The Activity of Aldose Reductase Is Elevated in Diabetic Mouse Heart

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Abstract. The importance of aldose reductase (AR) has been implicated in the pathogenesis of diabetic complications, although the alterations in the expression and activity of AR during hyperglycemia in the heart have not been well characterized. We investigated the expression and enzyme activity of AR in a murine diabetic model. Three weeks after the induction of hyperglycemia with streptozotocin, the level of AR mRNA was significantly reduced in the cardiac ventricles of BDF-1 mice. In contrast, the activity of AR was significantly elevated in the heart without any significant change in the protein level. In these mice, the level of cardiac thiobarbituric acid-reactive substances was unaltered, whereas the level of reduced glutathione (GSH) was significantly increased. Daily administration of insulin for 3 weeks completely normalized the level of AR mRNA and the enzyme activity. On the other hand, daily administration of an antioxidant, N-acetylcysteine significantly reduced the level of AR mRNA in the heart with a concomitant elevation in the enzyme activity. These results suggest that the activity of AR in the heart is affected by GSH dynamics. Augmented AR activity at the early stage of hyperglycemia may perturb glycolysis and affect cardiac performance.

Keywords: aldose reductase, diabetes, glutathione, heart, oxidative stress

Introduction

Cardiovascular complications are the leading cause of mortality in diabetic patients. Although the coronary artery is the major site affected in diabetes, impaired ventricular performance without coronary artery disease has been reported in experimental diabetic animals as well as in diabetic patients (1–4). In such functional impairment, the involvement of metabolic perturbation of myocytes has been suggested (5).

Aldose reductase (AR; EC 1.1.1.21) has been implicated in the pathogenesis of various diabetic complications (6). The enzyme catalyzes the reduction of various aldehydes, including the aldehyde form of glucose, using NADPH as a cofactor. AR converts glucose to sorbitol, which is next converted to fructose by sorbitol dehydrogenase (SDH) using the cofactor NAD⁺. This is the so-called polyol pathway, an alternate route of glucose metabolism. Under hyperglycemic conditions, acceleration of the polyol pathway leads to accumulation of sorbitol and osmotic disturbances in the lens (7). Fructose produced by the enhanced flux through the polyol pathway promotes non-enzymatic glycation (8). It is also proposed that the polyol pathway may elicit a decreased NAD⁺/NADH ratio, the condition illustrated as pseudohypoxia (9–11). Such metabolic perturbation is postulated to provoke early tissue damage in the ocular lens, retina, peripheral nerve, and the renal glomerulus, where insulin-independent uptake of glucose takes place.

In the heart, however, the involvement of AR in diabetic cardiac complications is controversial. AR catalyzes the reduction of such reactive aldehydes as 4-hydroxynonenal (HNE) and acrolein produced by oxidative damage to unsaturated fatty acids. During myocardial ischemia-reperfusion, the formation of HNE and accumulation of HNE-modified proteins result in tissue damage (12, 13). AR was thus suggested to act as a mediator of late-phase ischemic preconditioning by decreasing the accumulation of HNE (14). On the other hand, AR was reported to exacerbate ischemia-reperfusion injury of the heart. Inhibitors of AR mitigated ischemia-reperfusion injury and improved the
levels of intracellular sodium and calcium perturbed during ischemia (15–18). These observations suggest that AR plays opposing roles in the ischemic heart. Since the alteration of cardiac AR activity by hyperglycemia has not been well characterized, we set out to examine the expression and enzyme activity of AR in streptozotocin-induced diabetic mice. We report here increased AR activity at the early stage of hyperglycemia with concomitant down-regulation of cardiac AR gene expression. The increased enzyme activity observed in the heart suggests that AR protein is susceptible to GSH dynamics.

