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Evaluation of an aldose reductase inhibitor on lens metabolism, ATPases and antioxidative defense in streptozotocin-diabetic rats: an intervention study

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Abstract

Aims/hypothesis. Aldose reductase inhibitors (ARIs) prevent biochemical abnormalities associated with diabetic complications. We evaluated whether a short-term intervention with an adequate dose of ARI, introduced at the very early, precataractous stage, reversed diabetes-induced metabolic imbalances, down-regulation of ATPases and oxidative stress in the lens.
Methods. The groups included mature control and streptozotocin-diabetic rats treated with or without ARI sorbinil ($65\ mg \cdot kg^{-1} \cdot day^{-1}$, in the diet, for 2 weeks after 4 weeks of untreated diabetes). Free cytosolic NAD^+:NADH and $NADP^+$:NADPH ratios were calculated from the lactate dehydrogenase and malic enzyme systems. Concentrations of metabolites and adenine nucleotides, Na^+/K^+-ATPase, H^+-ATPase and Ca^{++}-independent Mg^{++}-ATPase activities and variables of oxidative stress were measured in individual lenses.
Results. Sorbinil treatment essentially corrected diabetes-induced sorbitol and fructose accumulation, *myo*-inositol depletion, decrease in free cytosolic NAD^+:NADH ratio and energy deficiency. Malondialdehyde accumulation, reduced glutathione depletion and the increase in oxidized glutathione:reduced glutathione ratio were partially corrected. Free cytosolic $NADP^+$:NADPH ratio and 4-hydroxyalkenal concentrations were similarly increased in diabetic rats treated with or without ARI. Sorbinil did not counteract diabetes-induced down-regulation of the three ATPase activities.
Conclusion/interpretation. All biochemical changes assessed in our study are known to be prevented by ARIs. Despite the essential normalization of the sorbitol pathway activity, only part of them were, however, *reversed* by the ARI treatment introduced at the very early, i. e. precataractous, stage of diabetes. Therefore, intervention studies can easily underestimate the importance of aldose reductase in the pathogenesis of diabetic complications and should be interpreted with caution. [Diabetologia (2000) 43: 1048–1055]

Keywords Aldose reductase, ATPase activities, energy metabolism, lens, NAD(P)-redox state, oxidative stress, rat, reversal study, sorbinil, streptozotocin-diabetes

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Abbreviations: AR: Aldose reductase, ARI: aldose reductase inhibitor, 4-HA: 4-hydroxyalkenals, GSSG: oxidized glutathione, GSH: reduced glutathione, MDA: malondialdehyde, P_i: inorganic phosphate, SDH: sorbitol dehydrogenase

The consequences of increased aldose reductase (AR) activity in tissue-sites for diabetic complications include down-regulation of membrane transporters and osmolyte depletion [1, 2], impaired ion homeostasis [1, 3], energy deficiency [4, 5], NAD-redox and NADP-redox changes [5], oxidative stress [6–8], protein kinase C activation [9, 10], accumulation of advanced glycation end-products [11, 12], impaired neurotrophism [13, 14], up-regulation of vascular endothelial growth factor [15] and other imbalances. Numerous studies in the animals models of di-

abetes and galactose feeding indicate that these biochemical changes and, most importantly, their outcome, i.e. diabetic complications, are prevented by structurally different AR inhibitors (ARIs) [1–6, 8–19]. In contrast, the results of clinical trials of ARIs on diabetic neuropathy [20] and retinopathy [21] were disappointing and created a number of questions regarding the trials' design, adequacy of the ARI doses as well as applicability of the AR concept to diabetic complications in humans in general and reversibility of the early and later stages of these complications.

