Inhibition of Aldehyde Dehydrogenase 2 by Oxidative Stress Is Associated with Cardiac Dysfunction in Diabetic Rats

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Left ventricular (LV) dysfunction is a common comorbidity in diabetic patients, although the molecular mechanisms underlying this cardiomyopathic feature are not completely understood. Aldehyde dehydrogenase 2 (ALDH2) has been considered a key cardioprotective enzyme susceptible to oxidative inactivation. We hypothesized that hyperglycemia-induced oxidative stress would influence ALDH2 activity, and ALDH2 inhibition would lead to cardiac functional alterations in diabetic rats. Diabetes was induced by intraperitoneal (i.p.) injection of 60 mg/kg streptozotocin. Rats were divided randomly into four groups: control, untreated diabetic, diabetic treated with *N*-acetylcysteine (NAC) and diabetic treated with a-lipoic acid (α-LA). Cardiac contractile function, oxidative stress markers and reactive oxygen species (ROS) levels were assessed. ALDH2 activity and expression also were determined. The role of ALDH2 activity in change in hyperglycemia-induced mitochondrial membrane potential ($\Delta\psi$) was tested in cultured neonatal cardiomyocytes. Myocardial MDA content and ROS were significantly higher in diabetic rats than in controls, whereas GSH content and Mn-SOD activity were decreased in diabetic rats. Compared with controls, diabetic rats exhibited significant reduction in LV ejection fraction and fractional shortening, accompanied by decreases in ALDH2 activity and expression. NAC and α-LA attenuated these changes. Mitochondrial $\Delta\psi$ was decreased greatly with hyperglycemia treatment, and high glucose combined with ALDH2 inhibition with daidzin further decreased $\Delta\psi$. The ALDH2 activity can be regulated by oxidative stress in the diabetic rat heart. ALDH2 inhibition may be associated with LV reduced contractility, and mitochondrial impairment aggravated by ALDH2 inhibition might reflect an underlying mechanism which causes cardiac dysfunction in diabetic rats.

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INTRODUCTION

Diabetes mellitus is a common metabolic disorder that can affect patient survival and quality of life because of acute and chronic complications (1–3). Cardiovascular complications, including diabetic cardiomyopathy, are the major causes of morbidity and mortality in diabetic patients. This common and serious comorbidity has an asymptomatic onset and is characterized by impaired contractility and relaxation of the left ventricle independent of coronary artery disease or hypertension (4,5). Approximately 30% of patients with type 1 diabetes have this specific cardiomyopathy (6). However, the molecular mechanisms underlying diabetic cardiomyopathy remain incompletely understood.

Shortly after the onset of hyperglycemia, the production of reactive oxygen species (ROS)—including superoxide anion, hydroxyl radicals and hydrogen peroxide (H_2O_2)—increases by glucose autoxidation, the electron transport chain in mitochondria and membrane-bound NADPH oxidase (7). Oxidative stress caused by these free radicals has been documented to play a crucial role in the pathogenesis of diabetic complications (7,8).

Mitochondrial aldehyde dehydrogenase 2 (ALDH2), the main enzyme responsible for acetaldehyde oxidation in ethanol metabolism, is considered responsible for oxidation and detoxification of aromatic and aliphatic aldehydes such as 4-hydroxy-2-nonenal (4-HNE) (9–11). 4-HNE is a highly cytotoxic aldehyde generated during oxidative stress as a result of lipid peroxidation (12,13). ALDH2 recently has been considered a key cardioprotective enzyme, probably because of its detoxification of reactive aldehydes. Transgenic overexpression of ALDH2 was found to attenuate ethanol exposure-induced myocardial dysfunction (14,15). Enhanced ALDH2 activity by ethanol preconditioning or the direct effect of the ALDH2 activator-1 led to

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cardioprotection against ischemia-reperfusion injury (16–18). Nonetheless, previous studies also indicated that ALDH2 had redox-sensitive thiol group in the active site of the enzyme and thus was prone to an oxidative-based inactivation (19–21). More specifically, long-term nitroglycerin treatment caused ALDH2 inactivation because of overproduction of mitochondrial ROS (19).

From this knowledge, we hypothesized that chronic hyperglycemia-induced oxidative stress would have an effect on ALDH2 activity, and that ALDH2 inhibition would involve left ventricular (LV) dysfunction of the diabetic heart. Accordingly, we evaluated cardiac contractile function, oxidative stress levels and ALDH2 activity and expression in the diabetic rat heart in this study.

MATERIALS AND METHODS

Animals and Induction of Diabetes

Male Wistar rats weighing 200 to 250 g were purchased from the Department of Experimental Animals of Shandong University (Jinan, China). The rats were fed normal chow and had free access to water. Housing was at a constant temperature of 21°C ± 1°C with a fixed 12-h light/dark cycle. All animal procedures were in accordance with NIH Guide and were approved by the Animal Use and Care Committee of Shandong University.

