Glyceraldehyde-3-Phosphate Dehydrogenase in Retinal Microvasculature: Implications for the Development and Progression of Diabetic Retinopathy

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Purpose. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been hypothesized as a mediator in the activation of multiple pathways implicated in the pathogenesis of diabetic retinopathy. The objective of this study was to understand the mechanism by which high glucose inactivates GAPDH in retinal microvascular cells.

Methods. Bovine retinal endothelial cells (BRECs), transfected with GAPDH, were incubated in 20 mM glucose. The effect of the overexpression of GAPDH on its activity, apoptosis, and upstream signaling pathways, protein kinase C, and hexosamine pathways was determined. The effect of the inhibitors of nitration and ribosylation on GAPDH activity, its nuclear translocation and reversal of glucose insult was also evaluated.

Results. High glucose decreased GAPDH activity, expression, and nuclear translocation. Overexpression of GAPDH prevented glucose-induced inhibition of its activity, nuclear translocation, apoptosis, and activation of protein kinase C and hexosamine pathways. Inhibitors of nitration and ribosylation ameliorated glucose-induced inhibition of GAPDH, and their addition during the normal glucose exposure that followed high glucose levels had a beneficial effect on GAPDH activity and the degree of nitration and ribosylation.

Conclusions. In hyperglycemia, GAPDH in retinal microvascular cells is inhibited by its covalent modifications, and this activates multiple pathways implicated in the pathogenesis of diabetic retinopathy. The agents that can directly target modification of GAPDH have potential in inhibiting the development and in arresting the progression of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2010;51:1765–1772) DOI: 10.1167/iovs.09-4171

Retinopathy is one of the most severe ocular complications of diabetes. Multiple effector pathways have been implicated in the pathogenesis of diabetic retinopathy, including activation of the hexosamine and protein kinase C (PKC) pathways, formation of advanced glycation end products (AGEs), and activation of the polyol pathway,[1] but the exact mechanism remains elusive.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is considered to provide a common link between hyperglycemia and activation of some of the major pathways associated with the pathogenesis of diabetic complications.[2,3] In addition to serving as a critical checkpoint in glycolysis, inhibition of GAPDH contributes to the diversion of upstream glycolytic intermediates to alternative pathways that could lead to the formation of AGEs, activation of PKC, and induction of the hexosamine and polyol pathways.[1] Our recent studies have demonstrated that GAPDH is reduced in the retina in diabetes and remains compromised even after good glycemic control is reinstituted; the enzyme is covalently modified and translocated to the nuclear fraction.[4] Nuclear translocation of GAPDH is shown to be closely associated with the induction of apoptosis,[5] and apoptosis of retinal microvascular cells precedes the histopathology characteristic of diabetic retinopathy.[6] However, how diabetes affects GAPDH in retinal microvascular cells, the target of histopathology, remains unclear.

Retinopathy is considered largely a microvascular complication of diabetes.[7,8] The retina is a complex tissue with multiple cell types, and microvascular and other nonvascular cells could contribute to the inhibition of GAPDH seen in the rat retina in diabetes. The overall objective of this study was to conclusively establish the role of GAPDH and its signaling pathway in the development and progression of diabetic retinopathy. With the use of isolated retinal microvascular cells (endothelial cells and pericytes), we have investigated the mechanism by which high glucose inactivates GAPDH and how the overexpression of GAPDH affects glucose-mediated metabolic abnormalities. Further, our recent studies have shown that reinstitution of good control in diabetic rats does not protect inhibition of retinal GAPDH. We also investigated the effect of reversal of a high glucose exposure to a normal glucose exposure on GAPDH activity and its covalent modification.

Methods

Retinal Endothelial Cells and Pericytes

Endothelial cells (BRECs) and pericytes were isolated from bovine retina and cultured on dishes coated with 0.1% gelatin.[9,10] BRECs were grown in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (heat inactivated), 5% growth medium supplement (Nu-Serum; BD Biosciences, Franklin Lakes, NJ), 50 μg/mL heparin, 50 μg/mL endothelial cell growth supplement, and 1% antibiotic/antimycotic. Pericytes were grown in DMEM containing 15% fetal bovine serum and 1% antibiotic/antimycotic. Cells were incubated in normal (5 mM) or high (20 mM) glucose media with or without 1 μM PJ34 (poly(ADP-ribose) polymerase; PARP inhibitor VIII; Calbiochem/EMD Chemicals, Inc., Gibbstown, NJ) or 2.5 μM FeTPPS (5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrinato Iron (III), Chloride; Calbiochem/EMD Chemicals, Inc.). All cells received fresh media every 48 hours. Nuclear and cytosol fractions were prepared by differential centrifugation, as previously described by us.[4] Briefly, the cells harvested by trypsinization were pooled from three to four culture dishes

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(60 mm). After removing the trypsin by rinsing the cells with phosphate-buffered saline, the pellet was homogenized in a glass homogenizer in 50 mM glycyl glycine buffer (pH 7.0) containing 10 mM EDTA, 100 mM sodium fluoride, 0.5 mM dithiothreitol, and protease inhibitors. The homogenate was centrifuged at 250*g* for 5 minutes to remove cell debris, and the supernatant was centrifuged at 5000*g* for 15 minutes to obtain the nuclear pellet. HEPES buffer (50 mM; pH 7.5) containing 1% triton X-100, 150 mM sodium chloride, 1 mM EDTA, and protease inhibitors was used to suspend the nuclear fraction. Cytosol fraction was prepared by centrifuging the supernatant at 105,000*g* for 90 minutes.[4] Proteins were quantified using the bicinchoninic assay (Sigma-Aldrich, St. Louis, MO).

