Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine

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Guo Z, Xia Z, Jiang J, McNeill JH. Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine. Am J Physiol Heart Circ Physiol 292: H1728–H1736, 2007.—We investigated the effect of N-acetyl-L-cysteine (NAC) on the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, antioxidant enzymes, and inflammatory markers in diabetic rat hearts. Metabolic parameters, free 15-F2t-isoprostane level, protein expression of NADPH oxidase, superoxide dismutase (SOD), heme oxygenase (HO-1), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) were analyzed in control and streptozotocin-induced diabetic rats treated with or without NAC in drinking water for 8 wk. The cardiac protein expression of p67phox and p22phox was increased in diabetic rats, accompanied by increased NADPH-dependent superoxide production. As a compensatory response to the increased NADPH oxidase, the protein expression of Cu-Zn-SOD and HO-1 and the total SOD activity were also increased in diabetic rat hearts. Consequently, cardiac free 15-F2t-isoprostane, an index of oxidative stress, was increased in diabetic rats, indicating that the production of reactive oxygen species becomes excessive in diabetic rat hearts. Cardiac inflammatory markers IL-6 and COX-2 were also increased in diabetic rats. NAC treatment prevented the increased expression of p22phox and translocation of p67phox to the membrane in diabetic rat hearts. Subsequently, the levels of cardiac free 15-F2t-isoprostane, HO-1, Cu-Zn-SOD, total SOD, IL-6, and COX-2 in diabetic rats were decreased by NAC. Consequently, cardiac hypertrophy was attenuated in diabetic rats treated with NAC. The protective effects of NAC on diabetic rat hearts may be attributable to its protection of hearts against oxidative damage induced by the increased NADPH oxidase and to its reduction in cardiac inflammatory mediators IL-6 and COX-2.

oxidative stress; streptozotocin-induced diabetes; nicotinamide adenine dinucleotide phosphate oxidase

CARDIOVASCULAR DISEASE is one of the major complications of diabetes mellitus, resulting in a high percentage of morbidity and mortality and producing significant costs for the health care system (2). Oxidative stress has been suggested to be involved in the development and progression of diabetes-induced cardiomyopathy (13). Activation of NADPH oxidase, autoxidation of glucose, and formation of advanced glycation end products seem to be relevant to the elevated oxidative stress in diabetes (40). NADPH oxidase consists of membrane-associated subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p40phox, p67phox, and Rac). Gp91phox and p22phox form the electron transfer component of the oxidase, and p47phox and p67phox are cytosolic components that interact with these two proteins to modulate their activity. When activated, p47phox and p67phox are phosphorylated, and the cytosolic components translocate to the membrane where they form a molecular cluster of the catalytically active oxidase. The enzyme catalyzes the one-electron reduction of molecular oxygen using NADPH as an electron donor-generating O2·− (4, 15, 46, 54). NADPH oxidase has been identified and characterized not only in neutrophils but also in endothelial cells, vascular smooth muscle cells, and cardiac myocytes (15, 29, 32, 47, 52).

An imbalance between reactive oxygen species (ROS) generation and antioxidant capacity favoring the former leads to oxidative stress and oxidative damage (30). Oxidative damage in various tissues may be controlled or prevented by enzymic and nonenzymic antioxidant defense systems, which include reduced glutathione (GSH), superoxide dismutase, catalase, glutathione peroxidase (1), and heme oxygenase (10). Heme oxygenase-1 (HO-1) is a heat shock protein induced by oxidative stress. HO-1 metabolizes the prooxidant heme to the antioxidant biliverdin, carbon monoxide, and free iron. Biliverdin is reduced to another antioxidant, bilirubin, by biliverdin reductase (10, 31, 33). SOD is another key antioxidant enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen (9, 22).

Heart failure, diabetes, and obesity are recognized as states of chronic inflammation. Inflammatory cytokines may play a role in all three of these conditions (52). Cyclooxygenase-2 (COX-2) is generally considered as an inflammatory mediator and is involved in inflammatory cell infiltration and fibroblast proliferation in hearts (26). It has been reported that high glucose and/or diabetes increased the expression of COX-2 in kidney (28), mesangial cells (23), monocytes (43), and vascular smooth muscle (16). However, it is unknown whether the expression of COX-2 is increased in diabetic rat hearts. IL-6 is secreted by cardiomyocytes and increased in failing hearts (18, 20). The increased IL-6 is involved in diabetic deterioration (21, 27) and may be associated with cardiac hypertrophy (19, 20).

