Banting Lecture 2004

The Pathobiology of Diabetic Complications

A Unifying Mechanism

Michael Brownlee

It's a great honor to join the exceptional club of Banting Award winners, many of whom were my role models and mentors. In addition, giving the Banting Lecture also has a very personal meaning to me, because without Frederick Banting, I would have died from type 1 diabetes when I was 8 years old. However, it was already apparent at the time I was diagnosed that for too many people like me, Banting's discovery of insulin only allowed them to live just long enough to develop blindness, renal failure, and coronary disease. For example, when I started college, the American Diabetes Association's Diabetes Textbook had this to say to my parents: "The person with type 1 diabetes can be reassured that it is highly likely that he will live at least into his 30s." Not surprisingly, my parents did not find this particularly reassuring.

At the same time we were reading this in 1967, however, the first basic research discovery about the pathobiology of diabetic complications had just been published in *Science* the previous year. In my Banting Lecture today, I am thus going to tell you a scientific story that is also profoundly personal.

I've divided my talk into three parts. The first part is called "pieces of the puzzle," and in it I describe what was learned about the pathobiology of diabetic complications starting with that 1966 *Science* paper and continuing through the end of the 1990s. In the second part, I present a unified mechanism that links together all of the seemingly unconnected pieces of the puzzle. Finally, in the third part, I focus on three examples of novel therapeutic approaches for the prevention and treatment of diabetic complications, which are all based on the new paradigm of a unifying mechanism for the pathogenesis of diabetic complications.

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AGE, advanced glycation end product; eNOS, endothelial nitric oxide synthase; FFA, free fatty acid; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; MnSOD, manganese superoxide dismutase; NFκB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, tricarboxylic acid; UCP, uncoupling protein.

PIECES OF THE PUZZLE

The general features of hyperglycemia-induced tissue damage are shown schematically in Fig. 1. The DCCT (Diabetes Control and Complications Trial) and the UKPDS (U.K. Prospective Diabetes Study) established that hyperglycemia, shown on the far left of the figure, is the initiating cause of the diabetic tissue damage that we see clinically, shown on the far right (1,2). Although this process is modified by both genetic determinants of individual susceptibility, shown in the top box, and by independent accelerating factors such as hypertension, shown in the bottom box, today I will concentrate on the inner boxes, the mechanisms that mediate the tissue-damaging effects of hyperglycemia.

When I refer to the tissue-damaging effects of hyperglycemia, of course, I mean damage to a particular subset of cell types: capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. What is distinct about these cells that makes them so vulnerable to hyperglycemia? We know that in diabetes, hyperglycemia is bathing all the cells of every tissue. So why does damage occur only in the few cell types involved in diabetic complications? The answer is that most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia, so that their internal glucose concentration stays constant. In contrast, the cells damaged by

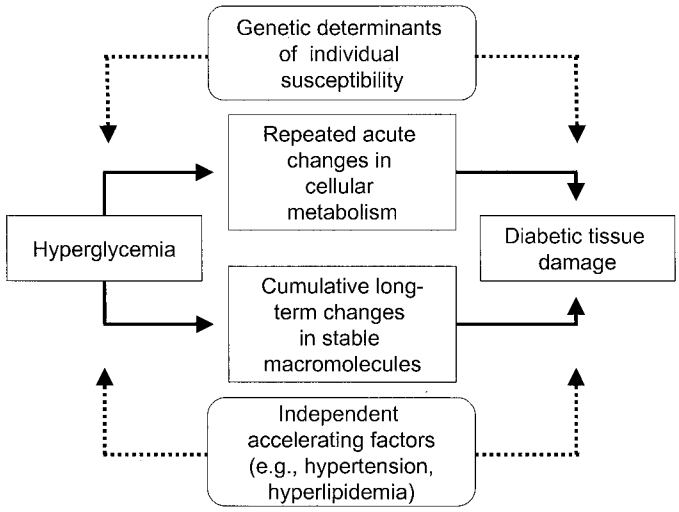

FIG. 1. General features of hyperglycemia-induced tissue damage.

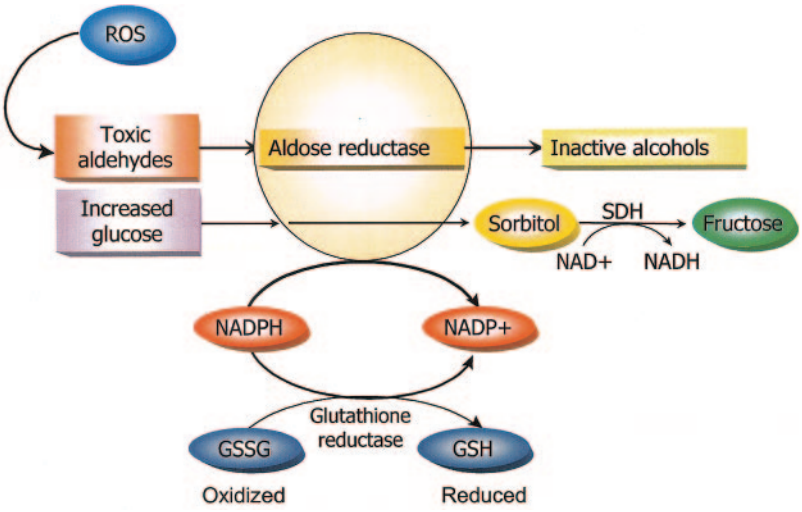

FIG. 2. Hyperglycemia increases flux through the polyol pathway. From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001.

hyperglycemia are those that cannot do this efficiently (3,4). Thus, diabetes selectively damages cells, like endothelial cells and mesangial cells, whose glucose transport rate does not decline rapidly as a result of hyperglycemia, leading to high glucose inside the cell. This is important, because it tells us that the explanation for what causes complications must involve mechanisms going on inside these cells, rather than outside.

