Role of tachykinin NK receptors on the local and remote injuries following ischaemia and reperfusion of the superior mesenteric artery in the rat

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1 Neuropeptides acting on tachykinin NK receptors play an important role in the amplification of inflammatory responses. We have assessed the effects of tachykinin NK receptor blockade on the injuries following intestinal ischaemia and reperfusion (I/R) in rats.

2 The tachykinin NK_1 receptor antagonist SR140333 dose-dependently (0.05 to 0.5 mg kg^{-1}) suppressed the local (intestine) and remote (lung) increases in vascular permeability and neutrophil recruitment following mild I/R injury. A structurally-distinct NK_1 receptor antagonist, CP99,994, but not tachykinin NK_2 or NK_3 receptor antagonists also suppressed mild I/R injury.

3 Neonatal pretreatment with capsaicin effectively depleted sensory neurons and abrogated the injuries following mild I/R.

4 Treatment with SR140333 (0.5 mg kg^{-1}) significantly reversed severe reperfusion-induced local and remote increases in vascular permeability, neutrophil recruitment, intestinal haemorrhage and blood neutropaenia, but did not prevent the lethality associated with severe I/R.

5 Post-ischaemic treatment with SR140333 significantly inhibited the elevations of TNF-α in the intestine and lung, but not serum, following severe I/R. The increase in the concentrations of IL-10 in the lung and serum were also suppressed.

6 Post-ischaemic blockade of tachykinin NK_1 receptors markedly inhibited the local and remote injuries, but not lethality, following reperfusion of the SMA in rats. Neuropeptides, possibly substance P, released from sensory nerves appear to account for the activation of these tachykinin NK_1 receptors. Antagonists of the tachykinin NK_1 receptor may be useful adjuncts in the treatment of the injuries which occur following reperfusion of an ischaemic vascular territory.
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Keywords: Capsaicin; IL-10; ischaemia and reperfusion injury; NK receptors; substance P; tachykinins; TNF-α

Abbreviations: I/R, ischaemia and reperfusion; MPO, myeloperoxidase; OD, optical density; SMA, superior mesenteric artery

Introduction

The reduction in blood flow, ischaemia, to an organ or a vascular bed can lead to significant tissue injury and cell death if it is prolonged. Thus, the restoration of blood flow, i.e. the 'reperfusion' of the ischaemic vascular bed, is necessary to save ischaemic tissue following an acute vascular event (Lefer & Lefer, 1996; Willerson, 1997). However, reperfusion may lead to recruitment and activation of leukocytes, release of mediators of the inflammatory process and injury to the affected vascular bed, limiting the beneficial effects of blood flow restoration (Lefer & Lefer, 1996; Willerson, 1997). Neutrophils and TNF-α are thought to be key players in the pathophysiology of ischaemia and reperfusion (I/R) injury (e.g. Kubes *et al*., 1995; Gilmont *et al*., 1996; Cornejo *et al*., 1997; Souza *et al*., 2000b). Thus,

strategies which limit neutrophil accumulation and pro-inflammatory cytokine production induced by the reperfusion process may be useful adjuncts in the treatment of ischaemic disorders in various organs.

Several studies have now demonstrated that the release and action of neuropeptides, especially substance P, may play an important role in the amplification of acute and chronic inflammatory responses (e.g. Bhatia *et al*., 1998; Bozic *et al*., 1996; reviewed by Quartara & Maggi, 1998). The main source of these neuropeptides in the periphery appears to be sensory nerve endings, but there is much evidence demonstrating the ability of inflammatory cells themselves to release neuropeptides (Maggi, 1997). The neuropeptides released appear to function mainly *via* the activation of tachykinin NK_1 receptors, but there is also evidence suggesting a role for both tachykinin NK_2 and NK_3 receptor activation (Cutrufo *et al*., 2000; Nenan *et al*., 2001). The role of tachykinin NK receptor activation during I/R injury is largely unknown, but acute ishemic events are accompanied by sensory nerve activation,

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significant sensation of pain and substance P release (Maggi, 1997).

We have recently described the local and remote reperfusion injuries which occur following ischaemia of the superior mesenteric artery (SMA) in the rat (Souza *et al*., 2000a, b). Following I/R of the SMA, we observed significant local (intestine and mesentery) and remote (lung) oedema and neutrophil accumulation, and marked systemic alterations, which included hypotension, neutropaenia and death, specially after more prolonged I/R periods (Souza *et al*., 2000a, b). Moreover, we detected significant pro-inflammatory cytokine production in tissues and serum after reperfusion of the ischaemic SMA (Souza *et al*., 2000b). Here, we have investigated the potential role of sensory nerve-derived tachykinins on the local and remote reperfusion injuries following ischaemia of the SMA. Initial experiments were designed to examine the dose-dependent effects of antagonists at the tachykinin NK receptors (NK$_1$, NK$_2$ and NK$_3$) on the injuries following mild I/R of the SMA. After demonstration that mild I/R was sensitive to capsaicin depletion and tachykinin NK$_1$ receptor blockade, we investigated the effects of treatment with the NK$_1$ receptor antagonist SR140333 on the local and systemic injuries following severe I/R injury.