Diabetes was induced in overnight-fasted rats by administering a single intraperitoneal (i.p.) injection of 60 mg/kg streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) freshly dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5). The control group was injected with a similar volume of sodium citrate buffer alone. We considered STZ-treated rats with blood glucose levels > 15.0 mmol/L after 72 h of injection as diabetic.

Experimental Protocol

Animals were randomly divided into four groups (n = 8): control, untreated diabetic, diabetic treated with *N*-acetylcysteine (NAC) and diabetic treated with α-lipoic acid (α-LA). One wk after diabetes induction, NAC and α-LA were administered to the diabetic treated groups by oral gavage for eight wks. The concentrations of NAC and α-LA were adjusted for a daily intake of 1.4 g/kg and 60 mg/kg, respectively, according to previous studies (22,23).

Measurement of Cardiac Function by Echocardiography

Transthoracic echocardiography was performed noninvasively with a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada). Rats were lightly anesthetized (0.3 mL of a cocktail containing 100 mg/mL ketamine and 10 mg/mL acepromazine given i.p.) for the duration of the recordings. The heart rate was monitored simultaneously by electrocardiography (ECG). Left ventricular (LV) end diastolic diameter (LVEDD) and end systolic diameter (LVESD) were used to calculate fractional shortening (FS) by the following formula: FS (%) = [(LVEDD – LVESD)/LVEDD] ×100%. LV end diastolic volume (LVEDV) and end systolic volume (LVESV) were calculated as described previously (24). Ejection fraction (EF) was calculated by the following formula: EF (%) = [(LVEDV – LVESV)/LVEDV] ×100%. All echocardiographically derived measures were obtained by averaging the readings of three consecutive beats.

Biochemical Measurements in Plasma and Heart Tissue

Plasma glucose concentration was measured by use of the Glucose Analyzer (Yellow Spring Instrument, Yellow Springs, OH, USA), and plasma Hb A_{1c} was determined by HPLC (Roche Diagnostics, Indianapolis, IN, USA). At the end of the experimental period, rats were killed, hearts were excised and heart tissues were weighed (wet weight) and homogenized in ice-cold PBS. The homogenates were centrifuged at 3,000*g* for 15 min at 4°C to obtain the supernatant. Total antioxidant concentration, content of malondialdehyde (MDA) (a reliable index of ROS-induced lipid peroxidation), glutathione (GSH) content and Mn-superoxide dismutase (Mn-SOD) activity were measured by commercially available kits according to the manufacturer's instructions (Jiancheng Co., Nanjing, China).

Measurement of Intracellular ROS Production in Isolated Rat Hearts

Intracellular ROS levels were monitored by flow cytometry by use of a peroxide-sensitive fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma) (25). Heart tissue was dissected and immediately frozen in liquid nitrogen. After rapid thawing, tissue was homogenized in 1 mL ROS buffer (150 mmol/L KCl, 20 mmol/L Tris, 0.5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 5 mmol/L glucose and 0.5 mmol/L octanoic acid, pH 7.4). The homogenate was exposed to 10 μmol/L DCFH-DA and incubated at 37°C for 30 min in the dark. After being washed twice with PBS, the homogenate was made into a single-cell suspension through a screen filter, and intracellular ROS levels were measured by use of FACS (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson).

Assessment of Mitochondria

Rat mitochondria were prepared from freshly excised hearts by differential centrifugation as described previously (26). Briefly, hearts were cut into small pieces and homogenized in HEPES buffer. The resulting homogenate was centrifuged at 1,500*g* for 10 min and 2,000*g* for 5 min at 4°C. The pellet at the bottom of the centrifuge tube was discarded and the supernatant was then centrifuged at 12,000*g* for 15 min. The resulting pellet was washed twice by resuspension in 1 mL HEPES buffer and centrifuged again at 12,000*g* for 15 min, followed by further purification by discontinuous gradient centrifugation using 30% (wt/vol.) Percoll. Mitochondrial purity was assessed by Western blot analysis with the mitochondrial marker prohibitin, the plasma membrane marker Na^+K^+ATPase and the cytosolic marker glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Protein concentration was determined by the bicinchoninic acid protein assay. The mitochondrial samples were frozen at –80°C until use.

ALDH2 Enzymatic Activity

The activity of ALDH2 in isolated mitochondria was determined by measuring the conversion of acetaldehyde to acetic acid and/or the conversion of propionaldehyde to propionic acid. The mitochondria were sonicated, centrifuged at 12,000*g* for 10 min at 4°C, and the supernatant was used to detect ALDH2 activity at room temperature by monitoring NADH formation from NAD^+ at 340 nm in a spectrophotometer (Beckman Coulter, Chaska, MN, USA). The assay mixture (0.2 mL) contained 100 mmol/L Tris-HCl (pH 8.5), 1 mmol/L NAD^+, 1 mmol/L 4-methylpyrazole and 50 μg protein. The reaction was started by the addition of 1 mmol/L acetaldehyde or propionaldehyde to the cuvette. Enzyme-specific activity was expressed as nmol NADH min^{-1} mg^{-1} protein.

