Abstract

Diabetes and oxidative stress concur to cardiac myocyte death in various experimental settings. We assessed whether N-acetyl-L-cysteine (NAC), an antioxidant and glutathione precursor, has a protective role in a rat model of streptozotocin (STZ)-induced diabetes and in isolated myocytes exposed to high glucose (HG). Diabetic rats were treated with NAC (0.5 g/kg per day) or vehicle for 3 months. At sacrifice left ventricle (LV) myocyte number and size, collagen deposition and reactive oxygen species (ROS) were measured by quantitative histological methods. Diabetes reduced LV myocyte number by 29% and increased myocyte volume by 20% compared to non-diabetic controls. NAC protected from myocyte loss (+25% vs. untreated diabetics, \(P<0.05\)) and reduced reactive hypertrophy (–16% vs. untreated diabetics, \(P<0.05\)). Perivascular fibrosis was high in diabetic rats (+88% vs. control, \(P<0.001\)) but prevented by NAC. ROS production and fraction of ROS-positive cardiomyocyte nuclei were drastically raised in diabetic rats (2.4- and 5.1-fold vs. control, \(P<0.001\)) and normalized by NAC.

In separate experiments, isolated adult rat ventricular myocytes were incubated in a medium containing high concentrations of glucose (HG, 25 mM) ± 0.01 mM NAC; myocyte survival (Trypan blue exclusion and apoptosis by TUNEL) and glutathione content were evaluated. The number of dead and apoptotic myocytes increased five and 6.7-fold in HG and glutathione decreased by 48% (\(P<0.05\)). NAC normalized cell death and apoptosis and prevented glutathione loss. NAC effectively protects from hyperglycemia-induced myocyte cell death and compensatory hypertrophy through direct scavenging of ROS and replenishment of the intracellular glutathione content.

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Keywords: Cell death; Oxidative stress; Isolated adult rat ventricular myocytes; Diabetes; N-acetylcysteine; Streptozotocin

1. Introduction

Diabetes is a major risk factor for the development of various cardiovascular complications, which have become the main causes of mortality in diabetic population [1,2]. Moreover, diabetics have worse outcome after myocardial infarction, with higher risk of heart failure [3]. Hyperglycemia is considered the main cause leading to generation of almost all chronic diabetic complications [4]. The diabetic heart shows abnormalities in intermediate metabolism [5,6], cardiac fibrosis [7], endothelial and vascular smooth muscle cell function [8] and contractile performance [5,9]. In addition, recent studies suggested that massive ventricular myocyte loss and hypertrophy of the remaining viable myocytes play a key role in diabetic cardiac damage in rodents [10,11] and humans [12].

Diabetes and its complications are associated with increased oxidative stress [13]. Several mechanisms have been proposed for the oxidative damage during chronic hyperglycemia including mitochondrial reactive oxygen species (ROS) overproduction [14], glucose autooxidation [15] and synthesis of advanced glycation end-products [16]. N-acetyl-L-cysteine (NAC), a thiol-containing radical scavenger and
glutathione precursor, has been successfully used in diabetes to attenuate renal damage and peripheral neuropathy in experimental models [17,18] but its effects on cardiac damage remains unknown. Experiments on isolated cells have shown that NAC reduces cell death induced by ROS in endothelial cells [19] and in isolated rat cardiomyocytes exposed to chelerythrine [20] or to anoxia and reoxygenation [21] but similar data on hyperglycemia-induced oxidative stress are still not available.

The objective of the present study was to verify whether NAC: (1) reduced ROS generation, attenuated cardiomyocyte loss and hypertrophy, in vivo, in a rat model of Type 1 diabetes; (2) protected adult rat LV ventricular myocytes from cell death when exposed to high glucose (HG) levels in vitro.

2. Materials and methods

2.1. Animals

Diabetes was induced in 20 male Sprague–Dawley rats weighing 175–200 g by a single intraperitoneal injection of 60 mg/kg body weight of streptozotocin (STZ, Sigma Chemical Company, St. Louis, Missouri), dissolved in sodium citrate saline buffer (pH 4.5). Another group of eight rats were injected with vehicle and served as controls. Diabetes was confirmed by measuring glycosuria 24 h after STZ injection, using glucotest strips (Roche Diagnostics). Four rats with glycosuria below 5% were not considered diabetic and excluded. Half of diabetic rats (n = 8) were treated with NAC (Hidonac®, Zambon Group S.p.A., Italy) from diabetic onset to sacrifice at the dose of 0.5 g/kg body weight per day in drinking water. Water consumption and body weight were monitored periodically to adjust the dose of NAC, whereas body weight was monitored weekly. Blood glucose (Accu-Check Instant test, Boehringer Mannheim) was assayed at sacrifice after 3 months of STZ or vehicle administration.