Methods

Animals

Male wistar rats (200 – 200 g) obtained from the Bioscience unit of our Institution were housed in standard conditions and had free access to commercial chow and water. All the procedures described had prior approval from the local animal ethics committee.

Ischaemia and reperfusion injury

Rats were anaesthetized with urethane (140 mg kg^{-1}, i.p.) and laparotomy was performed. The superior mesenteric artery (SMA) was isolated and ischaemia was induced by totally occluding the SMA for 30 or 120 min. After ischaemia, reperfusion was initiated by removal of the occlusion. Animals made ischaemic for 30 or 120 min were allowed to reperfuse for 30 (mild I/R) or 120 (severe I/R) min, respectively. The durations of I/R were based upon previous experiments (Souza *et al*., 2000a, b) and were optimal for mild and severe reperfusion injuries. Sham-operated animals or animals only made ischaemic were used as controls for the reperfusion-induced injury.

Initial experiments were carried out in the mild reperfusion injury model to examine the dose-dependent effect of tachykinin NK$_1$ (SR140333, 0.05 to 0.5 mg kg^{-1} and CP99,994, 4.0 mg kg^{-1}), NK$_2$ (SR48968, 0.6 to 6.0 mg kg^{-1}) and NK$_3$ (SR142801, 1.0 to 9.0 mg kg^{-1}) receptor antagonists. In these experiments, drugs were administered i.v. 5 min prior to the reperfusion of the SMA. We then tested the effects of the administration of SR140333 (0.5 mg kg^{-1}, i.v., 5 min prior to reperfusion) in the more severe I/R model. None of the drugs used in the present study had any significant effects on basal parameters (data not shown) and to simplify the graphs presented, basal data obtained in vehicle or drug treated animals have been pooled for presentation.

Neonatal capsaicin treatment

The protocol for capsaicin depletion was similar to that of Jancsó *et al*. (1977). Briefly, rats were pretreated on the second day of life with capsaicin, at which time they weighed approximately 200 g. As a positive control for the depletion of capsaicin-sensitive sensory nerves, rats received an intraplantar injection of formalin (3% in PBS; 100 μl/paw) and the composite licking behaviour during the first 10 min (early phase) and from 20 to 30 min (late phase) assessed (Shibata *et al*., 1989). Formalin-induced licking behaviour has been previously demonstrated to be dependent on the release of NK$_1$-acting neuropeptides from sensory nerves (Sakurada *et al*., 1993; Rupniak *et al*., 1996; Damas & Liegeois, 1999). To confirm the latter point, animals were treated with the tachykinin NK$_1$ receptor antagonist (0.5 mg kg^{-1}, i.p.) 30 min prior to the injection of formalin.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability (Saria & Lundberg, 1983; De Matos *et al*., 1999). Evans blue (20 mg kg^{-1}) was administered i.v. (1 ml kg^{-1}) *via* a femoral vein 2 min prior to reperfusion of the ischaemic artery. Thirty min (in the mild model) or 120 min (in the severe model) after reperfusion, segments of the duodenum (10 cm) were cut open and allowed to dry in a petri dish for 24 h at 37°C. The dry weight of the tissue was calculated and Evans blue extracted using 3 ml of formamide (24 h at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an ELISA plate reader. Results are presented as the amount of Evans blue per μg per 100 mg of tissue. The mesentery was also extracted *en bloc*, halved and a similar extraction procedure was performed. The right ventricle was flushed with 20 ml of phosphate buffered saline to wash the intravascular Evans blue in the lungs. The left lung was then excised and used for Evans blue extraction. The right lung was used for the determination of myeloperoxidase as described below.

Myeloperoxidase levels

The extent of neutrophil accumulation in the intestine, mesentery and right lung tissue was measured by assaying myeloperoxidase activity as previously described (De Matos *et al*., 1999). Briefly, a portion of duodenum, half the mesentery and the flushed right lung of animals that had undergone I/R injury were removed and snap frozen in liquid nitrogen. Upon thawing, the tissue (1 g of tissue per 19 ml of buffer) was homogenized in pH 4.7 buffer (NaCl 0.1 M NaPO$_4$ 0.02 M NaEDTA 0.015 M), centrifuged at $260 \times g$ for 10 min and the pellet subjected to hypotonic lysis (15 ml of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After a further centrifugation, the pellet was resuspended in 0.05 M NaPO$_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and re-homogenized. One ml aliquots of the suspension were transferred into 1.5 ml-Eppendorf tubes followed by three freeze – thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 min at $10,000 \times g$, the

pellet was resuspended to 1 ml and samples of intestine, mesentery and lung were diluted prior to assay. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H$_2$O$_2$ (0.5 mM). Results were expressed as total number of neutrophils by comparing the OD of tissue supernatant with the OD of rat peritoneal neutrophils processed in the same way. To this end, neutrophils were induced in the peritoneum of rats by injecting 3 ml of casein 5%. A standard curve of neutrophil (>95% purity) numbers versus OD was obtained by processing purified neutrophils as above and assaying for MPO activity.

