

Protective effects of relaxin in ischemia/reperfusion-induced intestinal injury due to splanchnic artery occlusion

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1 Splanchnic artery occlusion (SAO) followed by reperfusion causes endothelial injury and inflammation which contribute to the pathophysiology of shock. We investigated the effects of relaxin (RLX), known to afford protection against the deleterious effects of cardiac ischemia/reperfusion, given to rats subjected to splanchnic artery occlusion and reperfusion (SAO/R)-induced splanchnic injury.

2 RLX (30 ng kg⁻¹, 15 min. before reperfusion) significantly reduced the drop of blood pressure and high mortality rate caused by SAO/R. RLX also reduced histopathological changes, leukocyte infiltration (myeloperoxidase) and expression of endothelial cell adhesion molecules in the ileum. RLX counteracted free radical-mediated tissue injury, as judged by significant decrease in the tissue levels of peroxidation and nitration products (malondialdehyde, nitrotyrosine), DNA damage markers (8-hydroxy-2'-deoxyguanosine, poly-ADP-ribosylated DNA) and consumption of tissue antioxidant enzymes (superoxide dismutase). As a result, RLX led to a reduction of ileal cell apoptosis (caspase 3, terminal deoxynucleotidyltransferase-mediated UTP end labeling). The effects of RLX appear specific, as inactivated RLX substituted for the bioactive hormone had no effects.

3 In conclusion, these results show that RLX exerts a clear-cut protective effect in SAO/R-induced splanchnic injury, likely due to endothelial protection, decreased leukocyte recruitment and hindrance of free radical-mediated tissue injury leading to cell death, lethal complications and high mortality rate. Thus, RLX could be used therapeutically in intestinal ischemia.

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Abbreviations: ECAMs, endothelial cell adhesion molecules; ICAM-1, intercellular adhesion molecule 1; MDA, malondialdehyde; MPO, myeloperoxidase; NT, nitrotyrosine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PAR, poly-ADP-ribose; PARP, poly-ADP-ribosyl-polymerase; RLX, relaxin; SAO/R, splanchnic artery occlusion and reperfusion; SOD, superoxide dismutase; VCAM, vascular cell adhesion molecule

Introduction

In humans, intestinal ischemia usually results from impaired perfusion of blood to the bowel due to a variety of causes, including cardiac insufficiency, sepsis, vaso- and cardiodepressant drugs and complications of long-lasting surgery (Stoney & Cunningham, 1993; Mallick *et al.*, 2004). Complications of intestinal ischemia may be severe, ranging from persistent bleeding and symptomatic intestinal strictures to bowel perforation and peritonitis. Thus, surgical resection of the affected segment is usually necessary to minimize adverse outcomes (Sreenarasimhaiah, 2005). The pathophysiology of intestinal ischemia and its sequelae has been widely investigated using animal models of splanchnic artery occlusion (SAO) followed by reperfusion, which result in severe circulatory shock, characterized by hypotension, hemoconcentration, intestinal injury and a high mortality rate (Lefer & Lefer, 1993; Zimmermann *et al.*, 1993). An important

component of SAO-induced shock is endothelial dysfunction (Altura *et al.*, 1985; Carey *et al.*, 1992; Zingarelli *et al.*, 1992; Lefer & Lefer, 1993), yet induced by hypoxia during the ischemic phase (Harlan, 1985) and further exacerbated at reperfusion due to oxygen-derived free radicals generated from both endothelial cells (Ratych *et al.*, 1987; Lefer & Lefer, 1993) and activated adherent leukocytes (Granger *et al.*, 1981; McCord, 1981; Mullane *et al.*, 1988; Bittermann *et al.*, 1988). Endothelial dysfunction predisposes to vasospasm and increases adherence of platelets and neutrophils, which worsen the shock state. Normally, the endothelium inhibits adherence of leukocytes by producing antiadhesive factors, such as nitric oxide (NO) (Kubes *et al.*, 1991; Laroux *et al.*, 2000) and by expressing low levels of endothelial cell adhesion molecules (ECAMs), a generic term used to identify different types of surface glycoproteins based on their common function (Butcher, 1992; 1993; Sluiter *et al.*, 1993). Inflammatory mediators generated during ischemia/reperfusion can stimulate the expression of ECAMs (Granger, 1977). These molecules

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include early-phase proteins, such as P-selectin that is held responsible for leukocyte rolling, as well as late-phase proteins, such as E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule 1 (ICAM-1), which are involved in firm adhesion of leukocytes to the vascular endothelium that allows leukocyte extravasation into the tissue (Lawrence & Springer, 1991; Butcher, 1992; Laroux *et al.*, 2000). NO itself can reduce the expression of ECAMs, likely by interfering with the activation of the transcription factor NF- κ B or by binding to the ECAM gene promoters (Peng *et al.*, 1998; Spiecker & Liao, 1999). On the other hand, administration of NO synthesis inhibitors and targeted disruption of constitutive NO synthase (NOS) I and III genes cause an increased amount of leukocytes adherent to the vascular endothelium (Kubes *et al.*, 1991; Lefer *et al.*, 1999; Laroux *et al.*, 2000).

