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Antioxidant *N*-acetylcysteine restores systemic nitric oxide availability and corrects depressions in arterial blood pressure and heart rate in diabetic rats

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Abstract

Increased oxidative stress and reduced nitric oxide (NO) bioactivity are key features of diabetes mellitus that eventually result in cardiovascular abnormalities. We assessed whether *N*-acetylcysteine (NAC), an antioxidant and glutathione precursor, could prevent the hyperglycaemia induced increase in oxidative stress, restore NO availability and prevent depression of arterial blood pressure and heart rate *in vivo* in experimental diabetes. Control (C) and streptozotocin-induced diabetic (D) rats were treated or not treated with NAC in drinking water for 8 weeks, initiated 1 week after induction of diabetes. At termination, plasma levels of free 15-F_{2t}-isoprostane, a specific marker of oxygen free radical induced lipid peroxidation, was increased while the plasma total antioxidant concentration was decreased in untreated diabetic rats as compared to control rats ($P < 0.05$). This was accompanied by a significant reduction of plasma levels of nitrate and nitrite, stable metabolites of NO, ($P < 0.05$, D vs. C) and a reduced endothelial NO synthase protein expression in the heart and in aortic and mesenteric artery tissues. Systolic, diastolic and mean arterial blood pressures (SBP, DBP and MAP) and heart rate (HR) were reduced in diabetic rats ($P < 0.05$ vs. C) and NAC normalised the changes that occurred in the diabetic rats. The protective effects may be attributable to restoration of NO bioavailability in the circulation.

Keywords: *Oxidative stress, diabetes, nitric oxide, streptozotocin,* N-*acetylcysteine, arterial blood pressure*

Introduction

Hyperglycaemia increases oxidative stress [1–3] and compromises endothelial nitric oxide synthase (eNOS) activity or synthesis, resulting in reduced NO availability [4]. Increased production of reactive oxygen species and loss of endothelial NO bioactivity are key features of vascular disease states in diabetes that may eventually compromise cardiovascular homeostasis [5,6]. Depressions in SBP, MAP, HR and attenuated pressor responses to vasoactive agents are some of the earliest pathophysiological events associated with hyperglycaemia, particularly in animal models of Type 1 diabetes [7–9] and may be attributed to oxidative stress mediated impairment in cardiac chemoreflexes in diabetic rats [8]. The normalization of hyperglycaemia induced mitochondrial superoxide production has been shown to block several biochemical pathways that are involved in the pathogenesis of diabetic complications [10]. It is uncertain whether antioxidant therapy, aimed at preventing hyperglycaemia induced increases in oxidative stress, can restore NO bioavailability and prevent cardiovascular abnormalities *in vivo* in diabetes.

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NO is the only known endogenously formed radical that acts as a signaling messenger. It activates the enzyme soluble guanylate cyclase to produce the second messenger cyclic guanosine monophosphate (cGMP). NO can directly modulate cardiac contraction by accelerating relaxation and reducing diastolic tone [11–13]. Recent studies have shown that NO contributes to myocardial oxygen demand and supply balance and increases both left [14,15] and right [16] ventricular efficiency. However, NO bioavailability is decreased in diabetes, perhaps due to reduced expression of NO synthases [17] (largely eNOS) or as a consequence of increased destruction of NO by interactions with superoxide [18] (i.e. the formation of peroxynitrite from NO and superoxide) or resistance of vascular smooth muscle cells to normal or increased endothelial NO production [19]. We hypothesised that chronic treatment with *N*-acetylcysteine (NAC), an antioxidant and a glutathione precursor, could prevent the depression of arterial blood pressure and heart rate via mechanism(s) that may involve the restoration of NO bioavailability and antioxidant capacity.

Methods

Animals and induction of diabetes

Male Wistar rats weighing between 200 and 240 g were obtained from Charles River Laboratories Inc., Laval, Quebec and allowed to acclimatize in the local *vivarium* for 7 days. The rats were housed on 12-h light–dark cycle and were allowed free access to standard laboratory diet (Purina rat chow) and drinking water. Care was provided in accordance with the guidelines of the Canadian Council on Animal Care.

Diabetes was induced by a single intravenous tail vein injection (under halothane anaesthesia) of streptozotocin (STZ; Sigma, St Louis, MO, USA) (60 mg/kg). STZ was freshly dissolved in 0.9% saline immediately before use at a concentration of 60 mg/ml. An equivalent volume of saline was administrated by the same route to control animals. The rats were considered diabetic if they had hyperglycaemia (set at $\geq$15 mM) at 72 h after STZ injection. Blood glucose levels were determined using Accusoft glucose test strips read on a Glucometer (Roche Diagnostics, Laval, Quebec).

