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

Mamta Kanwar and Renu A. Kowluru*

Kresge Eye Institute, Wayne State University, Detroit, MI

*Corresponding Author: Renu A. Kowluru, Ph.D.
Kresge Eye Institute
Wayne State University
Detroit, MI 48201
E-Mail: rkowluru@med.wayne.edu

Key Words: Glycemic control, Glyceraldehyde-3-phosphate dehydrogenase, Metabolic memory, Retinopathy

ABSTRACT

Objective: Mitochondrial superoxide levels are elevated in the retina in diabetes, and MnSOD overexpression prevents the development of retinopathy. Superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which activates major pathways implicated in diabetic complications, including 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: Streptozotocin diabetic rats were either in poor control (PC, glycated hemoglobin>11%) for 12 months, or good control (GC, GHb<7) soon after induction of diabetes, or six months PC with six months GC (PC-GC). Retinal GAPDH, its ribosylation and nitration and AGEs and PKC activation were determined, and correlated with microvascular histopathology.

Results: In PC rats 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. Re-institution 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 unchanged. However, GAPDH and its translocation in GC 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 towards preventing its inhibition may inhibit development of diabetic retinopathy and arrest its progression.
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 (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 diabetic 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 as 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 as a likely possibility (14-16). How GAPDH contributes to the pathogenesis of diabetic retinopathy remains to be established.

Good glycemic control (GC) 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 re-establishment of GC. The imprinted effects of prior glycemic control either produce the long lasting benefits of GC, or resist the arrest of progression of diabetic retinopathy after re-institution of GC. Re-institution of GC after a profound period of poor glycemic control (PC) 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 six months when GC is initiated six 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 PC is reduced to two 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 have 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 PC before initiation of GC.

METHODS

Animals and glycemia. Lewis rats (male, 200g) were randomly assigned to normal or diabetic groups. Diabetes was induced with streptozotocin (55mg/kg BW), and rats were divided at random among three groups according to intended degree of glycemic
control. Rats in group 1 remained in PC for 12 months, in group 2 rats were in PC for six months followed by GC for six additional months (PC-GC), and in group 3 rats were subjected to GC soon after induction of diabetes (GC group). Some of the same rats have 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 PC group received a single injection of insulin (1-2 units) 4-5 times a week to prevent ketosis and weight loss, and the rats in GC 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 hour urine samples were measured daily and tested for glycosuria. Blood glucose was measured once a week using Elite Glucometer (Bayer Corporation, Tarrytown, NY), and glycated hemoglobin (GHb) every two months. The entire rat colony received powdered diet (PURINA 5001, TestDiet, Richmond, IN); and their food consumption was measured once and body weights 2-3 times every week. These experiments conformed to the ARVO Resolution on Treatment of Animals in Research, as well as to the specific Institutional Guidelines. The experiment was terminated 12 months after initiation of diabetes and the animals were sacrificed 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 micro-spatula.

Preparation of subcellular fractions. Retina was gently homogenized in a glass homogenizer in 50mmoles/L glycyl glycine buffer (pH 7.0) containing 10mmoles/L EDTA, 100mmoles/L sodium fluoride, 0.5mmoles/L dithiolthretol and protease inhibitors. The homogenate was centrifuged at 750xg for 5 minutes, and the supernatant was centrifuged at 5000xg for 15 min. The nuclear pellet was resuspended in 50mmoles/L HEPES buffer (pH 7.5) containing 1% triton X-100, 150mmoles/L sodium chloride, 1mmoles/L EDTA and protease inhibitors. The supernatant was further centrifuged at 105,000xg 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 (H2-B, nuclear marker) and lactate dehydrogenase (LDH, cytosolic marker).

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

Quantitative real-time PCR (Q-RT-PCR). RNA was isolated from the retina using TRIzol reagent, and 1μg RNA was converted to single stranded cDNA and quantified spectrophotometrically. Gene expression was measured using 90-300ng cDNA templates in 96 well plates in ABI-7500 sequence detection system (29, 30). Each sample was analyzed in triplicate, and the data normalized to β2-macroglobulin (B2-M) expression in each sample. GenBank accession numbers for the ABI TaqMan assays for GAPDH and B2-M used were NM-017008.3 and NM_012512.1 respectively. The fold change in gene expression relative to
normal was calculated using the 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, 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 re-probed with β-actin (Sigma-Aldrich, St Louis, MO). The band intensity was quantified using Un-Scan-It Gel digitizing software (Silk Scientific Inc, 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-ribose)ylation 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 re-probed for GAPDH.

**Quantification of GAPDH-mediated pathways.** Since inhibition of GAPDH activates major pathways that are implicated in the development of diabetic 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 previously described by us (23). Since addition of single O-linked β-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 Inc, Princeton, NJ). Bovine serum albumin 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 minutes 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 mid-retinal fields with one field adjacent to each of the 5 to 7 retinal arterioles radiating out from the optic disc, and expressed as per mm² of retinal area examined (5, 8, 26).

