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RESEARCH PAPER

Increased endothelin-1 vasoconstriction in mesenteric resistance arteries after superior mesenteric ischaemia-reperfusion

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ischaemia-reperfusion; endothelin-1; mesenteric resistance arteries; ET_A receptor; ET_B receptor

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BACKGROUND AND PURPOSE

Endothelin-1 (ET-1) plays an important role in the maintenance of vascular tone. We aimed to evaluate the influence of superior mesenteric artery (SMA) ischaemia-reperfusion (I/R) on mesenteric resistance artery vasomotor function and the mechanism involved in the changes in vascular responses to ET-1.

EXPERIMENTAL APPROACH

SMA from male Sprague-Dawley rats was occluded (90 min) and following reperfusion (24 h), mesenteric resistance arteries were dissected. Vascular reactivity was studied using wire myography. Protein and mRNA expression, superoxide anion ($O_2^{\bullet-}$) production and ET-1 plasma concentration were evaluated by immunofluorescence, real-time quantitative PCR, ethidium fluorescence and ELISA, respectively.

KEY RESULTS

I/R increased ET-1 plasma concentration, ET-1-mediated vasoconstriction and ET_B mRNA expression, and down-regulated ET_A mRNA expression. Immunofluorescence confirmed mRNA results and revealed an increase in ET_B receptors in the mesenteric resistance artery media layer after I/R. Therefore, the ET_B receptor agonist sarafotoxin-6 induced a contraction that was inhibited by the ET_B receptor antagonist BQ788 only in vessels, with and without endothelium, from I/R rats. Furthermore, BQ788 potentiated ET-1 vasoconstriction only in sham rats. Endothelium removal in rings from I/R rats unmasked the inhibition of ET-1 vasoconstriction by BQ788. Endothelium removal, N^{ω}-nitro-L-arginine methyl ester and superoxide dismutase abolished the differences in ET-1 vasoconstriction between sham and I/R rats. We also found that I/R down-regulates endothelial NOS mRNA expression and concomitantly enhanced $O_2^{\bullet-}$ production by increasing NADPH oxidase 1 (NOX-1) and p^{47phox} mRNA.

CONCLUSIONS AND IMPLICATIONS

Mesenteric I/R potentiated the ET-1-mediated vasoconstriction by a mechanism that involves up-regulation of muscular ET_B receptors and decrease in NO bioavailability.

Abbreviations

BQ123, *cyc*(DTrp-DAsp-Pro-D-Val-Leu); BQ788, *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarboyl-D-norleucine; ET-1, endothelin-1; I/R, ischaemia-reperfusion; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PFA, paraformaldehyde; PSS, physiological salt solution; SOD, superoxide dismutase; SMA, superior mesenteric artery

Introduction

Ischaemia/reperfusion (I/R) is widely associated with organ damage in various pathological conditions such as stroke (Lakhan *et al.*, 2009), and myocardial (Garcia-Dorado *et al.*, 2009), liver (Walsh *et al.*, 2009) and intestinal (Yasuhara, 2005; Paterno and Longo, 2008) ischaemia. The vascular system and the endothelium in particular are well known to be very sensitive to I/R-induced injuries (Gourdin *et al.*, 2009). Several factors have been described as contributing to vascular injury after I/R (Eltzschig and Collard, 2004) but the exact contribution of each mechanism remains unclear. Studies have reported an increase in plasma endothelin-1 (ET-1) concentration following cerebral (Brondani *et al.*, 2007; Giannopoulos *et al.*, 2008), cardiac (Brunner *et al.*, 2006), pulmonary (Kawashima *et al.*, 2003), renal (Camara-Lemarroy *et al.*, 2009) and intestinal (Oktar *et al.*, 2002; Guzmán-de la Garza *et al.*, 2009) I/R, and ET-1 has been postulated as one of the endothelial factors that most contribute to organ damage associated with I/R (Brunner *et al.*, 2006). Contractile responses to ET-1 have been described as increased in rat heart (García-Villalón *et al.*, 2008) and middle cerebral artery (MCA) (Stenman *et al.*, 2002) after I/R, while a decreased response in mesenteric arteries from deoxycorticosterone acetate salt hypertensive rats (Deng and Schiffrin, 1992) has been shown. Moreover, increased levels of plasma ET-1 concentration after intestinal I/R have been consistently reported (Kurtel and Ghandhour, 1999; Oktar *et al.*, 2002; Guzmán-de la Garza *et al.*, 2009), although very few studies have analysed the influence of I/R on ET-1 vasoconstrictor responses (Wood *et al.*, 1995, 1996; Ługowska-Umer *et al.*, 2008).

In mammals, ET-1 mediates its action via two receptors, ET_A and ET_B, both coupled to G-proteins (Masaki, 2004; Schneider *et al.*, 2007). The strong vasoconstrictor effect of ET-1 is mediated by the ET_A receptor, the dominant receptor subtype in the smooth muscle cells, while the ET_B receptor subtype is mainly found in the endothelial cells. Stimulation of endothelial ET_B receptors releases NO and/or COX metabolites, inducing vasodilatation on the underlying smooth muscle cells. Recently, smooth muscle ET_B receptors that participate in ET-1 vasoconstrictor responses have also been demonstrated (Böhm and Pernow, 2007; Schneider *et al.*, 2007). A few studies have linked altered ET-1 vascular responses after I/R with changes in the expression of the different ET receptor subtypes in rats, specifically after focal cerebral (Stenman *et al.*, 2002; Maddahi and Edvinsson, 2008), renal (Ramírez *et al.*, 2009) and hepatic (Yokoyama *et al.*, 2000) I/R.

The present study aims to evaluate if the occlusion (90 min) followed by 24 h reperfusion of the superior mesenteric artery (SMA) induces alterations in the ET-1-mediated responses, as well as the possibility of an endothelial vasodilator dysfunction in mesenteric resistance arteries. Furthermore, our studies evaluated the potential mechanisms responsible for the observed alterations.

We found that mesenteric I/R enhanced ET-1 plasma levels and induced a potentiation of ET-1 vasoconstriction in mesenteric resistance arteries without modifying the vasodilator response to ACh. The mechanisms associated with the potentiation of ET-1 responses were partially due to up-regulation of ET_B receptors in the smooth muscle cells. Despite a down-regulation of ET_A receptors, ET-1 responses mediated by these receptors were not modified. In addition, I/R induced an increase in superoxide anion ($O_2^{\bullet-}$) production and a decrease in endothelial NOS mRNA that could also participate in the alterations observed in ET-1 vasoconstriction.

Methods

Animals

Male Sprague-Dawley rats (310–350 g body weight) obtained from Harlan (Barcelona, Spain) were housed in a temperature-controlled room on a 12 h light–dark cycle and provided with access to food and water *ad libitum*. All animals and experimental protocols were in accordance with guidelines established by Spanish legislation (RD 1201/2005). Experiments were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Experimental mesenteric ischaemia

Rats were anaesthetized with sodium pentobarbitone (40 mg·Kg^{-1}; i.p.), and the ventral neck, abdomen and groin were shaved and washed with 10% povidone iodine. The abdominal cavity was accessed by a midline incision, and the SMA was visualized and clamped. Pulselessness of the mesenteric arterial branches was achieved immediately after complete occlusion of the SMA. Following 90 min ischaemia, the clamp was removed and the midline incision was closed. Rats were allowed to recover from anaesthesia and returned to their cages with free access to food and water. Sham animals underwent the same surgical procedure with the exception of the clamping. After 24 h reperfusion, rats were anaesthetized, decapitated, and the mesenteric arcade was removed and placed in physiological salt solution (PSS) of the following composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1, maintained at 4°C and continuously gassed with 95% O_2 and 5% CO_2.

Segments of third-order branches (vascular reactivity, $O_2^{\bullet-}$ production and immunofluorescence studies) and second- and third-order branches [real-time quantitative PCR (RT-qPCR)] of the mesenteric artery were dissected free of fat and connective tissue, and maintained in cold PSS. Vessels to be used for $O_2^{\bullet-}$ production were placed in PSS containing 30% sucrose overnight. Next, they were transferred to a cryomold (Bayer Química Farmacéutica, Barcelona, Spain) containing Tissue-Tek OCT embedding medium (Sakura Finetek Europe, Zoeterwoude, the Netherlands) for 20 min, and then they were immediately frozen in liquid nitrogen. For immunofluorescence studies, vessels were fixed with 4% phosphate buffered paraformaldehyde (PFA, pH = 7.4) for 1 h and washed in three changes of PBS (pH = 7.4). After being cleaned, arterial segments were placed in PBS containing 30% sucrose overnight, transferred to a cryomold containing Tissue Tek OCT embedding medium, and frozen in liquid nitrogen. Second- and third-order branches to be used for RT-qPCR were frozen in liquid nitrogen following dissection. All samples frozen in liquid nitrogen were kept at –70°C until the day of the experiments.

