N-acetylcysteine attenuates PKCβ2 overexpression and myocardial hypertrophy in streptozotocin-induced diabetic rats

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Abstract

Objective: Oxidative stress-mediated activation of protein kinase C (PKC) β2 in the myocardium has been implicated in the development of cardiomyopathy. Overexpression of PKCβ2 is associated with increased expression of connective tissue growth factor (CTGF) in myocardium, resulting in myocardial hypertrophy. We hypothesized that chronic treatment with the antioxidant N-acetylcysteine (NAC) would normalize oxidative stress-mediated overexpression of myocardial PKCβ2 and CTGF and attenuate the development of myocardial hypertrophy.

Methods: Control and streptozotocin-induced diabetic rats were treated with NAC in drinking water for 8 weeks. At termination rats were surgically prepared for hemodynamic measurement, subsequent to which their hearts were removed to evaluate cardiac performance and histological and biochemical changes. Further, the role of PKCβ2 in hyperglycemia-induced cardiomyocyte hypertrophy was tested in cultured neonatal rat cardiomyocytes.

Results: Myocardial hypertrophy, characterized by an increased ratio of ventricle weight to body weight and cardiomyocyte cross-sectional area was found to be higher in untreated diabetic rats. Further, in myocardium, increased levels of 15-F2t-isoprostane were accompanied by an increased expression of membrane-bound PKCβ2 and CTGF. N-acetylcysteine treatment not only attenuated these changes but also prevented hyperglycemia-induced hypertrophy in cultured neonatal rat cardiomyocytes.

Conclusions: The results suggest that PKCβ2 overexpression represents a mechanism causing hyperglycemia-mediated myocardial hypertrophy, which can be prevented by the antioxidant N-acetylcysteine.

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Keywords: N-acetylcysteine; Protein kinase C β2; Connective tissue growth factor; Myocardial hypertrophy; Diabetes

1. Introduction

Evidence suggests that chronic hyperglycemia is an independent cardiovascular risk factor which directly causes cardiac damage and leads to diabetic cardiomyopathy [1,2] that can occur independent of preexisting coronary artery disease and arterial hypertension. Cardiomyocyte hypertrophy and fibrosis, among the other important characteristics of diabetic cardiomyopathy which also include metabolic disturbances, abnormalities in regulation of calcium homeostasis, cardiac autonomic neuropathy and insulin resistance (see [3] for a review), are two very important characteristics of diabetic cardiomyopathy that lead to diabetic cardiac dysfunction. In humans [4,5] and in the streptozotocin (STZ) rat model of Type 1 diabetes [6], cardiomyopathy is associated with an initial diastolic dysfunction followed by altered contractile performance. However, the mechanisms
involved in this phenomenon are poorly understood and specific therapeutic strategies are currently undefined [7].

The current consensus is that hyperglycemia results in overproduction of reactive oxygen species (ROS) [8], leading to oxidative myocardial injury. Further, hyperglycemia could activate protein kinase C (PKC), which may, in turn induce PKC-dependent oxidative stress in diabetic vascular tissues and exacerbate diabetic cardiac and vascular abnormalities [9,10]. It has been demonstrated that STZ-diabetic rats exhibit a preferential activation of the PKCβ2 isomorph in the heart [10]. Targeted overproduction of the PKCβ2 isomorph in the myocardium resulted in cardiomyopathy that was associated with left ventricular hypertrophy and multifocal fibrosis in mice [11].

Connective tissue growth factor (CTGF) is rapidly upregulated in cardiac myocytes in response to hypertrophic stimuli and contributes to cardiac hypertrophy and fibrosis [12,13]. Concomitant overexpression of CTGF and PKCβ2 was demonstrated in STZ-diabetic rat hearts that exhibited cardiomyopathy [14]. We postulated that chronic hyperglycemia-induced oxidative stress may play a critical role in the development of diabetic cardiomyopathy that involves abnormal expression of myocardial PKCβ2 and CTGF. Therefore, we hypothesized that effective antioxidant therapy in diabetic rats should prevent or attenuate myocardial overexpression of PKCβ2 and CTGF and improve hemodynamic parameters with normalization of cardiomyocyte hypertrophy and cardiac fibrosis.