The first such mechanism that was discovered was the polyol pathway and increased polyol pathway flux, described in peripheral nerve in the 1966 *Science* paper I refer to above (5). This was the first piece of the puzzle. Then, ~10 years later, in the late 1970s, a second piece of the puzzle emerged: increased formation of advanced glycation end products (AGEs). In the late 1980s and early 1990s, a third piece of the puzzle was discovered: hyperglycemia-induced activation of protein kinase C (PKC) isoforms. And in the late 1990s, a fourth piece of the puzzle was discovered: increased hexosamine pathway flux and consequent overmodification of proteins by *N*-acetylglucosamine. I'm going to very briefly review each of these, because I think each one is an important piece of the puzzle.

Increased flux through the polyol pathway. The polyol pathway, shown schematically in Fig. 2, focuses on the enzyme aldose reductase. Aldose reductase normally has the function of reducing toxic aldehydes in the cell to inactive alcohols, but when the glucose concentration in the cell becomes too high, aldose reductase also reduces that glucose to sorbitol, which is later oxidized to fructose. In the process of reducing high intracellular glucose to sorbitol, the aldose reductase consumes the cofactor NADPH (6). But as shown in Fig. 2, NADPH is also the essential cofactor for regenerating a critical intracellular antioxidant, reduced glutathione. By reducing the amount of reduced glutathione, the polyol pathway increases susceptibility to intracellular oxidative stress.

How do we know that this piece of the puzzle is really important? From studies like one conducted by Ron Engerman and Tim Kern (7), in which diabetic dogs were treated for 5 years with an aldose reductase inhibitor. Nerve conduction velocity in the diabetic dogs decreased over time as it does in patients. In contrast, in diabetic dogs treated with an aldose reductase inhibitor, the diabetes-induced defect in nerve conduction velocity was prevented.

Intracellular production of AGE precursors. The second discovery listed on my pieces of the puzzle list is the intracellular production of AGE precursors. As shown schematically in Fig. 3, these appear to damage cells by three mechanisms. The first mechanism, shown at the top of the endothelial cell, is the modification of intracellular proteins including, most importantly, proteins involved in the regulation of gene transcription (8,9 and M.B., unpublished observations). The second mechanism, shown on the left, is that these AGE precursors can diffuse out of the cell and modify extracellular matrix molecules nearby (10), which changes signaling between the matrix and the cell and causes cellular dysfunction (11). The third mechanism, shown on the right of Fig. 3, is that these AGE precursors diffuse out of the cell and modify circulating proteins in the blood such as albumin. These modified circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology (12–21). Again, how do we know that this piece of the puzzle is really important? From many animal studies such as one done by Hans-Peter Hammes (22), showing that that pharmacologic inhibition of AGEs prevents late structural changes of experimental diabetic retinopathy.

PKC activation. The third mechanism on my "pieces of the puzzle" list was the PKC pathway. In this pathway, shown schematically in Fig. 4, hyperglycemia inside the cell increases the synthesis of a molecule called diacylglycerol, which is a critical activating cofactor for the classic isoforms of protein kinase-C, -β, -δ, and -α (23–26). When PKC is activated by intracellular hyperglycemia, it has a variety of effects on gene expression, examples of which are shown in the row of open boxes in Fig. 4. In each case, the things that are good for normal function are decreased and the things that are bad are increased. For example, starting from the far left of Fig. 4, the vasodilator producing endothelial nitric oxide (NO) synthase (eNOS) is decreased, while the vasoconstrictor endothelin-1 is increased. Transforming growth factor-β and plasminogen activator inhibitor-1 are also increased. At the bottom of the figure, the row of black boxes lists the pathological effects that may result from the abnormalities in the open boxes (26–30). Again, how do we know that this piece of

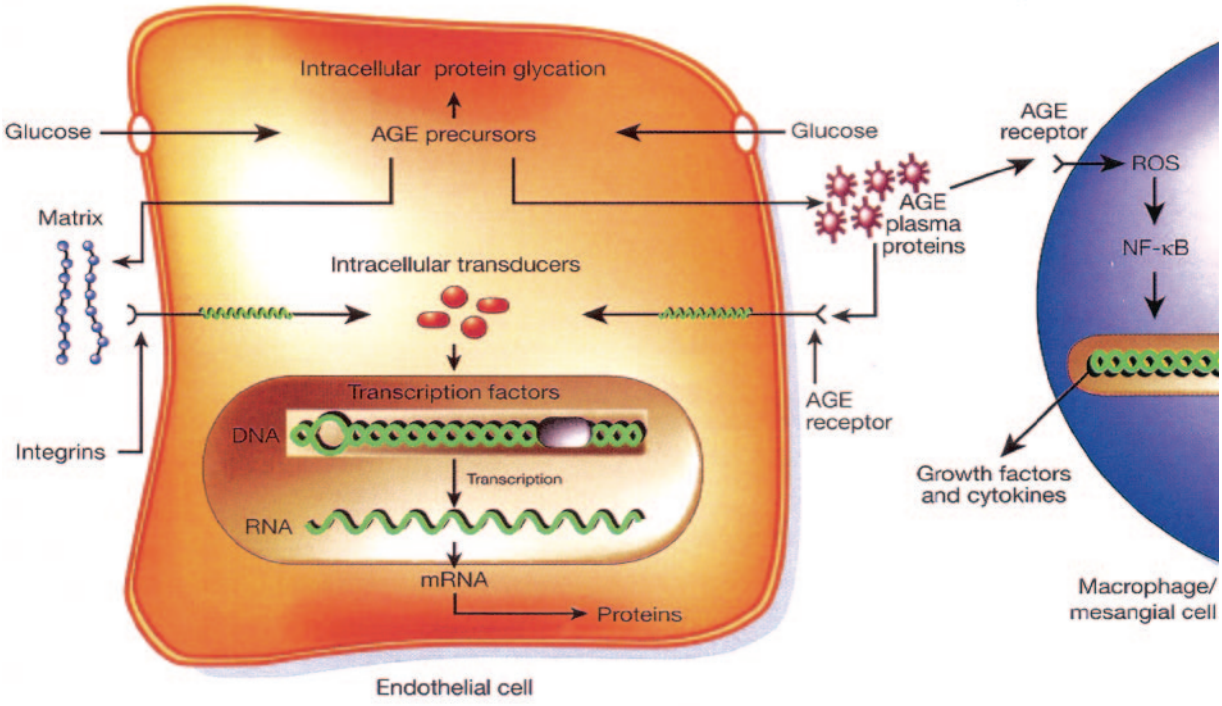

FIG. 3. Increased production of AGE precursors and its pathologic consequences. From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001.

the puzzle is really important? We know that this is important from many animal studies such as several published by George King, showing that inhibition of PKC prevented early changes in the diabetic retina and kidney (27,31,32).

