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M40403 prevents myocardial injury induced by acute hyperglycaemia in perfused rat heart

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Abstract

M40403 is a low-molecular-weight, synthetic manganese-containing biscyclohexylpyridine superoxide dismutase mimetic (SODm) that removes superoxide anions ($^{\cdot}O_2^-$) without interfering with other reactive species known to be involved in cardiovascular alterations (e.g. nitric oxide [NO] and peroxynitrite [$ONOO^-$]). As such, M40403 represents an important pharmacological tool to dissect the roles of O_2^- in functional and biochemical cardiovascular alterations induced by perfusion of high glucose concentrations into the heart.

Perfusion of a high glucose concentration of glucose into the heart elicited important cardiovascular alterations characterized by QT interval prolongation, increase in coronary perfusion pressure (CPP), lipid peroxidation, decrease in MnSOD activity and DNA damage. All parameters of cardiovascular alteration were attenuated by M40403 (1–10 mg/l). Furthermore, perfusion of a high of glucose concentration induced a significant formation of nitrotyrosine as well as an activation of poly(adenosine diphosphate [ADP]-ribose) synthetase (PARS), as determined by immunohistochemical analysis of heart tissue. The extent of staining for nitrotyrosine and PARS was reduced by M40403.

These results clearly indicate that O_2^- plays a critical role in the development of the functional and biochemical cardiovascular alterations induced by perfusion of a high concentration of glucose into the heart. Therefore, synthetic enzymes of SOD, such as M40403, offer a novel therapeutic approach for the management of various cardiovascular diseases where these radicals have been postulated to play a role.

Keywords: M40403; Glucose; Cardiovascular alteration

1. Introduction

Oxidative stress is an important component of diabetes and its complications (Ammar et al., 2000; Cakatay et al., 2000; Cameron et al., 1998; Cameron and Cotter, 1995, 1999; Gocmen et al., 2000; Haak et al., 2000; Ishi et al., 1998; Keegan et al., 1999; Obrosova et al., 2000; Pieper et al., 1993, 2000; Pieper and Siebeneich, 1997). Cardiovascular disease is a major complication of diabetes, leading to mortality in diabetic patients (Stamler et al., 1993). In the

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last few years, it has been demonstrated that hyperglycaemia may be one of the causes of the cardiovascular alterations (e.g. microvascular diseases, coronary heart disease and peripheral arterial disease) associated with diabetes (The DCCT Research Group, 1993; Kuusisto et al., 1994). Therefore, hyperglycaemia may represent an important predictive risk factor for hypertension and myocardial infarction in patients with and/or without diabetes (Capes et al., 2000). In this regard, it is not surprising that an increase in the glucose level can induce arrhythmias with QT prolongation, fatal in the case of infarction, and increases coronary constriction (D'Amico et al., 2001). Recently, it has been demonstrated that treatment with antioxidants reduces the oxidative stress and vascular dysfunction associated with experimental diabetes (Cameron et al., 1993, 1994, 1998; Cameron and Cotter, 1999; Karasu et al., 1995; Keegan et al., 1999). Recent studies have clearly demonstrated that an increased production of reactive oxygen species is responsible for the hyperglycaemia-induced electrophysiological alterations (Cervello et al., 2002). However, none of the previous studies identified the particular radical(s) involved in the hyperglycaemia-induced electrophysiological alterations. This stems from the fact that selective antioxidants have not been available. In various pathological situations, the use of native superoxide dismutase (SOD) enzymes both pre-clinically and clinically has shed light on the importance of O_2^- in disease and, thus, the therapeutic potential of exogenous SOD enzymes (Flohe, 1988; Huber et al., 1980; Uematsu et al., 1994). However, the native SOD enzyme has not been evaluated in hyperglycaemia-induced electrophysiological alterations. Thus, the role of superoxide in this condition has not been defined. There are drawbacks or problematic issues associated with the use of native enzymes as therapeutic agents (e.g., solution instability, immunogenicity of non-human enzymes, bell-shaped dose–response curves, high susceptibility to proteolytic digestion) and as pharmacological tools (e.g., they do not penetrate cells or cross the blood–brain barrier, limiting the dismutation of superoxide to the extracellular space or compartment). To overcome the limitations associated with native enzyme therapy, we have developed a series of SOD mimetics that catalytically remove O_2^-. M40403 is a prototypic example of a stable, low-molecular-weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native SOD enzymes, but with the advantage of being a much smaller molecule (Salvemini et al., 1999). An important property of these SOD mimetics is that they catalytically remove superoxide at a high rate without interacting with other biologically important reactive species including nitric oxide, peroxynitrite, hydrogen peroxide, oxygen or hydroxyl radicals (Riley et al., 1996, 1997). This property is not shared by other classes of SOD mimetics or scavengers, including several metalloporphyrins such as tetrakis-(*N*-ethyl-2-pyridyl)porphyrin and tetrakis-(benzoic acid)porphyrin, which interact with other reactive species, such as nitric oxide and peroxynitrite, that play important roles in inflammation (Patel and Day, 1999). Therefore, the purpose of our study was to evaluate whether interventions against O_2^- with M40403, a synthetic manganese-containing biscyclohexylpyridine SODm (Fig. 1), prevent the functional and biochemical cardiovascular alterations induced by perfusion of a high concentration of glucose into the heart. In particular, we investigated the effect of M40403 in isolated working rat hearts perfused with a high concentration of glucose on (i) QT interval prolongation, (ii) coronary perfusion pressure (CPP) increase, (iii) lipid peroxidation (malondialdehyde levels), (iv) superoxide dismutase (SOD) activity, (v) the nitration of tyrosine residues (an indicator of the formation of peroxynitrite) (by immunohistochemistry), (v) poly(adenosine diphosphate [ADP]-ribose) synthetase (PARS) activation and (vi) DNA damage (8 hydroxy-2′-deoxyguanosine formation).

