

Effect of Reinstitution of Good Glycemic Control on Retinal Oxidative Stress and Nitrative Stress in Diabetic Rats

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Clinical and experimental studies have shown that re-institution of good glycemic control (GC) after a period of poor glycemic control (PC) does not produce immediate benefits on the progression of retinopathy, and hyperglycemia is sufficient to initiate the development of diabetic retinopathy. In this study, the effect of reinstatement of GC on hyperglycemia-induced increased oxidative stress and nitrative stress was evaluated in the retina of rats maintained in PC before initiation of GC. In diabetic rats, 2 or 6 months of PC (GHb >11.0%) was followed by 7 months of GC (GHb <5.5%). Reinstatement of GC after 2 months of PC inhibited elevations in retinal lipid peroxides and NO levels by ~50%, but failed to have any beneficial effects on nitrotyrosine formation. However, reversal of hyperglycemia after 6 months of PC had no significant effect on retinal oxidative stress and NO levels ($P < 0.02$ vs. normal). In the same rats, inducible nitric oxide synthase expression and nitrotyrosine levels remained elevated by >80% compared with normal rats or rats kept in GC for the duration. This suggests that oxidative and nitrative modifications in retina occur early in the course of development of retinopathy in diabetes. These abnormalities are not easily reversed by reinstatement of GC, and the duration of PC before initiation of GC influences the outcome of the reversal. Characterization of the abnormalities responsible for the resistance of retinopathy to arrest after reinstatement of GC will help identify potential future therapies to inhibit progression of diabetic retinopathy. *Diabetes* 52:818–823, 2003

Hyperglycemia is the initiating event in the development of retinopathy, and studies have shown that improved glycemic control is associated with decreased development and progression of retinopathy in diabetes (1,2). However, reinstatement of normal glycemic control after a period of poor glycemic control does not produce immediate benefits on the progression of retinopathy (1–3), and the duration of poor glycemic control before initiation of good glycemic control plays a major role in the outcome of good

glycemic control (1,4,5). This suggests that some submicroscopic processes already begun in the retina during the initial period of high circulating hexoses and chronic elevation of blood aldohexose result in metabolic or physiological abnormalities that are not readily corrected by reestablishment of normoglycemia.

Hyperglycemia-induced metabolic disorders are postulated to initiate the sequence of events leading to the development of retinopathy. Many biochemical abnormalities have been identified in the retina in diabetes, including elevated oxidative stress, activation of protein kinase C, nonenzymatic glycation, polyol pathway, and increased nitric oxide (NO) (6–10), but which metabolic abnormalities may be critical in the etiology of diabetic retinopathy is unknown.

Diabetes-induced increased oxidative stress is postulated to play a significant role in the development of complications (7,11,12). The retina experiences increased oxidative stress and NO levels in diabetes, and antioxidants inhibit these retinal abnormalities and the development of retinopathy (8,13). Possible sources of oxidative stress in diabetes include increased generation of reactive oxygen species by auto-oxidation of glucose, decreased tissue concentrations of low-molecular weight antioxidants, and impaired activities of antioxidant enzymes (13,14).

Superoxides and NO can react and form peroxynitrite, a highly reactive intermediate, which can increase DNA damage, deplete intracellular reduced glutathione (GSH) levels, and initiate lipid peroxidation (15). Peroxynitrite modifies tyrosine in proteins to form nitrotyrosine, and nitration of proteins can inactivate mitochondrial and cytosolic proteins and damage of cellular constituents, resulting in nitrative stress (16). Nitrotyrosine levels are elevated in the retina in diabetes (17), and we have shown that they remain elevated when the pathology is developing in the retina (18,19).

In the present study, the effect of reinstatement of good glycemic control on hyperglycemia-induced increased retinal oxidative stress and nitrative stress was investigated in rats. The effect of duration of poor glycemic control before initiation of good glycemic control on these abnormalities was also investigated by allowing the rats to remain in poor glycemic control for 2 months, when metabolic abnormalities are present but cell death and pathology are not detectable in retinal vasculature (13), or for 6 months, when retinal vascular cell apoptosis is detectable (20), before initiation of good glycemic control.