2. Materials and methods

2.1. Animals and induction of diabetes

Male Wistar rats weighing between 200 and 240 g were obtained from Charles River Laboratories Inc., Quebec and allowed to acclimatize to the local vivarium. The rats were housed on 12-h light–dark cycle and were allowed free access to standard laboratory diet and drinking water. The animals were cared for in accordance with the principles and guidelines of the Canadian Council of Animal care. Diabetes was induced by a single tail vein injection (under halothane anesthesia) of STZ (60 mg/kg). The rats were considered diabetic and used for the study only if they had hyperglycemia (≥15 mM) at 72 h after STZ injection.

2.2. Experimental protocol

Animals were divided into four groups (n=8 each) that included control (C), control treated (CT), diabetic (D) and diabetic treated (DT) groups. One week after induction of diabetes, N-acetylcysteine (NAC), an antioxidant, was administered to the CT and DT groups in the drinking water for 8 weeks. NAC was dissolved in drinking water and the concentration of NAC was adjusted to give a daily intake of 1.4 - 1.5 g/kg (average 1.44±0.06 g/kg/day) in diabetic treated rats. This dose of NAC is relatively higher than reported in the literature, since NAC given at 0.5 g/kg/day did not completely prevent hyperglycemia-induced oxidative stress [15]. Blood samples were collected from the tail prior to and during NAC treatment, following 5-h fasting, for biochemical measurements.

2.2.a: Measurement of systolic and mean arterial blood pressure and heart rate

Systolic (SBP) and mean arterial blood pressure (MABP) and heart rate were determined in freely moving conscious rats using procedures as described previously [16]. After measurement of blood pressure, rats were anesthetized (pentobarbital 60 mg/kg) and blood was collected from the carotid artery. Subsequent to blood collection, the hearts were immediately removed and placed in ice-cold Krebs solution. Hearts and ventricles (left and right ventricles, LV and RV) were weighed. A portion of the left ventricle apex was fixed in 10% neutral-buffered formalin (NBF) and processed for immunohistochemical localization of PKCβ2. The remaining ventricular tissue was frozen in liquid nitrogen and stored at −70 °C until assayed for various parameters.

2.2.b: Measurement of diastolic and systolic function using isolated working heart procedure

In a separate study, using a similar experimental protocol and treatment groups, the diastolic function was evaluated in hearts using the isolated working heart procedure as described previously [17]. Left ventricular developed pressure as well as the rates of contraction (+dP/dT) and relaxation (−dP/dT) to varying left atrial filling pressures (from 3 to 11 mm Hg) and the t1/2 to maximum relaxation were determined in rats from all groups.

2.3. Biochemical measurements in plasma

Plasma glucose was analyzed using a Glucose Analyzer II (Beckman instruments, USA). Plasma insulin was measured with double antibody based radioimmunoassay kit (Linco Research Inc., St. Charles, MO). Plasma total antioxidant concentration was measured by using a commercially available kit (Calbiochem, San Diego, CA, USA). Superoxide dismutase (SOD) activity was measured by using a commercially available kit (Cayman chemical, Ann Arbor, USA) that measures all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

2.4. Plasma and heart tissue free 15-F₂t-isoprostane levels

Plasma and heart tissue free 15-F₂t-isoprostane (15-F₂t-Isop), an index of in vivo oxidative stress-induced lipid peroxidation, was measured by using an EIA kit (Cayman chemical, Ann Arbor, USA). Plasma samples or homogenized heart tissue (in PBS) were purified using Affinity Sorbent/Column (Cayman chemical) in the presence of 0.01% butylated hydroxytoluene (BHT) and then
processed for analysis of free 15-F_2t-IsoP as previously described [18,19].

2.5. Cardiomyocyte cross-sectional area

Paraffin-embedded left ventricular sections (1 to 2 μm) 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 suitable cross-sections were defined as having nearly circular capillary profiles and nuclei [20]. Each field was scanned together with a microscope by a CCD camera connected to a Macintosh computer and analyzed with image-analyzing software (NIH Image Version 1.61) by an observer who was blinded to treatment groups. A minimum of 150 cells per animal (no less than 50 cells per randomly chosen microscopic field) were analyzed.