During the last 5 years, a number of experimental studies were conducted to find out if the pathological process can be stopped and reversed after the whole spectrum of biochemical, functional and morphological changes has been initiated. The findings of at least two groups [17, 22] indicate that diabetes-induced neurovascular dysfunction, nerve conduction deficit and metabolic imbalances, characteristic for early experimental diabetic neuropathy, can be corrected by a very short (2 week) treatment with an adequate dose of ARI, i.e. the dose that completely blocked diabetes-associated increase in the sorbitol pathway activity. Retinal changes seemed, however, less prone to normalization. In particular, it was found that a 12-month treatment with an adequate dose of ARI did not correct early vascular abnormalities characteristic for diabetes-like retinopathy [23]. The results of longer ARI treatment from the same group [24] as well as the experiments with galactose withdrawal in the galactosaemic dog model (Kador et al., unpublished) provided, however, the evidence that the reversal of retinal vascular changes, at least at the stage of background retinopathy, can be achieved in principle.

The lens offers a number of advantages for studying AR-mediated metabolic imbalances because all major diabetes-associated metabolic changes, i.e. sorbitol pathway intermediate accumulation [6, 25–27], osmolyte depletion [26], down-regulation of ATPases [28, 29], energy deficiency [4, 5], NAD-redox and NADP-redox changes [5], antioxidant loss [6] and activation of lipid peroxidation [27] are clearly manifested in early diabetes. All these imbalances have been implicated in initiation (sorbitol accumulation) or progression of diabetic cataract formation and have been found preventable by ARIs [1, 4–6, 30]. The purpose of our study was to evaluate whether these diabetes-associated biochemical abnormalities can be *reversed* by a short-treatment with an adequate dose of ARI, introduced at the precataractous stage.

Materials and methods

The experiments were done in accordance with regulations specified by The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80–23) and the University of Michigan Protocol for Animal Studies.

Animals. Male Wistar rats (Charles River, Wilmington, Mass., USA), body weight 250–300 g, were fed a standard rat chow diet (ICN Biomedicals, Cleveland, Ohio, USA) and had free access to water. Diabetes was induced by a single injection of streptozotocin (Upjohn, Kalamazoo, Mich., USA, 55 mg/kg body weight, i.p.). Blood samples for glucose measurements were taken from the tail vein about 48 h after streptozotocin injection and the day before the rats were killed. Rats with blood glucose concentrations of 13.9 mmol/l or more were considered diabetic. The experimental groups included control and diabetic rats treated with or without ARI sorbinil (Pfizer, $65 mg \cdot kg^{-1} \cdot day^{-1}$, in the diet, for 2 weeks after 4 weeks of untreated diabetes).

Experimental procedure. Rats were sedated with carbon dioxide and killed by cervical dislocation. Both lenses were dissected by posterior incision, carefully separated from accompanying aqueous and vitreous humors, and frozen in liquid nitrogen. One lens from each rat was used for measurements of sorbitol pathway intermediates, malondialdehyde (MDA), MDA plus 4-hydroxyalkenals (4-HA) and total and ouabain-insensitive Na^+/K^+-ATPase activities. The second lens was used for measurements of reduced glutathione (GSH), oxidized glutathione (GSSG), pyruvate, lactate, malate, ATP, ADP and AMP. A separate set of lenses was used for assessment of H^+-ATPase and Ca^{++}-independent Mg^{++}-stimulated ATPase activities.

