Role of Glyceraldehyde 3-Phosphate Dehydrogenase in the Development and Progression of Diabetic Retinopathy

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OBJECTIVE—Mitochondrial superoxide levels are elevated in the retina in diabetes, and manganese superoxide dismutase overexpression prevents the development of retinopathy. Superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which activates major pathways implicated in diabetic complications, including advanced glycation end products (AGEs), protein kinase C, and hexosamine pathway. Our aim is to investigate the role of GAPDH in the development and progression of diabetic retinopathy and to elucidate the mechanism.

RESEARCH DESIGN AND METHODS—Rats with streptozotocin-induced diabetes were in a state of poor control (GHb >11%) for 12 months, good control (GHb <7) soon after induction of diabetes, or poor control for 6 months with 6 months' good control. Retinal GAPDH, its ribosylation and nitration, AGEs, and PKC activation were determined and correlated with microvascular histopathology.

RESULTS—In rats with poor control, retinal GAPDH activity and expressions were subnormal with increased ribosylation and nitration (25–30%). GAPDH activity was subnormal in both cytosol and nuclear fractions, but its protein expression and nitration were significantly elevated in nuclear fraction. Reinstitution of good control failed to protect inactivation of GAPDH, its covalent modification, and translocation to the nucleus. PKC, AGEs, and hexosamine pathways remained activated, and microvascular histopathology was unchanged. However, GAPDH and its translocation in good control rats were similar to those in normal rats.

CONCLUSIONS—GAPDH plays a significant role in the development of diabetic retinopathy and its progression after cessation of hyperglycemia. Thus, therapies targeted toward preventing its inhibition may inhibit development of diabetic retinopathy and arrest its progression. ***Diabetes*** **58:227–234, 2009**

Retinopathy is a multifactorial sight-threatening complication of diabetes. It is a progressive disease associated with chronic hyperglycemia (1). Although many glucose-induced retinal metabolic abnormalities are postulated to contribute to its development, the exact mechanism remains elusive (2–5). We have shown that in diabetes, retinal mitochondria experience increased oxidative damage and the mitochondrial enzyme that scavenges superoxide (manganese superoxide dismutase [MnSOD]) prevents vascular histopathology that is characteristic of diabetic retinopathy (6–8). Increased mitochondrial superoxide production inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in vascular endothelial cells, and inhibition of GAPDH is postulated to activate some of the key pathways that are associated with the development of diabetes complications, including increased formation of advanced glycation end products (AGEs) and activation of protein kinase C (PKC) and hexosamine pathway (9,10).

GAPDH is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate. Recent studies have shown that GAPDH is a protein with multiple cytoplasmic, membrane, and nuclear functions and is a major intracellular messenger mediating apoptosis of cells (11,12). GAPDH translocation to the nucleus is considered an important step in glucose-induced apoptosis of retinal Muller cells (13). The mechanism that initiates its translocation is not well understood; covalent modification by nitration/ribosylation is considered a likely possibility (14–16). How GAPDH contributes to the pathogenesis of diabetic retinopathy remains to be established.

Good glycemic control attenuates the development/progression of retinopathy in diabetic patients, but its effects on the progression of retinopathy are not immediate, and it takes years for retinopathy to halt progression after the reestablishment of good control. The imprinted effects of prior glycemic control either produce the long-lasting benefits of good control or resist the arrest of progression of diabetic retinopathy after reinstitution of good control. Reinstitution of good control after a profound period of poor glycemic control does not immediately benefit the progression of retinopathy. This suggests a "metabolic memory" phenomenon (17–20). Metabolic memory phenomenon is observed also in animal models of diabetic retinopathy (21–26); the formation of acellular capillaries, characteristic of early signs of diabetic retinopathy, does not stop for at least 6 months when good control is initiated 6 months after induction of diabetes in rats, and nitrotyrosine levels and oxidative stress remain elevated (24,26). These abnormalities are, however, partially inhibited if the duration of poor control is reduced to 2 months, suggesting the role of oxidative stress in the metabolic memory phenomenon (24). The role of GAPDH in metabolic imprinting remains to be elucidated.

In the present study, we investigated how GAPDH inhibition contributes to the development of retinopathy in diabetes and the mechanism(s) that could result in its inactivation. We have also explored the role of GAPDH in the metabolic memory phenomenon in diabetic rats by maintaining them in a state of poor control before initiation of a state of good control.

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RESEARCH DESIGN AND METHODS

Animals and glycemia. Lewis rats (male, 200 g) were randomly assigned to normal or diabetic groups. Diabetes was induced with streptozotocin (55 mg/kg body wt), and rats were divided at random among three groups according to intended degree of glycemic control. Rats in group 1 remained in poor control for 12 months; in group 2, rats were in poor control for 6 months followed by good control for 6 additional months (poor control–good control); and in group 3, rats were subjected to good control soon after induction of diabetes. Some of the same rats had been used by us in our previous studies (26,27).

