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The effect of *N*-acetylcysteine on cardiac contractility to dobutamine in rats with streptozotocin-induced diabetes

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Abstract

We examined if myocardial depression at the acute phase of diabetes (3 weeks after injection of streptozotocin, 60 mg/kg i.v.) is due to activation of inducible nitric oxide synthase and production of peroxynitrite, and if treatment with *N*-acetylcysteine (1.2 g/day/kg for 3 weeks, antioxidant) improves cardiac function. Four groups of rats were used: control, *N*-acetylcysteine-treated control, diabetic and *N*-acetylcysteine-treated diabetic. Pentobarbital-anaesthetized diabetic rats, relative to the controls, had reduced left ventricular contractility to dobutamine (1–57 μg/min/kg). The diabetic rats also had increased myocardial levels of thiobarbituric acid reactive substances, immunostaining of inducible nitric oxide synthase and nitrotyrosine, and similar baseline 15-F_{2t}-isoprostane. *N*-acetylcysteine did not affect responses in the control rats; but increased cardiac contractility to dobutamine, reduced myocardial immunostaining of inducible nitric oxide synthase and nitrotyrosine and level of 15-F_{2t}-isoprostane, and increased cardiac contractility to dobutamine in the diabetic rats. Antioxidant supplementation in diabetes reduces oxidative stress and improves cardiac function.

Keywords: Inducible nitric oxide synthase; Nitrotyrosine; Isoprostane; Cardiac contractility; Antioxidant

1. Introduction

Diabetes mellitus is associated with cardiac contractile dysfunction. Depression of left ventricular contractility has been observed in rats as early as four to eight weeks after the induction of type I diabetes by streptozotocin (Dai and McNeill, 1992; Dowell et al., 1986; Litwin et al., 1990; Okayama et al., 1994). Also, cardiac inotropic responses to noradrenaline and dobutamine are depressed in vivo (Paulson et al., 1986) and ex vivo (Smith et al., 1997) at eight weeks after the induction of streptozotocin-induced diabetes in rats. Furthermore, the shortening and relengthening phases of contraction are prolonged in isolated ventricular myocytes from rats with streptozotocin-induced diabetes for 4–6 days or 8 weeks (Ren and Davidoff, 1997). The cause of myocardial depression and reduced responses to adrenoceptor agonists at the acute phase of diabetes remains unclear.

Several studies have shown that the inducible isoform of nitric oxide synthase (iNOS) is activated at the acute and chronic phase of diabetes. Indeed, inducible nitric oxide synthase is detected in cardiac myocytes from diabetic rats at 4 to 8 weeks after injection of streptozotocin, but not in myocytes from control rats (Smith et al., 1997; El-Omar et al., 2003). Furthermore, non-selective inhibition of NOS by N^G-nitro-L-arginine methyl ester (L-NAME) has been shown to improve ventricular performance in isolated hearts from rats with streptozotocin-induced diabetes (Smith et al., 1997). These results suggest that the activation of inducible nitric oxide synthase in diabetes contributes to cardiac contractile dysfunction. Excessive production of nitric oxide leads to the formation of peroxynitrite ($ONOO^-$), a highly reactive oxidant produced by the interaction of the nitric oxide with the free radical superoxide (O_2^-) (Beckman and Koppenol, 1996; Ceriello et al., 2002b;

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Ischiropoulos, 1998). It is unclear what causes the induction of inducible nitric oxide synthase; however, there is an indication that hyperglycemia may be a factor. Indeed, one week of exposure of cultured human aortic endothelial cell to high glucose is associated with the formation of superoxide anion, peroxynitrite and nitrotyrosine (product of peroxynitrite-induced nitration of tyrosine) as well as cell apoptosis (Zou et al., 2002). In addition to oxidizing proteins, peroxynitrite causes lipid peroxidation which can lead to cellular damage. There is also evidence of increased peroxynitrite formation in the blood of patients with type II diabetes (Ceriello et al., 2001, 2002a) and platelets of patients with types I and II diabetes (Tannous et al., 1999). These observations suggest that myocardial depression at the early phase of diabetes is due to hyperglycemia-induced production of reactive oxygen species such as nitric oxide and peroxynitrite. If this is true, exogenous administration of antioxidants should reduce the formation of reactive oxygen species and improve cardiac performance in diabetes.

The first part of this study examined if rats with streptozotocin-induced diabetes for three weeks were associated with the activation of inducible nitric oxide synthase, formation of peroxynitrite, reduced myocardium antioxidative capacity, and depressed cardiac function. The second part examined if chronic treatment with *N*-acetylcysteine (antioxidant and inhibitor of inducible nitric oxide synthase) reduced the activation of inducible nitric oxide synthase, decreased the formation of peroxynitrite, increased antioxidative capacity and improved cardiac contractile function in rats with streptozotocin-induced diabetes. In this study, nitrotyrosine, 15-F_{2t}-isoprostanes and thiobarbituric acid reactive substances are used as markers of in vivo peroxynitrite-induced tyrosine nitration, lipid peroxidation and cellular lipid peroxidation damage, respectively (Mezzetti et al., 2000).

