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Protective effects of a new stable, highly active SOD mimetic, M40401 in splanchnic artery occlusion and reperfusion

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1 Splanchnic artery occlusion shock (SAO) causes an enhanced formation of reactive oxygen species (ROS), which contribute to the pathophysiology of shock. Here we have investigated the effects of M40401, a new *S*,*S*-dimethyl substituted biscyclohexylpyridine Mn-based superoxide dismutase mimetic (SODm, $k_{cat} = 1.2 \times 10^{+9}$ M^{-1} s^{-1} at pH = 7.4), in rats subjected to SAO shock.

2 Treatment of rats with M40401 (applied at 0.25, 2.5 or 25 μg kg^{-1}, 15 min prior to reperfusion), attenuated the mean arterial blood and the migration of polymorphonuclear cells (PMNs) caused by SAO-shock. M40401 also attenuated the ileum injury (histology) as well as the increase in the tissue levels of myeloperoxidase (MPO) and malondialdehyde (MDA) caused by SAO shock in the ileum.

3 Immunohistochemical analysis for nitrotyrosine revealed a positive staining in ileum from SAO-shocked rats. The degree of staining for nitrotyrosine was markedly reduced in tissue sections obtained from SAO-shocked rats which had received M40401. Reperfused ileum tissue sections from SAO-shocked rats showed positive staining for P-selectin and for anti-intercellular adhesion molecule (ICAM-1) in the vascular endothelial cells. M40401 treatment markedly reduced the intensity and degree of P-selectin and ICAM-1 in tissue sections from SAO-shocked rats. M40401 treatment significantly improved survival.

4 Additionally, the very high catalytic activity of this new mimetic (comparable to the native human Cu/Zn SOD enzyme and exceeding the activity of the human Mn SOD enzyme) translates into a very low dose ($\sim\mu$g kg^{-1}) required to afford protection in this SAO model of ischemia reperfusion injury.

5 Taken together, our results clearly demonstrate that M40401 treatment exerts a protective effect, and part of this effect may be due to inhibition of the expression of adhesion molecules and peroxynitrite-related pathways with subsequent reduction of neutrophil-mediated cellular injury.
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Abbreviations: ICAM-1, intercellular adhesion molecules; MDA, malonaldehyde; MPO, myeloperoxidase; NO, nitric oxide; O_2^- superoxide anions; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SAO, splanchnic artery occlusion; SOD, superoxide dismutase

Introduction

Occlusion of the splanchnic circulation followed by reperfusion (SAO) results in a severe form of circulatory shock, characterized by severe hypotension, hemoconcentration, intestinal injury and a high mortality rate (Lefer & Lefer, 1993; Zimmermann *et al*., 1993). An important component of SAO shock is endothelial dysfunction (Lefer & Lefer, 1993; Altura *et al*., 1985; Carey *et al*., 1992; Zingarelli *et al*., 1992) originally attributed to oxygen-derived free radicals released from both the reperfused endothelium (Lefer & Lefer, 1993; Ratych *et al*., 1987) and from activated adherent PMNs (Granger *et al*., 1981; McCord, 1981; Mullane *et al*., 1988; Bittermann *et al*., 1988). Endothelial dysfunction predisposes to vasospasm, platelet deposition, and increased neutrophil adherence, which exacerbates the shock state. Ischemia-reperfusion is a stimulus for leukocyte-endothelial interaction (Granger, 1977). Leukocyte-endothelial interaction involves a complex system of adhesion molecules including the selectins, β_2 integrins and the immunoglobulin superfamily (Butcher, 1993; Geng *et al*., 1990; von Andrian *et al*., 1991). Leukocyte interaction with the endothelium begins with leukocyte rolling, followed by adherence and transendothelial migration. P-selectin, a member of the selectin family of adhesion molecules, is believed to play a major role in the initial phase of leukocyte emigration, which is characterized by the rolling of leukocytes along the vascular endothelial surface. Although P-selectin is necessary for early neutrophil contact with the endothelium, P-selectin-mediated leukocyte-endothelial interaction is not sufficient to allow neutrophil

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emigration from the vessel. A firmer adherence of the neutrophil to the endothelial surface is required for transendothelial migration (Butcher, 1992). This firm adherence involves the interaction of β_2 integrins (i.e. CD11/CD18) on the PMN surface and intercellular adhesion molecule 1 (ICAM-1) on the endothelial cell surface (Butcher, 1992; Lawrence & Springer, 1991). Experimental studies have also showed that *in vivo* administration of antibodies raised against ICAM-1 reduced neutrophil infiltration into the inflamed lungs in the rabbit and protects the development of SAO-induced injury. Neutrophil activation at sites of injury results in a large production of superoxide anions ($\cdot O_2^-$) which in turn contributes to tissue damage seen post reperfusion in several ischemic organs including kidney (Morpurgo *et al*., 1996); stomach (Yoshikawa *et al*., 1990), intestine (Zimmermann *et al*., 1993), skin (Goossens *et al*., 1990) and heart (Ambrosio & Flaherty, 1992; Grill *et al*., 1992). Some important tissue damaging and pro-inflammatory roles attributed to $\cdot O_2^-$ include: endothelial cell damage and increased microvascular permeability (Droy-Lefaix *et al*., 1991; Haglind *et al*., 1994; Xia *et al*., 1995), formation of chemotactic factors such as leukotrienes B_4 (Fantone & Ward 1982; Deitch *et al*., 1990), recruitment of neutrophils at sites of inflammation (Boughton-Smith *et al*., 1993; Salvemini *et al*., 1996a; 1999a,b), lipid peroxidation and oxidation and DNA single-strand damage (Dix *et al*., 1996). In addition, $\cdot O_2^-$ by interacting with NO destroys the biological activity of this mediator attenuating important anti-inflammatory and tissue protective properties of NO namely: maintenance of blood vessel tone and platelet reactivity, cytoprotective effect in numerous organs (including heart, intestine and kidney), and release of anti-inflammatory and cytoprotective prostacyclin (*via* activation of the constitutive cyclo-oxygenase enzyme (Salvemini *et al*., 1993; 1996b). The product formed as a result of $\cdot O_2^-$ interacting with NO is peroxynitrite (ONOO-), a well described, potent cytotoxic and pro-inflammatory molecule (Salvemini *et al*., 1998; 1999a,b; Beckman *et al*., 1990; Ischiropoulos *et al*., 1992; Beckman & Crow 1993; Crow & Beckman, 1995; Misko *et al*., 1998). Therefore, removal of superoxide protects NO and reduces formation of the cytotoxic ONOO-.

