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# Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens

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**ABSTRACT** **Aldose reductase (AR) has been implicated in the etiology of diabetic cataract, as well as in other complications. However, the role of AR in these complications remains controversial because the strongest supporting evidence is drawn from the use of AR inhibitors for which specificity *in vivo* cannot be ascertained. To settle this issue we developed transgenic mice that overexpress AR in their lens epithelial cells and found that they become susceptible to the development of diabetic and galactose cataracts. When the sorbitol dehydrogenase-deficient mutation is also present in these transgenic mice, greater accumulation of sorbitol and further acceleration of diabetic cataract develop. These genetic studies demonstrated convincingly that accumulation of polyols from the reduction of hexose by AR leads to the formation of sugar cataracts.**

Diabetic complications such as neuropathy, nephropathy, retinopathy, and cataract, etc., occur in both insulin-dependent and noninsulin-dependent diabetes mellitus. Hyperglycemia has long been suspected as the cause of these manifestations, and the results of the Diabetic Control and Complications Trial (1) appear to confirm it. However, by what mechanism elevated blood glucose leads to these complications is unclear. One theory implicates the polyol pathway as a cause of diabetic cataract because of the discovery of polyols in cataractous lenses (2) and the identification of aldose reductase (AR) that reduces various sugars to their polyols (3, 4). AR reduces glucose to sorbitol, which is then converted to fructose by sorbitol dehydrogenase (SorD). Because sorbitol does not readily diffuse out of cells and its oxidation to fructose is slow, the accumulation of sorbitol under the hyperglycemic state would increase the intracellular osmotic pressure, leading to swelling and eventual rupture of the lens fiber cells (5). The involvement of AR in diabetic cataract is supported by the fact that animals such as rats and dogs that have high levels of this enzyme in their lenses are prone to develop diabetic cataract, whereas mice that have low lens AR activity are not (6). Rats and dogs also develop galactose-induced cataracts more readily than diabetes-induced cataracts (7). This fact agrees with the polyol model because galactose is a better substrate than glucose for AR *in vitro*, and its reduction product galactitol is not further converted to other metabolites, resulting in faster buildup of this polyol. Additional evidence for the polyol model came from the fact that several AR inhibitors could suppress cataract formation in experimentally induced diabetic animals (8–10). However, these drugs may inhibit AR by nonspecific hydrophobic interactions (11, 12), and their beneficial effects may be derived from the inhibition of other enzymes. The strongest challenge to the polyol model is the fact that kinetic analyses (13, 14) and x-ray crystallographic studies (15) indicated that AR has a very low affinity for glucose and galactose, and it has not been demonstrated directly that AR can reduce these hexoses *in vivo*.

In this report we show that transgenic mice expressing high levels of AR are susceptible to galactose and diabetic cataracts, providing the strongest evidence that accumulation of polyols is the main factor contributing to sugar cataracts.

## MATERIALS AND METHODS

**Generation of Transgenic Mice.** The entire 1.4-kb cDNA from a human AR (hAR) clone with 36-bp 5′ and 325-bp 3′ untranslated regions (16) was released from the Bluescript vector by *Xba* I and *Eco*RV digests and inserted into the *Xba* I and *Msc* I site of the pCAT-Basic vector (Promega) that contains the simian virus 40 splice site and poly(A) sequence. The −341 to +49 region of the mouse αA-crystallin promoter (17) was cloned by PCR amplification of BALB/c genomic DNA and inserted into the *Xba* I and *Xma* I sites at the 5′ end of hAR. A DNA fragment containing the αAcry–hAR hybrid gene was released from the vector by *Tha* I and *Nde* I digestions and injected into oocytes from CBA egg donors fertilized by C57BL males. Transgenic mice were identified by PCR screening of genomic DNA extracted from the tail by using the two primers as shown (Fig. 1) and then confirmed by Southern blot hybridization using hAR as a probe (data not shown).