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

**RESULTS**
Severity of hyperglycemia. Hyperglycemia, as reported earlier (26), was severe in the rats in PC group; GHb values were over 11% throughout the entire duration of the experiment (12 months). The rats in GC group maintained their GHb values similar to those in normal rats. In PC-GC group, GHb values before initiation of GC were not different from the PC group (GHb>11%), but became similar to those in normal group after initiation of GC (GHb<7%) (Table I). Average body weight and 24 hour urine volumes were similar in GC rats and normal rats.

Effect of diabetes on retinal GAPDH. Twelve months of PC had a marginal, but significant effect on the expression of GAPDH in the retina; protein expression was decreased by 15-20% in diabetic rats compared was decreased by about 20% (Figure 2).

Effect of diabetes on activation of retinal PARP. As shown in figure 3a, 12 months of PC significantly increased poly(ADP-ribosyl)ation of retinal proteins compared to normal rats suggesting 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. Since retinal cell apoptosis precedes the development of diabetic retinopathy (32, 33), 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 to the nuclear fraction, but 12 months of PC in rats resulted in reduction in its expression in the cytosol fraction with a concomitant increase in the nuclear fraction (Figure 4). The ratio of GAPDH expression in cytosolic and nuclear fraction was almost 4 to1 in normal rat retina, and decreased to 1.2 to1 in diabetic rat retina. In the same diabetic rats, despite increased expression of GAPDH, the glycolytic activity was decreased by almost 70% in the nuclear fraction (Figure 5a). GC group had similar expressions as normal control rats (data not shown).

Ribosylation and nitration have been shown to inhibit GAPDH activity (15, 31). We determined the levels of its ribosylation and nitration of retinal GAPDH. Twelve months of poor PC resulted in approximately 25% increase in ribosylation of GAPDH (Figure 3b) and 30% increase in nitration compared to the normal rat retina (Figure 3c). The rats in GC group had similar expressions as normal control rats (data not shown). The ratio of nitrated GAPDH was 2.5 to 1 in normal rat retina, and increased to almost 1:1 in PC group. However, in the nuclear fraction, nitrated GAPDH was about two fold higher in PC rats compared to normal rats (Figure 5b), 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 PC for six months before institution of GC, the protein and gene expressions of retinal GAPDH remained subnormal, and PARP remained activated; values obtained in PC and PC-GC groups were not different from each other (Figures 1, 2 and 3a). In addition, both ribosylation and nitration of GAPDH were also not different in the retina from PC group and PC-GC groups suggesting that six months of GC had no beneficial effect on the covalent modification of the enzyme (Figure 3b and c). The expression and activity of GAPDH in nuclear fraction of retina from PC-GC group were significantly different from those in normal rats, and the enzyme remained nitrated (Figures 4 and 5). However, these values were similar to those obtained from PC group, suggesting that six months of GC 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 GC soon after induction of diabetes (GC group), GAPDH gene expression was similar to that obtained from normal rat retina (Figure 2).

**Effect of reversal of hyperglycemia on GAPDH-mediated pathways.** As shown in figure 6a, multiple protein bands with increased AGEs were observed in the retina from rats in PC group compared with those from normal rats, and protein staining from the rats in PC-GC group was similar to those obtained from the rats in PC 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 (Figure 6b), the enzyme expression remained significantly elevated in both PC and PC-GC groups compared to the normal group of rats (p<0.05). Similarly, six months of GC 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 retinal vasculature (Figure 7) by about 4 fold compared with normal rats. Six months of GC that followed six months of PC failed to provide any protection, the number of acellular capillaries was similar in PC and PC-GC rats (average number of acellular capillaries/mm² retina in rats in normal, PC and PC-GC groups=1.5, 6.1 and 6.8 respectively).

**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 AGEs 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 hexosamine pathway) are activated. Our data suggests that the enzyme translocated to the nuclear fraction is covalently modified. Further, we provide exciting data demonstrating that re-institution of GC after six months of PC 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 re-institution of GC. Re-institution of GC also fails to provide any benefit to the covalent modification; the elevated levels of both ribosylation and nitration are sustained for at least six months after reversal of PC. 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 elevated after re-establishment of GC. 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 three months of diabetes 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 decreases GAPDH in vascular cells, putatively due to 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 as 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 as one of the mechanisms in the inhibition of GAPDH activity in hyperglycemic conditions (14). Our data show that PARP activity is significantly increased in PC group, and this is in accordance with the other 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), and these cells are one 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. Further, 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 increase in hydrophobicity due to its nitration is being postulated as 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 (13). Our results show that the expression of GAPDH is increased in nuclear fraction in the retina from diabetic rats compared to normal rats; however, the enzyme in the nuclear fraction appears to be in a more nitrated and inactivated state compared to 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 inactivated state.