In some rats, blood samples (1 mL) were collected by cardiac puncture under anaesthesia with pentobarbital after 3 and 24 h reperfusion. Plasma was separated by centrifuging at 12 000× *g*, 5 min, 4°C and stored at −70°C in aliquots.

Functional experiments

Vascular function was studied in mesenteric resistance arteries mounted on an isometric wire myograph (model 410 A, J.P. Trading, Aarhus, Denmark) filled with PSS and kept at 37°C according to the protocol previously described (Martinez-Revelles *et al.*, 2008). Thirty minutes after resting tension was established, mesenteric resistance arteries were maximally contracted with a K^+-depolarizing solution of the following composition (in mM): NaCl 16.7, KCl 100, KH_2PO_4 1.2, $MgSO_4$ 1.2, CaCl2 2.5, glucose 11.1 and $NaHCO_3$ 25. After washout and return to stable baseline, a concentration-response curve to agonists in the presence or absence of antagonists was performed.

When the influence of endothelium was evaluated, rings were first precontracted with phenylephrine (10 μM) and relaxed with ACh (10 μM) followed by a 30 min equilibration period. Only rings that relaxed more than 80% to ACh were used as endothelium intact; those that failed to relax to ACh were considered to have the endothelium removed.

Concentration-response curves to ET-1 (1 pM-0.1 μM) were performed in either the absence or the presence of endothelium. The influence of NO, COX metabolites and $O_2^{\bullet-}$ on ET-1-induced contraction was studied on rings with intact endothelium by 30 min incubation with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM), indomethacin (10 μM) or superoxide dismutase (SOD, 150 U·mL^{-1}) before the ET-1 concentration-response curve.

In a second series of experiments, responses to ACh (10 nM-3 μM) were obtained in arteries precontracted with phenylephrine (10 μM) after 30 min incubation with SOD (150 U·mL^{-1}), L-NAME (100 μM) or L-NAME (100 μM) plus indomethacin (10 μM). Contractile responses to phenylephrine (0.1–30 μM) were performed in parallel using rings from the same animal.

In a third series of experiments in rings with or without endothelium, a concentration-response curve was obtained for the selective ET_B receptor agonist sarafotoxin 6 (SC_6) alone or in the presence of *N-cis*-2,6-dimethylpiperidinocarbonyl-L-δ-methylleucyl-D-1-methoxycarboyl-D-norleucine (BQ788, 0.5 μM, 30 min), a selective ET_B receptor antagonist. Furthermore, a concentration-response curve to ET-1 was performed in the presence or the absence of endothelium after incubation with SC_6 (1 μM), which desensitizes and abolishes the ET_B responses (Cramer *et al.*, 1998).

Finally, the ET-1-induced responses were studied on endothelium intact and denuded rings, in the presence of *cyc*(DTrp-DAsp-Pro-D-Val-Leu) (BQ123) (0.5 μM, 30 min) or BQ788 (0.5 μM, 30 min), ET_A and ET_B receptor antagonists, respectively.

All the experiments were carried out on mesenteric resistance arteries from sham and I/R rats.

Determination of plasma ET-1 concentration

Plasma ET-1concentration was analysed by ELISA using commercially available kits (Biomedica Medizinprodukte GmbH & Co, Vienna, Austria) following the manufacturer's instructions. Results are expressed as pg ml^{-1}.

Immunofluorescence

Frozen transverse sections (14 μm) of mesenteric resistance arteries were processed as previously described (Martinez-Revelles *et al.*, 2008). Sections were incubated with a rabbit primary polyclonal antibody against ET_A receptor (1:100; Alomone Labs Ltd, Jerusalem, Israel), a sheep polyclonal antibody against the ET_B receptor (1:100; Abcam, Cambridge, UK) or a mouse monoclonal anti-eNOS (1:100; BD Transduction Laboratories, Lexington, UK). After being washed, rings were incubated with the secondary antibody: donkey anti-rabbit, anti-sheep or anti-mouse (1:200) IgG conjugated to Cy™3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Immunofluorescent signals were viewed using an inverted Leica TCS SP2 confocal laser scanning microscope (Leica, Heidelberg, Germany) with oil immersion lens (×63). The Cy™3-labelled antibody was visualized by excitation at 568 nm and detection at 600–700 nm. The specificity of the immunostaining was evaluated by omission of the primary antibody and processed as above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

Measurement of $O_2^{\bullet-}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate the production of $O_2^{\bullet-}$ *in situ*, as described previously (Martinez-Revelles *et al.*, 2008; Jiménez-Altayó *et al.*, 2009). Briefly, frozen tissue segments were cut into 14 μm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs–HEPES buffer (in mM): NaCl 130, KCl 5.6, CaCl2 2, MgCl2 0.24, HEPES 8.3 and glucose 11, pH 7.4. Fresh buffer containing DHE (2 μM) (Ex 488 nm and Em 610 nm) was applied topically onto each tissue section, cover slipped and incubated for 30 min in a light-protected humidified chamber at 37°C, and then viewed by fluorescent laser scanning confocal microscope (Leica TCS SP2; ×63), using the same imaging settings in each case. Parallel sections were incubated with polyethylene glycol SOD (PEG-SOD; 500 U·mL^{-1}). Quantitative analysis of $O_2^{\bullet-}$ production was performed with MetaMorph Image Analysis Software (Universal Imaging, Molecular Devices, Downingtown, PA, USA). Integrated optical densities in the target region were calculated. Four areas per ring were sampled for each experimental condition. All measurements were conducted blind.

Real-time quantitative PCR

Total RNA was obtained as previously described (Oliver *et al.*, 2009), quantified and analysed by running 1 μg of each sample by microfluidic electrophoresis using the Experion™ automated electrophoresis system (Bio-Rad, Madrid, Spain) following the manufacturer's recommendations. For cDNA synthesis, 500 ng of total RNA and oligo(dT)16 as primer (250 ng) in diethylpyrocarbonate-treated water were preheated to 70°C and cooled on ice. Reactions (20 μL) containing ImProm-II™ reaction buffer, 3 mM MgCl2, 20 U of Recombinant RNAsin® Ribonuclease Inhibitor (Promega Corp., Fitchburg, WI, USA), 0.5 mM of each deoxynucleoside

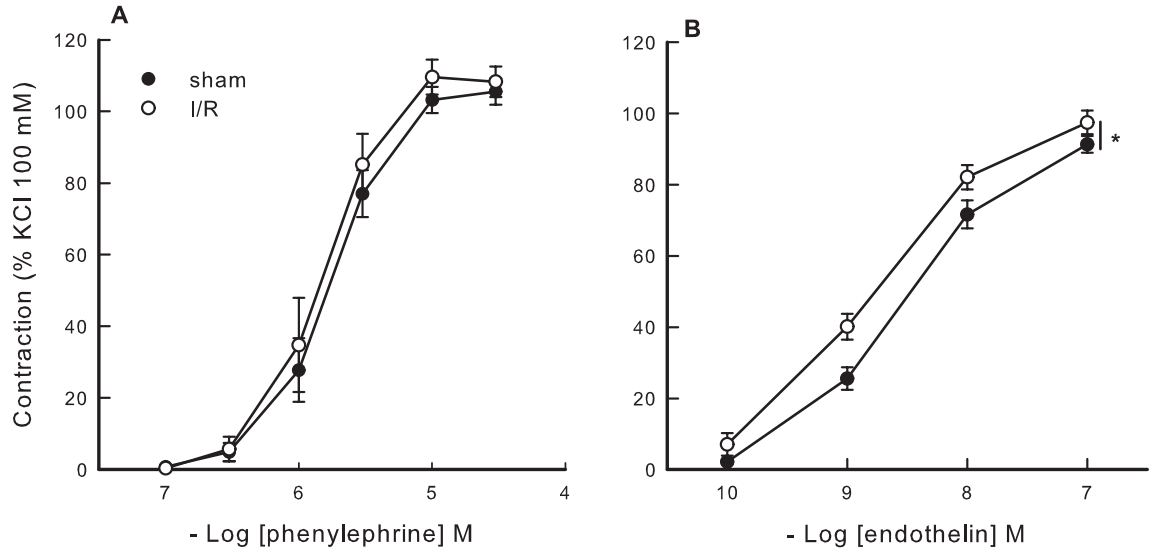

Figure 1

Concentration-response curves to phenylephrine (A) and ET-1 (B) in rings of endothelium-intact mesenteric resistance arteries from sham and I/R rats. Data are shown as mean ± SEM from 9 to 20 animals. *$P < 0.05$ sham versus I/R by two-way ANOVA.