We have recently shown that selective removal of superoxide by M40403 exerts beneficial effects in a model of SAO (Salvemini *et al*., 1999a,b). These new low molecular weight, synthetic manganese containing superoxide dismutase mimetic (SODm) represent a breakthrough in chemical design in that they are stable *in vivo*, possess high activity, and are selective for superoxide with no activity toward hydrogen peroxide (H_2O_2), ONOO-, NO, or hypochlorite (OCl^-). This novel selectivity resides in the nature of the manganese(II) center in these low molecular weight complexes. The resting oxidation state of the complex is the reduced state, Mn(II). As a consequence, the complex has no reactivity with reducing agents until it is oxidized to Mn(III) by superoxide. Since the complex is so difficult to oxidize (+0.75 v (SHE)) many oxidants will not oxidize these complexes, including nitric oxide and oxygen, and since they operate *via* a facile one-electron oxidation pathway other two-electron non-radical oxidants are also not able to oxidize the Mn(II) complex; e.g., $OONO^-$, H_2O_2, OCl^-. The unique selectivity of these complexes for superoxide in the presence of other ROS make it possible then to dissect the role of superoxide in disease models in which ROS are implicated. We have continued our computer-aided design and synthesis program so that we have recently developed M40401 (Figure 1, the *S*,*S*-dimethyl substituted derivative of the M40403 biscyclohexylpyridyl class of mimetic) which possesses a much higher catalytic activity at pH = 7.4. In fact, its catalytic rate exceeds $1 \times 10^{+9}$ M^{-1} s^{-1}, comparable to the native Cu/Zn SOD enzymes and about 100 times the activity of M40403 at pH = 7.4. As with M40403, M40401 has no catalase activity or reactivity with peroxynitrite.

The aim of the study reported here was to ascertain if such a highly superoxide specific catalyst with enhanced activity would be useful as a human therapeutic agent for reperfusion injury by assessing its activity in pharmacologically relevant animal models of reperfusion injury and to compare its *in vivo* activity to that of an SOD mimetic with similar stability and lipophilicity, but with the 100 fold less catalytic activity; i.e. to the precursor mimetic M40403. Such a study should enable us to determine the relative importance and role of superoxide-mediated injury in reperfusion in these models.

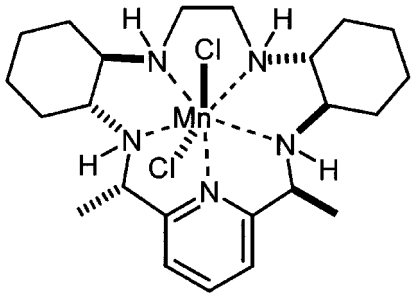
Methods

Animals

Male Sprague-Dawley rats (250–300 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L358/1 12/18/1986).

Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were allowed access to food and water *ad libitum*. The rats were anaesthetized with sodium pentobarbital (45 mg kg^{-1} i.p.). Following anaesthesia, catheters were placed in the carotid artery and jugular vein as described previously (Caputi *et al*., 1980). Blood pressure was monitored continuously by a Maclab A/D converter (Ugo Basile, Varese, Italy), and stored and displayed on a Macintosh personal computer. After



M40401

Figure 1 Structure of M40401.

midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution. Rats were observed for a 30 min stabilization period before either splanchnic ischemia or sham ischemia. SAO shock was induced by clamping both the superior mesenteric artery and the celiac trunk, resulting in a total occlusion of these arteries for 45 min. After this period of occlusion, the clamps were removed. In one study, the various groups of rats were sacrificed at 60 min for histological examination of the bowel and for biochemical studies, as described below.

Experimental groups

In the treated groups of animals ($n=10$), SOD mimetic (M40401) or corresponding volume of vehicle (26 mM sodium bicarbonate buffer, pH 8.1–8.3) was given as an intravenous bolus 0.25, 2.5 or 25 μg kg^{-1}, 15 min prior to reperfusion (SAO + M40401 groups). In separate groups of rats, surgery was performed identically to the SAO group, except that the blood vessels were not occluded (time-controlled sham group; Sham).

Measurement of nitrite/nitrate (NOx) in plasma

Nitrate/nitrate (NOx) levels, an indicator of NO synthesis, was measured in plasma samples from sham or SAO-shocked rats at 60 min after reperfusion as previously described (Cuzzocrea *et al*., 1997). First, nitrate in the plasma was reduced to nitrite by incubation with nitrate reductase (670 mu ml^{-1} and NADPH (160 μM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; vol. 1 : 1) to 100 μl samples. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA, U.S.A). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

Measurement of peroxynitrite production

The formation of peroxynitrite was estimated by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine, using a previously described method (Cuzzocrea *et al*., 1997). In separate groups, animals were injected with dihydrorhodamine 123 (2 μmol kg^{-1} in 0.3 ml saline i.v.) 40 min after reperfusion. Twenty minutes later, rats were sacrificed and plasma samples taken for rhodamine fluorescence evaluation using a Perkin-Elmer fluorimeter (Model LS50B; Perkin-Elmer, Norwalk, CT, U.S.A.) at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively). The rate of rhodamine formation, an index of peroxynitrite production, was calculated using a standard curve obtained with authentic rhodamine (1–30 nM) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