Determination of the concentration of circulating leukocytes

The total number of circulating leukocytes and neutrophils were evaluated in blood samples obtained *via* a cannula in the femoral artery. Samples were collected prior to ischaemia (time 0), 120 min after ischaemia and 30 and 120 min after reperfusion. The number of total circulating leukocytes was determined by counting leukocytes in a modified Neubauer chamber after staining with Turk's solution, and differential counts by evaluating the percentage of each leukocyte on blood films stained with May – Grunwald – Giemsa.

Measurement of haemoglobin concentrations

The determination of the concentrations of haemoglobin in tissue was used as an index of tissue haemorrhage. After washing and perfusing the intestines to remove excess blood in the intravascular space, a sample of approximately 100 mg of duodenum was removed and homogenized in Drabkin's colour reagent according to instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). This reagent measures the total haemoglobin content (in intact red blood cells or not) of a tissue. The suspension was centrifuged for 15 min at $3000 \times g$ and filtered using 0.2 μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of haemoglobin.

Measurement of cytokine concentrations in serum, intestine and lungs

TNF-α, IL-1β, IL-6 and IL-10 concentrations were measured in serum and intestine of animals using ELISA techniques previously described (Rees *et al.*, 1999a, b; Hagan *et al.*, 1993; Francischi *et al.*, 2000; Ball *et al.*, 2001). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at -20°C until further analysis. Serum samples were analysed at a 1:1 dilution in PBS. One hundred mg of duodenum or lung of sham-operated and reperfused animals were homogenized in 1 ml of PBS (NaCl 0.4 m and NaPO$_4$ 10 mM) containing anti-proteases (mM: PMSF 0.1; benzethonium chloride 0.1, EDTA 10 and aprotinin A 20 KI) and 0.05% Tween 20. The samples were then centrifuged for 10 min at $3000 \times g$ and the supernatant immediately used for ELISA assays at a 1:5 dilution in PBS. ELISA plates (Nunc MaxiSorb) were coated with sheep anti-rat TNF-α/IL-1β/IL-6 or IL-10 polyclonal antibodies (1–2 μg ml^{-1}) overnight. The plates were washed thrice and then blocked with 1% bovine serum albumin. After a further wash, plates were incubated with samples or

recombinant rat cytokine and incubated overnight. The biotinylated polyclonal antibodies were used at a 1:1000 to 1:2000 dilution and the assays had a sensitivity of 16 pg ml^{-1}.

Drugs and reagents

The following drugs were obtained from Sigma (U.S.A.): urethane, Evans blue, capsaicin, hexadecyltrimethylammonium bromide. SR140333, SR48968 and SR142801 were a kind gift of Sanofi Recherche, Lilly, France. CP99,994 was a gift of Pfizer Ltd., Sandwich, Kent.

Statistical analysis

Results are shown as the mean $\pm$ s.e.mean. Per cent inhibition was calculated by subtracting the background levels of Evans blue extravasation or myeloperoxidase (obtained in sham-operated animals) from control and treated animals. Differences were compared by using analysis of variance (ANOVA) followed by Student – Newman – Keuls *post-hoc* analysis. Results with a $P < 0.05$ were considered significant.

Results
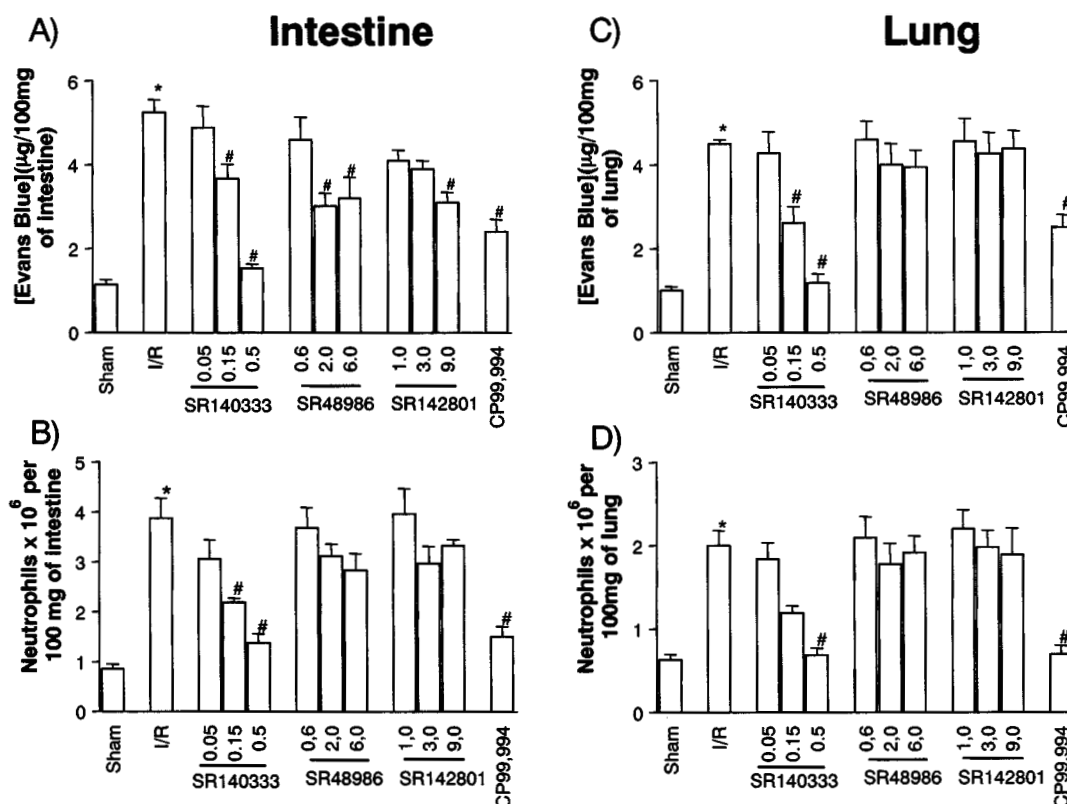
Dose-dependent effects of tachykinin NK receptor antagonists in a mild model of I/R injury