The degree of glycemia was achieved by adjusting the dose and frequency of insulin (NPH) administration. The rats in the poor-control group received a single injection of insulin (1–2 units) four to five times a week to prevent ketosis and weight loss, and the rats in good control group received insulin twice daily (7–8 units total) to maintain their blood glucose levels below 150 mg/dl and a steady gain in body weight (24,26). The entire rat colony was housed in metabolism cages: 24-h urine samples were measured daily and tested for glycosuria. Blood glucose was measured once a week using Elite Glucometer (Bayer, Tarrytown, NY), and GHb was measured every 2 months. The entire rat colony received a powdered diet (Purina 5001; TestDiet, Richmond, IN); their food consumption was measured once every week, and body weights were measured two to three times every week. These experiments conformed to the ARVO Resolution on Treatment of Animals in Research and to the specific institutional guidelines. The experiment was terminated 12 months after initiation of diabetes, and the animals were killed by an overdose of pentobarbital. Eyes were enucleated immediately; one eye was fixed in 10% buffered formalin, and from the other eye, the retina was immediately isolated by gently separating sensory retina from choroid using a microspatula.

Preparation of subcellular fractions. Retina was gently homogenized in a glass homogenizer in 50 mmol/l glycyl glycine buffer (pH 7.0) containing 10 mmol/l EDTA, 100 mmol/l sodium fluoride, 0.5 mmol/l dithiolthreitol, and protease inhibitors. The homogenate was centrifuged at 750*g* for 5 min, and the supernatant was centrifuged at 5,000*g* for 15 min. The nuclear pellet was resuspended in 50 mmol/l HEPES buffer (pH 7.5) containing 1% Triton X-100, 150 mmol/l sodium chloride, 1 mmol/l EDTA, and protease inhibitors. The supernatant was further centrifuged at 105,000*g* for 90 min to separate the cytosolic fraction (13,28). The purity of the fractions was determined by measuring the expressions of Histone 2-B (nuclear marker) and lactate dehydrogenase (LDH; cytosolic marker).

GAPDH enzyme activity. The enzyme activity was measured spectrophotometrically in a final assay volume of 100 μl containing 50 mmol/l triethanolamine buffer (pH 7.6), 50 mmol/l arsenate, 2.4 mmol/l glutathione, 250 μmol/l NAD, and cytosolic/nuclear protein (0–10 μg protein). The assay mixture was preincubated for 5 min at 37°C, and the reaction was initiated by 100 μg/ml glyceraldehyde-3-phosphate. Increase in NADH production was monitored at 340 nm. GAPDH activity was expressed as difference in absorbance in the presence/absence of glyceraldehyde-3-phosphate (28).

Quantitative real-time PCR. RNA was isolated from the retina using TRIzol reagent, and 1 μg RNA was converted to single-stranded cDNA and quantified spectrophotometrically. Gene expression was measured using 90- to 300-ng cDNA templates in 96-well plates in ABI-7500 sequence detection system (29,30). Each sample was analyzed in triplicate, and the data were normalized to β2-microglobulin (B2M) expression in each sample. GenBank accession numbers for the ABI TaqMan assays for GAPDH and B2M used were NM_017008.3 and NM_012512.1, respectively. The fold change in gene expression relative to normal was calculated using the delta delta cycles to threshold (ddCT) method.

Protein expression. Protein (15–30 μg) was separated by SDS-PAGE on a 4–16% gradient gel and blotted to nitrocellulose membrane. The membranes were blocked in 5% nonfat milk, incubated with the target primary antibody, washed, and followed by incubation with appropriate horseradish peroxidase–coupled secondary antibody. The target proteins were enhanced by ECL reagent and determined by autoradiography. The membranes then were stripped and reprobed with β-actin (Sigma-Aldrich, St. Louis, MO). The band intensity was quantified using Un-Scan-It Gel digitizing software (Silk Scientific, Orem, UT), and protein expression levels were calculated relative to β-actin in the same sample. Activity of poly(ADP-ribose) polymerase (PARP) was determined by measuring poly(ADP-ribosyl)ation of retinal proteins by separating them on SDS-PAGE gel.

Ribosylation and nitration of GAPDH. Covalent modification of GAPDH was determined by first immunoprecipitating protein (75–100 μg) with polyclonal anti-GAPDH antibody. Protein A/G plus agarose beads were used to collect GAPDH complexed with the antibody and analyzed by Western blot technique using monoclonal antibodies against poly(ADP-ribose) (PAR; Alexis Biochemicals, San Diego, CA) and nitrotyrosine (Upstate Biotechnology, Lake Placid, NY). To normalize for equal loading in each lane, the membranes were reprobed for GAPDH.

TABLE 1
Degree of glycemia in rats assigned to different states of glycemic control

	n	Duration (months)	Body wt (g)	GHb (%)	Urine volume (ml/24 h)
Normal	9	12	421 ± 35	6.7 ± 0.8	13 ± 6
PC	13	12	289 ± 37	12.7 ± 1.7	112 ± 39
GC	7	12	454 ± 41	6.9 ± 1.1	26 ± 10
PC	11	6	272 ± 27	13.1 ± 1.6	135 ± 19
↓		↓	↓	↓	↓
GC		6	409 ± 26	7.1 ± 0.9	19 ± 11

Data are means ± SD. The rats were weighed two times every week, and their food consumption was measured once every week. Body weight is the mean value during the entire duration of the intended metabolic control.