Materials and Methods

Animals

Seven-week-old BDF-1 male mice were maintained on a 12-h light/dark cycle and fed ad libitum. Hyperglycemia was induced by a single administration of streptozotocin (STZ, 200 mg/kg body weight) in the tail vein. The onset of hyperglycemia was verified 3 days after the injection of STZ by measuring the serum glucose level by the mutarotase glucose oxidase method. Insulin (INS, human insulin zinc suspension; Novartis Pharma K.K., Tokyo; 0.2 U/day, s.c.) or N-acetylcysteine (NAC; 500 mg/kg per day, i.p.) was then administered for 3 weeks. For administration of NAC, injection was started 3 days after the induction of hyperglycemia. The experimental procedure was approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Northern blot analysis

Total RNA was extracted from the whole heart or ventricles using the ISOGEN RNA extraction reagent (Nippon gene Co., Ltd., Tokyo) according to the manufacturer’s protocol. Twenty micrograms of total RNA were separated by electrophoresis on a 0.8% agarose/formamide gel and transferred onto a nylon membrane in 20× sodium chloride/sodium citrate (pH 7.0). The membrane was hybridized with [32P]-labeled DNA probes. The expression level of AR mRNA was quantified using a BAS2000 Bioimage Analyzer and normalized by the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and acidic ribosomal phosphoprotein PO (36B4) as internal controls.

Western blot analysis

Cardiac tissue was homogenized in 5 mM phosphate-buffered saline containing 10 mM 2-mercaptoethanol. After centrifugation of the homogenate for 40 min at 8,000×g, the supernatant fraction was used for Western blot analysis and the AR activity assay. Ten micrograms of protein in the supernatant fraction of the heart homogenate were subjected to SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-rat AR antibody (19) (a gift from Dr. N. Iwata, RIKEN, Wako) and then with alkaline phosphatase–conjugated goat anti-rabbit IgG antibody. Immunoreactive bands were detected with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chloride developing reagent (Invitrogen Corp., Carlsbad, CA, USA). The AR protein level was quantified using the NIH Image program.

Enzyme assay

AR activity was measured as described previously (20). Briefly, the reaction mixture consisted of 0.1 M phosphate buffer (pH 6.2), 75 µM NADPH, 10 mM DL-glyceraldehyde, and 40 µg of protein in a total volume of 100 µl. The assay was started by addition of an enzyme solution and carried out at 25°C. The substrate replaced by deionized water was used as a blank. The enzyme activity was estimated spectrophotometrically by calculating NADPH oxidation from the decrease in absorbance at 340 nm for 3 min. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol/min NADPH under the present assay conditions. In assays with heart samples, the reducing activity toward DL-glyceraldehyde was almost completely abolished in the presence of SNK-860, a specific inhibitor of AR. Thus, the activity demonstrated in the assay mostly reflects AR activity. Protein concentration in the supernatant fraction of cardiac tissue was measured by the method of Bradford using bovine serum albumin as a standard.

Measurement of TBARS and glutathione levels

Cardiac tissue was homogenized (10% wt/vol) in 1.15% KCl solution (pH 7.4) and thiobarbituric acid-reactive substances (TBARS) in the homogenate were measured according to the previously described method (21).

A Bioxytech GSH/GSSG-412 kit from Oxis Health Products was used to determine reduced and oxidized glutathione (GSH and GSSG). Cardiac tissue was homogenized (10% wt/vol) in 5% metaphosphoric acid with or without 33 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate, a scavenger of GSH. Total GSH and GSSG in the homogenate were determined according to the previously described method (22).

Statistical analyses

All values were expressed as means ± S.E.M. Group comparisons were analyzed by one-way analysis of
variance (ANOVA; KaleidaGraph 3.6 version 3.00; Synergy Software). All groups were analyzed simultaneously with Dunnett’s test. A difference with $P < 0.05$ was considered significant.

Results

Decreased expression of AR mRNA in the heart under hyperglycemia

The levels of serum glucose in STZ-treated mice were significantly elevated compared with those in the control group at 5 days as well as 3 weeks after the injection (Table 1). The relatively high level of serum glucose demonstrated in the control group may be due to the non-fasted condition at the time of measurement, and the strain of mice used in the study. BDF-1 is known to show higher blood glucose level compared to other strains. As shown in Fig. 1A, the expression of the AR transcript, detected as a 1.4-kb band, was significantly attenuated in STZ-treated mice at the same time. Normalized levels of AR mRNA are shown in Fig. 1B. Since hyperglycemia is known to alter the expression of GAPDH mRNA in some tissues or cell-lines (23, 24), we normalized them with the levels of other housekeeping genes to confirm the findings. Similar results were obtained when the level of AR mRNA was normalized by that of $\beta$-actin or 36B4. Since impaired ventricular performance was reported in diabetic patients (1, 2), we further isolated the left and right ventricles and examined the expression of AR mRNA. The levels of AR transcript in diabetic mouse ventricles were markedly reduced to approximately one-half of the control levels (Fig. 2A).

