Termination of Experimental Galactosemia in Rats, and Progression of Retinal Metabolic Abnormalities

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PURPOSE. To investigate the effect of termination of galactose feeding after a very short duration of experimental galactosemia on the biochemical abnormalities that are postulated to contribute to the development of retinopathy.

METHODS. Experimentally galactosemic rats (normal rats fed a 30% galactose-rich diet for 2 months) were fed a galactose-free diet for an additional 1 month. At the end of 3 months, retinas were removed to measure oxidative stress, nitric oxides (NOs), activity of PKC, and levels of nitrotyrosine. Data were compared between rats in the control group (fed a normal diet) and those in the experimentally galactosemic group (30% galactose diet for the entire 3 months).

RESULTS. Interruption of 2 months of galactose feeding by the withdrawal of galactose from the diet for 1 additional month had partially beneficial effects on retinal lipid peroxides, but the levels of an endogenous antioxidant, reduced glutathione (GSH), remained subnormal in the retina of galactose-withdrawal rats (P < 0.05 vs. normal and P > 0.05 vs. galactose group). Cessation of the galactose-rich diet had partially beneficial effects on NO levels in the retina, but the levels of nitrotyrosine, an indicator of the formation of peroxynitrite, and activation of PKC were not affected.

CONCLUSIONS. The results show that retinal dysmetabolism continues to progress after experimental galactosemia is terminated in rats. Particularly, antioxidant levels remain subnormal, and nitrotyrosine levels are elevated for at least 1 month. Identification of metabolic abnormalities associated with the progression of incipient retinopathy after hyperglycemia is normalized may help in the search for the cause of retinopathy. (Invest Ophthalmol Vis Sci. 2002;43:3287–3291)

Hyperglycemia has been found to be sufficient to initiate the development of diabetic retinopathy, but the mechanism by which hyperglycemia causes retinopathy is not clearly understood. 1,2 Multiple mechanisms are proposed to explain how hyperglycemia may cause the development of retinopathy, including, increased free radicals, nitric oxide (NOs), non-enzymatic glycation, elevated activity of the polyol pathway, and activation of protein kinase C (PKC).3–8 At present, however, a link between any particular abnormality of metabolism or physiology in the retina and the development of diabetic retinopathy is largely speculative, and observations to date raise a possibility that no one metabolic abnormality is the sole cause of the development of diabetic retinopathy. 9,10

Experimental galactosemia, induced by feeding normal animals (e.g., dogs, rats, or mice) a 30% to 50% galactose-supplemented diet, has been shown to reproduce the retinopathy and retinal metabolic abnormalities that occur in diabetes.8,11–13 Galactose-fed animals have elevated levels of blood hexoses, but do not develop other sequelae of insulin deficiency, such as alterations in lipids and protein metabolism.

It has been shown unambiguously that good glycemic control can retard the progression of diabetic retinopathy, and experimental studies support these findings.1,2,14 Clinical studies have shown that reinstitution of normal glycemic control after a period of poor glycemic control does not produce immediate results in slowing the progression of retinopathy.1,14 This resistance of incipient retinopathy to arrest is not peculiar to insulin therapy, but is characteristic of hyperglycemia. Dogs made galactosemic for 2 years and observed subsequently for up to 3 years after the galactosemia has been terminated show significant retinopathy,15 and the duration of poor glycemic control before initiation of good control plays a major role in the outcome of good control.16,17 This suggests that some submicroscopic processes already begun during the initial period of high circulating hexoses and chronic elevation of blood aldohexose results in metabolic or physiological abnormalities in retina that are not readily corrected by reestablishment of normoglycemia.

In the present study, we investigated the effect of termination of experimental galactosemia after a very short duration of increased blood hexoses (a duration at which cell death and histopathology are not detectable in retinal vasculature) on some of the biochemical abnormalities that are postulated to contribute to the development of retinopathy.

