Aldose Reductase Inhibitors and Prevention of Galactose Cataracts in Rats

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Our previous studies have shown that the aldose reductase inhibitor (ARI), sorbinil, prevents galactose-induced alterations and cataracts in rat lenses. We have now used sorbinil as well as another ARI, Eisai compound E-0722, to determine their potency in inhibiting aldose reductase- and galactose-induced alterations in lens morphology and Na⁺-K⁺-ATPase activity. Young Sprague Dawley rats were fed Purina Rat Chow plus 50% galactose, with or without 15 mg sorbinil, 0.15, 0.5, or 1.0 mg of E-0722/kg body weight per day. Controls were given Purina Rat Chow with or without ARIs. Lenses were studied for up to 60 days following the initiation of the diet using morphological, cytochemical and biochemical approaches to assess any alterations in the lens. While galactose-induced damage and cataracts were delayed by low doses (0.15 mg and 0.5 mg) of E-0722, they were completely prevented by the administration of 15 mg of sorbinil or 1 mg of E-0722/kg body weight per day. This study further showed that just 1 mg of E-0722 was more effective in preventing cataracts than 15 mg sorbinil. Thus it appeared that E-0722 was a more potent inhibitor of aldose reductase than sorbinil.


Since the report of Kinoshita et al demonstrating the delay of cataract development through the reduction in activity of aldose reductase, the effect of potential inhibitors of aldose reductase on sugar cataracts in experimental animals has been actively pursued. The search for more potent drugs to delay or prevent cataracts in humans continues. Studies from our laboratory have shown that the aldose reductase inhibitor, sorbinil (D-6-fluorospirochroman 4,4'-imidazolidine 2,5'-dione, CP-45,634, Pfizer, Groton, CT), inhibits galactose-induced alterations and opacity in rat lenses. Our studies showed that sorbinil added to galactose diet (50 mg added to a kilogram of 50% galactose diet) prevented alterations induced by galactose in morphology and activity of enzymes, such as Na⁺-K⁺-ATPase, acid phosphatase and arylsulfatase in the lens, and that no opacity developed even after 60 days. The animals on this diet ingested approximately 15 mg of sorbinil/day/kg body weight. Recently we have used a compound, (2R-4S)-6-fluoro-2-methyl-spirochroman-4,4'-imidazolidine-2',5'-dione (E-0722, M-79175, Eisai Co., Ltd., Tokyo, Japan), which is related to sorbinil but has been reported to be a more potent inhibitor of aldose reductase than sorbinil. This compound has been found to inhibit diabetes-related changes in many tissues and galactose-induced alterations in the lens (Shirai, T., Eisai Co. Ltd., personal communication). In this report we present our findings regarding the effect of three different doses of this aldose reductase inhibitor (E-0722) on the development of galactose-induced opacity, morphological alterations and alterations in the activity of Na⁺-K⁺-ATPase in rat lenses. For comparison, the inhibitory effects of sorbinil and Eisai E-0722 are included.

Materials and Methods

Sprague Dawley rats (Charles River, Wilmington, MA) weighing 50 g were used for this study. One group of rats was fed ground Purina Rat Chow mixed with 50% galactose and other groups of rats received a similar diet but with the addition of either 0.15 mg, 0.5 mg or 1 mg/day/kg body weight of aldose reductase inhibitor, Eisai E-0722, M-79175 (henceforth referred to as E-0722), or 15 mg sorbinil/day/kg body weight. The total dietary intake in both groups was monitored by the weight of the diet given to each animal before and after refills. The lenses were examined periodically with an ophthalmoscope and any observable changes in the lenses were recorded.
animals were sacrificed at desired time intervals following the initiation of diets. Eyes were enucleated and lenses were extracted by the posterior approach. Using methods routinely used in our laboratory, the lenses were processed for light microscopy, for transmission electron microscopy (TEM) or for the cytochemical localization and determination of the level of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (NPPase) activity. Experimental animals used in this study were treated in compliance with the ARVO Resolution on the Use of Animals in Research.