**Assay of AR Enzyme Activity.** Mouse lens AR crude extract was prepared, and activity was assayed as described (18). Briefly, lenses were isolated from 3-week-old mouse and homogenized in a sodium phosphate buffer at 4°C. Crude enzyme extract was obtained by spinning down the cell debris, and AR activity was measured spectrophotometrically by monitoring the rate of oxidation of NADPH at 340 nm. Reaction mix contains 67 mM sodium phosphate (pH 6.2), 5 mM DL-glyceraldehyde, 0.4 M lithium sulfate, and 200 μM NADPH. An aliquot of enzyme extract was added to initiate the reaction, which was done at 30°C. The unit of AR enzyme activity is defined as nmol of NADPH oxidized per min per mg of protein in the crude extract.

**Induction of Galactosemia and Diabetes.** To induce galactosemia, both transgenic mice and their normal littermates were fed with a diet of 50% galactose/50% Purina rat chow at the age of 3 weeks after birth. Hyperglycemia was induced by a single i.p. injection of streptozotocin at a dose of 200 mg/kg of body weight. Blood glucose was monitored by blood glucose test strips (HaemoGlukotest, Boehringer Mannheim), and those mice with blood glucose levels >500 mg/dl throughout the experimental period were included in this study. Lenses were examined by dilating the pupils with 1% tropicamide (Alcon, Puurs, Belgium), and the progression of cataract was divided into three stages as shown in Fig. 2.

**Measurement of Polyol Level in Mouse Lens.** To confirm the formation of galactitol in galactosemic mouse lens, 3-week-old

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Abbreviations: AR, aldose reductase; hAR, human AR; SorD, sorbitol dehydrogenase.

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transgenic mice from line CAR222 and their normal littermates were divided into two groups: (*i*) normal diet, in which mice were fed on normal Purina rat chow; (*ii*) galactose-feeding, in which mice were put on a diet of 50% galactose/50% Purina rat chow. Four mice from each group were sacrificed for polyol quantitation beginning 1 week after the diet and continuing for 3 consecutive weeks. Their lenses were homogenized, and polyols were extracted by ethanol precipitation, derivatized by phenylisocyanate, and separated by HPLC; their elution was then monitored by UV absorption at 240 nm (19). Similarly, the lens sorbitol level of diabetic heterozygous and homozygous transgenic mice was measured at the three time points.

**Crossing of AR Transgenic Mice with SorD-Deficient Mice.** Mice homozygous for human AR transgene ($hAR^{+/+}$) from two transgenic lines CAR222 and CAR648 were mated with homozygous SorD-deficient mice ($mSD^{-/-}$) called C57BL/LiA. The offsprings ($F_1$) of these crosses were then mated with each other, and their progenies ($F_2$) were induced to become diabetic and monitored for cataract development and sorbitol accumulation. The genotypes of the $F_2$ mice were determined as follows: hAR genotypes were determined by Southern blot hybridization using hAR cDNA as a probe. The hybridized hAR band in $hAR^{+/+}$ mice is twice as intense as $hAR^{+/-}$ mice and absent in $hAR^{-/-}$ mice. mSD genotypes were determined by enzyme assay. At the end of the experiment, mice were sacrificed, and SorD activity in the liver was measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm, as described (20). Briefly, mouse liver was homogenized, and crude enzyme extract was prepared by spinning down cell debris. Reaction mixture contained 0.1 M triethanolamine buffer (pH 7.4), 0.4 M fructose, and 0.4 mM NADH. The reaction was initiated by adding an aliquot of enzyme and was kept at 30°C. The unit of SorD activity is defined as nmol of NADH oxidized per min per mg of protein in the crude extract. The enzyme activity of $mSD^{+/-}$ and $mSD^{+/+}$ is 50.1 ± 4.7 and 105.6 ± 3.6 units, respectively. No SorD enzyme activity was detected in $mSD^{-/-}$ mice.

