macrophage growth by advanced glycation end products of the Maillard reaction. *J Immunol* 1994; **152**:1943–1949.

57 Higashi T, Sano H, Saishoji T, Ikeda K, Jinnouchi Y, Kanzaki T, *et al.* The receptor for advanced glycation end products mediates the chemotaxis of rabbit smooth muscle cells. *Diabetes* 1997; **46**:463–472.

58 Westwood ME, Thornalley PJ. Induction of synthesis and secretion of interleukin 1 beta in the human monocytic THP-1 cells by human serum albumins modified with methylglyoxal and advanced glycation endproducts. *Immunol Lett* 1996; **50**:17–21.

59 Abordo EA, Westwood ME, Thornalley PJ. Synthesis and secretion of macrophage colony stimulating factor by mature human monocytes and human monocytic THP-1 cells induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts. *Immunol Lett* 1996; **53**:7–13.

60 Abordo EA, Thornalley PJ. Synthesis and secretion of tumor necrosis factor-alpha by human monocytic THP-1 cells and chemotaxis induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts. *Immunol Lett* 1997; **58**:139–147.

61 Webster L, Abordo EA, Thornalley PJ, Limb GA. Induction of TNF alpha and IL-1 beta mRNA in monocytes by methylglyoxal- and advanced glycated endproduct-modified human serum albumin. *Biochem Soc Trans* 1997; **25**:250S.

62 Pugliese G, Pricci F, Romeo G, Pugliese F, Mené P, Gianni S, *et al.* Upregulation of mesangial growth factor and extracellular matrix synthesis by advanced glycation end products via a receptor-mediated mechanism. *Diabetes* 1997; **46**:1881–1887.

63 Smedsrod B, Melkko J, Araki N, Sano H, Horiuchi S. Advanced glycation end products are eliminated by scavenger-receptor-mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells. *Biochem J* 1997; **322**:567–573.

64 Horiuchi S, Higashi T, Ikeda K, Saishoji T, Jinnouchi Y, Sano H, *et al.* Advanced glycation end products and their recognition by macrophage and macrophage-derived cells. *Diabetes* 1996; **45**:S73–76.

65 Sano H, Higashi T, Matsumoto K, Melkko J, Jinnouchi Y, Ikeda K, *et al.* Insulin enhances macrophage scavenger receptor-mediated endocytic uptake of advanced glycation end products. *J Biol Chem* 1998; **273**: 8630–8637.

66 Vlassara H, Li YM, Imani F, Wojciechowicz D, Yang Z, Liu FT, *et al.* Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor complex. *Mol Med* 1995; **1**:634–646.

67 Ohgami N, Nagai R, Ikemoto M, Arai H, Kuniyasu A, Horiuchi S, *et al.* Cd36, a member of the class b scavenger receptor family, as a receptor for advanced glycation end products. *J Biol Chem* 2001; **276**:3195–3202.

68 Skolnik EY, Yang Z, Makita Z, Radoff S, Kirstein M, Vlassara H. Human and rat mesangial cell receptors for glucose-modified proteins: potential role in kidney tissue remodelling and diabetic nephropathy. *J Exp Med* 1991; **174**:931–939.

69 Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ. Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. *Proc Natl Acad Sci U S A* 1992; **89**:2873–2877.

70 Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, *et al.* Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem* 1992; **267**:14987–14997.

71 Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, *et al.* Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992; **267**:14998–5004.

72 Schmidt AM, Mora R, Cao R, Yan SD, Brett J, Ramakrishnan R, *et al.* The endothelial cell binding site for advanced glycation end products consists of a complex: an integral membrane protein and a lactoferrin-like polypeptide. *J Biol Chem* 1994; **269**:9882–9888.

73 Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, *et al.* Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994; **269**:9889–9897.

74 Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor foe advanced glycation end products trig-

gers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 1997; **272**:17810–17814.

75 Li J, Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *J Biol Chem* 1997; **272**:16498–16506.

76 Yamagishi S, Fujimori H, Yonekura H, Yamamoto Y, Yamamoto H. Advanced glycation endproducts inhibit prostacyclin production and induce plasminogen activator inhibitor-1 in human microvascular endothelial cells. *Diabetologia* 1998; **41**:1435–1441.