Briefly, for light microscopy, lenses were prefixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed for at least 2 days in 10% formalin, dehydrated and embedded in methacrylate (JB-4 embedding kit; Polysciences, Warrington, PA). Sections of 1 µm to 2 µm thickness were stained with hematoxylin-eosin or toluidine blue. Routine transmission electron microscopy was conducted on lenses prefixed in 1.7% glutaraldehyde, postfixed in 1% OsO	extsubscript{4} in 0.1 M cacodylate buffer and dehydrated in ethanol; small segments were embedded in Epon-araldite. Thin sections, stained with uranyl acetate and lead citrate, were observed under the Philips 4105L transmission electron microscope. Ultrastructural cytochemistry for the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase localization in the lenses was performed by incubating lenses in media containing p-nitrophenyl-phosphate (NPP disodium salt) as substrate. Following incubation and rinses, the lenses were processed for electron microscopy. For the assay of NPPase activity, lens homogenates were added to the assay medium and incubated (using the same procedure as used for cytochemistry). Following incubation, the enzyme reaction was terminated with trichloroacetic acid and centrifuged. Spectrophotometric analysis at 420 nm was conducted on the supernatant to determine the amount of released nitrophenol. Enzyme activity was then calculated for lenses in each dietary group.

Results

Light Microscopy

Our light microscopic studies showed that the morphology of lenses of rats receiving galactose in the diet for 20 days was considerably altered. Damage to the fibers in the form of vacuolation, swelling, disorganization and liquefaction was extensive (Figs. 1, 2). Sections from lenses of animals receiving galactose diet containing 15 mg/day/kg body weight sorbinil for 20 days exhibited normal organization and morphology of epithelium and fiber, except for slight swelling of superficial cortical fibers (Fig. 3). Sections from lenses of rats receiving 0.5 or 1 mg/day/kg body weight of E-0722 in the galactose diet appeared normal in every respect (Figs. 4, 5) and the morphology was comparable to what we have observed in normal laboratory chow-fed rats. Even after 60 days this normal appearance of lens was evident with 1 mg E-0722 diet.

Transmission Electron Microscopy and Ultrastructural Cytochemistry

In galactose-fed rat lenses, at 4 to 5 days, there was observable damage in the form of vacuolation and swelling of fibers. At 10 days these alterations were prominent in the equatorial and pre-equatorial regions (Fig. 6). The reaction product of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was minimal but when present it was visible between the epithelial and fiber cell membranes. In the first few days (5 to 10 days), the lenses of rats receiving sorbinil with galactose in their diet exhibited alterations similar to those observed in lenses of animals on the galactose diet. These alterations, in the form of vacuolation in epithelial and fiber cells, were limited in extent and less severe in the sorbinil
group (Fig. 7). The reaction product for Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was not significantly altered at this stage in the sorbinil group. At early stages, morphological alterations in the lenses of rats receiving 0.5 mg E-0722/day/kg body weight on galactose diet were essentially similar to those observed in groups fed a sorbinil plus galactose diet (Figs. 8, 9, compare with Fig. 7). However, in some lenses, at 10 days of galactose plus 0.5 mg E-0722 diet, focal disorganization of superficial cortical fibers with disruption of fiber membranes was evident. This alteration was localized in the first few fiber layers adjacent to the epithelium. In the deeper cortex fiber, the morphology appeared normal. With the inclusion of 1.0 mg E-0722/day/kg body weight in the galactose diet, the alterations in lens epithelium and fiber cell morphology that were observed at similar early stages in groups fed the galactose diet with either sorbinil or 0.5 mg E-0722 were not present (Fig. 10, compare with Figs. 7-9).

The alterations in lens morphology observed during the early stages of galactose feeding progressed anteriorly and posteriorly when the galactose diet was continued for longer periods. At 20 days both the epithelium and superficial cortical fibers exhibited vacuolation, swelling and cell membrane damage (Fig. 11). The reaction product of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was minimal. In comparison, lenses from rats receiving sorbinil with galactose in the diet for 20 days or longer demonstrated a minimum amount of galactose-induced damage. The level of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, as judged from the extent of reaction product, was unaffected in these lenses (Figs. 12, 13). In animals receiving 0.5 mg E-0722/day/kg body weight with galactose for 20 days, the alterations in lens morphology and the extent of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase reaction product were slightly more extensive than observed in lenses of animals receiving sorbinil and galactose for a similar period. However, these alterations were of lesser magnitude in lenses of animals receiving 0.5 mg E-0722 than in those receiving 0.15 mg E-0722 with galactose (Fig. 14, compare with Figs. 11-13). The ultrastructure of the lens epithelium and fiber cells and the extent of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in lenses from animals receiving 1 mg E-0722/day/kg body weight with galactose for 28 days or longer appeared normal and comparable to lenses from the galactose plus sorbinil group and the lab chow control group (Fig. 15).