At reperfusion, neutrophil activation at sites of injury results in a large production of superoxide anions ($\bullet\text{O}_2^-$), which give a major contribution to tissue inflammation and damage by causing endothelial cell damage and increased microvascular permeability (Droy-Lefaix *et al.*, 1991; Haglund *et al.*, 1994; Xia *et al.*, 1995), formation of chemotactic factors such as leukotriene B₄ (Fantone & Ward 1982; Deitch *et al.*, 1990), further neutrophil recruitment (Boughton-Smith *et al.*, 1993; Salvemini *et al.*, 1996; 1999a, b), membrane lipid peroxidation and DNA damage (Dix *et al.*, 1996). In addition, $\bullet\text{O}_2^-$ reacts with and inactivate NO, thereby reducing its anti-inflammatory and cytoprotective properties, including maintenance of blood vessel tone, decreased platelet and leukocyte reactivity, and increased generation of anti-inflammatory and cytoprotective prostacyclin *via* activation of constitutive cyclooxygenase (Salvemini *et al.*, 1993). The reaction product of $\bullet\text{O}_2^-$ and NO is peroxynitrite (ONOO⁻), a potent cytotoxic and proinflammatory molecule which further enhances reperfusion-induced tissue injury (Crow & Beckman, 1995; Misko *et al.*, 1998; Salvemini *et al.*, 1999a, b).

From the above grounds, it seems logical to assume that pharmacological strategies aimed at counteracting endothelial dysfunction could afford protection against ischemia/reperfusion-induced tissue damage. In this context, previous studies showed that the peptide hormone relaxin (RLX), best known for its effects on reproduction (Bani, 1997; Sherwood, 2004), is a potent cardiovascular effector (Bani, 1997; Dschietzig & Stangl, 2002; Samuel *et al.*, 2003; Nistri & Bani, 2005). RLX promotes vasodilatation in many target organs by inducing upregulation of endogenous NO production by cells of the vascular wall (Bani *et al.*, 1998a; Failli *et al.*, 2002). RLX is also capable to reduce the expression of ECAMs and to blunt leukocyte activation, thereby exerting anti-inflammatory effects (Nistri *et al.*, 2003; Masini *et al.*, 2004). Of note, our previous studies showed that RLX, given either preventively before ischemia or also at reperfusion, affords a clear-cut protection against ischemia/reperfusion-induced damage in the heart (Masini *et al.*, 1997; Bani *et al.*, 1998b; Perna *et al.*, 2005).

Aim of the current study is to investigate whether RLX could be useful as a therapeutic agent to blunt the adverse effects of ischemia/reperfusion in a well-assessed animal model of splanchnic artery occlusion and reperfusion (SAO/R) widely used for experimentation of potential new drugs for ischemia/reperfusion-induced splanchnic injury (Lefer & Lefer 1993; Zimmermann *et al.*, 1993; Cuzzocrea *et al.*, 2001).

Methods

Animals and reagents

Wistar albino 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 the use of animals for experimental and other scientific purposes (D.M. 116192), as well as with the current EC regulations (O.J. of E.C. L358/12/18/1986). Unless otherwise specified, all reagents and labware was from Sigma-Aldrich (Milano, Italy).

Surgical induction of splanchnic artery occlusion

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. Blood pressure was monitored continuously by a Maclab A/D converter (Ugo Basile, Varese, Italy), displayed and stored on a Macintosh personal computer. After 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 allowed to equilibrate for 30 min before further surgical treatments. SAO 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. Sham-operated animals, subjected to the same surgical procedure except for arterial clamping, were used as controls ($n = 10$).

Treatments

Rats undergoing SAO were divided in three experimental groups, 10 animals each. At 15 min before reperfusion, the rats were given an intravenous (i.v.) bolus of phosphate-buffered saline (PBS) containing the following substances: (i) highly purified porcine RLX, 30 ng kg⁻¹ (SAO/R + RLX); the hormone (2500–3000 IU mg⁻¹), prepared according to Sherwood & O'Byrne (1974), was generously donated by Dr O.D. Sherwood, University of Illinois, Urbana, IL, U.S.A.; (ii) inactivated RLX (iRLX), 30 ng kg⁻¹ (SAO/R + iRLX); iRLX was obtained by blockade of functional arginine residues by reaction with cyclohexanedione (Büllesbach & Schwabe, 1988); (iii) no drugs added (SAO/R + vehicle). The administration protocol was designed to reproduce a possible therapeutic use of RLX, that is, the drug being administered between the onset of ischemic symptoms and the surgical procedures inducing reperfusion. The half-life of RLX has been assessed to be about 2 h (Chen *et al.*, 1993), thus allowing us to reasonably assume that bioactive levels of the hormone were present in the blood of rats during the reperfusion period. Rats were killed at 60-min reperfusion, this time point being set based on the mean survival time. At laparotomy, an 8–10 cm-long ileal segment, 30 cm distal to the stomach was isolated, washed in saline and used for histological examination and for biochemical studies.