Initial experiments evaluating the dose-dependent effects of tachykinin NK receptor antagonists were conducted in a mild model of I/R injury. Post-ischaemic treatment of animals with the tachykinin NK$_1$ receptor antagonist SR140333 dose-dependently inhibited both the increase in vascular permeability and the recruitment of neutrophils in the intestine and lung following reperfusion of the ischaemic SMA (Figure 1). Maximal inhibition occurred at the dose of 0.5 m kg^{-1}. Post-ischaemic treatment with the tachykinin NK$_2$ receptor antagonist SR48968 had no effect on the recruitment of neutrophils into the lung or intestine following reperfusion injury (Figure 1). Moreover, SR48968 only marginally inhibited the increase in vascular permeability in the intestine, but not lung, of reperfused animals (Figure 1). In a similar manner, the tachykinin NK$_3$ receptor antagonist SR142801 had no effect on the increase in vascular permeability or neutrophil accumulation in the lung and intestine following mild I/R injury (Figure 1).

In order to confirm an important role for the tachykinin NK$_1$ receptor, animals were treated with CP99,994, a non-peptide tachykinin NK$_1$ receptor antagonist structurally distinct from SR140333. As seen with SR140333, post-ischaemic treatment with CP99,994 effectively inhibited both the increase in vascular permeability and the recruitment of neutrophils following mild I/R injury (Figure 1).

Effect of sensory nerves depletion by neonatal capsaicin treatment in a mild model of I/R injury

Sensory nerves are a major source of neuropeptides during inflammatory conditions (Holzer, 1991). In order to investigate the potential contribution of sensory nerves for the release of tachykinin NK$_1$-acting neuropeptides and consequent development of mild I/R injury, animals were treated neonatally with

Figure 1 Dose-dependent effects of the treatment with tachykinin NK receptor antagonists on the increase in vascular permeability and recruitment of neutrophils in the intestine (A,B) and lung (C,D) following mild ischaemia (30 min) and reperfusion (30 min) injury of the superior mesenteric artery. Changes in vascular permeability (A,C) were assessed by evaluating the extravasation of Evans blue dye and neutrophil recruitment (B,D) was assessed by evaluating the tissue levels of myeloperoxidase. Tachykinin NK$_1$ (SR140333, 0.05 – 0.5 mg kg^{-1} and CP99,994, 4.0 mg kg^{-1}), NK$_2$ (SR48986, 0.6 – 6.0 mg kg^{-1}) or NK$_3$ (SR142801, 1.0 – 9.0 mg kg^{-1}) receptor antagonists were given i.v. 5 min prior to reperfusion. Control animals (I/R) received phosphate buffered saline. Results are shown as μg Evans blue or as number of neutrophils per 100 mg of tissue and are the mean $\pm$ s.e.mean of 5 – 6 animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.05$ when compared to mild I/R animals.

capsaicin (Holzer, 1991). As a positive control for the effectiveness of neonatal capsaicin at depleting sensory nerves, we evaluated a behavioural effect, the licking response, after the intraplantar injection of formalin. Formalin induced a biphasic behavioural response that was sensitive to inhibition by SR140333. At the dose used (0.5 mg kg^{-1}), the inhibitory effects of SR140333 were of greater magnitude during the early phase (first 10 min) after formalin injection (82% inhibition) but persisted during the late phase (20 – 30 min) (56% inhibition, Table 1). Similarly to the effects of the tachykinin NK$_1$ receptor antagonist, neonatal treatment with capsaicin inhibited by 96 and 54%, the early and late phase, respectively (Table 1).

In capsaicin-treated animals, there was little increase in vascular permeability following mild I/R injury (Figure 2). Likewise, the recruitment of neutrophils following mild I/R was markedly inhibited in comparison to animals that were treated neonatally with vehicle (Figure 2).

Effects of a tachykinin NK$_1$ receptor antagonist on the local, remote and systemic injuries in a model of severe I/R injury

The next series of experiments was carried out in a model of severe I/R injury where in addition to the changes in vascular

permeability and neutrophil accumulation we could observe tissue haemorrhage, leukopenia, changes in cytokine levels in tissue and blood and significant lethality (Souza *et al.*, 2000b). For these experiments we chose to use the tachykinin NK$_1$ receptor antagonist SR140333 at a dose shown to be the maximally inhibitory in the mild I/R model (0.5 mg kg^{-1}). Post-ischaemic treatment with SR140333 virtually abrogated the increase in vascular permeability and neutrophil recruitment in the intestine and in the lung following severe I/R injury (Figure 3). Treatment with SR140333 also abrogated the intestinal increase of haemoglobin, a marker of tissue haemorrhage (Figure 4).