GAPDH Expression Plasmids and Transfection of Retinal Endothelial Cells

Total RNA isolated from BRECs with reagent (TRIzol; Invitrogen, Carlsbad, CA) was converted to cDNA using a reverse transcription kit (High-Capacity cDNA Reverse Transcription; Applied Biosystems, Foster City, CA) and served as the template for amplification of bovine GAPDH. PCR primers, designed using Primer-BLAST with GenBank accession number NM_001034034 were as follows: forward, 5′-CAT CAA GCT T**AG ACA AGA TGG TGA AGG TCG**-3′, which included the Kozak translation initiation site and a *Hin*dIII restriction site; reverse, 5′-GCT CGA G**CT CGT GCT CCT GCT GGG GCT GG**-3′, which included a *Xho*I restriction site. Bovine GAPDH-specific sequences are in bold. GAPDH was amplified in a reaction mixture containing 2.5 μM each primer, 0.2 mM dNTP, 1× colorless buffer containing 1.5 mM $MgCl_2$ and 2 U DNA polymerase (GoTaq; Promega, Madison, WI) using the following thermal cycling conditions: initial denaturation at 95°C for 3 minutes, 30 cycles of 95°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes; and a final extension of 72°C for 8 minutes. Agarose gel electrophoresis confirmed amplification of a single band of approximately 1040 bp. PCR products were purified (Wizard SV Gel and PCR Clean-Up System; Promega) before restriction enzyme digestion with *Xho*I and *Hin*dIII, and digested PCR products were ligated into pcDNA3.1 plasmid with T4 DNA ligase (Promega) before transformation (One Shot TOP10F' Chemically Competent *E. coli*; Invitrogen). Ampicillin-resistant bacterial colonies were screened by restriction digest with *Xho*I and *Hin*dIII for presence of the 1040-bp insert and were sequenced to confirm cloning of GAPDH. GAPDH containing plasmid was purified from cultures (PureYield Plasmid Midiprep system; Promega) and was quantified before transfection.

For transfection, BRECs (fourth to fifth passages) were incubated with 0.5 to 5.0 μg GAPDH plasmid, as previously described.[11] Transfection complexes were formed with transfection reagent (Effectene; Qiagen, Valencia, CA) and were incubated with BRECs for 8 hours before incubation in 5 mM or 20 mM glucose media for 4 days. Parallel incubations were carried out by incubating the cells in the transfection reagent alone for 8 hours followed by incubation in 5 mM or 20 mM glucose media. (Our previous studies have shown that control plasmid vectors [pGL3] or scrambled siRNA have no effect on retinal endothelial cells[11,12]). As shown in Figure 1a, the activity of GAPDH was significantly increased in BRECs overexpressing GAPDH compared with the nontransfected cells. The values obtained from the cells transfected with 2.5 μg GAPDH was 30% higher than that with 0.5 μg GAPDH and did not increase when the concentration of plasmid was increased to 5.0 μg GAPDH. Thus, 2.5 μg GAPDH plasmid was selected for all subsequent experiments.

GAPDH Enzyme Activity

Glycolytic activity of GAPDH was assayed in 2 to 5 μg protein according to a method recently used by us by quantifying difference in absorbance before and after the addition of GAPDH. Activity was adjusted for the amount of protein.[4]

Western Blot Analysis

Proteins (20–30 μg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was performed using antibodies against GAPDH, caspase-3, and PARP (Santa Cruz Biotechnology, Santa Cruz, CA) and β-*O*-linked N-acetylglucosamine (*O*-GlcNAc; Covance, Emeryville, CA). Membranes were stripped and reprobed with β-actin to evaluate the lane-loading control (for nuclear fractions, histone 2B was used as the loading control). Band intensities were quantified using gel digitizing software (Un-Scan-It; Silk Scientific Inc., Orem, UT).

