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# Interaction between Osmotic and Oxidative Stress in Diabetic Precataractous Lens

## STUDIES WITH A SORBITOL DEHYDROGENASE INHIBITOR

*Irina G. Obrosova,*† Lamia Fathallah* and Hans-Jochen Lang‡*

*Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109, U.S.A.; and ‡Hoechst Marion Roussel Deutschland GmbH, Chemical Research Frankfurt, D-65926 Frankfurt/Main, Germany

**ABSTRACT.** Both sorbitol accumulation-linked osmotic stress and "pseudohypoxia" [increase in NADH/$NAD^+$, similar to that in hypoxic tissues, and attributed to increased sorbitol dehydrogenase (l-iditol:$NAD^+$ 5-oxidoreductase; EC 1.1.1.14; SDH) activity] have been invoked among the mechanisms underlying oxidative injury in target tissues for diabetic complications. We used the specific SDH inhibitor SDI-157 [2-methyl-4(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino)pyrimidine] to evaluate the role of osmotic stress versus "pseudohypoxia" in oxidative stress occurring in diabetic precataractous lens. Control and diabetic rats were treated with or without SDI-157 (100 mg/kg/day for 3 weeks). Lens malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HA), MDA, GSH, and ascorbate levels, as well as the GSSG/GSH ratios, were similar in SDI-treated and untreated control rats, thus indicating that SDI-157 was not a prooxidant. Intralenticular osmotic stress, manifested by sorbitol levels, was more severe in SDI-treated diabetic rats (38.2 ± 6.8 vs 21.2 ± 3.5 μmol/g in untreated diabetic and 0.758 ± 0.222 μmol/g in control rats, $P < 0.01$ for both), while the decrease in the free cytosolic $NAD^+$/NADH ratio was partially prevented (120 ± 16 vs 88 ± 11 in untreated diabetic rats and 143 ± 13 in controls, $P < 0.01$ for both). GSH and ascorbate levels were decreased, while MDA plus 4-HA and MDA levels were increased in diabetic rats versus controls; both antioxidant depletion and lipid aldehyde accumulation were exacerbated by SDI treatment. Superoxide dismutase (superoxide:superoxide oxidoreductase; EC 1.15.1.1), GSSG reductase (NAD[P]H:oxidized-glutathione oxidoreductase; EC 1.6.4.2), GSH transferase (glutathione *S*-transferase; EC 2.5.1.18), GSH peroxidase (glutathione:hydrogen-peroxide oxidoreductase; EC 1.11.1.9), and cytoplasmic NADH oxidase activities were increased in diabetic rats versus controls, and all the enzymes but GSH peroxidase were up-regulated further by SDI. In conclusion, sorbitol accumulation and osmotic stress generated oxidative stress in diabetic lens, whereas the contribution of "pseudohypoxia" was minor. SDIs provide a valuable tool for exploring mechanisms of oxidative injury in sites of diabetic complications. BIOCHEM PHARMACOL **58**;12:1945–1954, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** lens; osmotic stress; oxidative stress; rat; sorbitol dehydrogenase inhibitor; streptozotocin-diabetes

Although the presence of both osmotic [1, 2] and oxidative [3] stress in the diabetic precataractous lens was established in the early 1980s, the interaction between the two major mechanisms implicated in diabetes-associated cataractogenesis has always been and still remains a subject of debate. In both the 1980s and 1990s, a number of studies [4–7] pointed to an association between a diabetes-induced increase in sorbitol pathway activity and oxidative injury, whereas others [8–10], on the contrary, regarded the two phenomena as independent and attributed the preventive effects of structurally different ARIs§ on lipid peroxidation and other manifestations of oxidative injury to the antioxidative properties of these compounds rather than to their ability to inhibit the first step of the sorbitol pathway. In spite of the fact that metal-chelating properties of a number of ARIs administered at high doses have been documented [11], the findings of exacerbation of oxidative stress with overexpression of the AR gene in the lens [12] as well as the development of a new potent ARI, WAY-121509 [13], which appears to ameliorate oxidative stress at doses far

† *Corresponding author*: Dr. Irina Obrosova, Division of Endocrinology and Metabolism, Department of Internal Medicine, The University of Michigan Health System, 1150 West Medical Center Drive, MSRB II, Rm. 5570, Ann Arbor, MI 48109-0678. Tel. (734) 936-5035; FAX (734) 662-2349; E-mail: iobrosso@medmail.med.umich.edu

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§ *Abbreviations*: AR, aldose reductase; ARI, aldose reductase inhibitor; DHAP, dihydroacetone phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GP, α-glycerophosphate; GSHPx, glutathione peroxidase; GSHTrans, glutathione S-transferase; GSSGRed, glutathione reductase; 4-HA, 4-hydroxyalkenals; MDA, malondialdehyde; SDH, sorbitol dehydrogenase; SDI, sorbitol dehydrogenase inhibitor; and SOD, superoxide dismutase.

below effective doses of potent biologically active antioxidants [14, 15], indicate that increased sorbitol pathway activity *per se* is somehow responsible for at least some manifestations of diabetes-induced oxidative injury.