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GC, good glycemic control; GSH, reduced glutathione; iNOS, inducible nitric oxide synthase; LPO, lipid peroxide; NO, nitric oxide; PC, poor glycemic control.

RESEARCH DESIGN AND METHODS

Animals. Wistar rats (male, 200 g) were randomly assigned to normal or diabetic groups. Diabetes was induced with intraperitoneal injection of streptozotocin (55 mg/kg). Diabetic rats were divided at random among four groups according to intended degree and duration of good glycemic control. Group 1 consisted of rats that were allowed to remain in poor glycemic control (PC) for 13 months, and in group 2 the rats remained in good glycemic control (GC) for the entire 13 months. The diabetic rats in group 3 were allowed to remain in PC for 2 months followed by GC for 7 months (PC2 GC), and in group 4 the rats remained in PC for 6 months followed by good glycemic control for 7 months (PC6 GC).

Glycemia. All diabetic rats received insulin (NPH) injections. The PC rats received a single injection of insulin (1–2 units) 4–5 times a week to prevent ketosis and weight loss, and the GC rats received insulin twice a day (8–10 units total) to maintain a steady gain in body weight and urine glucose <150 mg/24 h. Rats were housed in metabolism cages; 24-h urine samples were tested for glycosuria daily with Keto-Diastix (Bayer Corporation) and 3–4 times every week using quantitative methods (Glucose Kit, 510-A; Sigma Chemicals). Blood glucose was measured once a week (Glucometer Elite; Bayer Corporation), and GHb (kit 442-B; Sigma Chemicals) every 2 months. The entire rat colony received powdered diet (Purina 5001), and their food consumption and body weights were measured 2–3 times every week. These experiments conformed to the Association for Research in Vision and Ophthalmology (ARVO) Resolution on Treatment of Animals in Research, as well as to specific institutional guidelines. At the end of the desired duration of glycemic control, the animals were killed, and retinas were isolated by gently separating sensory retina from choroid using a microspatula under a dissecting microscope.

Lipid peroxides. Lipid peroxides (LPOs) in the retina were measured directly by redox reactions with ferrous ions, and the resulting ferric ions were detected using thiocyanate ion as the chromogen (21–23).

GSH. Cytosolic glutathione was measured fluorometrically using *O*-phthalaldehyde, and the fluorescence was determined at excitation and emission wavelengths of 350 and 420 nm, respectively (13).

NO. NO was quantitated by measuring the stable metabolites of NO (nitrate + nitrite) using a fluorometric assay kit (Cayman Chemical). Fluorescence generated by nitrite reaction with 2,3-diaminonaphthalene was measured at excitation and emission wavelengths of 365 and 450 nm, respectively (21–23).

Expression of inducible NO synthase. Expression of inducible NO synthase (iNOS) was determined in the retinal homogenate by Western blot analysis. Retinal protein (35 μ g protein) was separated on 8% reducing polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked in 5% milk, incubated with a polyclonal antibody against iNOS (Santa Cruz Biotechnology), washed, and incubated with anti-rabbit IgG horseradish peroxidase-conjugated antibody in blocking buffer for 1 h. The membranes were washed and developed using ECL-Plus Western blotting detection kit from Amersham Pharmacia Biotech. Kaleidoscope prestained molecular weight markers (BioRad) were run simultaneously on each gel. To ensure equal loading among the lanes, the expression of house-keeping protein GAPDH was determined. After blotting for iNOS, the membranes were incubated with stripping buffer (62.5 mmol/l Tris-HCl, pH 6.8, 100 mmol/l α -mercaptoethanol, 2% sodium dodecyl sulfate) at 50°C for 30 min, washed, and incubated with anti-GAPDH antibody (Biodesign International). The membranes were washed, incubated with horseradish peroxidase-conjugated secondary antibody, and developed using ECL-Plus Western blotting detection kit.