Biochemical measurements. Concentrations of glucose, sorbitol, fructose, GSH, GSSG, MDA, MDA plus 4-HA, pyruvate, lactate, malate and adenine nucleotides were assayed as described [26, 27, 31–33]. Free cytosolic NAD^+:NADH and $NADP^+$:NADPH were calculated from the steady-state metabolite concentrations and equilibrium constants of the lactate dehydrogenase and malic enzyme systems [34, 35]. For measurements of Na^+/K^+-ATPase, H^+-ATPase and Ca^{++}-independent Mg^{++}-stimulated ATPase activities, lenses were homogenized in 1.5 ml 0.1 mol/l sodium–phosphate buffer, pH 6.5. The homogenate was centrifuged at 3000 g to precipitate large particles and cell debris. The analytical mixture for Na^+/K^+-ATPase contained 0.15 mmol/l NADH, 1 mmol/l phosphoenolpyruvate, 1 mmol/l TRIS-ATP, 2 U of pyruvate kinase and 5 U of lactate dehydrogenase in 0.9 ml buffer containing 100 mmol/l NaCl, 10 mmol/l KCI, 2.5 mmol/l $MgCl_2$ and 30 mmol/l imidazole-HCl, pH 7.3. The reaction was initiated by addition of 0.1 ml supernatant and the total activity was measured spectrophotometrically for 15 min by monitoring a decrease in absorbance at 340 nm due to oxidation of NADH to NAD, stochiometric to hydrolysis of ATP to ADP and inorganic phosphate (P_i). Then 0.02 ml of 25 mmol/l ouabain was added and the reaction was followed for another 15 min. Ouabain-sensitive Na^+/K^+-ATPase activity was calculated as the difference between total and ouabain-insensitive Na^+/K^+-ATPase activities. For measurements of H^+-ATPase and Ca^{++}-independent Mg^{++}-stimulated ATPase activities, the lenses were homogenized in 1 ml buffer containing 10 mmol/l HEPES, 100 mmol/l KCl and 0.1 mmol/l EGTA, pH 7.4. The homogenates were centrifuged at 3000 *g* for 10 min. Then H^+-ATPase activity was assayed in the supernatant fractions as described

[36]. For measurements of Ca^{++}-independent Mg^{++}-stimulated ATPase activity, 0.25 mmol/l $MgCl_2$ was added to the analytical mixture used for H^+-ATPase [36]. The Ca^{++}-independent Mg^{++}-stimulated ATPase activity was calculated as the difference between total Ca^{++}-independent Mg^{++}-stimulated ATPase plus H^+-ATPase activity and H^+-ATPase activity.

Statistical analysis. The results are expressed as means ± SEM. Data were subjected to equality of variance *F* test and then to log transformation, if necessary, before one-way analysis of variance. When overall significance ($p < 0.05$) was attained, individual between-group comparisons were made using the Student-Newman-Keuls multiple range test. Significance was defined at *p* 0.05 or less. When between-group variance differences could not be normalized by log transformation, the data were analysed by the non-parametric Kruskal-Wallis one-way analysis of variance, followed by Fisher's PLSD test for multiple comparisons.

Results

The final body weights were lower in diabetic rats than in controls (300.1 ± 11.4 vs 428.3 ± 4.6 g, $p < 0.01$). The initial body weights were similar in control and diabetic groups. No difference was found between final body weights in diabetic rats treated with ARI (307.8 ± 16.8 g) and the untreated group.

Blood glucose concentration was increased 5.7-fold in diabetic rats compared with controls (18.1 ± 0.6 vs 3.2 ± 0.1 mmol/l, $p < 0.01$) and was not affected by ARI treatment (18.5 ± 0.6 mmol/l).

Lens glucose, sorbitol and fructose concentrations were 9.1-fold, 120-fold, and 9.4-fold higher in diabetic rats than controls (Table 1). Glucose concentrations were similar in diabetic rats treated with or without ARI. Sorbitol and fructose concentrations in diabetic rats were essentially normalized by the ARI treatment. Lens *myo*-inositol concentration was decreased 23.4-fold in diabetic rats compared with controls. This decrease was corrected by the ARI treatment.

Lens pyruvate and malate concentrations were decreased 1.7-fold and 1.8-fold in diabetic rats compared with controls (Table 2) and the decrease in both concentrations was corrected by the ARI treatment. Lens lactate concentrations were similar among the experimental groups. Free cytosolic NAD^+:NADH ratio was 2.5-fold lower in diabetic rats than in controls and this decrease was corrected by the ARI treatment. Free cytosolic $NADP^+$:NADPH ratios were similarly decreased in untreated and ARI-treated diabetic rats compared with controls.