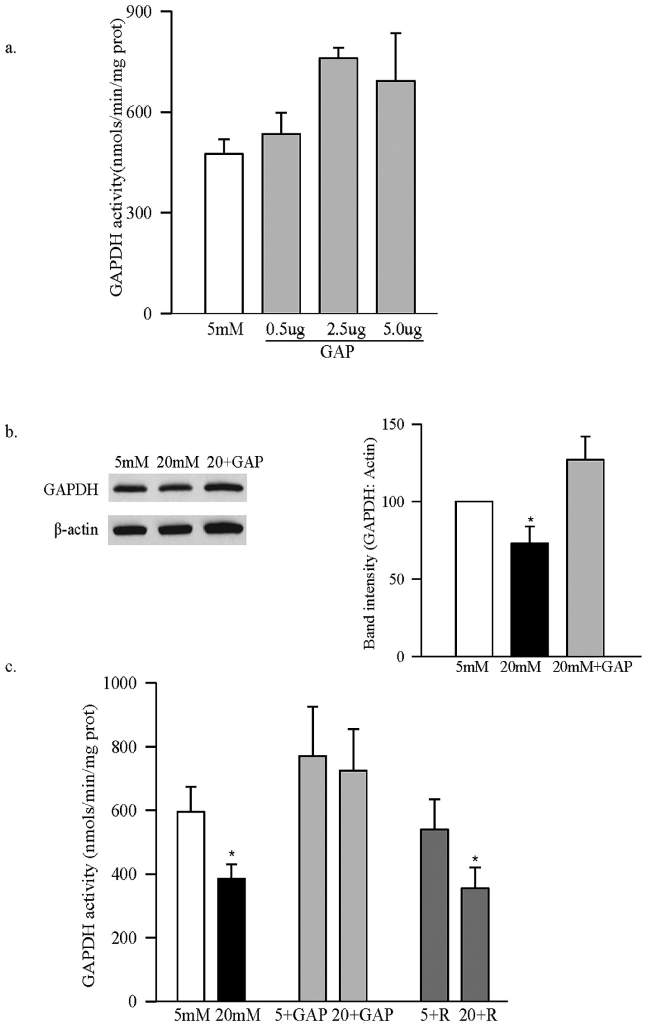

FIGURE 1. GAPDH in retinal endothelial cells. Retinal endothelial cells were transfected using 0.5 to 5.0 μg GAPDH expression plasmid for 8 hours. (**a**) GAPDH activity was measured spectrophotometrically to determine the degree of transfection. (**b**, **c**) BRECs were transfected with 2.5 μg GAPDH plasmid or no plasmid but with transfection reagent alone (R) for 8 hours and were incubated for 4 days in 5 mM or 20 mM glucose media. (**b**) GAPDH abundance was assessed by Western blot analysis, and (**c**) its glycolytic activity was assessed by measuring the production of NADH at 340 nm. Each parameter was measured in duplicate using four different cell preparations. Results are presented as mean ± SD of the values obtained from three different cell preparations, with each measurement made in duplicate. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 5+GAP and 20+GAP, GAPDH-transfected BRECs incubated in 5 mM or 20 mM glucose, respectively; 5+R and 20+R, BRECs treated with the transfection reagent alone, followed by incubation in 5 mM or 20 mM glucose, respectively. $^*P <$ 0.05 relative to 5 mM glucose.

Apoptosis

Apoptosis was determined by ELISA (Cell Death Detection ELISA^{PLUS} kit; Roche Diagnostics, Indianapolis, IN) and was confirmed by measuring the enzyme activity of caspase-3 and the cleavage of PARP.

For ELISA, mononucleosomes and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones, respectively, as described by us previously.[10,11] Absorbance generated by incubation with 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt (Roche Diagnostics) was measured at 405 nm.

Enzyme activity of caspase-3 was assayed by measuring the formation of p-nitroanilide by the cleavage of Ac-DEVD-pNA (Biomol Research Laboratory, Plymouth Meeting, PA) at 405 nm. Each sample was analyzed in duplicate.[9]

Cleavage of PARP into a 85-kDa subunit was determined by Western blot technique, as routinely used in our laboratory.[9]

Quantification of GAPDH Ribosylation and Nitration

Total cell protein (80–100 μg) was immunoprecipitated with 1 μg polyclonal anti-GAPDH (Santa Cruz Biotechnology) overnight at 4°C. The antibody-protein complexes were collected (Protein A/G Plus-Agarose; Santa Cruz Biotechnology) and subjected to SDS-PAGE. Covalent modification was analyzed using monoclonal antibodies against nitrotyrosine (Upstate Biotechnology, Lake Placid, NY) or poly (ADP-ribose) (PAR; Alexis Biochemicals, San Diego, CA). The membranes were stripped and reprobed with GAPDH to evaluate the lane-loading control.

Reversal of High-Glucose Exposure

Confluent BRECs from the fourth to fifth passages were incubated in either 5 mM glucose or 20 mM glucose media. At day 4, a group of cells incubated in 20 mM glucose were rinsed with DMEM and incubated for 4 additional days in 5 mM glucose medium in the presence or absence of 1 μM PJ34 or 2.5 μM FeTPPS (reversal group). Cells incubated in continuous 5 mM glucose or 20 mM glucose for the entire duration of 8 days served as controls.

Statistical Analysis

Data are presented as the mean ± SEM. Differences attributed to treatment were statistically analyzed using the Kruskal-Wallis test followed by the Mann-Whitney *U* rank sum test of paired comparisons. $P < 0.05$ was considered statistically significant.

Results

Endothelial Cells: Effect of High Glucose on GAPDH

Protein abundance of GAPDH was decreased in BRECs incubated in high glucose (Fig. 1b) with significant attenuation of its total glycolytic activity (Fig. 1c) compared with the cells incubated in normal glucose. High glucose also altered the subcellular distribution of GAPDH. As shown in Figure 2, nuclear accumulation of GAPDH was increased by more than 55% compared with the cells incubated in normal glucose. In addition, the glycolytic activity of GAPDH was decreased by 25% in the cytosol compared with >50% in the nuclear fraction in the cells incubated in high glucose compared with the cells incubated in normal glucose (Fig. 3).

Given that nuclear translocation of GAPDH is associated with proapoptotic activity, apoptosis was determined. As expected, cell apoptosis (quantified by ELISA) was increased by approximately 50% in the endothelial cells incubated in 20 mM glucose medium (Fig. 4a). In the same cells, the activity of apoptosis execution enzyme caspase-3 and the cleavage of PARP were also increased by approximately 30% to 40% compared with the cells incubated in 5 mM glucose (Figs. 4b, 4c). The signaling pathways that are postulated to be under the control of GAPDH (PKC and hexosamine pathways) were activated, as indicated by increased PKC βII expression (Fig. 5a) and *O*-GlcNAc modification of several proteins (Fig. 5b).