Nitrotyrosine. Nitrotyrosine, a biomarker of peroxynitrite formation, was measured in the retina by measuring nitrosylated proteins using immunochemical methods. Existing antibodies were removed from retinal homogenates (150 μ g protein per sample) by incubating first with protein A Sepharose, followed by overnight incubation with rabbit anti-nitrotyrosine antibody, and then protein A Sepharose to precipitate nitrotyrosine complexed with antibody. Proteins were separated on 8% denaturing polyacrylamide gels, followed by incubation with mouse anti-nitrotyrosine, and nitrotyrosine was detected using peroxidase-conjugated secondary antibody (17,19,22).

Tissue protein was measured by the Bradford method (24) using BSA as standard.

The results are reported as means \pm SD and were analyzed statistically using the nonparametric Kruskal-Wallis test followed by Mann-Whitney test for multiple group comparisons. Similar conclusions were reached also by using ANOVA with Fisher's or Tukey's test.

RESULTS

Hyperglycemia was, as expected, severe in the rats in the PC group: GHb values were >11% throughout the experi-

TABLE 1
Body weight and GHb in rats assigned to good glycemic control

	<i>n</i>	Duration (months)	Body weight (g)	GHb (%)
Normal	7	13	523 \pm 51	4.2 \pm 0.5
Poor control	6	13	339 \pm 26	11.6 \pm 0.9
Good control	6	13	499 \pm 66	5.1 \pm 2.3
Poor control		2	241 \pm 20	11.6 \pm 1.3
↓		↓	↓	↓
Good control	6	7	409 \pm 26	5.3 \pm 0.4
Poor control		6	306 \pm 40	11.4 \pm 0.7
↓		↓	↓	↓
Good control	7	7	484 \pm 32	4.9 \pm 0.4

Data are means \pm SD.

ment (13 months). In the rats in which GC was maintained for the entire duration, GHb values (5.1 \pm 2.3%) were similar to those of normal rats (4.2 \pm 0.5%; $P > 0.05$). In the PC2 GC and PC6 GC groups, glycemic control before initiation of good control was comparable to that in PC rats (GHb >11%), but the GHb values were significantly decreased (GHb <5.5%) at 2 months after initiation of good glycemic control (the first measurement made after initiation of good glycemic control), and the values remained unchanged for the entire 7 months of good glycemic control (Table 1).

Poor glycemic control resulted in increased retinal oxidative stress compared with that in age-matched normal rats, and this was demonstrated by both elevation in LPO values (normal rats, 411 \pm 69 nmol/mg protein; PC, 822 \pm 123) and decrease in GSH levels (64.4 \pm 7.6 to 46.3 \pm 6.3 nmol/mg protein; $P < 0.005$) (Fig. 1A and B). As shown in Fig. 2, in the same retina NO levels were elevated from 31.4 \pm 10.1 nmol/mg protein in normal rats to 62.8 \pm 17.5 in PC rats ($P < 0.02$), and the expression of iNOS was increased by >85% (Fig. 3). Peroxynitrite formation, as detected by measuring the major nitrosylated protein (80 kDa), was significantly higher ($P < 0.05$) in the retina of PC rats than in normal rats (Fig. 4).

Maintenance of rats in good glycemic control for the entire 13 months did not increase retinal oxidative stress, but increased NO by <20%; this was not significantly different ($P > 0.05$) from age-matched normal rats (Figs. 1 and 2). Retinal LPO, GSH, and NO levels from the GC group, however, were significantly different ($P < 0.02$) from those in the PC group. The expression of iNOS and nitrosylated protein was similar in GC and normal groups (data not shown).

Reinstitution of good glycemic control for 7 months after a short duration of poor glycemic control (PC2 GC group) had some beneficial effects on retinal oxidative stress. LPO levels were slightly (596 \pm 89 nmol/mg protein), but not significantly ($P > 0.05$) different from the normal group, and GSH levels remained significantly decreased ($P < 0.02$ vs. normal) (Fig. 1A and B). Intervention of this short-duration poor glycemic control inhibited elevations in retinal NO ($P > 0.05$ vs. normal) and iNOS expression by 70% (Figs. 2 and 3) but failed to have beneficial effects on nitrotyrosine levels (data not shown).

