Production of Fructose and Fructose-3-phosphate in Maturing Rat Lenses

Sundeep Lal, Benjamin S. Swergold, Anne H. Taylor, William C. Randall, Francis Kappler, and Truman R. Brown

Purpose. A large increase in glycation of crystallins between 1 and 8 months has been demonstrated in lenses obtained from aging rats. The objective of this study was to investigate if an age-associated increase in the levels of any of the phosphorylated and nonphosphorylated sugars in the aging rat lenses could be correlated with this increase.

Methods. Lenses were obtained from Sprague-Dawley rats ranging in age from 2 to 20 months. Trichloroacetic acid extracts of these tissues were analyzed by using \(^{31}P\)-NMR for sugar phosphates and high-pressure liquid chromatography equipped with an electrochemical detector for sugars and polyols.

Results. Although no elevation in the lenticular glucose levels was observed, an age-associated increase in the concentrations of polyol pathway-associated metabolites—sorbitol, fructose, sorbitol-3-phosphate, and fructose-3-phosphate—was detected. In contrast, no significant changes were observed in glycolytic or pentose shunt metabolites.

Conclusion. Aging lenses accumulate increased concentrations of fructose and fructose-3-phosphate. Because fructose-3-phosphate is a potent glycating agent and a potential in vivo source of 3-deoxyglucosone, its accumulation in the lens, along with fructose, may be a contributing factor in the age-associated increase of nonenzymatic glycation in rat lenses. Invest Ophthalmol Vis Sci. 1995;36:969–973.

The nonenzymatic glycation hypothesis\(^1\)–\(^3\) postulates that with age proteins become increasingly modified by sugars and that this process is important in many of the age-associated, post-translational modifications observed in mammalian tissue. This process starts when a sugar in its open chain carbonyl form reacts with an amino group of a protein to form a Schiff base, which then rearranges slowly to the more stable Amadori product.\(^4\)\(^,\)^\(^5\) Irreversible rearrangements of the Amadori product lead ultimately to the formation of advanced glycation end products (AGEs).\(^6\)\(^,\)^\(^7\) The relevance of these reactions to aging in mammalian lenses has been demonstrated by detection of an age-associated increase in the concentrations of glycated crystallins\(^8\) and AGEs.\(^9\)

Phosphorus nuclear magnetic resonance (\(^{31}P\)-NMR) has been a useful tool in the study of lenticular metabolism. Using this methodology, we previously identified fructose-3-phosphate (Fru-3-P) and sorbitol-3-phosphate (Sor-3-P) in mammalian lenses.\(^7\) These compounds are produced by a specific 3-phosphokinase that phosphorylates sugars at the C-3 hydroxyl position.\(^8\) Although the exact identity and function of this kinase will have to await its purification, it is clear that one of its products, Fru-3-P, a potent glycating agent in vitro, may play an important role in the formation of AGEs in the lens.\(^9\) We have proposed that as an intrinsic part of the glycation process, Fru-3-P dissociates by an amine-catalyzed \(\beta\)-elimination reaction to 3-deoxyglucosone, a reactive \(\alpha\)-dicarbonyl that appears to be a key intermediate in the formation of advanced glycation end products.\(^9\) In the present study, we have demonstrated that, as a function of age, rat lenses show an increased production of Fru-3-P, along with its precursor fructose.

METHODS. Animals and Materials. Sprague–Dawley rats were purchased from Taconic Laboratory (Germantown, NY). These were housed and cared for in accordance with recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols for the use of animals were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee. Blood glucose of the rats was measured using a glucose analyzer (One Touch II; Lifescan, Milpitas, CA) by sampling the blood from the saphenous vein. Rats were sacrificed by CO\(_2\) asphyxiation, and the eyes were dissected. Young and mature rabbit and bovine eyes were purchased from Pelfreeze (Rogers, AR). All chemicals were purchased from Sigma (St. Louis, MO).

Trichloroacetic Acid Extracts. Lenses were carefully dissected from the eyes and immediately frozen in liquid nitrogen, weighed, and stored at \(-70^\circ\)C until processed. Typically, a pool of six lenses was used for each data point in each age group so as to average any biologic variability in the samples. Frozen lenses were ground in a mortar chilled with liquid nitrogen, using approximately 8 \(\times\) weight-to-volume ratio of 5% trichloroacetic acid (TCA) to tissue weight. The resultant slurry was centrifuged at 8000g for 10 minutes. The supernatant was saved, and the precipitate was homogenized again in 5% TCA and centrifuged. The supernatants were pooled, and TCA was removed by fourfold extraction with diethyl ether. The resultant solution was subsequently concentrated to remove ether and lyophilized. The dried sample powder was reconstituted in 2.0 ml D\(_2\)O and 200 \(\mu\)l of 0.25 M CDTA (to chelate metal ions). The final pH of the extract was adjusted to 8.0.