Immunofluorescence localization for nitrotyrosine, P-selectin and ICAM-I

Indirect immunofluorescence staining was performed on 7 μm thick sections of unfixed rat ileum. Sections were cut in with a Slee and London cryostat at −30°C, transferred into clean glass slides and dried overnight at RT. Sections were permeabilized with acetone at −20°C for 10 min and rehydrated in PBS (phosphate buffered saline, 150 mM NaCI, 20 mM sodium phosphate pH 7.2) at RT for 45 min. Sections were incubated overnight: (1) with anti-nitrotyrosine rabbit polyclonal antibody (1 : 500 in PBS, v v^{-1}); or (2) with rabbit anti-human polyclonal antibody directed at P-selectin (CD62P) which react with rat and with mouse anti-rat antibody directed at ICAM-1 (CD54) (1 : 500 in PBS, v v^{-1}) (DBA, Milan, Italy). Sections were washed with PBS, and incubated with secondary antibody (TRITC-conjugated anti-rabbit and with FITC-conjugated anti-mouse (Jackson, West Grove, PA, U.S.A.) or with TRITC-conjugated anti-goat antibody (1 : 80 in PBS, v v^{-1}) for 2 h at RT. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a 40× oil objective.

Myeloperoxidase activity

Myeloperoxidase activity, an index of PMN accumulation, was determined as previously described (Mullane *et al*., 1988). Intestinal and lung tissues, collected 60 min after reperfusion, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of exchange in absorbance was measured by a spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37°C and was expressed in μ-units per gram weight of wet tissue.

Leukocyte count

Tail vein blood samples for leukocyte count were taken at 60 min after reperfusion. The number of leukocytes (WBC × 10^3 mm^3) is shown as mean ± s.d.

Malondialdehyde (MDA) measurement

Levels of malondialdehyde (MDA) in the plasma and in the intestinal tissues was determined as an index of lipid peroxidation, as described by Okhawa *et al*. (1979). Intestinal tissues, collected 60 min after reperfusion, were 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.

Measurement of cytokines

TNFα and IL-1β levels were evaluated in plasma samples at 60 min after reperfusion. The assay was carried out by using a colorimetric commercial kit (Calbiochem-Novabiochem Corporation, U.S.A.).

Light microscopy

For histopathological examination, biopsies of small intestine were taken 60 min after reperfusion. The tissue 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, N.J., U.S.A.). From each biopsy, 7 μm thick slices were obtained and stained with trichromic Van Gieson and studied using light microscopy (Dialux 22 Leitz).

Evaluation of survival

The various groups of rats were monitored for 4 h after SAO and reperfusion, and survival rates and survival times were evaluated.

Reagents

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, U.S.A.). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY, U.S.A.). Primary anti-PARP, anti-ICAM-I and anti-P-selectin were purchased from Santa Cruz (DBA, Milan, Italy). Secondary antibody (FITC-conjugated or Tric-conjugated) were obtained from Jackson (West Grove, PA, U.S.A.). Dihydrorhodamine 123 and rhodamine 123 were purchased from Molecular Probes (Eugene, OR, U.S.A.). All the other reagents were obtained from Sigma (Milan, Italy).

The complex M40401 is synthesized in a manner analogous to that previously reported for the SOD mimetic complexes M40403 and M40404 (Salvemini *et al*., 1998). Unlike M40404, which is inactive as an SOD mimetic and is the *R,R*-dimethyl substituted derivative of M40403, this new highly active mimetic is the *S,S*-dimethyl derivative, and its preparation in a stereocontrolled manner is accomplished *via* reduction using ammonium formate with Pd on charcoal catalyst in place of $NaBH_4$, which was used to generate the *R,R*-dimethyl derivative, M40404. Complete details of this synthetic procedure and the X-ray crystallographic characterization of this complex is the subject of a separate account to be submitted to *Inorganic Chemistry*.

Selectivity of M40401 In a previous series of papers (Riley *et al*., 1997), we have shown that the pentaaza macrocyclic ligand complexes of Mn(II) can not only be highly active catalysts for the dismutation of $\cdot O_2^-$, but that they are also highly selective. The complex biscyclohexyl complex, SC-55858, for example, has been shown to catalytically dismute $\cdot O_2^-$ at a rate exceeding 10^{+8} molecules of $O_2^{-\cdot}$ per molecule of complex per second at pH = 7.4 and 21°C, at a rate comparable to the native Mn SOD enzyme. Remarkably, this complex and others of this pentaaza macrocyclic ligand class, such as M40403 or M40401, do not react with hydrogen peroxide under the same conditions (Riley *et al*., 1997; Salvemini *et al*., 1999a,b), nor do they react with other biologically relevant oxidants such as ONOO- or nitric oxide. Thus, in our assays to assess catalytic catalase activity using oxygen electrodes (Marshall & Worsfold, 1978; Pasternack & Pysnik, 1983) in which total oxygen concentration evolved from the reaction of hydrogen peroxide with catalase (or any putative catalase mimic) is quantitatively monitored, no catalytic activity is observed between SC-55858, M40403, or M40401 and H_2O_2, and further, no stoichiometric reaction is observed to occur between these complexes and H_2O_2 as monitored *via* spectrophotometric or electrochemical (cyclic voltammetric) techniques. The stopped flow assay developed for monitoring peroxynitrite catalytic activity (Stern *et al*., 1996) was also utilized to assess the PN activity of these agents. No catalytic or stoichiometric reactivity of PN with these complexes is observed. These substrate specificities allow us to probe directly the biological roles that the free radical, $\cdot O_2^-$, plays by studying the effects that such selective catalysts exhibit *in vivo*.

Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired t-test. Non-parametric data were analysed with the Fisher's exact test. A P-value less than 0.05 was considered significant.