Increased hexosamine pathway activity. The last mechanism on the "pieces of the puzzle" list was increased flux through the hexosamine pathway. As shown schematically in Fig. 5, when glucose is high inside a cell, most of that glucose is metabolized through glycolysis, going first to glucose-6 phosphate, then fructose-6 phosphate, and then on through the rest of the glycolytic pathway. However, some of that fructose-6-phosphate gets diverted into a signaling pathway in which an enzyme called GFAT (glutamine:fructose-6 phosphate amidotransferase) converts the fructose-6 phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) *N*-acetyl glucosamine.

What happens after that is the *N*-acetyl glucosamine gets put onto serine and threonine residues of transcription factors, just like the more familiar process of phosphorylation, and overmodification by this glucosamine often results in pathologic changes in gene expression (33–35). For example, in Fig. 5, increased modification of the

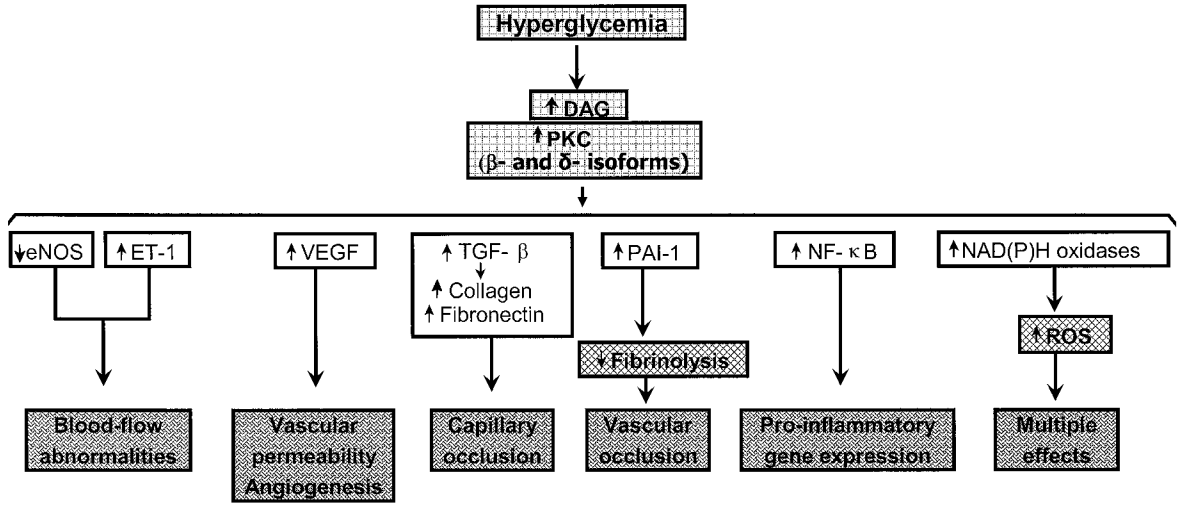

FIG. 4. Consequences of hyperglycemia-induced activation of PKC.

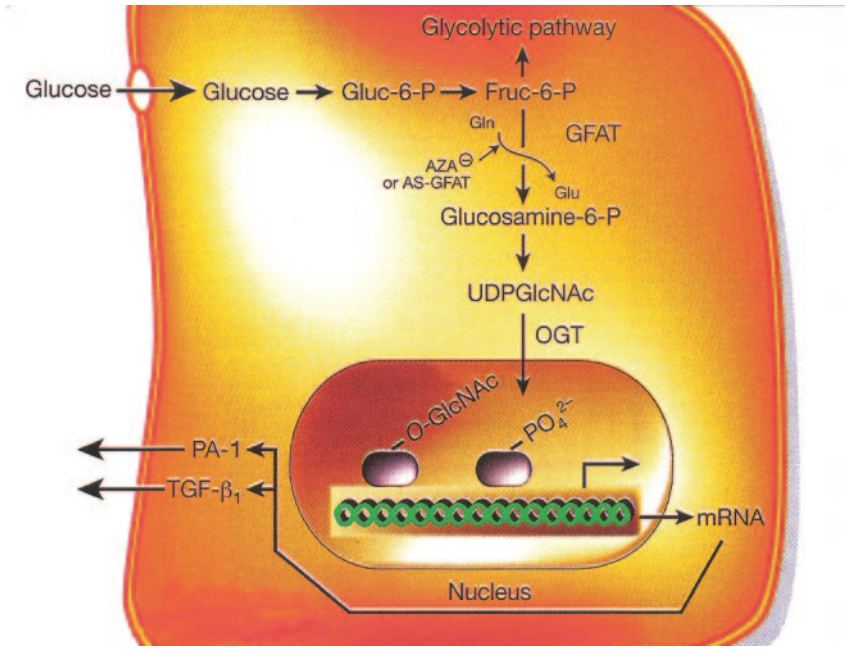

FIG. 5. Hyperglycemia increases flux through the hexosamine pathway. From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001.

transcription factor Sp1 results in increased expression of transforming growth factor-β1 and plasminogen activator inhibitor-1, both of which are bad for diabetic blood vessels (36). Again, how do we know that this piece of the puzzle is really important? Although this piece of the puzzle is the most recent to be recognized as a factor in the pathogenesis of diabetic complications, it has been shown to play a role both in hyperglycemia-induced abnormalities of glomerular cell gene expression (33) and in hyperglycemia-induced cardiomyocyte dysfunction in cell culture (37). In carotid artery plaques from type 2 diabetic subjects, modification of endothelial cell proteins by the hexosamine pathway is also significantly increased (38).