The mitochondria from control and diabetic hearts were sonicated and incubated with dithiothreitol (DTT) for 30 min at room temperature. Then, 1 mmol/L acetaldehyde or propionaldehyde, together with 1 mmol/L NAD^+ and 1 mmol/L 4-methylpyrazole were added, and absorbance changes were recorded.

Western Blot Analysis

Ventricular tissues were homogenized and sonicated in a lysis buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Equal amounts (20 μg) of proteins were separated by SDS-PAGE on a 12% gel and transferred to PVDF membrane. The membranes were blocked for 2 h in a solution of 5% (wt/vol) milk and then incubated overnight at 4°C with anti-ALDH2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-SOD2 (1:300; Santa Cruz Biotechnology). The same blot was stripped and reblotted with antibody to β-actin (1:1,000; Santa Cruz Biotechnology) as an internal control. Membranes were washed 3x and then incubated with 1:10,000 dilution of horseradish peroxidase–conjugated secondary antibody for 1 h. Protein bands were identified by a standard enhanced chemiluminescence method.

Table 1. General characteristics of experimental groups[a]

Parameter	Control (n = 8)	Diabetic (n = 8)	Diabetic + NAC treatment (n = 6)	Diabetic + α-LA treatment (n = 6)
Plasma glucose (mmol/L)	3.7 ± 0.2	21.7 ± 1.5[b]	16.1 ± 3.1[b,c]	18.2 ± 1.3[b,c]
Hb A_{1c} (% of Hb)	5.7 ± 0.3	8.8 ± 0.6[b]	7.2 ± 0.5[c]	6.8 ± 0.5[c]
Body weight (g)	448 ± 25	270 ± 21[b]	316 ± 15[b]	273 ± 17[b]
Heart weight (g)	1.17 ± 0.08	0.86 ± 0.05[b]	0.90 ± 0.05[b]	0.81 ± 0.04[b]
Heart weight/body weight (mg/g)	2.62 ± 0.10	3.25 ± 0.15[b]	2.86 ± 0.08	3.02 ± 0.16

[a]Data are presented as mean ± SEM.
[b]$P < 0.05$ versus control group.
[c]$P < 0.05$ versus diabetic group.

Immunohistochemistry

We performed immunohistochemistry for 4-HNE, a marker of lipid peroxidation and substrate of ALDH2 enzyme. Formalin-fixed myocardial sections were deparaffinized and rehydrated as described previously in detail (27). Primary antibody against 4-HNE (1:50; Santa Cruz Biotechnology) and biotinylated secondary antibody (1:200; Beyotime, Nanjing, China) were used. Images were captured using a microscope (Eclipse E800; Nikon, Tokyo, Japan) and digital sight camera (Nikon).

Studies of Neonatal Cardiomyocytes in Primary Culture

Neonatal rat cardiomyocytes were isolated from hearts of 1–3-day-old Wistar rat pups and cultured in medium containing 5.5 mmol/L glucose. Cardiomyocytes were treated for 48 h, with a normal concentration of glucose (5.5 mmol/L, LG); a high concentration of glucose (30 mmol/L, HG); LG combined with a selective ALDH2 activity inhibitor, daidzin (Sigma), at 20 μmol/L (LG + daidzin); or HG combined with 20 μmol/L daidzin (HG + daidzin).

After 48-h treatment, mitochondrial membrane potential (Δψ) was detected (28). Briefly, cardiomyocytes were suspended in HEPES buffer and incubated with 5 μmol/L JC-1 (Invitrogen, Carlsbad, CA, USA), a dual-emission mitochondrial potentiometric dye, for 30 min at 37°C. Then cells were washed 3x with HEPES buffer. The fluorescence intensity of each sample was analyzed (excitation 490 nm, emission 530 and 590 nm) using a spectrofluorimeter (Spectra Max Gemini; Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* analysis. Correlation analysis was performed using the Pearson correlation. $P < 0.05$ was considered statistically significant. Data were analyzed with use of GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

General Observations

STZ-administered rats showed characteristic symptoms of diabetes, including polydipsia, polyuria and increased food intake, along with reduced body weight gain as compared with controls (data not shown). At the end of the experiment, plasma glucose and Hb A_{1c} levels were markedly higher in diabetic than control rats (Table 1). Supplementation with NAC

Table 2. Plasma and tissue markers of oxidative stress in experimental groups[a]