Results

Protective effects of superoxide dismutase mimetic in sphlanchnic artery occlusion shock

Occlusion of the splanchnic arteries produced an increase in MAP which then decreased until death (Figure 2). The mean survival time was found to be 69 ± 3.7% min ($n = 27$) whereas control sham animals survived for the entire period of observation (4 h, $n = 24$; Table 1). Having established the survival time, in another series of experiments, animals were sacrificed either after the period of ischemia or 60 min post reperfusion in order to collect blood and tissues for biochemical analysis. Reperfusion of the ischemic splanchnic circulation led to the following events: a substantial increase in plasma and intestinal lipid peroxidation products as determined by increased levels of MDA (Figure 3), TNFα and IL1β (Figure 4) and a profound infiltration of neutrophils into the intestine and lung (Figure 5). These inflammatory events were triggered by the reperfusion phase since no changes were observed when blood or tissues were removed after the period of ischemia alone (data not shown).

The SOD mimetic, M40401, (0.25, 2.5 or 25 μg kg^{-1}, $n = 10$) when given i.v. 15 min prior to reperfusion, inhibited

in a dose dependent manner the increased plasma and ileum levels of MDA (Figure 3A,B) as well as TNFα and IL1β (Figure 4). M40401 significantly reduced the neutrophils infiltration into the ileum and in the lung (Figure 5). Also, M40401 ($n=10$) in a dose dependent manner prevented the fall in blood pressure (Figure 2) seen after reperfusion and increased the survival rate (100% survival at 4 h in M40401-treated rats *vs* 0% survival at 4 h in non-treated rats; Table 1).

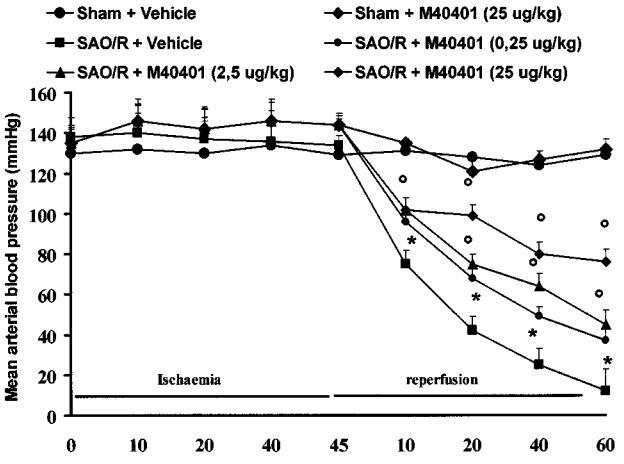


Figure 2 The fall in mean arterial blood pressure (MAP) in SAO rats ($n=6$) is blocked in a dose dependent manner by M40401. $^*P<0.05$ *versus* sham, $^\circ P<0.05$ *versus* IR.

Table 1 Effect of vehicle or M40401 on survival rate, percentage survival, and survival time in sham shocked rats or splanchnic artery occlusion (SAO) shocked rats

	Time after reperfusion (h)				
	2		*4*		*Survival*
Treatment	*Surviving*	*%*	*Surviving*	*%*	*time* (min)
Sham + vehicle	10/10	100	10/10	100	>240
Sham + M40401	10/10	100	10/10	100	>240
SAO + vehicle	0/10	0	0/10	0	69±3.7
SAO + M40401	10/10	100	10/10	100*	>240*

Animals received M40401 (25 μg/kg i.v. 15 min before reperfusion) an equal volume of vehicle (26 mM sodium bicarbonate buffer, pH 8.1–8.3). $^*P<0.01$ vs SAO + vehicle.

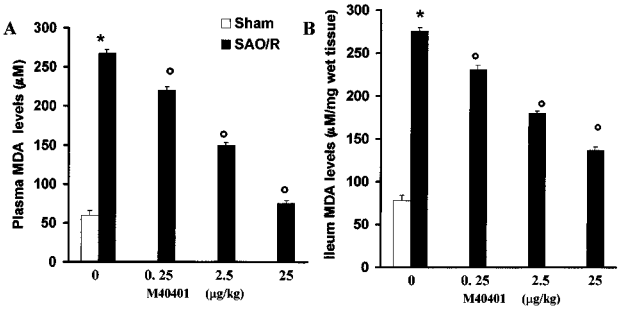


Figure 3 Reperfusion of the ischemic splanchnic circulation leads to profound increase in plasma (A) and ileum (B) levels of MDA and this is inhibited in a dose-dependent manner by M40401. Each point is the mean±s.e.mean for $n=10$ experiments. $^*P<0.05$ *versus* sham, $^\circ P<0.05$ *versus* IR.

NO and peroxynitrite production in splanchnic artery occlusion shock

There was no change in the plasma levels of nitrate/nitrite at 60 min of the reperfusion period (Figure 6A), in agreement

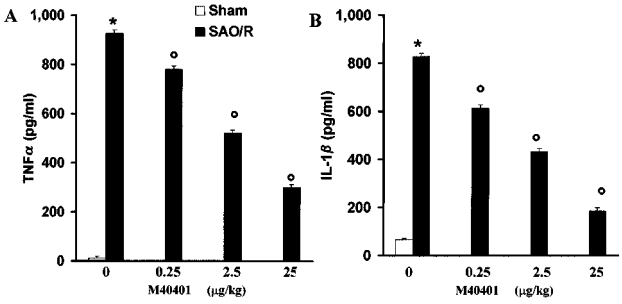


Figure 4 Reperfusion of the ischemic splanchnic circulation leads to profound increase in plasma TNFα (A) and IL1β (B) and this is inhibited in a dose-dependent manner by M40401. Each point is the mean±s.e.mean for $n=10$ experiments. $^*P<0.05$ *versus* sham, $^\circ P<0.05$ *versus* IR.