2.6. Immunohistochemical assay for PKCβ2

Paraffin-embedded left ventricular tissue blocks were sectioned at 3 μm. The sections were deparaffinized, rehydrated, treated with target retrieval buffer (DAKO, S1699), blocked with 3% hydrogen peroxide, washed with phosphate-buffered saline (PBS) and blocked with 5% normal goat serum in PBS for 30 min. The slides were then incubated with rabbit polyclonal primary PKCβ2 antibody (1:100, Santa Cruz) in PBS containing 1% normal goat serum over night at 4 °C. The slides were further incubated with secondary goat anti-rabbit labeled polymer (Envision+, K4003, DakoCytomation) after three washes in PBS. This was followed by development in DAB Black for 10 min. The sections were then incubated with nonspecific rabbit immunoglobins (IgG) and served as negative controls.

2.7. Protein expression of collagen Type I and III, CTGF and PKCβ2

Myocardial protein abundance of collagen Types I and III was determined by Western blot analysis utilizing an affinity purified goat polyclonal primary collagen I and collagen III antibodies (Santa Cruz Biotechnology, CA) and a donkey anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) as the secondary antibody. Myocardial CTGF protein was determined by Western blotting using biotin labeled human CTGF polyclonal antibody (R&D Systems) and peroxidase-conjugated streptavidin.

Because membrane translocation has been widely used as an indication of PKC activation [21], we examined the activity of PKCβ2 in rat heart tissue by characterization of the subcellular distribution. Left ventricular tissue was fractionated into membranous and cytosol fractions using methods similar to that described by Inoguchi et al. [10]. Briefly, samples were homogenized at 4 °C in buffer A (20 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol, 5 mM EDTA, 1 mM PMSF, 10 mM NaF, 25 μg/ml leupeptin, 0.1 mg/ml aprotinin) with a Polytron for 20 s and then homogenized again with 60 strokes of a Dounce homogenizer. The homogenate was then centrifuged at 100,000 × g for 60 min to separate the cytosol and particulate-bound (crude membrane) enzymes. The supernatant was labeled as the cytosol fraction. The pellet was then resuspended in buffer B (buffer A with 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. The resulting supernatant was retained as membrane fraction. Myocardial total protein was obtained by homogenizing the left ventricular tissue with buffer B and centrifuging at 100,000 × g for 60 min as mentioned above, and was used for the analysis of myocardial protein abundance of collagen Types I and III and CTGF. Both membranous and cytosolic fractions were subjected to SDS-PAGE and Western blot analysis using rabbit polyclonal primary PKCβ2 antibody and goat anti-rabbit IgG–HRP (Santa Cruz). The same blot was stripped and rebotted with antibodies to β-actin or GAPDH as internal controls. Immunoreactive protein bands were detected by enhanced chemiluminescence and measured by an imaging densitometer with image analysis software.

2.8. Studies on isolated ventricular myocytes in primary culture

Primary cultures of cardiomyocytes were prepared from the myocardium of neonatal Wistar rats (1–3 days old) essentially by a trypsin dispersion procedure according to the protocol of Simpson and Savion with minor modifications [22]. After dissociation of cardiomyocytes from the heart tissue with trypsin, cells were pre-plated for 2 h into culture flacons in Dulbecco’s modified Eagle medium (DMEM) with 20% newborn calf serum (NCS) to reduce the number

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
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</thead>
<tbody>
<tr>
<td>Water intake (ml/kg/day)</td>
<td>100±10</td>
<td>89±3</td>
<td>766±50*</td>
<td>610±30**</td>
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<tr>
<td>Food consumption</td>
<td>63±1</td>
<td>64±1</td>
<td>163±7*</td>
<td>140±5*,**</td>
</tr>
<tr>
<td>(g/kg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>507±8</td>
<td>495±14</td>
<td>372±20*</td>
<td>343±14*,**</td>
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<tr>
<td>Plasma glucose (mM)</td>
<td>8.3±0.1</td>
<td>8.3±0.3</td>
<td>33.2±2.0*</td>
<td>27.6±0.9*,**</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>1.3±0.2</td>
<td>1.0±0.1</td>
<td>0.2±0.1*</td>
<td>0.3±0.1*</td>
</tr>
</tbody>
</table>

Values were obtained at termination (9 weeks after STZ injection and 8 weeks of NAC treatment). All values are expressed as mean±S.E.M. (n=8 per group).