2. Materials and methods

The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

2.1. Induction of diabetes

Male Wistar rats (300–350 g, 11–12 weeks old) were injected with streptozotocin (60 mg/kg, i.v., Sigma) or the vehicle (0.9% NaCl) via the tail vein under light halothane anaesthesia. The rats were considered to be diabetic and used for the study if they had hyperglycemia (>15 mmol/l) at 48 h after injection of streptozotocin, as detected by AccuSoft test strips (Hoffmann-La Roche Ltd., Sambandam et al., 2000; Rodrigues et al., 1999). Less than 5% of rats were excluded from the experiment by this criteria. The rats were studied at three weeks after the injection of streptozotocin. No rats died after the administration of streptozotocin during the study period, and no insulin supplement was applied since 60 mg/kg of streptozotocin was shown to destroy ~90% of pancreatic β-cells and to induce stable diabetes in male Wistar rats without supplementation of insulin to enhance survival (Rodrigues et al., 1999). Plasma glucose level was measured by the glucose oxidase method (Sigma, Trinder 100 kit) via the use of a Spectrarainbow (ART F039039, Austria).

2.2. Administration of N-acetylcysteine

At 48 h after i.v. injection of streptozotocin, half of the control and diabetic rats were given *N*-acetylcysteine (Sigma, 1–2 g/l) which was added to the drinking water, and the remaining rats were given tap water. The concentration of the drug in the water was adjusted daily according to the amount of water consumed the previous day. The amount of *N*-acetylcysteine consumed by the rats during the study (19–21 days) was 1.2±0.1 g/day per kg body weight.

2.3. Preparation of rats

The rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), tracheotomised, and allowed to breath spontaneously in room air. Body temperature was maintained at 37 °C by a rectal thermometer and a heat lamp connected to a Thermistemp Temperature Controller (Model 71, Yellow Springs Instrument Co., Ohio, USA). Polyethylene cannulae (PE50), filled with heparinized saline (0.9% NaCl, 25 I.U./ml), were inserted into the left femoral vein for the administration of drugs, and left femoral artery for the measurement of mean arterial pressure by a pressure transducer (P23DB, Gould Statham, CA, USA) as well as the withdrawal of a blood sample for the measurement of blood glucose. Heart rate was derived from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). The right femoral artery was also cannulated for the withdrawal of a reference blood sample for cardiac output measurements by the microspheres technique (Pang, 1983). This technique also involved the insertion of a polyethylene (PE50) cannula into the left ventricle through the right carotid artery for the recording of left ventricular peak systolic pressure by a pressure transducer. All pressure recordings were displayed on a polygraph (Grass 7D, Grass Instruments, Quincy, MA, USA). A differentiator (Model 7P20C, Grass Instruments, Quincy, MA, USA) was used to derive the maximal rate of increase (+dP/dt) and decrease (−dP/dt) of left ventricular pressure during contraction and relaxation, respectively.

2.4. Immunostaining of nitrotyrosine, inducible nitric oxide synthase and endothelial nitric oxide synthase

The tissues were rinsed in phosphate-buffered saline (0.1 M) and cut into small blocks (5×5×5 mm). The tissue blocks were embedded in Tissue-Tek O.C.T, frozen in liquid nitrogen and stored at −70 °C. Sections (8 μm thick) of the tissues were cut at −20 °C, collected on slides, and fixed with liquid nitrogen-cooled acetone. Immunohistochemical staining for inducible nitric oxide synthase involved incubation of the sections for 3 min in hydrogen peroxidase (0.88 mol/l) to quench endogenous peroxidase activity, treatment with a blocking solution (Vectastain universal quick kit, Vector Laboratory) for 30 min, incubation with polyclonal anti-nitrotyrosine antibody (rabbit

anti-nitrotyrosine, 1:250, pH 7.2, containing 0.1% bovine serum albumin, Upstate Biotechnology) for 60 min, incubation with biotinylated panspecific universal secondary antibody for 10 min, and incubation with streptavidin–peroxidase complex (Vectastain universal quick kit, Vector Laboratory) for 20 min. A 5-min wash with phosphate-buffered saline (0.1 mol/l, pH 7.2) was performed between each step, and a solution of 3, 3′-diaminobenzidine (0.5 mg/ml in 0.1 mol/l phosphate-buffered saline, pH 7.2; Sigma) was used as the chromogen. Some sections were treated with mouse nonspecific immunoglobulin G (1:200, Vector Laboratory) instead of the primary antibody and they served as negative controls. The immunohistochemical staining protocols for inducible nitric oxide synthase and endothelial nitric oxide synthase (eNOS) were similar to that for nitrotyrosine except that the polyclonal anti-inducible nitric oxide synthase antibody (rabbit anti-inducible nitric oxide synthase, 1:500, pH 7.2, with 0.1% bovine serum albumin, BioMol) and the polyclonal anti-endothelial nitric oxide synthase antibody (rabbit anti-endothelial nitric oxide synthase, 1:500, pH 7.2, with 0.1% bovine serum albumin, BioMol), respectively, were used as primary antibodies.