Experimental protocol and haemodynamic measurement

Animals were divided into four groups ($n = 7$ each): control (C), control treated (CT), diabetic (D) and diabetic treated (DT). One week after induction of diabetes, NAC (NAC, Sigma-Aldrich, Inc., St Louis, MO) was administered to the CT and DT groups in the drinking water for a duration of 8 weeks. NAC was freshly dissolved in drinking water to give a daily intake of 1.4–1.5 g/kg body weight (average 1.44 $\pm$ 0.06 g/kg/day) in diabetic rats. Because of its poor oral bioavailability (4–10%) [20], NAC was given at a higher dosage in our study than has been reported previously [21]. At termination, blood pressure and heart rate (HR) were measured as described below.

Haemodynamic measurement: After pentobarbital anaesthesia (20 mg/kg), animals were surgically prepared on a sterilised surgical table equipped with an anaesthetic machine (Fluotec-3, Yorkshire, England) which provided a constant supply of oxygen and halothane anaesthesia. In addition, a local anaesthetic (Lidocaine) was injected at the site of incision. A fluid filled (heparinised saline, 20 U/ml) catheter (PE 50, Intramedic® Clay Adams, Becton Dickinson and Company, Sparks, MD) was placed in the left carotid artery for measurement of systolic, diastolic and mean arterial blood pressures (SBP, DBP and MAP) and HR. The catheter was exteriorised at the nape of the neck, passed through a harness and tether and connected to a swivel (Instech Lab Inc., PA) mounted above the cage to allow free movement of the animal. The surgical wounds were closed using wound clips and the animal was allowed to recover from surgery and anaesthesia in the cage before recording values of SBP, DBP, MAP and HR. The arterial catheter was connected, by a swivel, to a disposable pressure transducer (DTX®, Viggo-Spectramed, Oxnard, CA) that was connected to a Gould TA 2000 Thermal Array Recorder (Gould Instrument System Inc., Ohio, USA) and a computer for simultaneously monitoring of blood pressures and HR, using custom made data acquisition software. Rate and pressure product (RPP) was calculated as the product of HR (beat/min) and SBP (mmHg). In order to achieve normalization of cardiac baroreflexes, animals were allowed to recover from anaesthesia and surgery for at least 4 h before blood pressures and HR were recorded.

After the haemodymamic measurements, rats were anaesthetized (pentobarbital) and blood was collected from the carotid artery. The collected blood samples were immediately centrifuged at 3000*g* using a desktop centrifuge (Beckman Allegra 21R, Beckman Instruments Inc., Spinco Division, CA), plasma separated, aliquoted into small mirocentrifuge tubes and stored at -70°C until assayed. Subsequent to blood collection, thoracotomy was performed in each rat. The heart, aorta and SMA were immediately removed and placed in ice-cold Krebs solution (120 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$). Hearts and ventricles (left and right ventricles, LV and RV) were weighed. The aorta and mesenteric artery from each rat was fixed in 10% neutral buffered formalin (NBF) and processed

for immunohistochemical localization of eNOS. The remaining ventricular tissue was immediately frozen in liquid nitrogen and stored at −70°C until assayed.

Plasma glucose and insulin assay

Basal and biweekly blood samples were collected for glucose and insulin analyses from the tail vein following a 5 h fast. Plasma glucose was analysed using a Glucose Analyzer II (Beckman Instruments, USA). Plasma insulin was measured with a double antibody based radioimmunoassay kit (Linco Research Inc., St Charles, MO). The intra- and inter-assay coefficients of variation of the insulin assay were 4 and 12%, respectively.

Measurement of plasma total antioxidant status

Plasma total antioxidant concentration was measured using a commercially available kit (Calbiochem, San Diego, CA, USA). The assay is based on the ability of antioxidants in the plasma to inhibit the oxidation of ABTS® (2,2′-azino-di-[-3-ethylbenzodiazoline sulfonate]) to ABTS®+ by metmyoglobin (a peroxidase). The amount of ABTS®+ formed (which depends on the total antioxidant concentration in plasma) was read at 600 nm using a temperature controlled spectrophotometer (water bath maintained at 37°C).

Enzyme immunoassay (EIA) for plasma free 15-F_{2t}-isoprostane

Plasma 15-F_{2t}-isoprostane (old name: 8-isoprostane), a specific and reliable index for *in vivo* oxidative stress induced lipid peroxidation, was measured by using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, USA). Plasma samples were purified using an Affinity Sorbent/Column (Cayman Chemical) and then processed for 15-F_{2t}-isoprostane assay as previously described [22,23]. The assay relies on the competition between 8-isoprostane in the plasma (free) and an 8-isoprostane–acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane specific rabbit antiserum binding sites. The rabbit antiserum 8-isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The values of 15-F_{2t}-isoprostane are expressed as pg/ml plasma. The intra- and interassay coefficient of variance of the assay was 4 and 9%, respectively, in our study.