Control (C), Control + NAC treatment (CT), diabetes (D) and diabetes + NAC treatment (DT). HR, SBP and MABP indicate heart rate, systolic and mean arterial blood pressure, respectively.

* P<0.05 or 0.01 vs. C and CT.
** P<0.05 vs. D.
of non-myocyte cells. Cells that were not attached to the pre-plated flacons were reseeded into culture plates at a density of $1 \times 10^5$ cells/mm$^2$. $0.1$ mmol/l of BRDU was also added to the medium for the first $48$ h to inhibit the proliferation of non-cardiomyocytes. Cardiomyocytes cultures were maintained at $37$ °C in humidified air with $5\%$ CO$_2$. Observation of the autonomic beating under inverted phase contrast microscope (Leica) and immunocytochemical staining of $\alpha$ sarcomeric actin allowed identifying the cardiomyocytes. The cells other than cardiomyocytes in the cultures were limited to less than $10\%$ of the total cell number. The cells cultured for $3$ days were serum-starved in medium containing $1\%$ NCS for $24$ h before experiments. Cardiomyocytes were then treated, for $48$ h, either with a normal concentration of glucose ($5.5$ mmol/l, LG), a high concentration of glucose ($25.5$ mmol/l, HG), HG in the presence of $1$ mM NAC (HG + NAC) or the selective PKC$\beta_2$ inhibitor CGP53353 (CGP) (Sigma, USA) at $1/\mu$M (HG + CGP), or LG in the presence of the PKC activator phorbol-12-myristate-13-acetate (PMA) (Calbiochem, USA) at $10/\mu$M (LG + PMA), or LG in the presence of CGP53353 and PMA (LG+PMA + CGP), or LG in the presence of the PKC$\delta$ inhibitor rottlerin (RL) at $1/\mu$M and PMA (LG+ PMA+RL).


The incorporation of $[^3]$H-thymine was measured to test protein synthesis as previously described [23]. Briefly, after synchronization, cultured cardiomyocytes were treated with conditional medium for $12$ h, then $[^3]$H-thymine ($3.7 \times 10^5$ Bq/ml) was added to the culture medium for another $12$-h incubation. The cells were then washed with phosphate-buffered saline (PBS) and treated with trypsin and EDTA to fully digest the cells. After centrifugation, aliquots

### Table 2

<table>
<thead>
<tr>
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<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
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<tbody>
<tr>
<td>Total antioxidant concentration (mM)</td>
<td>1.20±0.05</td>
<td>1.61±0.06</td>
<td>0.78±0.10</td>
<td>1.15±0.08</td>
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<tr>
<td>Superoxide dismutase (U/ml)</td>
<td>4.11±0.19</td>
<td>3.53±0.29</td>
<td>2.36±0.27</td>
<td>3.22±0.20</td>
</tr>
<tr>
<td>Free 15-F$_2$-IsoP (pg/ml)</td>
<td>31±11</td>
<td>24±2</td>
<td>69±17</td>
<td>37±8</td>
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<tr>
<td>Free 15-F$_2$-IsoP (pg/g tissue)</td>
<td>584±62</td>
<td>743±74</td>
<td>928±84</td>
<td>605±119</td>
</tr>
</tbody>
</table>

Values were obtained at termination (9 weeks after STZ injection and 8 weeks of NAC treatment).

All values are expressed as mean±S.E.M. ($n=8$ per group).

Control (C), Control+NAC treatment (CT), diabetes (D) and diabetes+NAC treatment (DT).

* $P<0.05$ or $0.01$ vs. C.

** $P<0.05$ vs. D.

### Table 3

<table>
<thead>
<tr>
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<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight (g)</td>
<td>1.49±0.02</td>
<td>1.44±0.03</td>
<td>1.33±0.04</td>
<td>1.10±0.03</td>
</tr>
<tr>
<td>Ventricle weight (g)</td>
<td>1.20±0.02</td>
<td>1.19±0.02</td>
<td>1.14±0.03</td>
<td>0.94±0.03</td>
</tr>
<tr>
<td>Ventricle/Body weight (g/kg)</td>
<td>2.43±0.05</td>
<td>2.43±0.03</td>
<td>3.10±0.10</td>
<td>2.73±0.07</td>
</tr>
<tr>
<td>HR (beat/min)</td>
<td>410±9</td>
<td>415±8</td>
<td>332±9</td>
<td>378±15</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>146±4</td>
<td>148±3</td>
<td>120±3</td>
<td>136±5</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>129±6</td>
<td>124±4</td>
<td>110±3</td>
<td>122±4</td>
</tr>
</tbody>
</table>

Values were obtained at termination (9 weeks after STZ injection and 8 weeks of NAC treatment).