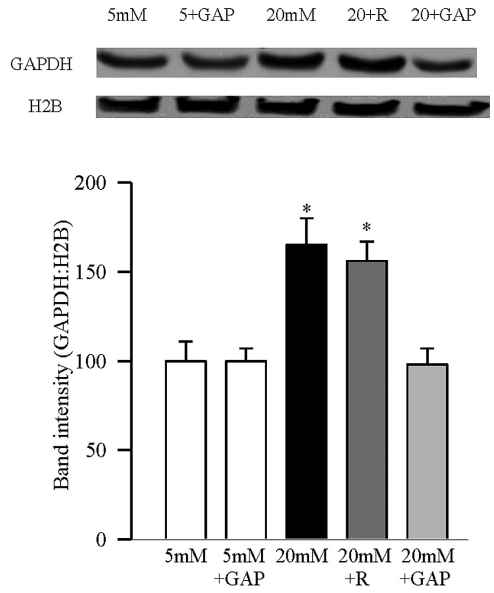

Figure 2. Subcellular distribution of GAPDH. BRECs either transfected with 2.5 μg GAPDH plasmid or left untransfected were incubated for 4 days in 5 mM or 20 mM glucose media. The nuclear fraction was prepared by centrifugation, and the expression of GAPDH was determined by Western blot analysis using histone 2B as a loading control. Western blot represents results obtained from three or four different experiments, with each measurement made in duplicate. Histogram represents mean ± SD. GAPDH band intensity adjusted to H2B band intensity, and the values obtained from BRECs incubated in 5 mM glucose are considered 100%. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 5+GAP and 20+GAP, GAPDH-transfected cells incubated in 5 mM or 20 mM glucose, respectively; 20+R, BRECs treated with the transfection reagent alone before incubation with 20 mM glucose. *$P <$ 0.05 compared to 5 mM glucose.

Covalent modifications of GAPDH are shown to inhibit its activity[4,13]; therefore, we investigated the effect of high glucose on these modifications. As shown in Figure 6, both nitration and ribosylation of GAPDH were increased by two-fold in BRECs incubated in high glucose compared with normal glucose.

Effect of Overexpression of GAPDH

Overexpression of GAPDH in BRECs protected a glucose-induced decrease in its glycolytic activity (Fig. 1b) and prevented its accumulation in the nucleus, as shown by its protein expression in the nuclear fraction by the Western blot technique (Fig. 2). This was accompanied by inhibition of the activation of the apoptotic machinery, as confirmed by ELISA (Fig. 4a), caspase-3 activity (Fig. 4b), and the cleavage of PARP (Fig. 4c). Further, the activation of PKC and hexosamine pathways was also ameliorated in the cells overexpressing GAPDH (Figs. 5a, 5b). However, the incubation of cells with just the transfection reagent, without any plasmids, had no effect on GAPDH activity (Fig. 1c) or their apoptosis (Fig. 4).

Reversal of Glucose Exposure

Four days of normal glucose levels that followed 4 days of high glucose levels had no beneficial effect on the activity of GAPDH in both cytosolic and nuclear fractions of BRECs; the activity remained subnormal (Fig. 3), and the enzyme continued to be covalently modified, as evidenced by its increased nitration and ribosylation (Fig. 6). Values obtained in the reversal group were not different from those obtained from the cells incubated in high glucose for the entire duration of the experiment.

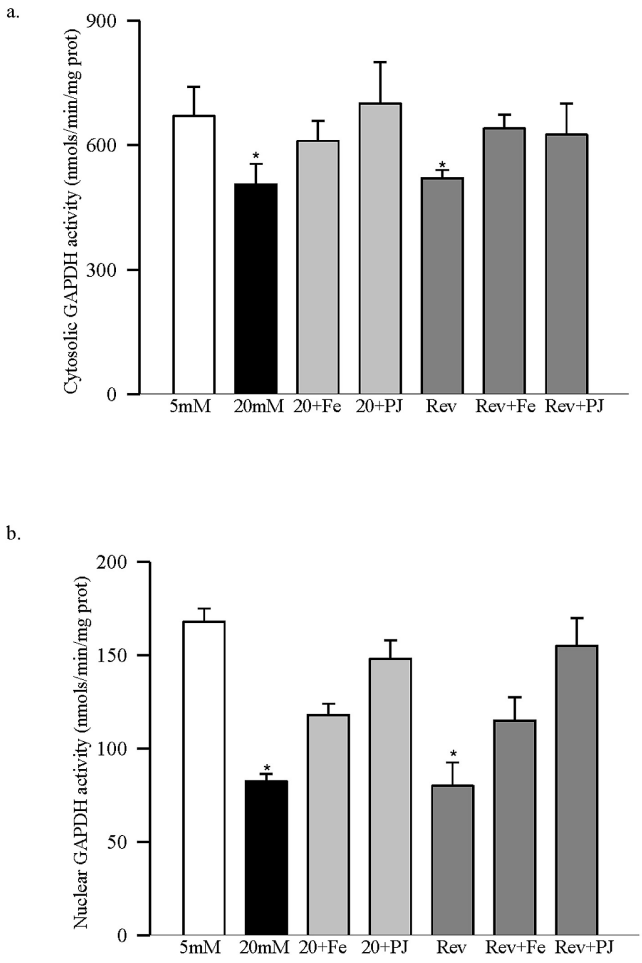

FIGURE 3. GAPDH activity in retinal endothelial cells. (**a**) Cytosol and (**b**) nuclear fractions of BRECs were prepared by differential centrifugation, and GAPDH activity was assessed with the use of 1 to 2 μg protein by measuring the production of NADH. Each measurement was made in duplicate using three or more cell preparations, and the values are represented as mean ± SD. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 20+Fe, 20 mM glucose and 2.5 μM FeTPPS; 20+PJ, 20 mM glucose and 1.0 μM PJ34; Rev, BRECs incubated in 20 mM glucose for 4 days followed by 5 mM glucose for another 4 days without any addition; Rev+Fe or Rev+PJ, 2.5 μM FeTPPS or 1.0 μM PJ34 addition, respectively, during only the 5 mM glucose exposure that followed 20 mM glucose exposure. $^*P < 0.05$ compared with 5 mM glucose. $\#P < 0.05$ compared with 20 mM glucose alone.

To determine the effect of direct inhibition of peroxynitrite or ribosylation on the metabolic memory phenomenon, FeTTPS or PJ34 was included during the normal glucose incubation period that followed 4 days of high glucose exposure. As shown in Figure 3, addition of FeTTPS during 4 days of normal glucose exposure had a beneficial effect on GAPDH activity in cytosol and nuclear fractions. The degree of nitration and ribosylation were also decreased, as determined by immunoprecipitation (Fig. 6). Thus, our results show in principle that the direct inhibition of covalent modification of GAPDH during the reversal phase has a more beneficial effect than the reversal of high glucose alone.