In the rats that were allowed to remain in poor glycemic control for 6 months before reinstatement of good glycemic control (PC6 GC group), retinal GSH remained subnormal

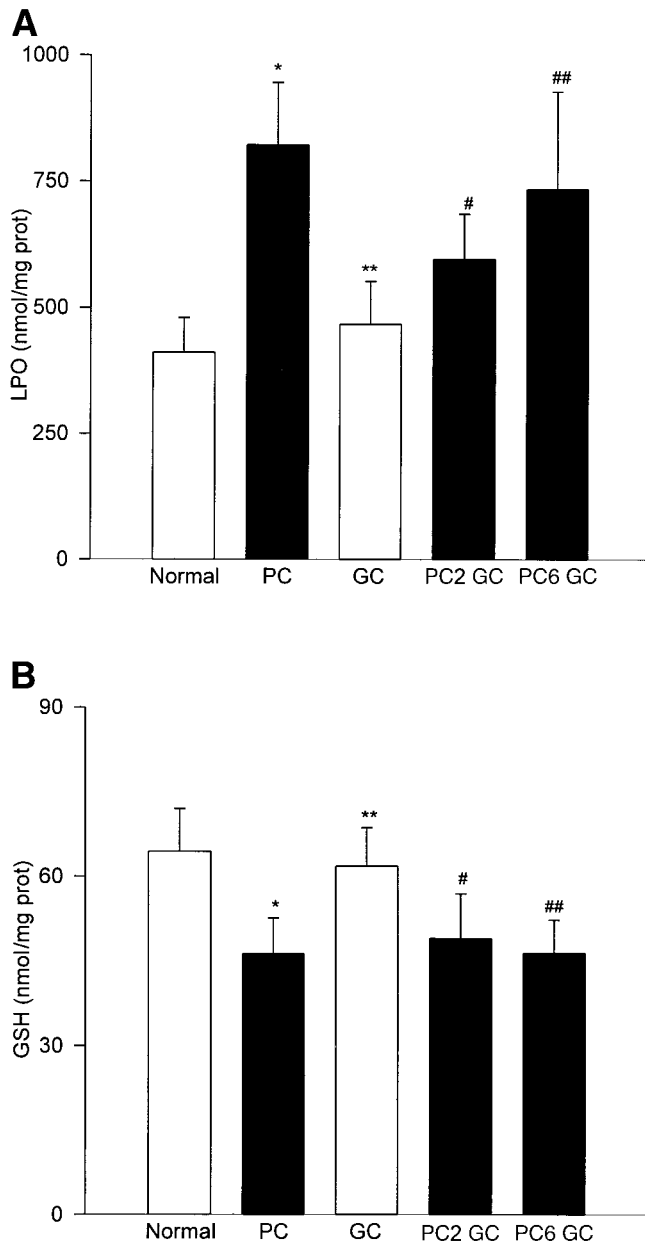


FIG. 1. Effect of reversal of hyperglycemia on retinal oxidative stress. **A:** LPOs: hydroperoxides in the retina were measured directly by the redox reactions with ferrous ions, and the resulting ferric ions were detected using thiocyanate ion as the chromogen. The values represent means \pm SD of six rats each in normal and GC groups, eight rats each in PC and 2PC GC groups, and seven rats in the 6PC GC group. * $P = 0.003$ vs. normal; 0.005 vs. GC. ** $P = 0.175$ vs. normal. # $P = 0.059$ vs. normal; 0.086 vs. GC. ## $P = 0.003$ vs. normal; 0.018 vs. GC. **B:** Glutathione: GSH was measured in the cytosol of retina by fluorometrically method using *O*-phthalaldehyde, and each measurement was made in triplicate. The values are means \pm SD of six to eight rats in each group. * $P = 0.003$ vs. normal; 0.010 vs. GC. ** $P = 0.083$ vs. normal. # $P = 0.012$ vs. normal; 0.031 vs. GC. ## $P = 0.002$ vs. normal; 0.008 vs. GC.

and NO levels elevated (Figs. 1B and 2); 7 months of additional good glycaemic control had no significant effect on cytosolic antioxidant and NO formation (both $P > 0.05$ vs. normal). LPO levels in the retina were decreased to 734 ± 193 nmol/mg protein versus 822 ± 123 nmol/mg protein in PC group (Fig. 1A) but were significantly higher than those obtained from normal rats ($P < 0.005$). Reinstatement of good glycaemic control had no effect on iNOS