2.2. Echocardiographic studies

Transthoracic echocardiographic studies were performed before sacrifice, 3 months after STZ (or vehicle) injection. Rats were sedated with diazepam (10 mg/kg i.p.) (Biologici Italia Lab, Italy) and xylazine (2.5 mg/kg i.p.) (Bayer, Italy). A commercial echocardiography system (Aloka SSD-5500), equipped with 13 MHz linear and 7.5 MHz phase array ultrasound transducers, was used. Parasternal long-axis images were acquired at the aorta and mitral valve with an appropriate angulation to identify the maximum LV length; the transducer angulation was then changed and a short axis view was recorded at the level of papillary muscles. After it was ensured that the image was on axis (based on the roundness of the LV shape), 2-D targeted M-mode tracings were recorded for four to eight cardiac cycles at a sweep speed of 100 mm/s and analyzed off-line.

Anterior, posterior end-diastolic and end-systolic wall thickness and LV internal dimensions were measured according to the American Society for Echocardiography leading-edge method [22]. Circumferential end-systolic wall stress (CESS) was calculated from systolic blood pressure (SBP) measured in conscious rats prior to sacrifice and echocardiographic variables according to the formula proposed by De Simone et al. [23].

2.3. Hemodynamics

Before sacrifice, SBP and heart rate (HR) were measured non-invasively in the conscious rats by the tail-cuff method (Letica LE 5002).

2.4. Tissue preparation

The heart was excised from the chest, trimmed of atria and large vessels and weighed. The LV, inclusive of the interventricular septum and the right ventricle, were dissected and their weights recorded. First a mid-ventricular section was immediately frozen in OCT for cryosectioning. Another mid-ventricular section of the LV was then cut perpendicularly to its longitudinal axis and fixed in phosphate-buffered 4% formaldehyde. Histological paraffin-embedded sections (5 µm) were then prepared.

2.5. Interstitial and perivascular collagen

LV sections were stained with Sirius red to measure interstitial and perivascular fibrosis [24]. Interstitial collagen was quantified at a final magnification of 200x with a microscope (Axioskop, Zeiss) connected to a video camera (XC-77CE CCD Video Camera Module, Sony). The resulting image was processed on Kontron KS300 image-analysis system (Kontron-Zeiss). The content of interstitial collagen (expressed as the fractional area of the entire cross-section) was averaged on nine fields selected across the wall thickness in the septum and free wall. The nature of the Sirius red-stained collagen deposit was confirmed by examining the sections under a microscope fitted with a linear cross-polarizing filter that renders collagen fibers birefringent [25].

Perivascular fibrosis was examined in an average of 15–20 coronary arterioles (lumen diameter <100 µm) in each LV after Sirius red staining, as previously described [26].

2.6. Morphometric determination of myocyte number and volume

LV sections were stained with hematoxylin and eosin. The total number and volume of myocytes were determined by
quantitative morphometric methods [27]. Myocytes positioned perpendicularly to the plane of the section with a visible nucleus and cell membrane clearly outlined and unbroken were then selected for the cross-sectional area measurement [26,28]. This area was determined by manually tracing the cell contour on a digitized image acquired on the image-analysis system at a magnification of 400x. A total of 50 myocytes were selected in the LV of each heart and analyzed by an observer blinded to the experimental treatment.

2.7. In situ detection of reactive oxygen species (ROS)

Dihydroethidium staining (DHE, Sigma–Aldrich) was used to evaluate the in situ concentration of ROS as previously described [29]. DHE is freely permeable to cells and in the presence of ROS is oxidized to fluorescent ethidium bromide, which is trapped intracellularly by intercalation into DNA. LV frozen sections (20 µm) were placed on pol- ysin glass slides and 7.5 µM DHE was applied at 37 °C for 30 min in a humidified chamber protected from light.