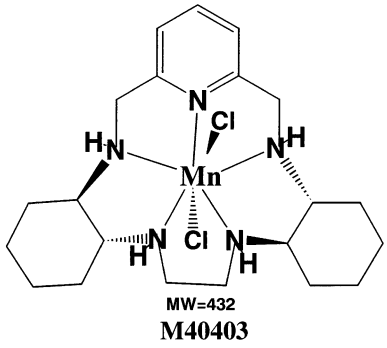

Fig. 1. Structure of M40403.

2. Methods

2.1. Cell culture

Human umbilical vein endothelial cells (BioWhittaker, Walkersville, USA) were cultured in Endothelial Growth medium Bullet Kit w/2% FBS (BioWhittaker). Cells were cultured in 96-well plates (200 μl medium/well) or in 12-well plates (3 ml medium/well) until 90% confluence. Cells were exposed to hydrogen peroxide (10 mM for 2 h) or peroxynitrite (1 mM for 30 min), in the presence or absence of M40403 (10–1 μM).

2.2. Measurement of mitochondrial respiration

Cell respiration was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mazzon et al., 2002). Cells in 96-well plates were incubated at 37 °C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in dimethyl sulfoxide (DMSO) (100 μl). The extent of reduction of MTT to formazan within cells was quantitated

by measuring OD_{550}. As previously reported (Mazzon et al., 2002), although the reduction of MTT appears to be mainly by the mitochondrial complexes I and II, it also may involve NADH- and NADPH-dependent processes that occur outside the mitochondrial inner membrane. Thus, this method cannot be used to separate the effect of free radicals, oxidants or other factors on the individual enzymes of the mitochondrial respiratory chain, but is useful to monitor changes in the general energy status of the cells (Mazzon et al., 2002).

2.3. Isolated hearts

Male Sprague–Dawley rats (4–6 months old and weighing on average 250 g) were anaesthetized with urethane (1.2 mg/kg/ i.p.) and then heparanized (sodium heparin, 250 IU, i.p., 10 min before heart excision). The hearts were rapidly excised and placed in ice-cold perfusion solution (constituents below) prior to coronary perfusion. Hearts were cannulated via the aorta and perfused retrogradely under constant flow (10 ml min^{-1}) using a calibrated roller pump (Gilson, Miniplus-2) with a buffer solution of the following composition: D(+)glucose, (mmol/l) 11.1; $CaCl_2$, 1.4; NaCl, 118.5; $NaHCO_3$, 25.0; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; and KCl, 4.0. The buffer solution was gassed with 95%O_2 and 5%CO_2 (pH 7.4) and perfused at 37 °C. Each experiment lasted 2 h.

A total of 41 hearts were used. Some were excluded because the preparations were unstable. A stable preparation was defined as having a sinus rate of >220 beats/min or a CPP>60 mm Hg between 5 and 15 min after the start of perfusion. Any heart not in sinus rhythm during the study was also excluded. The remaining hearts, which satisfied the criteria for a stable preparation, were used. Eight hearts were perfused with the buffer solution, as detailed above, containing D-glucose at a concentration of 11.1 mmol/l, and served as control hearts. Ten hearts were perfused with the buffer solution containing D-glucose at the concentration of 33.3 mmol/l (high glucose). Hearts (eight for each groups) were perfused with D-glucose (33.3 mmol/l) plus M40403 (1–10 mg/l).

For each heart, a unipolar electrocardiogram (ECG) was recorded by implanting a stainless steel wire electrode into the apex of the left ventricular muscle mass, with a second electrode connected to the aorta. This electrode arrangement gave clear P waves and ventricular complexes. A 3-min ECG (speed 50 mm/s) was recorded every 10 min for 2 h. The coronary perfusion pressure was monitored continuously during each study.

ECG analysis was carried out by a cardiologist who was unaware of the sequence of experiments. The following parameters were calculated: (1) heart rate (R–R interval), (2) the duration between onset of the P wave and onset of the ventricular complex (PR interval) and (3) the width of the ventricular complex. No separate T wave is seen in the rat ECG, thus conventional measurement of the QT interval is impracticable. This is because repolarization begins in the apex of the ventricles before depolarization is complete in other parts of the ventricles, secondary to the brief duration of the ventricular action potential. The width of the ventricular complex was measured at 100% repolarization and is defined as QT_{100}. The QT value of each experiment was calculated as the mean of the ECG readings performed every 10 min.

