Biochemistry and molecular cell biology of diabetic complications

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Diabetes-specific microvascular disease is a leading cause of blindness, renal failure and nerve damage, and diabetes-accelerated atherosclerosis leads to increased risk of myocardial infarction, stroke and limb amputation. Four main molecular mechanisms have been implicated in glucose-mediated vascular damage. All seem to reflect a single hyperglycaemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain. This integrating paradigm provides a new conceptual framework for future research and drug discovery.

All forms of diabetes are characterized by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve. As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end-stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke and limb amputation. Large prospective clinical studies show a strong relationship between glycaemia and diabetic microvascular complications in both type 1 and type 2 diabetes[1,2]. Hyperglycaemia and insulin resistance both seem to have important roles in the pathogenesis of macrovascular complications[2–5].

Diabetes-specific microvascular disease in the retina, glomerulus and vasa nervorum has similar pathophysiological features. Early in the course of diabetes, intracellular hyperglycaemia causes abnormalities in blood flow and increased vascular permeability. This reflects decreased activity of vasodilators such as nitric oxide, increased activity of vasoconstrictors such as angiotensin II and endothelin-1, and elaboration of permeability factors such as vascular endothelial growth factor (VEGF). Quantitative and qualitative abnormalities of extracellular matrix contribute to an irreversible increase in vascular permeability. With time, microvascular cell loss occurs, in part as a result of programmed cell death, and there is progressive capillary occlusion due both to extracellular matrix overproduction induced by growth factors such as transforming growth factor-β (TGF-β), and to deposition of extravasated periodic acid–Schiff-positive plasma proteins. Hyperglycaemia may also decrease production of trophic factors for endothelial and neuronal cells. Together, these changes lead to oedema, ischaemia and hypoxia-induced neovascularization in the retina, proteinuria, mesangial matrix expansion and glomerulosclerosis in the kidney, and multifocal axonal degeneration in peripheral nerves.

The pathogenesis of atherosclerosis in non-diabetics has been extensively described in recent reviews, and begins with endothelial dysfunction[6]. In diabetic arteries, endothelial dysfunction seems to involve both insulin resistance specific to the phosphatidylinositol-3-OH kinase pathway[7,8] and hyperglycaemia. Pathway-selective insulin resistance results in decreased endothelial production of the anti-atherogenic molecule nitric oxide, and increased potentiation of proliferation of vascular smooth muscle cells and production of plasminogen activator inhibitor-1 (PAI-1) via the Ras → Raf → MEK kinase → mitogen-activated protein (MAP) kinase pathway[7]. Hyperglycaemia itself also inhibits production of nitric oxide in arterial endothelial cells[9] and stimulates production of PAI-1 (ref. 10).

Both insulin resistance and hyperglycaemia have also been implicated in the pathogenesis of diabetic dyslipidaemia. The role of insulin resistance has been reviewed recently[5]. Hyperglycaemia seems to cause raised levels of atherogenic cholesterol-enriched apolipoprotein B-containing remnant particles by reducing expression of the heparan sulphate proteoglycan perlecan on hepatocytes[4]. Associations of atherosclerosis and atherosclerosis risk factors with glycaemia have been shown over a broad range of glucose tolerance, from normal to diabetic. Postprandial hyperglycaemia may be more predictive of atherosclerosis than is fasting plasma glucose level or haemoglobin A1c[11]. Whether postprandial hyperglycaemia is an independent risk factor is controversial and requires further study.

Mechanisms of hyperglycaemia-induced damage

How do these diverse microvascular and macrovascular pathologies all result from hyperglycaemia? Four main hypotheses about how hyperglycaemia causes diabetic complications have generated a large amount of data, as well as several clinical trials based on specific inhibitors of these mechanisms. The four hypotheses are: increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. Until recently there was no unifying hypothesis linking these four mechanisms.

Increased polyol pathway flux

Aldose reductase (alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21) is the first enzyme in the polyol pathway. It is a cytosolic, monomeric oxidoreductase that catalyses the NADPH-dependent reduction of a wide variety of carbonyl compounds, including glucose. Its crystal structure has a single domain folded into an eight-stranded parallel α/β-barrel motif, with the substrate-binding site located in a cleft at the carboxy-terminal end of the β-barrel[12]. Aldose

reductase has a low affinity (high K_m) for glucose, and at the normal glucose concentrations found in non-diabetics, metabolism of glucose by this pathway is a very small percentage of total glucose use. But in a hyperglycaemic environment, increased intracellular glucose results in its increased enzymatic conversion to the polyalcohol sorbitol, with concomitant decreases in NADPH. In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase, with NAD^+ reduced to NADH. Flux through this pathway during hyperglycaemia varies from 33% of total glucose use in the rabbit lens to 11% in human erythrocytes. Thus, the contribution of this pathway to diabetic complications may be very much species, site and tissue dependent (Fig. 1).