77 Tsuji H, Iehara N, Masegi T, Imura M, Ohkawa J, Arai H, *et al.* Ribozyme targeting of receptor for advanced glycation end products in mouse mesangial cells. *Biochem Biophys Res Commun* 1998; **245**:583–588.

78 Bierhaus A, Haslbeck KM, Humpert PM, Liliensiek B, Dehmer T, Morcos M, *et al*. Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J Clin Invest* 2004; **114**:1741–1751.

79 Vlassara H, Fuh H, Donnelly T, Cybulsky M. Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol Med* 1995; **1**:447–456.

80 Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, *et al.* Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice: a potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 1995; **96**:1395–1403.

81 Schmidt AM, Crandall J, Hori O, Cao R, Lakatta E. Elevated plasma levels of vascular cell adhesion molecule-1 (VCAM-1) in diabetic patients with microalbuminuria: a marker of vascular dysfunction and progressive vascular disease. *Br J Haematol* 1996; **92**:747–750.

82 Sengoelge G, Födinger M, Skoupy S, Ferrara I, Zangerle C, Rogy M, *et al.* Endothelial cell adhesion molecule and PMNL response to inflammatory stimuli and AGE-modified fibronectin. *Kidney Int* 1998; **54**:1637–1651.

83 Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, *et al.* Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. *J Clin Invest* 1996; **97**:238–243.

84 Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I, *et al.* Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest* 1998; **101**:1219–1224.

85 Hirata C, Nakano K, Nakamura N, Kitagawa Y, Shigeta H, Hasegawa G, *et al.* Advanced glycation end products induce expression of vascular endothelial growth factor by retinal Muller cells. *Biochem Biophys Res Commun* 1997; **236**:712–715.

86 Soro-Paavonen A, Watson AM, Li J, Paavonen K, Koitka A, Calkin AC, *et al.* Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes. *Diabetes* 2008; **57**:2461–2469.

87 Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, *et al.* Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005; **83**:876–886.

88 Rong LL, Gooch C, Szabolcs M, Herold KC, Lalla E, Hays AP, *et al.* RAGE: a journey from the complications of diabetes to disorders of the nervous system – striking a fine balance between injury and repair. *Restor Neurol Neurosci* 2005; **23**:355–365.

89 Koya D, King GL. Protein kinase C activation and the development of diabetic complications. *Diabetes* 1998; **47**:859–866.

90 Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* 1992; **89**:11059–11063.

91 Craven PA, Davidson CM, DeRubertis FR. Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. *Diabetes* 1990; **39**:667–674.

92 Shiba T, Inoguchi T, Sportsman JR, Heath WF, Bursell S, King GL. Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol* 1993; **265**:E783–793.

93 Scivittaro V, Ganz MB, Weiss MF. AGEs induce oxidative stress and activate protein kinase C-beta (II) in neonatal mesangial cells. *Am J Physiol* 2000; **278**:F676–683.

94 Derubertis FR, Craven PA. Activation of protein kinase C in glomerular cells in diabetes: mechanism and potential links to the pathogenesis of diabetic glomerulopathy. *Diabetes* 1994; **43**:1–8.

95 Xia P, Inoguchi T, Kern TS, Engerman Rl, Oates PJ, King GL. Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 1994; **43**:1122–1129.

96 Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JI. High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 1991; **261**:F571–577.

97 Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL. Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 1997; **100**:115–126.

98 Kikkawa R, Haneda M, Uzu T, Koya D, Sugimoto T, Shigeta Y. Translocation of protein kinase C alpha and zeta in rat glomerular mesangial cells cultured under high glucose conditions. *Diabetologia* 1994; **37**:838–841.

99 Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, *et al.* Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* 1996; **272**:728–731.

100 Craven PA, Studer RK, DeRubertis FR. Impaired nitric oxide-dependent cyclic guanosine monophosphate generation in glomeruli from diabetic rats: evidence for protein kinase C-mediated suppression of the cholinergic response. *J Clin Invest* 1994; **93**:311–320.

101 Ganz MB, Seftel A. Glucose-induced changes in protein kinase C and nitric oxide are prevented by vitamin E. *Am J Physiol* 2000; **278**:E146–152.

102 Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, *et al.* Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and *in vivo*: a specific vascular action of insulin. *Circulation* 2000; **101**:676–681.