Lens ATP concentration was 1.6-fold lower in diabetic rats than in controls whereas ADP and AMP concentrations were 1.6-fold and 2-fold higher (Table 3). Diabetes-induced decrease in ATP concentration was corrected by the ARI treatment whereas ADP and AMP concentrations tended to decrease being not significantly different from either the untreated diabetic group or controls. The ATP:ADP ratio and adenylate energy charge were 1.9-fold and 1.1-fold lower in diabetic rats than in controls. Both variables were corrected by ARI.

Total, ouabain-insensitive and ouabain-sensitive ATPase activities were 1.7-fold, 1.6-fold and 1.7-fold lower in diabetic rats than controls and none of these activities were affected by ARI (Fig. 1). In a similar fashion, H^+-ATPase activities were 2.3-fold and 1.5-fold lower in untreated and ARI-treated diabetic rats than controls (Fig. 2). The Ca^{++}-independent Mg^{++}-stimulated ATPase activity was decreased 3.4-fold in diabetic rats compared with controls. This activity tended to increase with the ARI treatment but the difference with the untreated diabetic group did not achieve statistical significance.

Table 1. Sorbitol pathway intermediate and *myo*-inositol concentrations (nmol/lens) in control and diabetic rats treated with or without ARI ($n = 8$)

	Control	Diabetic	Diabetic + ARI
Glucose	18.9 ± 2.2	172 ± 24[a]	261 ± 33[a]
Sorbitol	13.3 ± 2.8	1594 ± 152[a]	33.8 ± 7.7[b]
Fructose	21.0 ± 2.3	197 ± 19[a]	37.0 ± 6.4[b]
myo-Inositol	63.2 ± 2.7	2.7 ± 1.7[a]	67.5 ± 23.8[b]

[a] Significantly different compared with controls ($p < 0.01$); [b] significantly different compared with untreated diabetic rats ($p < 0.01$)

Table 2. Pyruvate, lactate and malate concentrations (nmol/lens) and free cytosolic NAD^+ : NADH and $NADP^+$: NADPH ratios in lens in control and diabetic rats treated with or without ARI ($n = 7$–8)

	Control	Diabetic	Diabetic + ARI
Pyruvate	6.7 ± 0.7	3.9 ± 0.6[a]	7.5 ± 1.0[c]
Lactate	310 ± 59	436 ± 66	487 ± 59
Malate	3.8 ± 1.3	2.1 ± 0.7[a]	3.9 ± 1.5[c]
NAD^+ : NADH	194 ± 26	79 ± 6[b]	155 ± 18[c]
$NADP^+$: NADPH	0.041 ± 0.005	0.061 ± 0.007[a]	0.062 ± 0.007[a]

[a,b] Significantly different compared with controls ($p < 0.05$ and < 0.01, respectively); [c] significantly different compared with untreated diabetic group ($p < 0.01$)

Table 3. Lens energy status in control and diabetic rats treated with or without ARI ($n = 6$–8)

	Control	Diabetic	Diabetic + ARI
ATP	133 ± 18	84 ± 9[a]	134 ± 19
ADP	14.7 ± 4.3	23.6 ± 7.5[a]	20.8 ± 3.6
AMP	3.9 ± 0.4	6.6 ± 0.8[b]	5.0 ± 0.5
ATP : ADP	7.7 ± 2.3	4.0 ± 1.5[a]	6.8 ± 1.1[c]
Adenylate charge	0.91 ± 0.01	0.84 ± 0.01[b]	0.90 ± 0.01[d]

ATP, ADP, AMP concentrations are expressed in nmol/lens
[a,b] Significantly different compared with controls ($p < 0.05$ and < 0.01, respectively); [c,d] significantly different compared with untreated diabetic group ($p < 0.05$ and < 0.01, respectively)