What is the biochemical mechanism(s) linking increased sorbitol pathway activity and oxidative stress in target tissues for diabetic complications, including the diabetic precataractous lens? Although several hypotheses have been proposed to answer this question, no consensus has been achieved. A number of studies indicate that both fructose, the product of the sorbitol pathway, and its derivatives, fructose 3-phosphate and 3-deoxyglucosone, are potent glycation agents [16, 17], and advanced glycation end-products are known to induce the formation of reactive oxygen species [18]. Others link oxidative injury to loss of the most important nonenzymatic antioxidants such as GSH and ascorbate, which is considered to occur due to severe sorbitol accumulation-linked osmotic stress [4, 7]. The alternative concept suggests that polyol accumulation *per se* does not contribute to oxidative injury, and such injury results from depletion of NADPH, a cofactor shared by AR and GSSGRed [12, 13, 19]. Finally, one group hypothesized [20] that neither sorbitol accumulation nor NADPH deficiency is important, and oxidative stress is a consequence of so-called "pseudohypoxia," i.e. increase in free cytosolic NADH/$NAD^+$, similar to that in hypoxic tissues, and attributed to increased NAD-dependent oxidation of sorbitol to fructose by the second half of the sorbitol pathway, SDH.

SDH was found to be specifically inhibited ($K_i = 9.2 \times 10^{-5}$ mol/L for the reaction: sorbitol + $NAD^+ \rightarrow$ fructose + NADH + $H^+$, for SDH from sheep liver) by the pyrimidine derivatives SDI-157 [2-methyl-4(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino)pyrimidine, pro-drug] and SDI-158 [2-hydroxymethyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino)pyrimidine, *in vitro* active compound], which were first synthesized and studied by Hoechst AG [21]. Both SDI-157 and SDI-158 [the same compound as CP-166,572 (Pfizer) and WAY-135706 (Wyeth-Ayerst)] dramatically increase sorbitol levels in diabetic precataractous rat lens [21–24], and thus potentiate intralenticular osmotic stress without affecting (at the doses applied in the aforementioned studies) consumption of NADPH by AR [24]. They also were reported to ameliorate lens NAD-redox imbalances, at least in short-term diabetes [23]. Thus, the SDIs provide a valuable tool for exploring some controversial questions related to biochemical mechanisms of diabetic complications. The present study was aimed at evaluating the role of sorbitol accumulation-linked osmotic stress versus "pseudohypoxia" in oxidative stress in diabetic precataractous rat lens, by assessing changes in markers of lipid peroxidation, levels of GSH and ascorbate, antioxidative defense enzymes, NADH oxidase activities, and NAD(P)-redox imbalances in response to SDH inhibition by a specific SDI.

## MATERIALS AND METHODS

The experiments were performed 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), body weight 250–300 g, were fed a standard rat chow diet (ICN Biomedicals) and had ad lib access to water. Diabetes was induced by a single injection of streptozotocin (Upjohn, 55 mg/kg body weight, i.p.). Blood samples for measurements of glucose were taken from the tail vein *ca.* 48 hr after streptozotocin injection and the day before the rats were killed. Rats with blood glucose $\geq$ 250 mg/dL were considered as diabetic. The experimental groups included control and 3-week diabetic rats treated with or without SDI-157 (Hoechst Marion Roussel, 100 mg/kg body weight/day, in the drinking water). The treatment of diabetic rats was started *ca.* 48 hr after streptozotocin injection.

### *Reagents*

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from the Sigma Chemical Co. Methanol (HPLC grade), perchloric acid, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific. Ethyl alcohol (200 proof dehydrated alcohol, U.S.P. punctilious) was purchased from the Quantum Chemical Co. Dihydroxyacetone phosphate dilithium salt monohydrate was purchased from Fluka BioChemika. β-D-Glucose, sorbitol, N.F., *myo*-inositol, C.P., and D-fructose, U.S.P., were purchased from Pfanstiehl Laboratories, Inc. Kits for MDA and 4-HA, and GSHPx assays were purchased from Oxis International.

## MATERIALS AND METHODS

Rats from each group were sedated with carbon dioxide and subsequently killed by cervical dislocation. Both lenses were dissected rapidly by posterior incision, carefully separated from any accompanying aqueous and vitreous humors by gentle rolling over a fine filter paper, and frozen in liquid nitrogen for subsequent biochemical analyses. One lens from each rat in the control, untreated diabetic, and SDI-treated diabetic groups was used for measurements of total MDA plus 4-HA, MDA, sorbitol pathway intermediates, and ascorbate levels, as well as activities of antioxidative defense enzymes [i.e. SOD, GSHTrans, GSSGRed, GSHPx] and NADH oxidase. The second lens was used for measurements of GSH, GSSG, G6P, F6P, DHAP, GP, lactate, pyruvate, and malate. The group of SDI-treated control rats was set up to determine whether SDI-157 had any prooxidant properties, and was used for measurements

of sorbitol pathway intermediates, total MDA plus 4-HA, MDA, GSH, GSSG, and ascorbate levels only.

### *Biochemical Measurements*

**(1) MEASUREMENTS OF SORBITOL PATHWAY INTERMEDIATES.** Lenses were weighed and homogenized in 1.5 mL of 0.1 M sodium phosphate buffer, pH 6.5. Then a 0.05-mL volume of 0.3 M zinc sulfate, followed by an equivalent of barium hydroxide, was added to 0.25 mL of the homogenate for protein precipitation. The samples were centrifuged at 4000 *g* for 10 min, and aliquots of the supernatant were taken for spectrofluorometric measurements of glucose, sorbitol, and fructose by enzymatic procedures as we described previously [15].