103 Glogowski EA, Tsiani E, Zhou X, Fantus IG, Whiteside C. High glucose alters the response of mesangial cell protein kinase C isoforms to endothelin-1. *Kidney Int* 1999; **55**:486–499.

104 Hempel A, Maasch C, Heintze U, Lindschau C, Dietz R, Luft FC, *et al.* High glucose concentrations increase endothelial cell permeability via activation of protein kinase C alpha. *Circ Res* 1997; **81**:363–371.

105 Williams B, Gallacher B, Patel H, Orme C. Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA

expression and peptide production by human vascular smooth muscle cells *in vitro*. *Diabetes* 1997; **46**:1497–1503.
106 Studer RK, Craven PA, DeRubertis FR. Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes* 1993; **42**:118–126.
107 Pugliese G, Pricci F, Pugliese F, Mene P, Lenti L, Andreani D, *et al.* Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. *Diabetes* 1994; **43**:478–490.
108 Craven PA, Studer RK, Felder J, Phillips S, DeRubertis FR. Nitric oxide inhibition of transforming growth factor-beta and collagen synthesis in mesangial cells. *Diabetes* 1997; **46**:671–681.
109 Feener EP, Xia P, Inoguchi T, Shiba T, Kunisaki M, King GL. Role of protein kinase C in glucose- and angiotensin II-induced plasminogen activator inhibitor expression. *Contrib Nephrol* 1996; **118**:180–187.
110 Pieper GM, Riaz-ul-Haq. Activation of nuclear factor-kappaB in cultured endothelial cells by increased glucose concentration: prevention by calphostin C. *J Cardiovasc Pharmacol* 1997; **30**:528–532.
111 Yerneni KK, Bai W, Khan BV, Medford RM, Natarajan R. Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes* 1999; **48**:855–864.
112 Sayeski PP, Kudlow JE. Glucose metabolism to glucosamine is necessary for glucose stimulation of transforming growth factor-alpha gene transcription. *J Biol Chem* 1996; **271**:15237–15243.
113 Kolm-Litty V, Sauer U, Nerlich A, Lehmann R, Schleicher ED. High glucose-induced transforming growth factor beta1 production is mediated by hexosamine pathway in porcine glomerular mesangial cells. *J Clin Invest* 1998; **101**:160–169.
114 Daniels MC, Kansal P, Smith TM, Paterson AJ, Kudlow JE, McClain DA. Glucose regulation of transforming growth factor-alpha expression is mediated by products of the hexosamine biosynthesis pathway. *Mol Endocrinol* 1993; **7**:1041–1048.
115 McClain DA, Paterson AJ, Roos MD, Wei X, Kudlow JE. Glucose and glucosamine regulate growth factor gene expression in vascular smooth muscle cells. *Proc Natl Acad Sci U S A* 1992; **89**:8150–8154.
116 Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system: role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem* 1991; **266**:4706–4712.
117 Rossetti L, Hawkins M, Chen W, Gindi J, Barzilai N. *In vivo* glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. *J Clin Invest* 1995; **96**:132–140.
118 Hawkins M, Barzilai N, Liu R, Hu M, Chen W, Rossetti L. Role of the glucosamine pathway in fat-induced insulin resistance. *J Clin Invest* 1997; **99**:2173–2182.
119 Chen YQ, Su M, Walia RR, Hao Q, Covington JW, Vaughan DE. Sp1 sites mediate activation of the plasminogen activator inhibitor-1 promoter by glucose in vascular smooth muscle cells. *J Biol Chem* 1998; **273**:8225–8231.
120 Hart GW. Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. *Annu Rev Biochem* 1997; **66**:315–335.
121 Kadonaga JT, Courey AJ, Ladika J, Tjian R. Distinct regions of Sp1 modulate DNA binding and transcriptional activation. *Science* 1988; **242**:1566–1570.
122 Haltiwanger RS, Grove K, Philipsberg GA. Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-beta-N-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate. *J Biol Chem* 1998; **273**:3611–3617.
123 Daniel S, Zhang S, DePaoli-Roach AA, Kim KH. Dephosphorylation of Sp1 by protein phosphatase 1 is involved in the glucose-mediated activation of the acetyl-CoA carboxylase gene. *J Biol Chem* 1996; **271**:14692–14697.
124 Daniel S, Kim KH. Sp1 mediates glucose activation of the acetyl-CoA carboxylase promoter. *J Biol Chem* 1996; **271**:1385–1392.
125 Yamagishi SI, Edelstein D, Du XL, Brownlee M. Hyperglycemia potentiates collagen-induced platelet activation through mitochondrial superoxide overproduction. *Diabetes* 2001; **50**:1491–1494.
126 Hart GW. Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. *Annu Rev Biochem* 1997; **66**:315–335.
127 Musicki B, Kramer MF, Becker RE, Burnett AL. Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by O-GlcNAc in diabetes-associated erectile dysfunction. *Proc Natl Acad Sci U S A* 2005; **102**:11870–11875.
128 Akimoto Y, Kreppel LK, Hirano H, Hart GW. Hyperglycemia and the O-GlcNAc transferase in rat aortic smooth muscle cells: elevated expression and alteredpatterns of O-GlcNAcylation. *Arch Biochem Biophys* 2001; **389**:166–175.
129 Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, *et al.* Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance *Nature* 2008; **451**:964–969.
130 Jiang ZY, He Z, King B, Kuroki T, Opland DM, Suzuma I, *et al.* Characterization of multiple signaling pathways of insulin in the regulation of vascular endothelial growth factor expression in vascular cells and angiogenesis. *J Biol Chem* 2003; **278**:31964–31971.
131 Engerman RL, Kern TS, Larson ME. Nerve conduction and aldose reductase inhibition during 5 years of diabetes or galactosaemia in dogs. *Diabetologia* 1994; **37**:141–144.
132 Sima AA, Prashar A, Zhang WX, Chakrabarti S, Greene DA. Preventive effect of long-term aldose reductase inhibition (ponalrestat) on nerve conduction and sural nerve structure in the spontaneously diabetic Bio-Breeding rat. *J Clin Invest* 1990; **85**:1410–1420.
133 Lee AY, Chung SK, Chung SS. Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens. *Proc Natl Acad Sci U S A* 1995; **92**:2780–2784.
134 Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* 1995; **46**:223–234.
135 Nishikawa T, Du Edelstein DXL, Yamagishi S, *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; **404**:787–790.
136 Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, *et al.* Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 2000; **97**:12222–12226.
137 Wallace DC. Disease of the mitochondrial DNA. *Annu Rev Biochem* 1992; **61**:1175–212.
138 Trumpower BL. The protonmotive Q cycle: energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. *J Biol Chem* 1990; **265**:11409–11412.
139 Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 1997; **416**:15–18.