Re-institution of GC after six months of PC failed to produce any significant beneficial effects on GAPDH, but if GC was initiated soon after induction of diabetes in rats and allowed to continue for 12 months,
GADPH and diabetic retinopathy

GAPDH remained similar to that obtained from age-matched normal rats. This strongly supports that GAPDH, in addition to being a central player in the development of diabetic retinopathy, 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 six months of GC. Sustained increases in nitration and ribosylation of retinal GAPDH after reversal of hyperglycemia in rats suggests that GC fails to provide any benefit to the covalent modifications of the enzyme. Further, GC did not prevent translocation of the retinal enzyme from cytosol to the nucleus, suggesting that GAPDH remains proapoptotic even after GC is re-established. Due to 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 re-institution of GC (25), and nuclear accumulation of covalently modified GAPDH could be one of the important factors associated with increased apoptosis and histopathology 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 its pro-apoptotic nature possibly contributing to the development of diabetic retinopathy. In addition to its role in the pathogenesis of diabetic retinopathy, GAPDH is also an important in metabolic memory phenomenon. Sustained nitration and ribosylation are possible mechanisms for resistance of GAPDH to reverse inhibition 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 on the development of retinopathy, the number of acellular capillaries remains comparable in the rats in PC and in PC-GC groups. This confirms that GAPDH-mediated pathways, and the process of apoptosis of retinal capillary cells that begins before histopathology can be detected in the retinal vasculature (25), continues 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 re-institution of GC, however, strongly suggests that GAPDH has a significant role in the development and progression of diabetic retinopathy.
after reversal of hyperglycemia. Therapies targeted towards 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.

ACKNOWLEDGMENTS

Authors thank Dr. Bindu Menon for technical assistance, and Yakov Shamailov and Divyesh Sarman for their help in maintaining the rats. This study was supported in part by grants from the National Institutes of Health, Juvenile Diabetes Research Foundation, The Thomas Foundation, and Research to Prevent Blindness.

REFERENCES


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

<table>
<thead>
<tr>
<th></th>
<th>Duration (months)</th>
<th>Body Weight (g)</th>
<th>GHb (%)</th>
<th>Urine Vol (ml/24 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (9)</td>
<td>12</td>
<td>421±35</td>
<td>6.7±0.8</td>
<td>13±6</td>
</tr>
<tr>
<td>PC (13)</td>
<td>12</td>
<td>289±37</td>
<td>12.7±1.7</td>
<td>112±39</td>
</tr>
<tr>
<td>GC (7)</td>
<td>12</td>
<td>454±41</td>
<td>6.9±1.1</td>
<td>26±10</td>
</tr>
<tr>
<td>PC (11)</td>
<td>6</td>
<td>272±27</td>
<td>13.1±1.6</td>
<td>135±19</td>
</tr>
<tr>
<td>GC</td>
<td>↓ 6</td>
<td>↓ 409±26</td>
<td>↓ 7.1±0.9</td>
<td>↓ 19±11</td>
</tr>
</tbody>
</table>

The rats were weighed two times every week and their food consumption once very week. Body weight is the mean value during the entire duration of the intended metabolic control. GHb was quantified. The values are means ± SD, and the numbers in parentheses represent number of rats in each group.
FIGURE LEGENDS

**Figure 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 5 different rats in each group, and the bars represent the mean ± SD of the adjusted band intensities obtained from those rats.

![Western Blot Image](image)

**Figure 2:** Gene expression of GAPDH in the retina and effect of re-institution of good glycemic control. Retinal gene expression was measured with Q-RT-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 100%. Data represent the mean ± SD from 8-10 rats in each of the 4 groups. *P<0.05 compared to normal, and #P>0.05 compared to PC.

![qrtPCR Image](image)
Figure 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-ribo)sylated 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 poly PAR or nitrotyrosine. To ensure equal loading, the membranes were re-probed for GAPDH. The histograms represent ribosylation (b) or nitration (c) of retinal GAPDH from 5-6 rats in each group, and the values from normal rat retina are considered as 100%. *P<0.05 compared to normal, and #P>0.05 compared to PC.

Figure 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 lactate dehydrogenase (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 as 100%. The values obtained are mean ± SD from 4 or more rats in each of the 4 groups. *P<0.05 compared to normal, and #P>0.05 compared to PC.
Figure 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 340nm. Each measurement was performed in duplicate and assay repeated three or more times. The activity obtained in the cytosol obtained from normal rat retina is considered as 100%. The values are mean ± SD from at least 6 rats in each of the 4 groups. *P<0.05 compared to normal, and #P>0.05 compared to PC.

b. Nitration of GAPDH was performed by first 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 4-5 rats in each group.
Figure 6: Activation of AGEs and PKC in retina: Total AGEs and PKC activation 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 4 or more rats in each group.
Figure 7: Histopathology in retinal microvasculature: Trypsin digested retinal microvasculature was stained with periodic acid-Schiff and hematoxylin. The number of acellular capillaries was counted in multiple mid-retinal 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 PC for 12 months.