triphosphate, and 1 μL of ImProm-IITM Reverse Transcriptase (Promega Corp., Madison, USA), were treated by an extension step at 42°C for 60 min, incubated at 25°C for 5 min (Anneal step), and finally at 70°C for 15 min (heat inactivation).

mRNA encoding the two endothelin receptors (ET_A and ET_B) and eNOS were quantified by TaqMan® RT-qPCR with a GeneAmp 7500 Fast System (Applied Biosystems, Carlsbad, CA, USA) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. mRNA for the subunits of NAD(P)H-oxidase, NOX-1 and p^{47phox}, were quantified by RT-qPCR based on Sybr-Green fluorescence using the 18S ribosomal subunit of RNA as internal control. The specific primer-probes were: ET_A (Ednra: Rn00561137_m1), ET_B (Ednrb: Rn00569139_m1), eNOS (NOS3: Rn02132634_s1), GAPDH (Rn99999916_s1) and 18S (Hs99999901 s1) (Applied Biosystems). Primer sequences for rodent NOX-1 (NM 053863) and p^{47phox} (NM 053734) were: NOX1 (upper 5′-CCTTCCATAAGCTGGTGGCAT-3′ and lower 5′-GCCATG GATCCCTAAGCAGAT-3′) and, p^{47phox}: (upper 5′-AGGAGAT GTTCCCCATTGAGG-3′ and lower 5′-GTCCCATGAGGCT GTTGAA-3′). RT PCR reactions were set to manufacturer's conditions, as previously described (Oliver *et al.*, 2009). Ct values obtained for each gene were referenced to GAPDH or r18S (ΔCt) and converted to the linear form using the term $2^{-\Delta Ct}$ as a value directly proportional to the copy number of mRNA (Livak and Schmittgen, 2001).

Drugs

The following drugs were used: ACh chloride, BQ123 sodium salt; BQ788 sodium salt, dihydroethidium, indomethacin, phenylephrine hydrochloride, L-NAME, polyethylene glycol SOD, SOD (Sigma-Aldrich, Madrid, Spain); ET-1 (Alexis Biochemicals, Notttingham, UK); and sarafotoxin 6 (AnaSpec, Fremont, CA, USA). All other chemicals used were of analytical grade. All drug and molecular target nomenclature follows Alexander *et al.* (2011).

Data and statistical analysis

Contractile responses are expressed as a percentage of 100 mM KCl. Relaxations to ACh are expressed as the percentage change from the phenylephrine precontracted level. All results are expressed as mean ± SEM of the number (n) of rats indicated in the figure legends. Differences between concentration-response curves were assessed by a two-way ANOVA with repeated measures on the concentration factor. Differences between sham and I/R rats for plasma ET-1 concentration, RT-qPCR, sensitivity (pEC_{50}), and maximal responses (E_{max}) were analysed by Student's unpaired t-test. A value of $P < 0.05$ was considered significant.

Results

Both sham (347 ± 10 g, $n = 46$) and I/R (347 ± 17 g, $n = 57$) rats presented a similar slight decrease in body weight (sham: 328 ± 15 g, $n = 46$; I/R: 332 ± 18 g, $n = 57$) 24 h after the surgical procedure.

Changes in plasma ET-1concentration after I/R

Plasma concentration of ET-1 increased ($P < 0.05$) after 3 h of reperfusion (9.14 ± 2.2 pg·mL^{-1}, $n = 7$) compared with sham (5 ± 0.68 pg·mL^{-1}, $n = 14$) rats. ET-1 plasma concentration returned to basal levels after 24 h of reperfusion (I/R: 3.40 ± 0.48 pg·mL^{-1}, $n = 7$; sham: 2.84 ± 0.57 pg·mL^{-1}, $n = 12$).

Changes in vasoconstrictor responses of mesenteric resistance arteries after I/R

Contractions to KCl (100 mM) did not differ between I/R (4.71 ± 0.1 mN·mm^{-1}, $n = 52$) and sham (4.87 ± 0.12 mN·mm^{-1}, $n = 39$) rats. Phenylephrine (Figure 1A) contracted endothelium-intact mesenteric resistance arteries in a concentration-related manner but no significant changes

Table 1

*p*EC50 and E_{max} values for ET-1 and SC_6 concentration-response curves in mesenteric resistance arteries from sham and I/R rats and changes observed after ET_B receptor desensitization with SC_6 (10 μM), or in the presence of BQ123 (50 μM) and BQ788 (50 μM)

Agonist	Endothelium intact (+E) pEC_{50}	Endothelium intact (+E) E_{max}	Endothelium removed (-E) pEC_{50}	Endothelium removed (-E) E_{max}
Endothelin control				
sham	8.49 ± 0.07 (18)	91.40 ± 2.30 (18)	9.47 ± 0.25^{+++} (19)	117.00 ± 2.56^{+++} (19)
I/R	8.79 ± 0.08** (19)	97.40 ± 3.31 (19)	9.41 ± 0.23^{+} (10)	125.10 ± 3.96^{+++} (16)
SC_6				
sham	ND	3.42 ± 1.10 (8)	ND	5.66 ± 1.50** (8)
I/R	8.96 ± 0.15 (14)	41.16 ± 9.49** (14)	9.44 ± 0.24 (15)	37.48 ± 7.70 (15)
Endothelin (SC_6)				
sham	9.19 ± 0.19°°° (8)	100.11 ± 5.62 (8)	9.34 ± 0.14 (8)	122.60 ± 6.11^{+} (8)
I/R	9.25 ± 0.12°° (7)	102.17 ± 6.57 (7)	9.24 ± 0.02 (6)	124.31 ± 7.69^{+} (6)
Endothelin + BQ123				
sham	7.66 ± 0.39†† (9)	90.48 ± 2.58 (9)	8.45 ± 0.13† (6)	119.90 ± 3.98 (6)
I/R	8.03 ± 0.26†† (9)	90.77 ± 4.92 (9)	8.60 ± 0.20† (6)	128.40 ± 3.60 (6)
Endothelin + BQ788				
sham	8.70 ± 0.10 (8)	112.03 ± 3.10 (8)	9.38 ± 0.14 (4)	112.8 ± 4.17 (4)
I/R	8.79 ± 0.41 (10)	94.18 ± 3.39 (10)	8.83 ± 0.09† (4)	116.21 ± 2.08 (4)

Data are shown as mean ± SEM. The number of animals is shown in parentheses. *$P < 0.05$, **$P < 0.01$ sham versus I/R; $^{+}P < 0.05$ $^{+++}P < 0.001$ +E versus –E; †$P < 0.05$, ††$P < 0.01$ antagonist versus control; °°$P < 0.01$, °°°$P < 0.001$ versus Endothelin control. I/R, ischaemia-reperfusion; ND, not determined.