GAPDH in Retinal Pericytes

Given that the loss of pericytes is considered one of the earliest morphologic changes seen during the development of diabetic retinopathy[14] and that the possible mechanism of their loss is apoptosis,[15] some key experiments were also performed in pericytes isolated from bovine retina. As observed with isolated retinal endothelial cells, high glucose inactivated GAPDH in both cytosol and nuclear fractions of retinal pericytes (Fig. 7), and PJ34 or FeTPPs were able to ameliorate glucose-induced inactivation of GAPDH and inhibited their apoptosis (Fig. 8). Incubation of pericytes in 5 mM glucose medium with PJ34 or FeTPPS did not produce any effect on either GAPDH activity or pericyte apoptosis.

In the same pericyte preparations, the inactivation of GAPDH was not reversed by 4 days of incubation with normal glucose that followed 4 days of high glucose, suggesting its role

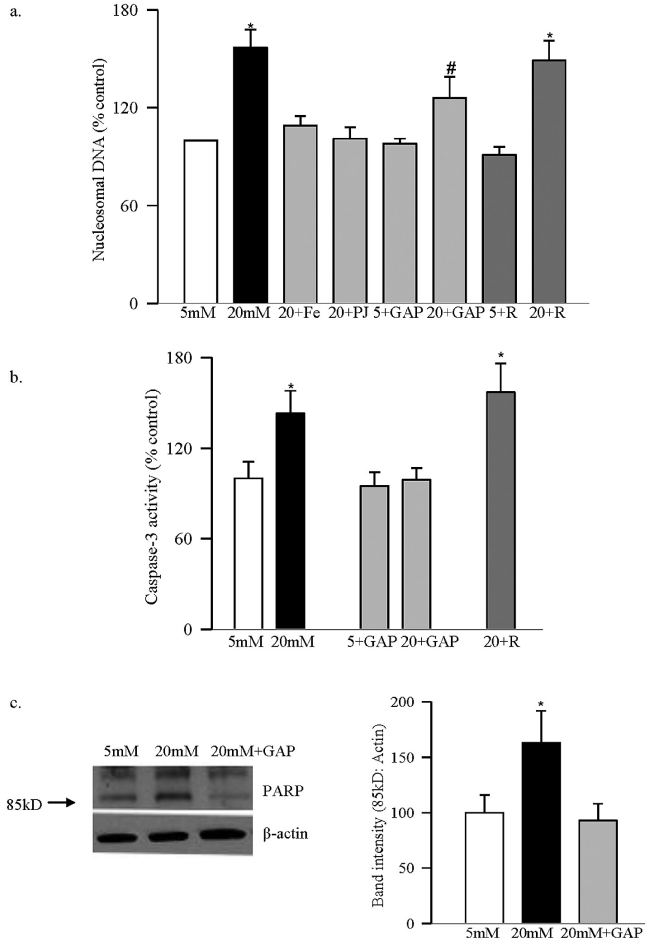

FIGURE 4. Effect of GAPDH overexpression on endothelial cell death: GAPDH-transfected BRECs (using 2.5 μg plasmid) or untransfected BRECs were incubated in 5 mM or 20 mM glucose for 4 days. (**a**) Apoptosis was determined by cytoplasmic histone-associated DNA fragments using ELISA. The graph shows the mean ± SD adjusted to the total DNA in each sample. (**b**) Activation of apoptosis execution enzyme caspase-3 was determined in the cells by measuring the cleavage of the substrate Ac-DEVD-pNA. Each experiment was repeated with at least three different BREC preparations, and measurements were made in duplicate. (**c**) The appearance of a 85-kDa band of PARP was determined by Western blot technique, and β-actin was used as a loading standard. Western blot is representative of four different experiments. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 20+Fe, 20 mM glucose and 2.5 μM FeTPPS; 20+PJ, 20 mM glucose and 1.0 μM PJ34; 5+GAP and 20+GAP, GAPDH-transfected cells incubated in 5 mM or 20 mM glucose, respectively; 5+R and 20+R, BRECs treated with the transfection reagent alone before incubation with 5 mM or 20 mM glucose, respectively. The values, represented as mean ± SD, obtained with 5 mM glucose were considered 100%. $^*P < 0.05$ compared with 5 mM glucose. $\#P < 0.05$ compared with 20 mM glucose alone.

