

# Protective effects of M40403, a selective superoxide dismutase mimetic, in myocardial ischaemia and reperfusion injury *in vivo*

<sup>1</sup>Emanuela Masini, <sup>2</sup>Salvatore Cuzzocrea, <sup>3</sup>Emanuela Mazzon, <sup>1</sup>Cosimo Marzocca, <sup>1</sup>Pier Francesco Mannaioni & <sup>\*,4</sup>Daniela Salvemini

<sup>1</sup>Department of Preclinical and Clinical Pharmacology, University of Florence, 50139 Florence, Italy; <sup>2</sup>Institute of Pharmacology, School of Medicine, University of Messina, 98100 Messina, Italy; <sup>3</sup>Department of Biomorphology, School of Medicine, University of Messina, Italy and <sup>4</sup>MetaPhore Pharmaceuticals, 1910 Innerbelt Business Center Drive, St Louis, Missouri, MO 63114, U.S.A.

**1** Myocardial injury caused by ischaemia and reperfusion comes from multiple pathogenic events, including endothelial damage, neutrophil extravasation into tissue, mast cell activation, and peroxidation of cell membrane lipids. These events are followed by myocardial cell alterations resulting eventually in cell necrosis. An enhanced formation of reactive oxygen species is widely accepted as a stimulus for tissue destruction and cardiac failure.

**2** In this study, we have investigated the cardioprotective effects of M40403 in myocardial ischaemia-reperfusion injury. M40403 is a low molecular weight, synthetic manganese containing superoxide dismutase mimetic (SODm) that selectively removes superoxide anion. Ischaemia was induced in rat hearts *in vivo* by ligating the left anterior descending coronary artery. Thirty minutes after the induction of ischaemia, the ligature was removed and reperfusion allowed to occur for at least 60 min. M40403 (0.1–1 mg kg<sup>-1</sup>) was given intravenously 15 min before ischaemia.

**3** The results obtained in this study showed that M40403 significantly reduced the extent of myocardial damage, mast cell degranulation and the incidence of ventricular arrhythmias. Furthermore, M40403 significantly attenuated, in a dose-dependent manner, neutrophil infiltration in the myocardium as well as the associated induction of lipid peroxidation. Calcium overload seen post-reperfusion of the ischaemic myocardium was also reduced by M40403.

**4** Immunohistochemical analysis for nitrotyrosine revealed a positive staining in cardiac tissue taken after reperfusion: this was attenuated by M40403. Moreover reperused cardiac tissue sections showed positive staining for P-selectin and for anti-intercellular adhesion molecule (ICAM-1) in the vascular endothelial cells. M40403 treatment markedly reduced the intensity and degree of P-selectin and ICAM-1 in these tissues. No staining for nitrotyrosine, P-selectin or ICAM-1 was found in cardiac tissue taken at the end of the ischaemic period.

**5** Overall, M40403 treatment reduced the morphological signs of myocardial cell injury and significantly improved survival.

**6** Taken together, these results clearly indicate that M40403 treatment exerts a protective effect against ischaemia-reperfusion-induced myocardial injury, supporting a key role for superoxide anion in reperfusion injuries. This suggests that synthetic enzymes of SOD such as M40403, offer a novel therapeutic approach for the treatment of ischaemic heart disease where superoxide anion plays a dominant role.

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**Keywords:** Ischaemia-reperfusion; rat heart; superoxide anion; peroxynitrite; superoxide dismutase mimetic; M40403

**Abbreviations:** ICAM-1, intracellular adhesion molecules; IR, ischaemia-reperfusion, MDA, malonyldialdehyde; MPO, myeloperoxidase; O<sub>2</sub><sup>-</sup>, superoxide anion; ONOO<sup>-</sup>, peroxynitrite; SODm, superoxide dismutase mimetic

## Introduction

Cardiac ischaemia results in endothelial injury followed by myocardial dysfunction and damage. Paradoxically, reperfusion of ischaemic heart, although providing the cells with oxygen and trophic substances, further exacerbates tissue damage since it promotes the accumulation of neutrophils and formation of oxygen-derived free radicals (McCord, 1985; Lefer *et al.*, 1991). In particular, endothelial cell injury caused by ischaemia enhances neutrophil adhesion and migration from the vasculature to the tissue (Bradford,

1976; Harlan, 1985; Salvemini *et al.*, 1993; 1999a). Leukocyte-endothelial cell interaction involves a complex system of adhesion molecules including selectins,  $\beta_2$  integrins and the immunoglobulin superfamily (Geng *et al.*, 1990; von Andrian *et al.*, 1991; Butcher, 1992). Neutrophil activation at sites of injury leads to the production of large amounts of oxygen-derived free radicals, the primary of which is the superoxide anion: overt production of superoxide anion extends endothelial and myocardial damage (Ratych *et al.*, 1987; Johnson III *et al.*, 1991; Lefer & Lefer, 1993). This process is in turn enhanced by platelet activation (Geng *et al.*, 1990). Resident mast cells also contribute to the dysfunction of the

\*Author for correspondence; E-mail: dsalvemini@metaphore.com

ischaemic-reperfused heart through the release of superoxide (Mannaioni *et al.*, 1991) and mediators such as histamine (Keller *et al.*, 1988; Masini *et al.*, 1990). These in turn contribute to the up-regulation of P-selectin and ICAM-1 expression on endothelial cells (Clark *et al.*, 1995) and to the development of arrhythmias (Wolff & Levi, 1986; Bani *et al.*, 1998). The release of histamine from mast cells is also amplified by the excessive release of superoxide and concomitant decrease in nitric oxide (NO), (Mannaioni *et al.*, 1991). The formation of superoxide anion is kept under tight control by endogenous superoxide dismutase (SOD) enzymes (Fridovich, 1995; Cuzzocrea *et al.*, 2001b). There are two forms of SOD: the Mn enzyme in mitochondria (SOD2) and Cu/Zn enzyme present in the cytosol (SOD1) or extracellular surfaces (SOD3). The importance of SOD2 is highlighted by the findings that in contrast to SOD1 (Reaume *et al.*, 1996) and SOD3 (Carlsson *et al.*, 1995), SOD2 knockout is lethal to mice (Lebovitz *et al.*, 1996; Melov *et al.*, 1999). In acute and chronic inflammation and in ischaemia and reperfusion injury, the production of superoxide anion is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence system to remove them. The result of such imbalance results in superoxide-mediated damage. Excessive release of superoxide anion contributes to tissue damage seen post-reperfusion in several ischaemic organs including kidney (Morpurgo *et al.*, 1996), stomach (Yoshikawa *et al.*, 1990), intestine (Zimmermann *et al.*, 1993; Salvemini *et al.*, 1999a, b; Cuzzocrea *et al.*, 2001a), skin (Goossens *et al.*, 1990) and heart (Ambrosio & Flaherty, 1992; Grill *et al.*, 1992). In the heart, a number of studies have shown that dismutation of superoxide with the native SOD enzyme (Burton, 1985; Ma *et al.*, 1992; Hangaishi *et al.*, 2001; Li *et al.*, 2001) or with overexpression of the human manganese SOD enzyme (Chen *et al.*, 1998; 2000) protects against myocardial ischaemia and reperfusion injury.