In another set of experiments, the different groups of rats (each group, $n = 20$) were monitored for 6 h after SAO/R to evaluate survival time.

Light microscopy

For conventional histopathological examination, ileal tissue specimens 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 specimen, 7- μm thick sections were cut and stained with trichromic Van Gieson.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity, an index of accumulation of inflammatory leukocytes, especially PMN, was determined as previously described (Mullane *et al.*, 1985). Ileal fragments were homogenized in a solution containing 0.5% hexadecyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at $20,000 \times g$ at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM H₂O₂. The rate of exchange in absorbance was measured by a spectrophotometer at 650 nm. MPO activity was defined as the enzyme amount degrading 1 μmol of peroxide min^{-1} at 37°C and was expressed in U g^{-1} of wet tissue.

Immunohistochemical analysis of P-selectin, ICAM-1, VCAM and E-selectin

P-selectin, ICAM-1, VCAM and E-selectin localization was detected in ileal sections by immunohistochemistry. Sections of Dietric-fixed, paraffin-embedded ileal fragments were dewaxed, rehydrated, permeabilized with 0.1% Triton X-100 in PBS for 20 min and incubated in 2% normal serum for 60 min. Sections were then incubated for 12 h at 4°C with antibodies directed to P-selectin (rabbit polyclonal, 1:500 in PBS; Pharmingen, San Diego, CA, U.S.A.), ICAM-1 (mouse monoclonal, 1:1000 in PBS; Santa Cruz/DBA, Milan, Italy), VCAM (mouse monoclonal, 1:500, Santa Cruz/DBA) and E-selectin (mouse monoclonal 1:1000, Santa Cruz/DBA). Labeling of antigen-antibody complexes was detected with appropriate peroxidase-conjugated secondary antisera, using diaminobenzidine as chromogen. Controls were sections incubated with nonimmune rabbit serum in the place of the primary antisera, as well as sections incubated without either the primary or the secondary antibody.

Measurement of malondialdehyde activity

Levels of malondialdehyde (MDA) in the ileal tissue was determined as an index of lipid peroxidation, as described by Ohkawa *et al.* (1979). Ileal fragments 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 of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at $3000 \times g$ for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm. Protein concentration was determined with the Bradford method. The values are expressed as nmol of thiobarbituric acid-reactive substances (MDA equivalents) mg^{-1} of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

Determination of 8-hydroxy-2'-deoxyguanosine

This was carried out on DNA isolated from ileal tissues, according to Lodovici *et al.* (2000). Briefly, samples were homogenized at 4°C, sonicated on ice (three times, 10 s) and diluted with 1 ml of 10 mM Tris-HCl buffer pH 8.0 containing 10 mM EDTA, 10 mM NaCl, 0.5% SDS. The samples were first incubated at 37°C for 60 min with RNase (20 $\mu\text{g ml}^{-1}$), then they were incubated at 37°C under argon in the presence of proteinase K (100 $\mu\text{g ml}^{-1}$). At the end of incubation, the mixture was added with chloroform/isoamyl alcohol (10:2 v v^{-1}) with 0.2 volumes of 10 M ammonium acetate and DNA was precipitated from the aqueous phase. DNA was solubilized in 100 μl of 20 mM acetate buffer pH 5.3 and denaturated at 90°C for 3 min. The total extracted DNA was supplemented with 10 IU of P1 nuclease in 10 μl and incubated for 60 min at 37°C with 5 IU of alkaline phosphatase in 0.4 M phosphate buffer, pH 8.8. All these procedures were performed under argon and the samples were protected from light. The hydrolyzed mixture was filtered by Amicon Micropure-EZ enzyme remover (Millipore, Milan, Italy) and 50 μl of samples were used for 8-hydroxy-2'-deoxyguanosine (8-OHdG) determination, using a Bioxytech®-EIA-kit following the manufacturer's protocol. Absorbance was read at 450 nm wavelength and the concentrations of 8-OHdG were determined by comparison with a standard curve. Values are expressed as $\text{ng } \mu\text{g}^{-1}$ of proteins, determined with the Bradford method.