A number of mechanisms have been proposed to explain the potential detrimental effects of hyperglycaemia-induced increases in polyol pathway flux. These include sorbitol-induced osmotic stress, decreased $(Na^+ + K^+)$ATPase activity, an increase in cytosolic $NADH/NAD^+$ and a decrease in cytosolic NADPH. Sorbitol does not diffuse easily across cell membranes, and it was originally suggested that this resulted in osmotic damage to microvascular cells. Sorbitol concentrations measured in diabetic vessels and nerves are, however, far too low to cause osmotic damage.

Another early suggestion was that increased flux through the polyol pathway decreased $(Na^+ + K^+)$ATPase activity. Although this decrease was originally thought to be mediated by polyol pathway-linked decreases in phosphatidylinositol synthesis, it has recently been shown to result from activation of PKC (see below). Hyperglycaemia-induced activation of PKC increases cytosolic phospholipase A_2 activity, which increases the production of two inhibitors of $(Na^+ + K^+)$ATPase — arachidonate and PGE_2 (ref. 13).

More recently, it has been proposed that oxidation of sorbitol by NAD^+ increases the cytosolic $NADH:NAD^+$ ratio, thereby inhibiting activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and increasing concentrations of triose phosphate[14]. Raised triose phosphate concentrations could increase formation of both methylglyoxal, a precursor of AGEs, and diacylglycerol (DAG) (through α-glycerol-3-phosphate), thus activating PKC (as discussed later). Although hyperglycaemia does increase the $NADH:NAD^+$ ratio in endothelial cells, this reflects a marked decrease in the absolute concentration of NAD^+ as a result of consumption by activated poly(ADP-ribose) polymerase (PARP), rather than reduction of NAD^+ to NADH[15]. Activation of PARP by hyperglycaemia is mediated by increased production of reactive oxygen species (T. Matsumura *et al.*, unpublished results). The source of hyperglycaemia-induced reactive oxygen species is discussed later.

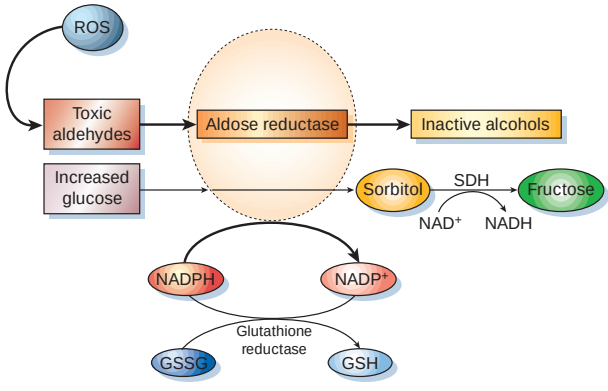

Figure 1 Aldose reductase and the polyol pathway. Aldose reductase reduces aldehydes generated by reactive oxygen species (ROS) to inactive alcohols, and glucose to sorbitol, using NADPH as a co-factor. In cells where aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented. Sorbitol dehydrogenase (SDH) oxidizes sorbitol to fructose using NAD^+ as a co-factor.

It has also been proposed that reduction of glucose to sorbitol by NADPH consumes NADPH. As NADPH is required for regenerating reduced glutathione (GSH), this could induce or exacerbate intracellular oxidative stress (Fig. 1). Decreased levels of GSH have in fact been found in the lenses of transgenic mice that overexpress aldose reductase, and this is the most likely mechanism by which increased flux through the polyol pathway has deleterious consequences[16]. This conclusion is further supported by recent experiments with homozygous knockout mice deficient in aldose reductase, which showed that, in contrast to wild-type mice, diabetes neither decreased the GSH content of sciatic nerve nor reduced motor nerve conduction velocity (S. K. Chung, personal communication).

Studies of inhibition of the polyol pathway *in vivo* have yielded inconsistent results. In a five-year study in dogs, aldose reductase inhibition prevented diabetic neuropathy, but failed to prevent retinopathy or thickening of the capillary basement membrane in the retina, kidney and muscle[17]. Several negative clinical trials have questioned the relevance of this mechanism in humans[18]. The positive effect of aldose reductase inhibition on diabetic neuropathy has, however, been confirmed in humans in a rigorous multi-dose, placebo-controlled trial with the potent aldose reductase inhibitor zenarestat[19].

Increased intracellular formation of advanced glycation end-products

AGEs are found in increased amounts in diabetic retinal vessels[20] and renal glomeruli[21]. They were originally thought to arise from non-enzymatic reactions between extracellular proteins and glucose. But the rate of AGE formation from glucose is orders of magnitude slower than the rate of AGE formation from glucose-derived dicarbonyl precursors generated intracellularly, and it now seems likely that intracellular hyperglycaemia is the primary initiating event in the formation of both intracellular and extracellular AGEs[22]. AGEs can arise from intracellular auto-oxidation of glucose to glyoxal[23], decomposition of the Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone (perhaps accelerated by an amadoriase), and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal[24]. These reactive intracellular dicarbonyls — glyoxal, methylglyoxal and 3-deoxyglucosone — react with amino groups of intracellular and extracellular proteins to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system[24]. All three AGE precursors are also substrates for other reductases[25].