## RESULTS

**Development of Transgenic Mice Over-expressing AR in Lens.** Mice have low levels of AR in their lenses, and they normally do not develop diabetic or galactose cataracts. We therefore developed transgenic mice with high levels of AR in their lens epithelial cells to see whether they would become susceptible to the development of these sugar cataracts. The cloned human AR cDNA (16), previously shown to encode for an active enzyme (21), was fused to the mouse αA-crystallin promoter that directs the expression of heterologous genes in the lens epithelial cells of transgenic mice (17) (Fig. 1). The hybrid gene αAcry–hAR was injected into mouse oocytes and allowed to develop to term in foster mothers. Five transgenic lines have been characterized, and their heterozygous offsprings all have increased AR activity in their lenses, ranging from 8- to 100-fold above normal (Table 1). The level of AR expression does not seem to correlate with the copy numbers of the hAR transgene, which in CAR222, -223, -435, -440, and -648 were estimated to be ≈30, 70, 100, 70, and 5, respectively (data not shown). Northern blot hybridization showed that the human AR transgene is only expressed in lens and is not expressed in brain, liver, testis, or muscle of these transgenic mice (data not shown). Under normal rearing conditions none of the transgenic mice developed cataract, indicating that over-expression of AR in the lens *per se* does not cause cataract.

Table 1. Lens AR enzyme level and rate of galactose cataract development

| Transgenic mouse line no. | Lens AR enzyme level, nmol/min per mg | Time to reach stages of galactosemic cataract (after 50% galactose diet), days | | |
|---|---|---|---|---|
| | | Stage I | Stage II | Stage III |
| CAR222 | 37.9 ± 3.57 | 2 | 14 | 21 |
| CAR223 | 11.1 ± 1.20 | N | N | N |
| CAR435 | 48.9 ± 3.75 | 1 | 14 | 21 |
| CAR440 | 11.1 ± 0.63 | N | N | N |
| CAR648 | 133.7 ± 8.50 | 1 | 10 | 14 |
| CAR222 + ARI* | | N | N | N |
| Normal mouse | 1.4 ± 0.09 | N | N | N |
| Normal rat | 37.4 ± 1.30 | 7 | 14 | 21 |

Enzyme activities of AR in lenses of five transgenic mouse lines were assayed at 3 weeks after birth, and values are expressed as mean ± SD from four mice. To induce galactose cataract, 3-week-old mice were fed with a 50% galactose diet, and the rate of cataract development is represented by the number of days that elapsed before the first appearance of the three stages of cataract, as defined in Fig. 2. Results from normal mice and rats are also included for comparison. To inhibit AR enzyme activity, AR inhibitor (AL01576, 0.5 mg/kg per day) was administered daily into the stomach of three mice from line CAR222 through a gastric tube throughout the experiment. N, no observable cataract.
*AR inhibitor.

**Galactose Cataract in Transgenic Mice.** When the 3-week-old heterozygous transgenic mice and their nontransgenic littermates were induced to become galactosemic by a 50% galactose diet, we noticed that the occurrence of cataract depended on the level of AR in their lenses (Table 1). Transgenic mice from lines CAR222, CAR435, and CAR648 with lens AR level comparable with or higher than that of the rats developed cataract, whereas nontransgenic mice and transgenic mice from lines CAR223 and CAR440 with AR levels below that of the rats failed to develop any observable cataract. The progression of cataract development was arbitrarily divided into three stages as shown in Fig. 2. The first stage is represented by the appearance of vacuoles at the periphery of the lens. Stage 2 occurs when the vacuoles cover the entire lens and fuse together, and when the lens becomes opaque, stage 3 has been reached. The transgenic mice reached the various cataract stages at a rate proportional to their lens AR level (Table 1),

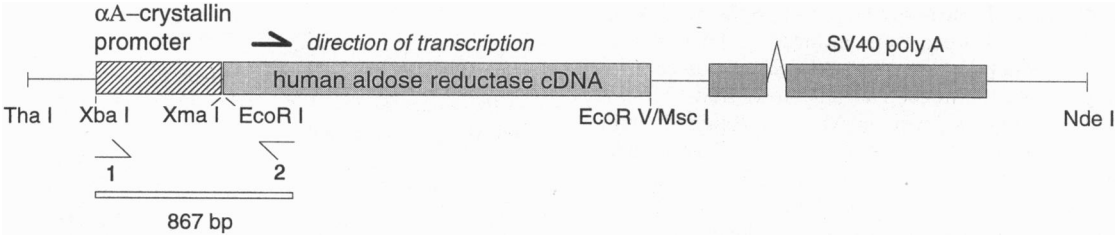


FIG. 1. Construction of the αAcry–hAR hybrid gene. The 3.3-kb αAcry–hAR hybrid gene was made by fusing the human AR cDNA with the lens-specific murine αA-crystallin promoter as described. The two half-arrows indicate positions of the pair of primers for PCR screening of transgenic mice; the size of the expected product is 867 bp. SV40, simian virus 40.