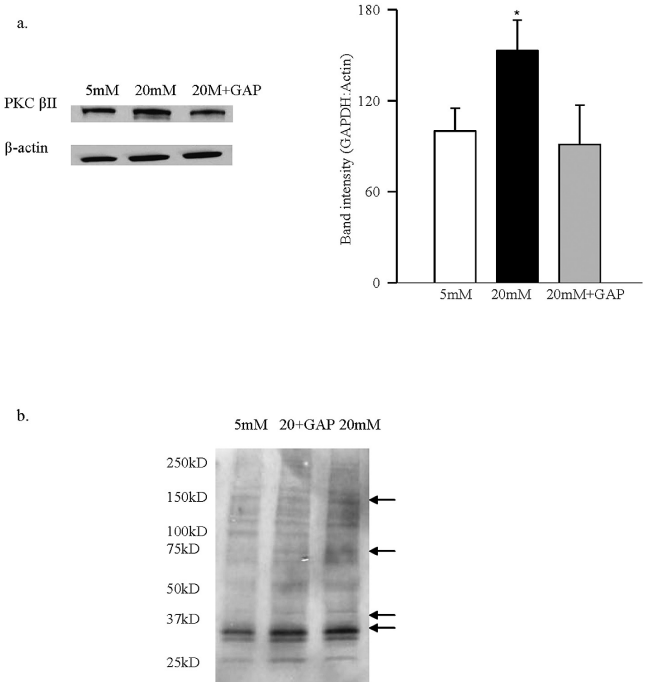

FIGURE 5. Activation of the signaling pathways upstream of GAPDH and the effect of GAPDH overexpression. Activation of (**a**) PKC and (**b**) hexosamine pathways were quantified in BRECs transfected with 2.5 μg GAPDH plasmid incubated in 5 mM or 20 mM glucose for 4 days by Western blot technique using polyclonal antibodies against PKC βII and *O*-GlcNAc, respectively. β-Actin was used as a loading standard. Blots are representative of four to five samples in each treatment group. *Arrows*: some of the bands that were increased in 20 mM glucose compared with 5 mM glucose. Western blot analyses are representative of three or more experiments. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 20-GAP, GAPDH-transfected BRECs incubated in 20 mM glucose. $^*P < 0.05$ compared with 5 mM glucose.

in the metabolic memory phenomenon (Fig. 7). However, supplementation with PJ34 or FeTPPs during the normal glucose period had a beneficial effect on glucose-induced inactivation of GAPDH.

DISCUSSION

GAPDH is considered a potential linking mechanism between hyperglycemia-induced oxidative stress and the major pathways implicated in the pathogenesis of diabetic complications.[2] In diabetes, retinal capillary cells experience increased oxidative stress, and their apoptosis is accelerated.[10,16,17] Our results, for the first time, demonstrate that high glucose exposure decreases the activity and abundance of GAPDH in retinal capillary cells. Translocation of the enzyme from cytosol to the nucleus is facilitated, allowing it to increase apoptosis, and the enzyme is covalently modified. However, when GAPDH is overexpressed in retinal endothelial cells, glucose-induced inhibition of GAPDH, its translocation to the nucleus, and the acceleration of apoptosis are ameliorated, and the activation of the signaling pathways upstream of GAPDH (PKC and hexosamine pathways) is inhibited. Our results also show that the reversal of high-glucose exposure of retinal microvascular cells by normal glucose does not prevent GAPDH from being covalently modified. If, however, the inhibitors of nitration or ribosylation are supplemented during the period of normal glucose that follows high glucose (reversal group), the inactivation of GAPDH and its modifications is ameliorated, suggesting that direct inhibition of the covalent modification during normal glycemic control has a better effect than does normal glycemic control alone. These results could have great clinical implications because the prevention of GAPDH covalent modification could help prevent activation of the major pathways that are important contributors to the development and progression of diabetic retinopathy.

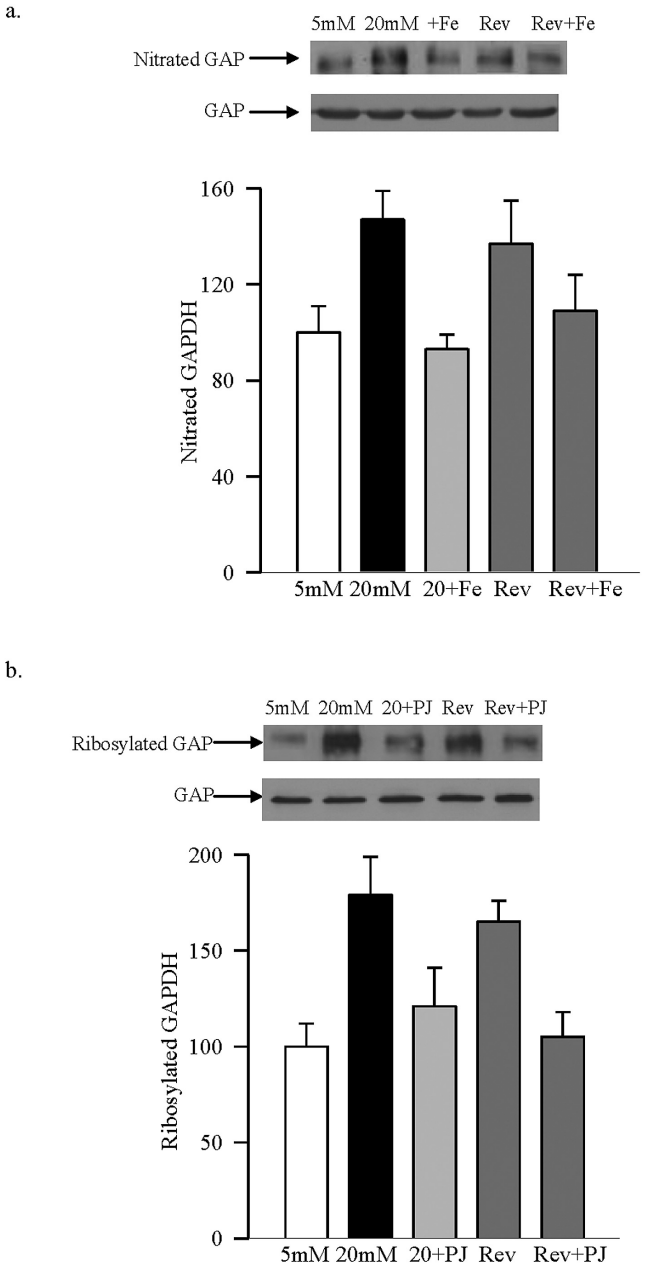

FIGURE 6. Covalent modification of GAPDH in BRECs. GAPDH was immunoprecipitated using 80 to 100 μg protein and was analyzed by Western blot for (**a**) nitration and (**b**) ribosylation. To ensure equal loading, the membranes were reprobed for GAPDH. The blots are representative of three different experiments each with two or more samples in all five different treatment groups. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; +Fe, 20 mM glucose supplemented with 2.5 μM FeTPPS; Rev, BRECs incubated in 20 mM glucose for 4 days followed by 5 mM glucose for 4 additional days without any addition; Rev+Fe or Rev+PJ, 2.5 μM FeTPPS or 1.0 μM PJ34 addition, respectively, during only the 5-mM glucose exposure that had followed 20-mM glucose exposure. Histograms present values as mean ± SD obtained from three different BREC preparations.