Quantification of GAPDH-mediated pathways. Because inhibition of GAPDH activates major pathways that are implicated in the development of diabetes complications, we investigated the effect of reversal of hyperglycemia on AGEs, PKC, and hexosamine pathways in the retina from the same set of animals used for GAPDH. Total AGEs formation was determined by Western blot using anti-AGE antibody (Wako Chemicals, Richmond, VA). PKC activation was determined by quantifying the expression of PKC βII, the isoform that is activated in diabetes, as we previously described (23).

Because addition of single *O*-linked ß-*N*-acetylglucosamine (O-GlcNAc) monosaccharides to serine or threonine residues on proteins is one of the processes coupled to the hexosamine pathway, we assessed hexosamine pathway by quantifying *O*-linked *N*-acetylglucosamine–modified proteins in the retina by Western blot using monoclonal antibody against O-GlcNAc (Covance, Princeton, NJ). BSA was used as a blocking medium (30).

Isolation of retinal microvessels and quantification of acellular capillaries. Microvessels were prepared from formalin-fixed eyes by trypsin digestion. The retina was isolated, rinsed in water overnight, and incubated with 3% crude trypsin containing 0.2 mmol/l sodium fluoride for 90 min at 37°C. Nonvascular cells were removed by gentle brushing, and the isolated vasculature was dried onto a microscope slide. The slides were stained with hematoxylin and periodic acid Schiff. The number of acellular capillaries (representing basement membrane tubes lacking cell nuclei) was counted in a masked manner in multiple midretinal fields with one field adjacent to each of the five to seven retinal arterioles radiating out from the optic disc and expressed as per square millimeter of retinal area examined (5,8,26).

Results are presented as means ± SD and analyzed statistically using the nonparametric Kruskal-Wallis followed by Mann-Whitney *U* test for multiple group comparison. Similar conclusions were achieved by using ANOVA with Fisher or Tukey tests.

RESULTS

Severity of hyperglycemia. Hyperglycemia, as reported previously (26), was severe in the rats in the poor-control group; GHb values were >11% throughout the entire duration of the experiment (12 months). The rats in the good-control group maintained their GHb values, which were similar to those in normal rats. In the poor control–good control group, GHb values before initiation of good control were not different from the poor-control group (GHb >11%) but became similar to those in the normal group after initiation of good control (GHb <7%) (Table 1). Average body weight and 24-h urine volumes were similar in good-control rats and normal rats.

Effect of diabetes on retinal GAPDH. Twelve months of poor control had a marginal but statistically significant effect on the expression of GAPDH in the retina; protein expression was decreased by 15–20% (Fig. 1) and gene expression by ~20% (Fig. 2) in diabetic rats.

Effect of diabetes on activation of retinal PARP. As shown in Fig. 3*A*, 12 months of poor control significantly increased poly(ADP-ribosyl)ation of retinal proteins

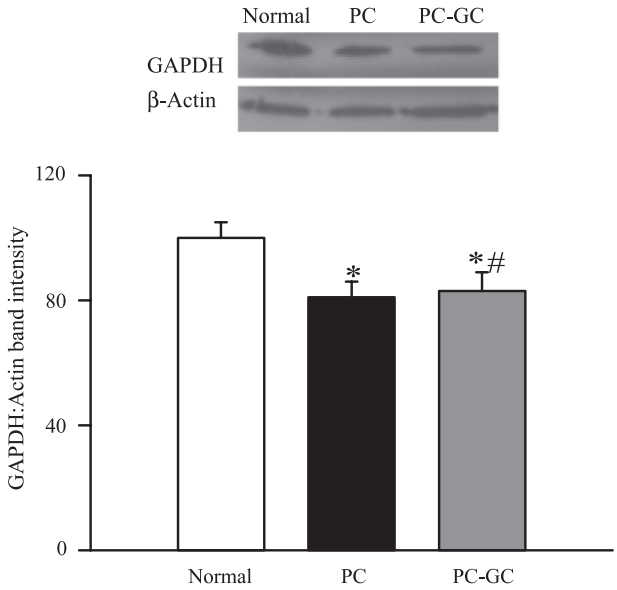

FIG. 1. Protein expression of GAPDH in diabetes and effect of reversal of glycemic control. GAPDH expression was determined in the retinal homogenate by Western blot technique using rabbit polyclonal GAPDH antibody. Equal loading of the sample in each lane was ensured by determining the expression of β-actin. The Western blots shown here are representative of at least five different rats in each group, and the bars represent the means ± SD of the adjusted band intensities obtained from those rats.

compared with that in normal rats, suggesting an increase in PARP activity. We did not, however, identify these ribosylated proteins (other than GAPDH, please see below), and that is beyond the focus of this study.