Assay of NPPase (Na\textsuperscript{+}-K\textsuperscript{+}-ATPase) Activity

Spectrophotometric analysis of the incubation medium, in which homogenates of lenses from rats fed laboratory chow, galactose plus sorbinil or galactose
plus 0.15 mg, 0.5 mg or 1 mg/day/kg body weight of E-0722 were incubated, was conducted to determine the nitrophenol released due to enzyme activity. The results obtained are presented in Table 1 and Figure 16 as the percent of unaffected NPPase activity. The enzyme activity in lenses of laboratory chow-fed rats was maintained at a constant level throughout the experiment and was considered as 100% in determining the alterations in enzyme activity in lenses of rats from the other dietary groups. There was a rapid drop in enzyme activity up to the ninth day in the lenses of galactose-fed rats. At approximately 12 days, the un-
affected enzyme activity was stabilized at about 54% of that observed in lenses from lab chow-fed rats. This observation is consistent with our previous studies. In animals fed the galactose diet containing 0.15 mg, 0.5 mg or 1 mg/day/kg body weight of E-0722 or 15 mg sorbinil, there was a drop in the enzyme activity for 9 to 11 days. However, the reduction was less significant than that observed when animals received a galactose diet without aldose reductase inhibitors. Moreover, in the E-0722-fed groups, the extent of the enzyme affected was dose-dependent. In 0.15 mg, 0.5 mg and 1 mg E-0722 groups 83%, 85% and 94%, respectively, of enzyme activity was retained at 10 to 12 days of diet. In the 0.15 mg E-0722 group, unaf-
ected enzyme activity dropped further to 65% by 32 days. In all other E-0722 and sorbinil groups the enzyme remained at the level observed at 12 days.

**Discussion**

Recent studies by Shirai (personal communication) have shown that the Eisai compound E-0722, which is related to sorbinil, inhibits diabetes-associated alterations in many tissues. This effect of E-0722 was observed with comparatively much lower doses than sorbinil. We undertook a systematic study to investigate the effect of this compound on galactose-induced morphological alterations and alterations at the site and level of Na\(^+\)-K\(^+\)-ATPase activity.
associated with normal lenses. Moreover, our studies included the dose effect of E-0722 and a comparison of this compound with sorbinil on galactose-associated lens alterations.

Our previous investigations described the morphological changes that were observed in lenses of galactose-fed rats. Briefly, alterations in lens morphology in lenses of galactose-fed rats in the form of vacuolation, swelling, disorganization and liquefaction of fibers began in the equatorial region and progressed toward the anterior and posterior cortical region with continuation of galactose diet. At 20 days of 50% galactose diet, the epithelium and cortical fibers exhibited considerable damage, including cell membrane disruption. Our previous investigations also showed a significant decrease in lens Na⁺-K⁺-ATPase...
activity and increases in both acid phosphatase and arylsulfatase activities with the progression of galactose cataracts.\textsuperscript{17,20-23} We have also shown that the inclusion of 15 mg sorbinil/day/kg body weight in a 50% galactose diet inhibited all of the above-stated galactose-induced alterations and cataracts.\textsuperscript{17-19} As many of the ARIs have side effects when administered systemically, the search continues for either new ARIs that do not produce side effects or for more potent compounds that at low doses can prevent cataract and the changes associated with diabetes.