Parameter	Control (n = 8)	Diabetic (n = 8)	Diabetic + NAC treatment (n = 6)	Diabetic + α-LA treatment (n = 6)
Total antioxidant concentration (mmol/L)	1.40 ± 0.20	0.73 ± 0.09[b]	1.18 ± 0.09[c]	1.16 ± 0.08[c]
Mn-SOD (U/mg prot)	64.53 ± 1.82	55.81 ± 2.71[b]	64.31 ± 2.70[c]	61.58 ± 2.41[c]
GSH (mg/g prot)	5.08 ± 0.28	3.40 ± 0.15[b]	4.40 ± 0.26[c]	4.12 ± 0.22[b,c]
MDA (nmol/mg prot)	2.10 ± 0.21	2.96 ± 0.27[b]	2.20 ± 0.10[c]	2.31 ± 0.17[c]

[a]Data are presented as mean ± SEM.
[b]$P < 0.05$ versus control group.
[c]$P < 0.05$ versus diabetic group.

or α-LA for 8 wks significantly ameliorated these changes. Body weight was significantly lower in diabetic than control rats ($P < 0.05$). NAC treatment increased the body weight of rats as compared with the untreated diabetic group, but the differences were not statistically significant. The body weight was significantly lower in NAC and α-LA treatment groups than in the control group (Table 1).

Plasma and Heart Tissue Markers of Oxidative Stress

Table 2 shows the results of oxidative stress markers in experimental groups. Plasma total antioxidant concentration was significantly lower in diabetic rats than in control rats ($P < 0.05$), in parallel with a significant reduction in the tissue activity of Mn-SOD. Both NAC and α-LA treatment restored total antioxidant concentration and Mn-SOD activity. The myocardial MDA content was significantly higher in diabetic than control rats, whereas the GSH content in diabetic rats was decreased. NAC and α-LA treatment attenuated these changes.

Myocardial Parameters and Systolic Function

As shown in Table 1, heart weight was significantly lower in diabetic than control rats ($P < 0.05$), whereas heart weight did not differ between NAC- and α-LA–treated rats and untreated diabetic rats. The ratio of heart-to-body weight was higher in diabetic than control rats. Although the ratio of heart-to-body weight decreased in NAC- or α-LA–treated rats as compared with untreated diabetic rats, the differences were not statistically significant. Heart rate was significantly lower in untreated diabetic than control rats (288.2 ± 18.3 versus 399.4 ± 24.8 beats/min), and antioxidant treatment enhanced the heart rate: 353.6 ± 28.9 beats/min for NAC-treated diabetic rats; 342.2 ± 19.8 beats/min for α-LA–treated diabetic rats ($P < 0.05$).

Compared with control, diabetic rats showed a significant reduction in EF and FS ($P < 0.05$) (Figure 1). Both NAC and α-LA treatment restored FS (Figure 1C).