2.5. 15-F_{2t}-isoprostane assay

2.5.1. Extraction

The extraction and enzyme immunoassay procedures used for the measurement of tissue 15-F_{2t}-isoprostane were as described by the manufacturer (8-Isoprostane extraction and enzyme immunoassay Kit, Cayman Chemical, Ann Arbor, MI), but with slight modifications as follows. The ventricular tissues (0.5 g) were homogenized in phosphate-buffered saline (1.0 ml). 15-F_{2t}-isoprostane in the homogenate was extracted with ethanol (2.0 ml) containing butylated hydroxytoluene (0.005%) to prevent oxidation, and centrifuged (2000 ×*g*) for 10 min. The supernatant was decanted into a test tube to which potassium hydroxide (15%) was added for the determination of total 15-F_{2t}-isoprostane by base hydrolysis. The samples were incubated at 40 °C for 1 h, and diluted 10-fold with ultra pure water. The pH was adjusted to 7.0–7.5 with HCl for purification.

2.5.2. Purification

Each sample was passed through a 4 ml 15-F_{2t}-isoprostane (8-isoprostane) affinity column (Cayman Chemical). The affinity column was first washed with column buffer (2 ml), UltraPure water (2 ml), then elution solution (2 ml) to elute 15-F_{2t}-isoprostane from the columns. The elution solution was collected in test tubes and evaporated to dryness under a stream of dry nitrogen. Afterwards, extraction and enzyme immunoassay buffer (0.5 ml) was added immediately to dissolve 15-F_{2t}-isoprostane before its quantification by extraction and enzyme immunoassay as described (Xia et al., 2003). In brief, standards (50 μl) and samples were added in duplicates to a 96-well plate, and this was followed by the addition of 15-F_{2t}-isoprostane acetylcholinesterase tracer and antibody. The prepared plates were incubated overnight at room temperature. The next day, the plates were washed five times with the wash buffer, followed by the addition of Ellman's reagent. After optimal development for 80–100 min, the plates containing 15-F_{2t}-isoprostane were read at 405 nm with the operator blinded.

The average sample recovery rate was 90±5 % (mean±S.E.M., $n=8$) as estimated using the 15-F_{2t}-isoprostane standards. All measured values were corrected by this recovery rate. Tissue 15-F_{2t}-isoprostane content was expressed as pg/g tissue.

2.6. Myocardium antioxidant capacity determination

Myocardial tissue antioxidant capacity was determined by exposure of the tissue homogenates to *t*-butylhydroperoxide (peroxidizing agent), and this resulted in the formation of lipid by-products, which formed chromogens when incubated with thiobarbituric acid (collectively termed thiobarbituric acid reactive substances). A lower tissue antioxidant capacity results in the formation of a greater amount of thiobarbituric acid reactive substances in the presence of *t*-butylhydroperoxide. The level of thiobarbituric acid reactive substances in the sample was estimated from the absorbance at 532 nm. Ventricular myocardium samples (300 mg) were thawed and homogenized on ice in Tris–EDTA buffer (0.05–0.1 mol/l) using a Polytron homogenizer (PT-10, Brinkman Instruments, Canada) for 30 s at 25% power. The homogenates were used for in vitro forced peroxidation using *t*-butylhydroperoxide and subsequent determination of thiobarbituric acid reactive substances, as described (Runzer et al., 2002). In brief, tissue homogenate (400 μl) was combined with *t*-butylhydroperoxide (400 μl in 0.9% saline with 2 mmol/l sodium azide to produce final concentrations of *t*-butylhydroperoxide, 0.5 to 5 mmol/l). The suspensions were incubated for 30 min at 37 °C, after which 400 μL of cold trichloroacetate (28% w/v)-sodium arsenite (0.1 mol/l) was added. The mixture was centrifuged at 12,000 ×g for 5 min at 4 °C. Supernatant (800 μl) was removed and added to thiobarbituric acid (400 μl of 0.5% in 25 mmol/l NaOH). The samples were boiled for 15 min, and the absorbance was measured spectrophotometrically at 532 nm. All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.7. Experimental protocol

2.7.1. Protocol I

The rats were divided into four groups ($n=6$ each) as follows: control rats, control rats given *N*-acetylcysteine, diabetic rats, and diabetic rats given *N*-acetylcysteine. Baseline readings of mean arterial pressure, heart rate, left ventricular pressure and left ventricular $+/-dP/dt$ were taken at 1 h after the completion of surgery. Dose–response curves of dobutamine (1–57 μg/min/kg, 1–2 min duration) were constructed in each group with a recovery period of 10–15 min between doses. Plateau responses were used for statistical analysis.

2.7.2. Protocol II

Another four groups of rats (as in Protocol I) were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). The hearts were excised and cleaned in cold saline. Myocardial samples were weighed and flash frozen in liquid nitrogen for 15-F_{2t}-isoprostane assay, and used for the determination of myocardium antioxidant capacity, and immunostaining of nitrotyrosine, inducible nitric oxide synthase and endothelial nitric oxide synthase using the Vectastain universal quick kit. All samples were stored at −70 °C until assayed.