A UNIFIED MECHANISM

Over 13,000 articles published since 1966 seemed to show that all of these pieces of the puzzle were important in the pathogenesis of diabetic complications, yet two things suggested that something major was missing. First, there was no apparent common element linking these mechanisms to each other. Second, clinical trials of inhibitors of these pathways in patients were all disappointing. Trying to make sense of all this, we hypothesized that all of these mechanisms were in fact linked to a common upstream event and that the failure to block all of the downstream pathways could explain the disappointing clinical trials with single-pathway inhibitors. What we discovered is that all of these different pathogenic mechanisms do reflect a single hyperglycemia-induced process and that this single unifying process is the overproduction of superoxide by the mitochondrial electron transport chain.

We began by asking the following question: What processes are increased by intracellular hyperglycemia in cells whose glucose transport rate is not downregulated by hyperglycemia but not increased in cells whose glucose transport rate is downregulated by hyperglycemia?

We discovered that a consistent differentiating feature common to all cell types that are damaged by hyperglycemia is an increased production of reactive oxygen species (ROS) (36,39). Although hyperglycemia had been associated with oxidative stress in the early 1960s (40), neither the underlying mechanism that produced it nor its consequences for pathways of hyperglycemic damage were known.

How does hyperglycemia increase superoxide production by the mitochondria? There are four protein complexes in the mitochondrial electron transport chain, called complex I, II, III, and IV (Fig. 6). When glucose is metabolized through the tricarboxylic acid (TCA) cycle, it generates electron donors. The main electron donor is NADH, which gives electrons to complex I. The other electron donor generated by the TCA cycle is $FADH_2$, formed by succinate dehydrogenase, which donates electrons to complex II. Electrons from both these complexes are passed to coenzyme Q, and then from coenzyme Q they are transferred to complex III, cytochrome-C, complex IV, and finally to molecular oxygen, which they reduce to water.

The electron transport system is organized in this way so that the level of ATP can be precisely regulated. As electrons are transported from left to right in Fig. 6, some of the energy of those electrons is used to pump protons across the membrane at complexes I, III, and IV. This generates what is in effect a voltage across the mitochondrial membrane. The energy from this voltage gradient drives the synthesis of ATP by ATP synthase (41,42). Alternatively, uncoupling proteins (UCPs; Fig. 6) can bleed down the voltage gradient to generate heat as a way of keeping the rate of ATP generation constant.

That's 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 (43), causing the electrons to back up to coenzyme Q, which donates the

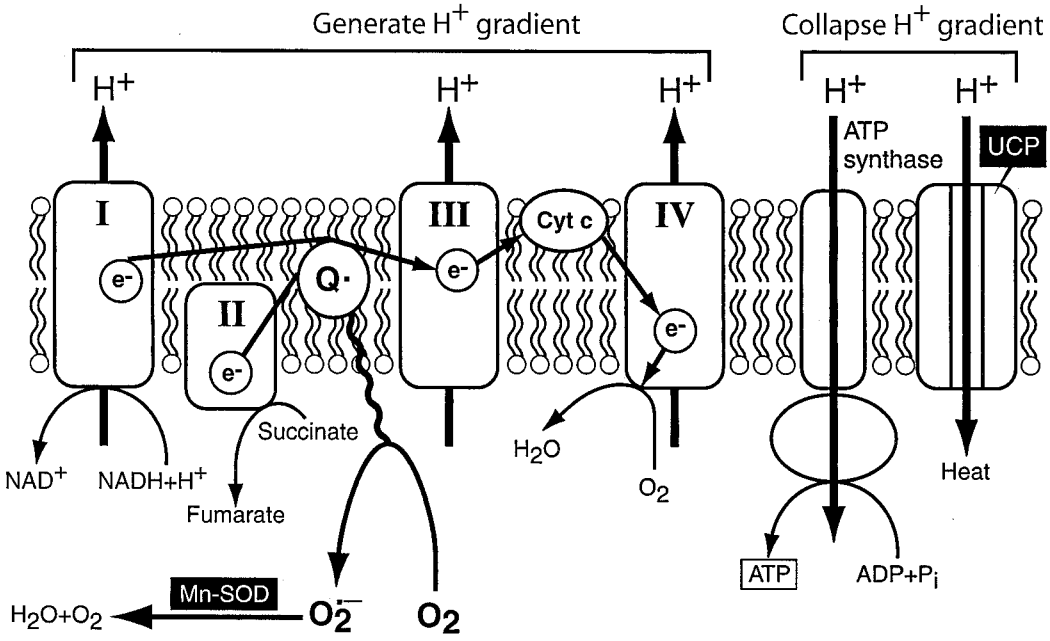

FIG. 6. Hyperglycemia-induced production of superoxide by the mitochondrial electron transport chain.

electrons one at a time to molecular oxygen, thereby generating superoxide (Fig. 6). 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.

How do we know that this really happens in cells known to be damaged by hyperglycemia? First, we looked at such cells with a dye that changes color with increasing voltage of the mitochondrial membrane and found that intracellular hyperglycemia did indeed increase the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation (44). In order to prove that the electron transport chain indeed produces superoxide by the mechanism we proposed, we examined the effect of overexpressing either UCP-1 or manganese superoxide dismutase (MnSOD) on hyperlglycemia-induced ROS generation (Fig. 7*A*). Hyperglycemia caused a big increase in production of ROS. In contrast, an identical level of hyperglycemia does not increase ROS at all when we also collapse the mitochondrial voltage gradient by overexpressing UCP (39). Similarly, hyperglycemia does not increase ROS at all when we degrade superoxide by overexpressing the enzyme MnSOD. These data demonstrate two things. First, the UCP effect shows that the mitochondrial electron transport chain is the source of the hyperglycemia-induced superoxide. Second, the MnSOD effect shows that the initial ROS formed is indeed superoxide.