140 Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest* 2001; **108**:1341–1348.

141 Yu T, Robotham JL, Yoon Y. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc Natl Acad Sci U S A* 2006; **103**:2653–2658.

142 Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; **404**:787–790.

143 DeRubertis FR, Craven PA, Melhem MF, Salah EM. Attenuation of renal injury in *db/db* mice overexpressing superoxide dismutase: evidence for reduced superoxide–nitric oxide interaction. *Diabetes* 2004; **53**:762–768.

144 Huang C, Kim Y, Caramori ML, Moore JH, Rich SS, Mychaleckyj JC, *et al.* Diabetic nephropathy is associated with gene expression levels of oxidative phosphorylation and related pathways. *Diabetes* 2006; **55**:1826–1831.

145 Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, *et al.* Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 2000; **97**:12222–12226.

146 Du X, Matsumura T, Edelstein D, Rossetti L, Zsengellér Z, Szabó C, *et al.* Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 2003; **112**:1049–1057.

147 Sawa A, Khan AA, Hester LD, Snyder SH. Glyceraldehyde-3-phosphate dehydrogenase: nuclear translocation participates in neuronal and nonneuronal cell death. *Proc Natl Acad Sci U S A* 1997; **94**:11669–11674.

148 Schmidtz HD: Reversible nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase upon serum depletion. *Eur J Cell Biol* 2001; **80**:419–427.

149 Diabetes Control and Complications Trial (DCCT) Research Group. Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. *Kidney Int* 1995; **47**:1703–1720.

150 Diabetes Control and Complications Trial Research Group. The effect of intensive diabetes therapy on the development and progression of neuropathy. *Ann Intern Med* 1995; **122**:561–568.