2.8. Statistical analysis

ED_{50} and E_{max} values were derived from analyses of dose–response curves using the GraphPad Prism program (GraphPad software, Inc, USA). To render the homogeneity of variances,

the ED_{50} data were log-transformed prior to statistical analysis. The data were analysed using analysis of variance (ANOVA) followed by multiple group comparison via Tukey test with $P<0.05$ selected as the criterion for statistical significance (SigmaStat, Jandel Scientific software, USA). The results are presented as mean±S.E.M.

3. Results

3.1. Baseline values in control and diabetic rats in the absence or presence of treatment with N-acetylcysteine

At 48 h after injection of streptozotocin, the rats had higher plasma concentration of glucose compared to the control rats (23.5 ±1.5 versus 5.7±0.8 mmol/l, $P<0.05$, $n=14–15$). At three weeks after injection of streptozotocin, the body weight of the diabetic rats was lower while plasma glucose was higher than those of the controls (Table 1). Baseline heart rate, left ventricular +dP/dt and −dP/dt readings of the diabetic rats were significantly ($P<0.05$) lower, but mean arterial pressure and left ventricular peak systolic pressure were not significantly different between the diabetic and control rats (Table 1).

Chronic administration of *N*-acetylcysteine did not significantly alter any baseline readings of body weight, plasma glucose, arterial pressure, heart rate or ventricular contractile function in the control rats. *N*-acetylcysteine also did not prevent changes in the body weight, plasma glucose and heart rate in the diabetic rats, but it partially restored left ventricular +dP/dt and −dP/dt in the diabetic rats (Table 1).

3.2. Cardiac contraction to dobutamine in control and diabetic rats

Dobutamine caused dose-dependent increases in left ventricular pressure, left ventricular +/−dP/dt and heart rate in the control rats and diabetic rats not treated with *N*-acetylcysteine; however, the increases were significantly greater in the control than diabetic rats (Fig. 1). Relative to the controls, the diabetic rats had significantly higher ED_{50} for left ventricular pressure (3.0-fold), +dP/dt (2.9-fold), −dP/dt (2.4-fold) and heart rate (3.9-fold) responses to dobutamine (Fig. 2), and significantly lower E_{max} values for left ventricular pressure (60% of controls),

Table 1
Plasma glucose, body weight and baseline values of mean arterial pressure (MAP), heart rate, maximal left ventricular systolic pressure (LVP), maximal rate of increase (+dP/dt) and decrease (−dP/dt) of LVP in four groups of pentobarbital-anaesthetized rats at 3 weeks following injection of streptozotocin (60 mg/ml/kg i.v., diabetic) or vehicle (0.9% NaCl, control), either with or without administration of *N*-acetylcysteine (NAC, 1.2±0.1 g/day) in the drinking water

	Control	Control +NAC	Diabetes	Diabetes +NAC
Body weight (g)	432±8	440±6	373±9[a]	362±15[a]
Plasma glucose (mM)	6.1±0.3	6.2±0.4	28.5±1.2[a]	27.0±2.9[a]
MAP (mm Hg)	102±4	102±2	99±3	106±5
Heart rate (beats/min)	347±8	348±6	316±9[a]	315±7[a]
LVP (mm Hg)	135±3	138±2	128±3	134±4
+dP/dt (mm Hg/s)	11,655±421	11,978±388	9729±371[a]	11,096±202
−dP/dt (mm Hg/s)	10,638±378	11,054±339	8335±210[a]	9935±141[b]

Results represent the mean±S.E.M., $n=6–7$ per group.
[a] $P<0.05$ versus corresponding values in the untreated control group.
[b] $P<0.05$ versus corresponding values in the diabetic untreated group.

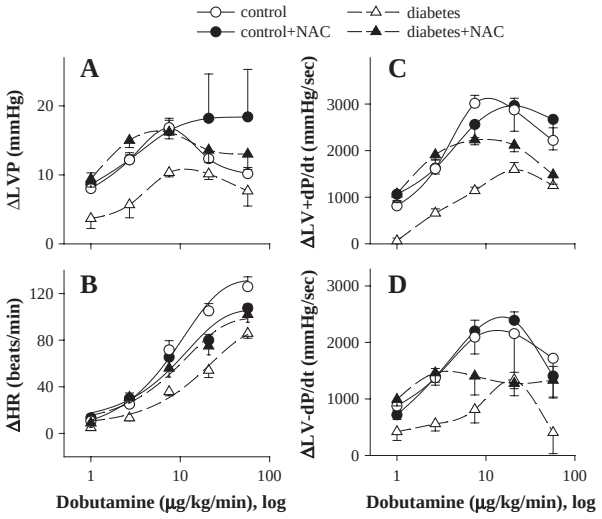

Fig. 1. Effects (mean±S.E.M., $n=6–7$ each) of chronic treatment of *N*-acetylcysteine (NAC, 1.2 g/kg per day) on actions of dobutamine (1–57 μg/kg/min) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR, B), maximal rate of rise (+dP/dt, C) and fall (−dP/dt, D) of LVP in pentobarbital-anaesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes.