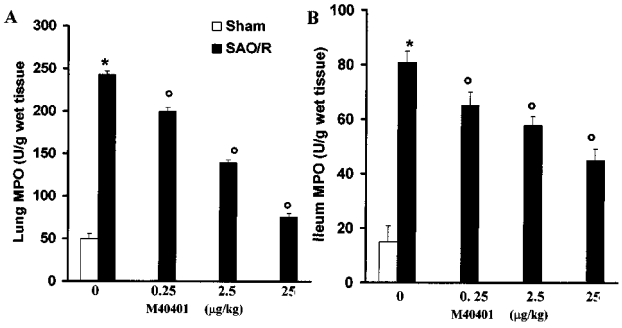


Figure 5 Reperfusion of the ischemic splanchnic circulation leads to the infiltration of neutrophils in the lung (A) and ileum (B). M40401 significantly inhibit in a dose-dependent manner the neutrophils infiltration. Each point is the mean±s.e.mean for $n=10$ experiments. $^*P<0.05$ *versus* sham, $^\circ P<0.05$ *versus* IR.

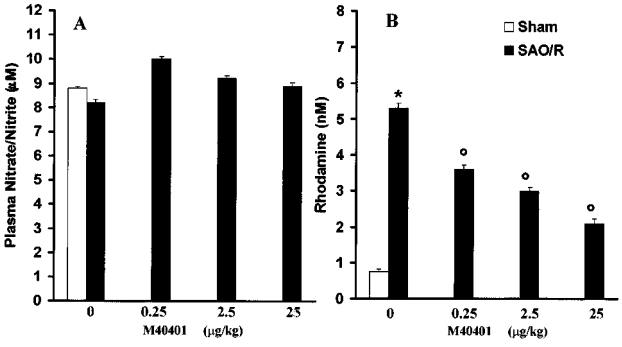


Figure 6 Plasma NOx levels (A); Plasma peroxynitrite production assessed by oxidation of dihydrorhodamine 123 to rhodamine (B). There was no change in the plasma levels of nitrate/nitrite during occlusion or 60 min of reperfusion period. Peroxynitrite production in the SAO-shocked rats were significantly increased *versus* sham group. M40401 significantly inhibit in a dose-dependent manner the elevation of the plasma peroxynitrite production. Each point is the mean±s.e.mean for $n=10$ experiments. $^*P<0.05$ *versus* sham, $^\circ P<0.05$ *versus* IR.

with previous observations where we have demonstrated that the current protocol of ischemia and reperfusion does not trigger the expression of the inducible isoform of NOS (iNOS) (Cuzzocrea *et al*., 1997). The treatment with M40401 did not affect baseline nitrite/nitrate levels (Figure 6A). In agreement with previous observations (Cuzzocrea *et al*., 1997), SAO shock caused a significant increase in the rhodamine fluorescence of plasma, indicative of peroxynitrite-induced oxidation of dihydrorhodamine during the reperfusion phase (Figure 6B). *In vivo* treatment with M40401 reduced in a dose dependent manner the oxidation of dihydrorhodamine 123 during reperfusion (Figure 6B).

At 60 min after reperfusion, ileum sections were taken from sham or shocked rats in order to determine the immunohistological staining for nitrotyrosine. While there was negligible staining in the intestinal sections of control animals (Figure 7A), immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining in the sub-mucosa vessels (Figure 7B, arrows). M40401 (25 μg kg^{-1}) treatment reduced the degree of immunostaining for nitrotyrosine (Figure 7C) in the reperfused intestine. In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. In this situation, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

Leukocyte count

The administration of M40401 did not modify the leukocyte count in sham-shocked rats. In contrast, SAO shock produced a marked leukopenia. Our data show that the leukocyte count was markedly decreased at 60 min after reperfusion. The administration of M40401 (25 μg kg^{-1}) significantly ameliorated leukopenia (Table 2).

Immunohistochemical localization of ICAM-1 and P-selectin in the reperfused intestine

Staining of ileum tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrows), demonstrating that ICAM-1 is constitutively expressed (Figure 8A). After 1 h of reperfusion, the staining intensity substantially increased in the vessels (Figure 8C; see arrows). Sections from M40401-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 8E; see arrows). Ileum tissue sections obtained from SAO-shocked rats undergoing 45 min of ischemia followed by 1 h reperfusion showed positive staining for P-selectin localized in the vessels (Figure 8C; see arrows). No staining was observed in sham-operated rats (Figure 8B). In tissue obtained at 1 h after reperfusion from M40401-treated rats, no positive staining for P-selectin was found (Figure 8F). 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.