Measurement of Mn-superoxide dismutase activities

Ileal samples were homogenized with 10 mM PBS, pH 7.4, sonicated on ice (three times, 20 s) and centrifuged at $100 \times g$ for 10 min. Superoxide dismutase (SOD) activity was measured in the supernatants as described by Nishida *et al.* (2002), with minor modifications. The assay is based on the inhibition of nitro blue tetrazolium conversion by SOD into a blue tetrazolium salt, mediated by superoxide radicals, which are generated by xanthine oxidase. The reaction was performed in sodium carbonate buffer 50 mM, pH 10.1 containing 0.1 mM EDTA, 25 μM nitro blue tetrazolium (Sigma, Milan, Italy), 0.1 mM xanthine, 2 nM xanthine oxidase (Boehringer, Germany). The rate of reduction of nitro blue tetrazolium was followed at 560 nm with a Perkin-Elmer spectrophotometer. The amount required to inhibit the rate of reduction of nitro blue tetrazolium by 50% was defined as one unit of enzyme activity. For measurement of MnSOD, enzyme activity was inhibited performing the assay in the presence of 2 mM NaCN after preincubation for 30 min.

Immunohistochemistry for nitrotyrosine and poly-(ADP-ribose)

Tyrosine nitration, an index of nitrosylation of proteins by peroxynitrite and/or ROS, and nuclear poly-ADP-ribosylated DNA, an activity marker for poly-ADP-ribosyl-polymerase (PARP), an enzyme involved in DNA repair, were determined by immunohistochemistry as previously described (Cuzzocrea *et al.*, 2001). Sections of Dietric-fixed, paraffin-embedded ileal fragments were dewaxed and rehydrated, endogenous peroxidase was quenched with 0.3% (v v^{-1}) hydrogen peroxide in 60% (v v^{-1}) methanol for 30 min. The sections were permeabilized with 0.1% (w v^{-1}) Triton X-100 in PBS for 20 min.

Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti nitrotyrosine (NT) rabbit polyclonal antibody (1:500 in PBS), or with anti-poly-ADP-ribose (PAR) goat polyclonal antibody (Jackson, Westgrave, PA, U.S.A.; 1:500 in PBS). Immune reaction was revealed by indirect immunoperoxidase method (Vectastain Elite kit, Vector, Burlingame, CA, U.S.A.), using 3,3'-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used. Negative controls for NT immunostaining were sections incubated with the primary antibody in the presence of excess NT (10 mM). Negative controls for PAR were sections incubated with only the primary or the secondary antibody. Quantitation of immunostaining was carried out by computer-aided densitometry on photomicrographs ($n=5$ per experimental group), using Optilab Graftek software on a Macintosh personal computer.

Determination of caspase-3 activity

The activity of caspase-3 was determined by use of a fluorescent substrate, according to Stennicke & Salvesen (1997). The Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Bachem, Germany) was used as a fluorescent substrate for caspase-3. Briefly, ileal fragments were homogenized with 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 42 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 2 $\mu\text{g ml}^{-1}$ leupeptin, and 1 $\mu\text{g ml}^{-1}$ pepstatin A. The homogenates were then centrifuged at 10,000 $\times g$ for 10 min. Caspase-3 activity was determined in the supernatant by fluorometric assay using the substrate, Ac-DEVD-AMC. Values are expressed as mU mg⁻¹ of proteins, determined with the Bradford method.

Terminal deoxynucleotidyltransferase-mediated UTP end labeling assay

Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit; DBA, Milano, Italy). Briefly, sections of Dietric-fixed, paraffin-embedded ileal fragments were dewaxed, rehydrated and incubated with 15 mg ml⁻¹ proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H₂O₂ for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with peroxidase-conjugated avidin and the signals were visualized with diaminobenzidine.

Statistical analysis

The reported data are expressed as the mean \pm s.e.m. of the reported number of animals per experimental group. Statistical

comparison of differences between groups was carried out using either two-way ANOVA for two variable analysis or one-way ANOVA followed by Student–Newman–Keuls multiple comparison test for single variable analysis. A P -value ≤ 0.05 was considered significant. Calculations were performed using the GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, U.S.A.).

Results

Reperfusion of the ischemic splanchnic circulation led to a slight increase in mean arterial pressure which then decreased until death. The mean survival time was 67 ± 4.7 min, whereas the sham-operated controls survived for the entire observation period (4 h).

Bioactive RLX (30 ng kg⁻¹), given i.v. 15 min before reperfusion, prevented the fall in blood pressure (Figure 1) seen during late reperfusion and increased the survival rate (88% survival at 4 h in RLX-treated rats vs 0% survival in vehicle-treated rats). iRLX was ineffective in preventing the fall in blood pressure and survival rate which was not different from vehicle-treated rats (Figure 1).