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In the present study, we investigated the effect of NAC, an antioxidant and glutathione precursor, on the expression of NADPH oxidase, HO-1, SOD, COX-2, and IL-6 in diabetic rat hearts.

MATERIALS AND METHODS

Induction of diabetes. Male Wistar rats weighing 220–240 g, purchased from Charles River (Laval, Quebec, Canada), were used in the study. All rats were housed in a temperature-controlled room (22°C–24°C) and kept on a 12-h:12-h light-dark cycle. They had free access to standard rat chow and water. All animals received humane care in accordance with the principles of the Canadian Council on Animal Care. The experimental protocol used in this study was reviewed and approved by the Animal Care Committee of the University of British Columbia. Diabetes was induced by a single tail-vein injection of streptozotocin (60 mg/kg body wt; Sigma, St. Louis, MO) under halothane anesthesia. Blood glucose was measured by using Accu-soft glucose test strips read on a glucometer (Roche Diagnostics, Laval, Quebec). Rats with glucose levels >15 mM at 72 h after streptozotocin injection were considered to be diabetic. Rats were randomly divided into four groups: control untreated (C, n = 8), control treated with NAC (CT, n = 8; Sigma-Aldrich), diabetic untreated (D, n = 8), and diabetic treated with NAC (DT, n = 8). One week after induction of diabetes, NAC was administered to the control-treated and diabetic-treated groups in the drinking water for 8 wk. NAC was dissolved in drinking water to give a daily intake of 1.4–1.5 g/kg body wt (average 1.44 ± 0.06 g·kg⁻¹·day⁻¹) in diabetic rats. Blood glucose levels were measured every 2 wk. The rats were weighed and then euthanized at termination (9 wk after the onset of diabetes) following anesthesia with an intraperitoneal injection of pentobarbital sodium (65 mg/kg). A blood sample was withdrawn from the inferior vena cava, and aliquots were stored at −80°C until use. Hearts were removed, washed, blotted dry, and weighed. A portion of the left ventricle from each rat was fixed in 10% neutral-buffered formalin and processed for histological analysis. The remaining ventricular tissue was immediately frozen in liquid nitrogen and stored at −80°C until assayed.

Determination of plasma glucose and insulin levels. Plasma levels of glucose were measured by using a glucose analyzer II (Beckman Instruments). Plasma insulin was measured by using a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO). The intra- and interassay coefficients of variation of the insulin assay were 4% and 12%, respectively.

Enzyme immunoassay for cardiac free 15-F₂-isoprostane. The homogenized heart tissue (in PBS) was purified by using an Affility kit (Cayman Chemical) in the presence of 0.01% butylated hydroxytoluene and then processed for analysis of free 15-F₂-isoprostane as previously described (50). The value of 15-F₂-isoprostane was expressed as tissue (ng/g). The intra- and interassay coefficients of variance of the assay were 7% and 11%, respectively, for tissue in our study.

Determination of cardiac total SOD activity and IL-6. Frozen heart tissues were pulverized and homogenized at 4°C in cold buffer and then centrifuged at 1,500 g for 5 min at 4°C. The supernatant was collected and stored at −80°C until use. The protein content of the samples was measured by the Bradford protein assay (6) with the use of Coomassie brilliant blue R-250 as the standard. Total SOD activity and IL-6 in cardiac tissue were measured by using commercially available ELISA kits (SOD kit from Cayman Chemical, Ann Arbor, MI; and IL-6 kit from R&D Systems, Minneapolis, MN).