The CPP in the aortic line was monitored with a Statham Spectramed pressure transducer connected to a chart recorder (Grass, 79E, Quincy, MA, USA). Air temperature was maintained by means of a heated (37 °C) water jacket. On establishing a stable CPP (20–30 min following cannulation), experiments were performed as previously described. CPP values used for statistical comparisons were calculated either as the mean of each 10-min value throughout the entire experiment or as the mean of the steady-state increase above baseline, if an increase in CPP was evident during an experiment.

Animal care was in compliance with Italian regulations for the protection of laboratory animals (D.M. 116/92), as well as with European Economic Community regulations (O.J. of E.C.L. 358/1 12/18/1986).

2.4. Measurement of heart tissue malondialdehyde

Malondialdehyde levels in the heart tissue were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). Heart tissue collected at the end of perfusion was homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% SDS, 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000×*g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.5. Immunofluorescence localization of nitrotyrosine and poly(ADP-ribose)

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or ROS, was determined by immunohistochemistry as previously described (Cuzzocrea et al., 2001a,b). At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in phosphate-buffered saline (PBS) for 20 min. Non-specific adsorption was minimized by incubating the sections in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with (1) anti-nitrotyrosine rabbit

polyclonal antibody (1:500 in PBS, v/v) or with anti-poly(ADP-ribose) goat polyclonal antibody rat (1:500 in PBS, v/v). Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA). In order to confirm that the immunoreaction for nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify binding specificity. To verify the binding specificity poly(ADP-ribose) for PAR, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out. Immunocytochemistry photographs (N=5) were assessed by densitometry as previously described (Cuzzocrea et al., 2001a,b), using Optilab Graftek software on a Macintosh personal computer.

2.6. Measurement of PARS activity

Heart tissues were placed in 0.5 ml of 56 mM HEPES buffer (pH 7.5) containing 28 mM KCI, 28 mM NaCl, 2 mM $MgC1_2$, 0.01% digitonin (to permeabilize plasma membranes) and 125 nM NAD^+ spiked with 0.25 μCi [^{3}H]NAD^+. PARS activity was then measured using a previously described method (Mazzon et al., 2002). Heart tissues were washed twice and incubated overnight in 2 N HCl, followed by neutralization in NaOH. The protein pellet was solubilized in 2% sodium dodecyl sulphate (SDS) in 0.1 mol/l NaOH and the [^{3}H]NAD^+ incorporation was determined by scintillation counting. The 3-aminobenzamide-inhibitable component of the [^{3}H]NAD^+ incorporation into proteins was considered as a specific indicator of PARS activity.

2.7. Determination of 8-hydroxy-2′-deoxyguanosine

Myocardial samples were frozen and kept at −80 °C until used. The samples were thawed at room temperature and cell DNA isolation was performed according to Lodovici et al. (2000) with a few modifications. Briefly, tissue samples were homogenized with a Polytron homogenizer at 0–4 °C, sonicated on ice for 30 s (10 s, three times) and then diluted with 1 ml of 10 mM Tris–HCl buffer pH 8.0 containing 10 mM EDTA, 10 mM NaCl, 0.5% SDS and incubated at 37 °C for 60 min with RNAse (20 μg/ml), then the samples were incubated at 37 °C in oxygen-free conditions with overnight insufflation of argon in the presence of proteinase K (100 μg/ml). At the end of the incubation period, the mixture was extracted with chloroform/isoamyl alcohol (10:2 v/v) with 0.2 volumes of 10 M ammonium acetate, and DNA was precipitated from the aqueous phase as reported previously (Lodovici et al., 2000). DNA was solubilized in 100 μl of 20 mM acetate buffer pH 5.3 and denaturated at 90 °C for 3 min. The entire extracted DNA was supplemented with 10 IU of P1 nuclease in 10 μl and incubated for 60 min at 37 °C with 5 IU of alkaline phosphatase in 0.4 M phosphate buffer pH 8.8. All these procedures were performed under argon and the samples were protected from light. The hydrolysed mixture was filtered through a Micropure-EZ enzyme remover (Amicon) and 50-μl samples were used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) determination using a Bioxytech®-EIA-kit following the protocol provided by the producer. In brief, the samples were incubated for 1 h at 37 °C with the primary antibody, then the assay plate was washed three times with PBS and the samples were incubated with the secondary antibody for 1 h at 37 °C. The plate was washed again three times and then the samples were incubated with the chromogen at room temperature for 15 min in the dark. The reaction was stopped by adding 100 μl of 1 M phosphoric acid and the absorbance was read at 450 nm wavelength. Concentrations were determined using a standard curve generated by plotting absorbance vs. log of 8-OHdG concentrations. The values are expressed as ng/μg of proteins.