Some important tissue damaging and pro-inflammatory roles attributed to superoxide anion include: endothelial cell damage and increased microvascular permeability (Hardy *et al.*, 1994; Okayama *et al.*, 1999; Tanita *et al.*, 1999; Koo *et al.*, 2001), formation of chemotactic factors such as leukotrienes B<sub>4</sub> (Fantone & Ward, 1982; Deitch *et al.*, 1990), recruitment of neutrophils at sites of inflammation (Boughton-Smith *et al.*, 1993; Salvemini *et al.*, 1996; 1999a), release of histamine from mast cells (Mannaioni & Masini, 1988), lipid peroxidation and oxidation and DNA single-strand damage (Dix *et al.*, 1996). In addition, superoxide anion by interacting with nitric oxide destroys the biological activity of this mediator (Gryglewski *et al.*, 1986). This attenuates important anti-inflammatory and tissue protective properties of nitric oxide namely: maintenance of blood vessel tone and platelet reactivity (Furchgott & Zawadzki, 1980; Radomski *et al.*, 1990), cytoprotective effect in numerous organs (including heart, intestine and kidney) (Moncada *et al.*, 1991; Alderton *et al.*, 2001), and release of anti-inflammatory and cytoprotective prostacyclin (*via* activation of the constitutive cyclo-oxygenase enzyme (Salvemini *et al.*, 1993). Furthermore, in addition to destroying beneficial nitric oxide, the product formed from the reaction of superoxide anion with nitric oxide is peroxynitrite, a well-described, potent cytotoxic and pro-inflammatory molecule (Beckman *et al.*, 1990; Ischiropoulos *et al.*, 1992; Beckman & Crow, 1993; Crow & Beckman, 1995; Salvemini *et al.*, 1999a). Substantial

evidence now exists to suggest that peroxynitrite accounts for the majority of nitric oxide-driven inflammatory events (Salvemini *et al.*, 1998), including myocardial injury post-ischaemia and reperfusion (Matheis *et al.*, 1992; Villa *et al.*, 1994; Naseem *et al.*, 1995; Schulz & Wambolt, 1995). Under these conditions, a reduction in peroxynitrite generation, markedly reduces reperfusion injury, as shown by reduced cardiac lipid peroxidation (Masini *et al.*, 2000) or improved myocardial mechanical performance (Schulz & Wambolt, 1995). Therefore, removal of superoxide protects nitric oxide and reduces formation of the cytotoxic peroxynitrite.

We have recently shown that selective removal of superoxide by SOD mimetics such as M40403 or M40401 exerts beneficial effects in models of ischaemia and reperfusion of the intestine as well as in models of acute inflammation (Salvemini *et al.*, 1999a; Cuzzocrea *et al.*, 2001a). These are stable, low molecular weight, manganese-containing, non-peptidic molecules possessing the function and catalytic rate of native SOD enzymes (Riley *et al.*, 1996; Salvemini *et al.*, 1999b; Aston *et al.*, 2001). An important property of these SODm is that they catalytically remove superoxide anion at a high rate without interacting with other reactive species including nitric oxide, peroxynitrite, hydrogen peroxide, oxygen or hypochlorite (Riley *et al.*, 1996; Salvemini *et al.*, 1999b). Thus, SODm can serve as selective probes for deciphering the role of superoxide in biological systems where other such relevant biological oxidants may be present. It is important to realize that this property is not shared by other 'so called and claimed classes of SOD mimetics' including several metalloporphyrins. This selectivity has not been demonstrated by other claimed SOD mimetic compounds such as the Mn<sup>III</sup> or Fe<sup>III</sup> porphyrins or the Mn<sup>III</sup> (Salen) which also react with other pertinent biological oxidants including nitric oxide and peroxynitrite (Patel & Day, 1999).

The aim of the study was to evaluate the role(s) of superoxide in myocardial ischaemia and reperfusion injury in rats and highlight potential mechanism(s) through which M40403 exerts its protective effects.

## Methods

### Animals

Eighty-four male albino rats, Wistar strain, weighing 250–300 g (Morini, Reggio Emilia, Italy) were used. They were quarantined for 7 days at 22–24°C with a 12 h light/12 h dark cycle before use. Standard laboratory chow (Rodentia, Bergamo, Italy) and water were available *ad libitum*. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the animal care committee of the University of Florence (Italy). The rats were randomly distributed in seven groups composed of 12–16 animals.

### Surgical procedure

The rats were anaesthetized by intraperitoneal injection of ketamine (Parke Davis, Milan, Italy; 150 mg kg<sup>-1</sup>). A cannula was inserted into the trachea and the animals were ventilated with air using a Palmer pump (U. Basile, Comerio,

Italy). Subcutaneous peripheral limb electrodes were inserted and an electrocardiogram (ECG) was continuously recorded for the entire duration of the experiment. Body temperature was maintained at  $38 \pm 1^\circ\text{C}$ . The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed Statham, Oxnard, CA, U.S.A.) to monitor mean arterial blood pressure (MAP). Heart rate (HR) and MAP were continuously recorded by a two channel polygraph recorder (AD Instruments). All rats underwent thoracotomy at the fifth left intercostal space, the pericardium was opened and a loose 00 braided silk suture was placed around the left anterior descending coronary artery approximately 1 to 2 mm below its origin. To facilitate the successive removal of the suture, a small silicon ring was inserted in the silk thread below the knot. Then, the chest was closed by a silk suture to minimize heart displacement, taking care to leave the ends of the coronary suture threads emerging from the surgical wound. Rats were allowed to equilibrate for 20 min to enable ECG values to stabilize. Ischaemia was induced by tightening the threads of the coronary suture and was maintained for 30 min. Reperfusion was obtained by reopening the chest and cutting the ligature around the coronary artery. The duration of reperfusion was predetermined to 60 min. In the animals that did not survive the entire reperfusion period, reperfusion lasted until cessation of the cardiac activity as revealed in ECG recordings. In all the animals, the survival time was recorded. To exclude that premature mortality of rats was caused by the surgical procedures or individual abnormalities, rats showing ECG signs of impaired cardiac function during the stabilization period before induction of ischaemia or soon after the coronary artery ligature were excluded from the experiments. M40403 (0.1, 0.3 and  $1 \text{ mg kg}^{-1}$ ), or an equivalent volume (0.5 ml) of vehicle (26 mM sodium bicarbonate buffer, pH 8.0–8.1) were injected i.v. in the dorsal penile artery 15 min before ischaemia. All rats were sacrificed at 60 min post-reperfusion for biochemical analysis.

### Experimental groups

**Group 1** This group consisted of 12 sham-operated rats. These rats were injected with 0.5 ml of vehicle and then underwent the same surgical procedures as above but without the tightening of the coronary sutures. They were used as controls (sham-operated) for biochemical and morphological analyses.

**Group 2** This group consisted of 12 sham-operated rats. These rats were injected with 0.5 ml of a solution containing  $1 \text{ mg kg}^{-1}$  of M40403 (the highest dose used). They were used as controls (sham operated plus M40403).

**Group 3** This group consisted of 16 rats undergoing ischaemia and reperfusion in the absence of SODm (IR).

**Group 4–6** These groups consisted of 12 rats treated with M40403 at 0.1, 0.3 and  $1 \text{ mg kg}^{-1}$  respectively 15 min before ischaemia and reperfusion.

**Group 7** This group consisted of eight rats that underwent ischaemia alone for 30 min and were then sacrificed at this time point. This group was used to determine the effects of ischaemia on the biochemical parameters studied.