Western blot analysis. Heart tissue (150 mg) was pulverized and homogenized using a Polytron homogenizer in 1.5 ml cold lysis buffer, which contained (in mM) 20 Tris·HCl (pH 7.5), 50 2-mercaptoethanol, 5 EGTA, 2 EDTA, 1 PMSF, and 10 NaF and (in μg/ml) 25 leupeptin and 2 aprotinin. The homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was collected and labeled as total preparation. The homogenate was centrifuged at 100,000 g for 60 min at 4°C. The supernatant was removed and labeled as cytosol preparation. The pellets were resuspended in buffer containing 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholic acid, homogenized again, incubated on ice for 30 min, and centrifuged at 100,000 g for 60 min at 4°C. The supernatant was removed and labeled as membrane preparation. The protein content of each fraction was measured by using the Bradford protein assay as described in Determination of cardiac total SOD activity and IL-6. Aliquot samples were stored at −80°C until use. After being boiled at 95°C for 5 min, samples (50 μg protein/lane) were electrophoresed on 10% or 15% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose membrane at 30 V overnight in the cold room. For blocking, the membrane was incubated in 5% nonfat milk in buffer (pH 7.4) containing 20 mM TrisHCl, 150 mM sodium chloride, and 0.05% Triton-X-100 until use. The protein content of each fraction was measured by using the Bradford protein assay as described in Determination of cardiac total SOD activity and IL-6. The antibody against HO-1 (1:2,000, Stress-Gen, Victoria, Canada); Cu-Zn-SOD (1:2,000, Santa Cruz Biotechnology); p22phox; p47phox; and p67phox (1:100, Santa Cruz Biotechnology); and COX-2 (1:500, Abcam, Cambridge, MA). After being washed three times with PBS-T, the blot was incubated with a horseradish peroxidase-conjugated secondary antibody [anti-rabbit for COX-2, HO-1, Cu-Zn-SOD (1:10,000), and p22phox (1:2,000); and anti-goat for p47phox and p67phox (1:2,000)] at room temperature for 1 h. After the final wash, the membrane was developed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and then exposed to X-ray film. All films were scanned by a densitometer, and the intensity of the bands was quantitated by using image analysis software. In all instances, the membranes were stained with Ponceau stain and reblotted with antibody against β-actin (1:500, Santa Cruz Biotechnology) after being stripped to verify the uniformity of protein loading and the transfer efficiency across the test samples. Protein standards (Precision Plus Protein Standards, Bio-Rad) were used to identify specific protein bands.

Quantification of ventricular O₂⁻ and NADPH oxidase-mediated O₂⁻ levels. Cardiac superoxide anion (O₂⁻) production was assessed in ventricular samples by the lucigenin chemiluminescence method. Chemiluminescence is produced by the reaction of lucigenin with O₂⁻ and only weakly with hydrogen peroxide, but not with mucloperoxidase. To prevent auto-oxidation of lucigenin, a low concentration (5 μmol/l) of lucigenin was used, as previously described (49), with the following modifications. Ventricular tissue (hearts cut transversely to include right and left ventricles) was cut in 10- to 15-mg blocks, rinsed in ice-cold PBS, and immediately powdered in liquid nitrogen. The ventricular powders were added to recording tubes containing ice-cold PBS and placed on ice for 10 min. Lucigenin was then added to the recording tubes and incubated in the dark for 5 min at room temperature. Luminometry on a BioOrbit 1250 luminometer (Turku, Finland) was used to measure the instantaneous velocity of O₂⁻ production at room temperature. Background measurements were performed with tubes containing only PBS and lucigenin (i.e., without tissue sample). Data were recorded (in mV/s). Measurements were recorded every 0.5 min until the respiratory burst subsided to <10% of peak activity (about 2–5 min) and averaged. The relative quantity of O₂⁻ production was expressed (as mV·10 mg wet tissue wt⁻¹·min⁻¹). To evaluate NAPDH oxidase activity, 100 μM NADPH was then added to the ventricular samples and luminescence was measured for an additional 2–5 min. In a separate study, ventricular samples were pretreated with apocynin (4 mM, Sigma), a specific NADPH oxidase inhibitor, before the addition of 100 μM NADPH, and luminescence was measured for 5 min thereafter.

Histological analysis. The left ventricle was immersion-fixed in 10% buffered formalin, and paraffin sections (1 to 2 μm) were cut. Left ventricle sections were stained with Masson’s trichrome. For analysis of ventricular myocyte cross-sectional area, microscopic
fields were randomly selected from both epicardial and endocardial portions of left ventricles and the images were acquired with a video camera (3 charge-coupled device color video camera KYF558B, Victor). The myocyte cross sections were traced, and the area was calculated with National Institute of Health Image 1.61 software (National Institutes of Health Service Branch). A minimum of 50 cells per animal was analyzed.