Colourimetric assay for plasma nitrite and nitrate (NOx) levels

Plasma NOx levels were determined using a commercially available colourimetric assay kit (Cayman Chemical Company, Ann Arbor, USA) based on the Griess reaction. The assay was performed according to the instructions of the manufacturer. Prior to the assay, plasma samples were ultrafiltered through a 30 kDa molecular weight cut-off filter (Ultrafree®-MC centrifugal filter units, Millipore Corporation, Bedford, MA) in order to reduce the background absorbance due to the presence of haemoglobin and to improve the colour formation. Briefly, 200 µl of plasma was placed in each ultra filtration unit (pre-rinsed with ultrapure water) and centrifuged for 40 min at 14,000 rpm (Beckman Allegra 21R, Beckman Instruments Inc., CA) at 5°C. The filtrate collected in the filtration tube was used for NOx analysis. For NOx assay, plasma nitrate was converted to nitrite using nitrate reductase and Griess reagent was added to convert nitrite into a deep purple azo compound. The absorbance was read at 540 nm. The intra- and interassay coefficient of variance of the assay was 6 and 8%, respectively, in our study.

Immunohistochemical assay for eNOS

Aortic and SMA samples were immediately fixed in 10% formalin solution for 24 h and embedded in paraffin. Paraffin embedded tissue blocks were sectioned at 3 µm and sections were mounted on positively charged slides. The slides were processed for eNOS staining using monoclonal eNOS antibody (1:600) and secondary antibody (Dako Envision®, Dakocytomation Inc., ON) as previously described [24]. Some sections incubated with nonspecific mouse and rabbit immunoglobulins (IgG) (Jacksons Immuno Research Laboratories, PA) served as negative controls.

Western blotting for myocardial eNOS

Myocardial protein abundance of eNOS was determined by Western Blot analysis utilizing a polyclonal rabbit anti-eNOS (1:500 dilution) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) as the second antibody. The immunoblots were probed using enhanced chemiluminescence dye (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) and autoradiographed. The same blot was stripped and reblotted with antibody to β-actin as an internal control. The intensity of the bands was determined by using densitometric analysis.

Enzyme immunoassay for heart tissue cyclic GMP

Quantitative determination of cGMP in rat heart tissue was performed using a cGMP EIA kit (Cayman Chemical, Ann Arbor, MI) according to the methods provided by the manufacturer with a minor modification. In brief, frozen heart tissue samples were thawed and homogenised on ice in phosphate buffered saline (PBS, pH 7.4) using a Polytron (PT-10,

Brinkman Instruments, Canada) homogenizer for 40 s (2 × 20 s) at 25% power. The homogenates were centrifuged at 1500*g* and the supernatant was used for cGMP assay. Fifty micro litre cGMP standards and samples were added in triplicate to the 96-well plate provided with the kit, followed by the addition of cGMP AchE Tracer and antiserum. The prepared plates were then incubated overnight (20 h) at 4°C. The next day, the plates were washed 5 times with the wash buffer, followed by addition of Ellman's reagent. After optimal development, 100–120 min under our conditions, the plates were read at 405 nm. Values of tissue cGMP were expressed as pmol/g wet tissue. The intra- and inter-assay coefficient of variations for this assay was 4.2 and 7.0%, respectively in our laboratory.

Histological analysis of myocyte cross sectional area

The left ventricle was immersion-fixed in 10% buffered formalin, and paraffin sections (1–2 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 CCD color video camera KYF55B, Victor). The myocyte cross sections were traced, and the areas were calculated with National Institute of Health (NIH) Image 1.61 software (National Institutes of Health Service Branch). A minimum of 50 cells per animal were analysed.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for the statistical analyses (GraphPad Prism 4, USA). Tukey's test was used for multiple comparisons of group means. The correlation relationships were evaluated by the Pearson test and $P < 0.05$ was considered significant.

Results

Baseline values and effects of NAC treatment

The initial body weight and water intake and food consumption did not differ among groups. All animals (100%) responded to the STZ induction of diabetes and no animals died or were excluded from the study. At 72 h after injection of STZ, the diabetic rats had higher plasma glucose levels (Diabetic: 23.7 ± 2.0 mM; Diabetic treated: 24.8 ± 1.6 mM) than in control rats (Control: 8.0 ± 0.2 mM; Control treated: 8.3 ± 0.3 mM) ($P < 0.01$). One week after STZ injection, plasma insulin levels in diabetic rats (Diabetic: 0.99 ± 0.31 ng/ml; Diabetic treated: 0.96 ± 0.16 ng/ml) were reduced as compared with control rats (Control: 2.23 ± 0.18 ng/ml; Control treated: 1.98 ± 0.18 ng/ml) ($P < 0.01$).

As shown in Table I, values of water intake, food consumption and plasma glucose levels were higher in group D than in control groups, and treatment with NAC for 8 weeks significantly reduced these changes. Plasma insulin levels in group DT and group D did not differ significantly. At termination, body weight in group D was lower than that in control groups, NAC treatment did not affect body weight gain significantly (Table II).

Heart and ventricular weights and physiological parameters

At sacrifice, the heart weight and the sum of left and right ventricle weights in group D was similar to that in the control groups irrespective of the significantly lower body weight in group D relative to the control groups (Table II). Consequently, a significant increase in heart weight to body weight ratio and the ventricle weight to body weight ratios were seen in group D as compared to control groups. In contrast, the heart weight and the sum of left and right ventricle weights in group DT were significantly lower than that in group D and control groups. Thus, treatment with NAC normalised the heart to body weight ratio. Ventricle weight to body weight ratio in group DT remained higher than group C, but was lower than that in group D. Cardiomyocyte cross sectional area was significantly increased in the untreated diabetic rats, but not in the NAC treated diabetic rats as compared to control rats (Table II).