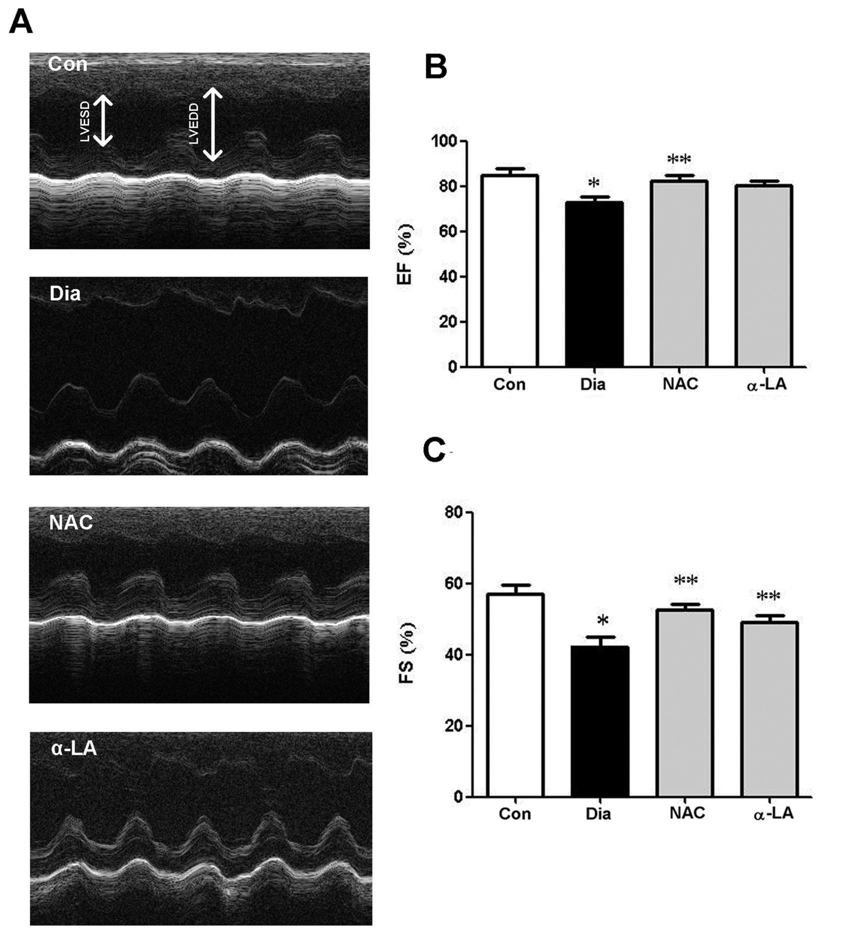

Figure 1. Left ventricular functional analysis with echocardiography. (A) Representative M-mode echocardiograms from control (Con), untreated diabetic (Dia), diabetic treated with NAC (NAC) and diabetic treated with α-LA (α-LA) rats. LVESD, left ventricular end systolic diameter; LVEDD, left ventricular end diastolic diameter. (B) The mean left ventricular ejection fraction (EF) from Con, Dia, NAC and α-LA rats. *$P < 0.05$ versus control group; **$P < 0.05$ versus diabetic group. Data are presented as mean ± SEM (n = 6–8). (C) The mean left ventricular fractional shortening (FS) from Con, Dia, NAC and α-LA rats. *$P < 0.05$ versus control group; **$P < 0.05$ versus diabetic group. Data are presented as mean ± SEM (n = 6–8).

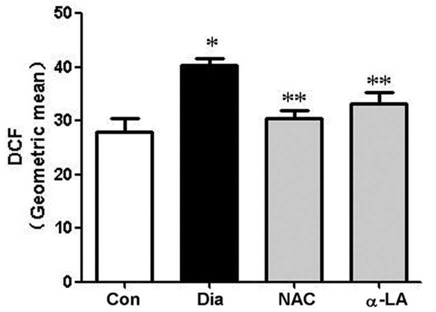

Figure 2. Reactive oxygen species (ROS) production in isolated rat hearts from control (Con), untreated diabetic (Dia), diabetic treated with NAC (NAC) and diabetic treated with α-LA (α-LA) rats. Intracellular ROS levels were determined by measuring the fluorescence of DCF with FACS (excitation 488 nm, emission 530 nm). *$P < 0.05$ versus control group; **$P < 0.05$ versus diabetic group. Data are presented as mean ± SEM of the geometric mean fluorescence (n = 6–8).

Only NAC treatment improved EF as compared with untreated diabetic rats (Figure 1B). The mean LVEDD and LVESD were significantly higher in diabetic than control rats (LVEDD, 6.04 ± 0.24 versus 5.45 ± 0.34; LVESD, 3.20 ± 0.31 versus 2.59 ± 0.31). NAC and α-LA treatment ameliorated these changes.

Intracellular ROS Production

To determine ROS levels, DCFH-DA was used because it permeates into cells and is oxidized to the fluorescent derivate DCF by ROS. Intracellular ROS was higher in diabetic than control hearts (Figure 2). The diabetes-associated increase in ROS was ameliorated markedly by NAC or α-LA treatment (Figure 2).

ALDH2 Enzymatic Activity Analysis

ALDH2 activity in diabetic rat heart mitochondria was decreased markedly, by approximately 40% and 30%, as compared with the control group, as measured by the conversion of propionaldehyde (Figure 3A) and acetaldehyde, respectively. NAC and α-LA treatment significantly improved mitochondrial ALDH2 activity, although it remained lower in treatment groups than in the control group (Figure 3A). Importantly, the decrease in ALDH2 dehydrogenase activity was correlated positively with decreased function of hearts, as indicated by the reduction in EF and FS ($r^2 = 0.680$, $P < 0.01$, and $r^2 = 0.733$, $P < 0.01$, respectively; Figure 4).