MATERIALS AND METHODS

Sprague-Dawley rats (male, 200 g) were randomly assigned to normal or galactosemic groups. Experimental galactosemia was induced in normal rats by feeding them a diet supplemented with 30% galactose. Average food consumption was measured daily and body weight two times each week. In a group of galactose-fed rats, after 2 months of galactose feeding, galactose supplementation was terminated, and the rats were fed normal diet for one additional month. All the rats were killed 3 months after initiation of the experiment, and the retinas were removed under a dissecting microscope and used for biochemical measurements. Glycated hemoglobin was measured 2 to 3 days before the experiment was terminated. Treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to specific institutional guidelines.

Oxidative stress was estimated in the retina by measuring the levels of thiobarbituric acid-reactive substances (TBARS), hydroperoxides, and reduced glutathione (GSH). TBARS were quantitated by measuring the absorbance at 535 nm of malonaldehyde-thiobarbituric acid adducts formed by acid hydrolysis at 100°C,13,15 and the level expressed relative to the protein concentration. Hydroperoxides were determined in the retina with an assay kit (Cayman Chemical Co., Ann Arbor, MI). Hydroperoxides were directly redox reacted with ferrous ions, and the resultant ferric ions were detected with thiocyanate ions used as the chromogen. GSH was measured fluorometrically in fresh retina with o-phthalaldehyde. Fluorescence was determined at excitation and emission wavelengths of 350 and 420 nm, respectively.19
Nitrotyrosine, a biomarker of peroxynitrite formation, was measured in the retina by immunochemical methods. Existing antibodies were removed from retinal homogenates by incubating, with protein A-agarose, followed by overnight incubation with rabbit anti-nitrotyrosine antibody, and then protein A-agarose to precipitate nitrotyrosine complexed with antibody. Proteins were separated by reducing polyacrylamide gel electrophoresis (10% gel), followed by incubation with mouse anti-nitrotyrosine. Nitrotyrosine was detected using peroxidase-conjugated secondary antibody.

Protein content was determined in the retina by the Bradford method, and bovine serum albumin was used as the standard. Results are reported as the mean ± SD and analyzed statistically using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test for multiple group comparisons. Similar conclusions were reached also by using ANOVA with the Fisher or Tukey test.

**RESULTS**

Experimental galactosemia in rats, as expected, resulted in increased oxidative stress in the retina, demonstrated by elevated levels of retinal TBARS and lipid hydroperoxides and decreased levels of GSH, compared with the age-matched normal rats (Fig. 1). Retinal NO levels were increased by more than 50% and PKC activity by approximately 40% in the rats fed a galactose-supplemented diet without interruption for 3 months compared with the normal rats (Figs. 2, 3). The protein expression of PKCβII (Fig. 5) and the levels of nitrotyrosine (an index of peroxynitrite formation) were significantly increased in the retina of rats fed galactose for the entire 3 months (Fig. 4).

 Interruption of 2 months of galactose feeding by withdrawal of galactose from the diet for 1 additional month had some beneficial effects on retinal lipid peroxides. Retinal TBARS and hydroperoxides were significantly (P < 0.05) and moderately (P > 0.05) decreased, respectively. However, the levels of the endogenous antioxidant GSH remained subnormal in the retina of galactose-withdrawal rats (P < 0.05 vs. normal and P > 0.05 vs. galactose-fed group), thus suggesting that the retina continued to undergo increased oxidative stress for some time, even after hyperglycemia was arrested (Fig. 1).