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31P-NMR Data Acquisition and Processing. The 31P-NMR data were acquired on a Bruker (Billerica, MA) AM-400 spectrometer at 162 MHz with 60° pulses and a 1.5-second repetition time. Typically, spectra were acquired in blocks of 20,000 scans for the rat lenses. Chemical shifts were referenced to glycerophosphorylcholine peak to 0.49 ppm. The identification of the metabolites was based on chemical shift analysis, spikes of the authentic compound, and enzymatic analysis. The metabolites were quantitated with the use of an external phosphate standard. The concentration of metabolites was normalized to the total weight of the pooled lenses and was expressed as μmol/g wet weight. The overall standard error of the mean of the metabolite concentrations was ±15%.

Quantitation of Lens Sugars. Ionic species from a 0.5-ml aliquot of TCA extracts of lenses were removed by passing it through a column containing an anion exchange resin (BioRad AG1-X8, Cl− form) and cation exchange resin (BioRad AG53-X8, H+ form). A 25-μl aliquot of a 2.0-mM solution of 2-deoxyglucose was added as an internal standard. The concentrations of the sugars were determined by high-pressure liquid chromatography (HPLC) equipped with a pulsed amperometric detector (DX-500) (Dionex, Sunnyvale, CA) using a Carbopac (MA-1) anion exchange column (Dionex) with isocratic elution by 600 mM NaOH. The peak areas were normalized to 2-deoxyglucose, and concentrations were determined from the standard curves run on the same day. All sugar estimates were performed in triplicate.

RESULTS. All rats used in this study were found to be normoglycemic. The rat lenses were studied from five different age groups ranging from 2 months to 20 months (2, 7, 10, 12, and 20 months). Figure 1 shows the 31P-NMR spectra from young (2 months) and mature (12 months) rat lenses. These spectra illustrate that the most prominent change with maturation in these lenses is the elevation of Fru-3-P and Sor-3-P. Whereas Fru-3-P and Sor-3-P are undetectable in the 2-month-old rat lenses, at 12 months they are present at a concentration of 190 nmol/g wet weight and 70 nmol/g, respectively. In contrast to Fru-3-P and Sor-3-P, no significant increases were found in other phosphorylated sugars, such as glucose-6-phosphate, sedoheptulose-7-phosphate, and α-glycerol phosphate (Table 1). The levels of Fru-3-P and Sor-3-P in lenses from the five age groups of rats is shown in Figure 2.

To understand the reasons for the increased production of Fru-3-P and Sor-3-P with maturation, concentrations of lenticular sugars relevant to the production of Sor-3-P and Fru-3-P (glucose, fructose, and sorbitol, as well as inositol) were measured. Figure 3 shows the HPLC chromatogram of these sugars from 2-month-old and 12-month-old rat lenses. Although no elevation was found in the levels of glucose with age, there was a significant increase in fructose concentration over those measured in lenses of the

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>2 Months</th>
<th>12 Months</th>
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<tbody>
<tr>
<td></td>
<td>Old (μmol/g)</td>
<td>Old (μmol/g)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.2 ± 0.05</td>
<td>0.96 ± 0.07</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.5 ± 0.04</td>
<td>1.23 ± 0.02</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.25 ± 0.03</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Inositol</td>
<td>2 ± 0.14</td>
<td>1.1 ± 0.013</td>
</tr>
<tr>
<td>Sorbitol-3-phosphate</td>
<td>ND</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Fructose-3-phosphate</td>
<td>ND</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Sedoheptulose-7-phosphate</td>
<td>0.25 ± 0.04</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>α-glycerol phosphate</td>
<td>0.29 ± 0.04</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Glucose-6-phosphate†</td>
<td>0.11 ± 0.02</td>
<td>0.123 ± 0.02</td>
</tr>
<tr>
<td>Fructose-6-phosphate‡</td>
<td>0.036</td>
<td>0.041</td>
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</table>

Metabolite concentrations estimated from pool of six lenses to minimize any biologic variability. The error in the nuclear magnetic resonance measurement was estimated to be ±15%; ND = not detectable. * Estimate represents the total of all anomeric forms of Fru-3-P. The resonance at 5.8 ppm is only 60% of total Fru-3-P present (Szwergold et al†). † Corrected for contributions from a coeluting anomeric form of sedoheptulose-7-phosphate. ‡ Estimated from the concentration of glucose-6-phosphate.
FIGURE 2. Age dependence of Fru-3-P and Sor-3-P levels in rat lenses.