Fluorescent images of ethidium bromide were obtained using an Olympus FV500 laser scanning confocal microscope with a 585-nm long-pass filter and analyzed with a image-analysis software (Scion Image Beta 4.02, Scion Corporation; MD, USA). The mean intensity fluorescence of myocyte nuclei was expressed as the fluorescence value of all the pixels in six randomly selected LV fields divided by the total number of pixels, obtained by confocal microscopy with identical laser and photomultiplier settings. In addition, the number of myocyte nuclei labeled by DHE was automatically counted in each field and reported as percentage of the total number of myocyte nuclei evaluated by quantitative morphometric method as previously described. To exclude the possibility of a direct quenching of ROS-induced ethidium bromide fluorescence by residual NAC, LV sections from untreated diabetic rat hearts were preincubated with a 20% NAC solution before DHE staining. No difference in signal intensity or percentage in labeled nuclei were found in presence or absence of exogenous NAC. In addition, fluoresce- nce intensity and the percentage of DHE-labeled myocyte nuclei were similar in LV sections of non-diabetic rats receiving NAC or not at the regimen used for diabetic rats (data not shown).

2.8. Isolated ventricular myocytes in primary culture

LV myocytes were isolated from naive adult normotensive rats [30]. Briefly, hearts were placed on a cannula for retro- grade perfusion through the aorta with collagenase buffer (selected type II, Worthington Biochemical Corp.), gassed with 85% O₂ and 15% N₂ at 37 °C. LV myocytes, were then isolated by mechanical dissociation, separated by differential centrifugation, and plated on 35 mm laminin-coated polystyrene tissue culture dishes in serum-free medium (SFM).

Contamination with non-myocyte cells accounted for 1–2%. After plating, the medium was changed and myocytes were cultured for 24 h in SFM containing 5.5 mM (normal glucose, NG) or 25 mM glucose (HG) in the absence of insulin. Hyperosmolarity was corrected by decreasing salt concentration, and acidosis by adding 20 mM HEPES buffer. To study the protective effect of NAC, cardiac myocytes were preincubated at a concentration of 0.01 mM for 1 h in a medium containing NG, then shifted to a HG medium (25 mM glucose). The hyperglycemic medium containing NAC 0.01 mM was renewed every 8 h.

2.9. Evaluation of cell viability

Trypan blue exclusion was used to determine myocyte viability [31]. Live isolated myocytes were incubated with 0.4% Trypan blue dye for 3 min. Approximately 200 cells in each of the eight dishes per experimental group were examined in a hemocytometer chamber under an inverted light microscope. Cells excluding the stain were considered viable and the percentage of non-blue cells was used as an index of viability. Myocytes were then fixed with 4% paraformaldehyde and processed for the determination of apoptosis.

2.10. In situ terminal deoxynucleotidyl transferase (TdT) assay for detection of apoptosis

Fixed myocytes (10 culture dishes per experimental group) obtained by isolation from two different rat hearts were covered with a solution containing 5 U of TdT, 1.5 mM CoCl₂, 0.2 M potassium cacodylate, 25 mM Tris–HCl, 0.25% BSA, and 0.5 mM biotin-16-dUTP, followed by 5 µg/ml FITC labeled Extravidin (Sigma-Aldrich). All nuclei were visualized with bisbenzimide [32]. Positive controls (myocytes treated with DNase I) and negative controls (omission of biotin-16-dUTP or TdT) were also included. The number of TdT-labeled cells was determined by counting at least 1000 isolated myocytes in each culture dish. Fluorescence microscopy and interference contrast was used to exclude apoptotic nuclei of non-cardiomyocyte origin from the count.

2.11. Glutathione content in isolated myocytes

Isolated cardiac myocytes were pre-incubated at a concentration of 0.01 mM NAC for 1 h in a normoglycemic medium, then shifted to a medium with HG (25 mM glucose) with or without NAC (0.01 mM). After 24 h, they were deproteinized in four volumes of 10% (w/v) 5-sulfosalicylic acid, left 30 min on ice, and centrifuged at 13 000 rpm for 5 min at 4 °C. Glutathione was measured in the supernatants by the glutathione reductase method [33].

In separate experiments, total glutathione content was measured with the same method in LV myocytes freshly isolated from a group of control Sprague–Dawley rats (n =
5), rats with 3 months of STZ-induced diabetes (D, $n = 4$) and diabetic rats treated with NAC for 3 months (D + NAC, $n = 4$). Results are expressed as $\mu$mol/10

2.12. Statistical analysis

Results are expressed as mean ± S.E.M. Differences between the three groups were tested by one-way ANOVA, if $F$ was significant (two-tail $P < 0.05$), multiple comparison between groups were tested by Bonferroni’s multiple comparison test.