To confirm these findings by an independent experimental approach, we depleted mitochondrial DNA from normal endothelial cells to form so-called ρ^0 endothelial cells, which lack a functional mitochondrial electron transport chain (Fig. 7*B*). When the mitochondrial electron transport chain is removed, the effect of hyperglycemia on ROS production is completely lost (M.B., unpublished observations). Similarly, in ρ^0 endothelial cells, hyperglycemia completely fails to activate the polyol pathway, AGE

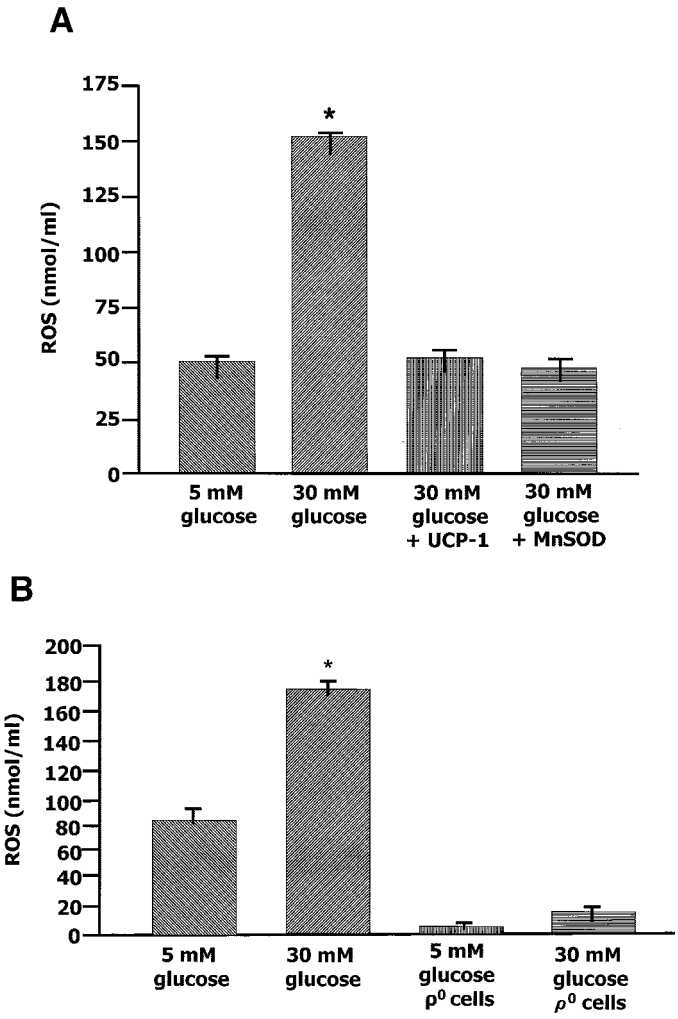

FIG. 7. Hyperglycemia-induced ROS production in wild type and ρ^0 endothelial cells. *A*: From ref. 39. *B*: From M.B., M.H. Zou, X. Du, D. Edelstein, unpublished data.

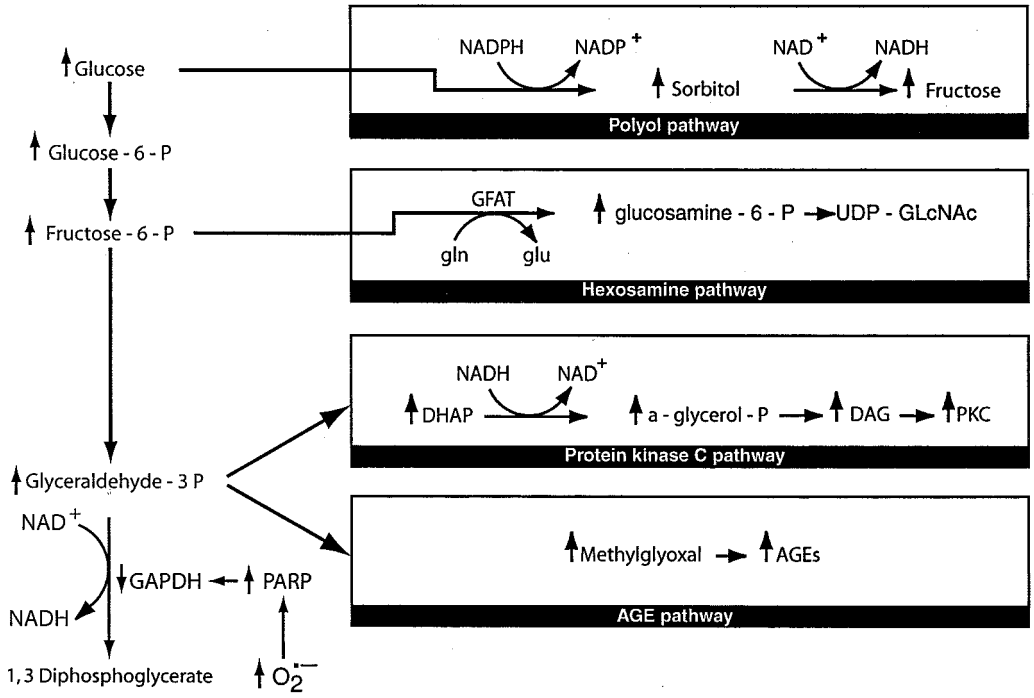

FIG. 8. Mitochondrial overproduction of superoxide activates four major pathways of hyperglycemic damage by inhibiting GAPDH. From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001.

formation, PKC, or the hexosamine pathway (M.B., unpublished observations).

We also looked at the effect of either UCP-1 overexpression or MnSOD overexpression on each of these four hyperglycemia-activated pathways. Hyperglycemia did not activate any of the pathways when either the voltage gradient across the mitochondrial membrane was collapsed by UCP-1 or when the superoxide produced was degraded by MnSOD (39). We have verified all of these endothelial cell culture experiments in transgenic mice that overexpress MnSOD (M.B., unpublished observations). When wild-type animals are made diabetic, all four of the pathways are activated in tissues where diabetic complications occur. In contrast, when MnSOD transgenic mice are made diabetic, there is no activation of any of the four pathways.

In endothelial cells, PKC also activates nuclear factor κB (NFκB), a transcription factor that itself activates many proinflammatory genes in the vasculature. As expected, hyperglycemia-induced PKC activation is prevented by either UCP-1 or MnSOD, both in cells and in animals.

Importantly, inhibition of hyperglycemia-induced superoxide overproduction using a transgenic approach (superoxide dismutase [SOD]) also prevents long-term experimental diabetic nephropathy in the best animal model of this complication: the *db/db* diabetic mouse (45).