**(2) PREPARATION OF PERCHLORIC EXTRACT.** Lenses were weighed, homogenized in 1.5 mL of ice-cold 6% $HClO_4$, and centrifuged at 4000 *g* for 10 min. After centrifugation, the samples were neutralized immediately with 5 M $K_2CO_3$ to pH 6–7 and were centrifuged again at 4000 *g* for 5 min to precipitate insoluble $KClO_4$.

**(3) MEASUREMENTS OF GSH, GSSG, AND ASCORBATE.** For measurements of GSH, we used the spectrofluorometric procedure with *O*-phthaldialdehyde [25] in our modification [26]. GSSG levels were assayed spectrofluorometrically by an enzymatic procedure as we have described [15, 26]. Ascorbate levels were measured colorimetrically with 2,6-dichlorophenolindophenol [27] after extraction of 0.2 mL of homogenate from *step 1* with 5% metaphosphoric acid.

**(4) MEASUREMENTS OF TOTAL MDA PLUS 4-HA AND MDA LEVELS.** Measurements of total MDA plus 4-HA levels as well as MDA alone were performed using kits from Oxis International (LPO-586 assay). The method is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-HA at 45°. Homogenate (0.2 mL) from *step 1* was used for measurements of total MDA plus 4-HA levels, and another 0.2 mL was used for measurements of MDA levels according to the procedure described in detail in the kit. The absorbance of chromogenic products was measured at 586 nm (in a Beckman DU 640 spectrophotometer) and was compared with the absorbance of corresponding standards.

**(5) MEASUREMENTS OF ANTIOXIDATIVE DEFENSE ENZYME AND CYTOPLASMIC NADH OXIDASE ACTIVITIES.** For measurements of antioxidative defense enzyme and NADH oxidase activities, the rest of the homogenate from *step 1* was centrifuged at 20,000 *g*, and the supernatant fraction was used for assays of enzymatic activities and protein content. Protein levels were quantitated with Pierce bicinchoninic acid protein assay kits. *SOD activity* was measured by following spectrophotometrically (at 480 nm) the autoxidation of (-)-epinephrine at pH 10.4 [28]. *GSHTrans activity* towards 1-chloro-2,4-dinitrobenzene was measured spectrophotometrically (at 340 nm) by monitoring the increase in absorbance due to formation of GSH–CDNB conjugate [29]. *GSSGRed activity* was measured spectrophotometrically (at 340 nm), by monitoring NADPH oxidation coupled to the reduction of GSSG to GSH [30]. *GSH peroxidase activity* towards *t*-butyl hydroperoxide was measured using kits from Oxis International, according to the procedure described in detail in the kit. *Cytoplasmic NADH oxidase activity* was measured by the method of Askar and Baquer [31] in our modification. The oxidation of NADH was followed spectrofluorometrically (λ excitation: 340 nm; λ emission: 460 nm) in an incubation mixture containing 0.9 mL of 50 mM phosphate buffer, pH 7.0, 2 μM rotenone, 7 μM NADH, and 0.1 mL of supernatant.

**(6) MEASUREMENTS OF GLYCOLYTIC INTERMEDIATES, MALATE, AND GP.** Levels of G6P, F6P, DHAP, pyruvate, lactate, malate, and GP were assayed in neutralized perchloric extracts from *step 2*, spectrofluorometrically by enzymatic procedures as described by Lowry and Passonneau [32].

**(7) CALCULATIONS OF FREE CYTOSOLIC $NAD^+$/NADH AND $NADP^+$/NADPH RATIOS.** According to classical publications of Krebs' laboratory [33, 34] and other studies [35], direct measurements of NAD, NADH, NADP, and NADPH do not provide information on compartmentalization of nicotinamide adenine nucleotides between cytosol and mitochondria and do not separate free from protein-bound forms (only free forms determine direction and free-energy changes of dehydrogenase reactions). The same studies proposed an alternative approach for assessment of free $NAD(P)^+$/NAD(P)H ratios in the cytoplasm and mitochondria from ratios of the concentrations of oxidized and reduced metabolites of suitable NAD(P)-linked dehydrogenase systems. Using this approach, free cytosolic $NAD^+$/NADH and $NADP^+$/NADPH ratios were calculated from the metabolite concentrations and the equilibrium constants of the lactate dehydrogenase and malic enzyme systems as follows [33, 34]:

$$\frac{[NAD^+]}{[NADH]} = \frac{[Pyruvate]}{[Lactate]} \times \frac{1}{K_1},$$

where $K_1$ is the equilibrium constant of lactate dehydrogenase ($1.11 \times 10^{-4}$ M).

$$\frac{[NADP^{+}]}{[NADPH]} = \frac{[Pyruvate] \times [CO_2]}{[Malate]} \times \frac{1}{K_2},$$

where $K_2$ is the equilibrium constant of malic enzyme ($3.44 \times 10^{-2}$ M) (the $CO_2$ concentration was taken to be 1.16 mM).