3. Results

3.1. Animal model

Water intake, food consumption and glycemia at sacrifice are shown in Table 1. Water intake in diabetic rats without (D) or with NAC (D + NAC) were, respectively, 6.2- and 5.9-fold higher than in controls. Similarly, food intake of D and D + NAC was significantly more elevated (+88% and +77%) than controls. Glycemia was comparable in the two groups of diabetic rats (Table 1).

3.2. Anatomical weights and physiological parameters

At sacrifice, body weights of diabetic rats were lower than controls (~40% for D and ~36% for D + NAC, Table 2). Likewise, diabetes caused a significant reduction of absolute heart weight and ventricular weights. In conscious D rats, SBP was elevated and HR was reduced 3 months after induction of diabetes. NAC did not affect SBP and HR in diabetic rats (Table 2).

3.3. LV dimensions and systolic function

Three months after STZ, echocardiography revealed a significant reduction of LV wall thickness (interventricular septum (IVSThd), anterior (AWThd) and posterior (PWThd)) and, as a consequence, a 21% decrease in echo-derived LV mass compared to controls (Table 3). A good correlation was found between LV mass determined by echocardiography and LV mass measured at autopsy (linear regression: slope=0.91 ± 0.07, $r^2 = 0.88$, data not shown). NAC supplementation did not affect LV wall thickness or LV mass in the D + NAC group. Neither LV chamber diameter (LVIDd), nor shortening fraction (SF) was significantly af-

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>D</th>
<th>D + NAC</th>
<th>ANOVA ($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water intake (ml/day)</td>
<td>47 ± 3</td>
<td>288 ± 19 *</td>
<td>277 ± 15 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Food consumption (g/day)</td>
<td>30 ± 1</td>
<td>56 ± 4 *</td>
<td>53 ± 2 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>137 ± 7</td>
<td>445 ± 21 *</td>
<td>437 ± 27 *</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values at sacrifice, after 3 months of STZ or vehicle administration. Eight rats per group. Control, diabetic (D), diabetic + NAC (D + NAC). * $P < 0.001$ vs. control (Bonferroni’s test).

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>D</th>
<th>D + NAC</th>
<th>ANOVA ($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>517 ± 15</td>
<td>311 ± 14 *</td>
<td>330 ± 21 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>1533 ± 41</td>
<td>1163 ± 41 *</td>
<td>1214 ± 59 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>1055 ± 21</td>
<td>802 ± 31 *</td>
<td>820 ± 38 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RV weight (mg)</td>
<td>289 ± 15</td>
<td>217 ± 11 *</td>
<td>222 ± 18 *</td>
<td>0.0044</td>
</tr>
<tr>
<td>Heart/body weight</td>
<td>2.97 ± 0.04</td>
<td>3.76 ± 0.09 *</td>
<td>3.73 ± 0.09 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>142 ± 1</td>
<td>162 ± 4 *</td>
<td>167 ± 3 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>350 ± 6</td>
<td>287 ± 7 *</td>
<td>271 ± 4 *</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Body and cardiac weights (LV, left ventricle; RV, right ventricle) were collected at sacrifice, after 3 months of STZ or vehicle. SBP and HR were measured non-invasively in conscious rats before sacrifice. Eight rats per group. Control, diabetic (D), diabetic + NAC (D + NAC). * $P < 0.05$, * $P < 0.001$ vs. control (Bonferroni’s test).

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>D</th>
<th>D + NAC</th>
<th>ANOVA ($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSThd (mm)</td>
<td>2.01 ± 0.03</td>
<td>1.56 ± 0.05 *</td>
<td>1.53 ± 0.07 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AWThd (mm)</td>
<td>1.99 ± 0.03</td>
<td>1.43 ± 0.03 *</td>
<td>1.56 ± 0.05 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PWThd (mm)</td>
<td>1.98 ± 0.02</td>
<td>1.68 ± 0.08 *</td>
<td>1.63 ± 0.07 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>1039 ± 17</td>
<td>825 ± 31 *</td>
<td>838 ± 25 *</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>6.9 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>7.2 ± 0.2</td>
<td>0.2163</td>
</tr>
<tr>
<td>SF (%)</td>
<td>57.3 ± 2.2</td>
<td>50.5 ± 2.9</td>
<td>51.0 ± 1.9</td>
<td>0.0976</td>
</tr>
<tr>
<td>CESS (kdynes/cm²)</td>
<td>45 ± 3</td>
<td>63 ± 9 *</td>
<td>74 ± 7 *</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