The potential importance of AGEs in the pathogenesis of diabetic complications is indicated by the observation in animal models that two structurally unrelated AGE inhibitors partially prevented various functional and structural manifestations of diabetic microvascular disease in retina, kidney and nerve[26–28]. In a large randomized, double-blind, placebo-controlled, multi-centre trial in type 1 diabetic patients with overt nephropathy, the AGE inhibitor aminoguanidine lowered total urinary protein and slowed progression of nephropathy, over and above the effects of existing optimal care. In addition, aminoguanidine reduced the progression of diabetic retinopathy (K. K. Bolton *et al.*, submitted).

Production of intracellular AGE precursors damages target cells by three general mechanisms (Fig. 2). First, intracellular proteins modified by AGEs have altered function. Second, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins (integrins) on cells. Third, plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species. This AGE receptor ligation activates the pleiotropic transcription factor NF-κB, causing pathological changes in gene expression.

In endothelial cells exposed to high glucose, intracellular AGE formation occurs within a week. Basic fibroblast growth factor is one

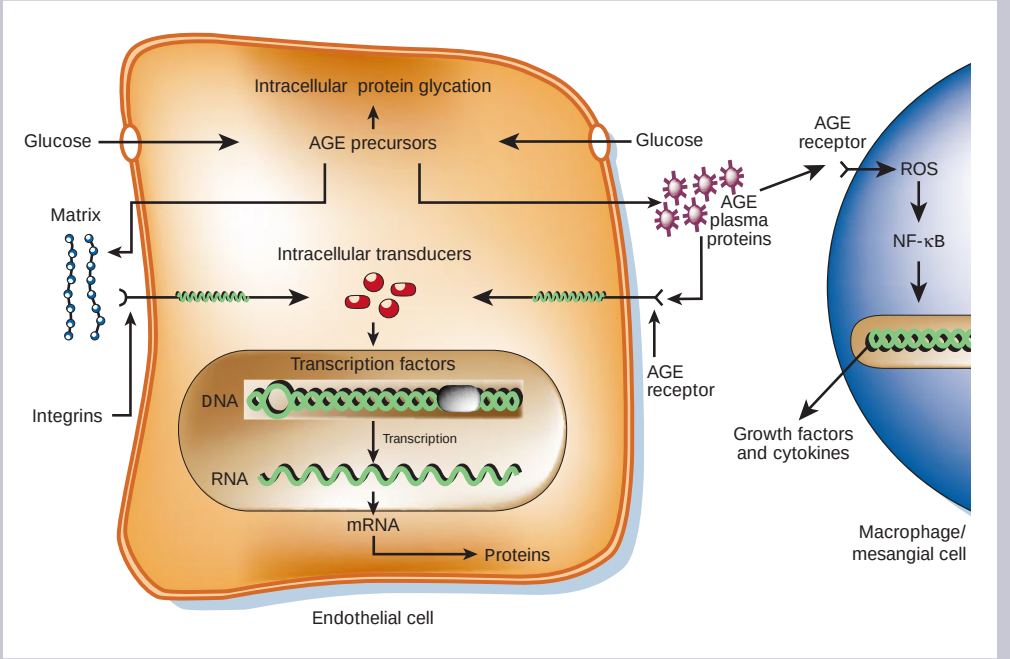

Figure 2 Mechanisms by which intracellular production of advanced glycation end-product (AGE) precursors damages vascular cells. Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages.

of the main AGE-modified proteins in endothelial cells[29]. Proteins involved in macromolecular endocytosis are also modified by AGEs, as the increase in endocytosis induced by hyperglycaemia is prevented by overexpression of the methylglyoxal-detoxifying enzyme glyoxalase I (ref. 30). Overexpression of glyoxalase I also completely prevents the hyperglycaemia-induced increase in expression of angiopoietin-2 in Muller cells (T. Matsumura *et al.*, unpublished results), a factor that has been implicated in both pericyte loss and capillary regression[31].

AGE formation alters the functional properties of several important matrix molecules. On type I collagen, intermolecular crosslinking by AGEs induces an expansion of the molecular packing[32]. These AGE-induced crosslinks alter the function of intact vessels. For example, AGEs decrease elasticity in large vessels from diabetic rats, even after vascular tone is abolished, and increase fluid filtration across the carotid artery[33]. AGE formation on type IV collagen from basement membrane inhibits lateral association of these molecules into a normal network-like structure by interfering with binding of the non-collagenous NC1 domain to the helix-rich domain[34]. AGE formation on laminin causes decreased polymer self-assembly, decreased binding to type IV collagen, and decreased binding to heparan sulphate proteoglycan[35].

AGE formation on extracellular matrix not only interferes with matrix–matrix interactions, but also interferes with matrix–cell interactions. For example, AGE modification of type IV collagen's cell-binding domains decreases endothelial cell adhesion[36], and AGE modification of a growth-promoting sequence of six amino acids in the A chain of the laminin molecule markedly reduces neurite outgrowth[37].