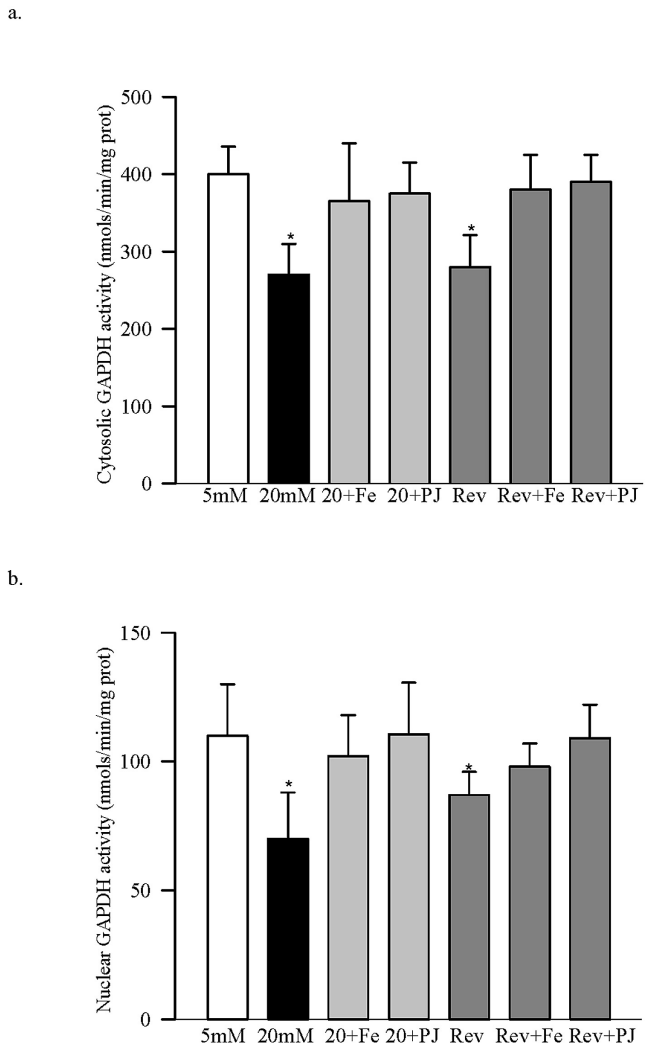

FIGURE 7. GAPDH activity in retinal pericytes. Cytosol and nuclear fractions of pericytes were isolated by differential centrifugation, and GAPDH activity was assessed in (**a**) 0.5 to 1 μg cytosol protein or (**b**) 3 to 5 μg nuclear protein by measuring the production of NADH at 340 nM. Each measurement was made in duplicate. Values are represented as mean ± SD obtained from three different pericyte preparations. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 20+Fe, 20 mM glucose and 2.5μM FeTPPS; 20+PJ, 20 mM glucose and 1.0 μM PJ34; Rev, pericytes incubated in 20 mM glucose for 4 days followed by 5 mM glucose for 4 additional days without any addition; Rev+Fe or Rev+PJ, 2.5 μM FeTPPS or 1.0 μM PJ34 addition, respectively, during only the 5-mM glucose exposure that followed the 20-mM glucose exposure. Values are presented as mean ± SD obtained from three to four different pericyte preparations, and each measurement was made in duplicate. $^*P < 0.05$ compared with 5 mM glucose.

GAPDH, a classic glycolytic enzyme, catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and serves as a critical checkpoint in glycolysis.[18] Inhibition of its glycolytic activity is postulated to contribute to the development of diabetic microvascular complications through the diversion of upstream glycolytic intermediates to alternative pathways that could lead to the formation of AGEs and the activation of PKC, hexosamine, and polyol pathways.[2] These pathways are considered some of the major metabolic pathways in the pathogenesis of diabetic retinopathy.[19–23] Recent studies have shown that GAPDH is inactivated in the retina in diabetes when histopathology findings characteristic of retinopathy can be observed in rats and its nuclear translocation and covalent modification are increased.[4] However, the retina has multiple cell types, and GAPDH has been shown to induce the apoptosis of retinal Muller cells in high-glucose conditions.[5] Our results strongly implicate that the microvasculature of the retina experiences compromised GAPDH, and they suggest a major role of this enzyme in the microvascular histopathology characteristic of diabetic retinopathy.

Our previous studies have shown that the nuclear accumulation of GAPDH in the retina is increased and its glycolytic activity is decreased in diabetic rats when histopathology findings are detectable. The results have suggested that although diabetes increases GAPDH translocation to the nucleus, it appears to be in covalently modified form; nitration of the enzyme is significantly increased in the nuclear fraction compared with the cytosol fraction.[4] In addition to its glycolytic capacity, GAPDH has nonglycolytic functions, and its translocation to the nucleus is considered to initiate apoptosis.[24] Here we show that high-glucose exposure of retinal endothelial cells increases the nuclear localization of GAPDH, and glycolytic activity is significantly decreased. This supports the role of GAPDH in capillary cell apoptosis, the phenomenon that precedes the histopathology observed in diabetic retinopathy. In addition, the inhibition of glucose-induced apoptosis of retinal capillary cells by the overexpression of GAPDH and the inhibitors of nitration and ribosylation further establishes its role in the development of retinal histopathology.