As shown in Table III, values of MAP, SBP, HR and DBP in diabetic rats were significantly lower than that in control rats. NAC treatment normalised these changes. Also, the value of RPP in group D was much

Table I. Water intake, food consumption, plasma glucose and insulin.

	C	C-T	D	D-T
Water intake (ml/kg/day)	108.4 ± 8.8	86.7 ± 2.17	759.9 ± 48.4⋆	613.9 ± 24.6⋆#
Food consumption (g/kg/day)	62.4 ± 0.9	65.4 ± 0.9	160.5 ± 7.6⋆	144.1 ± 5.1⋆#
Plasma glucose (mM)	8.3 ± 0.1	8.5 ± 0.3	33.7 ± 5.7⋆	27.3 ± 0.9⋆#
Plasma insulin (ng/ml)	1.4 ± 0.2	1.1 ± 0.1	0.2 ± 0.1⋆	0.3 ± 0.1⋆

Values were obtained after 9 weeks of STZ-induced diabetes or vehicle administration and following 8 weeks of NAC treatment. All values are means ± SEM, $n = 7$. Control (C), control + NAC treatment (CT), diabetic (D) and diabetic + NAC treatment (DT). ⋆$P < 0.05$ significantly different from control and control treated; #$P < 0.05$ significantly different from diabetic.

Table II. Body weight, heart weight, ventricular weight and myocyte cross sectional area.

	C	C-T	D	D-T
Body weight (g)	512 ± 6	484 ± 10	383 ± 19⋆	345 ± 15⋆
Heart weight (g)	1.48 ± 0.03	1.44 ± 0.03	1.32 ± 0.04	1.07 ± 0.03⋆#
LV + RV weight (g)	1.23 ± 0.02	1.20 ± 0.02	1.15 ± 0.03	0.92 ± 0.03⋆#
Heart/body weight (g/kg)	2.88 ± 0.04	2.98 ± 0.04	3.46 ± 0.03⋆	3.12 ± 0.07
LV + RV/body weight (g/kg)	2.39 ± 0.04	2.45 ± 0.03	3.05 ± 0.10⋆	2.69 ± 0.06⋆#
Myocyte cross sectional area (μm^2)	180 ± 40	190 ± 40	297 ± 72⋆	194 ± 35#

Values were obtained after 9 weeks of STZ-induced diabetes or vehicle administration and following 8 weeks of NAC treatment. All values are means ± SEM, $n = 7$. Control (C), control + NAC treatment (CT), diabetic (D) and diabetic + NAC treatment (DT). LV and RV indicate left ventricle and right ventricle, respectively. ⋆$P < 0.05$ significantly different from control and control treated; #$P < 0.05$ significantly different from diabetic.

lower than that in control groups. Treatment with NAC in diabetes partially restored the RPP value to a level that is higher than in group D but significantly lower than in control groups. Interestingly, rats in group DT maintained MAP, SBP, DBP and HR within normal ranges.

Oxidative stress

As shown in Figure 1A, plasma total antioxidant concentration was 1.20 ± 0.05 mM in untreated control rats and was decreased to 0.78 ± 0.1 mM in group D. NAC restored plasma total antioxidant concentration to 1.15 ± 0.08 mM in group DT, which is comparable to the value in group C. NAC also increased plasma total antioxidant concentration in control rats.

Figure 1B demonstrated a significant increase in plasma level of free 15-F_{2t}-isoprostane in group D ($P < 0.01$ vs. groups C or CT), indicative of increased oxidative stress and subsequent increase of reactive oxygen species induced lipid peroxidation. NAC treatment in diabetic rats prevented the increase in plasma free 15-F_{2t}-isoprostane.

Plasma and heart tissue levels of nitrate and nitrite (NOx)

At termination, plasma NOx was significantly decreased in diabetic rats (6.1 ± 0.9 μM) compared with control rats (11.5 ± 1.1 μM) (Figure 2A). NAC treatment restored plasma NOx to 10.1 ± 0.8 μM in group DT. However, NAC treatment in diabetic rats did not restore heart tissue levels of NOx, which was significantly decreased in diabetic rats as compared with control rats (Figure 2B).

Immunohistochemical staining and Western Blotting for eNOS protein

As shown in Figures 3 and 4, in non-diabetic rats, immunohistochemical studies showed positive eNOS immunostaining in the vascular endothelium of the thoracic aorta and mesenteric artery sections tested. In diabetic rats, eNOS expression was significantly decreased in LV as shown by immunostaining (data not shown) and was confirmed by Western Blot analyses (Figure 2C). Similarly, eNOS immunostaining in aortic (Figure 3) and mesenteric artery (Figure 4) vascular endothelium was decreased. Treatment with NAC in group DT prevented the loss of eNOS protein expression in LV seen in group D and improved eNOS expression in aortic and mesenteric artery vascular endothelium.