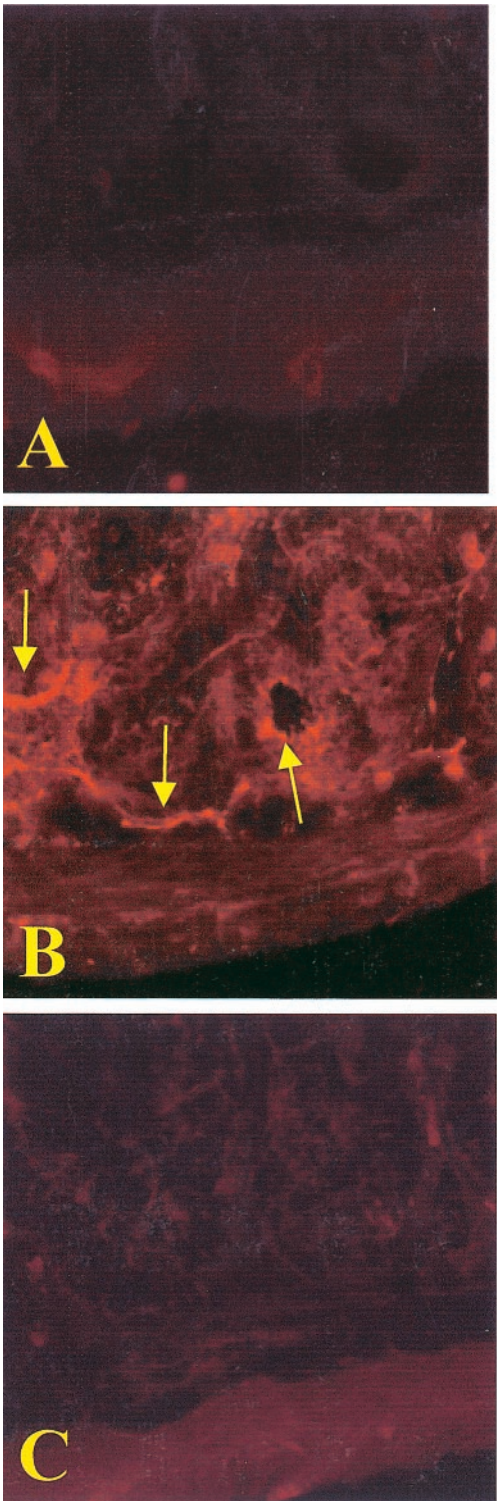


Figure 7 No positive staining for nitrotyrosine (A) was found in the ileum section from sham-administered rats. Sixty minutes after reperfusion immunohistochemical for nitrotyrosine (B) show positive staining localised in the vascular wall and in the inflammatory cells (arrows) in the injured area from a SAO-shocked rats. The intensity of the positive staining for nitrotyrosine (C) was significantly reduced in the ileum from M40401-treated rats. Original magnification: ×145. Figure is representative of at least three experiments performed on different experimental days.

Histological change

Histological examinations of the small intestine at 60 min of reperfusion (see representative sections in Figure 9) revealed pathologic changes. Ileum section showed inflammatory infiltration by inflammatory extending through the wall and concentrated below the epithelial layer (Figure 9B). M40401-treated rats show a significant reduction in organ injury (Figure 8C).

Discussion

The major goal of our work was to compare the efficacy of M40401, a recently developed SODm possessing a structure similar to, but having a much higher catalytic SOD activity than M40403 (Salvemini *et al*., 1999a,b) in splanchnic artery occlusion shock. Splanchnic artery occlusion shock is a well-described model of circulatory shock that arises after reperfusion of a prolonged ischemia of the splanchnic circulation (Lefer & Lefer 1993; Zimmermann *et al*., 1993; Cuzzocrea *et al*., 1997). The end result is a high and rapid mortality rate, with most animals dying within the first 2 h of reperfusion (Cuzzocrea *et al*., 1997). Some of the local alterations that occur in this model include upregulation of adhesion molecules (ICAM-1 and P-selectin (Butcher, 1992; Lawrence & Springer, 1991; Geng *et al*., 1990; Dreyer *et al*., 1991), neutrophil infiltration in the intestine, nitrotyrosine staining and PARP activation (Cuzzocrea *et al*., 1997) and a profound peroxidation of membranes leading to elevated plasma levels of lipid peroxidation products such as MDA (Salvemini *et al*., 1999a,b). Systemic alterations, on the other hand, include elevated plasma levels of the cytokines TNFα and IL1β, infiltration of neutrophils in lung and severe hypotension. The role of NO from the inducible form of iNOS is not involved in the pathogenesis of SAO shock (Cuzzocrea *et al*., 1998) whereas it seems that loss of NO from the constitutive enzyme (ecNOS) accounts at least in part for the damage seen in this model of reperfusion injury (Kanwar *et al*., 1994). This is not surprising in light of the beneficial effects ascribed to date to NO released from ecNOS (Gauthier *et al*., 1994) and from the protective effects seen in reperfusion injury with nitric oxide donors (Gauthier *et al*., 1994; Naseem *et al*., 1995). The role of superoxide as a key driver of the damage associated with reperfusion of the

Table 2 Effect of vehicle or M40401 on white blood cell count (WBC) of rats subjected to splanchnic artery occlusion (SAO) shock

	Time (min)	
	Basal 0	*Reperfusion* 60
Sham + Vehicle	5.2 ± 1	6.2 ± 1.5
Sham + M40401	5.9 ± 1.3	6.9 ± 1.4
SAO + Vehicle	6.4 ± 0.9	3.0 ± 0.5
SAO + M40401	6.1 ± 0.7	6.8 ± 0.3*

Animals received M40401 (25 μg/kg i.v. 15 min before reperfusion) an equal volume of vehicle (26 mM sodium bicarbonate buffer, pH 8.1–8.3). *$P<0.01$ vs SAO + vehicle.