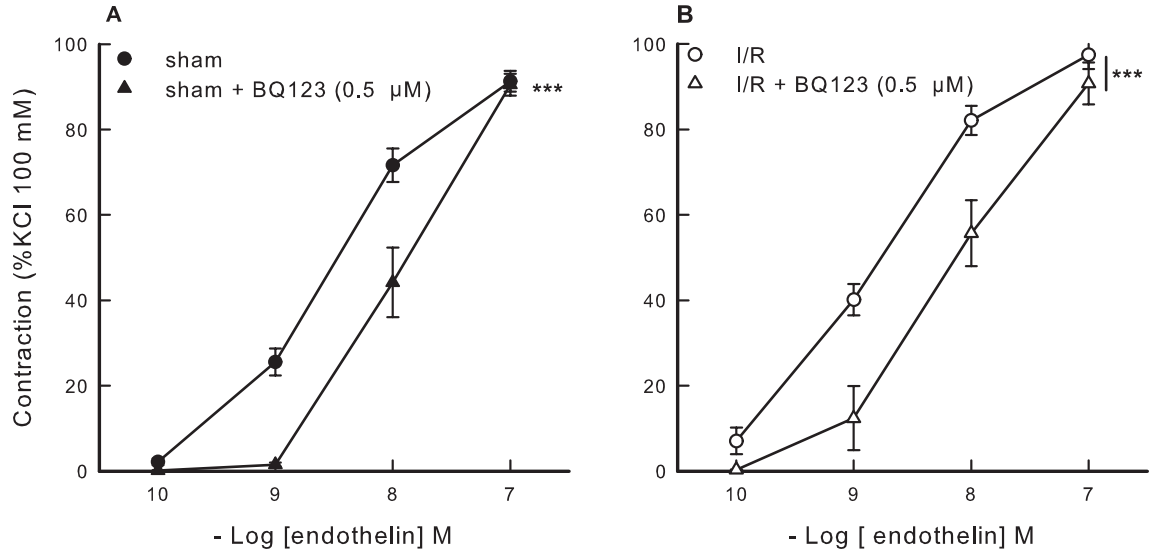

Figure 2

Effect of BQ123 on concentration-response curve for ET-1 obtained in rings of endothelium intact mesenteric resistance arteries from sham (A) and I/R (B) rats. Data are shown as mean ± SEM from 9 to 19 animals. ***$P < 0.001$ in the absence versus the presence of BQ123 by two-way ANOVA.

were observed after I/R (sham: $pEC_{50} = 5.80 \pm 0.17$, $n = 6$; I/R: $pEC_{50} = 5.9 \pm 0.18$, $n = 5$). Nevertheless, responses to ET-1 (Figure 1B) were significantly potentiated ($P < 0.05$) in endothelium intact rings from I/R rats when compared with sham animals (Table 1).

Role of ET_A and ET_B receptors on the potentiation by I/R of ET-1 vasoconstriction

Antagonists of the two described ET receptors were used to study the participation of both subtypes in the potentiation

of ET-1-induced vasoconstriction observed after I/R. BQ123, a selective ET_A receptor antagonist, shifted the ET-1 concentration-response curve to a similar extent to the right in rings from sham and I/R (Figure 2, Table 1) rats. The presence of BQ788, a selective ET_B receptor antagonist, potentiated the concentration-response curve to ET-1 in rings from sham (Figure 3A) but did not modify that from I/R rats (Figure 3B). After endothelium removal, BQ788 shifted the ET-1 concentration-response curve to the right in rings from I/R (Figure 3C, Table 1) but not from sham (Table 1) rats. In contrast, BQ123 shifted to a similar extent to the right the ET-1 concentration-response curve in endothelium-denuded rings from sham and I/R animals (Table 1).

To analyse in more depth the participation of endothelial and smooth muscle ET_B receptors in the observed potentiation of ET-1-induced vasoconstriction by I/R, responses to SC_6, a selective ET_B receptor agonist, were studied in endothelium intact (Figure 4A) and removed (Figure 4B) rings from both groups of rats. No contractile response to SC_6 was observed in either endothelium intact (Figure 4A) or endothelium removed (Figure 4B) rings from sham rats. In contrast, after I/R, SC_6 induced a marked contraction irrespective of the presence or absence of endothelium (Figure 4, Table 1). In addition, the presence of BQ788 abolished the SC_6 responses observed in endothelium intact (Figure 4A) and endothelium removed (Figure 4B) rings from I/R rats.

In another series of experiments, we observed that after ET_B receptor desensitization with SC_6, responses to ET-1 mediated by ET_A receptors were similar in mesenteric resistance arteries rings from I/R and sham rats irrespective of the presence (Figure 5A) or absence (Figure 5B) of endothelium. Although endothelium removal produced an increase ($P < 0.05$) in the maximal responses (E_{max}) to ET-1 mediated by ET_A receptors in both groups of rats, it did not modify the potency (Table 1). It is interesting to note that in endothelium-intact vessels, the concentration-response curve for ET-1 mediated only by ET_A receptors was significantly shifted to the left even though no changes in E_{max} were observed in comparison with the responses induced by the ET-1 control (Table 1).

To confirm the differential contribution of ET_A and ET_B receptors to the functional responses induced by ET-1 after I/R, mRNA and protein expression were studied. Protein expression for ET_A receptor was clearly detected by immunofluorescence in the media and adventitial layer of rings from sham animals and was reduced after I/R (Figure 6A, upper part). For ET_B receptors, we observed a slight fluorescence in endothelial and adventitial layer in sham animals (Figure 6A, lower part) and a marked increase after I/R. The expression of ET_B receptor was increased in the endothelium but more especially in the media layer (Figure 6A, lower part).

Analysis of the mRNA levels showed that both ET receptor subtypes were expressed in mesenteric resistance arteries (Figure 6B). I/R reduced the level of ET_A receptor expression but increased mRNA levels for ET_B receptors (Figure 6B).

Role of endothelial NO and $O_2^{\bullet-}$ on the potentiation of ET-1 response after I/R

Removal of endothelium significantly increased the pEC_{50} and the E_{max} to ET-1 (Table 1). After I/R, no changes in the ET-1 concentration-response curve were observed in endothelium-denuded vessels (Figure 7A), even in the pres-

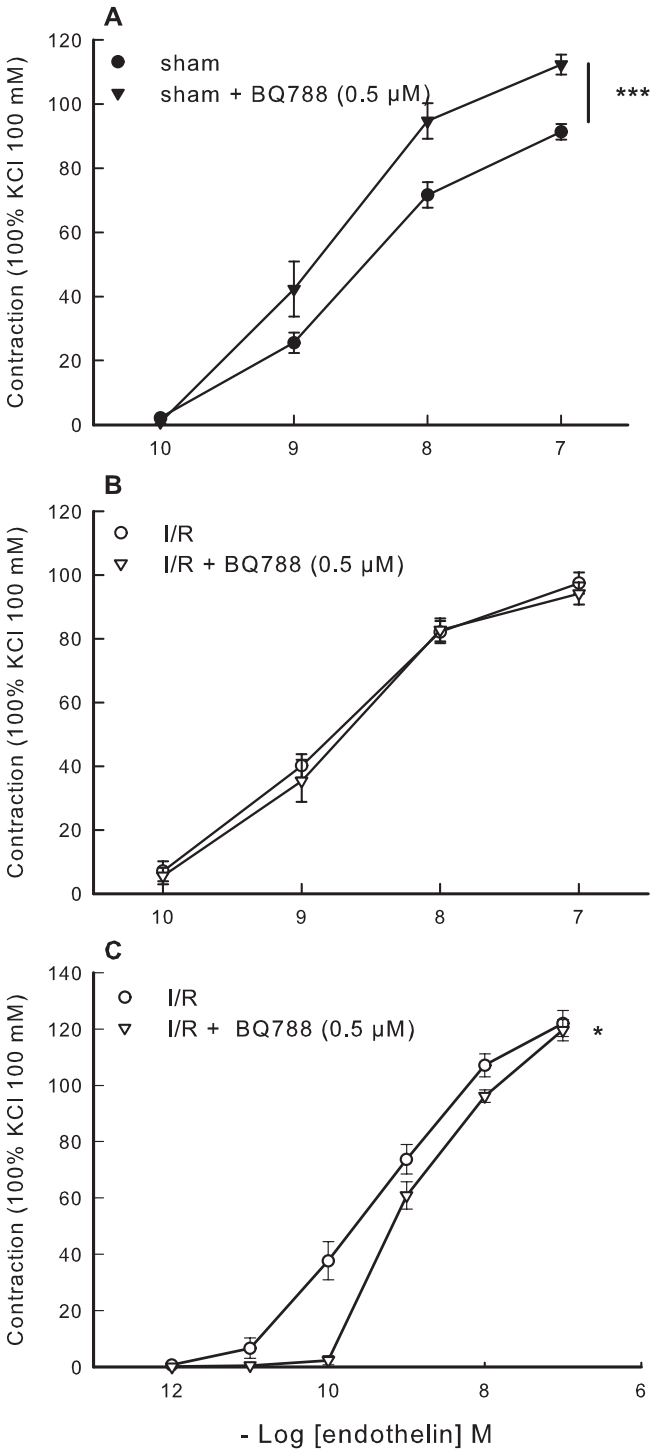

Figure 3

Effect of BQ788 on concentration-response curve for ET-1 obtained in rings of endothelium intact mesenteric resistance arteries from sham (A) and I/R rats with (B) or without endothelium (C). Data are shown as mean ± SEM from 5 to 18 animals. ***$P < 0.001$ in the absence versus the presence of BQ788 by two-way ANOVA.