All values are expressed as Mean±S.E.M. ($n=8$ per group).

Control (C), Control+NAC treatment (CT), diabetes (D) and diabetes+NAC treatment (DT).

* $P<0.05$ or $0.01$ vs. C and CT.

** $P<0.05$ vs. D.
of the supernatant solution were counted with a scintillation counter (TriCard 2000 CA, Packard). [\(^{3}\)H]-thymine uptake for treated cells was compared with control cultures. The experiment was repeated 6 times.

The incorporation of [\(^{3}\)H]-leucine was measured according to the method of Thaik et al. [24]. After synchronization, cultured cardiomyocytes were treated with condition medium and co-incubated with [\(^{3}\)H]-leucine (3.7 × 10^4 Bq/ml) for 48 h. The last steps were the same as for [\(^{3}\)H]-thymine incorporation.

2.10. Measurement of cell surface area by confocal immunofluorescence

Cultured myocytes were washed with PBS, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and

Fig. 2. Effect of NAC treatment on left ventricular myocyte cross-sectional area in control (C), diabetic (D), control treated (CT) and diabetic treated (DT) rats. Top panel: cross-sections of left ventricle stained with Masson’s trichrome. The slides A, B, C and D are representative left ventricular sections from control, control treated, diabetic and diabetic treated groups, respectively. Bottom, bar graph shows quantitative analysis of cross-sectional area. Amplifications ×200. Results are expressed as mean±S.E.M. n=8 per group. *P<0.05 vs. all other groups. Myocardial collagen was stained in blue with Masson’s trichrome. Collagen is abundant in diabetic rat hearts (top, slide C) as compared with other groups.
incubated with Texas red conjugated phalloidin (Molecular Probes, Eugene, OR) to visualize sarcomeres. Images were visualized with a confocal microscope (Bio-Rad MRC-1000) and captured with the attached digital camera. The cell surface area was determined with image analysis software (MetaMorph Imaging System, Meta Imaging Series 4.5) and calculated as mean of 200 cells from randomly selected fields from six experimental replicates.

2.11. Superoxide analysis in cultured cardiomyocytes

Superoxide (O$_{2}^{-}$) generation in cultured cardiomyocytes was estimated by dihydroethidium (DHE) staining as previously described [25]. DHE entered the cell and is oxidized primarily by O$_{2}^{-}$ to yield fluorescent products, such as ethidium, which intercalates into DNA, resulting in an increase in quantum yield. Briefly, cultured myocytes from various treatment groups as described above were loaded with DHE at a concentration of 10 $\mu$mol/l in HEPES buffer (2 mM HEPES–50 mM glucose in Hank balanced salt solution) for 30 min at 37 °C. At the end of the incubation, the cells were rinsed with Hank balanced salt solution and examined with an excitation/emission wavelength of 560/660 nm. Digital images were recorded on a SenSys digital camera. The integrated fluorescence intensity (expressed in arbitrary units) was quantified on a gray scale of 240–255 and measured with the HPIAS-1000 Image-Analysis System. The relative fluorescence intensity was calculated by dividing the total integrated optical density by the total number of cells in each field. Mean fluorescence intensity measurements were obtained from six separate experiments in each group.

2.12. Statistical analysis

Data are presented as mean±S.E.M. One-way or two-way analysis of variance (ANOVA) was used for statistical analyses (GraphPad Prism, USA) of data obtained within the same group of rats and between groups of rats, respectively, followed by Tukey’s test for multiple comparisons of group means. $P<0.05$ was considered statistically significant.