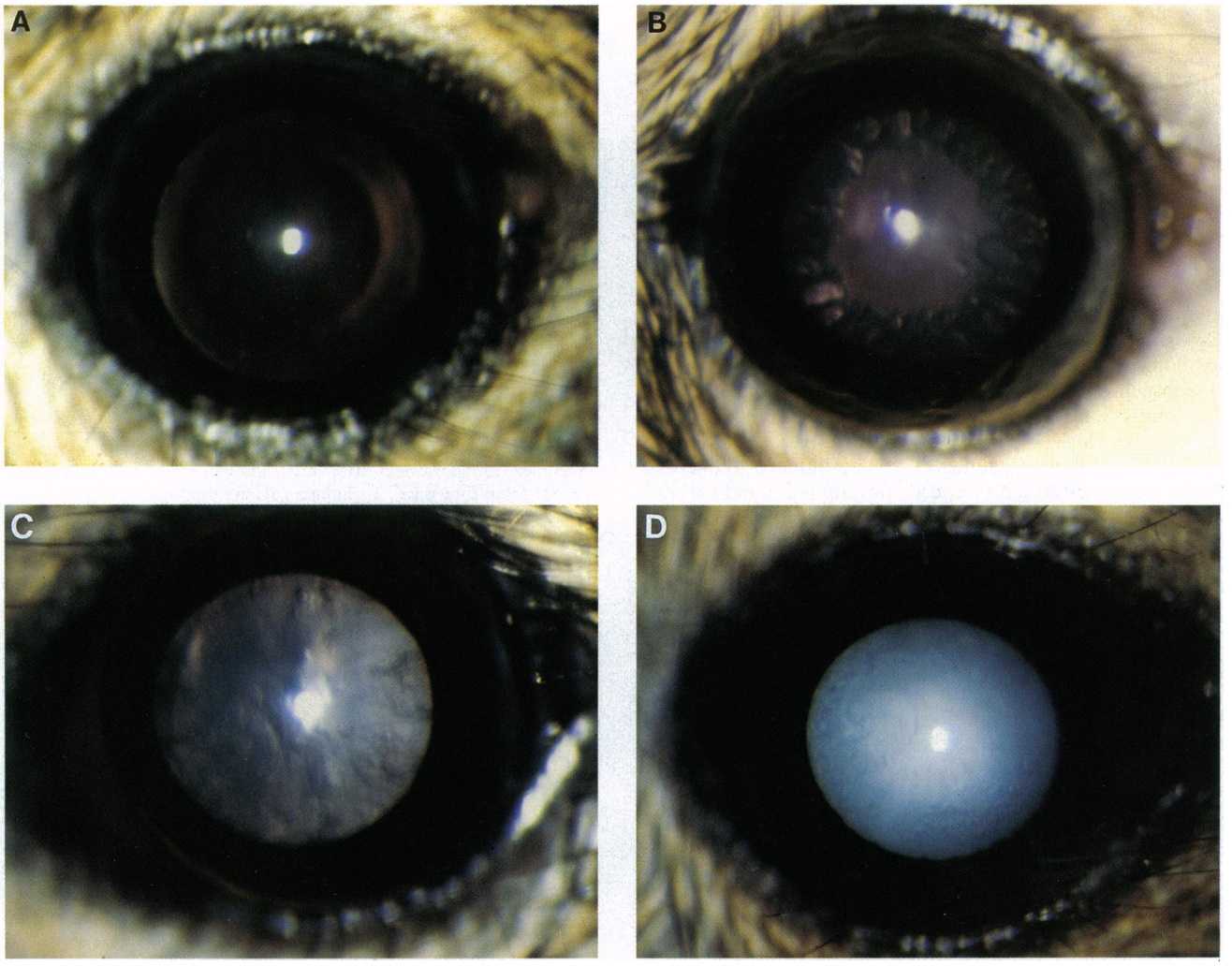


FIG. 2. Stages of cataract development in transgenic mice. (*A*) Eye showing normal lens with no sign of cataract. (*B*) Stage I: small vacuoles appear in the peripheral region of the lens. (*C*) Stage II: vacuoles cover the entire lens and fuse together so that individual vacuoles disappear. (*D*) Stage III: complete opacification throughout the entire lens.

direct evidence for the involvement of AR in galactose cataract. This result is further supported by the fact that an AR inhibitor (AL01576, Alcon, Fort Worth, TX) (22) administered orally could suppress the formation of these cataracts (Table 1).

**Polyol Accumulation in Galactose Cataract.** To find out whether AR indeed reduced galactose to its polyol, the lens galactitol in one of the transgenic lines, CAR222, which has AR activity comparable with that of the rats, was measured at various times after initiation of high galactose feeding. Table 2 shows that there was a 20- to 30-fold increase in galactitol in the transgenic mice, indicating that AR can indeed reduce galactose to galactitol *in vivo* and, most likely, the accumulation of the polyol causes galactose cataract.

**Diabetic Cataract in Transgenic Mice.** When the 3-week-old heterozygous transgenic mice were induced to become diabetic by streptozotocin injection, they did not develop cataract as readily as galactosemic mice. Table 3 shows that only line CAR648, with the highest level of AR, developed diabetic cataract (data on CAR223, CAR435, and CAR440 not shown). Homozygous CAR222 mice, with the lens AR twice that of their heterozygous siblings, were susceptible to diabetic cataract development, and homozygous CAR648 developed cataract much faster than their heterozygous counterparts, indicating that, similar to galactose cataract, susceptibility to diabetic cataract and the rate of cataract development are proportional to lens AR level. The lens sorbitol level under hyperglycemic conditions is much less than the galactitol level under galactosemic conditions, reflecting faster accumulation of the latter, as mentioned earlier.