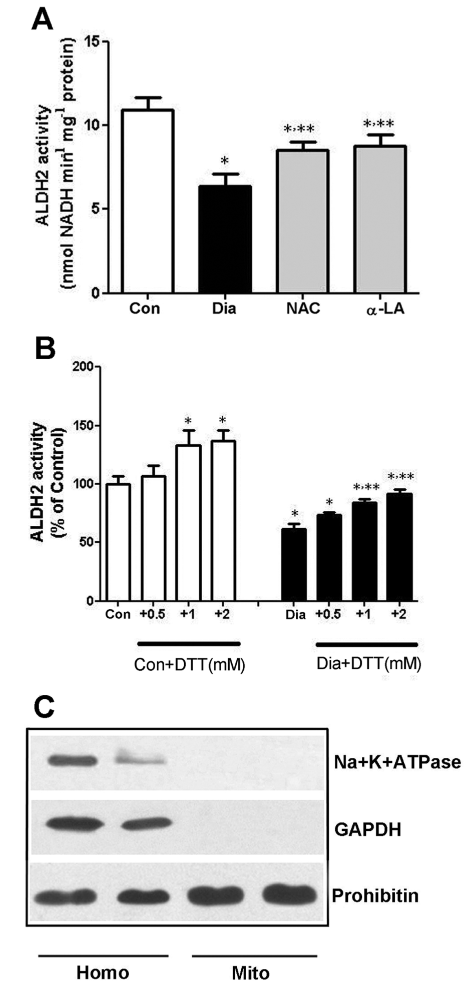

Figure 3. ALDH2 dehydrogenase activity analysis. (A) The effect of NAC and α-LA treatment on mitochondrial aldehyde dehydrogenase 2 (ALDH2) activity in isolated rat hearts from control (Con), untreated diabetic (Dia), diabetic treated with NAC (NAC) and diabetic treated with α-LA (α-LA) rats. ALDH2 activity was determined by measuring the conversion of propionaldehyde to its propionic acid product. *$P < 0.05$ versus control group; **$P < 0.05$ versus diabetic group. Data are mean ± SEM (n=6–8). (B) The effect of dithiothreitol (DTT) on ALDH2 activity in isolated heart mitochondria from control or diabetic rats. *$P < 0.05$ versus control group; **$P < 0.05$ versus diabetic group. Data are mean ± SEM (n=4). (C) Representative Western blot showing various cellular markers in total homogenate (Homo) and isolated mitochondria (Mito) prepared from rat hearts.

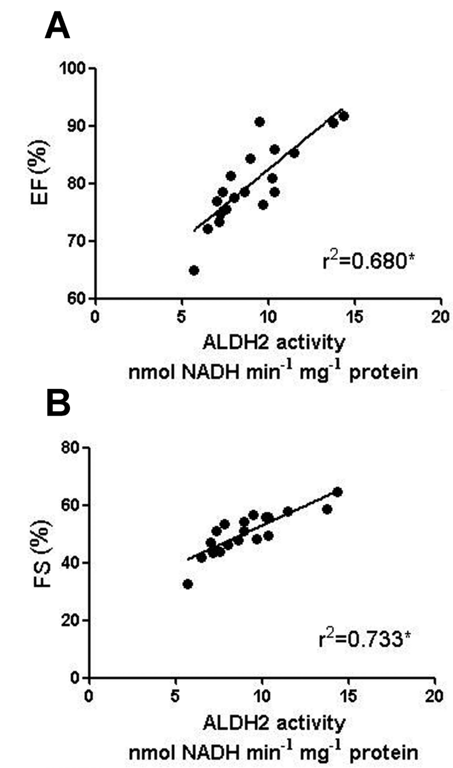

Figure 4. Correlation between myocardial ALDH2 activity and EF (A) and FS (B). Data were analyzed by linear regression. *$P < 0.05$.

To determine whether ALDH2 inactivation resulted from thiol-group oxidation, the heart mitochondrial fractions from diabetic and control rats were coincubated with the selective thiol-reducing agent DTT. ALDH2 dehydrogenase activity in diabetic and control groups was not significantly increased by 0.5 mmol/L DTT, but the activity was markedly enhanced in the presence of 1 or 2 mmol/L DTT in both groups (Figure 3B). In the diabetic group, the addition of 2 mmol/L DTT could not completely restore ALDH2 activity to the level of the control group, which suggests that other mechanisms account for enzymatic inac-