Measurement of diastolic function using isolated working heart procedure. In a separate study, with the use of a similar experimental protocol and treatment groups, the diastolic function was evaluated in hearts using the isolated working heart procedure as described previously (35). The left ventricular developed pressure and the rates of contraction (+dP/dt) and relaxation (−dP/dt) to varying left atrial filling pressures (3–11 mmHg) as well as the half time (t½) to maximum relaxation were determined in rats from all groups.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed by one-way ANOVA followed by Tukey’s post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Animal characteristics. Plasma glucose and insulin, heart weight-to-body weight ratio, and cardiomyocyte cross-sectional area were measured in control and diabetic rats at termination. As shown in Table 1, the plasma glucose level was significantly increased in diabetic rats compared with that in control rats. NAC treatment slightly but significantly reduced the plasma glucose level in diabetic rats. The plasma insulin level was significantly reduced in diabetic rats compared with that in control rats, and NAC treatment did not affect insulin level. The body weight was significantly reduced, but the heart weight and the sum of the weights of the left and right ventricles were unchanged in diabetic rats compared with those in control rats. Consequently, heart weight-to-body weight ratio and ventricle weight-to-body weight ratio were significantly increased in diabetic rats compared with those in control rats. NAC treatment had no effect on body weight in diabetic rats but significantly decreased the heart weight and ventricle weight-to-body weight ratio. To further examine whether true cardiac hypertrophy occurred in diabetic rats, we measured cardiomyocyte cross-sectional area. Cardiomyocyte cross-sectional area was significantly increased in diabetic rats (296.5 ± 72.2 μm²) compared with that in control rats (189.7 ± 34.6 μm²) and control rats treated with NAC (189.7 ± 39.8 μm²) (P < 0.05, n = 8). NAC treatment significantly reduced the cardiomyocyte cross-sectional area in diabetic rats (193.7 ± 34.6 in diabetic-treated vs. 296.5 ± 72.2 μm²) as well as the half time (t½) to maximum relaxation were determined in rats from all groups.

Expression of NADPH oxidase subunits. Because NADPH oxidase is the main source of ROS in the cardiovascular tissues (24, 29, 30, 32, 46), we next measured the protein expression of p22phox, p47phox, and p67phox in the hearts of control and diabetic rats. As shown in Fig. 1, the cardiac expression of p22phox protein in membrane fraction was significantly increased in diabetic rats compared with that in control rats. NAC treatment significantly reduced the cardiac expression of p22phox in membrane fraction in diabetic rats. As shown in Fig. 2, the cardiac p67phox protein expression was significantly increased in both membrane and cytosolic fractions in diabetic rats compared with that in control rats. NAC treatment significantly reduced the cardiac p67phox expression in membrane fraction but not in cytosolic fraction in diabetic rats (Fig. 2, A and B). The ratio of p67phox expression in membrane:cytosol was unchanged in diabetic rats compared with that in control rats but was significantly reduced in diabetic rats treated with NAC (Fig. 2C), indicating that NAC inhibited membrane translocation of p67phox. As shown in Fig. 3, there was no significant difference in p47phox protein expression in membrane and cytosolic fraction among four groups. These data suggest that the increased cardiac NADPH oxidase subunits may contribute to the increased ROS, which caused the cardiac damage in diabetic rats. NAC treatment attenuated the cardiac oxidative damage by decreasing the expression of NADPH oxidase.

Table 1. Body weight, cardiac weight, plasma glucose, and insulin

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<th>C</th>
<th>CT</th>
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<th>DT</th>
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<tr>
<td>Plasma glucose, mM</td>
<td>7.76±0.21</td>
<td>7.46±0.15</td>
<td>27.74±0.67*</td>
<td>25.34±0.66†</td>
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<td>Plasma insulin, ng/ml</td>
<td>1.36±0.15</td>
<td>1.06±0.09</td>
<td>0.23±0.05*</td>
<td>0.34±0.06*</td>
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<td>Body weight, g</td>
<td>526±10.5</td>
<td>532±7.3</td>
<td>424±9.2*</td>
<td>393±8.7*</td>
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<tr>
<td>Whole heart weight, g</td>
<td>1.71±0.09</td>
<td>1.66±0.04</td>
<td>1.63±0.06</td>
<td>1.38±0.03†</td>
</tr>
<tr>
<td>Ventricular weight, g</td>
<td>1.43±0.07</td>
<td>1.42±0.03</td>
<td>1.34±0.05</td>
<td>1.17±0.03*</td>
</tr>
<tr>
<td>Heart/body weight, g/kg</td>
<td>3.15±0.17</td>
<td>3.13±0.09</td>
<td>3.85±0.09*</td>
<td>3.49±0.09</td>
</tr>
<tr>
<td>Ventricular/body weight, g/kg</td>
<td>2.65±0.14</td>
<td>2.68±0.06</td>
<td>3.16±0.09*</td>
<td>2.81±0.05†</td>
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Values are means ± SE; (n = 8 samples/group), which were obtained at euthanasia after 9 wk of streptozotocin or vehicle administration with or without 8 wk of N-acetyl-L-cysteine treatment. C, control; CT, control treated; D, diabetic; DT, diabetic treated. *P < 0.05 or 0.01 vs. C; †P < 0.05 vs. D.