**(8) STATISTICAL ANALYSIS.** The results are expressed as means ± SD. Differences among experimental groups were evaluated by ANOVA, and the significance of differences

**TABLE 1. Lens glucose, sorbitol, and fructose in control and diabetic rats treated with or without SDI**

| | Glucose | Sorbitol (μmol/g wet weight) | Fructose |
|---|---|---|---|
| Control | 1.29 ± 0.159 | 0.758 ± 0.222 | 1.33 ± 0.315 |
| Control + SDI | 0.728 ± 0.105 | 6.7 ± 1.6* | 0.397 ± 0.076† |
| Diabetic | 11.5 ± 1.5* | 21.2 ± 3.5* | 8.6 ± 2.1* |
| Diabetic + SDI | 10.9 ± 0.7* | 38.2 ± 6.8*† | 2.6 ± 0.7†‡ |

Values are means ± SD; N = 7–18.
*,† Significantly different from controls: *$P < 0.01$, and †$p < 0.05$.
‡ Significantly different from untreated diabetic ($P < 0.01$).

between these groups was assessed by the Student–Newman–Keuls multiple range test. Significance was defined at $P < 0.05$.

## RESULTS

The final body weights were lower in diabetic rats than in controls (313.6 ± 25.7 vs 384.5 ± 24.3 g, $P < 0.05$). The initial body weights were similar in control and diabetic groups. SDI treatment did not affect weight gain in either control or diabetic rats, and the final body weights were similar in SDI-treated (360.4 ± 28.9 g) and untreated control rats, as well as in SDI-treated (308.5 ± 32.6 g) and untreated diabetic rats.

Blood glucose levels were increased markedly in diabetic rats compared with those in controls (334.1 ± 62.3 vs 74.8 ± 11.9 mg/dL, $P < 0.01$). Blood glucose levels in either control or diabetic rats were not affected by SDI treatment (77.5 ± 13.2 and 348.5 ± 87.0 mg/dL, respectively).

Lens glucose, sorbitol, and fructose levels in control and diabetic rats treated with or without SDI are presented in Table 1. Glucose levels were similar in control rats treated with or without SDI. Sorbitol levels were 8.8-fold higher and fructose levels 3.4-fold lower in SDI-treated versus untreated control rats. Glucose, sorbitol, and fructose levels in diabetic rats were increased 8.9-, 28.0-, and 6.5-fold, respectively, compared with those in controls. Glucose levels were similar in diabetic rats treated with or without SDI. Sorbitol levels in the SDI-treated diabetic rats were increased 1.8-fold compared with those in untreated diabetic rats, while fructose levels were reduced markedly but not normalized completely.

Total MDA plus 4-HA as well as MDA levels (Fig. 1A and B) were similar in SDI-treated and untreated control rats. Both total MDA plus 4-HA and MDA levels were increased in diabetic rats (2- and 2.7-fold vs controls, $P < 0.01$ and $P < 0.05$, respectively). The increase in both parameters in diabetic rats markedly progressed with SDI treatment. Total MDA plus 4-HA and MDA levels were 1.8- and 2.1-fold higher in the SDI-treated diabetic group than in untreated diabetic rats, and 3.5- and 6-fold higher than in controls ($P < 0.01$ versus both untreated diabetic and control rats for either parameter).

Lens GSH levels were similar in SDI-treated and untreated control rats. GSH levels were decreased markedly (4.6-fold) in diabetic rats versus controls and were lowered further by SDI (2.8-fold vs untreated diabetic rats and 12.9-fold vs controls, Table 2). Lens GSSG levels were similar in the control, SDI-treated control, and diabetic groups. The GSSG levels in diabetic rats tended to decrease with SDI treatment, but the difference from levels in untreated diabetic rats did not achieve statistical significance. Lens GSSG/GSH ratios were similar in SDI-treated