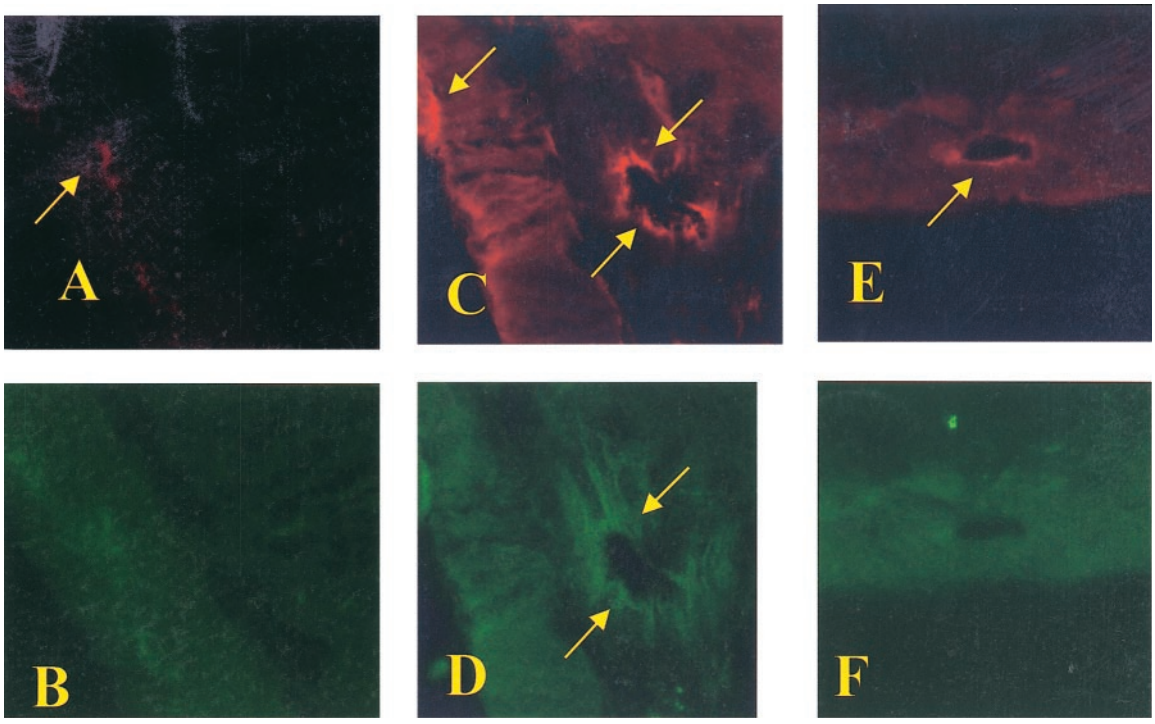


Figure 8 Staining of ileum tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining along vessels (arrow), demonstrating that ICAM-1 is constitutively expressed (A), no P-selectin staining was seen in sham animals (B). Section obtained from SAO shocked-rats showed intense positive staining (see arrows) for ICAM-1 (C) and for P-selectin (D) on vascular wall. The degree of endothelial staining for ICAM-1 (E) and for P-selectin (F) was markedly reduced in tissue section obtained from M40401-treated rats. Original magnification: $\times$145. Figure is representative of at least three experiments performed on different experimental days.

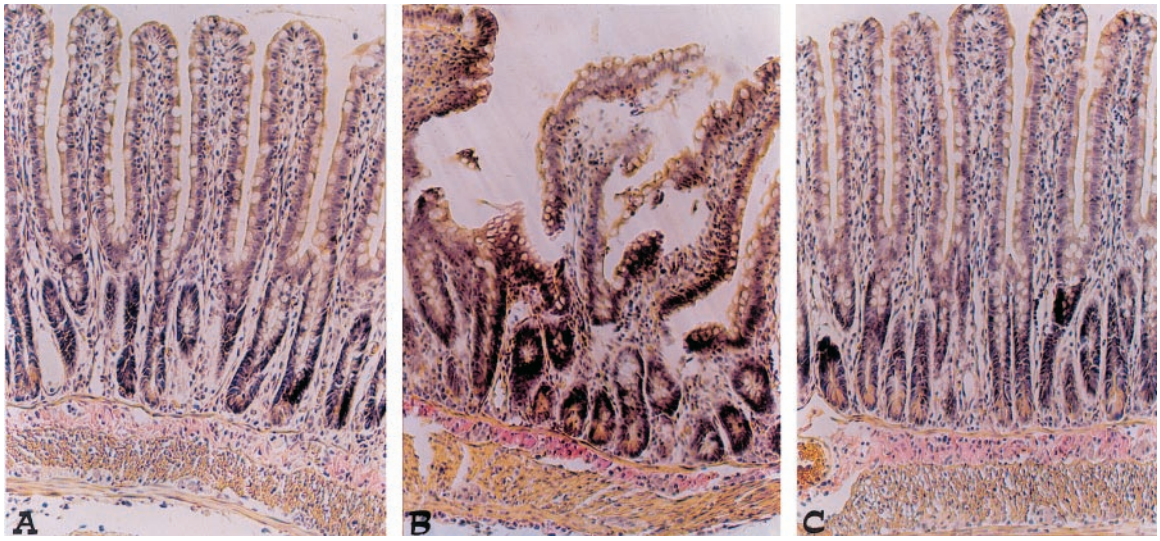


Figure 9 Distal ileum section from a sham rat demonstrating the normal architecture of the intestinal epithelium and wall (A). Distal ileum section from a SAO shocked-rats mice demonstrating oedema of the distal portion of the villi (B). Distal ileum from M40401-treated rats shows reduced SAO-induced organ injury (C) Original magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

ischemic sphlanchnic circulation has been demonstrated by the use of M40403, a stable superoxide dismutase mimetic which selectively removes $\cdot O_2^-$ without directly interacting with other reactive oxygen species that have been implicated in SAO such as ONOO-. Thus, M40403 at doses ranging from 0.1 3 μg kg^{-1} inhibited the infiltration of PMNs in lung and ileum, cytokines (TNFα and IL1β) release, lipid peroxidation, hypotension and improved survival at the highest dose tested (Salvemini *et al*., 1999a,b). Here we have clearly shown that M40401 had similar protective effects except at doses at least 100 fold lower. The magnitude of the potency difference directly correlates with the catalytic rate constant for the dismutation of superoxide which these two molecules possess. Thus, at pH = 7.4 the ratio of the two catalytic rate constants is approximately 80 fold. It is possible that the beneficial and protective effects seen with SODm are due at least in part to inhibition of TNFα and IL1β as well as attenuation of lipid peroxidation, since pharmacological inhibition of either TNFα/IL1β release or lipid peroxidation is protective in this model of SAO (Cuzzocrea *et al*., 1999; Squadrito *et al*., 1999; Sun *et al*., 1999; Campo *et al*., 1998). In this study we have also extended our previous finding by showing that M40401 inhibits peroxynitrite formation, nitration of tyrosine residues in the intestine and the upregulation of adhesion molecules.