### Determination area at risk and infarct size

Sixty minutes after reperfusion of the ischaemic myocardium, the left anterior descending coronary artery was re-ligated with a loose 00 braided silk suture in the same place of the previous ligature and 2 ml of Evans Blue (Sigma Co., St. Louis, MO, U.S.A.) was retrogradely injected with a thin catheter inserted into carotid artery to delineate the *in vivo* area at risk (AAR). At the end of reperfusion, or at the moment of cessation of the cardiac activity in the rats that did not survive the predetermined reperfusion period, the chest was re-opened and the hearts quickly removed. In all samples, the anterior left ventricular wall, 3-mm distal to the ligature (to exclude tissue areas damaged by the surgical procedure) was excised. This was cut into fragments, some of which, chosen at random, were used for morphological studies and the others for biochemical assays. The extension of the left ventricular myocardium undergoing damage caused by ischaemia-reperfusion was determined by the nitroblue tetrazolium dye exclusion method. The sham-operated hearts from the rats of group 1 and 2 were treated with the same method and were used as negative controls. On removal, the hearts were attached to a Langendorff's apparatus through a cannula introduced into the aorta and perfused with 10 ml of 1% nitroblue tetrazolium dissolved in a modified Tyrode solution, pH 7.4, at a constant pressure of 40 cm of water at  $37^\circ\text{C}$  for 20 min.

In this way, the aortic semilunar valve remains closed and the dye enters directly into the coronary arteries. Following this treatment, the normal myocardium shows an intense blue staining reaction because of the presence of dehydrogenase enzymes, whereas ischaemia-reperfusion-injured lesions remain unstained. Thus, the latter regions appear as clearly delineated, unstained zones. The hearts were detached from the cannula, weighed, fixed in buffered 4% formaldehyde for 12 h, and the ventricles sectioned in 1-mm transverse slices from the apex to the ligature. The 1-mm sections were placed in individual wells with the basal side exposed. Each slide was weighed and visualized under an Olympus microscope equipped with a CCTV television camera (Sony, Tokyo, Japan) interfaced with an Apple Macintosh LC III personal computer through a Videospigot card (Supermac, Sunnyvale, CA, U.S.A.). The left ventricular area, AAR, and the area of infarction for each slide were then determined by computer planimetry using 1.49 Image Analysis Program, National Institute of Health, Bethesda, MD, U.S.A.). The size of myocardial infarction was determined by the following equation  $(A_1 \times Wt_1) + (A_2 \times Wt_2) + (A_3 \times Wt_3) + (A_4 \times Wt_4) + (A_5 \times Wt_5)$ , where A is per cent area of infarction by planimetry from subscripted numbers 1–5 representing sections and Wt is the weight of the same numbered sections. In each heart, the total volume of the damaged myocardium was calculated as the sum of the partial values of the different slices. To allow a comparison of the extension of myocardial injury between hearts of different size, the total volume of the damaged myocardium was divided by the heart weight (grams).

### Analysis of electrocardiogram (ECG)

The development of disturbances of the cardiac rhythm, such as ventricular tachycardia (VT) and ventricular fibrillation

(VF), which are known to be associated with myocardial *ischaemia* and reperfusion (Fujimoto *et al.*, 1983), were evaluated in ECG recordings of all rats subjected to the study during both coronary artery occlusion and reperfusion as described (Gelvan *et al.*, 1991). Ventricular tachycardia was recognized as three or more consecutive premature ventricular contractions, and ventricular fibrillation was recognized as irregular modulating baseline. A heart was considered to be in normal sinus rhythm when normal sinus complexes occurring at a regular rate were observed. The values are reported as the number of rats with heart arrhythmia over the total number of animals of each group. Electrocardiogram analysis, which allows to identify the moment of the cessation of cardiac activity, was also used to evaluate the survival period of the rats during the post-*ischaemic* reperfusion phase.

#### *Immunohistochemical localization of nitrotyrosine*

Tyrosine nitration was detected as previously described (Cuzzocrea *et al.*, 1997) in cardiac sections by immunohistochemistry. Tissues were fixed in 10% buffered formalin and 8  $\mu$ m sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H<sub>2</sub>O<sub>2</sub> in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate buffered saline for 20 min. Non specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with 1 : 1000 dilution of primary anti-nitrotyrosine antibody (DBA, Milan, Italy) or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy).

#### *Immunohistochemical analysis of P-selectin and ICAM-1*

P-selectin and ICAM-1, localization was detected as previously described (Gauthier *et al.*, 1994) in cardiac sections by immunohistochemistry. Acetone-fixed frozen sections were permeabilized with 0.1% Triton X-100 in phosphate buffered saline for 20 min and incubated in 2% normal serum for 1 h in order to minimize non-specific adsorption. Sections were then incubated overnight at 4°C with rabbit anti-human polyclonal antibody directed at P-selectin (CD62P) which react with rat or mouse anti-rat antibody directed at ICAM-1 (CD54) at a dilution 1 : 500. Controls included buffer alone or non-specific purified IgG. Antibody binding sites were visualized using an avidin-biotin peroxidase complex immunoperoxidase technique (Vector Laboratories) with diaminobenzidine.

#### *Evaluation of myeloperoxidase activity*

Myeloperoxidase (MPO) activity can be used as a marker for neutrophil accumulation in tissues. MPO activity was evaluated according to Bradley *et al.* (1982). Frozen samples of left ventricular tissue weighing approximately 100 mg were

homogenized in 1.5 ml of 50 mmol l<sup>-1</sup> potassium phosphate buffer, pH 6. One millilitre of the homogenate was centrifuged at 10,000  $\times$  g for 10 min, and the pellet was suspended in 1 ml of potassium phosphate buffer (50 mmol l<sup>-1</sup>), pH 6, containing 0.5% hexadecyl-trimethylammonium bromide (Sigma) to negate peroxidase activity of haemoglobin and myoglobin (Miller *et al.*, 1993) and to solubilize membrane-bound MPO. The suspensions were treated with three cycles of freezing-thawing, sonicated on ice for 10 s, and centrifuged at 12,000  $\times$  g for 10 min. MPO activity was determined in the supernatants. Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of potassium phosphate buffer (50 nmol l<sup>-1</sup>), pH 6, containing 0.19 mg ml<sup>-1</sup> of  $\delta$ -dianisidine chloride and 0.0005% H<sub>2</sub>O<sub>2</sub> as a substrate for MPO. Oxidized  $\delta$ -dianisidine forms a stable chromophore absorbing at a 460-nm wavelength. The absorbance was determined spectrophotometrically over 2 min. The values of tissue MPO activity were obtained by comparison with standard concentrations of  $\delta$ -dianisidine in the presence of excess H<sub>2</sub>O<sub>2</sub>. One unit of MPO activity is defined as that required to degrade 1 micromol of hydrogen peroxide per minute at 25°C. Protein concentration was determined with the Bradford method (Bradford, 1976). The results are expressed as mU mg<sup>-1</sup> of proteins.