SAO/R also resulted in a marked infiltration of inflammatory leukocytes into the ileum, as shown by the increase in tissue MPO activity. Histopathological examination of ileal wall showed severe mucosal abnormalities, consisting of truncated, edematous villi, hemorrhage foci and loss of surface and gland epithelium. These events were triggered by reperfusion, as no changes were observed in preliminary experiments in which tissues were taken soon after ischemia (data not shown). Both MPO activity and histopathological abnormalities were prevented by pretreatment with RLX (Figure 2a and b). Conversely, iRLX was ineffective.

In the vehicle-treated rats subjected to SAO/R, leukocyte infiltration was accompanied by a rise in the local immunocytochemical expression of early-phase and late-phase ECAMs. In fact, examination of ileal sections from sham-operated rats showed faint immunostaining for ICAM-1 and no labeling for P-selectin, E-selectin and VCAM, indicating that, except

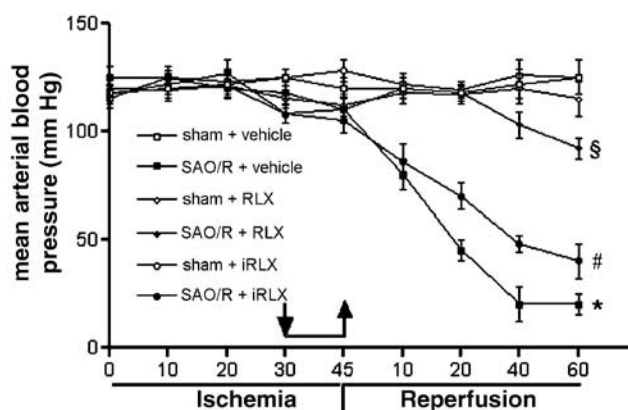


Figure 1 Trend of mean arterial pressure in SAO/R rats. RLX, but not iRLX, prevents the fall in mean arterial pressure in SAO/R rats. Arrows point at start and withdrawal of RLX, iRLX or vehicle. Significance of differences (two-way ANOVA): * $P < 0.001$ vs sham-operated animals; § $P < 0.001$ vs SAO/R + vehicle; # $P < 0.001$ vs SAO/R + RLX.

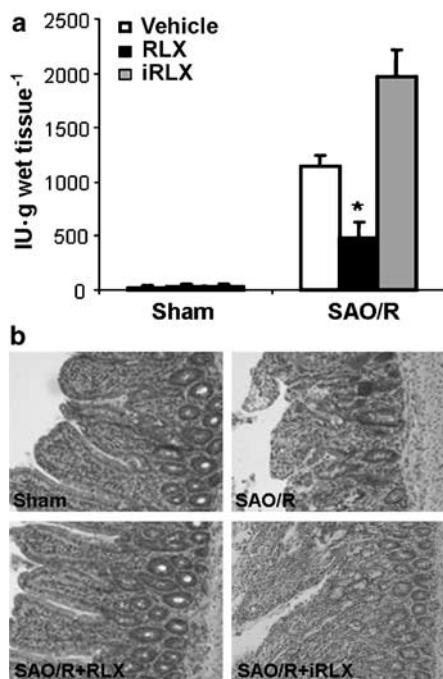


Figure 2 (a) MPO activity is significantly increased in the ileum of SAO/R rats. RLX, but not iRLX, significantly reduces MPO activity. Significance of differences (one-way ANOVA): * $P < 0.01$ vs SAO/R + vehicle and SAO/R + iRLX. (b) Representative histopathological features of the ileum in sham-operated rats and SAO/R rats treated with vehicle or RLX. Severe mucosal abnormalities, consisting of truncated, edematous villi, hemorrhage foci and loss of surface and gland epithelium, can be seen in SAO/R + vehicle rats. These abnormalities are absent in SAO/R + RLX rats.

ICAM-1, the other adhesion molecules were not constitutively expressed. In the vehicle-treated rats undergoing SAO/R, there was a strong immunostaining for all the adhesion molecules studied, especially in the blood vessels. Conversely, in the RLX-treated rats undergoing SAO/R, no appreciable immunostaining for P-selectin, E-selectin and VCAM was found, nor was any increase in the constitutive ICAM-1 immunocytochemical expression (Figure 3). No immunostaining was detected in the negative control sections (data not shown).

Local recruitment and activation of inflammatory cells in the vehicle-treated rats undergoing SAO/R led to a clear-cut increase in ileal tissue markers of oxidative stress, such as lipid peroxidation products (MDA), DNA damage (8-OHdG) and consumption of endogenous antioxidant enzymes, like MnSOD. RLX, but not iRLX, inhibited the increased ileum level of MDA and 8-OHdG and prevented the decrease of MnSOD content (Figure 4).