Heart tissue cyclic GMP

At termination, the heart tissue concentration of cGMP did not significantly differ between diabetic and control rats (Figure 5). Treatment of diabetic rats with NAC significantly increased heart tissue cGMP. NAC, however, did not alter the cGMP concentration in control rat hearts.

Table III. Haemodynamic parameters.

	C	C-T	D	D-T
SBP (mmHg)	148 ± 5	150 ± 3	121 ± 4⋆	136 ± 4#
DBP (mmHg)	117 ± 5	116 ± 4	101 ± 4⋆	112 ± 4
MAP (mmHg)	127 ± 5	128 ± 4	109 ± 3⋆	120 ± 4#
HR (beat/min)	414 ± 10	423 ± 8	334 ± 10⋆	376 ± 16#
RPP ($\times 10^3$)	61.3 ± 3.5	63.4 ± 1.7	40.5 ± 2.3⋆	51.2 ± 2.9⋆#

Values were obtained after 9 weeks of STZ-induced diabetes or vehicle administration and following 8 weeks of NAC treatment. SBP, DBP, MAP and HR were measured in conscious status before sacrifice through carotid artery cannulization. RPP indicates rate (HR) and pressure (SBP) product. All values are means ± SEM, $n = 7$. Control (C), control + NAC treatment (C-T), diabetic (D) and diabetic + NAC treatment (D-T). Statistical analysis was done by one-way ANOVA followed by Tukey's test. ⋆$P < 0.05$ significantly different from control and control treated; #$P < 0.05$ significantly different from diabetic.

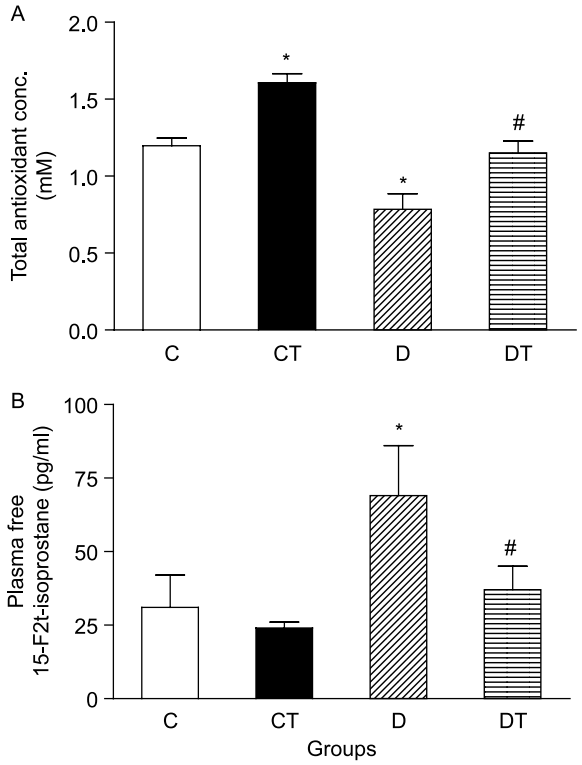

Figure 1. Plasma concentration of total antioxidants (A) and plasma free 15-F_{2t}-isoprostane (B) in control and diabetic rats. C, CT, D, DT indicate control, control NAC-treated, diabetic and diabetic NAC-treated groups, respectively. All values are means ± SEM, $n = 7$. $^{\star}P < 0.05$ significantly different from control and control treated; $^{\#}P < 0.05$ significantly different from diabetic.