We have previously shown an increase in the concentration of blood neutrophils during the ischaemic period, and a rapid drop in neutrophil levels once reperfusion occurs (Souza *et al.*, 2000b). As SR140333 was administered just prior to reperfusion, the concentration of circulating neutrophils at 120 min of ischaemia were similar and markedly greater than sham-operated animals (Figure 5). In vehicle-treated animals, reperfusion of the ischaemic SMA induced a rapid fall of circulating neutrophils to levels observed in sham-operated animals (Figure 5). Pretreatment with SR140333 partially reversed the rapid neutropenia with approximately 30% inhibition at 120 min (Figure 5).

Table 1 Effects of the treatment with neonatal capsaicin or the tachykinin NK_1 receptor anatagonist SR140333 on the behavioural response to intraplantar formalin injection in rats

| Pretreatment	Composite licking time (s)
First phase	
Vehicle	290 ± 7
SR140333	54 ± 7*
Vehicle	229 ± 8.3
Capsaicin	8 ± 21*
Second phase	
Vehicle	535 ± 10
SR140333	235 ± 23*
Vehicle	529 ± 17
Capsaicin	241 ± 9*

SR140333 (0.5 mg kg^{-1}) was given i.p. 30 min prior to injection of formalin and control animals (vehicle) received phosphate buffered saline. Capsaican (50 mg kg^{-1}) or the corresponding volume (50 μl) of capsaican-vehicle were given on the second day of life and rats were used after 10 weeks. Rats then received an intraplantar injection of formalin (3% in PBS; 100 μl/paw) and the licking behaviour during the first 10 min (early phase) and from $20-30$ min (late phase) assessed. Results are the mean$\pm$s.e.mean number of composite licking time (s) for six animals in each group. *$P < 0.01$ when compared to vehicle-treated animals.

The levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and the anti-inflammatory cytokine IL-10 are markedly elevated in serum and tissues after severe I/R injury in rats (Figure 6; Souza *et al*., 2000b). Post-ischaemic treatment with SR140333 significantly inhibited the elevations of TNF-α in the intestine and lung of severe I/R animals but failed to alter the elevation of the concentration of this cytokine in serum (Figure 6). The elevations of the concentration of IL-6 or IL-1β in tissue and serum were not affected by SR140333 treatment, with the exception of a 40% inhibition of the increase of IL-6 levels in the intestine of animals submitted to severe I/R injury (data not shown). Interestingly, SR140333 abrogated the increase in the concentrations of IL-10 in the lung and serum after severe I/R injury (Figure 6).

Reperfusion after 120 min of ischaemia of the SMA artery leads to the death of approximately 60% of the animals (Souza *et al*., 2000b). In the present series of experiments, treatment with SR140333 had no significant effect on the lethality in the severe I/R model (Vehicle-treated, 58% lethality after 120 min, $n = 12$; SR140333, 39% lethality, $n = 8$; $P > 0.05$).