We next examined the effect of insulin administration on the AR gene expression in the heart of normal and STZ-treated mice. Insulin treatment significantly restored the serum glucose level and body weight of STZ-treated mice (Table 2). As shown in Fig. 2A, the decrease in AR mRNA expression in the cardiac ventricles of diabetic mice was abolished by insulin administration. Insulin alone did not affect the AR expression in normal mouse ventricles. These results indicated that the decrease in the AR mRNA level in STZ-treated mice was highly correlated to the deficiency in insulin and increased blood glucose level.

Increased AR activity in the cardiac ventricles of hyperglycemic mice

To examine whether the change in AR mRNA expression affects the level of AR protein, immunoblot
analyses were performed. While the level of AR protein tended to decline in the cardiac ventricles of STZ-treated mice, there was no significant difference compared with that of normal mice. Administration of insulin did not affect the level of AR protein in these mice (Fig. 2B).

In contrast, the overall activity of AR in the cardiac ventricle (units/mg protein) was significantly elevated in diabetic mice (Fig. 2C). The increase in AR activity

![Image of Western blot analysis](image-url)

**Fig. 2.** Effects of hyperglycemia on expression and activity of AR. A: Effects of daily administration of insulin (INS) on AR gene expression in the cardiac ventricles of STZ-induced diabetic mice. Mice were sacrificed 3 weeks after the STZ injection. The AR/GAPDH ratio is expressed relative to that of the control. The values each represent a mean ± S.E.M. (n = 12). *P<0.05, **P<0.05. B: AR protein in the ventricles of STZ-induced diabetic mice. Ten micrograms of soluble protein were subjected to Western blot analysis. The values each represent a mean ± S.E.M., expressed as percent of the control (n = 6). C: Increased AR activity in the ventricles of STZ-induced diabetic mice. The values each represent a mean ± S.E.M., expressed as percent of the control (n = 12). The enzyme activity in the control group was 2.47 ± 0.11 munits/mg protein. *P<0.05, **P<0.05.

**Table 2.** Effects of insulin (INS) on body weight, heart weight, and serum glucose in STZ-induced diabetic mice (3 weeks)

<table>
<thead>
<tr>
<th></th>
<th>Body wt (g)</th>
<th>Heart wt (mg)</th>
<th>Heart wt × 10³ /Body wt</th>
<th>Serum glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.8 ± 0.6</td>
<td>140.8 ± 5.2</td>
<td>5.46 ± 0.17</td>
<td>237.6 ± 10.7</td>
</tr>
<tr>
<td>STZ</td>
<td>21.8 ± 0.9*</td>
<td>111.8 ± 7.3*</td>
<td>5.09 ± 0.24</td>
<td>745.2 ± 49.4*</td>
</tr>
<tr>
<td>INS</td>
<td>27.0 ± 0.5</td>
<td>132.0 ± 6.5</td>
<td>4.91 ± 0.25</td>
<td>263.1 ± 9.7</td>
</tr>
<tr>
<td>STZ + INS</td>
<td>24.2 ± 0.4*</td>
<td>122.9 ± 5.1</td>
<td>5.07 ± 0.17</td>
<td>476.3 ± 46.1*</td>
</tr>
</tbody>
</table>

The values each represent a mean ± S.E.M. (n = 12). *P<0.05 vs Control and **P<0.05 vs STZ.
was almost completely suppressed by insulin administration, suggesting that the alteration in AR activity was associated with the blood glucose level. Since AR activity was elevated despite no significant change in its protein level in STZ-treated mice, it was assumed that post-translational modification of AR protein might be involved in this process.

Increased GSH level in the cardiac ventricles of hyperglycemic mice

Reactive oxygen species have been documented to alter the expression of AR (25, 26) while AR possesses a redox-sensitive cysteine residue that modulates the enzymatic activity (27). Alterations in the levels of GSH, GSSG, and TBARS are thus determined to assess the extent of cellular oxidative stress in the cardiac tissue of diabetic mice. As shown in Fig. 3A, a significant increase in GSH was observed in the ventricles of mice under hyperglycemia for 3 weeks. The level of GSSG, the GSH/GSSG ratio, and the level of TBARS remained unchanged at this time point (Fig. 3: B – D). On the other hand, increased GSH level in the diabetic ventricle was restored by daily treatment with insulin. Although it is generally recognized that hyperglycemia causes oxidative stress (28), the cellular defense system against oxidative stress may be augmented to maintain the redox state in the myocyte at the early stage of STZ-induced hyperglycemia.