2.8. Measurement of Mn-SOD activities

The frozen samples were homogenized with 10 mM phosphate buffer saline (pH 7.4) in a Polytron homogenizer and then sonicated on ice for 1 min (20 s, three times). The sonicated samples were centrifuged at 100×g for 10 min. Supernatant was used for SOD measurement.

The assay of SOD activity was basically the same as that described by Beauchamp and Fridovich (1971) and Nishida (2002), with some modifications. In brief, the assay is based on the inhibition of nitro blue tetrazolium conversion by SOD into a blue terazolium salt, mediated by superoxide radicals generated by xanthine oxidase. The reaction was performed in sodium carbonate buffer 50 mM, pH 10.1 containing EDTA, 0.1 mM, nitro blue tetrazolium (NBT) 25 μM (Sigma, Milan, Italy), xanthine, 0.1 mM. Xanthine oxidase (Boehringer, Germany) was added in a concentration of 2 nM. The rate of reduction of NTB was followed at 560 nm with a Perkin Elmer spectrophotometer. The amount required to inhibit the rate of reduction of nitro blue tetrazolium by 50% was defined as one unit of activity.

2.9. Statistical analysis

Data are given as means±S.E.M. Coronary perfusion pressure and ECG values used for statistical comparisons are expressed either as the mean of each 3-min value throughout the entire experiment or as the mean of the steady-state increment above baseline, if an increase in coronary perfusion pressure or modifications of ECG parameters were evident during an experiment. The statistical analysis was performed with one-way analysis of variance between groups (ANOVA), followed by Duncan's

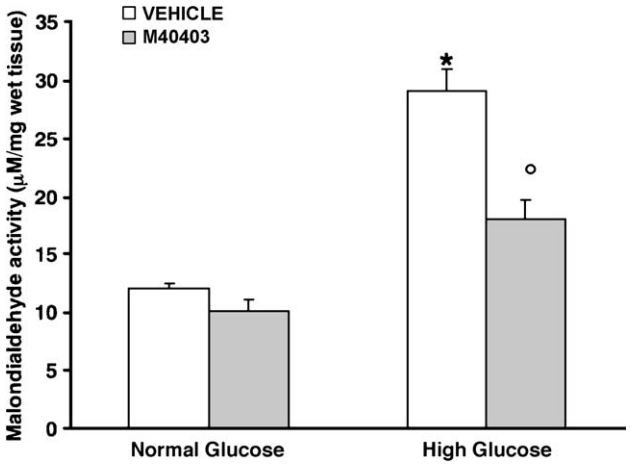

Fig. 2. Malondialdehyde activity in the hearts perfused for 2 h with a normal or high glucose concentration (33.3 mM) in the presence or absence of M40403. Malondialdehyde levels were significantly increased in the hearts from high glucose-treated rats in comparison those of hearts from rats exposed to normal glucose. M40403 significantly reduced the high glucose-induced increase in malondialdehyde levels. Data are means±S.E.M. for five rats for each group. *p<0.01 versus normal glucose; °p<0.01 versus high glucose.

multiple range test. A probability level of less than 5% was considered to be statistically significant.

2.10. Materials

M40403 was kindly supplied by Dr. Daniela Salvemini, Department of Biological and Pharmacological Research, MetaPhore Pharmaceuticals, 1910 Innerbelt Business Center Drive, St. Louis, MO 63114, USA. Primary anti-nitrotyrosine antibody was from Upstate Biotech (DBA).

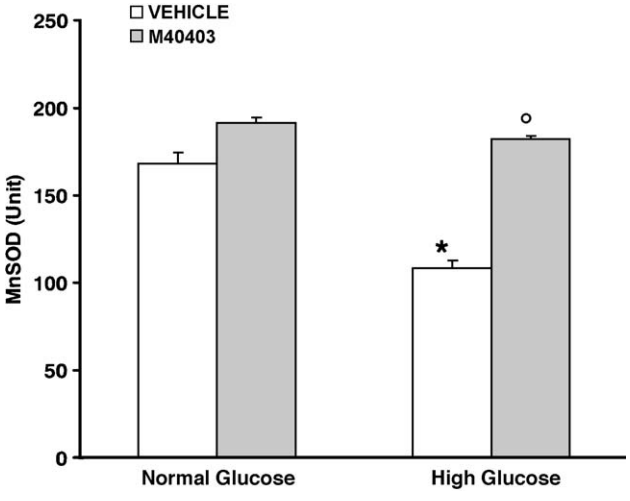

Fig. 3. MnSOD activity in hearts perfused for 2 h with a normal or high glucose concentration (33.3 mM) in the presence or absence of M40403. MnSOD activity was significantly reduced in the hearts from high glucose-treated rats in comparison with levels in hearts from normal glucose treated rats. M40403 treatment significantly prevented the high glucose-induced reduction in MnSOD levels. Data are means±S.E.M. for five rats for each group. *p<0.01 versus normal glucose; °p<0.01 versus high glucose.

All other reagents and compounds used were obtained from Sigma.