Discussion

The restoration of blood flow of an ischaemic vascular territory, i.e. reperfusion, is a major therapeutic objective
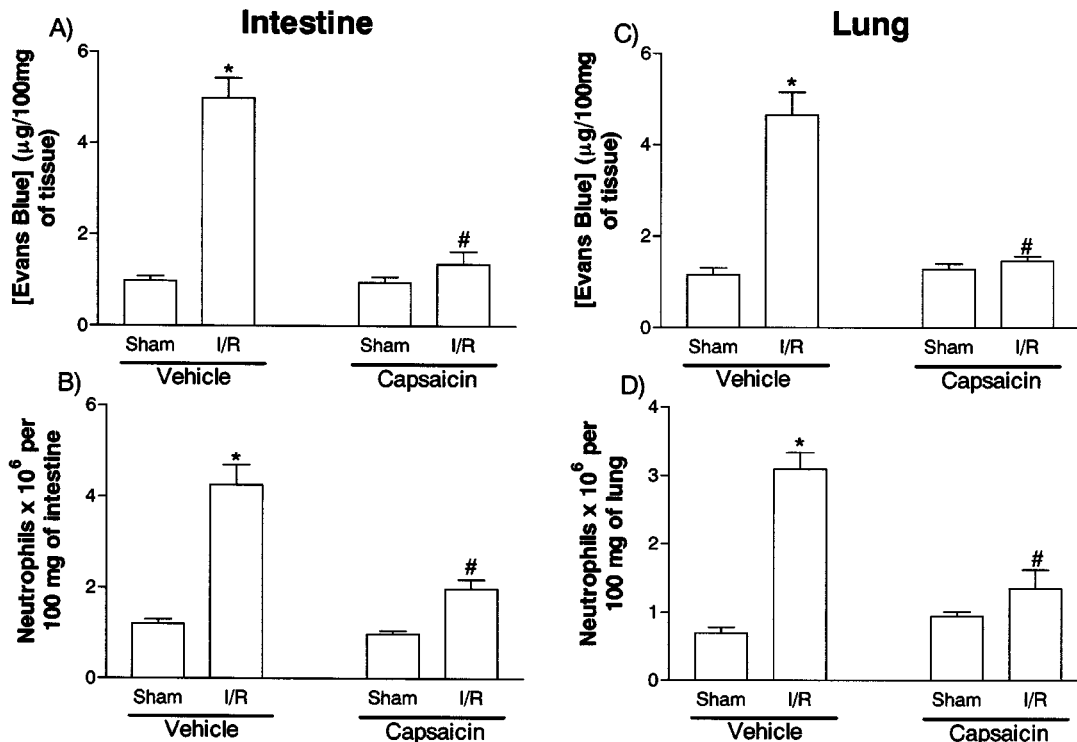
Figure 2 Effects of the neonatal treatment with capsaicin on the increase in vascular permeability and recruitment of neutrophils in the intestine (A,B) and lung (C,D) following mild ischemia (30 min) and reper (30 min) injury of the superior mesenteric artery. Changes in vascular permeability (A,C) were assessed by evaluating the extravasation of Evans blue dye and neutrophil recruitment (B,D) was assessed by evaluating the tissue levels of myeloperoxidase. Capsaicin (50 mg kg^{-1}) or the corresponding volume (50 μl) of capsaicin-vehicle were given on the second day of life and rats were used after 10 weeks. Results are shown as μg Evans blue or as number of neutrophils per 100 mg of tissue and are the mean$\pm$s.e.mean of six animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.05$ when compared to vehicle-treated animals.
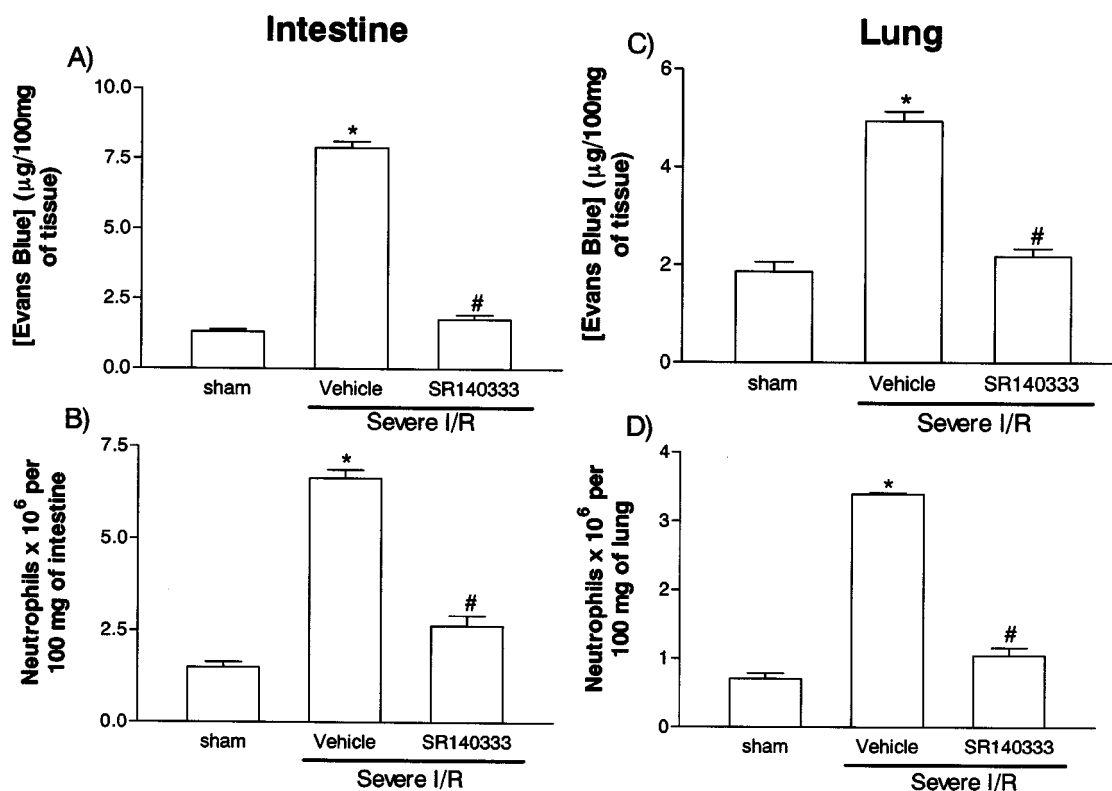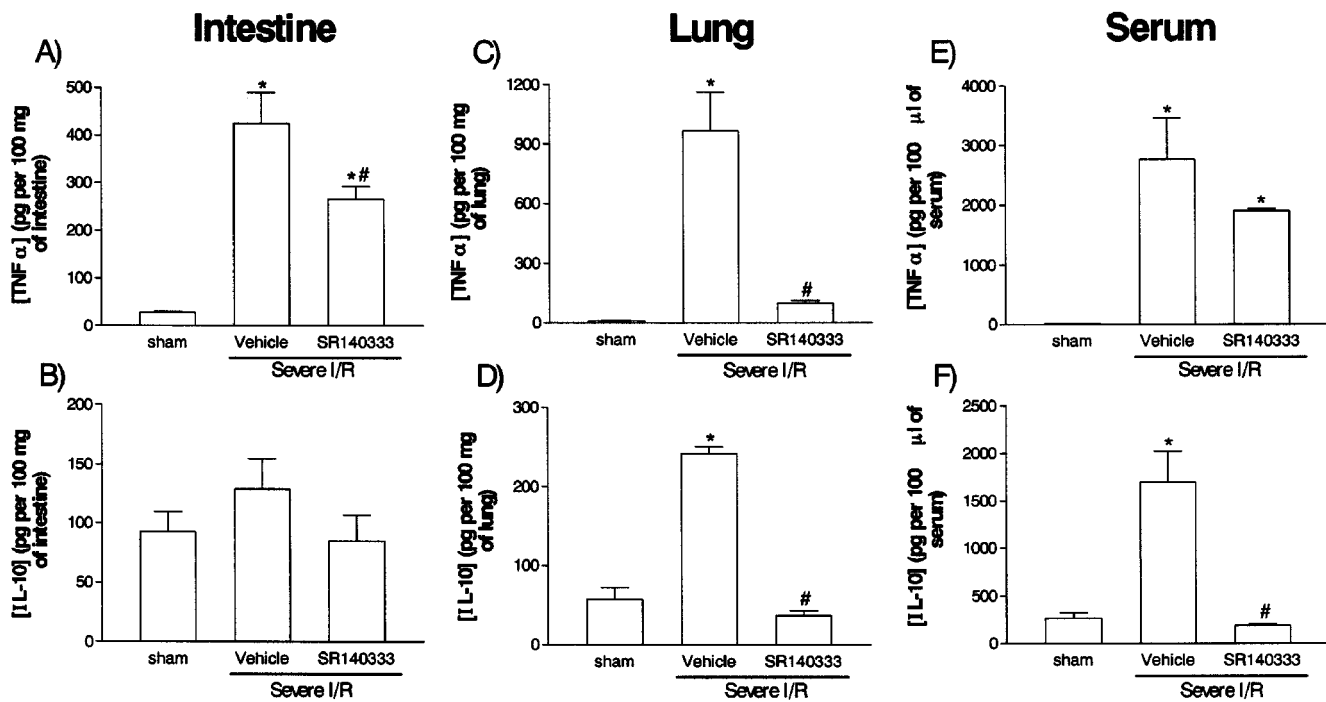
Intestine