Subcellular translocation of GAPDH and its covalent modification. Because retinal cell apoptosis precedes the development of diabetic retinopathy (31,32), we investigated the effect of diabetes on subcellular translocation of GAPDH in the retina. In normal rat retina, the protein expression of GAPDH was 35% higher in the cytosolic fraction compared with the nuclear fraction, but 12 months of poor control in rats resulted in reduction in its expression in the cytosol fraction, with a concomitant increase in the nuclear fraction (Fig. 4). The ratio of GAPDH expression in cytosolic and nuclear fraction was almost 4:1 in normal rat retina and decreased to 1.2:1 in diabetic rat retina. In the same diabetic rats, despite increased expression of GAPDH, the glycolytic activity was decreased by ~70% in the nuclear fraction (Fig. 5*A*). The good-control group had expressions similar to those of the normal control rats (data not shown).

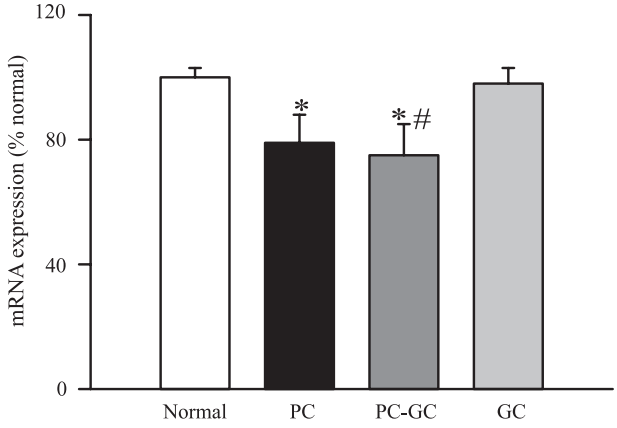

FIG. 2. Gene expression of GAPDH in the retina and effect of reinstitution of good glycemic control. Retinal gene expression was measured with quantitative real-time PCR, and the values were normalized to the expression of the housekeeping gene *B2M* in the same sample. The value obtained from the retina of age-matched normal rats is considered to be 100%. Data represent the means ± SD from 8–10 rats in each of the four groups. *$P < 0.05$ compared with normal; #$P > 0.05$ compared with poor control.

Ribosylation and nitration have been shown to inhibit GAPDH activity (15,33). We determined the levels of its ribosylation and nitration of retinal GAPDH. Twelve months of poor control resulted in an ~25% increase in ribosylation of GAPDH (Fig. 3*B*) and a 30% increase in nitration compared with the normal rat retina (Fig. 3). The rats in the good-control group had expressions similar to those of the normal-control rats (data not shown). The ratio of nitrated GAPDH was 2.5:1 in normal rat retina and increased to almost 1:1 in the poor-control group. However, in the nuclear fraction, nitrated GAPDH was about twofold higher in poor-control rats compared with normal rats (Fig. 5*B*), strongly suggesting that the enzyme in nuclear fraction is mainly in its covalently modified and inactivated form.

Effect of reversal of hyperglycemia on GAPDH. When diabetic rats were allowed to remain in poor control for 6 months before institution of good control, the protein and gene expressions of retinal GAPDH remained subnormal, and PARP remained activated; values obtained in poor-control and poor control–good control groups were not different from each other (Figs. 1, 2, and 3*A*). In addition, both ribosylation and nitration of GAPDH were also not different in the retina from the poor-control group and poor control–good control group, suggesting that 6 months of good control had no beneficial effect on the covalent modification of the enzyme (Fig. 3*B* and *C*). The expression and activity of GAPDH in nuclear fraction of retina from the poor control–good control group were significantly different from those in normal rats, and the enzyme remained nitrated (Figs. 4 and 5). However, these values were similar to those obtained from the poor-control group, suggesting that 6 months of good control did not prevent the enzyme from translocating to the nucleus and thus failed to protect the retina from apoptosis. But when the rats were maintained in good control soon after induction of diabetes (good-control group), GAPDH gene expression was similar to that obtained from normal rat retina (Fig. 2).

Effect of reversal of hyperglycemia on GAPDH-mediated pathways. As shown in Fig. 6*A*, multiple protein bands with increased AGEs were observed in the retina from rats in the poor-control group compared with those from normal rats, and protein staining from the rats in the poor control–good control group was similar to those obtained from the rats in the poor-control group. We, however, did not identify the retinal proteins that had increased AGEs. In the same retina samples, reversal of hyperglycemia had no effect on increased PKCβII (Fig. 6*B*); the enzyme expression remained significantly elevated in both the poor-control and the poor control–good control groups compared with that in the normal group of rats ($P < 0.05$). Similarly, 6 months of good control did not produce any reduction in O-GlcNAcylation of retinal proteins (data not shown), confirming that reversal of hyperglycemia had no beneficial effect on GAPDH-mediated downstream and upstream signaling pathways.