Oxidative stress. To test whether oxidative stress was increased in diabetic rat hearts, we measured the cardiac level of free 15-F2t-isoprostane, an index of oxidative stress, in control and diabetic rats by using radioimmunoassay. Free 15-F2t-isoprostane levels in the heart tissue were significantly increased in diabetic rats (0.93 ± 0.08 ng/g tissue) compared with controls (0.58 ± 0.06 ng/g tissue) (P < 0.05, n = 8), indicating an increased oxidative stress and subsequent lipid peroxidation in diabetic hearts. Diabetic rats treated with NAC had significantly decreased free 15-F2t-isoprostane levels in heart tissue (0.61 ± 0.12 ng/g tissue) compared with those in untreated diabetic rats (0.93 ± 0.08 ng/g tissue) (P < 0.05, n = 8). There was no significant difference in free 15-F2t-isoprostane levels in the heart tissue between untreated control rats (0.58 ± 0.06 ng/g) and control rats treated with NAC (0.74 ± 0.07 ng/g) (P > 0.05). The data suggest that oxidative stress is increased in diabetic rat hearts and that treatment with NAC prevents this change.

Total SOD activity and the protein expression of Cu-Zn-SOD and HO-1. To test whether there were any changes in antioxidant enzymes in diabetic hearts, we measured the total

AJP-Heart Circ Physiol • VOL 292 • APRIL 2007 • www.ajpheart.org
SOD activity and the protein expression of Cu-Zn-SOD and HO-1 in control and diabetic rat hearts by using ELISA and Western blot analysis. Total SOD activities were significantly increased in the hearts of diabetic rats (5.13 ± 0.48 U/mg protein) compared with those in control rats (3.19 ± 0.36 U/mg protein) (∗P < 0.01 vs. C; #P < 0.05 vs. D).

As shown in Fig. 4, Cu-Zn-SOD protein expression was significantly increased in the hearts of diabetic rats compared with that in control rats. NAC treatment significantly reduced Cu-Zn-SOD protein expression in the hearts of diabetic rats.

As shown in Fig. 5, HO-1 protein expression was significantly increased in diabetic rats compared with that in control rats. NAC treatment significantly decreased HO-1 protein expression in diabetic rats. These observations demonstrated that antioxidant enzymes, SOD and HO-1, were induced to protect the heart against oxidative damage in response to the increased NADPH oxidase.

Ventricular O2•− and NADPH oxidase-mediated O2•− levels. As shown in Fig. 6, ventricular lucigenin chemiluminescence was significantly increased in the myocardium of diabetic rats compared with that in control rats, indicating increased O2•− production. NAC treatment prevented the significant increase of O2•− production (P > 0.05, DT vs. C) (Fig. 6A). The quantity of NADPH-stimulated O2•− production was more than twofold in the diabetic rat hearts compared with that in the control rat hearts (P < 0.05, D vs. C or CT, Fig. 6B). NAC treatment significantly reduced NADPH-stimulated O2•− production in diabetic rat hearts (∗P < 0.01, DT vs. D, Fig. 6B). Apocynin blocked NADPH-stimulated O2•− production in diabetic rat hearts (P > 0.05, D vs. C, or D vs. DT, Fig. 6C).

IL-6 and COX-2 protein expression. To test whether diabetes and NAC produced an effect on the expression of cardiac IL-6 and COX-2, we measured the IL-6 and COX-2 protein levels in control and diabetic rat hearts using ELISA and Western blot analysis. As shown in Fig. 7, the cardiac COX-2 protein level was 2.7-fold higher in diabetic rats than in control rats.