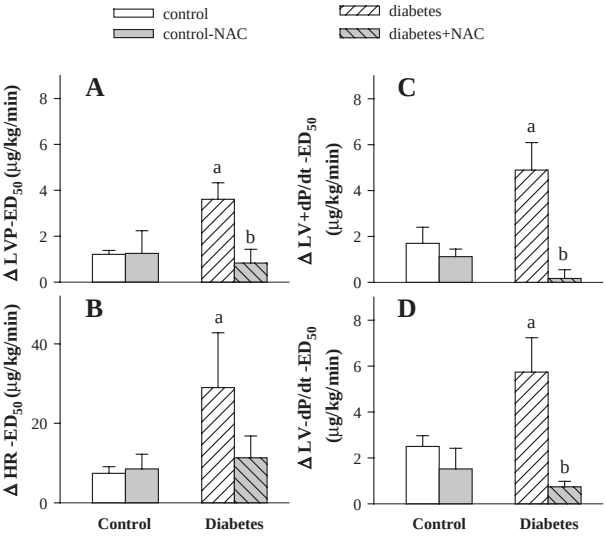

Fig. 2. Effects (mean±S.E.M., $n=6–7$ each) of chronic treatment of *N*-acetylcysteine (NAC, 1.2 g/kg per day) on potency (ED_{50}) of response to dobutamine (1–57 μg/kg/min) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR, B), maximal rate of rise (+dP/dt, C) and fall (−dP/dt, D) of LVP in pentobarbital-anaesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes. [a]Significantly different ($P<0.05$) from untreated control rats. [b]Significantly different from untreated diabetic rats.

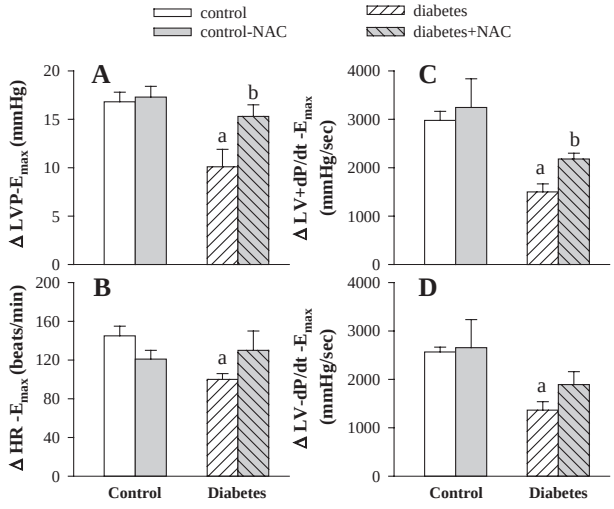

Fig. 3. Effects (mean±S.E.M., n=6–7 each) of chronic treatment of *N*-acetylcysteine (NAC, 1.23 g/kg per day) on maximum effects (E_{max}) of dobutamine (1–57 μg/kg/min) on left ventricular (LV) pressure (LVP, A), heart rate (ΔHR, B), maximal rate of rise (+dP/dt, C) and fall (−dP/dt, D) of LVP in pentobarbital-anaesthetized control rats and rats with streptozotocin (60 mg/kg, i.v.)-induced diabetes. [a]Significantly different (P<0.05) from untreated control rats. [b]Significantly different from untreated diabetic rats.

+dP/dt (50%), −dP/dt (53%) and heart rate (69%) to dobutamine (Fig. 3).

Treatment with *N*-acetylcysteine did not significantly alter cardiac contractile responses to dobutamine in the control rats, but increased contractile and heart rate responses to dobutamine in the diabetic rats (Fig. 1), such that the ED_{50} of left ventricular pressure, left ventricular +/−dP/dt and heart rate responses to dobutamine were similar in the diabetic rats relative to the corresponding readings in the control rats (Fig. 2). *N*-acetylcysteine also significantly augmented maximum left ventricular pressure and left ventricular pressure +dP/dt responses to dobutamine in the diabetic rats, but did not significantly alter maximum −dP/dt and heart rate responses to dobutamine in the diabetic rats (Fig. 3).

3.3. Immunohistochemistry of nitrotyrosine, inducible nitric oxide synthase and endothelial nitric oxide synthase in the hearts

In the absence of primary antibodies, no immunostaining was apparent in any groups (Fig. 4, negative control). Immunostainings of endothelial nitric oxide synthase (dark red dots, indicated by arrows) were of similar intensity in all four groups (Fig. 4). In contrast, immunostainings for nitrotyrosine and inducible nitric oxide synthase were clearly identified in the diabetic