Hyperglycemia-induced mitochondrial superoxide production activates the four damaging pathways by inhibiting GAPDH. Figure 8 shows the scheme we proposed for how all of these data link together. This model is based on a critical observation we made: diabetes in animals and patients, and hyperglycemia in cells, all decrease the activity of the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Inhibition of GAPDH activity by hyperglycemia does not occur when mitochondrial overproduction of superoxide is prevented by either UCP-1 or MnSOD (36). What happens when GAPDH activity is inhibited? As shown in Fig. 8, the level of all the glycolytic intermediates that are upstream of GAPDH increase. Increased levels of the upstream glycolytic metabolite glyceraldehyde-3-phosphate activates two of the four pathways. It activates the AGE pathway because the major intracellular AGE precursor methylglyoxal is formed from glyceraldehyde-3 phosphate. It also activates the classic PKC pathway, since the activator of PKC, diacylglycerol, 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, consuming NADPH in the process. To rule out the possibility that any other hyperglycemia-induced metabolic change accounted for these observations, we inhibited GAPDH activity using antisense DNA, so that the level of GAPDH activity in cells cultured in 5 mmol/l (90 mg/dl) glucose was reduced to that normally found in cells exposed to hyperglycemia. With reduced GAPDH activity the only perturbation in these cells, the activity of each of the four pathways in 5 mmol/l glucose was elevated to the same extent as that induced by hyperglycemia (46).

Hyperglycemia-induced mitochondrial superoxide production inhibits GAPDH by activating poly(ADP-ribose) polymerase. At this point, we knew that the way that hyperglycemia activates the four major pathways of hyperglycemic damage is by the overproduction of superoxide by the mitochondria, which then decreases GAPDH activity. In a test tube, superoxide itself directly inactivates GAPDH, but only at concentrations that far exceed levels found in hyperglycemic cells. We therefore asked the following question: In cells and tissues, how do ROS

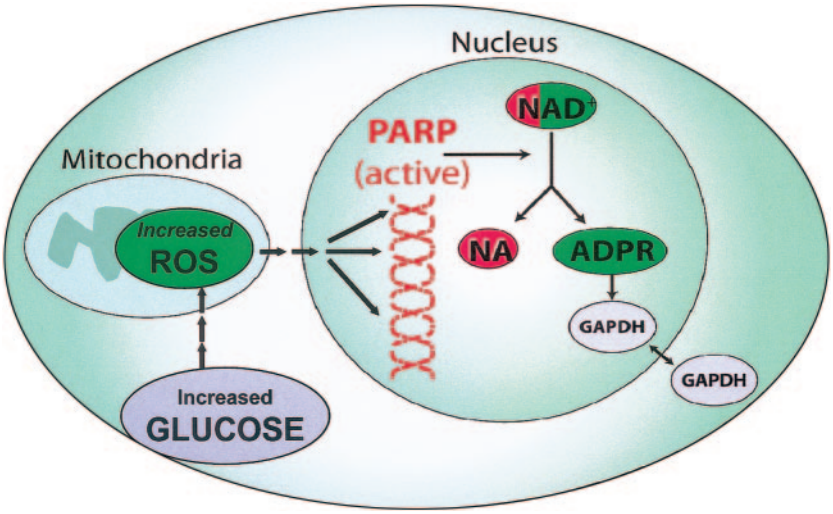

FIG. 9. ROS-induced DNA damage activates PARP and modifies GAPDH.

actually inhibit GAPDH activity? To answer this question, we looked for chemical modifications of GAPDH that correlated with the hyperglycemia-induced decrease in GAPDH activity. As shown in Fig. 9, we found that hyperglycemia-induced superoxide inhibits GAPDH activity in vivo by modifying the enzyme with polymers of ADP-ribose (46). By inhibiting mitochondrial superoxide production with either UCP-1 or MnSOD, we prevented both modification of GAPDH by ADP-ribose and reduction of its activity by hyperglycemia. Most importantly, both modification of GAPDH by ADP-ribose and reduction of its activity by hyperglycemia were also prevented by a specific inhibitor of poly(ADP-ribose) polymerase (PARP), the enzyme that makes these polymers of ADP-ribose. This established a cause-and-effect relationship between PARP activation and the changes in GAPDH.

How does hyperglycemia activate PARP, a DNA repair enzyme that's found exclusively in the nucleus, and how does nuclear PARP get together with GAPDH, a glycolytic enzyme commonly thought to reside in the cytosol?

Normally, PARP resides in the nucleus in an inactive form, waiting for DNA damage to activate it (Fig. 9). When increased intracellular glucose generates increased ROS in the mitochondria, we found that these free radicals induce DNA strand breaks, thereby activating PARP. Both hyperglycemia-induced processes are prevented by either UCP-1 or MnSOD (46). 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. What is GAPDH doing in the nucleus? Although GAPDH is commonly thought to reside exclusively in the cytosol, in fact it normally shuttles in and out of the nucleus, where it plays a critical role in DNA repair (47,48).

A schematic summary showing the elements of the unified mechanism of hyperglycemia-induced cellular damage is shown in Fig. 10. When intracellular hyperglycemia develops in target cells of diabetic complications, it causes increased mitochondrial production of ROS. The ROS cause strand breaks in nuclear DNA, which activate PARP. PARP then modifies GAPDH, thereby reducing its activity. Finally, decreased GAPDH activity activates the polyol pathway, increases intracellular AGE formation, activates PKC and subsequently NFκB, and activates hexosamine pathway flux.

How does the unifying mechanism explain diabetic macrovascular disease? We now had a unifying mechanism that explains the pathogenesis of diabetic microvascular disease. But then we thought: What about diabetic macrovascular disease? In contrast to diabetic microvascular disease, data from the UKPDS have shown that hyperglycemia is not the major determinant of diabetic macrovascular disease. For microvascular disease end points, there is a nearly 10-fold increase in risk as HbA_{1c} increases from 5.5 to 9.5%. In contrast, over the same

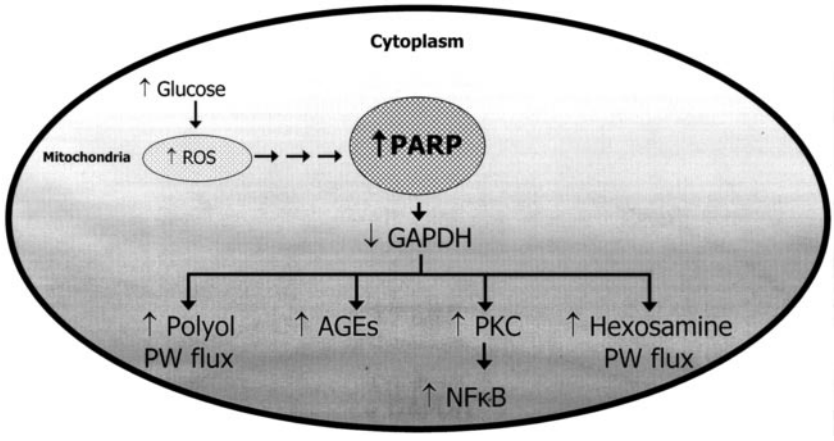

FIG. 10. The unifying mechanism of hyperglycemia-induced cellular damage.