Effect of reversal of hyperglycemia on the retinal histopathology. Poor glycemic control in rats for 12 months increased the number of acellular capillaries in the

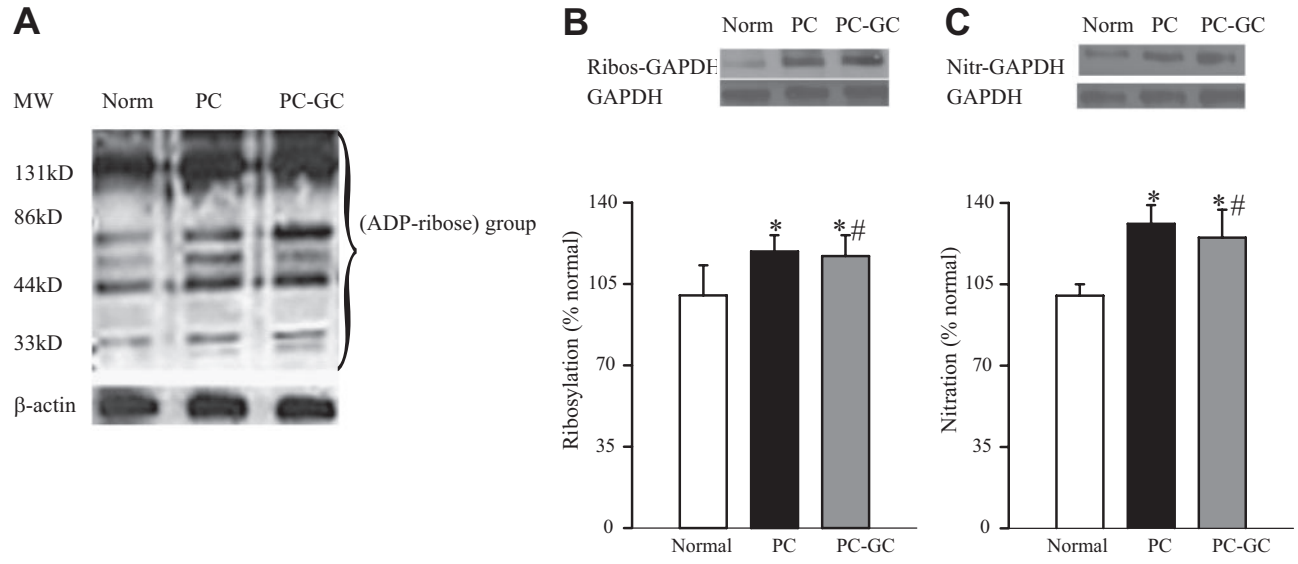

FIG. 3. PARP activity in the retina and covalent modification of GAPDH. *A*: PARP activity was determined in the retinal extract by Western blot technique. Poly(ADP-ribosyl)ated proteins were detected using antibody obtained from Santa Cruz Biotechnology. To determine covalent modification of GAPDH, it was immunoprecipitated from retinal proteins and analyzed by Western blot technique using monoclonal antibodies against either PAR or nitrotyrosine. To ensure equal loading, the membranes were reprobed for GAPDH. The histograms represent ribosylation (*B*) or nitration (*C*) of retinal GAPDH from five to six rats in each group, and the values from normal rat retina are considered to be 100%. *$P < 0.05$ compared with normal; #$P > 0.05$ compared with poor control.

retinal vasculature (Fig. 7) by about fourfold compared with that in normal rats. The 6 months of good control that followed 6 months of poor control failed to provide any protection; the number of acellular capillaries was similar in poor-control and poor control–good control rats (average number of acellular capillaries per millimeter squared of retina in rats in the normal, poor-control, and poor control–good control groups: 1.5, 6.1, and 6.8, respectively).

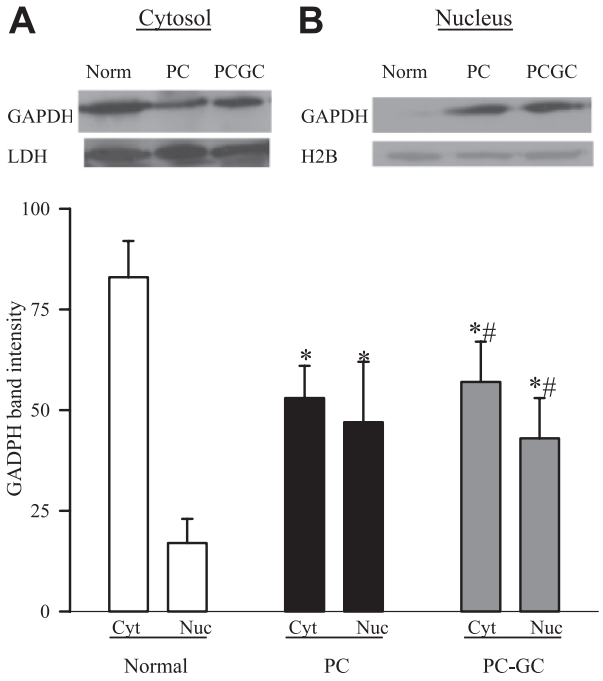

FIG. 4. Effect of diabetes on subcellular localization of GAPDH. Subcellular fractionation was performed on retinal homogenate by centrifugation. GAPDH expression was determined by Western blot. Histone 2B and LDH were used to determine the purity of nuclear and cytosolic fractions, respectively. The histogram represents the relative expression of GAPDH in cytosolic and nuclear fractions, and the total expression of GAPDH in cytosol and nuclear fraction is considered to be 100%. The values obtained are means ± SD from four or more rats in each of the four groups. *$P < 0.05$ compared with normal; #$P > 0.05$ compared with poor control.