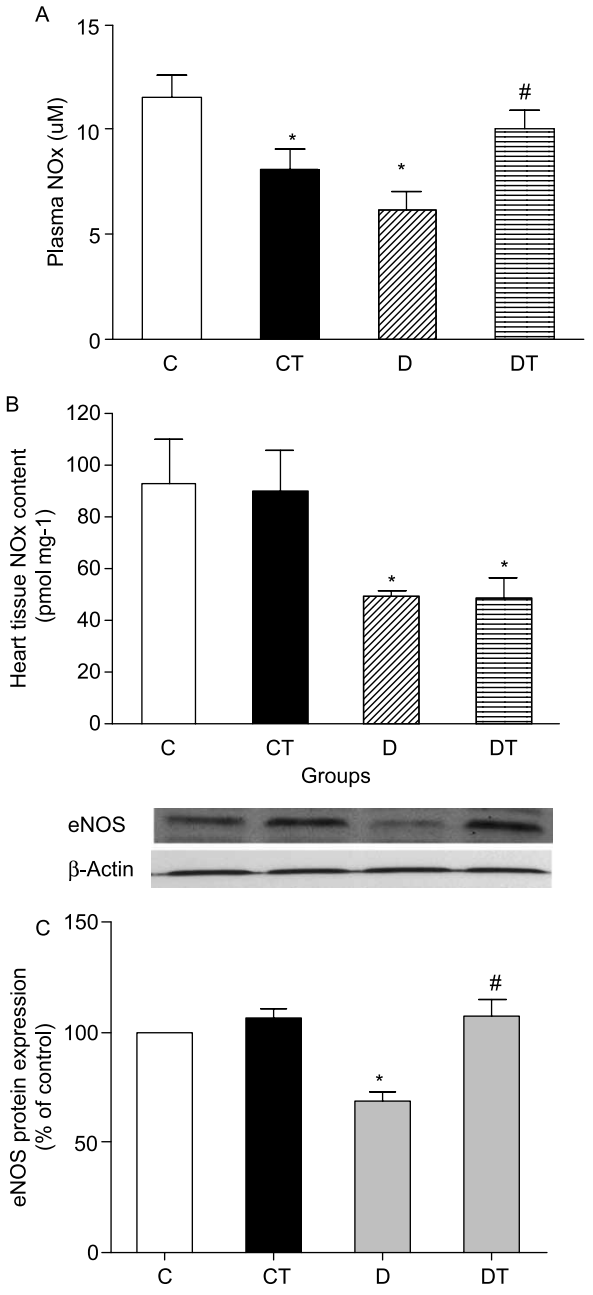

Figure 2. Plasma (A) and heart tissue (B) levels of NOx, stable metabolites of nitric oxide. C, CT, D, DT indicate control, control NAC-treated, diabetic and diabetic NAC-treated groups, respectively. Values are means ± SEM, $n = 7$. (C) Myocardial eNOS expression as quantified by Western Blotting analysis. C, D, CT and DT indicate control, diabetic, control NAC treated and diabetic NAC treated rats, respectively. Values are means ± SEM, $n = 4$. $^{\star}P < 0.05$ significantly different from control and control treated; $^{\#}P < 0.05$ significantly different from diabetic.

Correlation analysis

A positive correlation was demonstrated between plasma levels of NOx and heart tissue levels of cGMP in diabetic groups ($r = 0.60$, $n = 14$, $P < 0.03$, Pearson test), but not in control groups (data not shown). An inverse correlation was found between RPP and heart tissue levels of cGMP in control ($r = -0.78$, $n = 7$, $P = 0.04$) and in diabetic NAC treated rats ($r = -0.82$, $n = 7$, $P = 0.02$). Taken together, a significant inverse relationship between RPP and heart tissue cGMP existed in control and diabetic NAC-treated rats ($r = -0.80$, $n = 14$, $P = 0.0006$). Such a relationship did not exist in diabetic-untreated rats, nor did it exist in control NAC treated rats (data not shown).

Discussion

This study shows that STZ-induced diabetes is associated with enhanced oxidative stress and compromised plasma total antioxidant capacity, accompanied by reduced NO bioavailability following 9 weeks of diabetes. Arterial blood pressures (MAP, SBP) and heart rate were depressed in the STZ-induced diabetic rats. This result is in accordance with recent reports from our laboratory [24] and others [25,26] showing reductions in blood pressure and HR in rats following a moderate duration of STZ-induced diabetes. Antioxidant treatment with NAC not only normalised plasma total antioxidant capacity and prevented oxidative stress induced lipid peroxidation, but also simultaneously restored systemic NO bioavailability, normalised heart-to-body weight ratio