Increased peroxynitrite levels seen in the retina and its microvascular cells are considered to play an important role in the development of diabetic retinopathy.[25,26] Peroxynitrite induces the nitration of GAPDH,[27] and nitration inhibits its activity.[28] Nitration is also associated with the nuclear accumulation of GAPDH and is a factor in cell death independent of glycolytic impairment.[29] Further, increases in reactive oxygen species can lead to increased DNA damage,[30] which, in turn, can result in the activation of PARP.[31,32] The inhibition of PARP with PJ34 reverses aortic endothelial dysfunction in diabetic animals for several weeks.[33] Activation of PARP, which is shown to contribute to retinal capillary cell death, leukostasis, and the development of retinopathy in diabetic rats,[34–36] also regulates GAPDH.[3,4] Here we show that retinal microvasculature (endothelial cells and pericytes) is one of the targets of

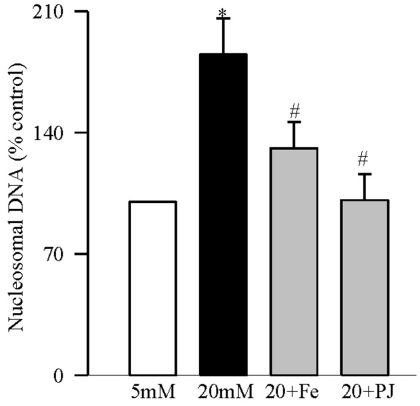

FIGURE 8. Effect of FeTPPS and PJ34 on the apoptosis of retinal pericytes. Apoptosis was measured by performing ELISA for cytoplasmic histone-associated DNA fragments. The graph represents mean ± SD obtained from the pericytes incubated in 20 mM glucose in the presence or absence of 2.5 μM FeTPPS or 1.0 μM, and these values were adjusted to the total DNA. Values obtained from the cells incubated in 5 mM glucose are considered as 100% (control). Results are presented as mean ± SD of the values obtained from three different cell preparations, with each measurement made in duplicate. $^*P < 0.05$ compared with 5 mM glucose. $^\#P < 0.05$ compared with 20 mM glucose.

such covalent modifications. Both nitration and ribosylation of GAPDH are increased in these cells in high-glucose conditions, and the addition of a nitrotyrosine breaker or a PARP inhibitor prevents glucose-induced inhibition of GAPDH and apoptosis, suggesting that covalent modification is one of the important mechanisms that regulate its activity and subcellular translocation.

GAPDH is shown to regulate multiple upstream pathways that are considered major contributors in the development of diabetic retinopathy. GAPDH increases flux through the hexosamine pathway by fructose-6 phosphate, increases AGEs formation by increasing methyglyoxal,[37] and activates PKC by the formation of diacyglycerol. GAPDH antisense oligonucleotides are shown to activate each of these pathways induced by high glucose in bovine aortic endothelial cells.[3] Here we show that high glucose increases PKC expression and activates the hexosamine pathway in retinal endothelial cells and that the overexpression of GAPDH inhibits such glucose-induced increases. We must acknowledge that our present study did not directly measure PKC activity; however, hyperglycemia activates PKC in the retina and its endothelial cells,[19] and reinstitution of normal glycemia after a period of poor glycemic control, in addition to failing to provide benefits to retinal GAPDH activity, also fails to normalize PKC expression,[4] suggesting a strong relationship between increased PKC activity and its expression. Thus, our data strongly imply that the inhibition of GAPDH has the potential to contribute to the development of diabetic retinopathy by modulating its upstream signaling pathways.

In the pathogenesis of diabetic retinopathy, along with the breakdown of the blood-retinal barrier,[38] early losses of pericyte and endothelial cells are also detected in the retina, which is followed by the development of acellular capillaries and microaneurysms.[6,15,38,39] Our results show that high glucose also inhibits GAPDH in retinal pericytes, the cells that provide support to the capillaries. The possible mechanism appears to be its covalent modification. This strongly implies that GAPDH could be important in both endothelial cell loss and pericyte ghost formation.

Good glycemic control provides great benefits in the progression of diabetic retinopathy,[4,25,40–43] and the benefits of prior glycemic control continue even after its termination, suggesting a metabolic memory phenomenon. Our laboratory has shown that the retina continues to experience oxidative and nitrosylative damage; GAPDH remains compromised and apoptosis-related genes remain upregulated, even when hyperglycemic insult is terminated in rats.[4] Their retinal microvasculature continues to undergo apoptosis, and many apoptosis-encoded genes continue to be upregulated.[40] Here we show that the exposure of retinal endothelial cells and pericytes to normal glucose after a period of high glucose does not provide any benefit to GAPDH activity, its translocation to the nucleus, and covalent modification. However, if the period of normal glucose that has followed high glucose is supplemented with the peroxynitrite scavenger or the inhibitor of ribosylation, inhibition of the enzyme activity and its covalent modification are ameliorated, suggesting that the normal glucose that follows high glucose exposure does not provide any benefit to alterations in GAPDH activity. When this normal glucose period is supplemented with FeTPPs or PJ34, however, alterations in GAPDH are not observed. These results imply that supplementation with the agents that can directly target covalent modification of GAPDH during good glycemic control have greater potential to arrest the progression of diabetic retinopathy than simply good glycemic control itself.

In summary, we show that GAPDH is one of the targets of hyperglycemia-induced changes in the retinal microvasculature and that the reversal from high glucose exposure to normal glucose is not enough to abrogate the effects of high glucose, but supplementation with the inhibitors of covalent modification during the normal glucose period provides protection. These could have immense clinical significance because the mechanisms involved in nitrosylation and ribosylation of GAPDH could serve as potential targets for future therapeutics to inhibit and also to aid glycemic control in regulating cytosolic GAPDH. This could help prevent accelerated apoptosis of retinal microvascular cells and activation of the major pathways that are important in the development and progression of diabetic retinopathy.

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