Apparently mice require a higher level of lens AR than rats to develop diabetic cataracts. One likely reason is that under hyperglycemic conditions rat lens AR level is substantially increased because AR expression is induced by a high glucose level (23), whereas AR level of the transgenic mice is not

Table 2. Lens galactitol level in normal and transgenic mice

| | Galactitol level in lens, μmol/g of wet wt | | |
|---|---|---|---|
| | 1 week* | 2 week* | 3 week* |
| CAR222 transgenic mice | | | |
| Normal diet | <0.08 | <0.08 | <0.08 |
| 50% galactose diet | 31.0 ± 3.50 | 43.2 ± 3.13 | 26.8 ± 2.71 |
| Nontransgenic littermates | | | |
| Normal diet | <0.08 | <0.08 | <0.08 |
| 50% galactose diet | 1.51 ± 0.28 | 1.28 ± 0.14 | 1.39 ± 0.03 |

The amount of galactitol in lenses of CAR222 transgenic mice and their nontransgenic littermates that were put on a normal or galactose diet was measured at the indicated time points, as described. Values of galactitol level are expressed as mean ± SD from four mice.
*Number of weeks after treatment.

Table 3. Rate of diabetic cataract formation and lens sorbitol level in heterozygous and homozygous transgenic mice

| Mouse line | Transgene genotype | Lens AR enzyme level, nmol/min per mg | Time to reach stages of diabetic cataract, days | | | Lens sorbitol after diabetes* | | |
|---|---|---|---|---|---|---|---|---|
| | | | Stage I | Stage II | Stage III | 1 week† | 2 weeks† | 3 weeks† |
| Transgenic | | | | | | | | |
| CAR222 | Heterozygous | 37.9 ± 3.57 | N | N | N | 1.3 ± 0.11 | 1.9 ± 0.40 | 3.0 ± 0.69 |
| | Homozygous | 75.4 ± 4.29 | 17 | 28 | ND | 5.2 ± 0.14 | 6.0 ± 0.48 | 7.4 ± 0.67 |
| CAR648 | Heterozygous | 133.7 ± 8.50 | 14 | 25 | 31 | 15.8 ± 1.31 | 17.5 ± 1.23 | 19.1 ± 1.86 |
| | Homozygous | 270.3 ± 15.26 | 7 | 12 | 20 | 20.5 ± 1.08 | 25.4 ± 1.54 | 18.9 ± 1.79 |
| Normal | | 1.4 ± 0.09 | N | N | N | 0.3 ± 0.12 | 0.3 ± 0.11 | 0.3 ± 0.02 |