Decreased mRNA level and increased activity of AR in NAC-treated mouse heart

AR protein has been shown to be sensitive to oxidants due to the presence of an active site cysteine residue (Cys-298) (29, 30). To examine whether AR activity is modulated by GSH levels, we treated mice with NAC, a precursor of GSH, and examined the expression and

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**Fig. 3.** Effects of daily administration of insulin (INS) or N-acetylcysteine (NAC) on the levels of GSH (A), GSSG (B), the GSH/GSSG ratio (C), and TBARS (D) in the cardiac ventricles of STZ-induced diabetic mice. Mice were sacrificed 3 weeks after the STZ injection. The values each represent a mean ± S.E.M. (n = 6). *P<0.05, #P<0.05.
activity of AR in the cardiac tissue.

Administration of NAC did not affect the serum glucose levels or body weight of control or diabetic mice (Table 3). On the other hand, a significant increase in the GSH/GSSG ratio along with a decrease in TBARS was demonstrated in diabetic mice treated with NAC (Fig. 3: C and D). Expression of AR mRNA was significantly attenuated not only in diabetic mice, but also in control mice treated with NAC (Fig. 4A). AR protein levels were unaltered in these mice, whereas the activity of AR

Table 3. Effects of N-acetylcysteine (NAC) on body weight, heart weight, and serum glucose in STZ-induced diabetic mice (3 weeks)

<table>
<thead>
<tr>
<th></th>
<th>Body wt (g)</th>
<th>Heart wt (mg)</th>
<th>Heart wt /Body wt × 10^3</th>
<th>Serum glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.2 ± 1.1</td>
<td>146.5 ± 7.5</td>
<td>5.62 ± 0.24</td>
<td>247.2 ± 17.6</td>
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<tr>
<td>NAC</td>
<td>26.6 ± 0.8</td>
<td>138.2 ± 3.2</td>
<td>5.21 ± 0.11</td>
<td>260.4 ± 6.5</td>
</tr>
<tr>
<td>STZ</td>
<td>23.8 ± 0.9</td>
<td>122.3 ± 7.2*</td>
<td>5.12 ± 0.20</td>
<td>767.4 ± 106.6*</td>
</tr>
<tr>
<td>STZ + NAC</td>
<td>23.2 ± 1.2</td>
<td>118.6 ± 6.4</td>
<td>5.12 ± 0.13</td>
<td>791.9 ± 36.5</td>
</tr>
</tbody>
</table>

The values each represent a mean ± S.E.M. (n = 6). *P<0.05 vs Control.

Fig. 4. Effects of N-acetylcysteine (NAC) on expression and activity of AR under hyperglycemia. A: Decreased expression of AR mRNA in the cardiac ventricles of NAC-treated mice. Mice were sacrificed 3 weeks after the STZ injection. B: AR protein in the ventricles of NAC-treated mice. C: Increased AR activity in the ventricles of NAC-treated mice. The values each represent a mean ± S.E.M., expressed as a percent of the control (n = 6). The enzyme activity in the control group was 2.96 ± 0.17 munits/mg protein. *P<0.05, #P<0.05.
was markedly augmented by NAC treatment (Fig. 4: B and C). Compared to the increase in AR activity observed in diabetic mice, the increase in the enzyme activity was much greater in the control or diabetic mice treated with NAC. These results suggest that the activity of AR in cardiomyocytes is partially suppressed under euglycemic conditions.

Discussion

In the STZ-induced diabetic mouse heart, we presently demonstrated alterations in the expression of AR mRNA as well as in the AR enzyme activity. The level of AR mRNA in the mouse heart was significantly decreased, while no significant change was observed in the AR protein level at 3 weeks of hyperglycemia. In contrast, the enzyme activity was significantly augmented. Since these alterations were reversed by insulin administration, metabolic changes caused by high blood glucose and/or insulin deficiency appeared to alter the expression and activity of AR.