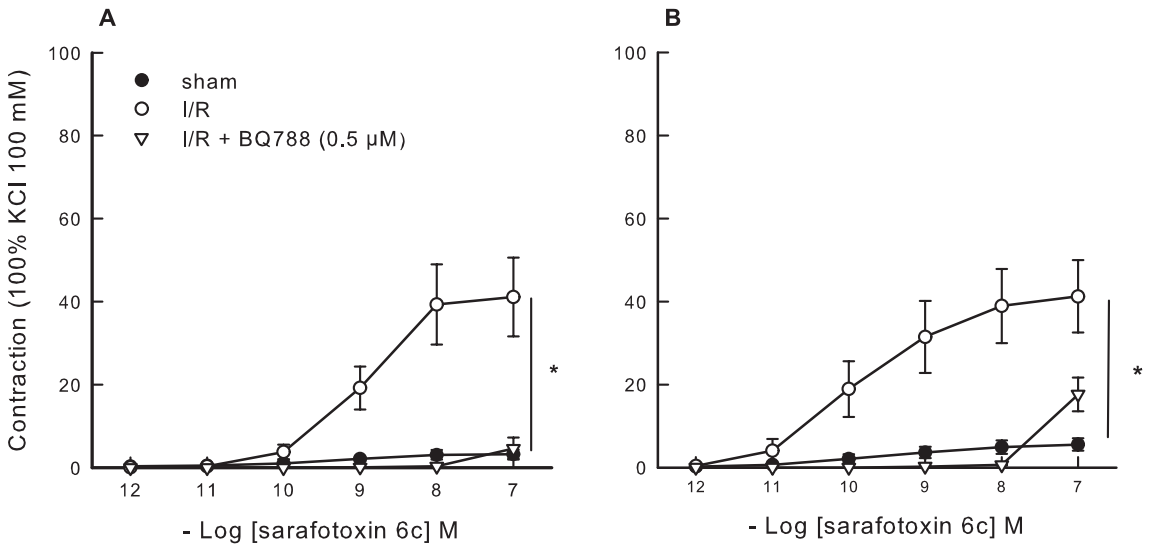

Figure 4

Concentration-response curve to SC_6 and effect of BQ788 in rings from endothelium intact (A) and denuded (B) mesenteric resistance arteries from sham and I/R rats. Data are shown as mean ± SEM from 6 to 8 animals. *$P < 0.05$ sham versus I/R by two-way ANOVA.

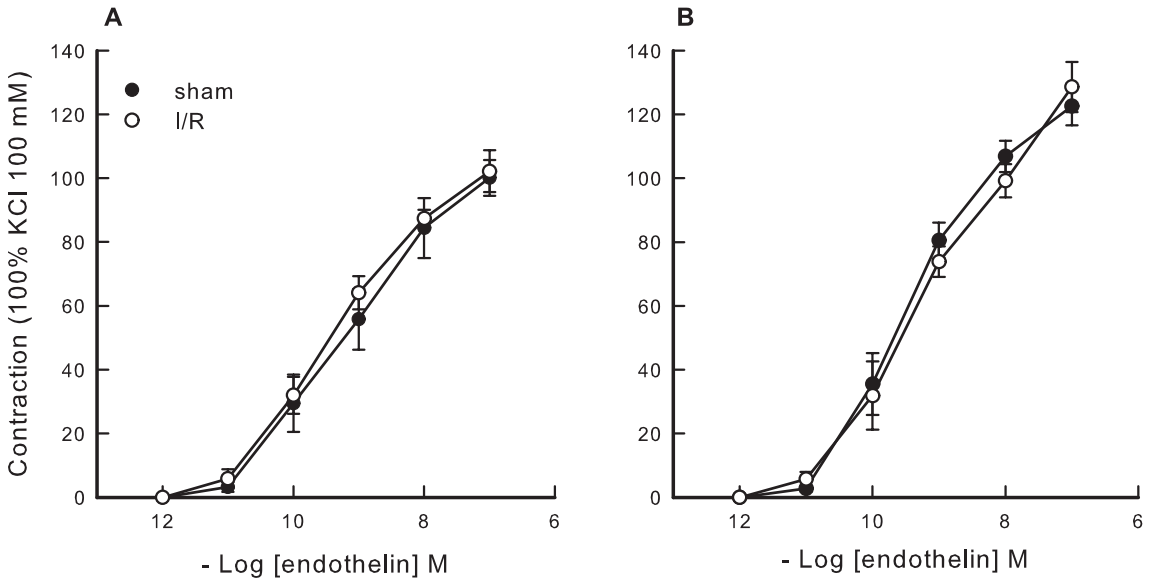

Figure 5

Concentration-response curve to ET-1 after ET_B receptor desensitization in rings from endothelium intact (A) and denuded (B) mesenteric resistance arteries from sham and I/R rats. Data are shown as mean ± SEM from 6 to 8 animals.

ence of L-NAME (Figure 7B). In contrast, incubation with indomethacin had no effect on vasoconstrictor responses to ET-1 (data not shown).

The presence of SOD had no effect on the ET-1 concentration-response curve in sham animals (data not shown), but it abolished the ET-1 potentiation observed after I/R (Figure 8).

ACh similarly relaxed the mesenteric resistance arteries from both groups of rats (Figure 9). Neither the pEC_{50} (sham: 7.00 ± 0.19, n = 5; I/R: 6.9 ± 0.3, n = 9) nor the maximum relaxation to ACh (sham: 95.00 ± 2.7%, n = 5; I/R: 97.02 ± 0.3%, n = 9) were affected by mesenteric I/R. The ACh responses were unaffected by SOD in sham and I/R animals. Furthermore, L-NAME alone or plus indomethacin inhibited, to a similar extent, the ACh-mediated responses in sham (Figure 9A) and I/R (Figure 9B) rats.

To clarify these observations, eNOS expression and $O_2^{\bullet-}$ formation were analysed. As can be seen in Figure 10B, the mRNA levels of eNOS were significantly reduced after I/R. In addition, the antibody for eNOS, which induced fluorescence