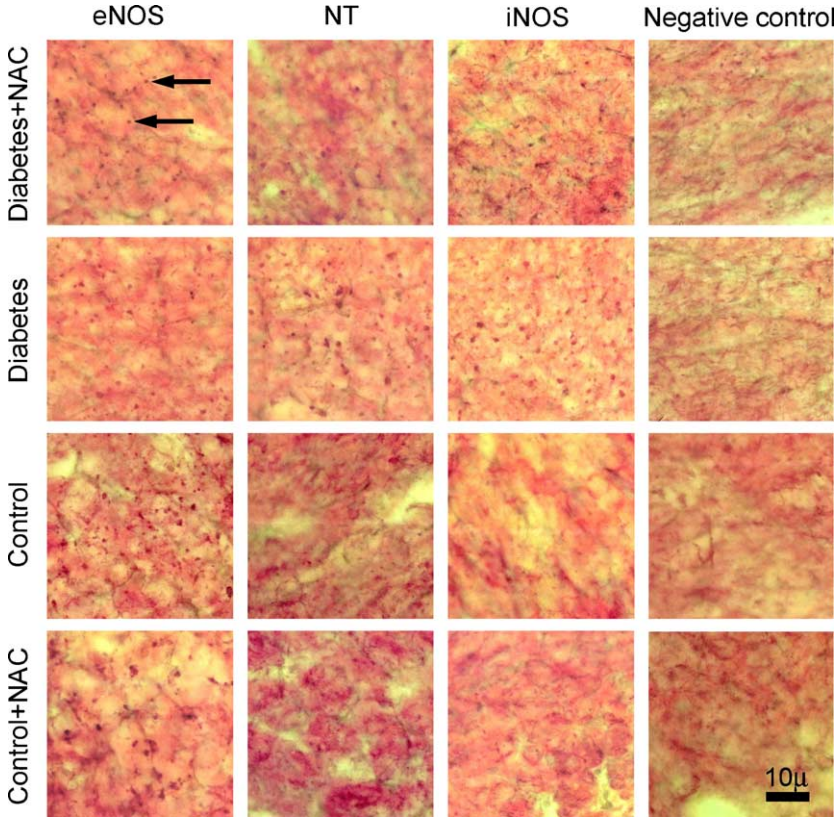

Fig. 4. Effect of chronic treatment with *N*-acetylcysteine (NAC, 1.2 g/kg per day) on immunohistochemistry of nitrotyrosine (NT), inducible (iNOS) and endothelial (eNOS) nitric oxide synthase in myocardial tissues from control and diabetic rats i.v. injected with saline (0.9% NaCl) or streptozotocin (60 mg/kg), respectively. Immunostaining was performed using anti-NT, anti-iNOS, or anti-eNOS antibodies at 3 weeks after i.v. injection of streptozotocin or saline (n=3). The negative control involved staining with mouse nonspecific immunoglobulin G instead of the primary antibody. Magnification: ×100.

group, but not in the control nor control +*N*-acetylcysteine group, and were infrequently seen in the diabetes +N-acetylcysteine group (Fig. 4).

3.4. Myocardial level of 15-F_{2t}-isoprostane

The level of 15-F_{2t}-isoprostane in the heart of the control rats was similar to that of the diabetic rats. The level of 15-F_{2t}-isoprostane was not altered by treatment with *N*-acetylcysteine in the control rats, but was markedly reduced to ≈50% in the diabetic rats (Fig. 5A).

3.5. Myocardium antioxidant capacity

The formation of thiobarbituric acid reactive substances following peroxidation challenge was similar in the control and diabetic rats at the lowest (0.5 mmol/l) and highest (5 mmol/l) concentrations of *t*-butylhydroperoxide (Fig. 5B). The formation of thiobarbituric acid reactive substances was significantly higher in the diabetic than control group at 1 or 2 mmol/l of *t*-butylhydroperoxide. These intermediate concentrations of *t*-butylhydroperoxide are within the optimal range for the detection of thiobarbituric acid reactive substances, but are sufficiently low to avoid non-specific bleaching of the colour produced by the thiobarbituric acid reaction that occurs at approximately 5 mmol/l of *t*-butylhydroperoxide (Xia et al., 2003; Runzer et al., 2002).

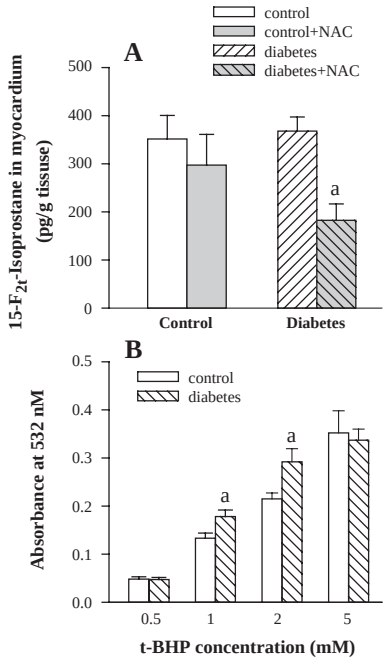

Fig. 5. A) Effects of chronic treatment with *N*-acetylcysteine (1.2 g/kg/day) on myocardial 15-F_{2t}-isoprostane level in control rats and streptozotocin (60 mg/kg, i.v.)-induced diabetic rats. B) Thiobarbituric acid-reactive substance formation (as reflected by absorbance at 532 nm) as a function of *t*-butylhydroperoxide (*t*-BHP) concentration in myocardium of control and diabetic rats. [a]Significantly different ($P<0.05$) between diabetic vs. control group, ($n=6–8$).

4. Discussion

The diabetic rats in the present study had reduced body weight, similar baseline mean arterial pressure, heart rate and left ventricular contractility at three weeks after injection of streptozotocin. Reductions of in vivo (Dowell et al., 1986; Litwin et al., 1990; Paulson et al., 1986; Banyasz et al., 1996) and in vitro (Okayama et al., 1994; Smith et al., 1997) cardiac contractile function have been reported in rats with streptozotocin-induced diabetes for a duration of 4 to 8 weeks.