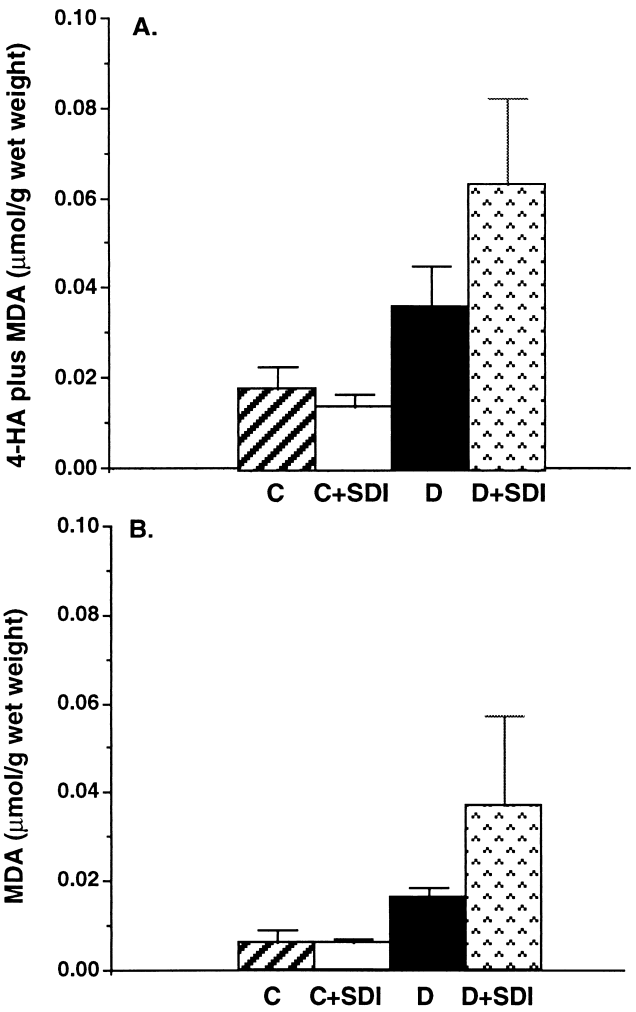


**FIG. 1. Lens total MDA plus 4-HA (A) and MDA (B) levels in control and diabetic rats treated with or without SDI (means ± SD, N = 7–18).**

**TABLE 2. Levels of non-enzymatic antioxidants and GSSG/GSH ratios in control and diabetic rats treated with or without SDI**

| | GSH (μmol/g wet wt) | GSSG (μmol/g wet wt) | GSSG/GSH | Ascorbate (μmol/g wet wt) |
|---|---|---|---|---|
| Control | 4.77 ± 0.54 | 0.161 ± 0.032 | 0.034 ± 0.007 | 0.190 ± 0.046 |
| Control + SDI | 4.86 ± 0.45 | 0.202 ± 0.036 | 0.041 ± 0.006 | 0.202 ± 0.023 |
| Diabetic | 1.04 ± 0.42* | 0.161 ± 0.047 | 0.202 ± 0.117* | 0.135 ± 0.025* |
| Diabetic + SDI | 0.37 ± 0.10*† | 0.123 ± 0.034‡ | 0.380 ± 0.220*§ | 0.075 ± 0.022*§ |

Values are means ± SD, N = 7–19.
* Significantly different from controls, $P < 0.01$.
† Significantly different from untreated diabetic rats, $P < 0.05$.
‡ Significantly different from controls, $P < 0.05$.
§ Significantly different from untreated diabetic rats, $P < 0.01$.

and untreated control rats. The GSSG/GSH ratio was increased 5.9-fold in diabetic rats vs controls and was increased further by SDI treatment (1.9-fold vs untreated diabetic rats and 11.2-fold vs controls). Lens ascorbate levels were decreased 1.4-fold in diabetic rats vs controls and were reduced further by SDI treatment (1.8-fold vs untreated diabetic rats and 2.6-fold vs controls). No difference in ascorbate levels was found between SDI-treated and untreated control rats.

G6P levels were 1.8-fold higher in diabetic rats vs controls, and were unaffected by SDI treatment (Table 3). F6P levels tended to increase in diabetic rats treated with or without SDI, but the difference from control levels did not achieve statistical significance for either group. DHAP levels were 1.5-fold higher in diabetic rats versus controls and were reduced by SDI treatment to levels that were not significantly different from those in either control or untreated diabetic rats. Pyruvate levels were decreased in diabetic rats versus controls and were unaffected by SDI treatment. Lactate levels were similar in control and diabetic rats and were lower in SDI-treated diabetic rats compared with untreated diabetic rats. Malate levels were decreased 2.1-fold in diabetic rats versus controls, and no difference was found between SDI-treated and untreated diabetic groups. GP levels were increased dramatically (7-fold) in diabetic rats versus controls. This increase was ameliorated but not prevented completely by SDI treatment.

Free cytosolic $NAD^+$/NADH ratios were lower in diabetic rats versus controls ($P < 0.05$), and this decrease was ameliorated but not prevented completely by SDI treatment (Fig. 2A). Free cytosolic $NADP^+$/NADPH ratios were elevated in diabetic rats treated with or without SDI versus controls ($P < 0.05$ for both comparisons) and were unaffected by SDI treatment (Fig. 2B).

SOD and GSSGRed activities tended to increase in the diabetic group, but the difference from those in controls did not achieve statistical significance (Table 4). SOD was up-regulated further by SDI treatment (2.4-fold vs untreated diabetic rats and 5.6-fold vs controls). GSSGRed was up-regulated further as well, although to a lesser extent (1.9-fold vs controls; the difference from untreated diabetic rats did not achieve statistical significance). GSHTrans activity was 1.6-fold higher in diabetic rats versus controls and was up-regulated further by SDI treatment (1.6-fold vs untreated diabetic rats and 2.6-fold vs controls). GSHPx activity was 2.2-fold higher in diabetic rats versus controls and remained unaffected by SDI treatment. NADH oxidase activity was 1.8-fold higher in diabetic rats versus controls and was up-regulated further by SDI treatment (1.6-fold vs untreated diabetic rats and 2.9-fold vs controls).