Withdrawal of the galactose dietary supplement resulted in partial inhibition of the galactose-induced increase in retinal NO levels (Fig. 2); however, the expression of the enzyme involved in increased NO, iNOS, remained elevated (data not shown).
addition, nitrotyrosine levels in the retina were not affected by the termination of galactose feeding in the rats (Fig. 4). The other metabolic abnormality that is postulated to be involved in the development of retinopathy, increased PKC activity, was not affected by the cessation of galactose feeding. PKC activity in the galactose-withdrawal group was significantly higher ($P < 0.05$) compared with that in age-matched normal rats and was not different ($P > 0.05$) from the rats fed galactose for the entire 3-month duration. Similarly, the protein expression of PKC was elevated by approximately 50% in both experimentally galactosemic and galactose-withdrawal groups, compared with the normal group (Fig. 3).

As expected, 3 months of galactose feeding resulted in significant elevations in glycated hemoglobin, and cessation of experimental galactosemia had a marginal, but statistically significant, effect on glycated hemoglobin values (Table 1).

**DISCUSSION**

This study, for the first time, provides evidence that many of the hyperglycemia-induced metabolic abnormalities in rat retina are not normalized for at least 1 month after a galactose-rich diet is replaced with a normal diet. The observed failure to reverse completely the galactosemia-induced metabolic abnormalities—increases in oxidative stress, NO levels, and activation of PKC—is of particular interest, because these abnormalities have been postulated to contribute to the development of retinopathy in diabetes.\(^3,4,7,8,13\)

Diabetes or experimental galactosemia is reported to increase oxidative stress,\(^13,22\) and increased serum lipid hydroperoxides are associated with the increased prevalence of retinopathy in diabetes.\(^23\) Possible sources of oxidative stress may include shifts in redox balances, due to carbohydrate and lipid dysmetabolism; decreased tissue concentrations of low-molecular-weight antioxidants (including GSH and vitamin E); and impaired activities of antioxidant defense enzymes.\(^4,12,24\) In hyperglycemia TBARS are increased, antioxidant defense systems are impaired, and GSH levels are decreased in the retina.\(^4,25,26\) Increased oxidative stress is postulated to play a role in loss of pericytes in diabetic retinopathy\(^27\) and is linked to increased thickening in the retinal basement membrane.\(^28\) Our recent results have shown that administration of a com-

**TABLE 1. Severity of Hyperglycemia in Galactose-Fed Rats**

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Food (g/d)</th>
<th>GHB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 9)</td>
<td>429 ± 33</td>
<td>34 ± 5</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Galactose (n = 7)</td>
<td>583 ± 17*</td>
<td>31 ± 3</td>
<td>6.4 ± 0.7*</td>
</tr>
<tr>
<td>Galactose-withdrawal (n = 9)</td>
<td>580 ± 17*</td>
<td>29 ± 2</td>
<td>5.6 ± 0.5†</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD, with number of rats in each group in parentheses. GHB, glycated hemoglobin.

$P < 0.05$ compared with *galactose, and †galactose-withdrawal groups.
preheparin mixture of antioxidants significantly inhibits both the development of acellular capillaries and the pericyte ghosts in diabetic rats and galactose-fed rats, suggesting a strong association between hyperglycemia-induced retinal oxidative stress and the development of pathologic histology in the retina. Termination of galactose feeding in rats had beneficial effects on retinal TBARS, but intracellular antioxidant GSH remained subnormal in the retina 1 month after galactose was withdrawn from the rats. This suggests that the reversal of hyperglycemia may have only very marginal effect on oxidative stress in the retina.

In our study, rat retinal NO levels remained elevated at a duration of hyperglycemia when histopathologic formations occur in the vasculature. Increased reactive oxygen species have been shown to activate nuclear transcriptional factor-B, which can activate NO, resulting in increased NO. NO is reported to play an important role in the regulation of retinal vascular functions and contributes to the pathophysiologic course of retinopathy. Interruption of galactose feeding in rats had partial beneficial effects on retinal NO levels, but had no effect on the enzyme involved in its synthesis, thus suggesting that the adverse effects of NO continued to progress.