Several cell-associated binding proteins for AGEs have been identified, including OST-48, 80K-H, galectin-3, the macrophage scavenger receptor type II and RAGE[38–41]. Some of these are likely to contribute to clearance of AGEs, whereas others may underlie the sustained cellular perturbations mediated by binding of the AGE ligands. In cell-culture systems, the AGE receptors identified seem to mediate long-term effects of AGEs on key cellular targets of diabetic complications such as macrophages, glomerular mesangial cells and vascular endothelial cells, although not all these receptors bind proteins with physiological AGE-modification levels. These effects include expression of cytokines and growth factors by macrophages and mesangial cells (interleukin-1, insulin-like growth factor-I, tumour necrosis factor-α, TGF-β, macrophage colony-stimulating factor, granulocyte–macrophage colony-stimulating factor and platelet-derived growth factor), and expression of pro-coagulatory and pro-inflammatory molecules by endothelial cells (thrombomodulin, tissue factor and the cell adhesion molecule VCAM-1)[42–47]. In addition, binding of ligands to endothelial AGE receptors seems to mediate in part the hyperpermeability of the capillary wall induced by diabetes, probably through the induction of VEGF[48].

Consistent with this concept, blockade of one such receptor, RAGE, a member of the immunoglobulin superfamily with three immunoglobulin-like regions on a single polypeptide chain, suppressed macrovascular disease in an atherosclerosis-prone type 1 diabetic mouse model in a glucose- and lipid-independent fashion[49]. Blockade of RAGE has also been shown to inhibit the development of diabetic nephropathy and periodontal disease, and to enhance wound repair in murine models. RAGE has been shown to mediate signal transduction, through generation of reactive oxygen species, which activates both the transcription factor NF-κB, and $p21^{Ras}$ (refs 50, 51). AGE signalling is blocked in cells by expression of RAGE antisense cDNA[52] or an anti-RAGE ribozyme[53].

Activation of protein kinase C

The PKC family comprises at least eleven isoforms, nine of which are activated by the lipid second messenger DAG. Intracellular hyperglycaemia increases the amount of DAG in cultured microvascular cells and in the retina and renal glomeruli of diabetic animals. It seems to achieve this primarily by increasing *de novo* DAG synthesis from the glycolytic intermediate dihydroxyacetone phosphate, through reduction of the latter to glycerol-3-phosphate and stepwise acylation[54]. Increased *de novo* synthesis of DAG activates PKC both in cultured vascular cells[55] and in retina and glomeruli of diabetic animals[54]. The β- and δ-isoforms of PKC are activated primarily, but increases in other isoforms have also been found, such as PKC-α and -ε isoforms in the retina[54] and PKC-α and -β in glomeruli[56] of diabetic rats. Hyperglycaemia may also activate PKC isoforms indirectly through both ligation of AGE receptors[57] and increased activity of the polyol-pathway[58], presumably by increasing reactive oxygen species.

In early experimental diabetes, activation of PKC-β isoforms has been shown to mediate retinal and renal blood flow abnormalities[59], perhaps by depressing nitric oxide production and/or increasing endothelin-1 activity (Fig. 3). Abnormal activation of PKC has been

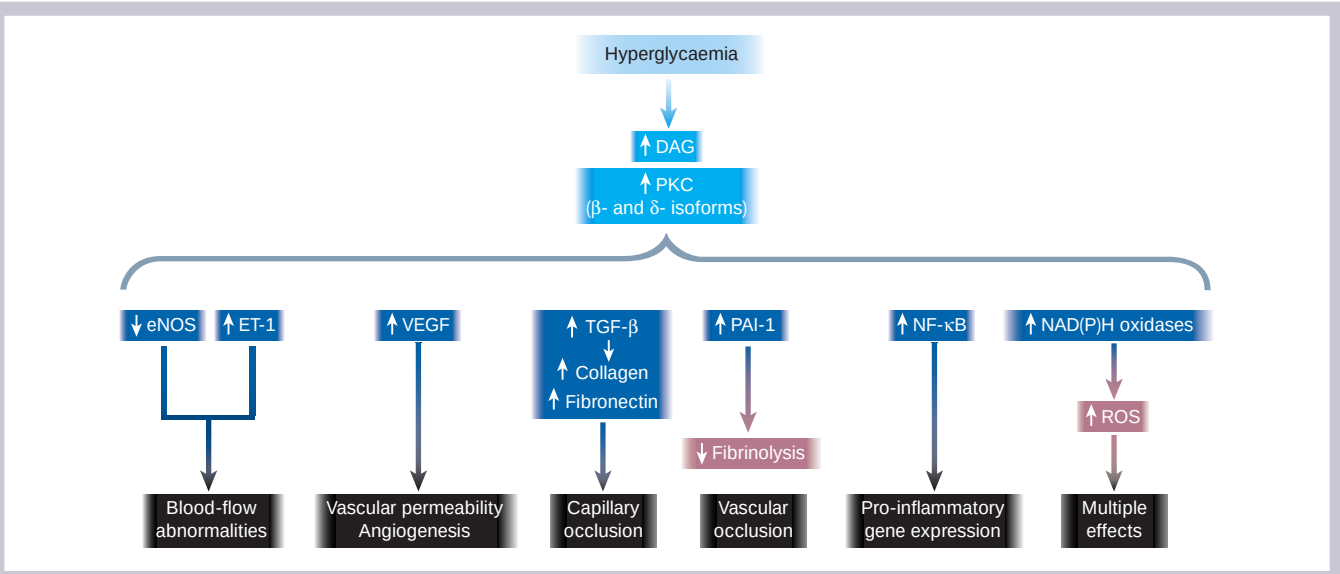

Figure 3 Consequences of hyperglycaemia-induced activation of protein kinase C (PKC). Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the β- and δ-isoforms. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-κB and NAD(P)H oxidases.