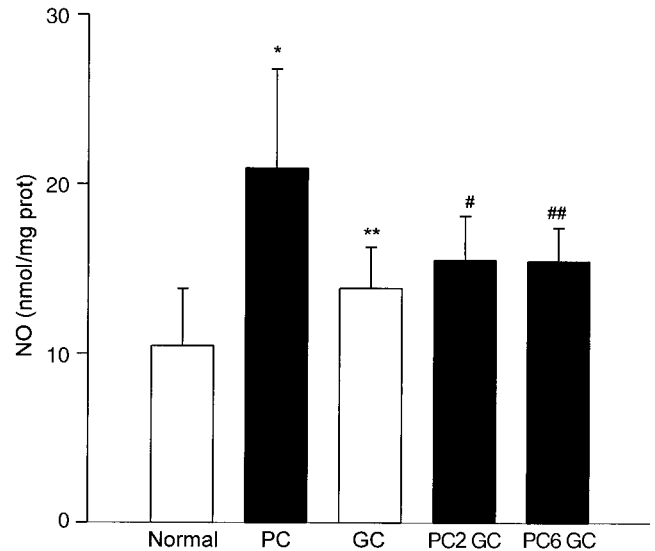


FIG. 2. NO in the retina and reversal of hyperglycemia. NO levels were estimated in the retina by measuring the stable metabolites of nitrate and nitrites, and each measurement was made in triplicate. Values represent means \pm SD of three separate experiments, and are from seven rats each in normal and PC groups, eight rats in the GC group, and six rats each in the two reversal groups. * $P = 0.008$ vs. normal; 0.016 vs. GC. ** $P = 0.065$ vs. normal. # $P = 0.060$ vs. normal; 0.523 vs. GC. ## $P = 0.012$ vs. normal; 0.028 vs. GC.

expression (Fig. 3) or nitrotyrosylated proteins in the retina (Fig. 4).

DISCUSSION

This is the first report providing evidence that initiation of good glycaemic control soon after induction of diabetes in rats prevents increases in oxidative stress and nitrate stress, but delay in the initiation of good glycaemic control for even 2 months has only partial beneficial effect on oxidative stress and nitrate stress. If the intervention with good glycaemic control is delayed for longer time (6 months), these abnormalities are not reversed. These results suggest that oxidative and nitrate modifications in the retina occur early in the course of development of retinopathy in diabetic rats and are not easily reversed by reinstatement of good glycaemic control. These results are in agreement with our previous studies with experimentally galactosemic rats (another model of diabetic retinopathy), which showed that retinal GSH remained subnormal and nitrotyrosine levels elevated for at least 1 month of galactose-free diet after 2 months of 30% galactose diet (22).

Oxidative stress is increased in the retina in diabetes and remains elevated when histopathology is developing (8,13,25). Increased oxidative stress is postulated to play a role in pericyte dropout in diabetic retinopathy (26) and is linked to increased retinal basement thickening (27). A strong association between hyperglycemia-induced retinal oxidative stress and the development of retinal histopathology is observed in both diabetic rats and galactose-fed rats; administration of multiple antioxidants significantly inhibits the development of both acellular capillaries and pericyte ghosts (8). The data presented here show that oxidative stress is only partially corrected if good glycaemic control is initiated after 2 months of poor glycaemic control and is not corrected if the reversal is initiated after 6