To further examine cardiac contractile function in diabetes, the rats were challenged with dobutamine, a β_1-adrenoceptor agonist. The results show that the diabetic rats had reduced inotropic and chronotropic responses to dobutamine, exemplified by decreases in the efficacy (reduced E_{max}) and potency (increased EC_{50}) of heart rate, and left ventricular developed tension as well as contractility (left ventricular $+/-dP/dt$) responses to dobutamine. Depressed in vitro (Smith et al., 1997) and in vivo (Heller et al., 1988) cardiac contractile responses to dobutamine have been reported in rats with streptozotocin-induced diabetes. These results are consistent with the observation that the number of β-adrenoceptors is decreased in cardiac membranes from diabetic rats at one to ten weeks after injection of streptozotocin (Beenen et al., 1997; Atkins et al., 1985; Ramanadham and Tenner, 1987; Nishio et al., 1988).

In the present study, reduced cardiac contractile function in the diabetic rats was associated with increased immunostaining of inducible nitric oxide synthase and nitrotyrosine but not endothelial nitric oxide synthase, and increased formation of thiobarbituric acid reactive substances upon peroxidation challenge; however, baseline myocardial level of 15-F_{2t}-isoprostane was not significantly changed. These results suggest that inducible nitric oxide synthase, but not endothelial nitric oxide synthase, is activated, and formation of peroxynitrite is increased at the acute phase of diabetes. At this time there was similar baseline lipid peroxidation (formation of 15-F_{2t}-isoprostane), but decreased tissue antioxidative capacity (increased thiobarbituric acid reactive substances formation upon peroxidation challenge).

The present results of the activation of iNOS in type I diabetes is in accordance with those of our previous study which shows that relative to the control rats, rats with streptozotocin-induced diabetes for three weeks had reduced cardiac contractile response to noradrenaline, as well as increased reverse transcription-polymerase chain reaction (RT-PCR) products of inducible nitric oxide synthase, increased immunostaining of iNOS and nitrotyrosine, and increased activity of iNOS in the myocardium

(Cheng et al., 2004). Furthermore, the acute administration of a selective inhibitor of inducible nitric oxide synthase improved in vivo cardiac contractility to noradrenaline (Cheng et al., 2004). These results of the detrimental effect of inducible nitric oxide synthase on contractile function in diabetes are consistent with the results of Marfella et al. (2004) which show that the presence of inducible nitric oxide synthase in mice ($iNOS^{+/+}$) with streptozotocin-induced diabetes (relative to the $iNOS^{-/-}$diabetic mice) is associated with increased myocardial infarct size following ischemia-reperfusion injury, and higher myocardial content of nitrotyrosine, proinflammatory cytokines and apoptosis in the pre-infarction area.

It is well known that *N*-acetylcysteine and its metabolites (cysteine and inorganic sulfite) can increase intracellular concentrations of glutathione, part of the antioxidative defence system. *N*-acetylcysteine also acts directly as a free radical scavenger (Kelly, 1998). It is of interest that *N*-acetylcysteine has also been shown to inhibit in vivo activation of inducible nitric oxide synthase in rats treated with lipopolysaccharide, but has no effect when the enzyme is already induced; the mechanism may involve inhibition of the expression of the inducible nitric oxide synthase protein (Bergamini et al., 2001) or/and DNA binding activity of nuclear transcription factor-κB (Rota et al., 2002).

Chronic (3 weeks) treatment with *N*-acetylcysteine did not affect any baseline measurements or responses to dobutamine in the control rats. In the diabetic rats, *N*-acetylcysteine did not alter body weight, plasma glucose and heart rate, but partially restored baseline left ventricular contractility (left ventricular $+/-dP/dt$). *N*-acetylcysteine also markedly restored contractile response to dobutamine, such that the potency of left ventricular pressure and left ventricular $+/-dP/dt$ and heart rate responses to dobutamine were similar in the *N*-acetylcysteine-treated diabetic rats relative to the corresponding readings in the control rats. Moreover, maximum left ventricular pressure and left ventricular $+dP/dt$ response to dobutamine were also increased significantly. These results show that chronic antioxidative therapy with *N*-acetylcysteine improves cardiovascular function at the acute phase of diabetes.

Further experiments were undertaken to find out if chronic treatment with *N*-acetylcysteine alter the antioxidative status in the rats. *N*-acetylcysteine did not alter endothelial nitric oxide synthase immunostaining in the control or diabetic rats. In the diabetic rats, *N*-acetylcysteine reduced the staining of inducible nitric oxide synthase as well as nitrotyrosine (protein nitration) and decreased myocardial level of 15-F_{2t}-isoprostane, which indicates the inhibitions of inducible nitric oxide synthase activation and peroxynitrite-mediated cytotoxicity. It is therefore logical to speculate that improved cardiac performance by *N*-acetylcysteine in the diabetic rats is due to the suppression of oxidative stress, which results in the preservation of proteins, such as receptors, enzymes, transport proteins and structural proteins, as well as reduction of cellular damage through lipid peroxidation.