Detection of nuclear poly-ADP-ribosylated DNA sites, a marker for PAR-polymerase (PARP) activity, an enzyme involved in DNA repair, in sections from SAO/R, vehicle-treated rat ileum showed an intense immunostaining mostly located at the epithelium and at the inflammatory infiltrate in the underlying mucosa. In the RLX-pretreated animals there was negligible PAR immunostaining (Figure 5). No immunostaining was detected in the negative control sections (data not shown).

In sections from ileal tissue specimens taken after SAO/R, we observed a strong immune reaction for NT, located mainly in submucosal vessels. Pretreatment with RLX, but not with iRLX, reduced the degree of NT immunostaining. Labeling

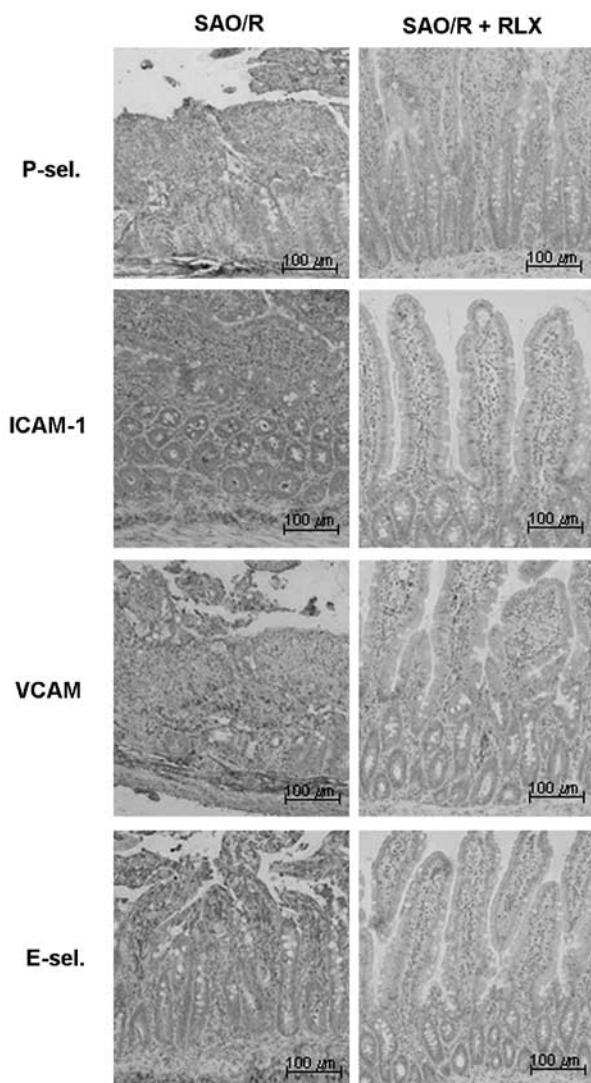


Figure 3 Immunostaining for ECAMs of ileal tissue sections from SAO/R rats treated with vehicle or RLX. RLX reduces the overexpression of the adhesion molecules investigated.

was nearly absent in sections from the sham-operated rats (Figure 6). Specificity of the immune reaction for NT was confirmed by the almost complete inhibition of the labeling upon pretreatment of the primary antibody with excess NT (data not shown).

Inflammation-induced damage of cells in the ileal wall was assessed by analysing the occurrence of apoptosis through the measure of the activity of caspase-3, a key enzyme in early apoptotic cascade, and by detection of fragmented chromosomal DNA by TUNEL assay (Figure 6a and b). In the rats undergoing SAO/R, as compared with the sham-operated animals, caspase-3 activity was significantly increased and TUNEL-positive nuclear staining was markedly enhanced. Pretreatment with RLX inhibited caspase-3 activation and reduced the amount of TUNEL-positive nuclei, whereas iRLX had no effect.

Discussion

The current findings show that RLX, given i.v. to rats subjected to SAO-induced, prolonged ileal ischemia 15 min