Nitrotyrosine, which is a stable end product of peroxynitrite and has been shown involved in the pathogenesis of retinopathy in diabetes, are elevated at 12 to 14 months of hyperglycemia in diabetic rats and galactose-fed rats. The elevation in PKC activity in hyperglycemia can be attributed to part to excessive production of diacylglycerol (DAG), or to increased reactive oxygen species that can directly increase the activity. Elevation of retinal activity of PKC can have effects that are characteristic of changes observed in diabetic retinopathy, including stimulation of neovascularization and endothelial proliferation, increased vascular permeability, stimulation of apoptosis, and contribution to hyperglycemia-induced oxidative stress. The results presented herein clearly show that termination of galactose feeding for 1 month after 2 months of 30% galactose feeding was terminated. Hyperglycemia (diabetes and experimental galactosemia) increases the activity of PKC in the retina and in retinal microvessels. We have shown that the activity of PKC remains elevated at 12 to 14 months of hyperglycemia in diabetic rats and galactose-fed rats. The elevation in PKC activity in hyperglycemia can be attributed to part to excessive production of diacylglycerol (DAG), or to increased reactive oxygen species that can directly increase the activity. Elevation of retinal activity of PKC can have effects that are characteristic of changes observed in diabetic retinopathy, including stimulation of neovascularization and endothelial proliferation, increased vascular permeability, stimulation of apoptosis, and contribution to hyperglycemia-induced oxidative stress.

The results in the present study were obtained from experimentally galactosemic rats, an animal model of diabetic retinopathy. However, recent studies have shown some differences in the retinal changes in galactose-fed and diabetic rats. The activation pattern of caspases in diabetic mice and galactose-fed mice are different, and administration of aminoguanidine inhibits both retinal capillary apoptosis and histopathologic disorders in diabetic rats, but fails to have any effect in galactose-fed animals. The reasons for these differences are not known, but in galactose-fed animals, retinal histopathologic appearance is indistinguishable from that of diabetic rats, and the metabolic abnormalities, postulated to be involved in the pathogenesis of retinopathy in diabetes, are observed in galactose-fed animals. Our results identify the metabolic abnormalities that fail to reverse after cessation of galactose feeding, and these studies are consistent with previous reports of long-term studies showing that cessation of galactose feeding in dogs and rats does not immediately inhibit the progression of retinopathy. Moreover, in rats, withdrawal of galactose after 4 or 8 months of galactose feeding does not prevent the progression of thickening of the basement membrane until after 16 to 20 months, and administration of an aldose reductase inhibitor or amino-guanidine after 12 months of 50% galactose feeding has no effect on the development of retinopathy. Clinical studies have shown that instituting tight glycemic controls in insulin-dependent diabetic humans does not produce immediate beneficial effect. Progression of retinopathy remains unchanged (or even worsen in 5%–10% of patients) for almost 2 years after initiation of tight control of diabetes. Similarly, in rats, the improvement of glycemic control by islet transplantation after several months of diabetes has been shown to arrest the progression of retinopathy less effectively than if intervention is started after only a few weeks of diabetes. The retinal metabolic abnormalities that are not promptly reversed by elimination of hyperglycemia may play an important role in the progression of retinopathy that occurs after correction of hyperglycemia.

The rats used in our experiments were fed galactose-supplemented diets for 2 months, followed by a galactose-free normal diet for 1 additional month. Intervention in hyperglycemia by galactose withdrawal from the rats previously fed a galactose-supplemented diet is expected to normalize the elevated tissue levels of aldohexoses promptly. Thus, the resistance of these metabolic abnormalities to ready reversal after galactosemia is terminated cannot account for the failure to achieve good hexose levels in these rats.

Our studies show that decreased intracellular antioxidant levels, increased nitrosylated proteins, and activation of PKC persist for some time, even after the reestablishment of normoglycemia. Characterization of the metabolic abnormalities responsible for the progression of incipient retinopathy after normalization of hyperglycemia is important for understanding the pathogenesis and identifying potential future therapies for retinopathy in diabetes.

References


