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The missing link: A single unifying mechanism for diabetic complications

TAKESHI NISHIKAWA, DIANE EDELSTEIN, and MICHAEL BROWNLEE

Department of Metabolic Medicine, Kumamoto University School of Medicine, Kumamoto, Japan, and Albert Einstein College of Medicine, Diabetic Research Center, Bronx, New York, USA

The missing link: A single unifying mechanism for diabetic complications. A causal relationship between chronic hyperglycemia and diabetic microvascular disease, long inferred from various animal and clinical studies, has now been definitely established by data from the Diabetes Control and Complications Trial (DCCT), a multicenter, randomized, prospective, controlled clinical study. A relationship between chronic hyperglycemia and diabetic macrovascular disease in patients with non-insulin-dependent diabetes mellitus (NIDDM) is also supported by the Kumamoto study. How does hyperglycemia induce the functional and morphologic changes that define diabetic complications? Vascular endothelial cells are a major target of hyperglycemic damage, but the mechanisms underlying this damage remain incompletely understood. Three seemingly independent biochemical pathways are involved in the pathogenesis: glucose-induced activation of protein kinase C (PKC) isoforms; increased formation of glucose-derived advanced glycation end products; and increased glucose flux through the aldose reductase pathway. The relevance of each of these three pathways is supported by animal studies in which pathway-specific inhibitors prevent various hyperglycemia-induced abnormalities. Hyperglycemia increases reactive oxygen species (ROS) production inside cultured bovine aortic endothelial cells. In this paper, we show that ROS may activate aldose reductase, induce diacylglycerol, activate PKC, induce advanced glycation end product formation, and activate the pleiotropic transcription factor nuclear factor-kappa B (NF-κB). These data demonstrate that a single unifying mechanism of induction, increased production of ROS, serves as a causal link between elevated glucose and each of the three major pathways responsible for diabetic damage.

A causal relationship between chronic hyperglycemia and diabetic microvascular disease, long inferred from various animal and clinical studies [1], has now been definitely established by data from the Diabetes Control and Complications Trial (DCCT), a multicenter, randomized, prospective, controlled clinical study. The DCCT study demonstrated conclusively that the relative risks for the development of diabetic complications increased with increasing levels of mean hemoglobin (Hb) A_{1c}. Patients with type 1 diabetes whose intensive insulin therapy produced HbA_{1c} levels 2% lower than those receiving conventional insulin therapy had a 76% lower incidence of retinopathy, a 54% lower incidence of nephropathy, and a 60% reduction in neuropathy. A relationship between levels of chronic hyperglycemia and diabetic microvascular and macrovascular disease in patients with type 2 diabetes has also been found in several studies [2–4]. Thus, hyperglycemia is the primary initiating factor in the pathogenesis of diabetic complications.

The distinguishing characteristic of cells that have been damaged by hyperglycemia is their lack of down-regulation of glucose transport when extracellular glucose is elevated. Vascular smooth muscle cells that have not been damaged by hyperglycemia show an inverse relationship between glucose concentration and glucose transport measured as 2-deoxyglucose uptake. In contrast, vascular endothelial cells, a major target of hyperglycemic damage, show no significant change in glucose transport when glucose concentration is elevated. Thus, intracellular hyperglycemia appears to be the major determinant of diabetic tissue damage [5].

Intracellular hyperglycemia causes tissue damage by mechanisms that can be grouped into two categories. One category of mechanisms involves repeated acute changes in cellular metabolism that are reversible when euglycemia is restored. Another category of mechanisms involves cumulative changes in long-lived macromolecules that persist despite restoration of euglycemia. These mechanisms are influenced by genetic determinants of susceptibility or resistance to hyperglycemic damage.

Four major hypotheses about how hyperglycemia causes diabetic complications have generated a large amount of data, as well as several clinical trials based on specific inhibitors of these mechanisms: formation of reactive oxygen species; increased activity of aldose reductase; activation of protein kinase C (PKC) isoforms; and increased formation of advanced glycation end products. No current unifying hypothesis links these four mecha-

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nisms, but either polyol pathway-induced redox changes [decreased nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)/nicotinamide adenine dinucleotide phosphate ($NADP^+$) and increased nicotinamide adenine dinucleotide (reduced form) (NADH)/nicotinamide adenine dinucleotide (NAD^+) ratios] or hyperglycemia-induced formation of reactive oxygen species could potentially account for all of the other biochemical abnormalities.