151 Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. *N Engl J Med* 2000; **342**:381–389.

152 Vaissière T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutat Res* 2008; **659**:40–48.

153 Probst AV, Dunleavy E, Almousni G. Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* 2009; **10**:192–206.

154 Göndör A, Ohlsson R. Replication timing and epigenetic reprogramming of gene expression: a two-way relationship? *Nat Rev Genet* 2009; **10**:269–276.

155 El-Osta A, Brasacchio D, Yao D, Pocai A, Jones PL, Roeder RG, *et al.* Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *J Exp Med* 2008; **205**:2409–2417.

156 Brasacchio D, Okabe J, Tikellis C, Balcerczyk A, George P, Baker EK, *et al.* Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that co-exist on the lysine tail. *Diabetes* 2009; **58**:1229–1236.

157 Miao F, Smith DD, Zhang L, Min A, Feng W, Natarajan R. Lymphocytes from patients with type 1 diabetes display a distinct profile of chromatin histone H3 lysine 9 dimethylation: an epigenetic study in diabetes. *Diabetes* 2008; **57**:3189–3198.

158 Reddy MA, Villeneuve LM, Wang M, Lanting L, Natarajan R. Role of the lysine-specific demethylase 1 in the proinflammatory phenotype of vascular smooth muscle cells of diabetic mice. *Circ Res* 2008; **103**:615–623.

159 Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. *Proc Natl Acad Sci U S A* 2008; **105**:9047–9052.

160 Seaquist ER, Goetz FC, Rich S, Barbosa J. Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 1989; **320**:1161–1165.

161 Quinn M, Angelico MC, Warram JH, Krolewski AS. Familial factors determine the development of diabetic nephropathy in patients with IDDM. *Diabetologia* 1996; **39**:940–945.

162 Agardh D, Gaur LK, Agardh E, Landin-Olsson M, Agardh CD, Lernmark A. HLA-DQB1*0201/0302 is associated with severe retinopathy in patients with IDDM. *Diabetologia* 1996; **39**:1313–1317.

163 Oates PJ, Mylari BL. Aldose reductase inhibitors: therapeutic implications for diabetic complications. *Expert Opin Investig Drugs* 1999; **8**:2095–2119.

164 Szaflik JP, Majsterek I, Kowalski M, Rusin P, Sobczuk A, Borucka AI, *et al.* Association between sorbitol dehydrogenase gene polymorphisms and type 2 diabetic retinopathy. *Exp Eye Res* 2008; **86**:647–652.

165 Tong Z, Yang Z, Patel S, Chen H, Gibbs D, Yang X, *et al.* Promoter polymorphism of the erythropoietin gene in severe diabetic eye and kidney complications. *Proc Natl Acad Sci U S A* 2008; **105**:6998–7003.

166 Ewens KG, George RA, Sharma K, Ziyadeh FN, Spielman RS. Assessment of 115 candidate genes for diabetic nephropathy by transmission/disequilibrium test. *Diabetes* 2005; **54**:3305–3318.

167 Diabetes Control and Complications Trial Research Group. Clustering of long-term complications in families with diabetes in the diabetes control and complications trial. *Diabetes* 1997; **46**:1829–1839.

168 Al-Kateb H, Boright AP, Mirea L, Xie X, Sutradhar R, Mowjoodi A, *et al.* Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Multiple superoxide dismutase 1/splicing factor serine alanine 15 variants are associated with the development and progression of diabetic nephropathy: the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Genetics study. *Diabetes* 2008; **57**:218–228.

169 Drumm ML, Konstan MW, Schluchter MD, Handler A, pace R, Zou F, *et al.* Genetic modifiers of lung disease in cystic fibrosis. *N Engl J Med* 2005; **353**:1443–1453.

170 Bremer LA, Blackman SM, Vanscoy LL, *et al.* Interaction between a novel TGFB1 haplotype and CFTR genotype is associated with improved lung function in cystic fibrosis. *Hum Mol Genet* 2008; **17**:2228–2237.

171 Ceradini DJ, Yao D, Grogan RH, Callaghan MJ, Edelstein D, Brownlee M, *et al*. Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice. *J Biol Chem* 2008; **283**:10930–10938.

172 McGill JB. Improving microvascular outcomes in patients with diabetes through management of hypertension. *Postgrad Med* 2009; **121**:89–101.