3. Results

3.1. General characteristics and effects of NAC treatment

Administration of STZ resulted in characteristic symptoms of diabetes including hyperglycemia, hypoinsulinemia, decreased body weight gain along with increased food and fluid intake when compared to age-matched controls (Table 1). At 72 h after injection of STZ, diabetic rats had higher plasma glucose levels (23.7±2.0 mM in group D, and 24.8±1.6 mM in DT) than in control rats (8.0±0.2 mM in C and 8.3±0.3 mM in CT groups). All animals responded to STZ treatment and no animal died or was excluded from the study. One week after STZ injection, the plasma insulin levels in group D (0.99±0.31 ng/ml) and group DT (0.96±0.16 ng/ml) were lower compared to the C (2.23±0.18 ng/ml) and CT groups (1.98±0.18 ng/ml). At termination, fluid intake, food consumption and plasma glucose levels were higher in diabetic rats compared to control rats, and treatment with NAC for 8 weeks moderately but significantly attenuated these changes (Table 1). Body weight in the D group was lower than that in control groups and NAC treatment did not have a significant effect on body weight gain in the DT group. Plasma insulin levels however, did not improve in the DT group.

3.2. Effect of NAC treatment on plasma and tissue markers of oxidative stress

Plasma and cardiac levels of free 5-F$_{2t}$-IsoP were significantly increased in diabetic rats as compared to control
NAC treatment prevented these changes. The plasma total antioxidant concentration was significantly lower in diabetic rats than in control rats, in parallel with a significant reduction in the plasma activity of SOD, a major endogenous antioxidant enzyme. NAC restored plasma total antioxidant concentration and SOD activity in the diabetic rats to levels comparable to that in the control rats. NAC also increased the plasma total antioxidant concentration in control rats ($P < 0.05$, group CT vs. group C), but did not further increase plasma SOD activity in the control rats.

### 3.3. Hemodynamic and myocardial parameters.

At termination the SBP, MABP and heart rate were significantly lower in untreated diabetic rats compared to control groups. Treatment with NAC restored both blood pressure and heart rate in the DT group. Further, heart weight and ventricular weight in the D group were similar to that of control groups irrespective of the significant lower body weights in the D group relative to the control groups (Table 3). Consequently, a significant increase in the ventricular weight to body weight ratio was observed in the D group compared to control groups. NAC treatment significantly reduced ventricle weight in the DT group compared to the D group. The ventricular weight to body weight ratio in the DT group remained higher than the C group, but was lower than that in the D group.

### 3.4. Effect of NAC treatment on diastolic and systolic function

In control hearts, there was a progressive increase in $-\frac{dP}{dT}$ values in response to increases in left atrial filling pressure (Fig. 1). However, in untreated diabetic hearts, there was a significant impairment both in terms of rate of relaxation and also in the $t/2$ relaxation values. Treatment...
with NAC moderately improved the diastolic function in diabetic rats by reducing τ/2 and improving the rate of relaxation without affecting these variables in control rat hearts. Also, the values of area under curve (AUC) of +dP/ dT in diabetic untreated hearts (37910±1975) were significantly lower than that in the control (50870±3270) and control treated (48545±2480) hearts (P<0.05), which was improved by NAC treatment (41350±3360 in group DT, P>0.05 vs. group C or CT). The changes in left ventricular developed pressure basically mirrored the changes of +dP/ dT (data not shown).

3.5. Effects of NAC on cardiomyocyte hypertrophy and cardiac fibrosis

Myocyte cross-sectional area (an indication of cardiomyocyte hypertrophy) was significantly increased in diabetic rats compared to control rats and this was accompanied by increased abundance in myocardial collagen (an indication of cardiac fibrosis). NAC treatment prevented these changes (Fig. 2). Western blot analysis indicated increased myocardial collagen I (Fig. 3A) and collagen III (Fig. 3B) protein expression in diabetic rat hearts that was attenuated by NAC treatment in the DT group.

3.6. Protein expression of PKCβ2 and CTGF

Immunostaining showed an increase in PKCβ2 (Fig. 4, bottom panel) expression in diabetic rat hearts compared to control rats, which was confirmed by Western blotting analysis (Fig. 4, top panel). Further, Western blots revealed that the overexpression of PKCβ2 seen in the diabetic hearts was a result of an increase in PKCβ2 protein only in the particulate fraction. This is because myocardial cytosolic PKCβ2 protein expression was not different between control and diabetic hearts (Fig. 4, central panel). Left ventricular CTGF protein expression was increased in diabetic rat heart and was attenuated by NAC treatment (Fig. 5).