Hyperglycemia increases intracellular reactive oxygen species (ROS) and lipid peroxidation. In aortic endothelial cells, 30 mmol/L glucose increased ROS formation [measured as dichlorofluorescein (DCF)] by 250% within 24 hours, and resultant lipid peroxidation (measured as malondialdehyde) by 330% by 168 hours. Thus, hyperglycemia rapidly increases intracellular ROS production in cells affected by diabetic complications [6].

The enzyme aldose reductase converts various aldehydes (such as 2-oxo-aldehydes and those derived from lipid peroxidation) to inactive alcohols. NADPH is the cofactor in both this reaction and also in the regeneration of glutathione by glutathione reductase. The activity of aldose reductase is reversibly down-regulated by nitric oxide modification of a cysteine residue in the enzyme's active site. ROS appear to reduce nitric oxide levels and thus may activate aldose reductase [7–9]. In a euglycemic environment, oxidant stress (i.e., ROS) increases the concentration of toxic aldehydes. At the same time, nitric oxide levels are reduced, converting aldose reductase to a higher activity form. Glutathione levels are unaffected, and the reactive aldehydes are detoxified.

In a hyperglycemic environment, oxidant stress (ROS) increases, and the reactive aldehydes are then detoxified. However, in contrast to the euglycemic state, increased intracellular glucose concentration results in increased enzymatic conversion of glucose to the polyalcohol sorbitol, with concomitant decreases in NADPH and glutathione. In cells in which aldose reductase activity is sufficient to deplete glutathione, hyperglycemia-induced oxidative stress is augmented. Sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH). In cells in which sorbitol dehydrogenase activity is high, this may result in an increased NADH : NAD^+ ratio. This could increase de novo synthesis of diacylglycerol (DAG) by inhibition oxidation of triose phosphates, thereby activating PKC.

The effect of aldose reductase inhibition is most striking on diabetes-induced decreases in nerve conduction velocity. In dogs with diabetes, conduction became significantly less than normal within 42 months [10]. Conduction velocity in aldose reductase inhibitor-treated dogs remained statistically equal to normal throughout the 5-year study. In contrast, aldose reductase inhibition had no effect on the development of diabetic retinopathy, perhaps due to the low aldose reductase activity in retinal vascular cells [10].

The third mechanism by which intracellular hyperglycemia damages tissue is PKC activation. Hyperglycemia increases DAG content, in part by de novo synthesis and possibly also by phosphatidylcholine hydrolysis. Increased DAG activates PKC, primarily the β and δ isoforms. Activated PKC increases the production of cytokines and extracellular matrix (ECM), the fibrinolytic inhibitor plasminogen activator inhibitor (PAI-1), and the vasoconstrictor endothelin-1. PKC is also a mediator of vascular endothelial growth factor activity. These changes contribute to basement membrane thickening, vascular occlusion, increased permeability, and activation of angiogenesis [11]. ROS also activate PKC in vascular endothelial cells. As ROS-producing H_2O_2 increases, it activates PKC. The mechanism appears to involve direct or indirect activation of phospholipase D, which hydrolyzes phosphatidylcholine to produce DAG. ROS could also increase DAG through increased de novo synthesis resulting from ROS inhibition of the enzyme glyceraldehyde phosphate dehydrogenase [12, 13].

The significance of this mechanism has been established by studies of an isoform-specific PKC inhibitor in diabetic animals. Diabetes increased mean retinal circulation time in rats from 0.67 second to 1.40 seconds. Treatment of diabetic rats with the PKCβ inhibitor LY 333531 reduced the time to 0.87 second at the highest dose tested. Similarly, diabetes increased glomerular filtration rate (GFR) in rats from a mean of 3.0 to 4.6 mL/min. Treatment of diabetic rats with the PKCβ inhibitor LY 333531 normalized the mean glomerular filtration rate (GFR) at the highest doses tested. Similarly, diabetes increased the albumin excretion rate in rats from a mean of 1.6 to 11.7 mg/day. Treatment of diabetic rats with the PKCβ inhibitor LY 333531 reduced the mean albumin excretion rate to 4.9 mg/day at the highest dose tested [14].