implicated in the decreased glomerular production of nitric oxide induced by experimental diabetes[60], and in the decreased production of nitric oxide in smooth muscle cells that is induced by hyperglycaemia[61]. Activation of PKC also inhibits insulin-stimulated expression of the messenger RNA for endothelial nitric oxide synthase (eNOS) in cultured endothelial cells[62]. Hyperglycaemia increases endothelin-1-stimulated MAP-kinase activity in glomerular mesangial cells by activating PKC isoforms[63]. The increased permeability of endothelial cells induced by high glucose in cultured cells is mediated by activation of PKC-α, however[64]. Activation of PKC by raised glucose also induces expression of the permeability-enhancing factor VEGF in smooth muscle cells[65].

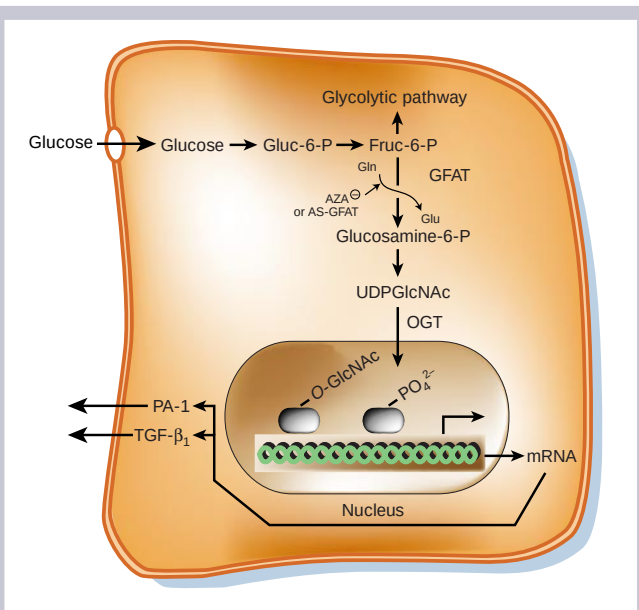

Figure 4 The hexosamine pathway. The glycolytic intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of *N*-acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme *O*-GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors as PAI-1 and TGF-β1. AZA, azaserine; AS-GFAT, antisense to GFAT.

In addition to affecting hyperglycaemia-induced abnormalities of blood flow and permeability, activation of PKC contributes to increased microvascular matrix protein accumulation by inducing expression of TGF-β1, fibronectin and type IV collagen both in cultured mesangial cells[66] and in glomeruli of diabetic rats[67]. This effect seems to be mediated through inhibition of nitric oxide production by PKC[68]. But hyperglycaemia-induced expression of laminin C1 in cultured mesangial cells is independent of PKC activation[69]. Hyperglycaemia-induced activation of PKC has also been implicated in the overexpression of the fibrinolytic inhibitor PAI-1 (ref. 70), the activation of NF-κB in cultured endothelial cells and vascular smooth muscle cells[71,72], and in the regulation and activation of various membrane-associated NAD(P)H-dependent oxidases.

Treatment with an inhibitor specific for PKC-β significantly reduced PKC activity in the retina and renal glomeruli of diabetic animals. Concomitantly, treatment significantly reduced diabetes-induced increases in retinal mean circulation time, normalized increases in glomerular filtration rate and partially corrected urinary albumin excretion. Treatment of a mouse model of type 2 diabetes (*db*/*db*) with a β-isoform-specific PKC inhibitor ameliorated accelerated glomerular mesangial expansion[73].

Increased flux through the hexosamine pathway

Shunting of excess intracellular glucose into the hexosamine pathway might also cause several manifestations of diabetic complications[74]. In this pathway, fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions that require UDP-*N*-acetylglucosamine, such as proteoglycan synthesis and the formation of *O*-linked glycoproteins (Fig. 4). Inhibition of the rate-limiting enzyme in the conversion of glucose to glucosamine — glutamine:fructose-6-phosphate amidotransferase (GFAT) — blocks hyperglycaemia-induced increases in the transcription of TGF-α, TGF-β1 (ref. 74) and PAI-1 (ref. 10). This pathway is also important role in hyperglycaemia-induced and fat-induced insulin resistance[75,76].

The mechanism by which increased flux through the hexosamine pathway mediates hyperglycaemia-induced increases in gene

transcription is not certain, but the observation that binding sites for the transcription factor Sp1 regulate hyperglycaemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells[77] suggested that covalent modification of Sp1 by *N*-acetylglucosamine (GlcNAc) might explain the link between activation of the hexosamine pathway and hyperglycaemia-induced changes in transcription of the gene for PAI-1. Glucosamine itself was subsequently shown to activate the PAI-1 promoter through Sp1 sites in glomerular mesangial cells[78]. The glycosylated form of Sp1 seems to be more transcriptionally active than the deglycosylated form[79]. A fourfold increase in *O*-acetylglucosaminylation of Sp1 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, supporting the concept that *O*-acetylglucosaminylation and phosphorylation compete for the same sites on this protein[80].