#### *Determination of malonyldialdehyde production*

Malonyldialdehyde (MDA) is an end-product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals and is considered a reliable marker of myocardial cell damage (Rao *et al.*, 1983). MDA levels in cardiac tissues was determined by measurement of the chromogen generated from the reaction of MDA with 2-thiobarbituric acid as described previously (Aruoma *et al.*, 1989). Approximately 100 mg of myocardial tissue were homogenized with 1 ml of 50 mmol l<sup>-1</sup> Tris-HCl buffer containing 180 mmol l<sup>-1</sup> KCl and 10 mmol l<sup>-1</sup> EDTA, final pH 7.4, using a tissue homogenizer (Ing. Terzano, Milan, Italy). 0.5 ml of 2-thiobarbituric acid (1% w v<sup>-1</sup>) in 0.05 mol l<sup>-1</sup> NaOH and 0.5 ml of HCl (25% w v<sup>-1</sup> in water) were added to 0.5 ml of sample. The mixture was placed in test tubes, sealed with screw caps, and heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3 ml of 1 butanol, and the organic phase was separated by centrifugation at 2000  $\times$  g for 10 min. The absorbance of the organic phase was read spectrophotometrically at a 532-nm wavelength. Protein concentration was determined according to the Bradford (1976) assay. The values are expressed as nmoles of thiobarbituric acid-reactive substances (MDA equivalents) per mg of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

#### *Evaluation of calcium content*

Excessive calcium influx is a critical event, accompanying irreversible injury in myocardial *ischaemia-reperfusion* (Bourdillon & Poole-Wilson, 1981). The calcium content of the myocardial tissue was measured by atomic absorption spectrometry as described previously (Bani *et al.*, 1998). Briefly, tissue fragments weighing approximately 30 mg were rinsed thoroughly in calcium-free-buffered solution, dried in an oven at 80°C, and digested overnight with 65% HNO<sub>3</sub>

(100  $\mu\text{l}$  10  $\text{mg}^{-1}$  of dry tissue). After addition of 32% HCl (150  $\mu\text{l}$  10  $\text{mg}^{-1}$  dry tissue) the samples were dried at 45°C under nitrogen. At the moment of the assay the samples were suspended in 50  $\mu\text{l}$  of 32% HCl and added with lanthanum chloride ( $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ ) to provide a final concentration of 1% lanthanum. The amounts of calcium in the samples were read in an atomic absorption spectrophotometer (Perkin-Elmer, Überlingen, Germany) at a 422 nm wavelength. The relevant values were determined by comparison with a standard curve obtained with increasing concentrations of  $\text{CaCl}_2$  and expressed as ng of calcium per mg of tissue, dry weight.

### Computer-assisted mast cell morphometry

Evaluation of light transmittance across mast cells was performed according to the methods described previously (Bianchi & Mugnai, 1991; Masini *et al.*, 1997). The mast cells were viewed by a CCTV television camera (Sony) applied to a Reichert-Jung Microstar IV light microscope (Cambridge Instruments Inc., Buffalo, NY, U.S.A.) with a  $\times 100$  oil immersion objective and interfaced with an Apple Macintosh LCIII personal computer through a Videospigot card (Supermac). The card allows for the light transmitted across the microscopic slide to be determined within a range of 256 grey levels, which are comprised between 0 (black level) and 255 (white level). The card also allows for a digitized image of mast cells to be reproduced on the basis of the values estimated. Measurements of transmittance were carried out using a 1.49 Image analysis program (National Institute of Health, Bethesda, MD, U.S.A.). In each experimental group, the transmittance of 100 different mast cells, 10 from each animal of the group (five per fragment), was analysed and the mean transmittance value (mean  $\pm$  s.e.m.) was then calculated.

### Light microscopy for morphological observations

For histopathological examination, biopsies of left ventricular tissues were taken at the end of the reperfusion period. The tissues were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room-temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, U.S.A.). From each sample, 7  $\mu\text{m}$  thick slices were obtained and stained with ematosylin eosin and studied using light microscopy (Dialux 22 Leitz).

### Statistical analysis

The reported data are expressed as mean  $\pm$  s.e.m. For ECG analysis, significance of difference between the experimental groups was assayed by the  $\chi^2$  test. In the biochemical and morphometric assay, and in the evaluation of survival of the rats during reperfusion, the distribution of the measured values in the different experimental groups was assessed to be gaussian. Statistical analysis was performed by either one-way analysis of variance (ANOVA) test followed by Student-Newman-Keuls multiple comparison test or by Student's *t*-test for unpaired values. Calculation were carried out using a GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, U.S.A.).  $P < 0.05$  was considered significant.

### Materials

M40403 was synthesized by MetaPhore, as described previously (Salvemini *et al.*, 1999b). Primary anti-nitrotyrosine, anti-ICAM and anti P-Selectin antibodies were from Upstate Biotech (DBA, Milan, Italy). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

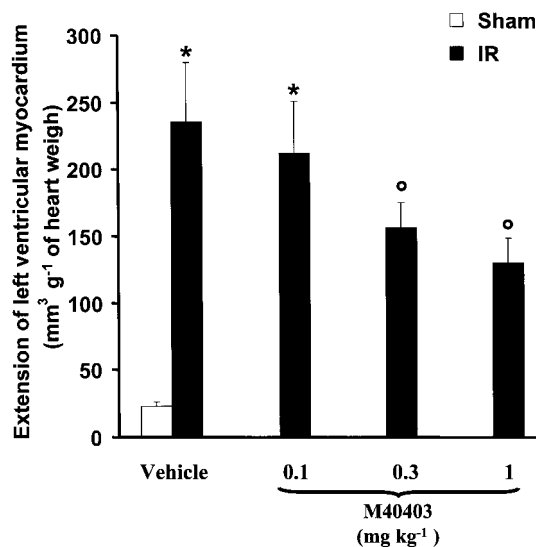
### Results

#### Extension of myocardial infarct size caused by regional *ischaemia* and reperfusion

The results of the computer-assisted morphometry on the *ischaemic* reperfused hearts stained with nitroblue tetrazolium showed that M40403 dose-dependently reduced the extension of myocardial damage (Figure 1). The mean values for the area at risk (AAR) ranged from  $56 \pm 3\%$  to  $59 \pm 4\%$  and were similar in all animal groups studied ( $P = \text{ns}$ , data not shown). The summary data for the infarct size, expressed as per cent of AAR, following 30 min coronary occlusion and 60 min of reflow are shown in Figure 2. M40403 did not modify the extension of at risk area per left ventricle (LV), ( $P = \text{ns}$ ), while the portion of this area rendered necrotic was significantly reduced ( $P < 0.01$ ) in the animals treated with M40403 at the dose of 1  $\text{mg kg}^{-1}$ . Sham-operation did not result in a significant degree of infarction (data not shown).

#### Haemodynamic parameters: ECG analysis and duration of survival during the post *ischaemic* reperfusion phase

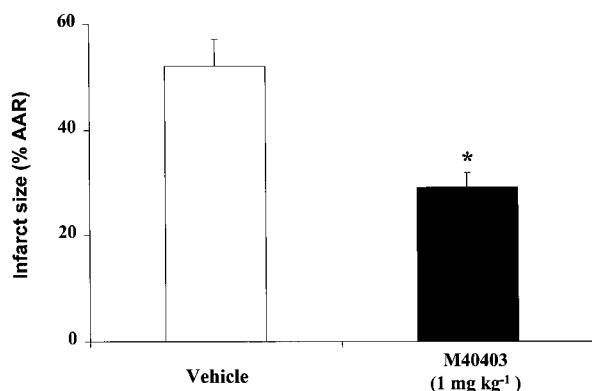
Mean values for MAP and HR measured during the course of the experiments are reported in Table 1. Baseline haemodynamic data were similar ( $P = \text{ns}$ ) in all groups studied. In sham operated rats, infusion of vehicle for M40403, 1  $\text{mg kg}^{-1}$  had



**Figure 1** Extension of left ventricular myocardium with *ischaemic* reperfusion-induced injury as evaluated by computer-assisted morphometry on heart stained with nitroblue tetrazolium. Significance of difference between groups (one-way ANOVA: each group is the mean  $\pm$  s.e.m. of at least 12 experiments; \*n.s.; ° $P < 0.01$ ).