The final mechanism by which intracellular hyperglycemia damages susceptible tissues is by increasing the formation of advanced glycation end products (AGEs). AGEs can arise from autooxidation of glucose to glyoxal, decomposition of the Amadori product to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate to methylglyoxal. These reactive dicarbonyls react with amino groups of proteins to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system. All three AGE precursors are also substrates for other reductases as well [15, 16]. Intracellular production of AGE precursors damages target cells by three general mechanisms. First, intracellular protein glycation alters protein function. Second, ECM modified by AGE precursors has abnormal functional properties. Third, plasma proteins modified by AGE precursors bind to AGE receptors on adjacent cells such as macrophages, thus inducing recep-

tor-mediated ROS production that activates nuclear factor-kappa B (NF-κB) and expression of pathogenic gene products, including cytokines and hormones [17].

An example of intracellular protein glycation is the increase in macromolecular endocytosis in endothelial cells by the AGE-precursor methylgloxal. Exposure to 30 mmol/L glucose increased macromolecular endocytosis by GM7373 endothelial cells stably transfected with neo 2.2-fold. In contrast, when increased methylglyoxal accumulation was prevented by overexpressing the enzyme glyoxalase I in these cells, 30 mmol/L glucose did not increase macromolecular endocytosis [15]. An example of matrix modification by AGE-precursors is the increased permeability to albumin of glomerular basement membrane modified by AGEs. Ultrafiltration of albumin (J_s) by AGE-modified glomerular basement membrane is significantly increased compared with ultrafiltration of albumin by unmodified glomerular basement membrane over a range of different filtration pressures [18].

Five AGE-binding proteins have been identified: the receptor for advanced glycation end products (RAGE), p60, p90, galectin-3, and the scavenger receptor type II. RAGE is a novel member of the immunoglobin superfamily, whose ligation generates ROS and activates the pleiotropic transcription factor NF-κB. p60 exhibits 95% identity to OST-48, a component of the oligosaccharyltransferase complex in microsomal membranes, and p90 has significant sequence homology with human 80K-H, a PKC substrate. Galectin-3, a carbohydrate-binding protein, also binds AGEs. The type II macrophage scavenger receptor binds AGEs and mediates their uptake by endocytosis. To date, only RAGE has actually been shown to transduce signals initiated by AGE-ligand binding. The signal transduction pathway involves the generation of ROS, which then activate the pleiotropic transcription factor NF-κB [19–22].

AGE-modified protein binding to specific receptors on macrophages and endothelial cells cause pathologic changes in diabetic blood vessels by inducing the expression of pathogenic gene products. On macrophages and mesangial cells, binding stimulates production of tumor necrosis factor α, (TNF-1) interleukin-1, insulin-like growth factor-1, and granulocyte-macrophage colony-stimulating factor at levels that increase proliferation of smooth muscle cells and increase matrix production. On endothelial cells, binding induces changes in gene expression that are procoagulatory, and increases expression of leukocyte-binding vascular adhesion molecule-1 [23].

The significance of AGEs as mediators of glucose-induced pathology in diabetic target tissues has been established by using an inhibitor of AGE formation. The effects of an AGE inhibitor (aminoguanidine) on diabetic pathology have been investigated in retina, kidney, nerve, and artery. In experimental animals, development of all pathognomonic abnormalities examined was inhibited 85% to 90%. These include the development of retinal acellular capillaries and retinal microaneurysms, increased urinary albumin excretion and mesangial fraction volume, decreased motor and sensory nerve conduction velocity and action-potential amplitude, and diminished arterial elasticity and increased arterial fluid filtration [17].