Recently, hyperglycaemia was shown to induce a 2.4-fold increase in hexosamine pathway activity in aortic endothelial cells, resulting in a 1.7-fold increase in Sp1 *O*-linked GlcNAc and a 70–80% decrease in Sp1 *O*-linked phosphothreonine and phosphoserine[10]. Concomitantly, hyperglycaemia resulted in a 3.8-fold increase in expression from an 85-base-pair truncated PAI-1 promoter–luciferase reporter DNA containing two Sp1 sites, but failed to increase expression when the two Sp1 sites were mutated[10].

Modification of Sp1 by GlcNAc may regulate other glucose-responsive genes in addition to that for PAI-1. As virtually every RNA polymerase II transcription factor examined has been found to be *O*-acetylglucosaminylated[81], it is possible that reciprocal modification by *O*-acetylglucosaminylation and phosphorylation of transcription factors other than Sp1 may function as a more generalized mechanism for regulating glucose-responsive gene transcription.

In addition to transcription factors, many other nuclear and cytoplasmic proteins are dynamically modified by *O*-linked GlcNAc, and may show reciprocal modification by phosphorylation in a manner analogous to Sp1 (ref. 81). One example relevant to diabetic complications is the inhibition of eNOS activity by hyperglycaemia-induced *O*-acetylglucosaminylation at the Akt site of the eNOS protein[82]. Other examples might be various PKC isoforms, which are activated by glucosamine without membrane translocation (H. J. Goldberg, C. J. Whiteside & G. Fantus, personal communication).

Thus, activation of the hexosamine pathway by hyperglycaemia may result in many changes in both gene expression and protein function, which together contribute to the pathogenesis of diabetic complications.

A common element linking hyperglycaemia-induced damage

Although specific inhibitors of aldose reductase activity, AGE formation, PKC activation and the hexosamine pathway each ameliorate various diabetes-induced abnormalities in cell culture and animal models, there has been no apparent common element linking the four mechanisms of hyperglycaemia-induced damage[17,26–28,59,83]. This issue has now been resolved by the recent discovery that each of the four different pathogenic mechanisms reflects a single hyperglycaemia-induced process: overproduction of superoxide by the mitochondrial electron-transport chain[10,84]. Many studies have shown that diabetes and hyperglycaemia increase oxidative stress[85], but neither the underlying mechanism nor the consequences for other pathways of hyperglycaemic damage were known.

To understand how hyperglycaemia increases the production of reactive oxygen species inside cultured aortic endothelial cells[86], a brief overview of glucose metabolism is helpful (Box 1). When the electrochemical potential difference generated by the proton gradient across the inner mitochondrial membrane is high, the lifetime of superoxide-generating electron-transport intermediates such as ubisemiquinone is prolonged (Fig. 5). There seems to be a threshold value above which superoxide production is markedly increased[87]. Du *et al.*[82] have found that hyperglycaemia increases the proton gradient above this threshold value as a result of overproduction of electron donors by the TCA cycle. This, in turn, causes a marked increase in the production of superoxide by endothelial cells. Overexpression of manganese superoxide dismutase (MnSOD), the mitochondrial form of superoxide dismutase, abolished the signal generated by reactive oxygen species, and overexpression of uncoupling protein-1 (UCP-1) collapsed the proton electrochemical

Box 1
Overview of glucose metabolism

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 by way of two shuttle systems, or it can reduce pyruvate to lactate, which exits the cell to provide 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 $FADH_2$. Mitochondrial NADH and $FADH_2$ provide energy for ATP production through oxidative phosphorylation by the electron-transport chain.

Electron flow through the mitochondrial electron-transport chain (Fig. 5) is carried out by four inner membrane-associated enzyme complexes, plus cytochrome *c* and the mobile electron carrier ubiquinone. NADH derived from both cytosolic glucose oxidation and mitochondrial TCA cycle activity donates electrons to NADH:ubiquinone oxidoreductase (complex I). Complex I 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 ubisemiquinone radical-generating Q cycle. Electron transport then proceeds through cytochrome *c*, cytochrome *c* oxidase (complex IV) and, finally, molecular oxygen (O_2). Electron transfer through complexes I, III and IV generates a proton gradient that drives ATP synthase (complex V).

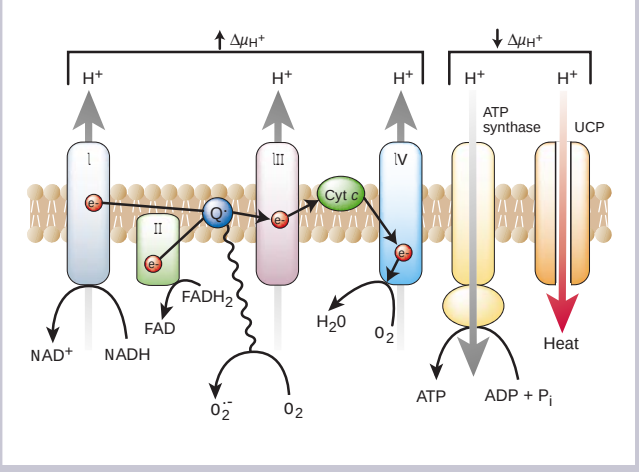

Figure 5 Production of superoxide by the mitochondrial electron-transport chain. Increased hyperglycaemia-derived electron donors from the TCA cycle (NADH and $FADH_2$) generate a high mitochondrial membrane potential ($\Delta\mu_{H+}$) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce O_2 to superoxide.

gradient and prevented hyperglycaemia-induced overproduction of reactive oxygen species.