IVSThd : diastolic interventricular septum thickness; AWThd : diastolic anterior wall thickness; PWThd : diastolic posterior wall thickness; LVIDd : LV internal diastolic diameter; SF : shortening fraction; CESS : circumferential end-systolic wall stress. Eight rats per group. Control, diabetic (D), diabetic + NAC (D + NAC). * $P < 0.05$ vs. control. * $P < 0.01$ vs. control; * * $P < 0.001$ vs. control (Bonferroni’s test).
fected by NAC in diabetic rats. CESS, an index of myocardial afterload, was significantly increased in D rats \( (P < 0.05 \text{ vs. controls, Table 3}) \), but again NAC did not affect this change.

3.4. Interstitial and perivascular fibrosis

Interstitial collagen content in LV myocardium was similar in control \( (3.2 \pm 0.1\%) \), D \( (2.9 \pm 0.1\%) \) and D + NAC \( (3.1 \pm 0.2\%) \) groups (ANOVA; \( P = 0.47; \) Fig. 1a). On the other hand, diabetes (D) caused a 88% increase of perivascular fibrosis around intramural coronary arterioles compared to controls (from \( 1.62 \pm 0.08\% \) to \( 3.04 \pm 0.24\% \)); this increase was significantly attenuated by NAC \( (D + NAC = 2.01 \pm 0.08\%; P < 0.001 \) vs. D (Bonferroni’s test).

3.5. Myocyte number and size

To establish whether the reduced cardiac mass in diabetic rats was accompanied by myocyte loss and/or atrophy, we measured the total number and size of myocytes in the LV.

The number of LV myocytes was reduced by 29% in D compared to controls (from \( 37.6 \pm 1.5 \times 10^6 \) to \( 26.6 \pm 1.9 \times 10^6 \), \( P < 0.05 \), Fig. 2). NAC significantly reduced this loss, with \( 33.4 \pm 1.7 \times 10^6 \) LV myocytes in the D + NAC group \( (P < 0.05 \text{ vs. D}) \). Diabetes also induced hypertrophy of LV myocytes. Their computed volume increased by 20% compared to controls \( (P < 0.05, \) Fig. 3a). The same tendency was observed when cross-sectional area was used as a second independent index of hypertrophy \( (P < 0.01 \text{ control vs. D, Fig. 3b}) \). Volume and cross-sectional area of myocytes were fully normalized in the D + NAC group \( (P < 0.05 \text{ vs. D}) \), suggesting an important role of NAC in preventing cardiomyocyte hypertrophy in this model of Type 1 diabetes.
3.6. ROS production

As illustrated by representative images (Figs. 4a–c) and semi-quantitative analysis (Fig. 4d), the fluorescent intensity of DHE staining, index of ROS production, was enhanced more than twofold in myocyte nuclei of diabetic rats from 15.2 ± 0.3 a.u. in control to 36.9 ± 3.7 a.u. in D (P < 0.05). NAC drastically reduced by 38% the red fluorescent intensity of ethidium bromide intercalated into DNA (22.9 ± 2.2 a.u, P < 0.01 vs. D), reflecting a reduction of ROS generation. Similarly, diabetes was associated to a fivefold increase in the fraction of myocyte nuclei positive for the DHE labeling (from 15 ± 4% in control to 76 ± 8% in D; P < 0.001; Fig. 4e). NAC reduced by 43% the number of DHE-labeled nuclei (43 ± 8%; P < 0.01 vs. D).