Heterozygous and homozygous transgenic mice from lines CAR222 and CAR648 were induced to diabetes by streptozotocin injection. The rate of cataract progression was recorded, and sorbitol level was measured weekly as described. N, no observable cataract during course of experiment; ND, not determined.

*Amount of sorbitol is expressed in μmol per g (wet wt) of lens, and the values are mean ± SD from four mice.

†Time period represents duration of diabetes.

increased in the diabetic state (data not shown) because the αA-crystallin promoter used to drive the AR transgene is not induced by hyperglycemia. The differences in the level of hexokinase and SorD between the rat and mouse lens may also contribute to their different susceptibilities to diabetic cataract.

**Effect of SorD Deficiency on Diabetic Cataract.** The results of our experiments clearly showed that AR is involved in diabetic and galactose cataract formation. However, whether the polyols or other metabolites are responsible remains a question. Fructose (24), converted from sorbitol by SorD, and its phosphorylated derivative fructose 3-phosphate (25) are thought able to crosslink proteins and thus cause diabetic lesions. Further, the change in the redox potential as a result of decrease in NADPH level, the cofactor of AR in hexose reduction, has been suggested to lead to diabetic complications (26). If the fructosylation model is valid, then blocking the conversion of sorbitol to fructose should retard cataractogenesis. If the change in redox potential causes diabetic lesions, then blocking the conversion of sorbitol to fructose should not affect the rate of cataract development. On the other hand, if cataract formation is due to the accumulation of sorbitol, then inactivating SorD should hasten cataract development. To test these models homozygous mice from lines CAR222 and CAR648 were crossed with homozygous SorD-deficient mice called C57BL/LiA (27). This strain of mice was found to be deficient in SorD in all tissues tested, including lens (27), and the lack of SorD in liver and lens was confirmed in our laboratory (data not shown). The siblings of these crosses ($F_1$) were mated with each other, and their offsprings ($F_2$) were induced to become diabetic by streptozotocin injection. The rate of cataract development of the $F_2$ mice of nine different genotypes is shown in Table 4. Mice deficient in SorD clearly accumulated higher levels of sorbitol and developed cataract faster than those with a normal level of SorD, indicating that it is the accumulation of sorbitol that causes diabetic cataract.

## DISCUSSION

Several conclusions can be drawn from these experiments. (*i*) They convincingly show that AR is involved in diabetic and galactose cataracts and validate the previous studies using AR inhibitors to demonstrate the involvement of AR in this disease. (*ii*) Notwithstanding the predictions from kinetic studies and structural analyses, we show that AR can reduce glucose and galactose to their respective polyols *in vivo*. The buildup of these polyols under the slow rate of synthesis may reflect the slow dissipation rate of these metabolites in the lens. On the other hand, there could also be ancillary proteins to assist AR in reducing hexoses in the lens cells, which is not reflected in the *in vitro* situations. More accurate measurement of the rate of synthesis, oxidation, and leakage of these polyols in lens culture or lens cell culture may resolve these issues. (*iii*) Polyol accumulation is the major contributing factor for sugar cataracts, not fructosylation or the reduction of NADPH level. This is in agreement with the findings that SorD inhibitors increased the lens sorbitol levels in diabetic rats and accelerated cataract development (28).