Inhibition by MnSOD or UCP-1 of hyperglycaemia-induced overproduction of mitochondrial superoxide completely prevented an increase in polyol pathway flux, increased intracellular AGE formation, increased PKC activation and an increase in hexosamine pathway activity in endothelial cells.

As hyperglycaemia-induced overproduction of mitochondrial superoxide induces a 66% decrease in GAPDH activity[10], the effect of hyperglycaemia on polyol pathway flux may reflect the accumulation of glycolytic metabolites, including glucose, upstream of GAPDH (Fig. 6). Although the reversible inhibition of GAPDH by reactive oxygen species has been well described, the inhibition of GAPDH by hyperglycaemia-induced reactive oxygen species may reflect the resulting activation of PARP and depletion of NAD^+ (ref. 15), rather than direct oxidative inactivation of the enzyme, as intracellular GSH levels remain high.

In regard to AGEs, methylglyoxal-derived AGE, the primary intracellular AGE induced by hyperglycaemia[30], is formed by fragmentation of triose phosphates. Thus the effect of hyperglycaemia on intracellular AGE formation also probably reflects increased triose phosphate levels resulting from inhibition of GAPDH by mitochondrial overproduction of reactive oxygen species (Fig. 6)[10]. This hypothesis is supported by the observation that GAPDH antisense oligonucleotides caused identical intracellular increases in AGE in the absence of hyperglycaemia (M.B. *et al.*, unpublished results).

Hyperglycaemia activates PKC by increasing the *de novo* synthesis of DAG[54], so the effect of hyperglycaemia on PKC activation probably reflects increased dihydroxyacetone phosphate levels resulting from inhibition of GAPDH by reactive oxygen species (Fig. 6)[10]. That GAPDH antisense oligonucleotides also caused activation of PKC in the presence of physiological glucose concentrations (M. B. *et al.*, unpublished results) supports this hypothesis.

Because hyperglycaemia increases hexosamine pathway flux by providing more fructose-6-phosphate for GFAT — the rate-limiting enzyme of the pathway — the effect of hyperglycaemia on hexosamine pathway flux probably reflects increased fructose-6-phosphate levels, resulting from inhibition of GAPDH by reactive oxygen species (Fig. 6)[10]. GAPDH antisense oligonucleotides also caused identical increases in hexosamine pathway flux in the absence of hyperglycaemia (M. B. *et al.*, unpublished results). Hyperglycaemia-induced activation of the redox-sensitive pleiotropic transcription factor NF-κB was also prevented by inhibition of mitochondrial superoxide overproduction[84].

Overexpression of UCP-1 or MnSOD corrects a variety of hyperglycaemia-induced phenotypes in target cells of diabetic complications. In cultured glomerular mesangial cells, overexpression of MnSOD suppresses the increase in collagen synthesis induced by high glucose[88]. In dorsal root ganglion (DRG) neurons from both wild-type and $MnSOD^{+/-}$ mice, overexpression of MnSOD decreases hyperglycaemia-induced programmed cell death, and in embryonic rat DRG neurons, overexpression of UCP-1 inhibits cleavage of programmed cell death effector caspases (J. W. Russell, personal communication). In aortic endothelial cells, overexpression of either UCP-1 or MnSOD completely blocks hyperglycaemia-induced monocyte adhesion (J. L. Nadler & C. C. Hedrick, personal communication), prevents hyperglycaemia-induced inhibition of the anti-atherogenic enzyme prostacyclin synthetase, and prevents hyperglycaemia-induced inhibition of peroxisome proliferator-activated receptor-γ activation (M. B. *et al.*, unpublished data). Overexpression of either MnSOD or UCP-1 also prevents inhibition of eNOS activity by hyperglycaemia[82]. In platelets, chemical uncouplers or SOD mimetics both prevent potentiation by hyperglycaemia of collagen-induced platelet activation and aggregation[89].