DISCUSSION

GAPDH, a classic glycolytic enzyme, is implicated in diverse cytoplasmic, membrane, and nuclear activities and has been shown to play a significant role in cell death (12), and its inhibition is considered to activate major pathways of endothelial cell damage, including activation of PKC, hexosamine pathway flux, and AGE formation (9,10). Here, we show that diabetes inhibits GAPDH activity in the retina, and its expression becomes subnormal. The enzyme is translocated from cytosol to the nucleus, and GAPDH-mediated downstream and upstream signaling pathways (AGEs, PKC, and the hexosamine pathway) are activated. Our data suggest that the enzyme translocated to the nuclear fraction is covalently modified. Furthermore, we provide exciting data demonstrating that reinstitution of good control after 6 months of poor control does not produce any beneficial effects on the inactivation of retinal GAPDH. The enzyme remains inactive with its expression elevated in the nucleus, suggesting that inhibition of GAPDH and its subcellular translocation resist reversal after reinstitution of good control. Reinstitution of good control also fails to provide any benefit to the covalent modification; the elevated levels of both ribosylation and nitration are sustained for at least 6 months after reversal of poor control. In the same animals the signaling pathways that are in direct control of GAPDH remain activated in the retina, and the number of acellular capillaries remain elevated after reestablishment of good control. These novel and exciting observations strongly suggest a role for GAPDH in the development and progression of diabetic retinopathy.

Although others have reported decreased retinal GAPDH activity at 3 months of diabetes duration in rats (34), ours is the first report showing that the enzyme remains inhibited at duration of diabetes in rats when signs of retinopathy can be detected. High glucose de-

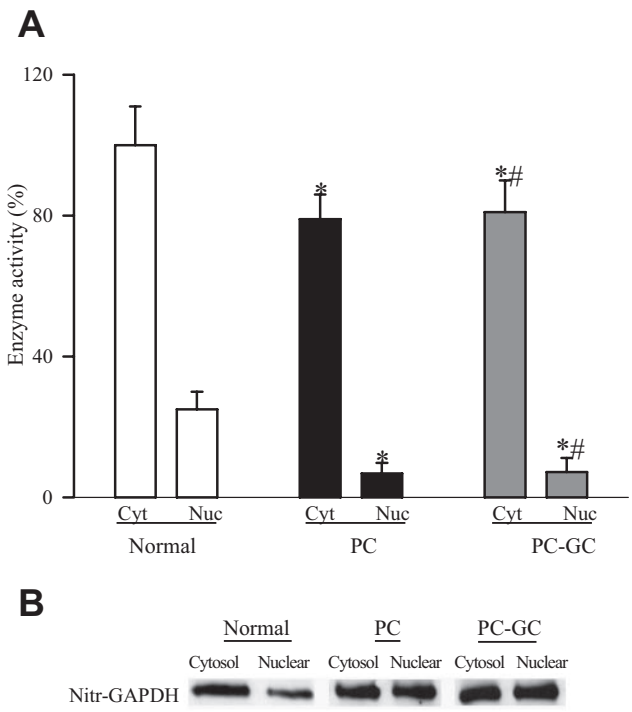

FIG. 5. Glycolytic activity and covalent modification of GAPDH in cytosolic and nuclear fractions. *A*: The enzyme activity of GAPDH was measured in the cytosolic and nuclear fractions of the retina by measuring the increase in the production of NADH at 340 nm. Each measurement was performed in duplicate, and assay was repeated three or more times. The activity obtained in the cytosol obtained from normal rat retina is considered to be 100%. The values are means ± SD from at least six rats in each of the four groups. *$P < 0.05$ compared with normal; #$P > 0.05$ compared with poor control. *B*: Nitration of GAPDH was performed by immunoprecipitating GAPDH from the cytosolic and nuclear fractions of the retina, followed by separation on SDS-Gel. The nitrated GAPDH was identified using antibody against nitrotyrosine from Upstate Biotechnology. The blots are representative of four to five rats in each group.