3.7. [3H]-Thymine and [3H]-leucine incorporation and cardiomyocyte surface area

To test the effect of hyperglycemia and treatments of NAC or PMA (a PKC activator) on DNA and protein synthesis in cardiac myocytes, we measured the [3H]-thymine and [3H]-leucine incorporations, respectively. No significant difference in [3H]-thymine incorporation was found among groups (Fig. 6B), suggesting that neither hyperglycemia nor NAC or PKC activation affect DNA synthesis in cultured cardiac myocytes. Furthermore, the number of cardiomyocytes did not significantly differ among groups (not shown). However, both hyperglycemia and PMA significantly increased [3H]-leucine incorporation compared to normal glucose control group (Fig. 6A). Both NAC and PKCβ2 inhibitor CGP, decreased hyperglycemia-induced increase in [3H]-leucine incorporation. Further, CGP, but not rottlerin, prevented PMA stimulated [3H]-leucine incorporation. Cardiomyocyte surface area was significantly higher in HG (768±68 μm²) and LG+PMA (750±72 μm²) groups compared with LG control (500±13 μm²) (P<0.01). NAC and CGP prevented or attenuated HG-induced increase in cardiomyocyte surface area (HG+NAC: 529±8 μm², P>0.05 vs. LG control; HG+CGP: 590±12 μm², P<0.05 vs. HG or LG control). However, CGP, but not rottlerin, prevented PMA-induced increase in cardiomyocyte surface area (LG+PMA+CGP: 570±25 μm², P>0.05 vs. LG control; LG+PMA+RL: 695±61 μm²; P<0.05 vs. LG control).

3.8. Superoxide generation in cardiomyocytes

In cultured cardiomyocytes, fluorescence intensity was increased 2.6- and 2.2-fold, respectively, by hyperglycemia and PMA treatment as compared with normal glucose control (Fig. 7H). Both NAC and CGP significantly reduced, but did not completely prevent hyperglycemia-induced increase in fluorescence intensity. However, the PKCβ2 inhibitor CGP, but not the PKCδ inhibitor rottlerin, prevented PMA-induced significant increase in fluorescence intensity (P>0.05, LG+PMA+CGP vs. LG, Fig. 7H).

4. Discussion

In this study, we show that STZ-induced Type 1 diabetes is associated with increased oxidative stress as indicated by a significant increase in both plasma and myocardial tissue levels of free 15-F_{2t}-isoprostane, which is a reliable index of ROS-induced lipid peroxidation [26] and also an independent risk factor for cardiac diseases [27,28]. Enhanced levels of oxidative stress are accompanied by compromised SOD activity and total antioxidant concentrations in plasma. Further, diabetic rat hearts exhibited increased expression of PKCβ2 and CTGF with concomitant myocyte hypertrophy and cardiac fibrosis, a finding that is similar in nature to that reported by Way et al. [14]. However, to our knowledge, this is the first study showing that chronic antioxidant therapy, with NAC at a dose sufficient to prevent hyperglycemia-induced oxidative stress, prevented myocardial overexpression of PKCβ2.
and CTGF and subsequent myocardial hypertrophy and fibrosis. The results support our hypothesis that hyperglycemia-induced oxidative stress plays a pivotal role in the development of diabetic cardiomyopathy and involves the abnormal expression of myocardial CTGF and PKCβ2 proteins.

The effect of NAC on hyperglycemia-induced cardiomyopathy is likely dose-dependent. Fioridaiso et al. recently reported that NAC attenuated hyperglycemia-induced cardiomyocyte hypertrophy in vitro and in vivo [15]. In their in vivo study, NAC given at a dosage of 0.5 g/kg/day (~1/3 of dose used in the current study), for 3 months, prevented the increase in perivascular fibrosis seen in diabetic rat hearts and reduced hypertrophy. However, NAC at 0.5 g/kg/day did not attenuate the significant increase in heart/body weight ratio in diabetes, nor did it completely prevent the increase in oxidative stress in the diabetic myocardium [15]. In the current study, NAC treatment prevented the increase in myocardial and plasma free 15-F2-isoprostane observed in diabetes and attenuated the increase in ventricle/body weight ratio in the diabetic rats. Furthermore, NAC treatment reduced myocyte cross-sectional area and myocardial collagen content, an indication of improvement in cardiomyocyte hypertrophy and cardiac fibrosis respectively. These results suggest that NAC should be provided at a dose/concentration sufficient to prevent oxidative stress.