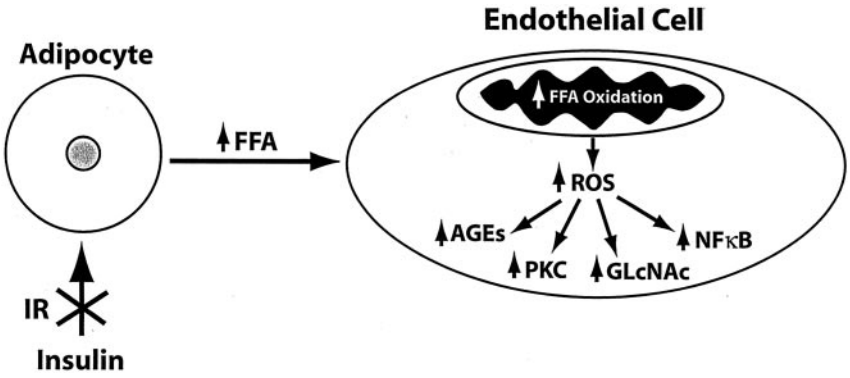

FIG. 11. Insulin resistance causes mitochondrial overproduction of ROS in macrovascular endothelial cells by increasing FFA flux and oxidation. From Hofmann S, Brownlee M: Biochemistry and molecular cell biology of diabetic complications: a unifying mechanism. In *Diabetes Mellitus: A Fundamental and Clinical Text*. 3rd ed. LeRoith D, Taylor SI, Olefsky JM, Eds. Philadelphia, Lippincott Williams & Wilkins, p. 1441–1457, 2004.

HbA_{1c} range, macrovascular risk increases only about twofold (2).

If hyperglycemia is not the major determinant of diabetic macrovascular disease, what about the constellation of risk factors associated with insulin resistance and the metabolic syndrome? In order to separate increased macrovascular disease risk due to insulin resistance and its associated abnormalities from increased risk due to hyperglycemia, the San Antonio Heart Study studied men without diabetes or impaired glucose tolerance (49). Not surprisingly, high insulin resistance increases cardiovascular risk by 2.5-fold. What is surprising, though, is that after adjustment for 11 known cardiovascular risk factors, including LDL, HDL, triglycerides, systolic blood pressure, and smoking, the insulin-resistant subjects still had a twofold increased risk of cardiovascular disease. This suggests that a large part of cardiovascular disease risk due to insulin resistance reflects a previously unappreciated consequence of insulin resistance.

Using both cell culture and animal models, we found that the unappreciated consequence of insulin resistance is increased free fatty acid (FFA) flux from adipocytes into arterial endothelial cells, shown schematically in Fig. 11. In macrovascular, but not in microvascular endothelial cells, we found that this increased flux results in increased FFA oxidation by the mitochondria. Since both β-oxidation of fatty acids and oxidation of FFA-derived acetyl CoA by the TCA cycle generate the same electron donors (NADH and $FADH_2$) generated by glucose oxidation, increased FFA oxidation causes mitochondrial overproduction of ROS by exactly the same mechanism described above for hyperglycemia. And, as with hyperglycemia, this FFA-induced increase in ROS activates the same damaging pathways: AGEs, PKC, the hexosamine pathway (GlcNAc), and NFκB. In insulin-resistant nondiabetic animals models, inhibition of either FFA release from adipocytes or FFA oxidation in arterial endothelium prevents the increased production of ROS and its damaging effects (M.B., unpublished observations).

While more work certainly needs to be done, these data support a major role for the unifying mechanism in the pathogenesis of diabetic macrovascular, as well as microvascular, complications.

NOVEL THERAPEUTIC APPROACHES

In the last part of my talk, I'm going to describe three examples of novel therapeutic approaches for the prevention and treatment of diabetic complications, all based on the new paradigm of a unifying mechanism for the pathogenesis of diabetic complications.

Transketolase activators. The first new class of potential therapeutic agents is transketolase activators. This concept originated from an obvious feature of the unifying mechanism (Fig. 8). When increased superoxide inhibits GAPDH activity, the glycolytic intermediates above the enzyme accumulate and are then shunted into the four pathways of hyperglycemic damage. We noted that 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 (50). Although this pathway is traditionally taught as flowing from pentose phosphates to glycolytic intermediates, in fact it can also flow from glycolytic intermediates to pentose phosphates, depending on the concentrations of substrate presented to the transketolase enzyme. Since we know that in diabetes, the concentration of the glycolytic intermediates is high, we reasoned that if we could activate transketolase, then we could decrease the concentration of these two glycolytic metabolites and thus divert their flux away from three of the damaging pathways normally activated by hyperglycemia.

But how could we activate transketolase? Since this enzyme requires the vitamin thiamine as a cofactor, we tried different thiamine derivatives and measured their effects. While thiamine itself only activated transketolase ~25% in arterial endothelial cells, the thiamine derivative called benfotiamine activated transketolase 250% in arterial endothelial cells. Based on such cell culture experiments, we treated diabetic rats for 9 months with benfotiamine and then evaluated the effect of this treatment in the retina. After 9 months of diabetes, there was a threefold increase in hexosamine pathway activity. In contrast, in diabetic animals treated with benfotiamine, there was a complete prevention of hexosamine pathway activation. The results were identical for diabetes-induced increases in intracellular AGE formation, PKC activation, and NFκB activation. Most importantly, benfotiamine treatment completely prevented the major structural lesion of both human nonproliferative retinopathy and experimental diabetic retinopathy: acellular capillaries (50).