no significant haemodynamic effects (Table 1). In rats subjected to coronary occlusion and reperfusion, mean values for MAP significantly decreased throughout the experimental period, but there was no alteration in heart rate. The mean values of MAP and HR of rats and subjected to IR and treated with M40403 were not different from the IR group treated with vehicle (Table 1). Examination of ECG recordings from the rats subjected to myocardial ischaemia and reperfusion showed that M40403 ( $1 \text{ mg kg}^{-1}$ ) reduced the occurrence of ventricular arrhythmias. During ischaemia, VT occurred in six out of 16 control animals, whereas no ventricular arrhythmias were found in the 12 rats treated with  $1 \text{ mg kg}^{-1}$  M40403 ( $P < 0.003$ ). During reperfusion, VT and VF occurred in 12 out of the 16 rats treated with the vehicle, whereas these arrhythmias were present in seven out of the 12 rats treated with  $0.1 \text{ mg kg}^{-1}$  of M40403 ( $P < 0.01$ ), in five out of the 12 rats treated with  $0.3 \text{ mg kg}^{-1}$  of M40403 and only in one of the 12 rats treated with the highest of M40403. In the sham operated groups treated with vehicle or with M40403 at the dose of  $1 \text{ mg kg}^{-1}$ , VT was observed only in one animal, starting 45 min from placement of the silk thread around the left coronary artery.

During ischaemia, none of the animals died; while in the post-ischaemic reperfusion period, some animals died in both



**Figure 2** Graph of infarct size expressed as per cent of area at risk (AAR) after 30 min of ischaemia and 60 min of reperfusion. Each group is the mean  $\pm$  s.e.m. of at least nine experiments. Legend for each bar is shown underneath. \* $P < 0.001$ .

the M40403-treated and untreated groups. However, the number of animals that survived was higher and the duration of survival was significantly longer in the M40403-treated groups than in the untreated one (Table 2). All sham-operated rats survived the entire experimental period (90 min).

#### Computer-assisted morphometry of mast cells

Morphometric analysis of cardiac mast cells showed that light transmittance across mast cells, which is inversely related to the amount of secretory granules, was significantly higher in the ischaemic-reperfused hearts in comparison with the sham-operated ones (Figure 3). Conversely, in the rats treated with the highest dose ( $1 \text{ mg kg}^{-1}$ ), the light transmittance across cardiac mast cells was nearly similar to the sham-operated ones (Figure 3).

#### Biochemical studies

The results of the biochemical assays for the markers of ischaemia-reperfusion-induced myocardial injury showed that in sham operated rats malonyldialdehyde (MDA) production, an index of peroxidation of cell membrane lipids and calcium content, was very low. In the ischaemic-reperfused hearts, MDA production, and calcium content were significantly elevated (Figures 4 and 5). These parameters were significantly reduced in a dose dependent manner by M40403 (Figures 4 and 5).

#### Immunohistology measurements of nitrotyrosine

In sham-operated hearts, little if any positive staining for nitrotyrosine was observed (Figure 6A). In the post-ischaemic myocardium, however, strong positive staining for nitrotyrosine was observed within myocytes (Figure 6B). M40403 treatment ( $1 \text{ mg kg}^{-1}$ ) reduced the degree of immunostaining for nitrotyrosine (Figure 6C) in the reperfused heart.

#### Histological changes, myeloperoxidase activity and immunohistochemical localization of ICAM-1 and P-selectin

At 60 min after reperfusion, neutrophil infiltration and activation of vascular endothelial cells were assessed by the

**Table 1** Effect of vehicle or M40403 on mean arterial pressure (MAP) and heart rate (HR) in sham operated rats or ischaemic reperfusion rats

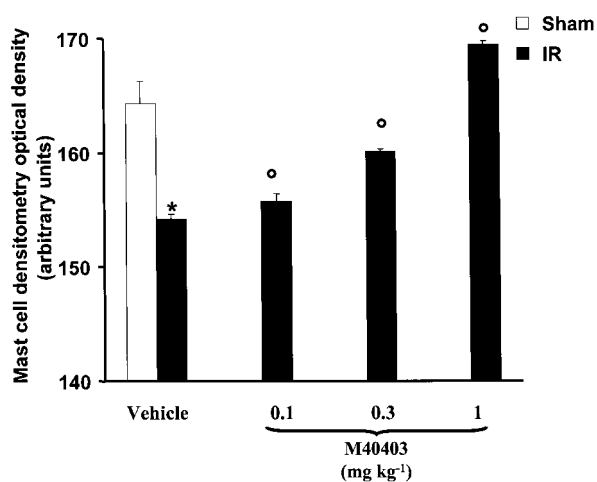
Group	n		Baseline	Occlusion (min)		Reperfusion (min)	
				15	30	30	60
Sham + vehicle	12	MAP	128 $\pm$ 7	126 $\pm$ 4	119 $\pm$ 3	110 $\pm$ 6	102 $\pm$ 4
		HR	427 $\pm$ 14	429 $\pm$ 16	426 $\pm$ 20	434 $\pm$ 16	429 $\pm$ 7
Sham + M40403 1 mg kg <sup>-1</sup>	12	MAP	124 $\pm$ 6	120 $\pm$ 6	116 $\pm$ 5	98 $\pm$ 6	98 $\pm$ 9
		HR	420 $\pm$ 2	427 $\pm$ 3	426 $\pm$ 4	424 $\pm$ 7	432 $\pm$ 8
IR + vehicle	16	MAP	128 $\pm$ 8	120 $\pm$ 6	114 $\pm$ 4	94 $\pm$ 7	95 $\pm$ 7
		HR	456 $\pm$ 11	432 $\pm$ 11	438 $\pm$ 12	429 $\pm$ 10	431 $\pm$ 10
IR + M40403 0.1 mg kg <sup>-1</sup>	12	MAP	126 $\pm$ 7	120 $\pm$ 5	118 $\pm$ 7	96 $\pm$ 6	96 $\pm$ 9
		HR	430 $\pm$ 10	434 $\pm$ 9	426 $\pm$ 8	417 $\pm$ 7	426 $\pm$ 8
IR + M40403 0.3 mg kg <sup>-1</sup>	12	MAP	129 $\pm$ 6	125 $\pm$ 6	125 $\pm$ 8	95 $\pm$ 7	95 $\pm$ 8
		HR	432 $\pm$ 8	430 $\pm$ 8	428 $\pm$ 10	426 $\pm$ 8	430 $\pm$ 6
IR + M40403 1 mg kg <sup>-1</sup>	12	MAP	117 $\pm$ 6	109 $\pm$ 9	106 $\pm$ 10	98 $\pm$ 12	98 $\pm$ 9
		HR	435 $\pm$ 39	440 $\pm$ 37	442 $\pm$ 38	422 $\pm$ 36	426 $\pm$ 35

Animals received M40403 or an equal volume of vehicle (26 mM sodium bicarbonate buffer, pH 8.1–8.3). All data are mean  $\pm$  s.e.m. of  $n$  observations.