The most common functional aberrations of diabetic cardiomyopathy in experimental animals are impaired force of contraction coupled with delayed relaxation and inadequate ventricular filling which is observed in both chemically induced as well as spontaneously diabetic rats [29,30]. A study from our laboratory has shown that diabetic rats develop cardiomyopathy 6 weeks after injection of STZ [29]. In the present study, at termination, both the rate of contraction (+dP/dT) and the rate of relaxation (−dP/dT) were impaired in untreated diabetic rats, which were associated with lower MABP and SBP. NAC treatment improved the rate of relaxation and pressure development and restored MABP and SBP, a reflection that is probably due to an improvement in myocardial hypertrophy and fibrosis.

Our in vitro study results suggest that nonspecific activation of PKC isoforms is sufficient to induce the same degree of hypertrophy as did hyperglycemia. This is demonstrated by increased [3H]-leucine incorporation and cell size in response to general PKC activator (PMA) and high glucose concentrations, in cultured neonatal cardiomyocytes. It has been shown that hyperglycemia predominantly activates PKCβ2, and to a less degree PKCδ in cultured neonatal cardiomyocytes [31]. We tested the relative role of PKCβ2 and PKCδ in PKC-induced myocyte hypertrophy. PMA-induced hypertrophy was prevented by specific inhibition of PKCβ2, but not by inhibition of PKCδ, suggesting that PKCβ2 is the major pro-hypertrophy signal. However, it is interesting to note that specific inhibition of PKCβ2 attenuated, but did not completely prevent the hyperglycemia-induced cardiomyocyte hypertrophy, suggesting that PKCβ2 plays an important, but not mandatory, role in hyperglycemia-induced cardiomyocyte hypertrophy. It is also worth noting that in cultured cardiomyocytes, NAC completely prevented hyperglycemia-induced cardiomyocyte hypertrophy despite an incomplete inhibition of superoxide formation (Fig. 7G). This suggests that NAC may have conferred its effects through mechanisms other than as a superoxide scavenger, and merits further study. That CGP could prevent PMA-induced superoxide formation (Fig. 7G) suggests the PKCβ2 isoform activation represents a mechanism of superoxide formation in the diabetic cardiomyocytes.

It is noteworthy that NAC treatment (DT group) significantly reduced the plasma glucose level as compared with the diabetic group (P<0.05, Table 1), which may be attributable to a NAC effect in preventing hyperglycemia-induced insulin resistance [32], since NAC did not significantly increase plasma insulin levels in the diabetic rats. The effect of NAC in reducing plasma glucose may have contributed, in part, to the suppressive effect of NAC on oxidative stress and may have potential impact on differences in cardiac alterations, PKCβ2 and/or CTGF that were observed between diabetic groups.

Despite the evidence now available about the possible role of oxidative stress in the development of diabetic complications [33], classical antioxidants, in particular vitamin E, have failed to show convincing beneficial effects [34]. A possible explanation is that the antioxidant therapy with vitamin E or other antioxidants is limited to scavenging already formed oxidants and may, therefore, be considered a `symptomatic’ rather than a causal treatment for oxidative stress [33]. In addition, the patient haptoglobin genotype difference may play a role in determining the benefit that may be derived from antioxidant therapy (see [35] for a review). It has been suggested that interrupting mitochondrial superoxide overproduction would normalize the pathways involved in the development of diabetic complications [36]. NAC effect in enhancing endogenous SOD activity (Table 2), which may have resulted from its capability in attenuating tumor necrosis factor-alpha-induced reduction of SOD activity in vascular endothelial cells [37], should have increased its antioxidant potential. The plasma level of tumor necrosis factor-alpha is increased in diabetes [38]. In addition, the abovementioned partial “glucose-lowering effect” of NAC may make it a causal therapy for oxidative stress in diabetes, hence a promising antioxidant in the treatment of diabetic complications.

In summary, we have shown that NAC treatment prevented hyperglycemia-induced myocardial PKCβ2 and CTGF protein overexpression and the subsequent cardiomyocyte hypertrophy and cardiac fibrosis that was associated with improved cardiac function in diabetic rats. NAC, an effective antioxidant and a precursor of GSH, demonstrated beneficial effects in treatment of diabetic cardiomyopathy.
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