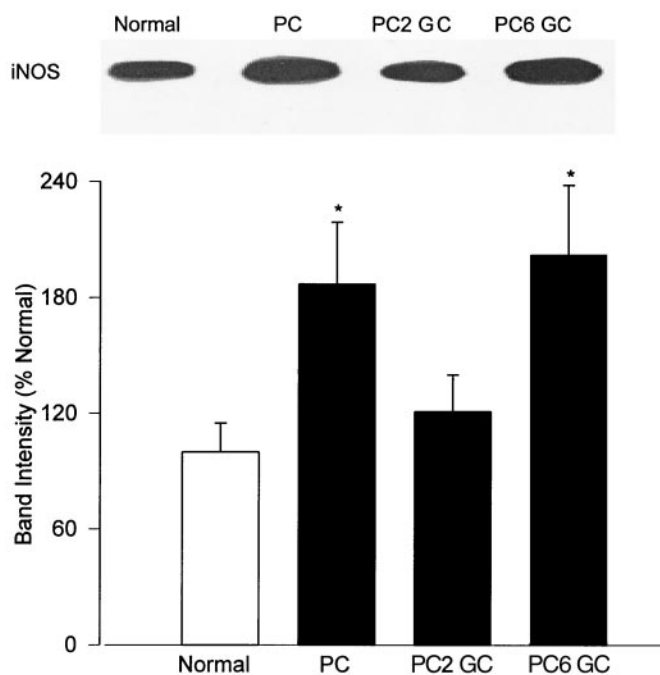


FIG. 3. Expression of iNOS in the retina of rats with different glycemic control. iNOS expression was determined in the retinal homogenates by Western blot. The proteins were separated on 8% SDS gel, and the band intensity was adjusted to GAPDH expression. The values obtained from the normal rats are 100%. The Western blots are representative of four different rats in each group, and the bars show means \pm SD of the band intensities obtained from those four rats. * $P < 0.05$ vs. normal.

months of poor glycemic control. These results are supported by previous reports suggesting that mitochondrial superoxide production is one of the factors for the failure of retinopathy to arrest after reinstatement of normoglycemia (28). Thus, oxidative stress plays an important role not only in the development of retinopathy in diabetes, but also in the resistance of retinopathy to arrest after good glycemic control is initiated.

In the pathophysiology of diabetic retinopathy NO plays a role in the regulation of retinal vascular functions and vascular damage (29), and hydroxy-L-arginine levels are elevated in the aqueous humor of diabetic patients (30). We have shown that NO levels remain elevated in the retina at a duration of diabetes when histopathology can be seen in the vasculature (8). The present study shows that intervention of good glycemic control in rats had partial beneficial effects on retinal NO levels when good glycemic control was initiated after 2 months of poor glycemic control, but failed to have any beneficial effect if good control was preceded by 6 months of poor glycemic control, thus suggesting that adverse effects of NO continue to progress.

Retinal endothelial cells and pericytes synthesize iNOS under stimulated conditions (31), and iNOS levels are elevated in the retina when retinopathy is developing in diabetic rats (19). iNOS is shown to play a crucial role in retinal apoptosis in ischemic proliferative retinopathy (32), and inhibition of NO synthase reduces the systemic and ocular hemodynamic reactivity (33). The data presented here show that diabetes-induced increased expression of iNOS is not reversed completely even if good

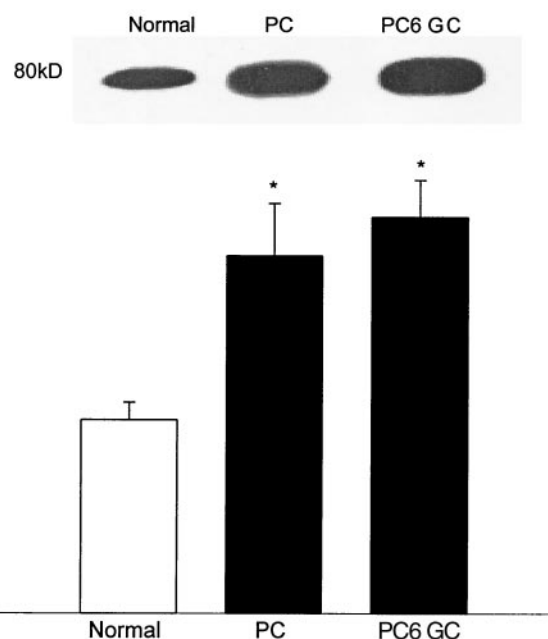


FIG. 4. Effect of reversal of hyperglycemia on nitrotyrosylated proteins. Nitrotyrosine was detected in the retina by immunochemical methods using rabbit anti-nitrotyrosine antibody. The Western blots are representative of at least three different rats in each group, and the histogram shows the means \pm SD of the absorbance of the 80-kDa band obtained from three rats/group. * $P < 0.05$ vs. normal.

glycemic control is initiated after a short duration of diabetes.