Lung

Figure 3 Effects of the treatment with the tachykinin NK$_1$ receptor antagonist SR140333 on the increase in vascular permeability and recruitment of neutrophils in the intestine (A,B) and lung (C,D) following severe ischaemia (120 min) and reperfusion (120 min) injury of the superior mesenteric artery. Changes in vascular permeability (A,C) were assessed by evaluating the extravasation of Evans blue dye and neutrophil recruitment (B,D) was assessed by evaluating the tissue levels of myeloperoxidase. SR140333 (0.5 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animals (vehicle) received phosphate buffered saline. Results are shown as μg Evans blue or as number of neutrophils per 100 mg of tissue and are the mean$\pm$s.e.mean of 5–6 animals in each group. $*P<0.01$ when compared to sham-operated animals and $\#P<0.05$ when compared to mild I/R animals.
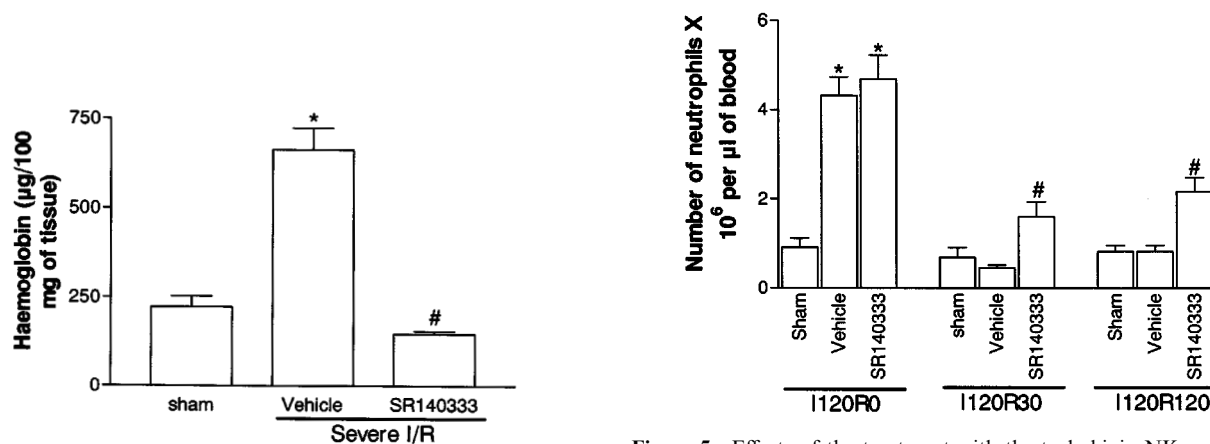
Figure 4 Effects of the treatment with the tachykinin NK$_1$ receptor antagonist SR140333 on the haemorrhage observed in the intestine following severe ischemia (120 min) and reperfusion (120 min) of the superior mesenteric artery. Tissue haemorrhage was assessed by evaluating the tissue levels of haemoglobin. SR140333 (0.5 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animals (vehicle) received phosphate buffered saline. Results are shown as μg haemoglobin per 100 mg of tissue and are the mean$\pm$s.e.mean of 5–6 animals in each group. $*P<0.01$ when compared to sham-operated animals and $\#P<0.01$ when compared to severe I/R animals.