One mechanism by which M40401 will attenuate the damage seen after reperfusion of the ischemic intestine is by reducing ONOO- formation by simply removing $\cdot O_2^-$ before it reacts with NO. This is important since the pro-inflammatory and cytotoxic effects of ONOO- are numerous (Beckman *et al*., 1990; Ischiropoulos *et al*., 1992; Beckman & Crow, 1993; Crow & Beckman 1995; Salvemini *et al*., 1998; Misko *et al*., 1998). The production of ONOO- has been demonstrated in the perfused heart (Matheis *et al*., 1992; Schulz & Wambolt, 1995), liver (Ma *et al*., 1995), kidney (Morpurgo *et al*., 1996), intestine (Cuzzocrea *et al*., 1997; 1998; Squadrito *et al*., 1999), brain (Casevieille *et al*., 1993) and lung (Kooy *et al*., 1995). Furthermore, removal of ONOO- by agents such as FeTMPS, a porphyrin-containing molecule which increases the rate of isomerization of ONOO- to nitrate (Misko *et al*., 1998; Salvemini *et al*., 1998), significantly attenuates damage associated with SAO (Cuzzocrea *et al*., 2000). Peroxynitrite (and the products of the reactions of both nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide, Halliwell, 1997) will nitrate tyrosine residues in proteins. The nitrotyrosine formation, as detected by immunofluorescence, is considered to be an indicator of 'increased nitrosative stress' (Eiserich *et al*., 1998). Consistent with previous results (Cuzzocrea *et al*., 1997; 1998; Squadrito *et al*., 1991) we have found that increased immunohistochemical expression of nitrotyrosine is mostly localized on vessels and in the areas of infiltrated inflammatory cells, suggesting that ONOO- or other nitrogen derivatives and oxidants are formed *in vivo* and may contribute to tissue injury. Indeed, we have found a marked increase in the plasma levels of peroxynitrite after reperfusion of the ischemic splanchnic circulation. This was inhibited in a dose-dependent manner by M40401, which also attenuated nitrotyrosine staining. Overall these findings indicate that part of the beneficial effect of M40401 is through inhibition of ONOO- formation, attenuation of nitrosative stress, and lack of an effect on NO released from ecNOS.

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 (Szabó & Dawson, 1998). Furthermore, substantial evidence exists to support the fact that PARP activation is important in damage associated with reperfusion injury of the ischemic gut. Thus, Szabo and coworkers provided evidence that formation of ONOO- following reperfusion of the ischemic splanchnic circulation, damages DNA which in turn leads to PARP activation and endothelial cell injury (Cuzzocrea *et al*., 1997; Szabó *et al*., 1997). PARP inhibitors such as nicotinamide and 3-aminobenzamide attenuate damage (Cuzzocrea *et al*., 1997;

Chaterjee *et al*., 2000). Furthermore, recent studies have demonstrated that $PARP^{-/-}$ mice are significantly protected from SAO shock when compared with $PARP^{+/+}$ mice (Liaudet *et al*., 2000). Thus, we may propose that the observed beneficial effects of M40403 and M40401 are (at least in part) due to the prevention of the activation of PARP.

Inhibition of superoxide prevents the infiltration of neutrophils at inflamed sites as shown by the use of the native SOD enzyme, experiments performed in transgenic mice that overexpress the human CuZnSOD enzyme (Li *et al*., 1995; Lebovitz *et al*., 1996; Melov *et al*., 1999), and by SODm such as SC-55858 (Salvemini *et al*., 1999a,b; Lowe *et al*., 1996, M40403 and M40401 (Salvemini *et al*., 1999a,b and this study). Endothelial cells appear to be major regulators of neutrophil trafficking, regulating the process of neutrophil chemotaxis, adhesion and emigration from the vasculature to the tissue. During the early phase of reperfusion, P-selectin is rapidly released to the cell surface from preformed storage pools after exposure to certain stimuli, such as hydrogen peroxide, thrombin, histamine, or complement, and allows the leukocytes to roll along the endothelium (Geng *et al*., 1990). ICAM-1 constitutively expressed on the surface of endothelial cells, is then involved in neutrophil adhesion (Bittermann *et al*., 1988; Dreyer *et al*., 1991). Hypoxic endothelial cells synthesize proinflammatory cytokines, which can up-regulate endothelial expression of the constitutive adhesion molecule ICAM-1 in autocrine fashion (Farhood *et al*., 1995). The expression of P-selectin and ICAM-1 corresponds with the induction of neutrophil recruitment, which is maximal within the first hour of reperfusion, and persists at a lower rate in the late phase of reperfusion (Shreeniwas *et al*., 1992). In accordance with these findings, we observed that a 45-min occlusion of the splanchnic artery followed by 1-h reperfusion induced the appearance of P-selectin on the endothelial vascular wall and upregulated the surface expression of ICAM-1 on endothelial cells. Treatment with M40401 reduced proinflammatory cytokines production (Figure 4) and abolished the expression of P-selectin and the upregulation of ICAM-1 (Figure 8E,F), while not affecting the constitutive levels of ICAM-1 on endothelial cells (data not shown). Our data suggest that superoxide production contributes to the regulation of neutrophil infiltration through the upregulation of these adhesion molecules. Cytokines are most likely involved in this process, since it is known that TNFα induces the upregulation of ICAM-1 and P-selectin (Farhood *et al*., 1995; Shreeniwas *et al*., 1992).

Finally, histological examination of the ileum revealed significant preservation of the architecture by M40401.

In conclusion, we have observed that M40401 exerts its protective effects at doses considerably lower than that of M40403. Such increased efficacy may be due to increased catalytic rate or differential tissue distribution. This lends additional credence to the conclusion that superoxide anion plays a major contributory role in the pathogenesis of ischemia-reperfusion injury and thus points to the potential clinical use of SOD mimetics, such as M40401, in ischemia-reperfusion injury.

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