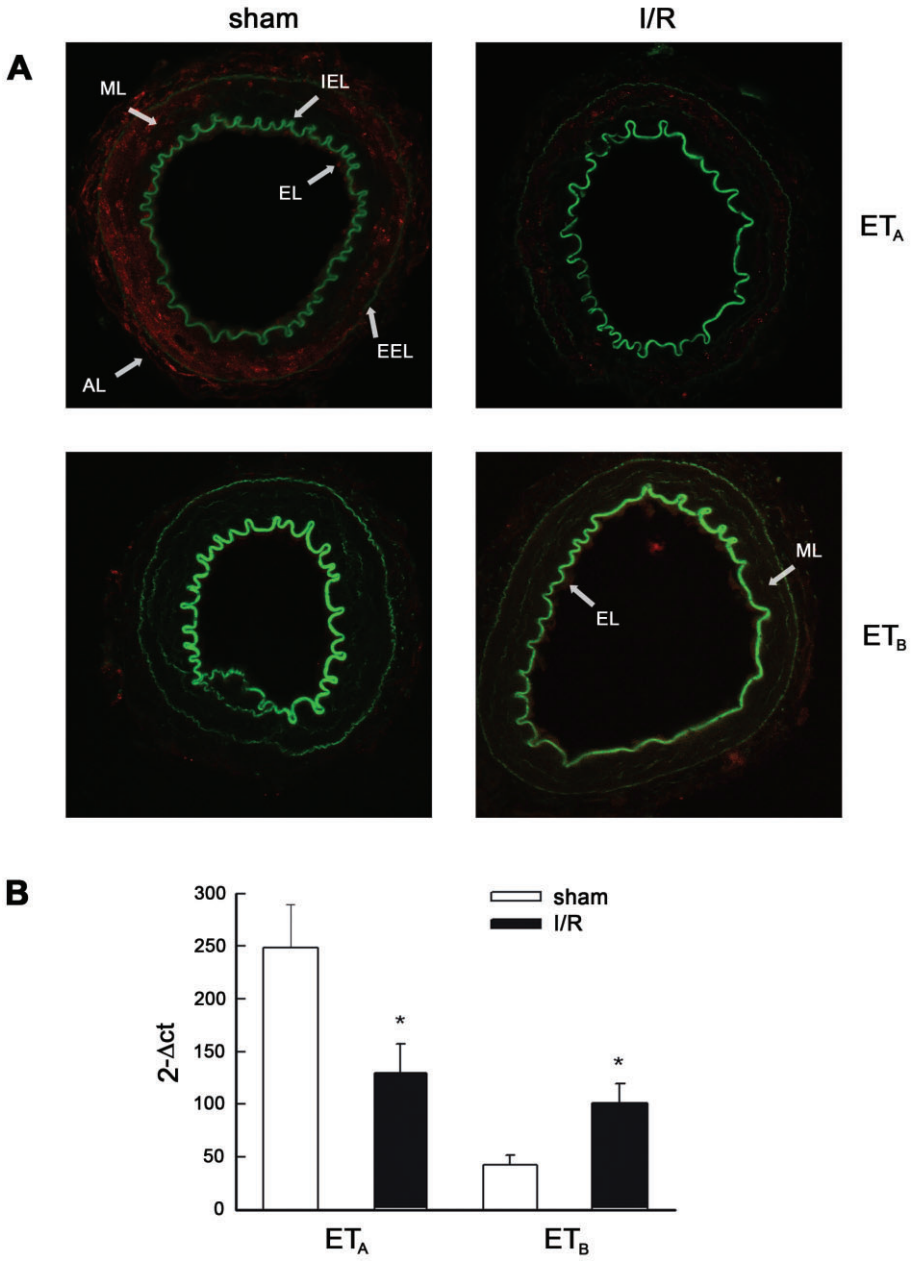

Figure 6

Representative images of ET_A and ET_B receptor fluorescence (A) and ET_A and ET_B receptor mRNA levels of mesenteric resistance arteries from sham and I/R rats (B). Graph shows mRNA level expressed as $2^{-\Delta ct}$ using GADPH as a housekeeping gene. Arrowheads show positive staining. AL, adventitial layer; EL, endothelial layer; IEL, internal elastic lamina; ML, media layer. Data are shown as mean ± SEM from 5 animals. *$P < 0.05$ sham versus I/R by Student's unpaired *t*-test.

only in the endothelial layer of mesenteric resistance arteries, detected lower levels of fluorescence due to eNOS expression 24 h after I/R (Figure 10A).

Weak ethidium bromide fluorescence was observed in the media and endothelial layer of mesenteric resistance arteries from sham rats (Figure 11A). However, after I/R, there was an increase in fluorescence in all three layers of the vessel wall (Figure 11A) suggesting increased $O_2^{\bullet-}$ production by I/R. Analysis of mRNA levels of NAD(P)H-oxidase subunits (major source of vascular $O_2^{\bullet-}$) shows that NOX-1 and p^{47phox} were expressed in mesenteric resistance arteries (Figure 11B) and markedly enhanced after I/R.

Discussion

Restoring blood flow to ischaemic tissue, a major therapeutic goal in the treatment of arterial occlusion, often leads to both

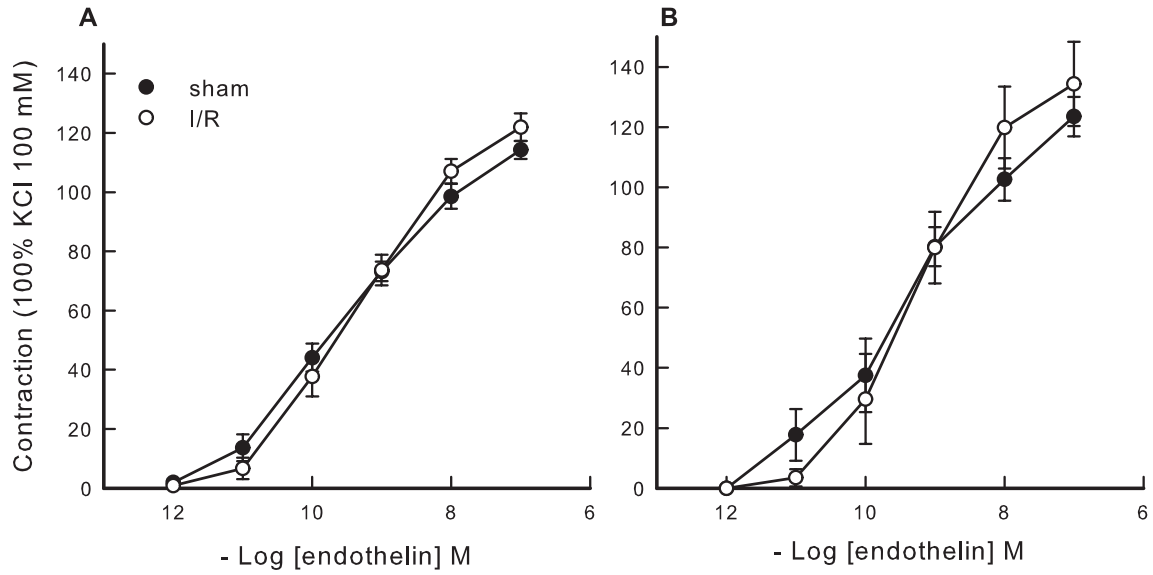

Figure 7

Effect of endothelium removal (A) or L-NAME (B) on concentration-response curves to ET-1 obtained in rings of mesenteric resistance arteries from sham and I/R rats. Data are shown as mean ± SEM from 9 to 20 animals. *$P < 0.05$ control versus INDO or L-NAME and ***$P < 0.001$ control versus endothelium removal by two-way ANOVA.

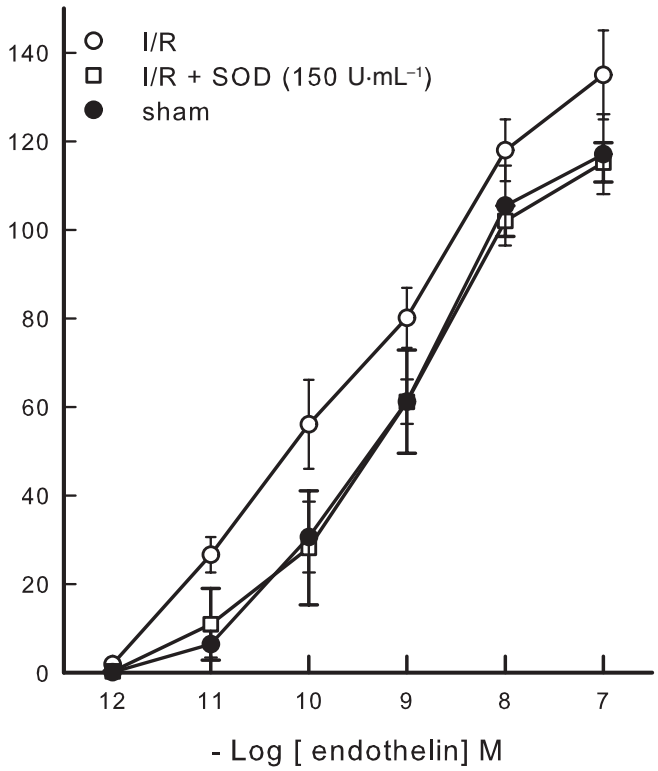

Figure 8

Effect of SOD on concentration-response curves to ET-1 obtained in rings of mesenteric resistance arteries, endothelium intact, from sham and I/R rats. Data are shown as mean ± SEM from 4 to 5 animals. *$P < 0.05$ sham versus I/R by two-way ANOVA.

local and ischaemic organ damage, called I/R injury (Zimmerman and Granger, 1992). There is evidence that ET-1 levels increase in the first few hours of reperfusion (Ługowska-Umer *et al.*, 2008) and seem to play an important role in the disturbances following I/R (Brunner *et al.*, 2006). Increased ET-1 plasma concentration has been reported after I/R in humans (Ziv *et al.*, 1992; Brondani *et al.*, 2007) and in rat models of cerebral (Barone *et al.*, 1993), cardiac (Brunner *et al.*, 2006) and mesenteric (Büyükgebiz *et al.*, 1995; Oktar *et al.*, 2002; Wang *et al.*, 2006, Guzmán-de la Garza *et al.*, 2009) ischaemia. After SMA occlusion, we found that ET-1 plasma concentration was elevated 3 h after reperfusion and returned to basal levels 24 h after reperfusion, as previously described in Wistar rats (Ługowska-Umer *et al.*, 2008).

Several studies have reported enhanced vascular reactivity to ET-1 after cerebral (Stenman *et al.*, 2002), hepatic (Kawamura *et al.*, 1995) and mesenteric ischaemia (Wood *et al.*, 1995). Corroborating these studies, we observed that vasoconstrictor responses to ET-1 were potentiated by I/R, whereas those to phenylephrine and KCl were unaffected, indicating that the effects of I/R were selective to this peptide and could be attributed to changes in ET receptor population and/or function.