3. Results

3.1. Effects of high glucose on heart function

Heart rate (RR interval, 230±19 ms) and atrioventricular conduction time (PR interval, 59±9 ms) were similar for all groups.

In the hearts perfused with the control buffer solution, QT was 110±7 ms and remained steady throughout all the perfusion. D-Glucose (33.3 mmol/l) caused a significant prolongation of QT (202±19 ms, p<0.001 vs. control).

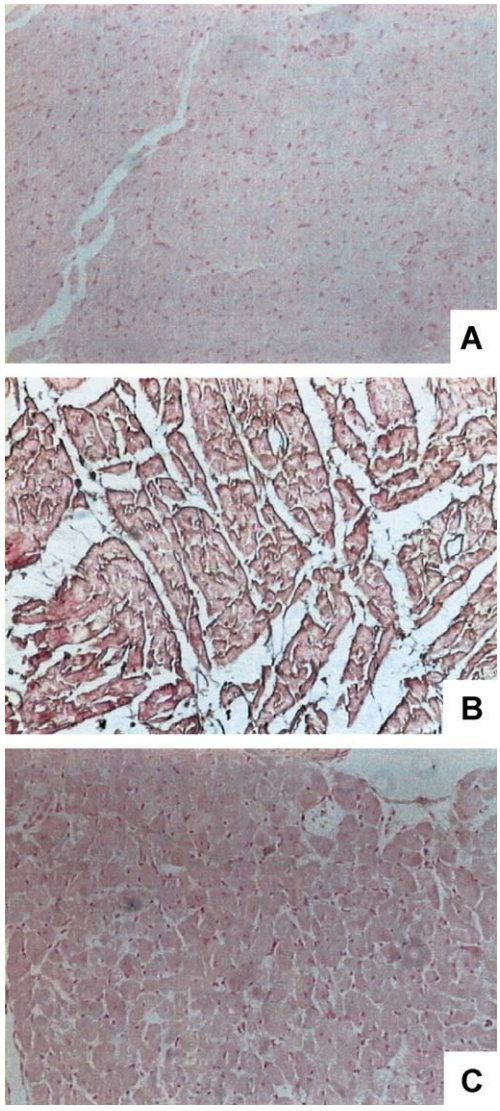

Fig. 4. (A) No positive staining for nitrotyrosine (NT) was found in normal glucose-perfused hearts. (B) Immunohistochemistry for NT shows positive staining along the endothelium and in myocardial cells from high glucose-treated hearts. (C) The intensity of the positive staining for NT was significantly reduced in hearts from M40403-treated rats. Original magnification: ×145. Figure is representative of at least five experiments.

The effect of high glucose was completely prevented by M40403 10 mg/l (QT, 107±11 ms), but unaffected by M40403 1 mg/l.

In hearts perfused with the control buffer solution, CPP was 72±9 mm Hg. This value was steady throughout perfusion. D-Glucose (33.3 mmol/l) caused a significant increase in CPP evident 60 min after the start of perfusion and persisting until 120 min (116±15 mm Hg, $p<0.001$ vs. control study). The effect of high glucose was completely prevented by M40403: 10 mg/l, CPP values (68±9 mm Hg) were not significantly different from those observed during the control study. M40403 at the concentration of 1 mg/l was without effect.

3.2. Effects of high glucose on MnSOD, malondialdehyde production and nitrotyrosine formation

Hearts perfused with high glucose (33.3 mM) exhibited a substantial increase in the production of malondialdehyde (Fig. 1) as well as a significant decrease in the activity of MnSOD (Fig. 3). M40403 (10 mg/l)-treated hearts showed a significant attenuation of the increase in malondialdehyde (Fig. 2) as well as a significant prevention of the loss of MnSOD activity (Fig. 2) caused by high glucose. No effects were seen with 1 mg/l M40403. There was no increase in malondialdehyde production in normal glucose (11.1 mM)-perfused rat hearts. Sections of heart perfused with the control buffer solution did not reveal any immunoreactivity for nitrotyrosine (Figs. 4A and 5) within the normal architecture. Positive staining for nitrotyrosine (Figs. 3 and 4) was localized in the endothelium and myocardial cells in tissue from hearts perfused with high glucose. M40403 10 mg/l reduced the staining for nitrotyrosine (Figs. 4C and 5).

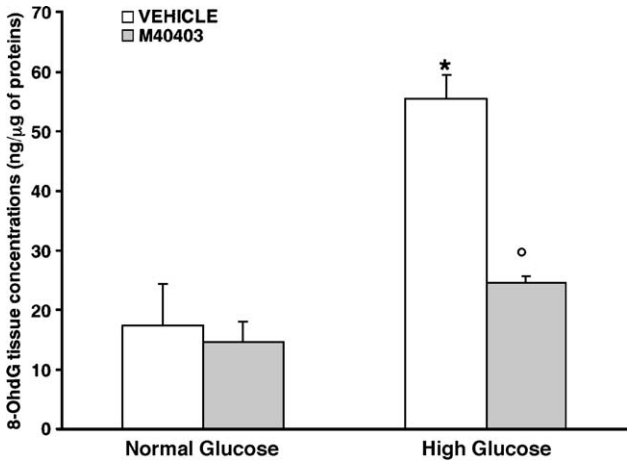

Fig. 6. 8-OHdG tissue concentration, index of DNA damage, in hearts perfused for 2 h with a normal or high glucose concentration (33.3 mM) in the presence or absence of M40403. Hearts perfused with high glucose (33.3 mM) exhibited a substantial increase in DNA damage. The treatment of hearts with M40403 (10 mg/l) resulted in a significant attenuation of the 8-OHdG tissue formation induced by high glucose perfusion. Data are means±S.E.M. for five rats for each group. *$p<0.01$ versus normal glucose; °$p<0.01$ versus high glucose.