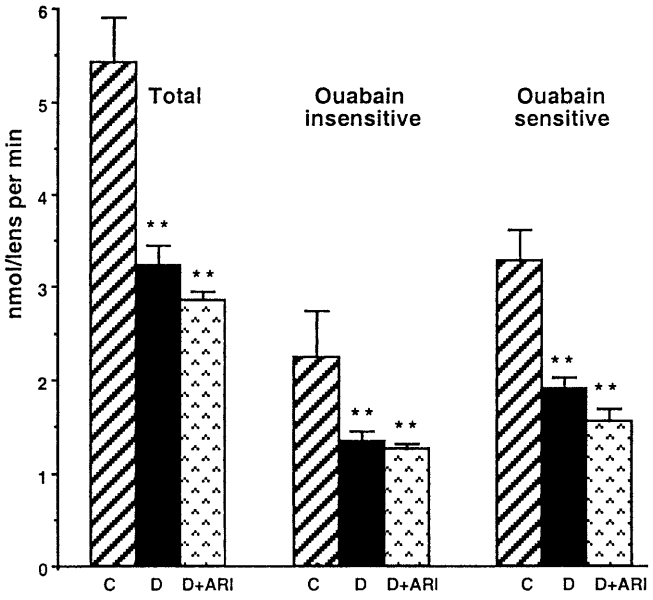

Fig. 1. Lens total, ouabain-insensitive and ouabain-sensitive Na^+/K^+-ATPase activities in control (C) and diabetic (D) rats treated with or without ARI (means ± SEM, $n = 8–10$). **; significantly different vs controls ($p < 0.01$)

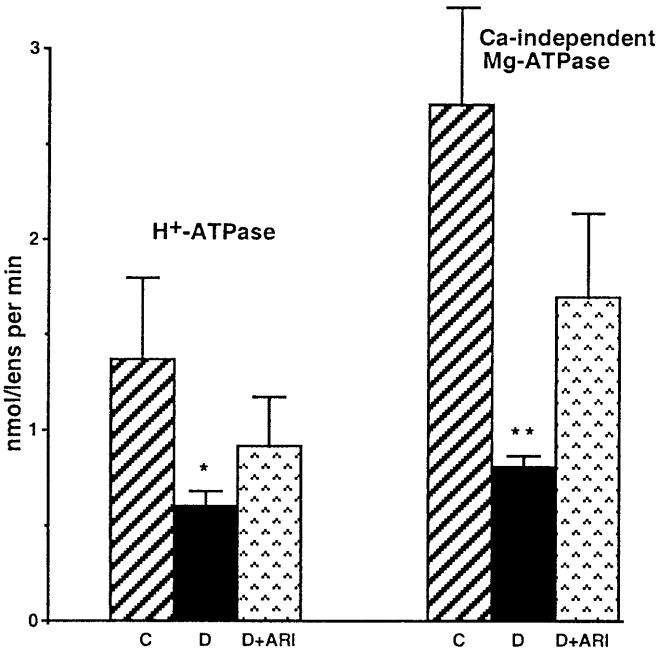

Fig. 2. Lens H^+-ATPase and Ca^{++}-independent Mg^{++}-stimulated ATPase activities in control (C) and diabetic (D) rats treated with or without ARI (means ± SEM, $n = 7–10$). *,**; significantly different vs controls ($p < 0.05$ and < 0.01, respectively).