Peroxynitrite, formed by the reaction between superoxides and NO, modifies tyrosine in proteins to form nitrotyrosine, and this stable end product is involved in inactivation of mitochondrial and cytosolic proteins, resulting in damage of cellular constituents (16). It can increase DNA damage, deplete intracellular GSH levels, and initiate lipid peroxidation (15). Peroxynitrite is elevated in the retina early in diabetes and remains elevated at 14 months of diabetes in rats, and administration of antioxidants or aminoguanidine to diabetic rats significantly inhibits diabetes-induced nitration of proteins (17–19). Nitration of proteins is postulated to be involved in the apoptosis of retinal cells (32), can disrupt protein assembly and functions with possible pathological consequences (34), and results in oxidation of protein sulfhydryls (15). The data presented here show that in retina, nitrosylated proteins remain significantly elevated after intervention of poor glycemic control with good glycemic control, and these results are similar to those reported with galactose-withdrawal experiments in rats (22). This strongly suggests that some of the retinal proteins are nitrosylated early in the pathogenesis of retinopathy in diabetes and are resistant to reversal even after 7 months of good glycemic control in rats.

The rats in the PC2 GC group were killed at 9 months of age versus 13 months of age in other groups, but the partial reversal of the retinal abnormalities by good glycemic control cannot be accounted for by the differences in the age of rats, since duration of diabetes and the age of the rat (2–14 months) have no significant effect on the increase in retinal oxidative stress and NO levels (8,19,23).

Diabetic retinopathy continues to progress for a consid-

erable period even after hyperglycemia is corrected, and retinal vascular lesions (e.g., pericyte loss and acellular capillaries) are considered irreversible lesions. Clinical studies have shown that instituting tight glycemic control in insulin-dependent diabetic humans does not necessarily immediately benefit the progression of retinopathy; retinopathy remains unchanged or even worsens in 5–10% of patients for almost 2 years, after which patients in tight control begin to show slowing of retinopathy progression (2). Studies with diabetic animals have demonstrated that good glycemic control can prevent the development of diabetic retinopathy if the good glycemic control is initiated within a few weeks after the onset of diabetes, but that intervention with good glycemic control after many months of poor control is much less effective in inhibiting retinopathy (1,35). Similarly, islet transplantation in rats after several months of diabetes arrests the progression of retinopathy less effectively than if intervention occurs after only a few weeks of diabetes (36), and preexisting damage at the time of intervention is considered a primary factor in determining the outcome of a therapy (5). The data presented here show that if good glycemic control is initiated soon after induction of diabetes in rats, retinal oxidative stress and nitrate stress are not elevated, but if poor glycemic control is maintained for 2 months before instituting good glycemic control, these abnormalities are only partially reversed. This suggests that some of the proteins are oxidatively modified or nitrosylated early in the course of development of retinopathy in diabetes. Similarly, in galactose-fed rats, withdrawal of galactose after 6 months of 50% galactose feeding is shown to neither arrest nor reverse the progression of retinopathy (4), and withdrawal of galactose after 4 or 8 months does not prevent the progression of basement membrane thickening until after 16–20 months (5). However, Kador et al. (37) have recently reported that removal of galactose from diet at the initial stages of background retinopathy delays the progression of retinopathy in dogs. The results of the present study show that the retina escapes increased oxidative and nitrate stress only if good glycemic control is instituted soon after the induction of diabetes, suggesting that oxidatively modified and nitrosylated proteins, once formed, are not easily reversible even after a short duration of poor glycemic control, and might be contributing to the progression of retinopathy after reinstatement of good glycemic control.

The data presented here suggest that hyperglycemia-induced formation of oxidatively modified and nitrosylated proteins are early events in the development of diabetic retinopathy and cannot be easily corrected after reestablishment of good glycemic control in rats. Understanding the mechanisms responsible for the resistance of retinopathy to arrest after reinstatement of good glycemic control in diabetes may reveal novel means for inhibiting the progression of retinopathy.

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