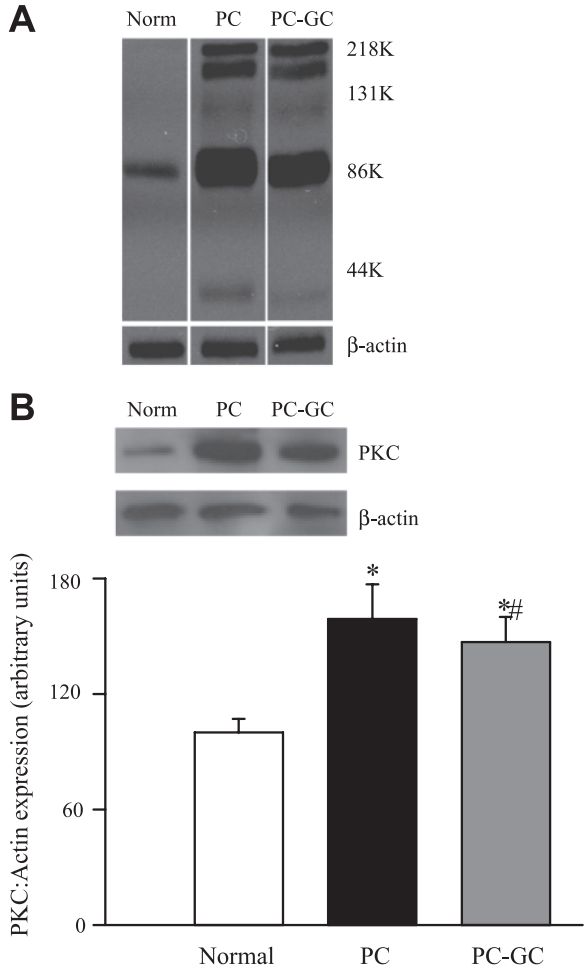

FIG. 6. Activation of AGEs and PKC in retina. Total AGEs (*A*) and PKC activation (*B*) were measured in the retinal homogenate by Western blot technique using antibodies against anti-AGE and PKC βII, respectively. β-Actin was used as a loading standard. These blots are representative of four or more rats in each group.

creases GAPDH in vascular cells, putatively because of overproduction of superoxide by mitochondrial electron transport chain (9). Retinal mitochondria are dysfunctional in diabetes, and superoxide levels are elevated (7,8,35); and complex III is considered one of the sources of increased superoxide (8). Overexpression of MnSOD that is shown to prevent glucose-induced inhibition of GAPDH in vascular cells (9) also prevents the development of retinopathy in diabetic mice (8). Thus, taken together, data strongly implicate the role for GAPDH in the pathogenesis of retinopathy in diabetes.

Mitochondrial superoxide break DNA strand and activate PARP, and PARP-mediated poly(ADP-ribosyl)ation of GAPDH is considered one of the mechanisms in the inhibition of GAPDH activity in hyperglycemic conditions (14). Our data show that PARP activity is significantly increased in the poor-control group, and this is in accordance with another published report (36). Increased PARP activity plays an important role in diabetes-induced retinal capillary cell death, and inhibitors of PARP prevent retinal leukostasis, oxidative stress, and retinopathy in diabetic rats (36–39). Here, we show that increased ribosylation of retinal GAPDH could be one of the mechanisms responsible for its inactivation.

GAPDH is susceptible to nitration by peroxynitrite (formed by reaction between nitric oxide and superoxide); steady-state exposure of GAPDH to low doses of peroxynitrite in rat astrocytes results in its inhibition (15,16). GAPDH is also a target for inactivation by nitric oxide in endothelial cells (40), which are some of the microvascular cells in the retina that present pathology of diabetic retinopathy. Nitric oxide and peroxynitrite levels are elevated in the retina and its capillary cells, and these levels remain elevated at duration when vascular histopathology characteristic of retinopathy is developing in diabetic rats (5,6,25,41–43). Our results clearly show that diabetes increases nitration of retinal GAPDH, implying that nitration is associated with its inhibition. In support, covalent modification of GAPDH by nitration is also observed in other pathological conditions associated with inflammation (44), and diabetic retinopathy is believed to be a low-grade chronic inflammatory disease (45–48). Peroxynitrite itself damages DNA and triggers activation of PARP (49); we clearly show that ribosylation of GAPDH is also increased in diabetes. Furthermore, reduction in retinal GAPDH expression (gene and protein) suggests that, in addition to its covalent modification, the gene transcript of GAPDH is decreased in diabetes.

GAPDH is also a major intracellular messenger that mediates cell death via apoptosis. Covalent modification of GAPDH is suggested to trigger its translocation from cytosol to the nuclear fraction (12,13). During nuclear translocation, its activity is lost (50), and an increase in hydrophobicity due to its nitration is being postulated as