Younger animals (Fig. 4). These lenticular levels of fructose correlated significantly with Fru-3-P (r = 0.88), confirming a precursor-product relationship. This increase in fructose was accompanied by an increase in concentrations of sorbitol and depletion of inositol in these tissues (Fig. 4, Table 1).

Qualitatively similar production of fructose and Fru-3-P with age was seen in lenses from Fisher rats and two other mammalian species, rabbit and bovine. However, because only approximate rabbit and bovine ages could be gauged, it was not possible to quantify precisely these changes in the metabolite levels with respect to age.

DISCUSSION. The first step in nonenzymatic glycation of crystallins is the formation of a Schiff base that can rearrange to form a more stable ketoamine. These subsequently lead to the formation of covalent linkages through the formation of complex heterocyclic products. Because the Schiff base adducts and ketoamines represent only an intermediate step in the accumulation of AGEs, the rate of formation of AGEs will be dependent on the amount of glycated protein, which in turn will be directly proportional to the concentration of the glyating agent(s). Initially, the non-enzymatic glycation hypothesis focused mainly on glucose as the predominant glycating agent. However, it is now well recognized that other sugars and sugar phosphates can contribute to glycation. This recognition is significant because glucose is among the poorest of all intracellular glycating agents and is present at low concentrations in the lens in comparison to plasma. Prominent among the other glycating agents proposed to be important in the lens are fructose, produced by the polyol pathway, and ascorbate, thought to decompose to the glycating agents dehydroascorbate and threose.

It has been demonstrated that the most significant increase in glycation of crystallins from rat occurs between 1 and 8 months. The objective of this study was to investigate if an age-associated increase in the levels of any of the phosphorylated and nonphosphorylated sugars in rat lenses could correlate with this...
increase in glycation. Of all the phosphorylated and nonphosphorylated sugars measured in this study, the carbohydrates that could be considered as glycating agents are glucose, fructose, fructose-6-phosphate, glucose-6-phosphate, sedoheptulose-7-phosphate, and Fru-3-P. Although over time all these sugars will contribute to some extent to the total glycation in the lens, the only sugars that demonstrated an age-associated increase in concentrations were Fru-3-P and fructose. The time course of the increase in these carbohydrates is consistent with the previously reported increase in the glycation of crystallins from rat lenses. Although the extent of involvement of F3P and fructose still must be evaluated, these observations argue that they are likely to be important contributors to the total nonenzymatic glycation observed in this model system.

An interesting aspect of these findings is the correlation between production of fructose and Fru-3-P. This suggests that the age-associated increase in Fru-3-P is in large measure due to a concomitant increase in fructose levels rather than to an activation of the 3-phosphokinase. This finding raises questions regarding the mechanism of the increased production of fructose with age. Although the most likely source of fructose in these tissues is the polyol pathway, the mechanism by which the activity of this pathway increases with age is unclear. One likely possibility for this phenomenon could be an age-associated alteration of the properties of the enzymes constituting the polyol pathway. Alternatively, a change in redox status of the lens may also account for this observation.

Alterations in levels of Fru-3-P and Sor-3-P in the maturing lens are of interest because, in the past, production of these metabolites has been observed only in lenses of diabetic rats in which, within 2 weeks of induction of diabetes, Fru-3-P achieves a steady state concentration of approximately 1 μmol/g. However, unlike the diabetic lens in which an increase in Fru-3-P is accompanied by the elevation of a number of other sugars and sugar phosphates, the increase in the levels of Fru-3-P and fructose in the aging rat lens occurs without increases in glucose, glycolytic, and pentose shunt metabolites. Consequently, the aging rat lens may prove to be a useful model for investigating the specific role of Fru-3-P and fructose in nonenzymatic glycation of crystallins.

In conclusion, the production of Fru-3-P in the aging lens offers a new perspective for understanding the age-associated increase of nonenzymatic glycation in rat lenses. Because qualitatively similar trends also
were observed in rabbit and bovine lenses, the production of these metabolites may have relevance to other species as well. However, the extent of contribution of Fru-3-P and fructose in glycating crystallins, in comparison to other potential glycating agents, still must be evaluated. To this end, assays for glycated or crosslinked crystallin adducts unique to a specific glycating agent will have to be developed.

Key Words
glycation, lens senescence, nuclear magnetic resonance

References