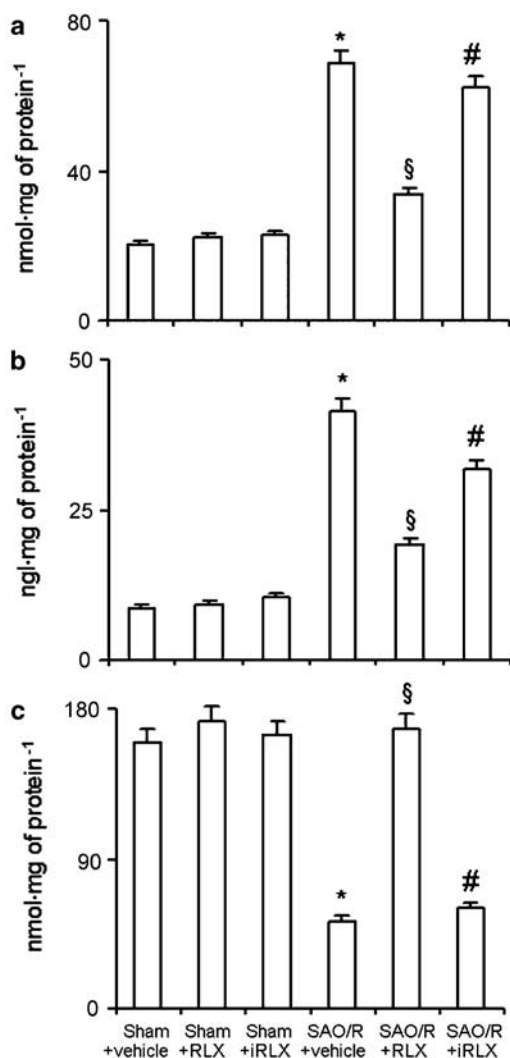


Figure 4 MDA (a) and 8-OHdG (b) are increased, while MnSOD activity (c) is decreased in ileal tissue of SAO/R + vehicle rats. RLX, but not iRLX, significantly prevents the occurrence of the above changes. * $P < 0.01$ vs sham-operated, § $P < 0.01$ vs SAO/R + vehicle, # $P < 0.01$ vs SAO/R + RLX.

before reperfusion, affords protection against reperfusion-induced tissue injury. Compared with rats given PBS or iRLX, which underwent overt SAO/R-induced shock and severe ileal inflammation and damage, administration of bioactive RLX resulted in a marked, statistically significant reduction of the accumulation of inflammatory leukocytes into the ileum (tissue MPO activity), likely due to the observed decrease in the expression of early-phase P-selectin and late-phase E-selectin, VCAM and ICAM-1 by the ileal endothelium, as well as to a direct inhibitory effect of RLX on circulating neutrophils (Masini *et al.*, 2004).

The marked reduction of the local inflammatory infiltrate caused by RLX treatment could likely account for the decrease in ileal tissue markers of oxidative stress, such as lipid peroxidation products (MDA), DNA damage (8-OHdG) and consumption of the endogenous antioxidant MnSOD (Crow & Beckman, 1995; Dix *et al.*, 1996; Misko *et al.*, 1998; Salvemini *et al.*, 1999a,b), observed in the RLX-treated rats. This also fits well with the observed reduction of nuclear poly-ADP-

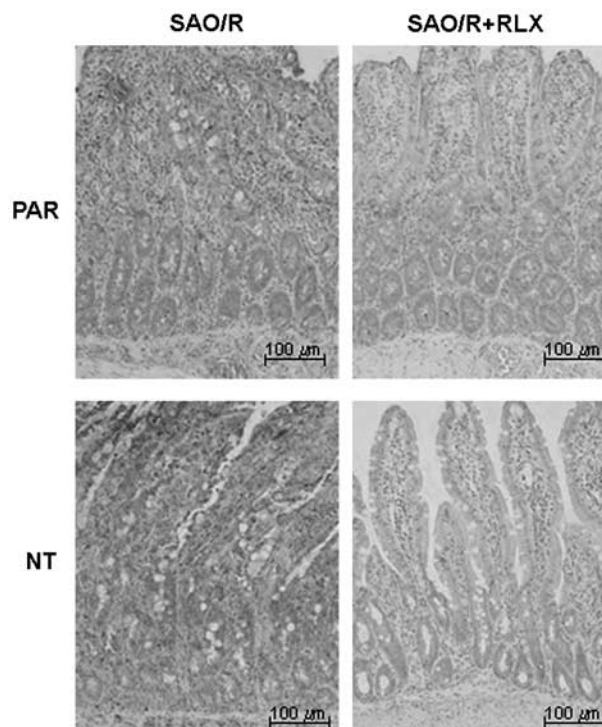


Figure 5 Immunocytochemical detection of Poly-ADP-ribosylated DNA (PAR) and NT in ileal tissue sections from SAO/R rats treated with vehicle or RLX. RLX reduces the expression of both PAR and NT.