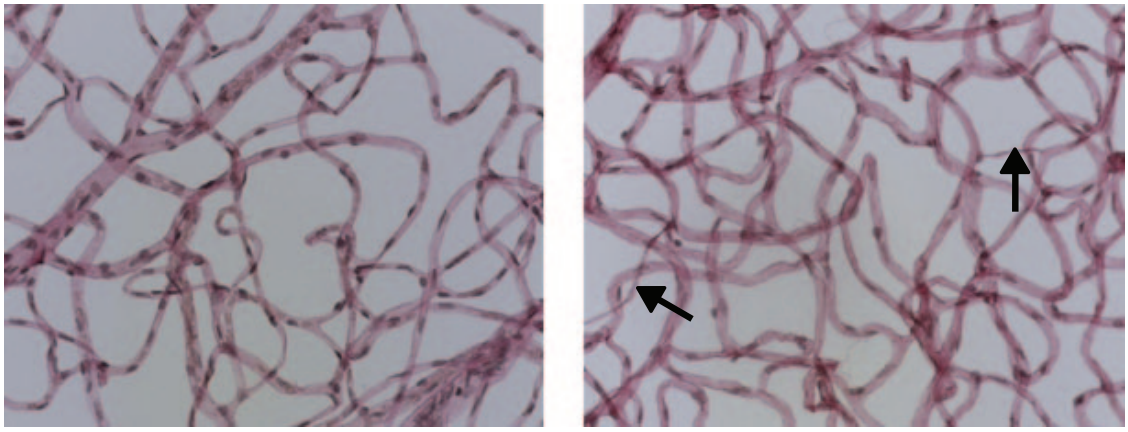

FIG. 7. Histopathology in retinal microvasculature of normal rats (*left panel*) and those in a state of poor control (*right panel*). Trypsin-digested retinal microvasculature was stained with periodic acid Schiff and hematoxylin. The number of acellular capillaries was counted in multiple midretinal fields and standardized to retinal area (per square millimeter). The arrows indicate acellular capillaries in the trypsin-digested microvessels obtained from a rat that was maintained in poor control for 12 months. (Please see http://dx.doi.org/10.2337/db08-1025 for a high-quality digital representation of this figure.)

one of the mechanisms (16). Nuclear translocation of GAPDH is suggested to play a role in retinal glial cell apoptosis in hyperglycemic conditions (13), and increased apoptosis of retinal capillary cells precedes the development of retinal pathology associated with diabetic retinopathy (32). Our results show that the expression of GAPDH is increased in nuclear fraction in the retina from diabetic rats compared with normal rats; however, the enzyme in the nuclear fraction appears to be in a more nitrated and inactivated state compared with the cytosol fraction. This implies that although diabetes increases GAPDH translocation to the nucleus, it is covalently modified before being translocated from the cytosol and is in an inactivated state.

Reinstitution of good control after 6 months of poor control failed to produce any significant beneficial effects on GAPDH, but if good control was initiated soon after induction of diabetes in rats and allowed to continue for 12 months, GAPDH remained similar to that obtained from age-matched normal rats. This strongly supports that in addition to being a central player in the development of diabetic retinopathy, GAPDH is important in the metabolic memory phenomenon. Reversal of hyperglycemia failed to provide any benefit to the activation of PARP; the enzyme remained activated even after 6 months of good control. Sustained increases in nitration and ribosylation of retinal GAPDH after reversal of hyperglycemia in rats suggests that good control fails to provide any benefit to the covalent modifications of the enzyme. Furthermore, good control did not prevent translocation of the retinal enzyme from cytosol to the nucleus, suggesting that GAPDH remains proapoptotic even after good control is reestablished. Because of tissue availability, apoptosis of retinal capillary cells was not measured in this study; however, in support of continued apoptosis, our previous studies have shown that the apoptosis execution enzyme caspase-3 remains active in the retina even after reversal of hyperglycemia in rats. The process of caspase-3 activation that starts before the appearance of retinal histopathology resists reversal by reinstitution of good control (25), and nuclear accumulation of covalently modified GAPDH could be one of the important factors associated with increased apoptosis and histopathology of diabetic retinopathy.

Inhibition of GAPDH increases the levels of glycolytic metabolite glyceraldehyde 3- phosphate, and this activates the major pathways implicated in diabetes complications, including AGEs formation, PKC activation, and hexosamine pathway (10). Here, we show that the reversal of hyperglycemia in rats, in addition to failing to provide any benefit to the inhibition of retinal GAPDH and its nuclear translocation, has no significant effect on GAPDH-mediated pathways. Thus, both the downstream and upstream consequences of GAPDH inhibition persist for at least 6 months after hyperglycemia is terminated, further strengthening the role of GAPDH in the development/progression of diabetic retinopathy.

Data presented here clearly show that the rats that presented no effect of reversal of hyperglycemia on GAPDH and its translocation to the nucleus also showed no effect on the development of retinopathy; the number of acellular capillaries remains comparable in the rats in the poor-control and poor control–good control groups. This confirms that GAPDH-mediated pathways and the process of apoptosis of retinal capillary cells that begins before histopathology and can be detected in the retinal vasculature (25) continue to progress even after hyperglycemia is terminated. The analyses of GAPDH, its translocation and covalent modification, and the consequences of its inhibition on the pathways were performed in the whole-retina samples, and this approach did not allow us to identify the specific cell type. The failure to reverse the development of retinal vascular histopathology by reinstitution of good control, however, strongly suggests that GAPDH has a significant role in the development and progression of diabetic retinopathy.

In conclusion, we have provided strong evidence demonstrating that GAPDH is inhibited and its downstream and upstream signaling pathway activated in the retina in diabetes at a duration when histopathology characteristic of retinopathy can be observed in rats. The covalent modification of the enzyme is increased. Diabetes accelerated translocation of retinal GAPDH into the nucleus, suggesting that its proapoptotic nature possibly contributes to the development of diabetic retinopathy. In addition to its role in the pathogenesis of diabetic retinopathy, GAPDH is also important in the metabolic memory phenomenon. Sustained nitration and ribosylation are possi-

ble mechanisms for resistance of GAPDH to reverse inhibition after reversal of hyperglycemia. Therapies targeted toward preventing GAPDH inhibition by blocking its covalent modification should help in the development and also in arresting the progression of diabetic retinopathy, a sight-threatening complication of diabetes.

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