Aldose Reductase in Early Streptozotocin-Induced Diabetic Rat Lens

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The present study investigates the status of lens aldose reductase in the early streptozotocin-induced diabetic rat. Aldose reductase protein concentration, protein synthesis, and enzyme activity was assayed at 3 days and 14 days post-streptozotocin injection. Our results indicated that there was no significant difference between normal (control) and diabetic rat lenses in these parameters during the time frame of the study. Results from whole lens analysis were supported by results of the examination of the isolated capsule-epithelium layer of these lenses. It is concluded from this study that in the initial stage of the diabetic cataract model, increase in enzyme protein or activity does not play a significant role in cataractogenesis, but rather that the hyperglycemic condition in combination with existing enzyme levels is sufficient to cause the cataractogenic changes. Invest Ophthalmol Vis Sci 30:2370-2375, 1989

It has been established that the hyperglycemic condition in experimental animal models, including the streptozotocin (STZ)-induced diabetic rat, causes lenticular changes. These changes, which include altered membrane permeability,1,2 loss of glutathione1,3 and inositol,4,5 and increased sugar alcohol,6 lead eventually to cataract formation. Kinoshita7 hypothesized that the initiating event of these phenomena is an increase in osmolarity of the lens. The osmolarity change is caused by the accumulation of sugar alcohol, which is formed by the action of the enzyme aldose reductase (AR) on sugar. This mechanism has been supported by the evidence that aldose reductase inhibitors (ARI) prevent the accumulation of sorbitol in the lens even though plasma, aqueous, and lenticular glucose levels were unaltered.7 AR has been implicated in causing other complications of diabetes mellitus besides cataracts, including retinopathy8,9 and possibly nephropathy10 and neuropathy.11 ARI such as AL01576 (Alcon Labs, Fort Worth, TX) and Sorbinil (Pfizer, Groton, CT) have been shown to eliminate the complications of cataract formation in experimental animals, and also to attenuate physiological and biochemical changes in the retina, sciatic nerve, and kidney.1,10,11

There is much controversy in the literature on the mechanism of elevated sorbitol production in relation to the status of tissue AR in the hyperglycemic condition. Increased enzyme activity,1,2 increased enzyme protein production,13 and activation of AR in the presence of high sugar14 have all been shown to occur in lenses of various hyperglycemic animal models as well as in human diabetic lenses. Other studies, however, have failed to demonstrate any increase in either the enzyme activity17,18 or protein.19,20 The present study further investigated this topic by examining the status of lens aldose reductase in the initial stages of STZ-induced diabetes in the rat. In order to examine early lenticular AR changes in a model which is initially less severe, the current study used 2-3-month-old rats (STZ dose = 50 mg/kg body weight), in contrast to other studies13,15,18 which used 3-4-week-old rats (STZ dose = 65-100 mg/kg body weight). As a continuation of our previous research efforts in the understanding of lens biochemical changes in the diabetic rat1 we report here the evaluation of lens AR protein distribution, protein synthesis, and enzyme activity in the early stage of the diabetic rat model and the effect of treatment of these rats with an ARI.

Materials and Methods

Induction of Diabetes

Male Sprague-Dawley rats (Harlen Co., Houston, TX) weighing approximately 250 g were made diabetic by injection with STZ (ICN Biochemicals, Inc., Cleveland, OH), 50 mg/kg body weight, into the tail vein. Animals with a blood glucose level of 375 mg/dl
and above were considered diabetic. ARI-treated groups were dosed orally (4.0 mg/kg body weight/day) with AL01576. The rats were kept diabetic with no insulin treatment for the duration of each study. Animals were sacrificed at intervals of 3 days, 7 days, and 14 days. Lenses were surgically removed and were either cultured immediately or processed for enzyme assay and SDS-PAGE studies. All chemicals for which source is not noted were reagent-grade. The current investigations conformed to the ARVO Resolution on the Use of Animals in Research.

**Lens Protein Biosynthesis in Organ Culture**

Rats were sacrificed by CO₂ asphyxiation at 3 days and 14 days postinjection. Lenses were surgically removed and cultured immediately in modified TC-199 medium at 37°C in a humidified atmosphere of 5% CO₂ according to the method of Sigler and Hess.²¹ ³⁵S-Methionine (sp. act. 1000 Ci/mmol; New England Nuclear, Cambridge, MA) was added (20 µCi/lens) to the culture media, and the lenses were pulsed for 16 hr. The lenses were thoroughly rinsed with cold medium, blotted, weighed, and homogenized for protein analysis.