As previously described, ET_A receptors located in the muscular layer mediate vasoconstriction, whereas vasodilatation is mainly attributed to endothelial ET_B receptors that release NO and/or COX metabolites (Masaki, 2004). Using immunofluorescence, we showed that ET_A receptors are located on the media layer of mesenteric resistance arteries and their contractile role was confirmed by the finding that BQ123 shifted the ET-1 concentration-response curve to the right. However, a change in their expression/function does not seem to play a major functional role in ET-1 response after I/R, since inhibition of the ET_A receptor by BQ123 was similar in rings from I/R

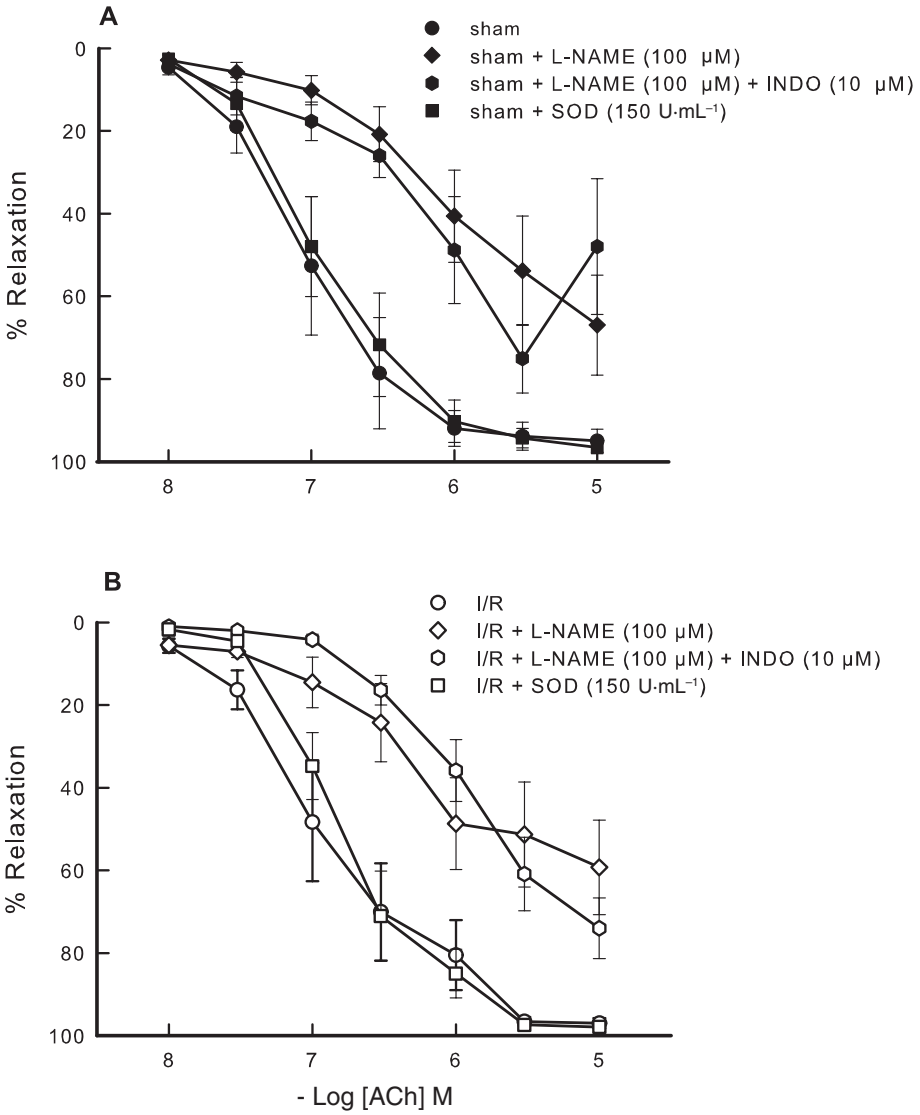

Figure 9

Concentration-response curves to Ach in the absence and in the presence of SOD, L-NAME or L-NAME plus indomethacin in endothelium intact mesenteric resistance arteries rings from sham and I/R rats. Data are shown as mean ± SEM from 5 to 9 animals.

and sham rats, and mRNA levels and protein expression of ET_A receptors in the media layer were decreased after I/R.

On the other hand, down-regulation in the endothelial ET_B receptor expression/function could also participate in the potentiation of ET-1 response induced by I/R. Surprisingly, a significant increase in ET_B receptor mRNA and protein expression was observed after I/R. Nevertheless, in mesenteric resistance arteries from I/R rats, the ET_B receptors were located not only in the endothelium as observed in sham rats but also in the media layer.

The use of a selective ET_B agonist (SC_6) and antagonist (BQ788) in functional studies clearly confirmed the participation of muscular ET_B receptors in the modulation of ET-1-mediated vasoconstriction after I/R. As expected, after blockade of ET_B receptors, the contractile response to ET-1 increased in endothelium intact rings from sham animals, confirming the participation of vasodilator ET_B receptors. In contrast, responses to ET-1 from I/R rats were unaffected by BQ788, but removal of endothelium revealed the presence of contractile ET_B receptors in smooth muscle cells because the ET-1 concentration-response curve was shifted to the right by BQ788. In addition, SC_6-induced vasoconstrictor responses after I/R were abolished by BQ788, corroborating the presence of muscular ET_B receptors that mediate contraction in rings from I/R but not from sham rats. These results agree with those observed in the MCA in a rat model of focal cerebral ischaemia (Stenman *et al.*, 2002) and are corroborated by the visualization of the ET_B receptor protein in the muscular layer and the increased ET_B receptors mRNA level after I/R.

To further analyse the contribution of the ET_B receptor to the ET-1 response after I/R, we studied the consequences of ET_B receptor desensitization by means of a high concentration of SC_6 (Cramer *et al.*, 1998). When the ET-1 vasoconstric-

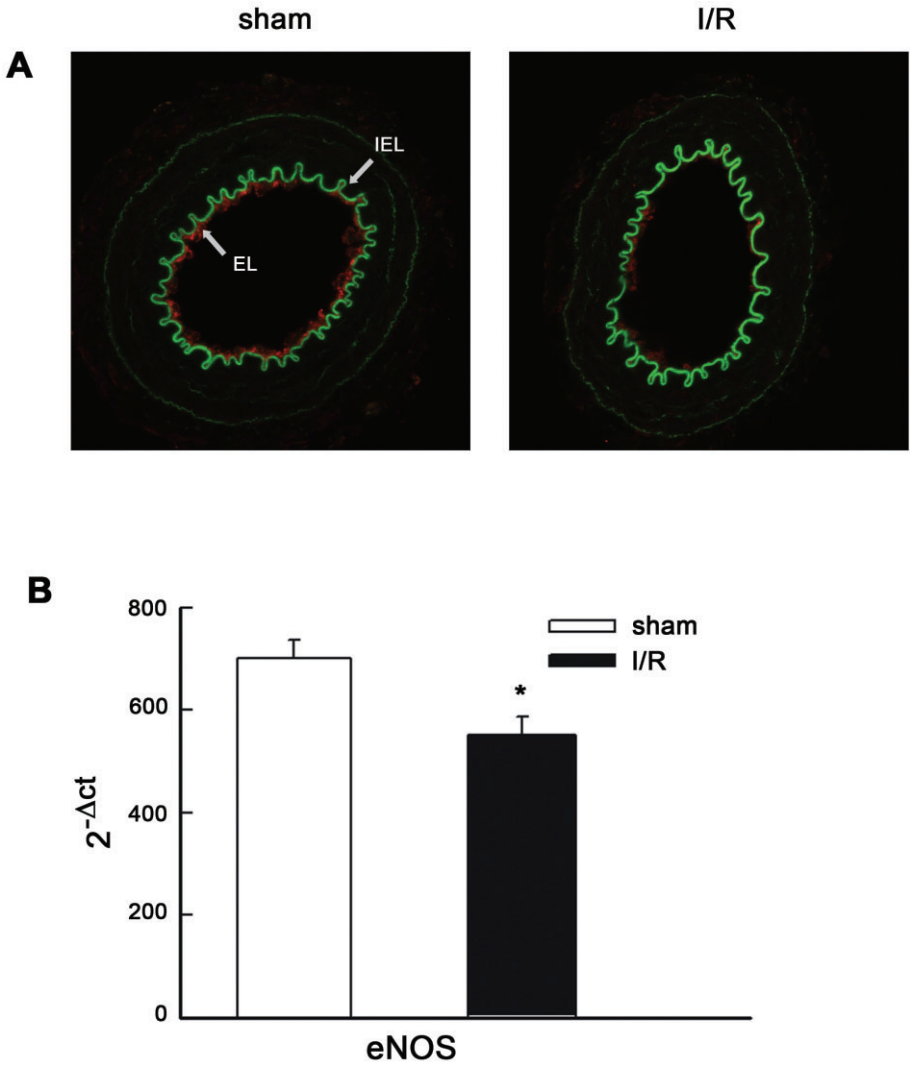

Figure 10

Representative images of eNOS fluorescence (A) and eNOS mRNA levels of mesenteric resistance arteries from sham and I/R rats (B). Graph shows mRNA level expressed as $2^{-\Delta ct}$ using GADPH as a housekeeping gene. Arrowheads show positive staining. EL, endothelial layer; IEL, internal elastic lamina. Data are shown as mean ± SEM from 5 animals.*$P < 0.05$ sham versus I/R by Student's unpaired *t*-test.

tor response was mediated only by ET_A receptors, no differences were seen between I/R and sham rats, once more indicating the essential role of ET_B receptors in the change in ET-1 responses after I/R.