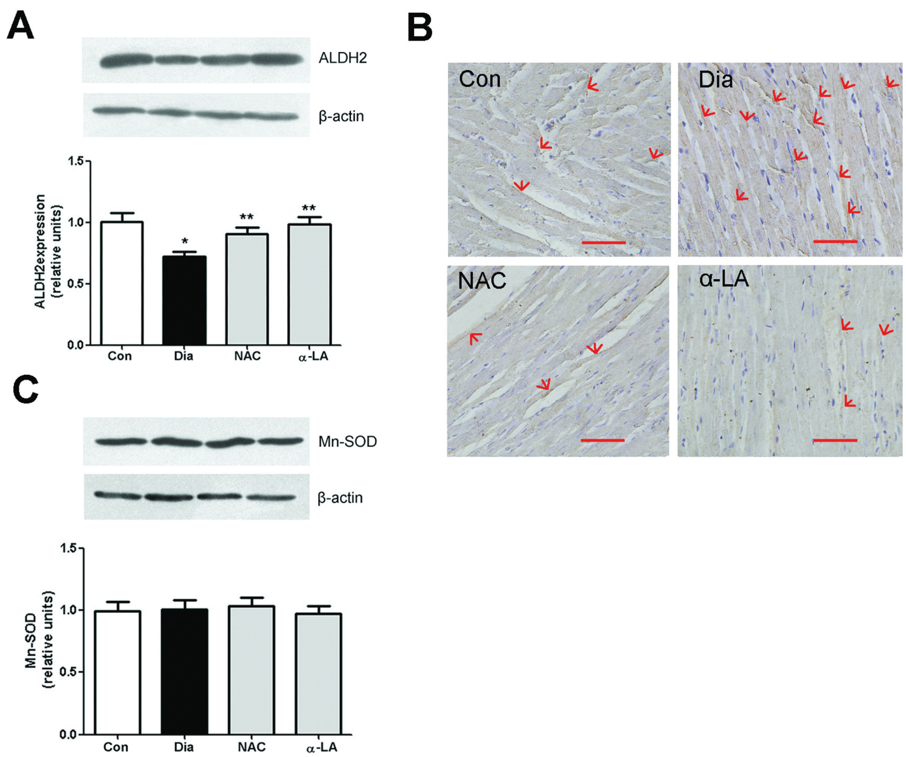

Figure 5. (A) Western blots of ALDH2 expression from control (Con), untreated diabetic (Dia), diabetic treated with NAC (NAC) and diabetic treated with α-LA (α-LA) rats. β-actin was an internal control. $^{*}P < 0.05$ versus control group; $^{**}P < 0.05$ versus diabetic group. Results are presented as mean ± SEM (n=6-8). (B) Representative immunohistochemistrical staining for 4-hydroxy-2-nonenal (HNE)-protein adducts from Con, Dia, NAC and α-LA rats. Scale bar, 50 μm. (C) Western blots of Mn-SOD protein expression from Con, Dia, NAC and α-LA rats. β-actin was an internal control. $^{*}P < 0.05$ versus control group; $^{**}P < 0.05$ versus diabetic group. Results are presented as mean ± SEM (n = 6–8).

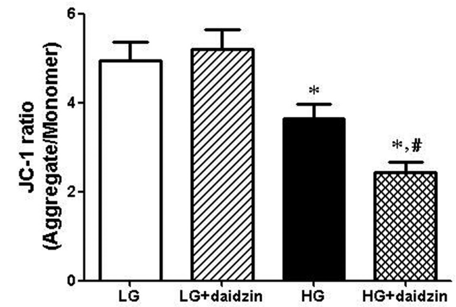

Figure 6. The change in Δψ was quantified from normal glucose (LG), normal glucose+daidzin (LG+daidzin), high glucose (HG) and high glucose+daidzin (HG+daidzin) group. $^{*}P < 0.05$ versus LG group; $^{\#}P < 0.05$ versus HG group. Data are presented as mean ± SEM (n = 4).

tivation. Western blot analysis of mitochondrial preparations from rats with different cellular markers revealed a high degree of mitochondrial enrichment, as indicated by the mitochondrial marker prohibitin, and the absence of contamination with cellular membrane or cytosol as shown by $Na^{+}K^{+}$ATPase and GAPDH results (Figure 3C).

ALDH2 Expression

Western blot analysis revealed a decrease in ALDH2 expression in diabetic rat hearts as compared with control hearts (Figure 5A), accompanied by an increase in the formation of HNE-protein adducts as shown by immunohistochemistrical staining results (Figure 5B). NAC or α-LA treatment ameliorated these changes in diabetic treatment groups. Western blots of Mn-SOD expression did not differ between diabetic and control rats (Figure 5C). NAC or α-LA treatment did not influence the production of Mn-SOD.

Study of Cultured Neonatal Cardiomyocytes

Given that mitochondrial function is essential to cardiac function (28,29), we investigated cardiomyocyte mitochondrial function using a lipophilic and cationic dye JC-1. The result was expressed as the ratio between red (aggregated JC-1) and green (monometric form of JC-1) fluorescence. Quantitative analysis showed cardiomyocyte Δψ greatly decreased during treatment with HG as compared with LG. Daidzin supplementation in LG medium did not have a significant effect on Δψ, but daidzin combined with HG treatment produced a further decrease in Δψ as compared with HG treatment alone (Figure 6).

DISCUSSION

The aim of this study was to examine whether chronic hyperglycemia-induced oxidative stress would have an effect on ALDH2 activity and expression in diabetic rat hearts, and whether ALDH2 inhibition would involve impaired LV function, using a STZ-induced diabetic rat model. Diabetic rat hearts showed a significant increase in myocardial tissue content of MDA, as well as intracellular ROS production. Enhanced MDA and ROS were accompanied by compromised Mn-SOD activity, GSH content and total antioxidant concentration. These findings are similar to those reported in the previous literature (22,30), indicating increased levels of oxidative stress in diabetic rats.