There is evidence that antioxidant therapy offers protection against cellular damage in various animal models of diabetes and in diabetic patients. Chronic treatment of alloxan-induced diabetic mice with *N*-acetylcysteine reduced the activation of the nuclear transcription factor-κB in the pancreas, and this has the potential of reducing the production of proinflammatory cytokines such as interleukins and inducible nitric oxide synthase (Ho et al., 1999). Acute administration of *N*-acetylcysteine also improved vasodilatation response to electrical stimulation or to substance P in rats with streptozotocin-induced diabetes (Bassirat and Khalil, 2000). Chronic treatment with *N*-acetylcysteine prevented the development of peripheral neuropathy in streptozotocin-induced diabetic rats (Sagara et al., 1996), as well as ultrastructure changes in the erythrocytes of patients with type II diabetes (Straface et al., 2002). Furthermore, Tossios et al. (2003) reported that *N*-acetylcysteine prevented reactive oxygen species mediated myocardial stress (decreased formation of 15-F_{2t}-isoprostane and nitrotyrosine in left ventricular cardiomyocytes) of patients undergoing cardiac surgery in a randomized, double-blind, placebo-controlled clinical trial. Also, chronic treatment with FP15, a porphyrinic decomposition catalyst (tetrakis 2-(triethylene glycol monomethyl ether) pyridyl porphyrin) that decomposes peroxynitrite to form nitrate, inhibited tyrosine nitration in the pancreas of a mouse given multiple low-doses of streptozotocin (Szabo et al., 2002). Oral administration of FP15 also reduced left ventricular end-diastolic pressure and increased left ventricular $-dP/dt$ in mice given a high dose of streptozotocin (Szabo et al., 2002). Moreover, rats with streptozotocin-induced diabetes for 12 weeks had increased myocardial level of thiobarbituric acid reactive substances, and this increase was attenuated following 6 weeks of treatment with insulin, and was abolished following 12 weeks of treatment with the antioxidant vitamin A (Zobali et al., 2002).

It is of interest that there was no change in baseline myocardial 15-F_{2t}-isoprostane, an in vivo indicator of oxidative stress, in the diabetic rats relative to the controls. Our results are in agreement with the observations of similar levels of F_2-isoprostane in the kidneys of control and diabetes rats at 3 weeks after the induction of diabetes, but reduction in the level of 15-F_{2t}-isoprostane in the kidneys of the diabetic rats after treatment with the antioxidant DL-α-lipoic acid (Obrosova et al., 2003). It has been suggested that the tissue level of 15-F_{2t}-isoprostane depends not only the rate of lipid peroxidation, but on arachidonic acid abundance, the rate of arachidonate metabolism to prostaglandin $F_2\alpha$, and prostaglandin $F_2\alpha$ clearance, and these processes are altered in diabetes mellitus (Naveh-Floman et al., 1984; Miinea et al., 2002; Tajiri et al., 1994). Indeed, there was a trend towards an accumulation of 15-F_{2t}-isoprostane level in

diabetic renal cortex when 15-F_{2t}-isoprostane concentration was normalized as per ng/μg arachidonate (Obrosova et al., 2003). As well, although baseline plasma levels of 15-F_{2t}-isoprostane were similar in the control and diabetic subjects, plasma 15-F_{2t}-isoprostane increased significantly during myocardial ischemia following heart surgery in high-risk patients with types I and II diabetes, relative to the levels in low-risk patients (Ansley et al., 2002). It has been shown that increased plasma levels of 15-F_{2t}-isoprostane is inversely correlated with post-operative cardiac function (Ansley et al., 2003). The present results also show that chronic treatment with *N*-acetylcysteine decreased the total amount of 15-F_{2t}-isoprostane in the myocardium to ~50% in the diabetic rats but not the controls, thereby suggesting that *N*-acetylcysteine enhanced the capacity of endogenous antioxidant, which was revealed as increased thiobarbituric acid reactive substances formation following peroxidation challenge in the diabetic rat hearts.

To summarize, the acute phase of streptozotocin-induced diabetes in rats was associated with activation of inducible nitric oxide synthase, increased peroxynitrite-mediated nitration of tyrosine, and depressed cardiac contractile response to dobutamine. Reduced antioxidative capacity (increased thiobarbituric acid reactive substances formation upon peroxidation challenge) also occurred at this time, even though baseline lipid peroxidation (formation of 15-F_{2t}-isoprostane) in the myocardium was not altered. Treatment with the antioxidant *N*-acetylcysteine reduced the activation of inducible nitric oxide synthase, decreased peroxynitrite-mediated nitration of tyrosine and lipid peroxidation, and augmented cardiac contractility to dobutamine. To our best knowledge, this is the first evidence that the antioxidant *N*-acetylcysteine improved cardiac function and concurrently reduced the level of myocardium 15-F_{2t}-isoprostane at three weeks after the induction of diabetes. Our results may highlight the necessity for antioxidant supplementation in the management of cardiac contractile dysfunction in diabetes mellitus even when baseline levels of lipid peroxidation products are not yet elevated.

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