3.3. Effects of high glucose on DNA damage and poly(ADP-ribose) formation

Hearts perfused with high glucose (33.3 mM) exhibited a substantial increase in DNA damage, assessed by the evaluation of 8-OHdG tissue concentration in the cardiac tissue (Fig. 6). Treatment of the heart with M40403 (10 mg/l) caused a significant attenuation of the 8-OHdG tissue formation induced by high glucose perfusion (_Hlt76912573[Fig. 6). Perfusion of hearts with high glucose caused a significant increase in PARS activity (Fig. 7). Treatment of hearts with M40403 (10 mg/l)

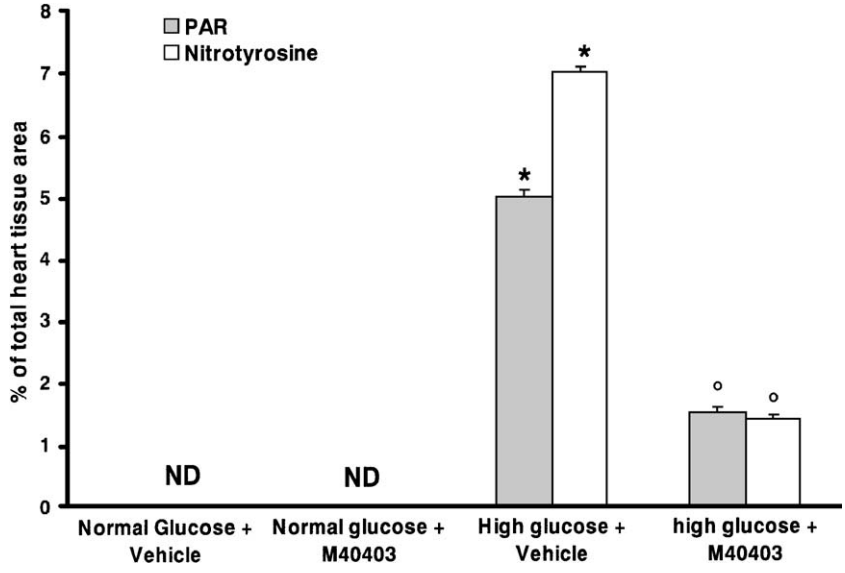

Fig. 5. Typical densitometry evaluation. Densitometry analysis of immunocytochemistry photographs ($n=5$) for nitrotyrosine and PAR from hearts was assessed. The assay was carried by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. *$p<0.01$ versus normal glucose; °$p<0.01$ versus high glucose.

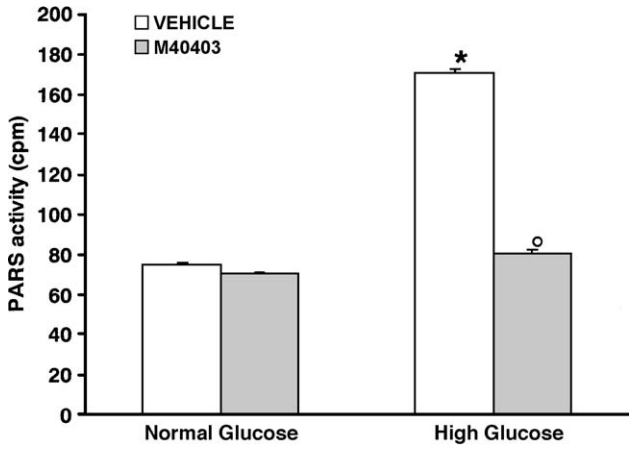

Fig. 7. PARS activity in the hearts perfused for 2 h with a normal or high glucose concentration (33.3 mM) in the presence or absence of M40403. PARS activity was significantly increased in the hearts from high glucose-treated rats in comparison with levels in hearts from normal glucose treated rats. M40403 significantly reduced the high glucose-induced increase in PARS activity. Data are means±S.E.M. for five rats for each group. *$p<0.01$ versus normal glucose; °$p<0.01$ versus high glucose.

caused a significant inhibition of PARS activation in response to high glucose (Fig. 7). At the end of the perfusion time, heart sections were taken for immunohistological staining for PAR. Sections of heart perfused with the control buffer solution did not reveal any immunoreactivity for PAR (Figs. 5 and 8A) within the normal architecture. Positive staining for PAR (Figs. 5 and 8B) was localized in the endothelium and myocardial cells in tissue from heart perfused with high glucose. M40403 10 mg/l reduced the staining for PAR (Figs. 5 and 8C).