In this study, light microscopic and transmission electron microscopic observations indicated that in the initial stages the presence of either E-0722 or sor-
binil in a galactose diet at the doses studied, had a less significant effect on galactose-induced alterations such as lens fiber swelling and vacuolation in the equatorial region than was observed at later stages. The preventive effect of E-0722 on continued galactose-induced injury to the lens was dose-dependent. In comparing the observations on lenses of animals fed the galactose diets containing various amounts of E-0722 or sorbinil for 20 days, those receiving 0.5 mg E-0722/day/kg body weight demonstrated more extensive alterations in lens morphology and in the extent of distribution of Na^+-K^+-ATPase reaction product than those animals fed sorbinil, but demonstrated significantly less extensive alterations than those animals from the 0.15 mg E-0722 group. The lens ultrastructure and the Na^+-K^+-ATPase activity was unaffected when either sorbinil or 1 mg E-0722 was included in the galactose diet for up to 60 days. Lens morphology in lenses from these two groups was comparable to those from lab chow-fed animals. These observations indicated that the inclusion of even a low dose of E-0722 provided some protection to the lens but that a higher dose of E-0722 was required to prevent morphological alterations associated with galactose.

It is difficult to quantitate precisely changes in enzyme activity through cytochemical studies. Because of this difficulty we conducted the assay of Na^+-K^+-ATPase activity using an approach similar to that used for cytochemistry. Our observations regarding the effects of both of the ARIs studied on galactose-induced alterations in Na^+-K^+-ATPase activity agreed with the light microscope and ultrastructural cytochemical observations. There was an initial drop in Na^+-K^+-ATPase activity even with the presence of ARIs in the galactose diet; however, this reduction was less significant than that observed in the galactose diet without ARIs. Moreover, in the lenses of animals fed different doses of E-0722, the initial decrease in Na^+-K^+-ATPase was dose-dependent. The reduction in enzyme activity was comparatively more significant in the 0.15 mg group than in the 1 mg E-0722 group. Furthermore, at the low dose of 0.15 mg, the enzyme activity continued to drop with continuation of the galactose diet, whereas in the higher E-0722 dose groups and in the sorbinil group, the enzyme activity stabilized after the initial drop.

These light microscopic, TEM and ultrastructural cytochemical studies show that either 15 mg of sorbinil or 1 mg of E-0722 in the galactose diet inhibited morphological alterations and protected Na^+-K^+-ATPase activity to a considerable extent. Lens morphology in these two groups of animals was similar to that of normal lenses and Na^+-K^+-ATPase activity was retained at a near normal level even after an extended period (up to 60 days) on the diets containing galactose. Our observations also indicated that the effect of E-0722 on galactose-induced alterations was dose-dependent. Lower doses of E-0722 (0.15 mg, 0.5 mg) delayed but did not totally inhibit alterations, while the higher 1 mg dose did inhibit galactose-induced alterations in lenses. Finally, our obser-
vations indicated that the aldose reductase inhibitor, E-0722, at a low dose, was indeed a potent inhibitor of galactose-induced lens damage and cataract development.

Although several reports in the literature indicate that ARIs inhibit sugar cataracts through the inhibition of enzyme aldose reductase, it is not clear if this is the only mechanism through which ARIs prevent cataract development. The question, therefore, remains whether or not the inhibitory effect of ARIs also is mediated through its effect on other enzyme systems, such as Na⁺-K⁺-ATPase, that have been shown by us and others to be altered during cataractogenesis. The recent report by Garner et al.24 and our recent preliminary studies indicate that ARIs slightly stimulate Na⁺-K⁺-ATPase activity. We observed that in animals fed E-0722 with rat chow (without galactose) the lens Na⁺-K⁺-ATPase activity was approximately 10% higher than that observed in lenses of rat chow-fed (without E-0722) animals. The extent of this enzymic stimulation, however, did not account for the loss of Na⁺-K⁺-ATPase activity observed in galactose-fed animals. This observation suggests dual cataract-preventive mechanisms of ARIs: one through its inhibitory effect on aldose reductase activity and a second through its stimulation of the pump (Na⁺-K⁺-ATPase). This dual effect would prevent both sugar alcohol formation and alteration in the normal lenticular levels of sodium and potassium observed to precede the development of opacity. It is suggested that the primary preventive effect of ARIs on sugar cataract development is through the inhibition of aldose reductase; however, it probably also intervenes secondarily through stimulation of Na⁺-K⁺-ATPase. We are currently in the process of further evaluating the effect of ARIs on Na⁺-K⁺-ATPase activity.

Key words: galactose cataracts, cataract inhibition, Na⁺-K⁺-ATPase activity, lens ultrastructure, aldose reductase inhibitors

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References