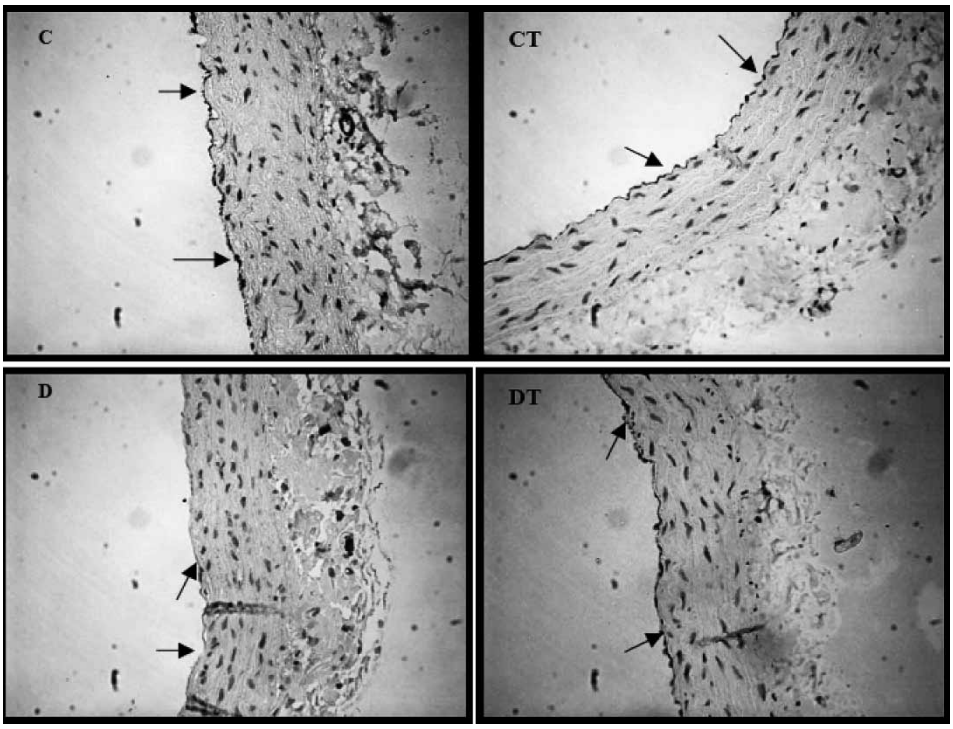

Figure 3. Effects of NAC treatment on eNOS expression in the aorta. Representative microphotographs of eNOS immunostaining in aorta endothelium of 9 weeks C, D, CT and DT. Magnification (× 25). eNOS staining (brown) can hardly been seen in diabetic aortic endothelium (D). Treatment with NAC normalised eNOS expression in DT aorta endothelium.

and restored the compromised arterial blood pressure and HR to normal. These results suggest that the increase in oxidative stress and the consequent reduction in NO bioavailability may have adversely affected cardiac efficiency and/or vascular reactivity *in vivo*, resulting in depression of arterial blood pressures in diabetic rats.

It is of particular interest that diabetic rats treated with NAC could maintain MAP, a major haemodynamic parameter, in the normal range while keeping the values of RPP, an indirect index of myocardial oxygen demand [27,28], at a relatively lower level than that in control rats (Table III). NAC may have

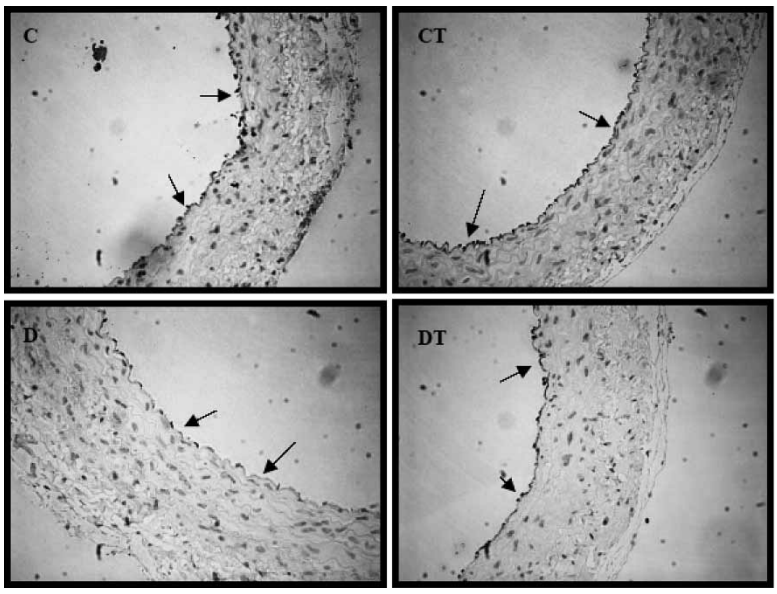

Figure 4. Effects of NAC treatment on eNOS expression in the mesentery artery. Representative microphotographs of eNOS immunostaining in mesentery artery endothelium of 9 weeks C, D, CT and DT. Magnification (× 25). eNOS staining (brown) was evidently reduced in diabetic mesentery artery endothelium (D). Treatment with NAC normalised eNOS expression in DT mesentery artery endothelium.

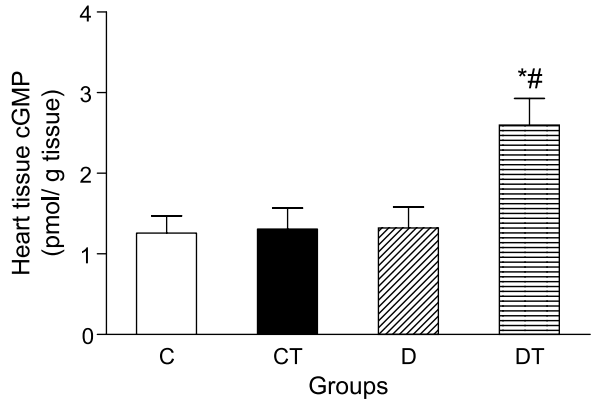

Figure 5. Heart tissue levels of cGMP. C, CT, D, DT indicate control, control NAC-treated, diabetic and diabetic NAC-treated groups, respectively. Values are means ± SEM, $n = 7$. $^{*}P < 0.05$ significantly different from control and control treated; $^{\#}P < 0.05$ significantly different from diabetic.

enhanced myocardial oxygen utilization efficiency through a mechanism yet to be elucidated. It is known that dilatation of coronary vessels is impaired in diabetic patients when myocardial metabolic demand is increased and that coronary microvascular adaptation to myocardial metabolic demand can be restored by inhibition of oxygen free radical formation [29]. Results from our current study suggest that restoration of the availability of NO, a potent vasodilator, and/or the subsequently increased activation of myocardial cGMP may represent a mechanism whereby NAC may have enhanced myocardial oxygen utilization efficiency in diabetic rat hearts, contributing in part, to the normalization of arterial blood pressure and heart rate.