Malondialdehyde plus 4-HA and MDA concentrations were 3.9-fold and 5.3-fold higher in diabetic rats than in controls (Fig. 3). Malondialdehyde concentration was 2-fold reduced, but not normalized, by the ARI treatment. MDA plus 4-HA concentration tended to decrease, due to reduced accumulation of

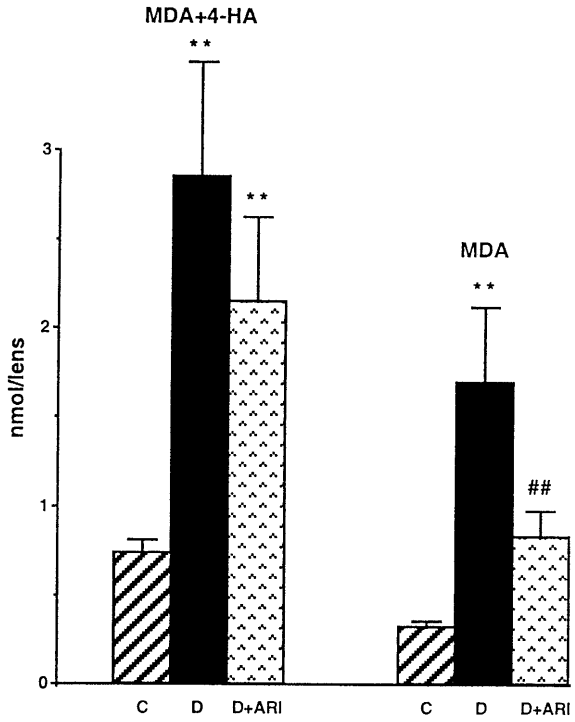

Fig. 3. Lens total MDA plus 4-HA and MDA concentrations in control (C) and diabetic (D) rats treated with or without ARI (means ± SEM, $n = 6–8$). **; significantly different vs controls ($p < 0.01$). ##; significantly different vs untreated diabetic group ($p < 0.01$)

MDA, but not 4-HA, in the ARI-treated rats. The difference with the untreated diabetic group did not, however, achieve statistical significance.

Lens GSH concentration was decreased 3.5-fold in diabetic rats compared with controls, whereas GSSG concentrations and the GSSG:GSH ratio were increased 2.5-fold and 12.2-fold (Fig. 4). Reduced glutathione concentration was increased, but not normalized, by the ARI treatment. Oxidized glutathione concentration tended to decrease being not significantly different from either the untreated diabetic group or controls. The GSSG:GSH ratio was 2.9-fold lower in ARI-treated diabetic rats than in untreated diabetic groups but was still 4.2-higher than in controls.

Discussion

The 2-week sorbinil treatment, started after 4 weeks of untreated diabetes, effectively inhibited increased sorbitol pathway activity in diabetic precataractous lens. Sorbitol and fructose accumulation was corrected by 99% and 94%, respectively. The higher efficacy of ARIs in correcting sorbitol compared with fructose accumulation has been reported for several tissues [37, 38] and suggests that, in addition to the sorbitol dehydrogenase (SDH) reaction, fructose can be

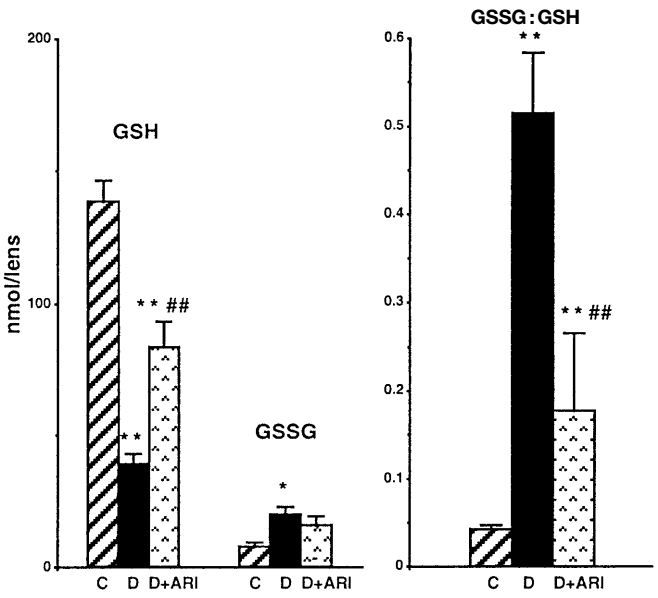

Fig. 4 A, B. Lens GSH and GSSG concentrations (**A**) and GSSG:GSH ratios (**B**) in control (C) and diabetic (D) rats treated with or without ARI (means ± SEM, $n = 6–8$). *,**; significantly different vs controls ($p < 0.05$ and < 0.01, respectively). ##, significantly different vs untreated diabetic group ($p < 0.01$)

formed in other pathways, e. g. by dephosphorylation of the glycolytic product, fructose 6-phosphate.