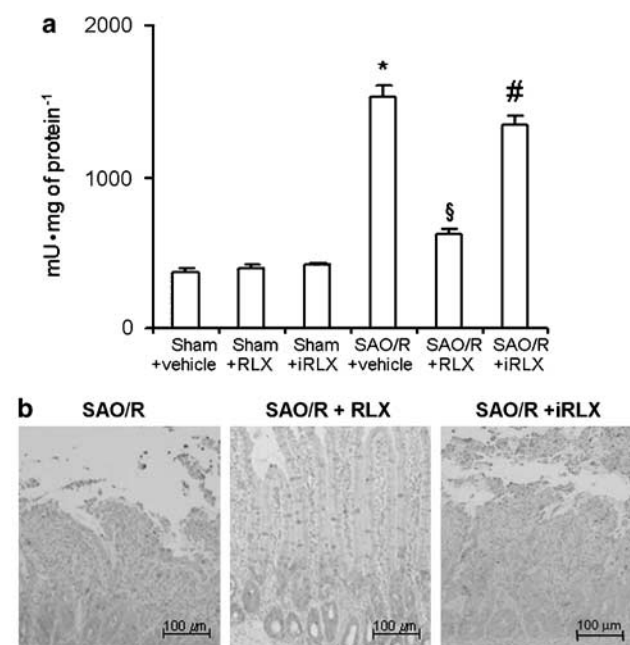


Figure 6 (a) Caspase-3 activity is increased in ileal tissue of SAO/R + vehicle rats. This effect is prevented by RLX, but not by iRLX. * $P < 0.01$ vs sham-operated animals, $P < 0.01$ vs SAO/R + vehicle, # $P < 0.01$ vs SAO/R + RLX. (b) TUNEL-positive cells in ileal tissue sections from sham-operated rats and SAO/R rats treated with vehicle or RLX. Numerous apoptotic cells can be seen in the ileum of SAO/R + vehicle as well as in the SAO/R + iRLX rats. In contrast, only few apoptotic cells can be detected at the villum tips in the samples from SAO/R + RLX rats.

ribosylated DNA sites and of NT immunostaining in the RLX-treated animals as compared with those given PBS or iRLX, these markers being generated by interaction of free radicals produced by oxidative stress with DNA and proteins, respectively (Inoue & Kawanishi, 1995; Salgo *et al.*, 1995; Eisserich *et al.*, 1998). In turn, PARP activation at reperfusion can paradoxically worsen cell damage and endothelial dysfunction (Szabó & Dawson, 1998): thus, prevention of PARP activation may have an additional cytoprotective effect in the SAO/R ileum, as reported previously (Cuzzocrea *et al.*, 1997; Szabó *et al.*, 1997).

The RLX-dependent protection of ileal tissue from the adverse effects of inflammation and oxidative stress results in a clear-cut, statistically significant reduction of cell apoptosis, as shown by decreased caspase-3 activity and TUNEL-positive cells. Overall, all these beneficial effects of RLX on SAO/R ileum may account for the striking increase in the percentage of rats that survived until term reperfusion (4 h).

The present findings extend the current knowledge on the unique property of RLX to blunt ischemia/reperfusion-induced damage, previously investigated in animal models of myocardial infarction (Masini *et al.*, 1997; Bani *et al.*, 1998b; Perna *et al.*, 2005). This property appears to depend on multiple, synergistic actions on vascular endothelial and smooth muscle cells (Bani *et al.*, 1998a; Failli *et al.*, 2002; Nistri *et al.*, 2003) as well as on inflammatory cells playing a key role in reperfusion-induced tissue injury, such as neutrophils (Masini *et al.*, 2004), likely mediated by a mechanism involving stimulation of NO production by the target cells (Nistri & Bani, 2005). In this context, it is worth noting that defective endogenous NO contributes substantially to SAO/R injury (Kanwar *et al.*, 1994), whereas pharmacological increase of NO bioavailability by administration of NO donors, such as SNAP (Gauthier *et al.*, 1994) or FK409 (Kalia *et al.* 2002), or

of the NOS substrate L-arginine (Ward *et al.*, 2000) has been shown to improve SAO/R-induced shock, especially by reducing endothelial dysfunction and leukocyte adhesion and recruitment. Compared with classical NO upregulating molecules, RLX could have additional, NO-independent beneficial effects: for instance, RLX has been recently ascribed anti-inflammatory properties by a glucocorticoid-like, plasma membrane receptor-independent action (Dschiezig *et al.*, 2004).

At present, no definite medical therapy for intestinal ischemia exists, as several drugs have been anecdotally proven but not carefully studied. Many substances, including antioxidants and free radical scavengers, NO donors, cyclooxygenase and lipoxygenase blockers, leukocyte inhibitors and antichemotaxis agents, anti-ECAM monoclonal antibodies, etc., have shown protective effects in experimental models, but none of them is currently in clinical use (Mallick *et al.*, 2004). On the other hand, numerous, recent experimental studies indicate ischemic preconditioning as the most promising strategy against intestinal ischemia, appearing to increase the tolerance of the intestine to reperfusion injury (Mallick *et al.*, 2004). Research focused on the application of novel drugs that can mimic the effects of ischemic preconditioning to manipulate the cellular events during SAO/R of the intestine is required: in this context, RLX has a broad range of beneficial actions, such as leukocyte and platelet inhibition, endothelial protection and ECAM downregulation, which could effectively reproduce many of the effects of ischemic preconditioning. This issue affords RLX – or putative synthetic RLX agonists – the nomination among the novel drugs to be tested for possible therapeutic use in intestinal ischemia.

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