## DISCUSSION

Comparison of total lipid aldehyde (MDA plus 4-HA) and MDA levels indicates that both in control and diabetic rat lenses, levels of MDA were quite similar to those of α,β-unsaturated lipid aldehydes 4-HA, the most toxic products of lipid peroxidation [36–40]. Exacerbation of diabetes-induced accumulation of MDA and 4-HA with SDI treatment is indicative of enhanced oxidative stress, and can be invoked as a factor contributing to SDI-induced acceleration of diabetes-associated cataractogenesis [21, 24]. The effect of SDI on MDA and 4-HA in the diabetic

**TABLE 3. Levels of glycolytic intermediates, malate, and GP (μmol/g wet weight) in lens in control and diabetic rats treated with or without SDI**

| | G6P | F6P | DHAP | Pyruvate | Lactate | Malate | GP | DHAP/GP |
|---|---|---|---|---|---|---|---|---|
| Control | 0.127 ± 0.012 | 0.058 ± 0.013 | 0.044 ± 0.016 | 0.123 ± 0.021 | 7.73 ± 0.73 | 0.106 ± 0.015 | 0.234 ± 0.026 | 0.195 ± 0.084 |
| Diabetic | 0.225 ± 0.030* | 0.078 ± 0.028 | 0.066 ± 0.019† | 0.080 ± 0.009* | 8.19 ± 0.48 | 0.051 ± 0.013* | 1.643 ± 0.438* | 0.041 ± 0.010* |
| Diabetic + SDI | 0.218 ± 0.017* | 0.076 ± 0.023 | 0.049 ± 0.014 | 0.094 ± 0.008* | 7.12 ± 0.58‡ | 0.053 ± 0.010* | 0.445 ± 0.071‡ | 0.106 ± 0.032*§ |

Values are means ± SD, N = 7–9.
*† Significantly different from controls: *$P < 0.01$, and †$P < 0.05$.
‡§ Significantly different from untreated diabetic groups: ‡$P < 0.01$, and §$P < 0.05$.

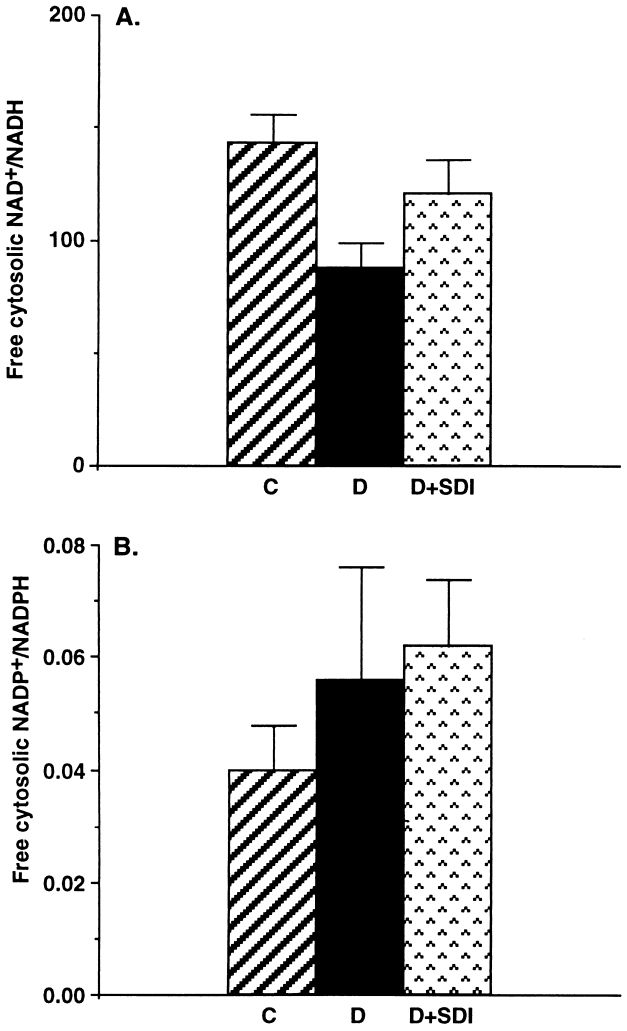


**FIG. 2. Lens $NAD^+$/NADH (A) and $NADP^+$/NADPH (B) ratios in control and diabetic rats treated with or without SDI (means ± SD, N = 7–9).**

lens cannot be attributed to intrinsic prooxidant properties of SDI-157, as the studies in control animals revealed the absence of any effect of the compound on lipid aldehyde levels. Therefore, increased lipid peroxidation in SDI-treated diabetic rats resulted from inhibition of lenticular SDH.

One of the most important metabolic consequences of SDH inhibition in the diabetic lens is a dramatic increase of sorbitol accumulation and a severe potentiation of intralenticular osmotic stress [21–24, and present study]. Sorbitol accumulation parallels depletion of GSH, the major biological antioxidant, which neutralizes reactive oxygen species initiating lipid peroxidation. Besides, GSH plays a major role in the metabolism of 4-HA [41]. As it was demonstrated by Lou *et al.* [4], the diabetes-induced loss of GSH could result at least partially from an impaired ability of the lens to concentrate amino acids required for GSH biosynthesis, coupled to a faster GSH efflux under hyperosmotic conditions. These findings are in agreement with reduced [$^{35}$S]cysteine uptake into the hyperosmotic lens [42]. In addition, it has been revealed [42] that osmotic stress can affect lens GSH levels indirectly, via a decrease in hepatic cysteine uptake and GSH biosynthesis, with concomitant reduction in plasma (and aqueous humor) GSH concentrations and down-regulation of $Na^+$-dependent GSH transporter-mediated [43] GSH uptake by lens epithelial cells. The osmotic origin of diabetes-induced GSH depletion is supported by a further dramatic decrease in lens GSH levels in response to potentiation of osmotic stress with SDH inhibition, found both by Geisen *et al.* [21] and in the present study. The observations of Kador *et al.* [24], demonstrating a negligible effect of SDI-157 on lens AR activity and, therefore, NADPH consumption (consistent with the unaffected free cytosolic $NADP^+$/NADPH ratio in the present study), exclude a potential contribution of AR-mediated NADPH deficiency and failure of the glutathione redox cycle to the mechanisms responsible for exacerbation of lens GSH depletion with SDI treatment. Therefore, sorbitol accumulation-linked osmotic stress *per se* induces GSH depletion in the diabetic lens.