**Table 2** Effect of vehicle or M40403 survival rate, percentage survival, and survival time in sham operated rats or ischaemic reperfused rats

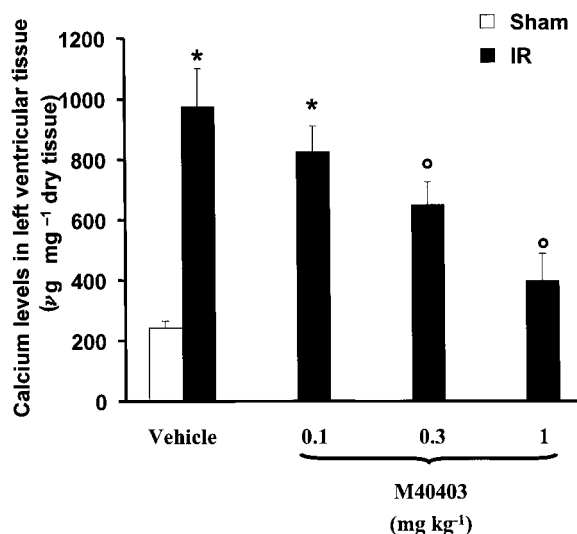
Treatment	Surviving	%	Survival time (min)
Sham + vehicle	12/12	100	> 60
Sham + M40403 1 mg kg <sup>-1</sup>	12/12	100	> 60
IR + vehicle	16/16	57	37 ± 3.7
IR + M40403 0.1 mg kg <sup>-1</sup>	6/12	50	47 ± 3.7
IR + M40403 0.3 mg kg <sup>-1</sup>	8/12	70*	54 ± 3.7*
IR + M40403 1 mg kg <sup>-1</sup>	10/12	84*	58 ± 3.7*

Animals received M40403 or an equal volume of vehicle (26 mM sodium bicarbonate buffer, pH 8.1–8.3). \* $P < 0.01$  vs IR + vehicle. The survival time represents the survival time during the reperfusion period.

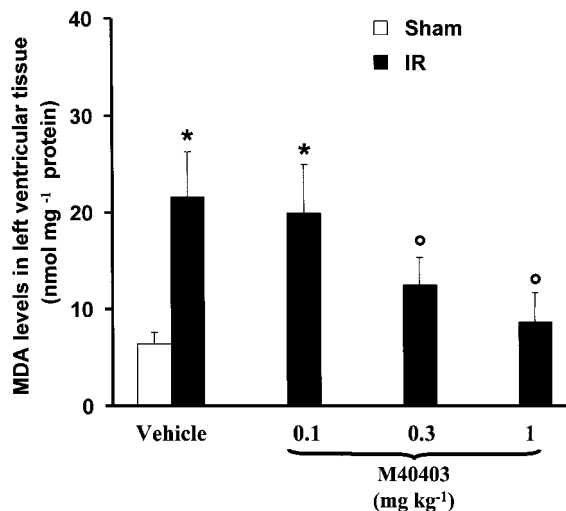


**Figure 3** Mast cell densitometry evaluated as light transmittance across left ventricular mast cells. Compared with sham-operated heart (group 2), the ischaemic-reperfused hearts show a significant increase in light transmittance indicating a decrease in intracellularly secretory granules. This effect is dose dependently reverted by M40403 treatments. Values are mean ± s.e.m. of eight experiments.  $P < 0.001$  vs sham operated hearts,  $P < 0.05$  one-way ANOVA.

measurement of tissue myeloperoxidase activity and by ICAM-1 and P-selectin immunostaining. As shown in Figure 7, myeloperoxidase activity significantly increased ( $P < 0.001$ ) in left ventricular tissue of ischaemic-reperfused rats. Histological examination of cardiac tissue (see representative sections in Figure 8) revealed pathological changes that correlated closely with the increase in myeloperoxidase activity. In particular, in sham-operated hearts, the myocardium had a normal appearance without intracellular oedema or blood vessel dilation and neutrophils adherent to the coronary endothelium or in the intramuscular connective tissue (Figure 8A). In the ischaemic-reperfused hearts, neutrophil infiltration was observed (Figure 8B). Intracellular oedema was also seen (Figure 8B). In contrast, in the ischaemic-reperfused hearts from rats treated with M40403, (1 mg kg<sup>-1</sup>), neutrophil infiltration was an occasional finding and the increase in myeloperoxidase activity induced by ischaemia-reperfusion was significantly reduced ( $P < 0.01$ )



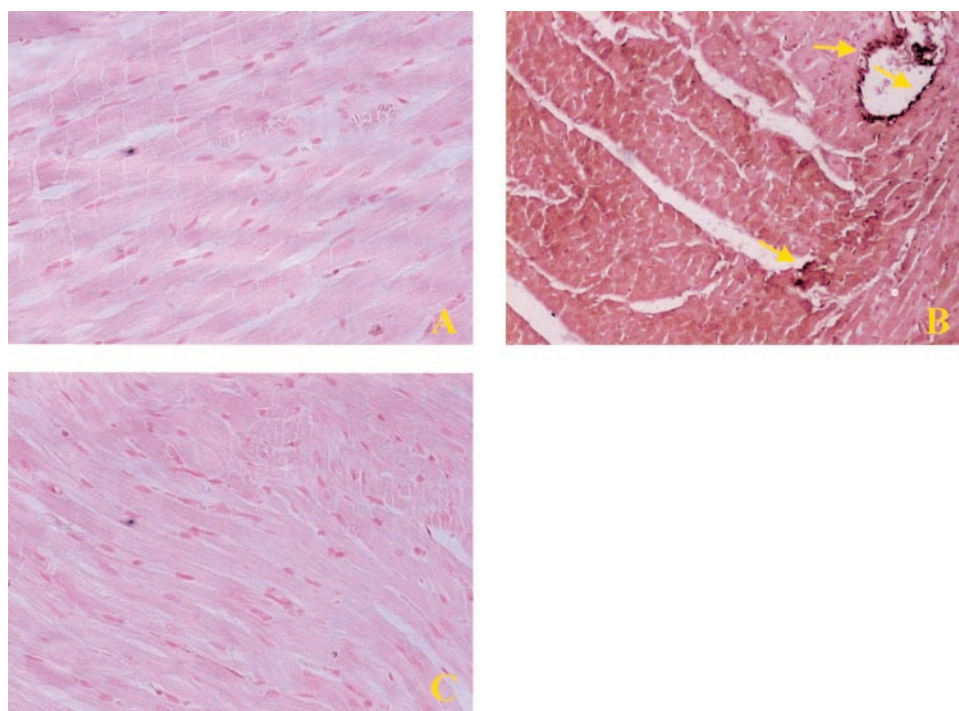
**Figure 4** Reperfusion of the ischaemic myocardium leads to enhanced release of calcium which was inhibited in a dose dependent manner by M40403 (0.1–1 mg kg<sup>-1</sup>). Values are mean ± s.e.m. of eight experiments. \*n.s.; <sup>o</sup> $P < 0.01$  one-way ANOVA.



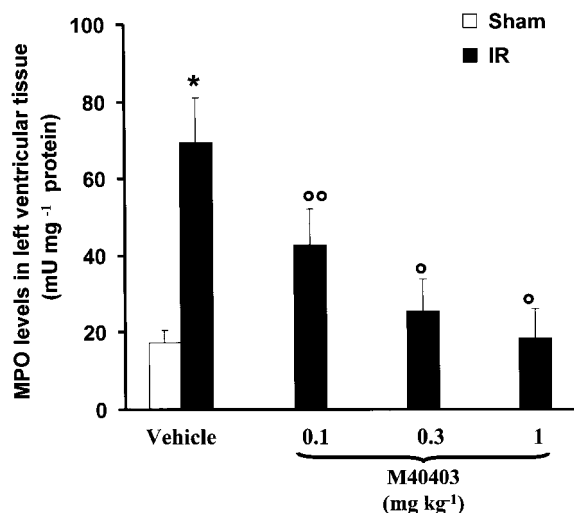
**Figure 5** Reperfusion of the ischaemic myocardium leads to enhanced release of calcium which was inhibited in a dose dependent manner by M40403 (0.1–1 mg kg<sup>-1</sup>). Values are mean ± s.e.m. of eight experiments. \*n.s.; <sup>o</sup> $P < 0.01$  one-way ANOVA.