In transgenic mice overexpressing human cytoplasmic Cu^{2+}/Zn^{2+} SOD, and in which diabetes was induced by streptozotocin (STZ) treatment, albuminuria, glomerular hypertrophy and glomerular

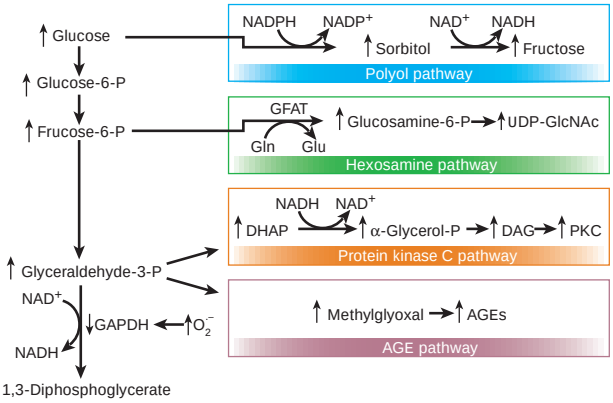

Figure 6 Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage. Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilization. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-*N*-acetylglucosamine increases modification of proteins by *O*-linked *N*-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH.

content of TGF-β and collagen type IV were all attenuated compared to wild-type littermates after 4 months of diabetes. The wild-type STZ-diabetic mice developed modest increases in fractional mesangial volume after 8 months of diabetes, and this change was also suppressed in the SOD transgenic diabetic mice[90]. Overexpression of the human SOD1 transgene in *db*/*db* diabetic mice similarly attenuated the extensive expansion of the glomerular mesangial matrix that was otherwise evident by age 5 months in the non-transgenic *db*/*db* littermates (F. R. DeRubertis, P. A. Craven, M. F. Melhem, H. Liachenko & S. L. Phillips, unpublished observations).

Future directions

The discovery that each of the four main mechanisms implicated in the pathogenesis of diabetic complications reflects a single hyperglycaemia-induced process provides a new conceptual framework for future research, although clinical trials will be necessary to show that the results from cell culture and animal studies are applicable to humans. Three general areas are of great importance to a more complete understanding of the molecular and cell biology of diabetic complications.

First is the phenomenon of so-called hyperglycaemic memory. This refers to the persistence or progression of hyperglycaemia-induced microvascular alterations during subsequent periods of normal glucose homeostasis. The most striking example of this occurred in the eyes of diabetic dogs during a post-hyperglycaemic period of euglycaemia[91]. After 2.5 years of elevated glucose, the eyes were histologically normal. But after a subsequent 2.5-year period of normal glycaemia, the eyes developed severe retinopathy. Results from the Epidemiology of Diabetes Interventions and Complications study indicate that hyperglycaemic memory also occurs in human patients. The effects of intensive and conventional therapy on the occurrence and severity of post-study retinopathy and nephropathy were shown to persist for four years after the Diabetes Control and Complications Trial (DCCT), despite nearly identical glycosylated haemoglobin values during the 4-year follow-up[92]. Hyperglycaemia-induced mitochondrial superoxide production may provide an explanation for the development of complications

during post-hyperglycaemic periods of normal glycaemia. Hyperglycaemia-induced increases in superoxide would not only increase polyol pathway flux, AGE formation, PKC activity and hexosamine pathway flux, but might also induce mutations in mitochondrial DNA. Defective subunits of the electron-transport complexes encoded by mutated mitochondrial DNA could eventually cause increased superoxide production at physiological concentrations of glucose, with resulting continued activation of the four pathways despite the absence of hyperglycaemia.

The second general area concerns the genetic determinants of susceptibility to both microvascular and macrovascular complications. Their role in microvascular complications is supported by familial clustering of diabetic nephropathy and retinopathy. In two studies of families in which two or more siblings had type 1 diabetes, if one diabetic sibling had advanced diabetic nephropathy, the other diabetic sibling had a nephropathy risk of 83% or 72%, whereas the risk was only 17% or 22% if the index case did not have diabetic nephropathy[93]. The DCCT reported familial clustering of retinopathy with an odds ratio of 5.4 for the risk of severe retinopathy in diabetic relatives of retinopathy-positive subjects from the conventional treatment group compared with subjects with no retinopathy[94]. For macrovascular complications, coronary artery calcification (an indicator of subclinical atherosclerosis) also shows familial clustering, with an estimated heritability of at least 40% (ref. 95). Thus, gene-mapping studies designed to identify genes that predispose to complications, as well as the interaction of these genes with metabolic factors, are warranted.

Finally, the paradigm discussed in this review suggests that interrupting the overproduction of superoxide by the mitochondrial electron-transport chain would normalize polyol pathway flux, AGE formation, PKC activation, hexosamine pathway flux and NF-κB activation. But it might be difficult to accomplish this using conventional antioxidants, as these scavenge reactive oxygen species in a stoichiometric manner. Thus, although long-term administration of a multi-antioxidant diet inhibited the development of early diabetic retinopathy in rats[96], and vitamin C improved endothelium-dependent vasodilation in diabetic patients[97], low-dose vitamin E failed to alter the risk of cardiovascular and renal disease in patients with diabetes[98]. New, low-molecular-mass compounds that act as SOD or catalase mimetics have the theoretical advantage of scavenging reactive oxygen species continuously by acting as catalysts with efficiencies approaching those of the native enzymes[99]. Such compounds normalize diabetes-induced inhibition of aortic prostacyclin synthetase in animals (M. B. *et al.*, unpublished results), and significantly improve diabetes-induced decreases in endoneurial blood flow and motor nerve conduction velocity[100]. These and other agents discovered using high-throughput chemical and biological methods might have unique clinical efficacy in preventing the development and progression of diabetic complications. □

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