Similarly to GSH, lens ascorbate levels were decreased in diabetic rats versus controls and were depleted further by SDI treatment. The mechanisms underlying ascorbate depletion in the diabetic lens remain unclear. It is known that GSH and other cellular thiols (such as dihydrolipoic acid, the reduced form of α-lipoate) play an important role in vitamin C homeostasis by regenerating ascorbate from dehydroascorbate and semiascorbyl radicals [44–46] and that lens ascorbate levels are reduced by L-buthionine(*S,R*)-sulfoximine, an inhibitor of glutathione biosynthesis [44]. Thus, both diabetes-induced decrease in lens ascorbate levels and further depletion of ascorbate with SDH inhibition could either be direct consequences of osmotic compensation under conditions of severe intralenticular sorbi-

**TABLE 4. Activities of antioxidative defense enzymes and HADH oxidase in control and diabetic rats treated with or without SDI**

| | SOD | GSSGRed | GSHTrans (nmol/mg protein per min) | GSHPx | NADH oxidase |
|---|---|---|---|---|---|
| Control | 55 ± 33 | 0.180 ± 0.097 | 1.03 ± 0.31 | 2.91 ± 0.73 | 4.93 ± 0.95 |
| Diabetic | 130 ± 46 | 0.248 ± 0.094 | 1.63 ± 0.43* | 6.31 ± 2.94* | 9.05 ± 2.42† |
| Diabetic + SDI | 307 ± 122*‡ | 0.336 ± 0.032† | 2.67 ± 0.36*‡ | 5.48 ± 1.71* | 14.35 ± 5.55*‡ |

Values are mean ± SD, N = 5–8.

*† Significantly different from controls: *$P < 0.01$, and †$P < 0.05$.

‡ Significantly different from untreated diabetic rats, $P < 0.01$.

tol accumulation, or secondary phenomena occurring due to depletion of GSH.

The activities of antioxidative defense enzymes measured in the present study were increased by diabetes (consistent with the results of Khanna *et al.* [47]), and all of them, with the exception of GSHPx, were up-regulated further by SDI. Up-regulation of NADH oxidase (a superoxide-generating enzyme) in untreated and SDI-treated diabetic rats probably is offset by a comparable activation of SOD. Therefore, depletion of major nonenzymatic antioxidants rather than compromised defense by antioxidative enzymes is the central event underlying enhanced oxidative stress in the diabetic lens and its further exacerbation by SDH inhibition. With this in mind, we have performed additional studies to estimate whether mechanisms other than osmotic stress could be implicated in SDI-induced loss of nonenzymatic antioxidants by the diabetic lens.

Studies in SDI-treated control rats did not reveal any difference from the untreated control group in lens GSH and ascorbate levels. Therefore, the SDI-induced depletion of both nonenzymatic antioxidants in the diabetic rats did not result from unidentified effects of SDI-157 on GSH biosynthesis or metabolic disposition of GSH and ascorbate.

It has been suggested* that the progressive depletion of a number of cell constituents (*myo*-inositol [23], GSH [21, 23, and the present study], GSSG [21], ADP [23], and AMP [23]) in the lenses of SDI-treated diabetic rats results from the disruption of lenticular cytoplasmic membranes rather than reflects complex osmotically-mediated phenomena affecting membrane transporters and ion homeostasis [48–50]. Such an assumption, however, is not supported by selective loss of the aforementioned metabolites with preservation of others. Besides, it does not agree with the findings of Kador *et al.* [24], who observed vacuole formation but not cataractous changes, associated with lens leakiness [2, 51], in SDI-157-treated diabetic rats 21 days after the beginning of SDI treatment, in spite of the use of younger (50 g) rats, which, according to Sasaki *et al.* [52], are more prone to sugar cataract formation than the mature (250–300 g) rats used in our study. Furthermore, the assumption regarding the loss of lenticular membrane integrity is not supported by our evaluation of G6P and F6P levels in SDI-treated versus untreated diabetic rats. Both metabolites, like other phosphorylated glycolytic intermediates, do not cross the intact cytoplasmic membrane. Moreover, neither G6P nor F6P is affected by osmotic stress, NAD(P)-redox imbalances, or relatively mild oxidative stress in diabetes [3, 15, 23, 53], and for these reasons, similar levels of the two metabolites in SDI-treated and untreated diabetic rats can be regarded as evidence of an absence of disruption of lenticular cytoplasmic membranes in spite of severe potentiation of intralenticular osmotic stress by SDH inhibition.