173 Zannad F. Implications of the ADVANCE study for clinical practice. *J Hypertens* 2008; **26**(Suppl 3):S31–34.

174 Earle KA, Morocutti A, Viberti G. Permissive role of hypertension in the development of proteinuria and progression of renal disease in insulin-dependent diabetic patients. *J Hypertens* 1997; **1**:191–196.

175 Gnudi L, Thomas SM, Viberti G. Mechanical forces in diabetic kidney disease: a trigger for impaired glucose metabolism. *J Am Soc Nephrol* 2007; **18**:2226–2232.

176 Gnudi L, Viberti G, Raij L, Rodriguez V, Burt D, Cortes P, *et al*. GLUT-1 overexpression: Link between hemodynamic and metabolic factors in glomerular injury? *Hypertension* 2003; **42**:19–24.

177 Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, *et al*. Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 2003; **9**:294–299.

178 Berrone E, Beltramo E, Solimine C, Ape AU, Porta M. Regulation of intracellular glucose and polyol pathway by thiamine and benfotiamine in vascular cells cultured in high glucose. *J Biol Chem* 2006; **281**:9307–9313.

179 Marchetti V, Menghini R, Rizza S, Vivanti A, Feccia T, Lauro D, *et al*. Benfotiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/FoxO signaling. *Diabetes* 2006; **55**:2231–2237.

180 Du X, Edelstein D, Brownlee M. Oral benfotiamine plus alpha-lipoic acid normalises complication-causing pathways in type 1 diabetes. *Diabetologia* 2008; **51**:1930–1932.

181 Rabbani N, Alam SS, Riaz S, Larkin JR, Akhtar MW, Shafi T, *et al*. High-dose thiamine therapy for patients with type 2 diabetes and-microalbuminuria: a randomised, double-blind placebo-controlled pilot study. *Diabetologia* 2009; **52**:208–212.

182 Thornalley PJ. The potential role of thiamine (vitamin B1) in diabetic complications. *Curr Diabetes Rev* 2005; **1**:287–298.

183 Thornalley PJ, Babaei-Jadidi R, Al Ali H, Rabbani N, Antonysunil A, Larkin J, *et al*. High prevalence of low plasma thiamine concentration in diabetes linked to a marker of vascular disease. *Diabetologia* 2007; **50**:2164–2170.

184 Szabó C, Biser A, Benko R, Böttinger E, Suszták K. Poly(ADP-ribose) polymerase inhibitors ameliorate nephropathy of type 2 diabetic Leprdb/db mice. *Diabetes* 2006; **55**:3004–3012.

185 Ilnytska O, Lyzogubov VV, Stevens MJ, Drel VR, Mashtalir N, Pacher P, *et al*. Poly(ADP-ribose) polymerase inhibition alleviates experimental diabetic sensory neuropathy. *Diabetes* 2006; **55**:1686–1694.

186 Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Macarthur H, Misko TP, *et al*. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science* 1999; **286**:304–306.

187 Vincent AM, Russell JW, Sullivan KA, Backus C, Hayes JM, McLean LL, *et al*. SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. *Exp Neurol* 2007; **208**:216–227.

188 Otero P, Bonet B, Herrera E, Rabano A. Development of atherosclerosis in the diabetic BALB/c mice. Prevention with Vitamin E administration. *Atherosclerosis* 2005; **182**:259–265.

189 Shen X, Zheng S, Metreveli NS, Epstein PN. Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. *Diabetes* 2006; **55**:798–805.

190 Zhang Y, Wada J, Hashimoto I, Eguchi J, Yasuhara A, Kanwar YS, *et al*. Therapeutic approach for diabetic nephropathy using gene delivery of translocase of inner mitochondrial membrane 44 by reducing mitochondrial superoxide production. *J Am Soc Nephrol* 2006; **17**:1090–1101.

191 Kowluru RA, Kowluru V, Xiong Y, Ho YS. Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress. *Free Radic Biol Med* 2006; **41**:1191–1196.

192 DeRubertis FR, Craven PA, Melhem MF. Acceleration of diabetic renal injury in the superoxide dismutase knockout mouse: effects of tempol. *Metabolism* 2007; **56**:1256–1264.