**Lens Fractionation and Protein Separation**

Fresh lenses or lenses after culture were each processed to isolate water soluble (WSF), urea soluble (USF), and membrane (MF) fractions according to the method of Russell et al.²² Culture media samples were used directly without any processing. Equal amounts of material on a per-lens basis were compared in all experiments. Chemicals for SDS-PAGE were obtained from BioRad Laboratories, Richmond, CA. Purified AR was a gift from Dr. Peter Kador of NEI. HRP-conjugated rabbit antigoat IgG was obtained from Zymed Laboratories, Richmond, CA. Gels were incubated in 25 mM Tris, 129 mM glycine, pH 8.3, containing 20% methanol for 16 hr at 4°C. The separated proteins were electroblotted to nitrocellulose paper for three hr at 110 V (0.3–0.5 A) at 4°C. The blots were then subjected to a blocking solution of 20 mM Tris, 500 mM sodium chloride, pH 7.5, containing 3% gelatin. Both the primary (anti-AR) and secondary (HRP-conjugated antigoat IgG) antibodies were used at 1:1000 dilution. The antibody probe was visualized by the reaction of hydrogen peroxide and 4-chloro-1-naphthol catalyzed by horseradish peroxidase conjugated on the secondary antibody.

**Assays of AR Activity**

_Conversion of glyceraldehyde to glycerol._ The rate of conversion of glyceraldehyde to glycerol by AR was measured spectrophotometrically by monitoring the oxidation of NADPH according to the method of Kador et al.²⁴ An aliquot of rat lens homogenate was added to the reaction mixture containing 0.1 M potassium phosphate buffer, pH 6.8, 0.2 mM NADPH, and 10 mM glyceraldehyde to yield a final volume of 1.5 mL. The reaction was allowed to proceed for six min at room temperature, and the change in absorbance at 340 nm was observed.

_Conversion of xylose to xylitol._ In this assay, 10 mM xylose was used as the substrate in the presence of 1 mM NADPH, an NADPH-generating system, and an aliquot of rat lens homogenate. At the end of a 16-hr incubation, the reaction was stopped by the addition of 0.6 M zinc sulfate to achieve a final sulfate concentration of 0.3 N. An equal volume of 0.3 N barium hydroxide was added, and the mixture was centrifuged at 15000 rpm. The supernatant was quantitatively removed and lyophilized. The reaction product (xylitol) was quantitated by gas liquid chromatography (GLC) according to the method of Hayman and Kinoshita.²⁵

**Results**

All SDS-PAGE profiles shown, including Coomassie blue staining, autoradiographs, and western blots, are confined to the molecular weight region of 38,000 d, which is the molecular weight of AR, to avoid the confusion of other irrelevant protein bands in this particular study.

**Aldose Reductase Protein in Whole Lens**

Protein profiles of the WSF, USF, and MF of lenses from control and two-week diabetic rats are com-
Fig. 1. Coomassie blue stained SDS-PAGE separation of whole lens proteins of normal control (1) and 2-week post-STZ diabetic (2) rats. MF = membrane fraction; WSF = water soluble fraction; USF = urea soluble fraction; AR = purified rat lens aldose reductase; MW = molecular weight markers (in daltons).

pared in Figure 1. AR, which has a molecular weight of 38 kd, is not a major protein in any of the fractions, even in the WSF, and constitutes less than 1% of the total protein. No increase was observed in the staining intensity of the AR protein band in the diabetic samples over the control normal lens samples.

Figure 2 represents autoradiographs of the protein biosynthesis profiles in lens WSF and USF from two-week diabetic and normal control rats. (Duplicate samples are from two separate animals.) These lenses were cultured in $^{35}$S-methionine containing media, and the incorporation of the radioisotope into individual polypeptides was visualized by autoradiography. The autoradiographs were examined for a specific increase in incorporation in the AR band, the intensity of other noncrystallin polypeptide bands remaining equal (Fig. 2, higher molecular weight bands). Polypeptides of molecular weight similar to AR did not show any specific increase in incorporation in the diabetic lens, indicating that there was no specific induction or increase in synthesis or turnover of this polypeptide.