The short-term treatment with an adequate dose of ARI seemed sufficient to correct diabetes-induced decrease in the free cytosolic NAD^+:NADH ratio. This is consistent with prevention studies in both diabetic [39] and galactose-fed [5] rats. The correction is probably mediated through decreased lipid peroxidation and inhibition of SDH activity. Our studies with 5% taurine supplementation [31] and SDH inhibitor [27] showed that both oxidative stress and increased flux through SDH are involved in the shift towards a more reduced state of free cytosolic NAD-couple in the diabetic lens.

The diabetes-induced increase in free cytosolic $NADP^+$:NADPH was not affected by the ARI treatment. The latter is the most surprising because increased accumulation of the sorbitol pathway intermediates, an indicator of NADPH consumption by AR, was essentially corrected. The lens free cytosolic $NADP^+$:NADPH ratio is, however, maintained, in addition to AR, by at least seven enzymes, i. e. NADP-dependent glucose 6-phosphate [40, 41] and 6-phosphogluconate [40, 42] dehydrogenases, malic enzyme [43], isocitrate dehydrogenase [43] and NADPH-dependent glutathione reductase [44, 45], NAD(P)H-oxidase [46] and NADPH-diaphorase [47]. The post-translational regulation of the above-mentioned enzymes in the lens, with the exception of glucose 6-phosphate dehydrogenase [40, 41], has not been studied in detail.

The diabetes-induced decrease in ATP concentration, ATP:ADP ratio and adenylate energy charge were corrected by short-term ARI treatment. This agrees with the early findings [48] showing a role for polyol accumulation and resulting osmotic stress in diabetes-induced decay of lens ATP and later reports of prevention of lens energy failure with AR inhibition in both diabetic [4] and galactosaemic [5] rats.

The mechanisms underlying decreased Na^+/K^+-ATPase pumping activity in sites for diabetic complications are not clear. One concept implies that diabetes-induced *myo*-inositol depletion, followed by disturbances in phosphoinositide turnover, interferes with the normal structural and functional properties of plasma membranes and is ultimately responsible for down-regulation of Na^+/K^+-ATPase [49]. Other findings [50] dissociate decreased Na^+/K^+-ATPase pumping activity and *myo*-inositol depletion in diabetic tissues. The preservation of diminished total, ouabain-insensitive and ouabain-sensitive Na^+/K^+-ATPase activities despite the complete normalization of lens *myo*-inositol concentrations in the ARI-treated diabetic rats in our study is consistent with the lack of close association between Na^+/K^+-pump function and *myo*-inositol abundance. The modulation of Na^+/K^+-ATPase activity by oxidative stress [51], C peptide [52], ATP depletion [53], nerve growth factor [54], protein kinase C inhibitor [55], thrombin [56] and endothelin-1 [57] reflects the complex regulation of this enzyme. Despite this complexity, the diabetes-induced down-regulation of Na^+/K^+-ATPase is, however, completely prevented by structurally different ARIs [29, 58]. There must be some component(s) in the enzyme regulation that undergoes irreversible, at least by the short-term ARI treatment, changes in the diabetic lens.