Alterations in the expression and activity of AR under hyperglycemia have not been well characterized in the heart, although a wealth of data has been collected on AR expression in the ocular lens and in the kidney, target organs of diabetic complications (31 – 34). In these studies, increased expression of AR mRNA under hyperglycemia or hypergalactosemia was documented. Characterization of the promoter region of the AR gene further depicted the presence of an osmotic response element responsible for up-regulation of the AR gene expression (35 – 37). On the other hand, we reported that AR expression is augmented by reactive oxygen and nitrogen species in vascular smooth muscle cells as well as in a macrophage cell line (25, 26). Since hyperglycemia is known to enhance oxidative stress, it was assumed that the level of AR mRNA is elevated in the mouse heart at 3 weeks of hyperglycemia. Contrary to this assumption, the level of AR mRNA was significantly decreased in the STZ-induced diabetic mouse heart.

Apparantly, there is a counter-regulatory response to attenuate oxidative stress in cardiac tissue at the early phase of hyperglycemia. It is well known that prolonged hyperglycemia increases oxidative stress in the tissue. On the other hand, time course studies revealed that the state of tissue oxidative stress varies with duration of hyperglycemia (38, 39). Cardiac tissue exposed to ambient high glucose may initially mount a defense response by augmenting the antioxidant system. In fact, the levels of GSH in STZ-induced or insulin-dependent spontaneously diabetic rats were elevated in the heart (40, 41), although there are some reports showing the opposite results (42, 43). Such discrepancy in the cardiac GSH among the diabetic models may be due to the duration of hyperglycemia at the time when the GSH level was measured. Since reactive oxygen species are known to augment the expression and activity of γ-glutamylcysteine synthetase, a rate-limiting enzyme of GSH synthesis (44), the de novo synthesis of GSH may be transiently augmented in response to oxidative stress evoked by hyperglycemia. Furthermore, augmented activities of anti-oxidative enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were documented in the heart at the early stage of diabetes without any change in the level of TBARS (39). Recently, we also observed increased expression of an anti-oxidative enzyme, glutathione peroxidase-3, in the cardiac ventricles of STZ-induced diabetic mice under the same experimental conditions (45). As expression of the AR gene is regulated by the cellular redox state via the transcription factor Nrf2 (25, 46, 47), the level of AR mRNA may be down-regulated by transient increase in GSH along with augmented activity of anti-oxidative enzymes at the early stage of hyperglycemia.

No correlation was observed among the levels of AR mRNA, protein, and the enzyme activity in the present investigation. Administration of NAC reduced the level of AR mRNA, whereas it increased enzyme activity without affecting the level of AR protein. The mechanisms underlying such discrepancy in STZ-treated mouse heart are unclear. However, the level of mRNA does not necessarily reflect the level of protein, as the level of protein is maintained by a dynamic turnover. On the other hand, augmented AR activity observed in NAC-treated mice suggests that the enzyme is affected by changes in GSH levels or GSH/GSSG ratio. In fact, AR possesses a redox-sensitive cysteine residue that modifies the enzymatic activity (29, 30). The direct modification and activation of AR protein may therefore be one of the mechanisms underlying the increased AR activity in STZ-treated mouse heart. Under euglycemic conditions, AR protein in the heart may be partially inactivated by modification of a cysteine residue, resulting in suppression of the enzyme activity. In this context, AR activity is postulated to be highly susceptible to the cellular GSH dynamics.

One of the important findings in this study is that the AR activity in the mouse heart was increased at the early stage of hyperglycemia. The role of AR in the pathogenesis of diabetic complications is supported by extensive evidence showing that inhibition of the enzyme prevents and/or delays the development of diabetic cataracts, neuropathy, and nephropathy (6). Furthermore, increased AR activity followed by enhanced flux through the polyol pathway has been documented to
accelerate the development of neuropathy in transgenic mice overexpressing AR (48–50). Thus, the increase in AR activity at the early stage of hyperglycemia may exacerbate the coronary artery-independent cardiomyopathy by perturbing various metabolic pathways. Recently, we investigated ischemia-reperfusion injury in the heart of these transgenic mice overexpressing AR, compared with that in the littermate mice (17, 18). In accord with the findings obtained by Hwang et al. and us (17, 18), increased ischemic injury and poor functional recovery following reperfusion were demonstrated in transgenic mice, which were ameliorated by treatment with AR inhibitors. Accordingly, high AR activity in cardiac tissue may aggravate ischemia-reperfusion injury and consequently increase the mortality rate. Thus, pharmacological inhibition of AR may reduce the risk of heart failure in patients with hyperglycemia.

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