(Figure 7). Overall, organ injury was significantly attenuated (Figure 8C).

Staining of cardiac tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrow), demonstrating that ICAM-1 is constitutively expressed (Figure 9A). After 1 h of reperfusion, the staining intensity substantially increased in the vessels (Figure 9B; see arrows). Sections from M40403-treated rats did not reveal any up-regulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Figure 9C; see arrow). Left ventricular tissue sections obtained from rats undergoing 30 min of ischaemia followed by 1 h reperfusion showed positive staining for P-selectin localized in the vessels (Figure 9E; see arrows). No staining was observed in sham-operated rats (Figure 9D) and



**Figure 6** No positive staining for nitrotyrosine (A) was found in the left ventricular section from sham-operated rats. Sixty minutes after reperfusion immunohistochemical analysis for nitrotyrosine (B) show positive staining localized in the vascular wall (see arrows) and in the cardiomyocyte in the injured area. The intensity of the positive staining for nitrotyrosine (C) was significantly reduced in the left ventricular section from M40403-treated rats. Original magnification:  $\times 150$ . Figure is representative of at least three experiments performed on different experimental days.



**Figure 7** Reperfusion of the ischaemic myocardium leads to neutrophil recruitment into the myocardium (as evidenced by increased MPO levels). Neutrophil recruitment was in turn inhibited in a dose dependent manner by M40403 (0.1–1 mg kg<sup>-1</sup>). Values are mean  $\pm$  s.e.m. of eight experiments. \*n.s.; <sup>o</sup> $P < 0.01$  one-way ANOVA.

in tissue obtained at 1 h after reperfusion from M40403-treated rats (Figure 9F). To verify the binding specificity for ICAM-1 or P-selectin, 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.

#### *Biochemical parameters in hearts taken from animals that underwent ischaemia alone*

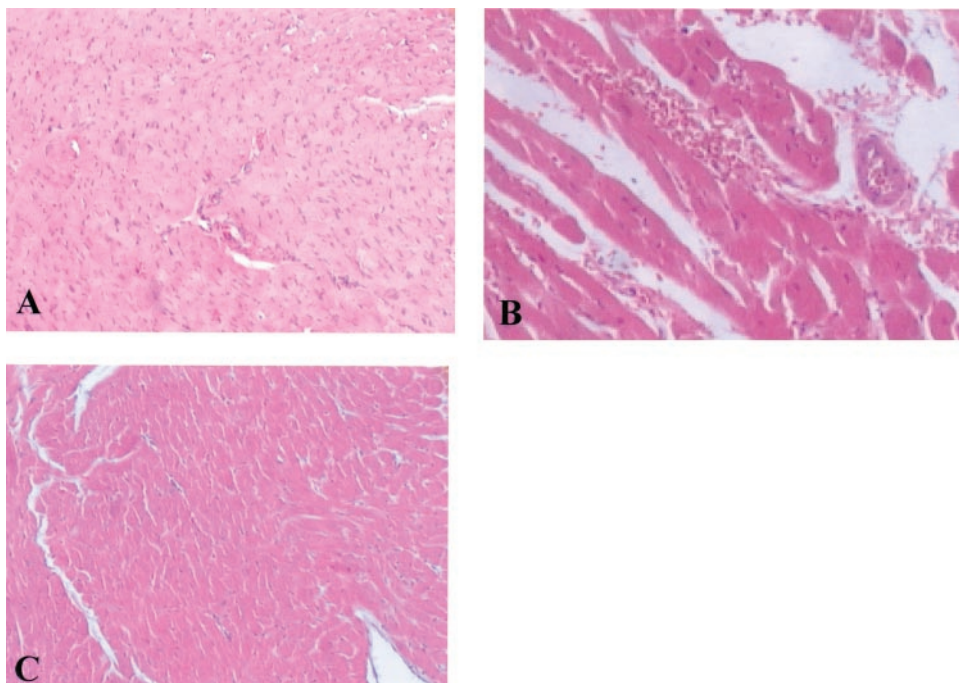
No increase in levels of MPO activity, MDA production and calcium contents were seen in tissues obtained from animals that underwent a period of ischaemia (30 min) alone (group 7, data not shown).

## Discussion

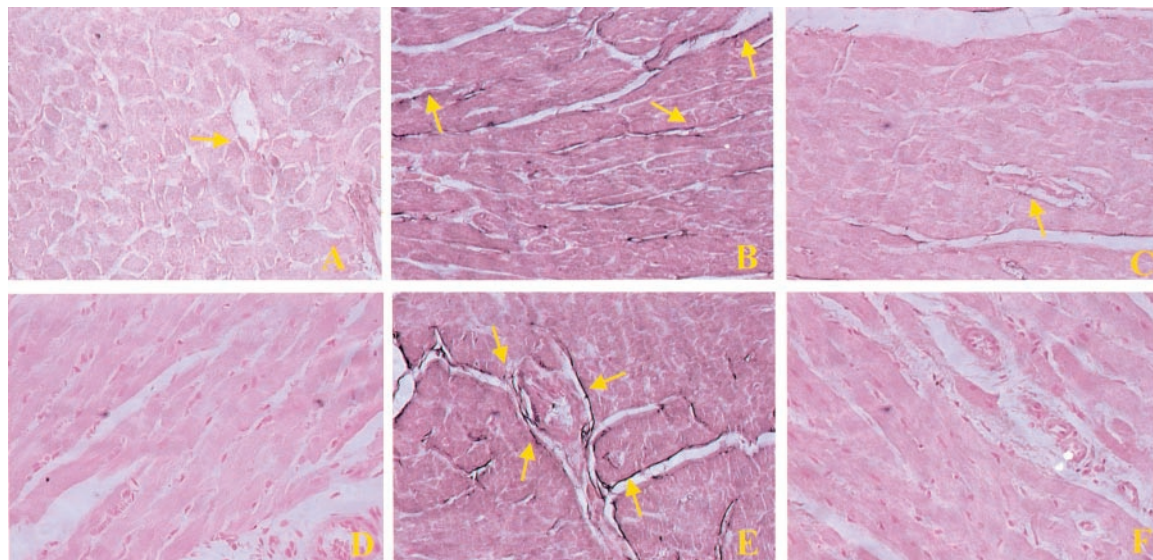
The results of this study show that, M40403 exerts a significant cardioprotective effect in the ischaemic and reperfused rat heart. The biochemical and histological alterations observed in this study were dependent upon reperfusion of the ischaemic myocardium since no alterations were observed in hearts that underwent a period of ischaemia alone. This is consistent with numerous reports that indicate that reperfusion is the key trigger of many of the events that lead to myocardial dysfunction associated with ischaemia and reperfusion injury (Braunwald & Kloner, 1985). Superoxide anions are in fact formed in excess during reperfusion of the ischaemic organ and not during the ischaemic phase (Cuzzocrea *et al.*, 1997; 2001a, b; Salvemini *et al.*, 1999a, b). M40403 exerts its beneficial effects by removing superoxide generated during reperfusion (Salvemini *et al.*, 1999a, b).