The relation between AR and oxidative stress is still the controversial area. Numerous reports indicate that the effects of ARIs and antioxidants are unidirectional and that both types of agents prevent or delay diabetic cataract [1, 3, 16, 59, 60], neuropathy [1, 17, 61], retinopathy [1, 15, 18, 19, 24, 62] and nephropathy [1, 63]. Conversely, some recent studies [64] suggest that AR, which metabolises the most toxic products of lipid peroxidation, 4-HA, protects from the development of diabetic complications. The findings of exacerbation of diabetes-induced lipid peroxidation and glutathione depletion with overexpression of the gene coding for AR protein in the lens [7] as well as the arrest of MDA and 4-HA accumulation by structurally different ARIs [8, 30] indicate, however, that increased AR activity results in, rather than protects from, oxidative injury in tissue sites for diabetic complications. This is supported by this intervention study showing the decrease, but not normalization, of MDA accumulation in ARI-treated rats compared with the untreated diabetic group. In contrast to the observations in the diabetic kidney [64],

sorbinil did not stimulate additional accumulation of 4-HA in the diabetic lens.

The decreased accumulation of lipid peroxidation products is consistent with the higher GSH concentration and the lower GSSG:GSH ratio in the ARI-treated diabetic rats compared with the untreated group. Our findings are consistent with numerous reports of prevention of diabetes-induced GSH depletion with ARI treatment [6, 8, 30]. The studies of our group and others with both ARI [8] and SDH inhibitor [27, 65] suggest that sorbitol accumulation and resulting osmotic stress, rather than AR-mediated NADPH deficiency and associated slowing of the glutathione redox cycle, non-enzymatic glycation of glutathione reductase or enzymes of GSH biosynthesis or NAD^+:NADH redox changes, are responsible for the loss of non-enzymatic antioxidants and increased lipid peroxidation in the diabetic rat lens. The osmotic origin of oxidative stress is, however, not supported by findings in the diabetic SDH-deficient mice that contained lower MDA and higher GSH concentrations in the lens than the diabetic mice with normal SDH activity [7]. Because of these interspecies differences, the mechanisms, underlying exacerbation of lenticular oxidative stress under conditions of increased AR activity in humans [66], require specific studies.

Recently, one group of investigators [67] suggested that oxidative stress is a cause rather than a consequence of increased AR activity and that sorbitol accumulation is downstream from oxidative stress in the pathogenesis of diabetic complications. This concept is not supported by the findings of our group [8, 26, 31] and others [68, 69]. In particular, the potent antioxidant DL-α-lipoic acid effectively counteracted oxidative stress in the diabetic lens [26], peripheral nerve [8, 61] and retina [70] but did not decrease accumulation of the sorbitol pathway intermediates in any of these tissues. Intralenticular sorbitol and fructose concentrations were not affected by taurine which decreased lens MDA accumulation about two-fold compared with the untreated diabetic group [31]. Butylated hydroxytoluene [68] and probucol [69] have been found ineffective on the sorbitol pathway intermediates in the diabetic nerve. These findings, together with the afore-mentioned reports of exacerbation of lipid peroxidation with overexpression of the gene coding for AR protein [7] and prevention or correction of tissue oxidative stress by ARIs [6, 8], clearly indicate that increased AR activity is a cause, but not a consequence, of oxidative stress in sites for diabetic complications.

All diabetes-induced metabolic changes in the lens, assessed in our study, are known to be *prevented* by ARIs. Despite the essential normalization of the sorbitol pathway activity, only part of them was, however, *reversed* by the ARI treatment introduced at the very early, i.e. precataractous, stage of diabetes. It is not clear from our study if "the point of no return" occurs as early as at the precataractous stage of diabetes or longer sorbinil treatment would correct the whole spectrum of biochemical abnormalities in the lens. It is, however, obvious that the intervention approach alone, especially with suboptimal doses of ARIs applied so far in clinical trials, can easily underestimate the importance of AR in the pathogenesis of diabetic complications. The only clinical trial of ARIs on diabetic ocular complications, i.e. sorbinil retinopathy trial [21], included patients with microaneurysms on both eyes treated with a relatively low dose of sorbinil.

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