3.7. Myocyte viability and apoptosis

In vitro experiments were performed to determine whether NAC could antagonize HG-induced myocyte death. Isolated adult rat ventricular myocytes were incubated for 24 h in a medium supplemented with HG, in the presence or absence of 0.01 mM NAC. Myocyte survival was evaluated with Trypan blue exclusion, whereas myocyte apoptotic death was measured with TUNEL staining on formalin-fixed cells. The percentage of myocyte permeable to Trypan blue increased fivefold in HG medium, from 1.9 ± 0.19% to 9.44 ± 0.84 (P < 0.001, Fig. 5). NAC totally normalized the percentage of Trypan blue stained myocytes in hyperglycemic conditions (HG + NAC = 2.44 ± 0.19%, P < 0.001 vs. HG).
Similarly, HG incubation stimulated myocyte apoptosis: the number of TdT-labeled myocytes increased 6.8-fold, from 0.58 ± 0.05% in NG to 3.93 ± 0.25% in HG (\(P < 0.001\), Fig. 5). Again, NAC normalized apoptosis to control values (HG + NAC = 0.82 ± 0.1%).

3.8. Glutathione content

To investigate whether a change in oxidative status in myocytes accounted for the protective effect of NAC on HG-induced cell death, glutathione levels were measured in adult rat ventricular myocytes. Exposure of myocytes to HG for 24 h produced a 48% reduction of total myocyte glutathione content compared with NG (20.8 ± 2.6 vs. 10.9 ± 1.5 µmol/10⁶ myocytes for NG and HG, respectively) (Fig. 6). Addition of NAC to the hyperglycemic medium prevented this decline in glutathione (HG + NAC = 23.0 ± 2.4 µmol/10⁶ myocytes). Myocytes cultured in NG did not significantly increase their glutathione content when pretreated with NAC (data not shown). Although it did not reach statistical difference, the ratio of GSH/GSSG, indicative of cell redox status, also decreased with HG concentration (1.03 ± 0.5 for controls vs. 0.32 ± 0.3 for HG) and NAC supplementation tended to restore it (HG + NAC = 0.86 ± 0.31).

These results, obtained in cardiac myocytes isolated from naïve rats and cultured in HG, were confirmed in separate experiments carried out in myocytes isolated from diabetic rats treated or not with NAC for 3 months. Total glutathione content was 15.6 ± 2.9 µmol/10⁶ myocytes in control rats and decreased by 37% in D rats (9.7 ± 2.9 µmol/10⁶ myocytes, \(P > 0.05\) vs. control). Chronic treatment of diabetic rats with NAC significantly increased total glutathione content to 30.0 ± 4.4 µmol/10⁶ myocytes (\(P < 0.01\) vs. D, ANOVA: \(P = 0.0063\)).

4. Discussion

The experiments performed in this study lead to two main results: (1) in a rat model of Type 1 diabetes, NAC attenuates LV myocyte loss (and compensatory hypertrophy) and blunts the generation of ROS, (2) NAC also protects from HG-induced apoptotic death of isolated adult rat ventricular myocytes, and restores their glutathione content. These two independent lines of observation concur to suggest that, similarly to what described in other organs affected by diabetes, NAC exerts a cardioprotective role, at least in part by antagonizing myocyte cell death induced by hyperglycemia.

4.1. N-acetylcysteine inhibits the cardiac generation of ROS and replenishes cellular glutathione stores in hyperglycemic conditions

In the present study, the intensity and nuclear distribution of the fluorescent DHE staining were significantly elevated in the diabetic myocardium. The same staining has been recently used to evaluate the in situ concentration of ROS in myocardium during severe hyperglycemia [29] and after ischemia and reperfusion injury [34]. Our results are indicative of an excess of myocardial ROS, a mechanism already involved in the physiopathology of several complications of diabetes, including diabetic cardiomyopathy [35]. NAC reduced both the intensity of ROS production in the single myocyte nucleus and the percentage of DHE-labeled nuclei. Hence, a novel finding of our study is that NAC, possibly through the attenuation of oxidative stress [36], drastically reduced myocyte loss, providing protection against the effect of STZ-induced diabetes on myocyte viability and suggesting a strategy for reducing myocardial damage in diabetes.