We used two antioxidants, NAC and α-LA, to investigate the effect of oxidative stress *in vivo* in rats. Interestingly, these two antioxidants reversed ALDH2 protein expression in treated diabetic animals but had no effect on Mn-SOD expression. In fact, we found Mn-SOD expression did not change with either treatment. Results in the literature showed a varied effect of hyperglycemia on Mn-SOD expression (31–33), and it is difficult to explain this discrepancy currently. From our findings, we speculate that the changes in Mn-SOD activity in experimental groups we found are not due to the alteration of its expression. However, a posttranslational modification might explain at least in part the observed changes in Mn-SOD activity. In addition, supplementation with NAC and α-LA moderately reduced plasma glucose. Both antioxidants previously were found to prevent hyperglycemia-induced insulin resistance or improve insulin sensitivity in diabetic rats (34,35). The partial lowering of glucose levels by these two antioxidants might be attributable to such effects.

A key finding of the current investigation is the significant inhibition of the myocardial ALDH2 activity in the diabetic heart. Of note, propionaldehyde and acetaldehyde oxidation was markedly lower, by about 40% and 30%, respectively, in diabetic than control rat hearts. Because ALDH2 functions as an antioxidant enzyme, this protein may be easily inactivated by free radicals. Recently, ALDH2 was identified as a target for oxidative modification during glyceryl trinitrate tolerance (19,21) and hepatic ischemia-reperfusion (36). Therefore, oxidative modification of the thiol group may be responsible for ALDH2 inactivation under hyperglycemic conditions. We further hypothesized that inhibition of ALDH2 activity might be due to an alteration in its expression. Indeed, we found the ALDH2 protein level significantly downregulated in the diabetic heart as compared with the control group, and this might explain the observed decrease in ALDH2 activity in response to hyperglycemia.

In this study, we observed that ALDH2 activity was improved significantly, but only partially restored by NAC or α-LA. *In vitro*, DTT dose-dependently improved ALDH2 activity in isolated mitochondria from diabetic and control rats but also moderately rescued ALDH2 activity in diabetic rats. The literature contained reports of various ALDH2 activity changes in response to antioxidants. Song *et al*. showed that incubation with reducing agents such as DTT restored the suppressed ALDH2 activity in alcohol-fed rats (37). However, in other reports, ALDH2 activity was restored only partially by DTT (38,39). A possible explanation for incomplete normalization of ALDH2 activity in this study could be that oxidants cause irreversible modification of the thiol group by prolonged exposure to hyperglycemia. In addition, modification of ALDH2 other than oxidation may contribute to enzymatic inactivation; one example is phosphorylation (39), which needs further investigation.

The present study suggests that reduced ALDH2 activity is associated with impaired cardiac contractile function in diabetic rats, consistent with previous observations (16,17). Chen *et al*. found that ALDH2 activity was correlated inversely with cardiac infarct size in rat hearts subjected to ischemia and reperfusion *ex vivo* (16). Increasing ALDH2 activity by ethanol or other treatments was accompanied by a reduction in infarct size, suggesting the involvement of ALDH2 in cardioprotection. Because of the protective role of ALDH2 in the metabolism of toxic aldehydes, inactivation of ALDH2 likely causes accelerated accumulation of aldehydes, and thus leads to increased susceptibility to myocardial damage. 4-HNE, for example, which was significantly increased in the diabetic rat hearts in our study, reacts with cysteine, hidtidine and lysine residues and forms protein adducts, thus resulting in inhibition of proteins such as the GAPDH (40), Na^+K^+ATPase (41) and 20S proteosome (42). At high concentrations, 4-HNE also directly inhibits ALDH2 activity (43), thus contributing to a vicious cycle. To further investigate the role of ALDH2 activity in the development of myocardial injury, we examined the decrease in ALDH2 activity by use of the selective inhibitor daidzin on mitochondrial function in cultured neonatal cardiomyocytes. Reduced ALDH2 activity contributed to the disrupted membrane potential caused by high glucose. Mitochondrial morphology and function have been reported to be controlled by the $\Delta\psi$ across the inner membrane (44,45). The decrease in $\Delta\psi$ will lead to changes in mitochondrial Ca^{2+} and AT*P* content, with deleterious effects. These data might reflect an underlying mechanism by which hyperglycemia leads to cardiac dysfunction. However, given the nature of the associated rather than causal relationship of our results, further study is warranted to better elucidate the role of ALDH2 in mitochondrial and cardiac function.

In summary, we have shown that hyperglycemia-induced oxidative stress could reduce the activity and expression of ALDH2 in the STZ-induced diabetic rat heart, and antioxidants ameliorated these changes. We also suggest that ALDH2 inhibition aggravates mitochondrial impairment in response to hyperglycemia, which might represent a mechanism underlying LV contractile dysfunction in diabetic rats. These findings might provide new knowledge about diabetic cardiomyopathy.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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