Systemic administration of M40403 results in a marked reduction of the myocardial areas damaged by post-ischaemic reperfusion as well as in a substantial reduction in the occurrence of severe ventricular arrhythmias. The current study also shows that M40403 acts at multiple levels in the





**Figure 8** Left ventricular section from sham rats demonstrating the normal architecture of the myocardial tissue (A). In the ischaemic-reperfused hearts, a neutrophil infiltration was observed (B). Intracellular oedema was also seen. Myocardium from M40403-treated rats shows reduced tissue injury (C). Original magnification:  $\times 150$  (for A and C)  $\times 300$  (for B). Figure is representative of at least three experiments performed on different experimental days.



**Figure 9** Staining of left ventricular section obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrow), demonstrating that ICAM-1 is constitutively expressed (A), no P-selectin staining was seen in sham animals. (D). Section obtained from IR shocked-rats showed intense positive staining (see arrows) for ICAM-1 (B) and for P-selectin (E) on vascular wall. The degree of endothelial staining for ICAM-1 (C) and for P-selectin (F) was markedly reduced in tissue section obtained from M40403-treated rats. Original magnification:  $\times 150$ . Figure is representative of at least three experiments performed on different experimental days.

cascade of events that lead to myocardial injury. These events include endothelial dysfunction, up-regulation of adhesion molecules such as ICAM-1 and P-selectin; (Geng *et al.*, 1990; Dreyer *et al.*, 1991; Lawrence & Springer, 1991; Butcher, 1992) neutrophil accumulation in the myocardium (Smith III *et al.*, 1988; Reynolds & McDonagh, 1989; Bani *et al.*, 1998), lipid peroxidation (Bani *et al.*, 1998), platelet and mast cell

activation (Keller *et al.*, 1988; May *et al.*, 1991; Bani *et al.*, 1998), generation of peroxynitrite (Matheis *et al.*, 1992; Naseem *et al.*, 1995; Schulz & Wambolt, 1995; Wang & Zweier, 1996) and calcium overload that eventually leads to myocardial cell damage and death (Duncan *et al.*, 1992).

M40403 decreased the recruitment of neutrophils from the circulation to the myocardium, as indicated by the decrease

in myeloperoxidase activity, a marker of neutrophil accumulation in tissues (Mullane *et al.*, 1985). This effect was confirmed histologically. Endothelial cells appear to be one of the major regulators of neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion, and migration into the tissue. P-selectin is rapidly released during the early phase of reperfusion, after exposure to certain stimuli, such as histamine, or complement, allowing the leukocytes to roll along the endothelium (Geng *et al.*, 1990; Lorant *et al.*, 1991; Patel *et al.*, 1991; Lorant *et al.*, 1993). ICAM-1, which is constitutively expressed on the surface of endothelial cells, is then involved in neutrophil adhesion (Wertheimer *et al.*, 1992; Farhood *et al.*, 1995). Reperfusion of the ischaemic tissue induces the appearance of P-selectin on the endothelial vascular wall and up-regulates the expression of ICAM-1. ICAM-1 and P-selectin expression was attenuated by M40403 providing an explanation as to the possible mechanism through which M40403 blocks neutrophil infiltration.

Moreover, inhibition of neutrophil infiltration by M40403 correlated well with an inhibition of lipid peroxidation (as measured by MDA) supporting the notion that release of superoxide from activated neutrophils is responsible at least in part to peroxidation of lipid membranes in the myocardium (Rao *et al.*, 1983).

In cardiac ischaemia and reperfusion injury, superoxide, produced during reperfusion, rapidly reacts with nitric oxide to form peroxynitrite (Matheis *et al.*, 1992; Naseem *et al.*, 1995; Schulz & Wambolt, 1995). A possible and most likely mechanism by which M40403 reduces ischaemia-reperfusion injury is by reducing peroxynitrite formation by simply removing superoxide before it reacts with nitric oxide. This has two fundamentally important consequences: sparing of the beneficial nitric oxide and removal of toxic peroxynitrite. The release of nitric oxide from the constitutive enzyme has numerous beneficial and cytoprotective roles in the heart (Gaballa & Goldman, 1999; Gaballa *et al.*, 1999) and nitric oxide donors exert beneficial effects in myocardial ischaemia and reperfusion injury (Johnson III *et al.*, 1991). Peroxynitrite has several pro-inflammatory and cytotoxic effects (Squadrito *et al.*, 1995) and its removal by agents such as FeTMPS, a porphyrin-containing molecule which increases the rate of isomerization of peroxynitrite to nitrate (Stern *et al.*, 1996) is cytoprotective and anti-inflammatory (Stern *et al.*, 1996; Salvemini *et al.*, 1996; Misko *et al.*, 1998; Cuzzocrea *et al.*, 2000). Furthermore, peroxynitrite has been implicated in myocardial ischaemia-reperfusion injury (Matheis *et al.*, 1992; Naseem *et al.*, 1995; Schulz & Wambolt, 1995). Peroxynitrite also nitrates tyrosine residues in proteins and nitrotyrosine formation, detected by immunofluorescence, has been used as a marker of endogenous formation of peroxynitrite (Beckman, 1996) or as an indicator of 'increased nitrosative stress' (Eiserich *et al.*, 1998). We have found that nitrotyrosine is indeed present in heart sections taken after ischaemia and reperfusion (but not in sections that underwent ischaemia alone) and that M40403 reduced the staining in

these tissues. Based on these findings, we conclude that the reperfusion of ischaemic myocardium evoked, at least in part, a superoxide-driven peroxynitrite formation that was, in turn responsible for the formation of nitrotyrosine.

Superoxide and peroxynitrite can also cause DNA single-strand damage that is the obligatory trigger for PARP activation (Inoue & Kawanishi, 1995; Salgo *et al.*, 1995), which ultimately leads to cell injury (Docherty *et al.*, 1999). Furthermore, recent studies have demonstrated the PARP<sup>-/-</sup> mice are significantly protected from myocardial ischaemia when compared with PARP<sup>+/+</sup> mice (Zingarelli *et al.*, 1998; Yang *et al.*, 2000).

Calcium leakage occurs during reperfusion (Masini *et al.*, 1989a, b) and a loss of calcium homeostasis with an excess calcium influx is thought to be a critical event underlying irreversible myocardial injury (Hayashi, 2000). M40403, was found in these studies to prevent calcium overload in the myocardial tissue and to strongly reduce myocyte alterations like those previously described in conditions of excess intracellular calcium (Bani *et al.*, 1998).

An additional contribution to the cardioprotective effect of M40403 against ischaemia-reperfusion injury may rely on its effect on cardiac mast cells. In fact, resident mast cells undergo degranulation during post-ischaemic reoxygenation of the heart, releasing powerful mediators, such as histamine, serotonin and leukotrienes (Keller *et al.*, 1988; Masini *et al.*, 1990). These substances can affect vascular resistance and permeability, thus increasing tissue oedema, up-regulate adhesion molecules and induce cardiac arrhythmias (Wolff & Levi, 1986; Bani *et al.*, 1998; Masini *et al.*, 1999). The results of the current study show that M40403, inhibits ischaemia-reperfusion-induced mast cell degranulation and hence the release of granule-stored mediators, including histamine, thus contributing to the myocardial salvage. Our own studies had shown that superoxide anion is able to evoke mast cell degranulation and subsequent histamine release (Mannaioni & Masini, 1988).

The protective effects of M40403 were supported by histological examination and accompanied by overall improved survival rate.

In conclusion, the results of the present study clearly indicate that superoxide anion generated during the reperfusion phase plays a critical role in the development of cardiac reperfusion injury. M40403, a low molecular weight SOD mimetic, which removed superoxide anion selectively without interfering with other reactive oxygen species, may offer a novel therapeutic approach for the treatment of ischaemic heart disease.

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