**Fig. 1.** Western blot analysis of cardiac NADPH oxidase subunit p22phox in membrane fraction. Actin was used to normalize for loading variations. Bar graphs are summary data of normalized densitometric ratios. The four experimental groups were control (C), control + N-acetyl-L-cysteine (NAC) treatment (CT), diabetic (D), and diabetic + NAC treatment (DT). NAC was administered in the drinking water for 8 wk. Data are expressed as means ± SE (n = 6 samples/group). ∗P < 0.01 vs. C; #P < 0.05 vs. D.

**Fig. 2.** Western blot analysis of cardiac NADPH oxidase subunit p67phox in membrane and cytosolic fraction. Actin was used to normalize for loading variations. Bar graphs are summary data of normalized densitometric ratios. A: representative blot and densitometry analysis of p67phox protein in membrane. B: representative blot and densitometry analysis of p67phox protein in cytosol. C: ratio of p67phox protein expression in membrane:cytosol. NAC was administered in the drinking water for 8 wk. Data are expressed as means ± SE (n = 7 samples/group). ∗P < 0.01 or 0.05 vs. C; #P < 0.05 vs. D.
rats. Similarly, the protein expression of IL-6 was also increased in diabetic rat hearts compared with that in control rats (Fig. 8). Treatment with NAC significantly decreased both expressions in diabetic rat hearts. These results indicate that oxidative stress induced by hyperglycemia increases the expression of cardiac IL-6 and COX-2 in diabetic rats.

Effect of NAC treatment on diastolic function. In agreement with a recent study on a similar model (51), cardiac performance was assessed by measuring left ventricular function in response to changes in left atrial filling pressure. The area under curve values for left ventricular developed pressure and \(\frac{dP}{dt}\) in diabetic untreated hearts were significantly lower than those in control hearts. Treatment of diabetic rats with NAC improved the decreases in these parameters (data not shown). Similarly, \(\frac{dP}{dt}\) was significantly reduced in diabetic rat hearts (26,700 ± 1,246 mmHg/s) compared with nondiabetic control (35,631 ± 2,200 mmHg/s, \(P < 0.05\)) and diabetic NAC-treated hearts (29,781 ± 2,361 mmHg/s, \(P < 0.05\) vs. D; \(P > 0.05\) vs. nondiabetic control). NAC improved ventricular relaxation by significantly reducing the increased \(\frac{t}{2}\) to maximum relaxation seen in diabetic untreated rat hearts (66.3 ± 0.8 ms in D vs. 59.0 ± 0.9 ms in DT, \(P < 0.05\)). The \(\frac{t}{2}\) to maximum relaxation in diabetic NAC-treated group did not differ from that in the control (53.7 ± 1.3 ms) or control NAC-treated group (53.4 ± 0.6 ms) (\(P > 0.1\)).

DISCUSSION

It has become evident that ROS play a crucial role in the development of diabetic complications. NADPH oxidase has emerged as the main source of ROS in the cardiovascular tissues with contributions from other sources, such as xanthine oxidase and mitochondrial respiration (24, 29, 30, 32, 46). Recent studies have provided evidence for increased levels of NADPH oxidase subunits in blood vessels (17, 47) and kidney from diabetic rats (3, 5, 14). Bhatti et al. (5) also reported that the antioxidant \(\alpha\)-lipoic acid attenuated the increased expression of NADPH oxidase subunits p22phox and p47phox in the kidney of streptozotocin-induced diabetic rats. The main new findings of this study are that NAC treatment prevents the membrane translocation of p67phox and the increased expres-
sion of p22phox and reduced myocardial superoxide formation in the diabetic rat hearts. We showed by Western blot analysis that the cardiac protein expression of p67phox and p22phox was enhanced in diabetic rats. This is consistent with the recent report that the NADPH oxidase activity in membrane fractions of diabetic hearts is significantly increased (47). Furthermore, since apocynin inhibition blocked NADPH-stimulated in-
creases of superoxide production (Fig. 6), these data would suggest that the increase in NADPH oxidase activity is a major cause of diabetic myocardial superoxide production. In the present study, NAC reduced the expression of p67phox in the membrane but not in the cytosolic fraction, indicating that it inhibited translocation of the p67phox subunit to the membrane fraction. The increased expression of p22phox, one membrane-bound subunit, was also prevented by NAC. Another critical subunit, p47phox, did not change significantly with diabetes and was not altered by NAC. Thus a mechanism of NADPH oxidase inhibition by NAC may be the suppression of translocation of the cytosolic p67phox component to the membrane fraction, with associated suppression of its anchor, p22phox.