It has also been demonstrated that organ injury during intestinal ischaemia is exacerbated by vasoconstriction of the mesenteric vessels caused by impaired production of NO by the damaged endothelial cells (Yasuhara, 2005). Thus, the next question to clarify was the role of endothelial NO in the potentiation of ET-1-induced vasoconstriction after I/R. Removal of the endothelium and NOS inhibition by L-NAME, but not COX inhibition by indomethacin, potentiated the responses to ET-1 in mesenteric resistance arteries from sham and I/R animals, confirming a modulator role of endothelial NO that is independent of I/R. Nevertheless, the differences seen between I/R and sham rats in ET-1 responses disappear after endothelium removal or L-NAME treatment, suggesting a decrease in endothelial NO bioavailability after I/R.

Several studies have reported that reperfusion increases $O_2^{\bullet-}$, which reacts with NO to produce peroxynitrite, diminishing NO bioavailability. In our study, an increase in $O_2^{\bullet-}$ paralleled by up-regulation of NOX-1 and p^{47phox} mRNA was observed. These data confirm the increase in oxidative stress after I/R and establish NAD(P)H-oxidase as the major source of $O_2^{\bullet-}$ in this pathophysiological condition. The fact that the potentiation of ET-1-mediated vasoconstriction after I/R was reversed by SOD treatment validates the functional role of $O_2^{\bullet-}$ in this change.

All these results, together with the observed decrease in mRNA and protein expression of eNOS after I/R, lead us to speculate that a decrease in the production and bioavailability of NO participates in the observed potentiation of ET-1 response.

On the other hand, endothelium-dependent vasodilatation has been demonstrated to be more affected by I/R injuries than vasoconstriction and endothelium-independent

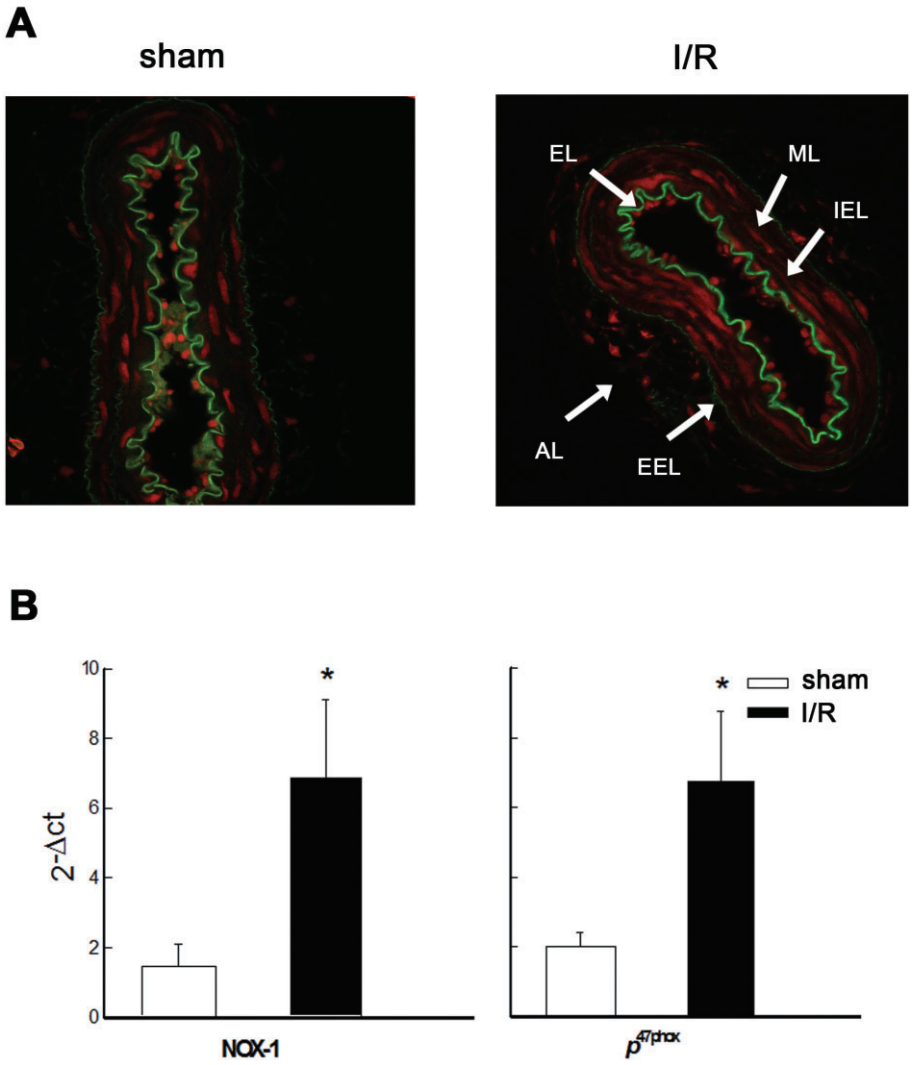

Figure 11

Representative images of $O_2^{\bullet-}$ production (A) and NOX-1 and p^{47phox} mRNA levels (B) of mesenteric resistance arteries from sham and I/R rats (B). Graph shows mRNA level expressed as $2^{-\Delta ct}$ using 18S as a housekeeping gene. Arrowheads show positive staining. EL, endothelial layer; EEL, external elastic laminae; IEL, internal elastic laminae; AL, adventitial layer; ML, media layer. Data are shown as mean ± SEM from 5 animals. *$P < 0.05$ sham versus I/R by Student's unpaired *t*-test.

vasodilatation (Kedzierski and Yanagisawa, 2001; Gourdin *et al.*, 2009), although discrepancies have been reported (Koksoy *et al.*, 2000; Seal and Gewert, 2005; Gourdin *et al.*, 2009; Soydan *et al.*, 2009). However, in our study, vasodilatation to ACh was similar in sham and I/R rats.

Our data do not provide a clear explanation for the lack of endothelial vasodilator dysfunction, despite increased $O_2^{\bullet-}$ and decreased eNOS expression, but some compensatory mechanism by other endothelium-derived factors can be proposed. Several studies have reported that in rat mesenteric resistance arteries, not only NO but also the endothelium-derived hyperpolarizing factor (EDHF) plays an important role in the vasodilatation to ACh (Hwa *et al.*, 1994; Shimokawa *et al.*, 1996; Sekiguchi *et al.* 2002; Burger *et al.*, 2009). The response to ACh was unaffected by SOD and only partially inhibited by L-NAME or L-NAME plus indomethacin, supporting a role for EDHF in mesenteric resistance arteries. However, the lack of difference between I/R and sham rats in the vasodilator response to ACh in the presence of L-NAME plus indomethacin excludes the possibility that augmented EDHF activity compensated for decreased NO bioavailability. Thus, our results indicate that the changes due to I/R are specific to ET-1 and do not affect ACh-induced vasodilatation.

For vascular tissue, it is well known that ET-1 is the main vasoconstrictor peptide released by endothelial cells (Schneider *et al.*, 2007). The observed increase in plasma ET-1concentration after I/R validates the integrity of the endothelium and focuses attention on changes in ET_B, eNOS expression and increased $O_2^{\bullet-}$ formation in mesenteric resistance arteries from I/R rats as responsible for the observed potentiation of ET-1 vasoconstriction.

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Conflict of interest

The authors state no conflict of interest.

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