3.4. Effects of M40403 on the impairment in mitochondrial respiration caused by hydrogen peroxide and peroxynitrite in human endothelial cells

Exposure of endothelial cells to hydrogen peroxide (10 mM for 2 h), or peroxynitrite (1 mM for 30 min) caused a substantial impairment of mitochondrial respiration (Fig. 9). M40403 (10–1 μM) treatment had no effect on the reduction of mitochondrial respiration caused by hydrogen peroxide or peroxynitrite.

4. Discussion

The present study demonstrates, for the first time, that the cardiovascular effects elicited by acute exposure of isolated rat hearts to high glucose could be prevented by infusion of M40403. In fact, the study shows that M40403, which is already reported to be beneficial in models of acute and chronic pathologies (Cuzzocrea et al., 2001a,b; Masini et al., 2002; Salvemini et al., 2001), reverses the cardiac QT interval prolongation and CPP increase induced by a high concentration of glucose. Since M40403 is a SODm that selectively removes O_2^- (Riley et al., 1997), essentially the study indicates that the inactivation of glucose-induced production of superoxide anions cardiac cells represents a crucial mechanism for reducing the impairment of cardiac function under hyperglycaemic conditions. In fact, multiple molecular cell changes have been described to occur concurrent with the cardiovascular damage generated by exposure of the heart to elevated concentrations of glucose (1) reduction of Na^+/K^+-ATPase activity (Gupta et al., 1992), (2) inhibition of Ca^{2+}-ATPase activity (Davis et al., 1985), (3) depressed Na^+/Ca^{2+} exchanger activity (Schaffer et al., 1997), (4) activation of Na^+/H^+ antiport (Williams and Howard, 1994) and (5) increase in cytosolic-free calcium (D'Amico et al., 2001). However, the increase in O_2^-

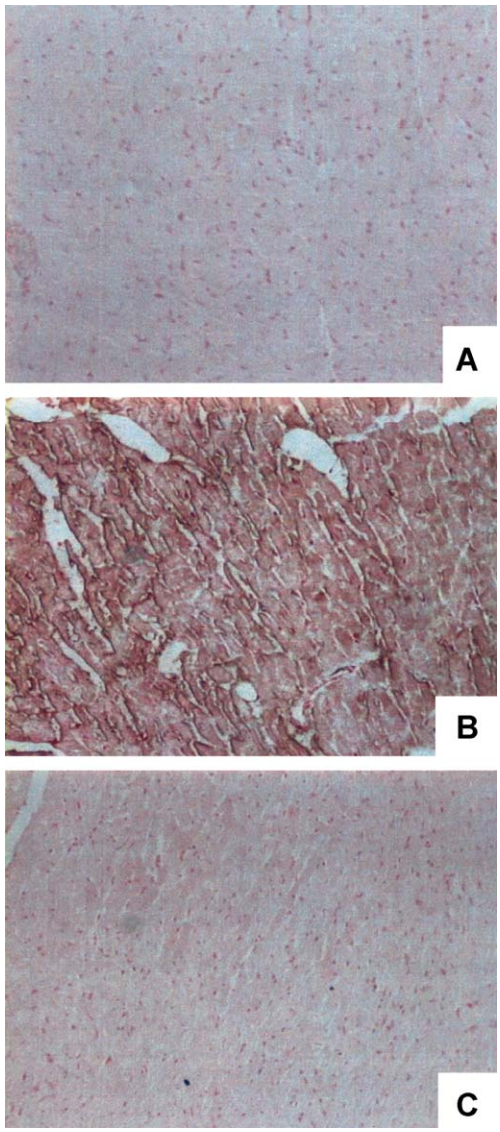

Fig. 8. No positive staining for PAR (A) was found in heart sections from normal glucose-perfused rat hearts. Immunohistochemistry for PAR (B) shows positive staining along the endothelium and in myocardial cells from high glucose-treated hearts. The intensity of the positive staining for PAR was significantly reduced in hearts from M40403-treated rats (C). Original magnification: ×145. Figure is representative of at least five experiments.