Fig. 6. Myocardial levels of superoxide production (A) and NADPH oxidase-mediated superoxide production in the absence (B) and presence (C) of apocynin as measured by lucigenin chemiluminescence. NAC was administered in the drinking water for 8 wk. Data are expressed as means ± SE (n = 5–7 samples/group). *P < 0.05 vs. C; #P < 0.05 vs. D; †P < 0.05 vs. CT.

Fig. 7. Western blot analysis (representative blot and densitometry analysis) of cardiac cyclooxygenase-2 (COX-2) in control and diabetic rats treated with or without NAC. Actin was used to normalize for loading variations. Bar graphs are summary data of normalized densitometric ratios. NAC was administered in the drinking water for 8 wk. Data are expressed as means ± SE (n = 7 samples/group). *P < 0.05 vs. C; #P < 0.05 vs. D.

Fig. 8. Cardiac IL-6 measured by ELISA in control and diabetic rats treated with or without NAC. NAC was administered in the drinking water for 8 wk. Data are expressed as means ± SE (n = 7 samples/group). *P < 0.05 vs. C; #P < 0.05 vs. D.
where it forms a molecular cluster to activate NADPH oxidase. Because NADPH oxidase is the major source of ROS, we speculate that the increased cardiac NADPH oxidase subunits may contribute to the increased ROS which cause the cardiac damage and result in cardiac hypertrophy in diabetic rats. This is demonstrated by the reports that p67phox and p22phox are upregulated in cardiac hypertrophy (29, 32). NAC treatment attenuated the cardiac oxidative damage and cardiac hypertrophy by decreasing the expression or the translocation of NADPH oxidase subunits, namely p22phox and p67phox. The mechanism by which NAC regulated p22phox and p67phox expression is not clear. Given that NAC is a precursor of glutathione and that an increased efflux of glutathione conjugate has been reported in acutely diabetic cardiomyocytes which were accompanied by increased oxidative stress (12), then supplementation of glutathione by NAC treatment might be a major mechanism leading to the suppression of NADPH oxidase subunits. However, further study is needed to address the related mechanism(s).

The enhanced expression of the NADPH oxidase subunits results in the increase of ROS in diabetic rat hearts. In response to the increased ROS, antioxidant enzymes such as HO-1 and SOD, which act as a defense system, are induced to protect against cellular and tissue injury (7, 10, 25). Our study showed that the total SOD activity and the protein expression of Cu-Zn-SOD and HO-1 were upregulated in diabetic rat hearts, which may be a compensatory response in the face of elevated NADPH oxidase-derived ROS. The reason why the increase in HO-1 was accompanied by an increase in SOD may be that an increase in HO-1 in diabetic rats brought about a robust increase in extracellular SOD, one of three kinds of SOD, and the cytoprotective mechanism of HO-1 against oxidative stress required an increase in extracellular SOD (45). Oxidative stress is determined by the balance between the generation of ROS and the antioxidant defense system (24). The increased cardiac free 15-F_2t-isoprostane, an index of oxidative stress (39), in diabetic rats in this study suggested that the increase in NADPH-derived ROS may greatly exceed the increase in antioxidant enzymes HO-1 and SOD. This may result in the increased oxidative stress leading to cardiac hypertrophy in diabetic rats. Increased antioxidant enzyme expression in diabetic rat hearts does not prevent but may slow the progression of diabetic cardiomyopathy. To prevent the progression of diabetic cardiomyopathy, enhanced antioxidant capacity may be required. This is demonstrated by our study in that treatment with NAC, an antioxidant, significantly reduced the expression of NADPH oxidase subunits, p22phox and p67phox, in diabetic rat hearts. The compensatory increased expression of cardiac HO-1 and SOD induced by NADPH-derived ROS was decreased following NAC treatment in diabetic rats. Although NADPH oxidase and antioxidant enzymes were simultaneously decreased in diabetic rats treated with NAC, the total antioxidant capacity relative to the generation of NADPH-derived ROS was greatly improved by NAC treatment, which resulted in a decreased oxidative stress as shown by the reduced cardiac free 15-F_2t-isoprostane in treated diabetic rats.