Although AR is also implicated in diabetic neuropathy, nephropathy, and retinopathy, the increase in sorbitol in these tissues under hyperglycemic conditions is not as dramatic as that in lens and no swelling of the cells is seen (29–31). Perhaps hyperglycemia and AR damage these tissues through a different mechanism. This hypothesis is supported by the fact that SorD inhibitors that accelerated cataract development actually normalized motor nerve conduction velocity and glomerular filtration rate in diabetic rats and did not affect diabetic

Table 4. Effect of SorD deficiency on the rate of diabetic cataract development and lens sorbitol level in $F_2$ mice

| Transgenic line number | Genotypes of $F_2$ mice | Time to reach stages of diabetic cataract, days | | | Amount of lens sorbitol 1 week after diabetes* |
|---|---|---|---|---|---|
| | | Stage I | Stage II | Stage III | |
| CAR222 | $hAR^{-/-}$ $mSD^{+/+}$ | N | N | N | 0.24 ± 0.01 |
| | $hAR^{-/-}$ $mSD^{+/-}$ | N | N | N | 0.39 ± 0.05 |
| | $hAR^{-/-}$ $mSD^{-/-}$ | N | N | N | 0.57 ± 0.04 |
| | $hAR^{+/-}$ $mSD^{+/+}$ | N | N | N | 1.38 ± 0.11 |
| | $hAR^{+/-}$ $mSD^{+/-}$ | N | N | N | 1.81 ± 0.09 |
| | $hAR^{+/-}$ $mSD^{-/-}$ | N | N | N | 2.74 ± 0.27 |
| | $hAR^{+/+}$ $mSD^{+/+}$ | 17 | 28 | ND | 5.00 ± 0.13 |
| | $hAR^{+/+}$ $mSD^{+/-}$ | 15 | 25 | ND | 5.43 ± 0.41 |
| | $hAR^{+/+}$ $mSD^{-/-}$ | 13 | 21 | ND | 6.82 ± 0.16 |
| CAR648 | $hAR^{-/-}$ $mSD^{+/+}$ | N | N | N | 0.25 ± 0.06 |
| | $hAR^{-/-}$ $mSD^{+/-}$ | N | N | N | 0.40 ± 0.03 |
| | $hAR^{-/-}$ $mSD^{-/-}$ | N | N | N | 0.57 ± 0.07 |
| | $hAR^{+/-}$ $mSD^{+/+}$ | 14 | 25 | ND | 16.07 ± 1.42 |
| | $hAR^{+/-}$ $mSD^{+/-}$ | 14 | 21 | ND | 16.96 ± 0.92 |
| | $hAR^{+/-}$ $mSD^{-/-}$ | 12 | 20 | ND | 17.35 ± 2.16 |
| | $hAR^{+/+}$ $mSD^{+/+}$ | 7 | 12 | 20 | 20.79 ± 0.66 |
| | $hAR^{+/+}$ $mSD^{+/-}$ | 4 | 7 | 14 | 22.56 ± 1.17 |
| | $hAR^{+/+}$ $mSD^{-/-}$ | 2 | 6 | 10 | 26.51 ± 1.98 |

The $F_2$ mice (progenies from sibling matings of $hAR^{+/-}$ $mSD^{+/-}$ mice) were induced to become diabetic by streptozotocin injection. Monitoring of cataract development, measurement of lens sorbitol level, and determination of the genotype of the $F_2$ mice were done as described. $hAR^{+/+}$ and $hAR^{+/-}$ are homozygous and heterozygous, respectively, for the hAR transgene. $hAR^{-/-}$ are the nontransgenic littermates. $mSD^{-/-}$ and $mSD^{+/-}$ are homozygous and heterozygous, respectively, for SorD deficiency. $mSD^{+/+}$ are the wild-type littermates for SorD. N, no observable cataract during course of experiment; ND, not determined.

*Amount of sorbitol is expressed in μmol per g (wet wt) of lens, and the values are mean ± SD from three to six mice.

retinopathy (28). Targeted increase of AR expression in relevant tissues may resolve some of these issues. Clinical trials using AR inhibitors to treat various diabetic complications were largely unsuccessful (32–34). Confirmation of the role of AR in these complications should provide an impetus to develop better AR inhibitors and, more importantly, provide a rationale for treating the patients with these drugs early to arrest the early steps of these complications.

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