The Western blot analysis of WSF and USF proteins are represented in Figure 3. No difference in the intensity of immune reaction staining was found between the AR in WSF samples of normal and diabetic rats (NC lane 1 and D lane 1 in Fig. 3). The AR staining in USF also showed considerable intensity, indicating that the enzyme may also be associated with the insoluble fraction. The association of AR in USF appeared to be a function of age. As shown in lanes 1 and 2 of NY and NO in Figure 3, the AR staining in USF was greater in 2-year-old rat and lower in 1-month-old rat. The distribution of AR between WSF and USF fractions was similar in normal control and diabetic rat lenses (lanes 1 and 2, NC and D), thereby disqualifying the possibility that AR is increasingly associated with insoluble proteins in the diabetic condition.

Fig. 2. Autoradiograph of $^{35}$S-methionine-labeled whole lens water soluble (WSF) and urea soluble (USF) proteins of normal control (N) and 2-week post-STZ diabetic (D) rats. Arrows indicate position of AR.

Aldose Reductase Protein in Epithelial Layer

Since it is known that AR protein and activity are present in greater concentration in the lens epithelium, we therefore compared the AR activity and protein biosynthesis in the lens epithelial layer of normal and diabetic rats in an effort to examine a more concentrated preparation of AR. Figure 4A shows a Coomassie blue stained SDS-PAGE protein profile of epithelial cells obtained from rats of 3 days and 14 days post-STZ injection, along with the normal controls. The protein band at 38 kd is detectable, but no increase in its staining intensity was found, which indicates that AR protein was not elevated. In agreement with the whole lens protein synthesis data, we did not observe any induction of specific AR synthesis in the epithelial cells of the diabetic rat lenses (Fig. 4B). There is an alteration in the differential
Fig. 4. (A) Coomassie blue stained SDS-PAGE separation of epithelial layer proteins of normal control (N) and diabetic (D) rat lens, 3 days and 14 days post-streptozocin. AR = purified rat lens aldose reductase; MW = molecular weight markers (in daltons). (B) Autoradiograph of 35S-methionine-labeled epithelial layer proteins shown in Figure 4A. Arrow indicates position of AR.

protein synthesis in epithelial proteins at 14 days post-STZ (Fig. 4B), resulting in an apparent increase in AR protein synthesis, along with synthesis of other proteins, of molecular weight > 38 kd. The apparent increase in protein biosynthesis in epithelium may be more significant if one assumes a lowered amino acid uptake in the diabetic lens. This observed differential synthesis will be the subject of discussion in a subsequent report.

The AR antibody reaction in the epithelium (Fig. 5) showed no difference between the normal controls and the 3-day and 14-day post-STZ injected rat lens samples. Laser densitometric scans of the AR reaction band showed no quantitative difference (scans not shown). Figure 5 also shows the AR antibody probed reaction of the WSF compared to the capsule epithelium (C-E) samples, and indicates clearly that for equal amounts of total protein loaded on the gel, the reaction product is several-fold greater in the C-E than in the lens WSF, supporting the theory of high AR distribution in the epithelium.

Aldose Reductase Activity

In order to verify whether the diabetic condition can induce aldose reductase enzyme activity in the whole lens, we assayed the lens AR activity in rats of 3 days, 7 days, 10 days, and 14 days post-STZ injection. As shown in Table 1, the results indicate that there was no significant difference in AR activity between the lenses of control and diabetic rats at various stages. Similar studies were carried out with isolated lens epithelial layers from these animals and showed no difference in these tissues (results not shown).

In a separate AR activity assay, we quantitated the reaction product (xylitol) and the substrate (xylose) by using GLC. As shown in Figure 6, the lens AR at 3 days or 14 days post-STZ injection did not convert more xylose to xylitol, indicating that there was no increase in enzyme activity. Also shown in Figure 6 is the inhibition effect of AL01576, an ARI, on the production of lens polyol in diabetic rats.