NO has been proposed to play a significant role in experimental and human diabetes mellitus [30–32], but its role is still controversial [33]. The duration and the severity of diabetes and differences in experimental diabetic models may explain the discrepancy. A study from our laboratory has found that plasma total antioxidant concentration and the extent of lipid peroxidation as well as plasma levels of NO are near normal in STZ-induced diabetic rats at 3 weeks of diabetes (Xia et al. unpublished data). However, in the current study, at 9 weeks of diabetes, plasma total antioxidant concentration was decreased, accompanied by a significant increase in plasma free 15-F_{2t}-isoprostane, a sensitive and reliable index of *in vivo* oxidative stress-induced lipid peroxidation and an independent risk marker of coronary heart disease [34,35]. Also, the plasma levels of NO were significantly reduced in diabetic rats. NAC treatment in the diabetic rats restored plasma total antioxidant concentration and plasma levels of NO, suggesting that reduction in NO is a consequence of hyperglycaemia induced oxidative stress.

NO has both cytoprotective and cytotoxic effects. In the circumstance of increased oxidative stress with compromised endogenous antioxidant capacity such as in diabetes of considerable duration, NO can react with superoxide anion to form the very reactive peroxynitrite ($ONOO^{-}$) [36]. This reaction reduces NO bioavailability. Peroxynitrite will cause tyrosine nitration in proteins and stimulate nuclear factor-kappaB (NF-kappaB) mediated gene expression, thus enhancing systemic inflammatory reactivity [36,37]. Furthermore, increased peroxynitrite may cause myocardial contractile dysfunction [38].

The significant correlation between plasma levels of NO and heart tissue levels of cGMP in rats from diabetic groups suggests that NAC restoration of plasma NO may contribute to the significant increase in heart tissue cGMP seen in the diabetic treated group. It was unexpected that the heart tissue cGMP levels in the diabetic untreated group remained in the normal range (comparable to that in control groups, Figure 5), despite a significant reduction in plasma and heart tissue levels of NO. Therefore, it may be argued that the increased heart tissue cGMP in DT rats may represent a mechanism by which the antioxidant NAC exerts its protective effects. A study has shown that the endogenous B-type natriuretic peptide (BNP) may serve to prevent acute hypertrophic responses in the diabetic rat heart via the cGMP pathway and the antihypertrophic and cGMP stimulatory actions of BNP are preserved in diabetic rat hearts [39]. In fact, the BNP level has been reported to be increased in diabetes in response to left ventricular volume expansion and pressure overload [40]. Therefore, the potential increase in BNP levels in the diabetic untreated group may have served to stimulate heart tissue cGMP.

It is of interest that NAC did not restore tissue NO availability in diabetic rat hearts despite a restoration of myocardial eNOS protein expression. The mechanism is not clear and deserves further study. One possible explanation is that the NAC action could be tissue specific and the NAC dose used in the current study might not be sufficient to prevent the formation of peroxynitrite in the myocardium. Peroxynitrite has been shown to cause eNOS uncoupling [41], resulting in decreases in NO synthesis. In addition, hyperglycaemia has been shown to inhibit bovine aortic endothelial eNOS activity by posttranslational modification at the protein kinase Akt/PKB site [42] and inhibit eNOS activity and NO production without reducing eNOS Ser1177 phosphorylation in human aortic endothelial cells [43]. Therefore, hyperglycaemia may have compromised eNOS activation in the myocardium of the diabetic rats. Alternatively, NAC has been shown to decrease *in vivo* NO production by inhibiting inducible NO synthase [44] and down regulate inducible NO synthase gene expression *in vitro* in human hepatocytes [45]. NAC could also negatively modulate NO production, independent of

inducible NO synthase, through inhibition of NF-kappaB activation [46]. Further study is needed to address whether NAC treatment can restore eNOS activity in the diabetic myocardium.

It is noteworthy that NAC treatment (DT group) significantly reduced the plasma glucose level as compared with the diabetic group (D group, $P < 0.01$, Table I), which may be attributable to a NAC effect in preventing hyperglycaemia induced insulin resistance [47], 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 to the reduction of water-intake in diabetic rats (Table I).

In summary, the present study confirms that decreases in arterial blood pressure and heart rate occur in the STZ-induced diabetic rats. The mechanisms for the depressions in arterial blood pressure and heart rate are uncertain, but may be attributable, in part, to diminished arterial pressure reactivity to sympathetic stimulation [48,49]. Restoration of circulatory NO bioavailability and the subsequently increased activation of myocardial cGMP may represent a mechanism by which the antioxidant NAC normalised arterial blood pressures and HR in the diabetic rats. Our recent study indicates that NAC can increase cardiac contractility in response to dobutamine stimulation *in vivo* in STZ-diabetic rats [50], suggesting NAC may restore arterial pressure reactivity to sympathetic stimulation in this model. Further study is needed to address the effects of NAC on hyperglycaemia induced pathological changes in the cardiomyocytes and myocardial endothelial cells.

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