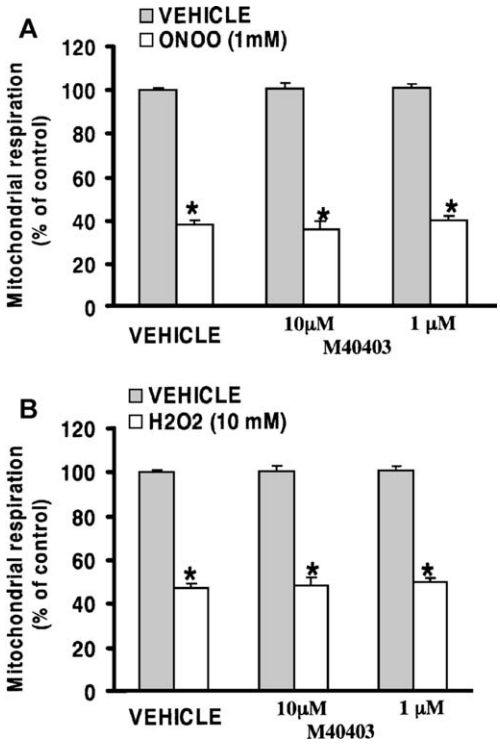

Fig. 9. Effect of M40403: on the peroxynitrite-induced (1 mM) (A) or H_2O_2-induced (10 mM) (B) suppression of mitochondrial respiration in human umbilical vein endothelial cells. Data represent means±S.E.M. of n=9 wells. *p<0.05 represents a significant suppression of mitochondrial respiration.

generation following exposure of the heart to elevated concentrations of glucose (Cervello et al., 2002) seems to be an event to approach first. O_2^- inactivation may prevent a series of phenomena leading to the above molecular alterations and to cardiac dysfunction. Among these is peroxynitrite formation. Recently, several lines of evidence have suggested, that vascular dysfunction may be, in fact, related to peroxynitrite production. First, the cytotoxic effect of peroxynitrite on the vascular endothelium has been directly demonstrated in perfused hearts (Villa et al., 1994) and in cultured human umbilical vein endothelial cells (Mazzon et al., 2002). Second, in the presence of superoxide generators, agonist-induced release of NO from ecNOS exerts autocrine cytotoxic effects via the generation of peroxynitrite, which can be prevented by inhibition of endothelial NOS in vitro (Az-Ma et al., 1996). Lastly, data from in vivo/ex vivo experiments suggest that under certain conditions, e.g. during hypercholesterolemia and atherosclerosis (White et al., 1994), endothelium-derived NO can combine with superoxide, and the resulting formation of peroxynitrite causes an impairment of endothelial function. In addition, as shown in vitro in cardiac cells from high glucose-perfused rat hearts (Cervello et al., 2002), and in vivo in plasma and tissues from diabetic patients (Aydin et al., 2001; Frustaci et al., 2000; Lyall et al., 1998), peroxynitrite accounts for some of the deleterious effects associated with raised O_2^- production (Beckeman and Koppenol, 1996; Guidarelli et al., 2000). ROS are cytotoxic via a number of mechanisms, including: (1) the initiation of lipid peroxidation, (2) the inactivation of a variety of enzymes (most notably mitochondrial respiratory enzymes and membrane pumps) (Crow and Beckman, 1995; Crow et al., 1995), (3) depletion of glutathione (Phelps et al., 1995) and (4) consumption of the endogenous antioxidant vitamins C and E (Novelli, 2002). Moreover, peroxynitrite can also cause DNA damage (Inoue and Kawanishi, 1995; Salgo et al., 1995) resulting in activation of the nuclear enzyme PARS, in depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP), and ultimately in cell death (Szabò and Dawson, 1999). Markers of these phenomena are nitrotyrosine formation, increased malondialdehyde production and detection of high levels of PARS activity in tissues (Cuzzocrea et al., 2001a,b).

In the present in vitro experimental model of high glucose-induced cardiovascular damage, the SODm M40403 was associated with a decreased detection of nitrotyrosine in cardiac cells, which accounts for the decreased generation of peroxynitrite and the improved cardiac function, as evidenced by the recovery of the normal QT interval and normal CPP. Furthermore, since addition of M40403 to the medium of the high-glucose perfused hearts caused a decrease in the level of malondialdehyde found in the cardiac tissue exposed to high glucose, one could deduce that M40403 also possesses the ability to reduce cardiac cell membrane lipid peroxidation in this model. The beneficial anti-oxidant effects of M40403 in hearts exposed to high glucose, therefore, are due, at least in part, to the prevention of peroxynitrite formation and lipid peroxidation. However, it is noteworthy that M40403 also prevents DNA damage, as evidenced by the reduced levels of PARS activity in cardiac cells. This enzyme activity is triggered by energy-consuming DNA repair mechanisms following peroxynitrite-produced DNA strand breaks and plays an important role in inflammatory injury (Cuzzocrea et al., 1998; Szabò et al., 1998). Therefore, the combined actions exerted by M40403 on NT, PARS expression, DNA damage and lipoperoxidation underline the concept that multiple factors are responsible for the cardiovascular damage induced by high glucose in the rat heart. These mechanisms begin with peroxynitrite generated following exposure to high glucose and elevated O_2^-. Bearing in mind the previously described molecular changes associated with high glucose-induced cardiovascular dysfunction, the action of M40403 affects the O_2^- downstream in the cascade.

In conclusion, we have shown that a high glucose concentration causes significant haemodynamic and electrical changes in isolated working rat hearts. We propose that the alterations induced by changes in the oxidative state of cells can be prevented by using the SODm M40403. The anti-oxidant effects of the SODm M40403 include (1) inhibition of $ONOO^-$ formation, (2) decrease in malondial-

dehyde activation and (3) prevention of the activation of PARS.

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