HG promotes cell death in adult isolated ventricular myocytes in response to activation of the local renin–angiotensin system [37] and membrane translocation of protein kinase C-δ [38]. In both cases this effect is mediated by an increase in oxidative stress. We observed here five and 7.6-fold increase in cell death and apoptosis, respectively, in adult rat
ventricular myocytes exposed for 24 h to a medium containing 25 mM glucose, equivalent to plasma levels observed in experimental diabetes. NAC fully protected against this HG-induced cell death and apoptosis. After 24 h of hyperglycemia, almost half the myocyte glutathione content was lost and in the remaining pool, the GSH/GSSG ratio was markedly diminished. Supplementation of the hyperglycemic medium with NAC, a thiol-containing precursor of glutathione, totally prevented the depletion of intracellular glutathione stores and tended to increase the GSH/GSSG ratio. The same tendency was noted in myocytes isolated from diabetic rats treated or not with NAC for 3 months. These data are in line with the well known glutathione-replenishing properties of NAC, that can be due both to its role as a stable precursor of cysteine, which can then enter in the glutathione synthesis cycle, and to its glutathione-sparing effect due to its antioxidant properties. In fact, glutathione depletion in disease can be due to a series of mechanisms including decreased cysteine availability (as it was suggested for instance for AIDS [39] or malnutrition [40]) and glutathione depletion by oxidative stress.

4.2. N-acetylcysteine limits cardiomyocyte loss and cellular remodeling induced by high glucose in vivo and in vitro

Diabetes causes death of various cell types in several organs [17,41,42]. At the cardiac level, increase in myocyte apoptosis has been reported in diabetic patients [12], mice [11] and rats [10]. In the latter model, ~30% of the initial number of LV myocytes are lost 4 weeks after the induction of diabetes by STZ [10]. Similarly, we observed a 29% decrease in LV myocyte number in diabetic rats 3 months after STZ injection. Although our data refer to a longer duration of diabetes, the comparable myocyte loss can be explained by the fact that myocyte apoptosis, the main form of cell death in this model, reaches its peak at 3 days and decreases by 36% and 78% at 10 and 28 days, respectively [10].

Diabetes is accompanied by cellular, structural and functional changes in LV [2]. Echocardiography showed a significant thinning of the LV wall myocardium in untreated diabetic rats, with a corresponding reduction of LV mass in the absence of depression of LV function. LV tended to be more dilated in diabetic rats. NAC limited, but did not fully reverse, the diabetes-induced myocyte loss.

Myocyte hypertrophy of the remaining cells characterizes the diabetic heart, mimicking cardiac myopathies in humans and animals [43]. We found that volume and area of myocytes, analyzed by two independent methods, were significantly larger in diabetic rats, as recently shown by Shiomi et al. [44], and NAC totally prevented this hypertrophic response. Thus, the compensatory hypertrophy of remaining myocytes in diabetic rats, did not occur in NAC-treated rats. The net balance of the two compensatory mechanisms (myocyte loss and reactive hypertrophy in untreated diabetic rats vs. marginal myocyte loss and absence of reactive hypertrophy in NAC-treated diabetic rats) probably explains why LV mass was similar in the two groups.

Cardiac remodeling in diabetes involves not only the muscular component, but also the extracellular matrix [45] that maintains the architecture and mechanical characteristics of the myocardium. The changes in interstitial collagen deposition in diabetes are still debated [44,46]. We did not find any changes in interstitial collagen content in the LV myocardium of untreated diabetic rats. Accordingly, the volume occupied by myocytes in the LV was similar in control and diabetic rats, suggesting that the observed increase in myocyte death did not induce a pathological response of the cardiac interstitium in terms of abnormal interstitial collagen deposition. The observation is in agreement with the prevalence of apoptosis over necrosis as a mechanism of cardiomyocyte death in diabetes [10]. Conversely, we observed a 47% increase in collagen deposition around intramural coronary arterioles of the diabetic rats 3 months after induction of diabetes as recently found [7]. In a model of aldosterone-induced inflammation in the rat heart [47], histochemical detection of fibrillar collagen revealed an accumulation of collagen within the perivascular space of intramural coronary arteries, which was completely blocked by NAC. In our experimental setting NAC supplementation almost totally prevented perivascular fibrogenesis induced by diabetes. Since oxidative stress has been held responsible for the development of perivascular fibrosis and the impairment of endothelium-dependent regulation of coronary flow [48], we hypothesized that the antioxidant effect observed with NAC might be also helpful in improving coronary flow with consequent reduction in myocyte loss and compensatory hypertrophy in diabetic rats.

4.2.1. Study limitation

This study focused primarily on the alterations induced by HG level at the myocardial level, and did not explore alterations at the vascular/endothelial levels that are known to contribute significantly to the cardiac complications of diabetes. We did not investigate the hemodynamic alterations induced by STZ in vivo, but provided indirect evidences of increased wall stress in the diabetic animals.

Acknowledgements

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References