It has been proposed that a potential relationship may exist between polyol pathway-induced redox changes and the three other biochemical mechanisms underlying diabetic complications. In this scheme, increased glucose flux through the polyol pathway could decrease the NADPH : $NADP^+$ ratio, thereby reducing glutathione reductase activity and increasing oxidative stress. In those cells where significant oxidation of sorbitol to fructose occurs, increased sorbitol flux through this pathway could also increase the NADH : NAD^+ ratio, thereby blocking glycolysis at the level of triose phosphates and increasing formation of α-glycerol phosphate, a precursor of DAG. In addition, increased triose phosphate concentrations produce more of the most potent AGE-precursor, methylgloxal. The difficulties with this attempt at a unifying theory of diabetic complications is that aldose reductase activity is low in several major target cells damaged in diabetes, such as endothelial cells. In addition, experimentally, aldose reductase inhibitors have no effect on hyperglycemia-induced increases in endothelial cell DAG, contrary to what is predicted [24, 25]. An alternative unifying mechanism is hyperglycemia-induced ROS production. ROS may activate aldose reductase, induce DAG, activate PKC, induce advanced glycation end product formation, and activate the pleiotropic transcription factor NF-κB [6–8, 12, 26, 27].

Glucose-induced damage in cells of arteries may be further accelerated by insulin resistance. Insulin resistance may damage arteries indirectly by exacerbating known risk factors for vascular damage. Insulin resistance alone or combined with hyperinsulinemia is associated with atherogenic changes in plasma lipoproteins, increased PAI-1, and hypertension. This association has been termed "Syndrome X" [28]. Alternatively, we have proposed that insulin resistance in vascular cells may promote damage by inhibiting antiatherogenic gene expression. Selective resistance to insulin action in the phosphatidyl inositol 3-kinase signaling pathway may reduce antiproliferative nitric oxide production and interfere with insulin's inhibitory effect on TNF and angiotensin II's stimulation of PAI-1 and intracellular adhesion molecule expression [9].

The susceptibility to damage by hyperglycemia, at least for the kidney and retina, is determined by unknown genetic factors. For example, in patients with type 1 diabetes, the cumulative incidence of overt proteinuria levels off at 27%. After 34 years of diabetes, the cumulative incidence of end-stage renal disease in affected patients is 21.4%. These data suggest that only a subset of

patients are susceptible to the development of clinical nephropathy [29]. Familial clustering of diabetic nephropathy also strongly suggests a major genetic effect. In one study, the nephropathy risk for diabetic siblings of an affected case was 83%, whereas the risk for diabetic siblings of an unaffected patient was 17%. In another study, the risks were 33% and 10%, respectively [30].

A major feature of hyperglycemic damage that is unaccounted for by current theories of the pathogenesis of diabetic complications is the so-called "hyperglycemic memory." This term refers to the development of retinopathy during posthyperglycemic normoglycemia. In the study that initially described this, normal dogs were compared with dogs with diabetes with either poor control for 5 years, good control for 5 years, or poor control for 2½ years ($P \rightarrow G_a$) followed by good control for the next 2½ years ($P \rightarrow G_b$). HbA_1 values for both the good control group and the $P \rightarrow G_b$ group were identical to the normal group. Quantitation of retinal microaneurysms and acellular capillaries in this study showed that lesions of diabetic retinopathy developed during 5 years of poor control. Good control prevented nearly all of this pathology. After 2½ years of poor control, retinopathy was absent. However, despite the institution of good control in this group after 2½ years, retinopathy developed over the next 2½ years to an extent almost equal to the 5-year poor control group [31].

A potential mechanism for hyperglycemic memory could involve two stages: induction and perpetuation. Induction could be explained by hyperglycemia-induced increases in ROS, which may be a consequence of increased reducing equivalents generated from increased glucose metabolism flowing through the mitochondrial electron transport chain. Increased ROS would not only cause cellular dysfunction but also induce mutations in mitochondrial DNA [32, 33]. Perpetuation (i.e., hyperglycemic memory) would occur because mitochondrial DNA mutated by hyperglycemia-induced ROS would encode defective electron transport chain subunits. These defective subunits would cause increased ROS production by the electron transport chain at physiologic concentrations of glucose and glucose-derived reducing equivalents. Such a mechanism could explain the observation that in human patients with diabetes with functioning pancreatic transplants, renal pathology continues to progress for at least 5 years after diabetes has been cured [31].

If future work substantiates this hypothesis for a unifying mechanism of glucose-accelerated aging of vascular and neurologic tissues, it may provide the basis for the development of new pharmaceutical agents for the treatment of these problems.

Reprint requests to Michael Brownlee, Albert Einstein College of Medicine, Diabetic Research Center, 1300 Morris Park Ave, Bronx, NY 10461, USA

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