PARP inhibitors. The second new class of potential therapeutic agents based on the unified mechanism is PARP inhibitors. Since we had shown that increased superoxide produced by the mitochondria in response to both hyperglycemia and increased FFA activates PARP,

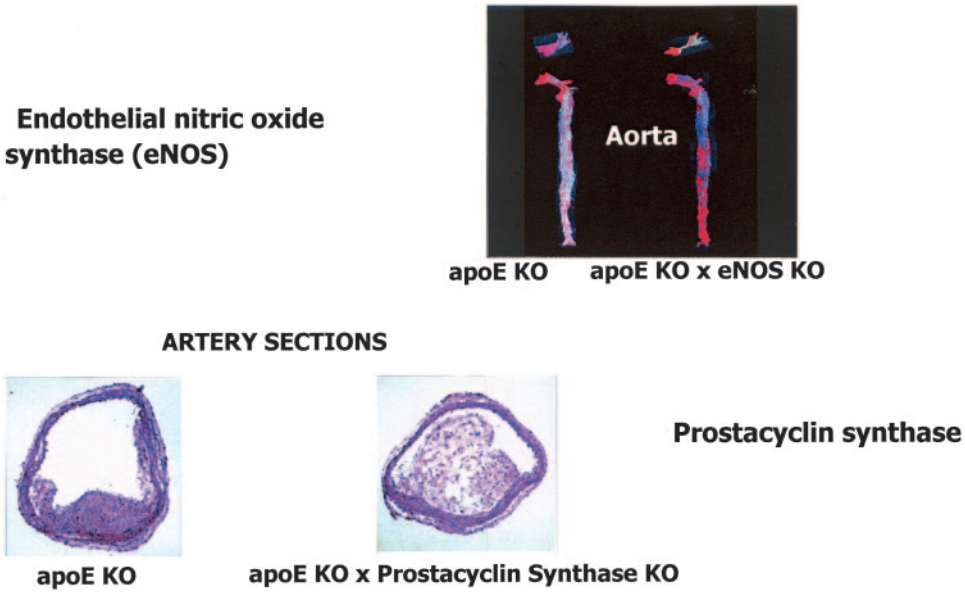

FIG. 12. Excess superoxide independently inhibits activity of two critical antiatherogenic enzymes without involvement of the four pathways of hyperglycemic damage. From refs. 51 and 52.

and that this PARP activation then modifies and inhibits GAPDH (Fig. 9), we predicted that that PARP inhibition would block the four major pathways of hyperglycemic damage that are activated by GAPDH inhibition. In cultured endothelial cells, a specific PARP inhibitor prevents hyperglycemia-induced activation of PKC, NFκB, intracellular AGE formation, and the hexosamine pathway (46). In long-term experimental diabetes, treatment with a PARP inhibitor also completely prevented the major structural lesion of both human nonproliferative retinopathy and experimental diabetic retinopathy: acellular capillaries (H.-P. Hammes, M.B., unpublished observations).

Catalytic antioxidants. Although the four damaging pathways, which I have described as "pieces of the puzzle," have been the major focus of complications research over the past 40 years, 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 (Fig. 12). Both are dramatically inhibited in diabetic patients and diabetic animals.

eNOS is a very important antiatherogenic enzyme with great relevance to diabetic macrovascular disease. This is illustrated in Fig. 12. The upper panel of Fig. 12 shows that in the aorta of a standard mouse model of atherosclerosis, the *apoE* knockout mouse, increased lipid-rich atherosclerotic lesions (red staining) are evident by week 16. However, when the *apoE* knockout mouse is crossed with an *eNOS* knockout mouse, the early atherosclerotic lesions are nearly doubled simply by loss of eNOS activity (51).

Prostacyclin synthase is another critical endothelial antiatherosclerotic enzyme. At the bottom of Fig. 12, long-term mature atherosclerotic plaques are shown in a vessel from an *apoE* knockout mouse and in a vessel from an *apoE* knockout mouse crossed with a prostacyclin synthase knockout mouse. The increase in lesion volume caused by lack of prostacyclin synthase is dramatic and very relevant to diabetic macrovascular disease (52).

To prevent direct oxidative inactivation of these key enzymes, we must directly reduce the amount of superoxide. However, conventional antioxidants are unlikely to do this effectively. Why? 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 (53), that works continuously like the enzymes for which these compounds are named.

Hyperglycemia-induced reactive oxygen overproduction directly reduces eNOS activity in diabetic aortas by 65%. However, when these diabetic animals are treated with an SOD/catalase mimetic, there is no reduction in activity of this antiatherogenic enzyme. 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.

These data strongly suggest that therapeutic correction of diabetes-induced superoxide production may be a powerful new approach for preventing diabetic complications.

CONCLUSION: PERSONAL REFLECTIONS

In this Banting Lecture, I've told you a scientific story about diabetic complications. I'd like to close by telling you a human story about diabetic complications. Paul Abercombie was an acquaintance of mine. Like all of us, he had hopes, fears, and dreams about the future. But unfortunately, Paul was different from many of us here because he also had type 1 diabetes. He did very well for a number of years, but then developed one complication after another, and finally, he died.

Like Paul, I and my family have struggled with diabetes for nearly all of my life. That struggle occupied a large part of my childhood, where everything I ate was weighed and calculated on a gram scale, and the doses of the few insulins that were then available were adjusted without the ability to know what my blood glucose values were. My parents did this despite the fact that most doctors

believed that hyperglycemia had nothing to do with the pathogenesis of diabetic complications, because my parents believed that abnormal glucose must be bad. I am here today, and have accomplished what I have, in large part because of them. As a doctor, I've also had to struggle with the additional burden of knowing too much about diabetic complications. Both my wife Karen and my sister Martha taught me how to fully appreciate each day, despite my knowledge, and they both continue to fill my life with joy and grace.

Today, I have never been more excited, more optimistic, or more certain that we can, and will, solve the remaining important scientific questions about diabetic complications, so that all people with diabetes, now and in the future, can live full lives free of the fears and uncertainties that Paul Abercrombie knew all too well.

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