Discussion

The early diabetic stages used in this study include 3 days and 14 days post-STZ injection. The lenses at this stage were completely clear, with no vacuoles observable by slit-lamp examination. Biochemical changes, however, such as a decrease in glutathione or inositol and an increase in sorbitol have been documented.1 These changes have been shown to be prevented by the ARI AL01576.1 The results of our study strongly suggest that in the STZ-induced diabetic rat model, the early changes in the lens biochemistry may not be related to an increase in the AR enzyme protein, synthesis, or enzyme activity. The concentration or activity of the enzyme contained in the rat lens was apparently sufficient to react with the ample supply of substrate and cause sorbitol accumulation in these lenses. This was further substantiated by the evidence that to prevent cataractous changes under high-glucose conditions effectively,
Table 1. Activity of aldose reductase as a function of time after streptozotocin injection

<table>
<thead>
<tr>
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<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0.1804 + 0.0062</td>
<td>0.1758 + 0.0094</td>
<td>0.1849 + 0.0093</td>
<td>0.1728 + 0.0085</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 3)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.1728 + 0.0004</td>
<td>0.1804 + 0.0071</td>
<td>0.1671 + 0.0128</td>
<td>0.1557 + 0.0029</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 3)</td>
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Results are expressed as mmoles NADPH × min⁻¹ g protein⁻¹. No significant difference was observed between values for normal and diabetic for any of the time points (P > 0.05, student t-test). NADPH was monitored according to the method of Kador et al.²³

AR is inhibited by ARI AL01576 to an activity level much lower than that seen in the control lens (Fig. 6).

In contrast to the report by some laboratories¹³ but in agreement with others,¹⁹ our studies in the hyperglycemic model showed that there was neither an increase in lens AR protein concentration (Figs. 1, 3) nor an increase in AR protein synthesis (Fig. 2). The results were consistent in the whole lens preparations as well as in the isolated epithelial cell preparations. In agreement with previous findings²⁶⁻²⁷ we also found a high concentration of AR located in the epithelial cell layer (Fig. 5). The lack of increase in AR concentration, shown by the protein and antibody stain data, indicate that AR protein had not accumulated to a higher concentration by 14 days post-STZ in the diabetic rat lens. The differential increase in the synthesis of higher molecular weight proteins (≥38 kd) seen at 14 days post-STZ (Figure 4B) may indicate an increased accumulation of epithelial proteins beyond the 14-day stage. However, in the time course of the present study, increased accumulation due to synthesis is not a consideration. Both the radiolabel incorporation and the antibody probe analysis are highly sensitive techniques, and the results obtained were very reproducible.

Although we did not find any alteration in the AR activity or synthesis in the model we used, some other significant changes in the lens in this early diabetic rat model had already occurred. Many of these changes were membrane-related lesions.¹ Earlier work from other laboratories²⁸ as well as from ours¹ has shown that the uptake of amino acids is altered at very early stages. Decrease in amino acid uptake was observed as early as 3 days post-injection, while leakage increase became noticeable at 5 days post-injection. Membrane lipid turnover or biosynthesis was altered by 2 weeks post-injection.²⁹

We believe that the loss in membrane function and possibly in structural integrity was the result of the osmotic stress induced by a sudden elevation of lens sorbitol. Thus, the leaky membrane can explain some of the histochemical evidence reported, such as the increased AR staining in the cortex¹⁵ or the change in AR distribution in the lens.²⁶ The osmotic-stress-induced hydration in the cortical region may damage the cells and thereby cause a diffusion of intracellular material, including the proteins, with the result that staining towards the inner cortex apparently increases. This loss in normal membrane permeabilities at early stages in the galactosemic cataract has been suggested by Kuwabara et al.³⁰

A noteworthy observation made in this study is that the epithelial cell layer, containing the highest AR concentration, maintained normal protein synthesis in a diabetic lens. This phenomenon has been shown in other osmotic cataracts²⁰⁻³¹ and is possibly due to increased cell proliferation²¹ or increased metabolic activity in the epithelium. The underlying fiber cells, although much lower in enzyme activity, lost their cellular integrity.³⁰ This difference suggests that in the hyperglycemic osmotic cataract, the fiber cells, unlike the more active epithelial layer, are unable to counteract the results of osmotic stress, and consequently demonstrate the observed changes in membrane and cytosolic parameters.

We therefore conclude that the AR activity normally present in the rat lens is sufficient to cause major biochemical and morphologic damage to the lens. In our opinion, the current model demonstrates that the increase in enzyme observed by other laboratories¹³⁻¹⁵,²⁰ cannot be a significant factor in promot-
ing cataractogenesis in the diabetic rat lens. We cannot rule out the possibility that there may be an increase in enzyme activity or AR protein accumulation 2 weeks post-streptozocin injection, but because our interest is currently confined to studying early changes, such experiments were beyond the scope of this study.

Key words: aldose reductase, streptozotocin-diabetic rat, cataract, lens, protein synthesis

Acknowledgments

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References