Hyperglycemia increases ROS production. Inflammatory markers such as IL-6 and COX-2 are produced by the myocardium in heart disease and might be stimulated by ROS (36, 38). As an early inflammatory response factor, COX-2 is induced by cardiac allograft rejection (53), myocardial infarction (42), and heart failure (48). The increased oxidative stress in diabetes has been shown to induce COX-2 (8, 23, 28). The antioxidant Tempol, a SOD mimetic, reduced renal COX expression and activity in experimental diabetes in the rat (28). In this study, we demonstrated that the protein expression of

\[ \text{Diabetes} \quad \text{Hyperglycemia} \]

\[ \text{NAC(+) \quad NAC(-)} \]

\[ \text{NAD(P)H oxidase} \quad \text{Antioxidant enzymes} \]

\[ \text{Reactive oxygen species} \quad \text{Inflammatory factors} \]

\[ \text{Cardiac oxidative stress} \quad \text{Cardiac oxidative damage} \]

\[ \text{Cardiac hypertrophy} \]

Fig. 9. Summary of the mechanism of NAC protective effect on the heart of streptozotocin-induced diabetic rats. Hyperglycemia in diabetes increases the production of cardiac NADPH oxidase, which results in the increased cardiac reactive oxygen species (ROS) generation. As a compensatory response to the increased NADPH oxidase, antioxidant enzymes, HO-1 and SOD, are also increased in diabetic rat hearts. An imbalance between ROS generation and antioxidant capacity favoring the former leads to the increased oxidative stress that causes oxidative damage in heart and leads to cardiac hypertrophy. Hyperglycemia, ROS, and oxidative stress stimulate the production of inflammatory factors, IL-6 and COX-2, which also contribute to cardiac hypertrophy. NAC treatment leads to the decreased production of cardiac NADPH oxidase and ROS, which is accompanied by the decreased antioxidant enzymes and inflammatory factors. The improved total antioxidant capacity relative to ROS generation by NAC treatment results in the decreased oxidative stress, which is followed by the decreased cardiac oxidative damage, inflammatory responses, and cardiac hypertrophy.
IL-6 and COX-2 was significantly increased in diabetic rat hearts. Treatment with an antioxidant, NAC, significantly decreased both expressions in diabetic rat hearts. This may be due to the decreased expression of NADPH oxidase subunits, the main sources of the ROS production in diabetic rat hearts treated with NAC. Takeda et al. (44) recently reported that transient glucose deprivation increased the generation of ROS and resulted in the increased expression of COX-2 in cardiac fibroblasts. The increase could be prevented by pretreatment with NAC (44). The upregulation of COX-2 and IL-6 in diabetic rat hearts may also contribute to the development of cardiac hypertrophy. It has been reported that COX-2 and its products induced cardiac hypertrophy (26, 34) and that COX inhibition reduced cardiac hypertrophy and fibrosis and improved cardiac function in a model of myocardial infarction (26, 41, 42). Transgenic mice double overexpressing IL-6 and IL-6 receptor also demonstrate myocardial hyper trophy (47). IL-6 may act as a nitric oxide-dependent cardiac depressant (11).

NAC treatment produced a slight but significant reduction in the plasma glucose level in diabetic rats. This reduction could not have affected the parameters assessed because of the marked hyperglycemia in the treated diabetic rats. The underlying mechanisms of the reduced hyperglycemia by NAC treatment are not clear but could be related to the suppressive effect of NAC on oxidative stress in some tissues such as muscle and fat. Because NAC treatment was given 1 wk after the development of diabetes when β-cell damage was virtually completed and because there was no significant increase in plasma insulin level in diabetic rats after 8 wk of NAC treatment, it is unlikely that any protection of pancreatic β-cells against streptozotocin by NAC treatment could have led to the reduction of blood glucose.

In summary, we demonstrate that both the oxidative stress system and antioxidant defense system are concomitantly up-regulated by hyperglycemia in diabetic rat hearts. NAC treatment reduces the ventricular hypertrophy and improved ventricular relaxation subsequent to the prevention of the increased oxidative stress induced by hyperglycemia and the upregulation of NADPH oxidase subunits and the resultant NADPH oxidase-mediated increase of superoxide production and inflammatory markers in diabetic rat hearts. These data support the hypothesis that oxidative stress contributes to the pathogenesis of diabetic cardiomyopathy and that the use of antioxidants may provide a promising therapy in attenuating diabetic cardiomyopathy as outlined in Fig. 9.

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REFERENCES


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