Several studies also suggest that diabetes-induced loss of GSH occurs due to glycation of GSSGRed [54, 55] or enzymes of GSH biosynthesis [56]; others, however, did not find any amelioration of GSH depletion by treatment with aminoguanidine, an inhibitor of nonenzymatic glycation [57]. In any case, the glycation mechanism is not involved in exacerbation of diabetes-induced GSH and ascorbate depletion with SDI treatment, for the reason that lens glucose levels were similar in untreated and SDI-treated diabetic rats, whereas lens fructose levels were 3.3-fold lower in the SDI-treated group. From the depletion of fructose (and, theoretically, products of its metabolism, fructose 3-phosphate and 3-deoxyglucosone) one would expect glycation to be decreased rather than increased in the SDI-treated group versus untreated diabetic rats. Nevertheless, lens antioxidant loss was more profound in the SDI-treated diabetic group and thus did not correlate with intensity of nonenzymatic glycation.

The metabolic consequences of SDH inhibition include amelioration of diabetes-induced lens NAD-redox imbalances, manifested by increased (versus the untreated diabetic group) free cytosolic $NAD^+$/NADH and DHAP/GP ratios. SDH activity was implicated in diabetes- and hyperglycemia-induced lens NAD-redox imbalances in 1985–1988 by McLean *et al.* and Hothersall *et al.* [58, 59] and by Cheng *et al.* [60]. In 1993, the concept was extrapolated to other targets for diabetic complications by Williamson *et al.* [20], who hypothesized that the SDH-mediated NAD-redox imbalances constitute a universal mechanism responsible for the development of diabetic complications, whereas other AR-mediated mechanisms, and in particular sorbitol accumulation *per se* and the resulting osmotic stress, are unimportant. In particular, the concept [20] implies that diabetes-induced oxidative stress would be ameliorated by SDH inhibition due to correction of NAD-redox imbalances and in spite of increase in sorbitol accumulation and potentiation of osmotic stress. The present study, demonstrating that the amelioration of diabetes-induced lens redox imbalances by SDI-157 treatment was associated with exacerbation instead of prevention of oxidative injury, indicated that the relative importance of osmotic versus NAD-redox-mediated mechanisms in oxidative stress in diabetic precataractous rat lens was quite the opposite of that implied by the "pseudohypoxia" premise. Diabetes-induced changes of all the parameters of oxidative stress and antioxidative defense (except GSHPx) assessed in our experiments progressed under conditions of potentiation of osmotic stress with SDH inhibition. Even NADH oxidase (which, theoretically, was thought to be controlled directly by the $NAD^+$/NADH ratio [61]) appeared to be further activated in SDI-treated rats, thus indicating that the regulation of this enzyme under diabetic conditions is not understood properly.

Theoretically, diabetes-induced NAD-redox imbalances should favor oxidative stress through several mechanisms

* Discussion at the Session on Ocular Complications, Annual Meeting of the European Association for the Study of Diabetes, Vienna, Austria, 1996.

including accumulation of triose phosphates (DHAP and GP), with their subsequent autoxidation and formation of methylglyoxal [62], which generates free radicals and impairs antioxidative defense [63]. It appears, however, that the importance of this mechanism in diabetic precataractous rat lens is fairly minor. The findings of Lee and Chung [12] demonstrating less severe oxidative stress (as reflected by both lens GSH and MDA levels) in SDH-deficient ($AR^{+/-}SD^{+/-}$) diabetic mice [versus diabetic mice with normal levels of SDH ($AR^{+/-}SD^{+/+}$)] imply that the NAD-redox mechanism could be more important for development of oxidative stress in other animal models. The question of which (adverse or beneficial) effects of SDH inhibition on oxidative stress prevail in the human diabetic lens will probably remain open, as based on the findings of an acceleration of diabetic cataract formation with SDH inhibition in both rat [21, 24] and mouse [12, 64] models, contradictory information on the effects of SDI on peripheral diabetic neuropathy [21, 22, 65], and a striking worsening of the parameters of autonomic neuropathy [66], one could hardly expect that this class of compounds will ever undergo a clinical trial for treatment of chronic diabetic complications. The role for SDH in oxidative stress, however, could be assessed in human lens epithelial cells cultured in SDI-containing high glucose medium or transfected with the SDH gene. Meanwhile, it is known that in spite of a rather low AR activity in the human lens [67], osmotically induced hydration precedes diabetic cataract formation [68], and that human lens epithelial cells respond to polyol accumulation and osmotic stress by the loss of nonenzymatic antioxidants [69], i.e. in a similar fashion as the diabetic hyperosmotic rat lens.

In conclusion, SDH inhibition in the diabetic precataractous lens results in severe osmotic stress linked to sorbitol accumulation followed by a dramatic loss of major nonenzymatic antioxidants, GSH and ascorbate. The latter leads to exacerbation of lipid peroxidation, which occurs in spite of amelioration of lens redox imbalances and compensatory activation of a number of antioxidative defense enzymes. The potentiation of oxidative stress by SDH inhibition is not due to intrinsic prooxidant properties of SDI-157, and occurs without intervention of glycation or glutathione redox cycling mechanisms. Sorbitol accumulation *per se* generates oxidative stress in the diabetic precataractous lens.

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