Figure 5 Effects of the treatment with the tachykinin NK$_1$ receptor antagonist SR140333 on the concentration of circulating neutrophils following severe ischaemia (120 min) and reperfusion (120 min) of the superior mesenteric artery. Neutrophils were counted in May–Grunwald–Giemsa-stained blood smears just prior to (I120R0) and at 30 (I120R30) and 120 (I120R120) min after reperfusion. SR140333 (0.5 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animals (vehicle) received phosphate buffered saline. Results are shown as the number of neutrophils $\times 10^6$ per μl of blood and are the mean$\pm$s.e.mean of 5–6 animals in each group. $*P<0.01$ when compared to sham-operated animals and $\#P<0.01$ when compared to severe I/R animals.

Figure 6 Effects of the treatment with the tachykinin NK$_1$ receptor antagonist SR140333 on the concentrations of TNF-α and IL-10 in the intestine, lung and serum following severe ischaemia (120 min) and reperfusion (120 min) of the superior mesenteric artery. TNF-α (A,C,E) and IL-10 (B,D,F) were assessed in the intestine (A,B), lung (C,D) and serum (E,F) by using specific ELISA. SR140333 (0.5 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animals (vehicle) received phosphate buffered saline. Results are shown as pg TNF-α or IL-10 per ml of plasma or as pg TNF-α or IL-10 per 100 mg of tissue and are the mean±s.e.mean of 5–6 animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.05$ when compared to severe I/R animals.

following ischaemia of an organ or tissue. However, the reperfusion of an ischaemic territory may activate the cascade of inflammation locally and systemically, limiting the potential benefits of blood flow restoration. Thus, strategies which limit the injury caused by the reperfusion process may be useful adjuncts in the treatment of acute ischaemic disorders in various organs. Here, we have evaluated the involvement of neuropeptides and the potential use of tachykinin NK receptor antagonists during intestinal I/R injury. Intestinal ischaemia is a common clinical syndrome of medical importance with a high mortality rate (Endean et al., 2001). Surgery is the treatment of choice and reperfusion injury may follow these surgical approaches.

Initial dose-response experiments were carried out in a mild model of I/R injury (Souza et al., 2000a). Post-ischaemic treatment of animals with SR140333, a tachykinin NK$_1$ receptor antagonist, inhibited both the increase in vascular permeability and neutrophil recruitment following mild I/R injury. Similarly, pretreatment with a structurally-unrelated tachykinin NK$_1$ receptor antagonist, CP-99,994, markedly inhibited mild I/R injury. Of note, not only were local (intestine) injury inhibited, but there was also a striking inhibition of remote (lung) reperfusion injury. In contrast, pretreatment with antagonists of tachykinin NK$_2$ or NK$_3$ receptors had little or no effect on the increase in vascular permeability or neutrophil influx following mild I/R injury. In fact, only a marginal inhibition of vascular permeability in the intestine was observed following treatment with SR48986, a tachykinin NK$_2$ receptor antagonist. In addition

to inhibiting mild I/R injury, post-ischaemic treatment with SR140333 effectively suppressed the reperfusion-induced increase in vascular permeability, neutrophil influx, tissue haemorrhage and blood neutropaenia. Moreover, SR140333 inhibited local (intestine) and remote (lung) increase in the concentrations of the pro-inflammatory cytokine TNF-α observed after severe I/R injury. The concentration of TNF-α in the serum of reperfused animals was not significantly affected. Similarly to our findings with phosphodiesterase type 4 inhibitors (Souza et al., 2001), the lack of effect of the tachykinin NK$_1$ receptor antagonist on serum concentrations of TNF-α correlated with the lack of effect of the drug on reperfusion-induced lethality. Overall, the above results are first to demonstrate an important role of the activation of tachykinin NK$_1$ receptors in the development of the local, remote and systemic injuries following intestinal I/R. Of note, the antagonists were given prior to reperfuse and at the end of the ischaemic period. This time frame of drug administration keeps in mind the real clinical situation when patients are seen with the ischaemic syndrome and will undergo a procedure to reperfusion the ischaemic vascular bed. In support of our present findings, tachykinin NK$_1$ receptor antagonism has been shown to reduce oxidative injury and improve functional recovery following in vitro myocardial I/R injury in magnesium-deficient animals (Kramer et al., 1997). Similarly, 'myocardial stunning' was prevented by tachykinin NK$_1$ receptor antagonism in an in vitro guinea-pig model (Chiao & Caldwell, 1996).

There are at least two possibilities that may explain the beneficial effects of tachykinin NK$_1$ receptor antagonists in our I/R model – inhibition of neutrophil influx and/or inhibition of pro-inflammatory cytokine release. As neutrophil recruitment underlies both local and remote injury (Souza *et al.*, 2000a, b), the prevention of the recruitment of this cell type may explain the anti-inflammatory effects of tachykinin NK$_1$ receptor antagonists. This possibility is in line with other studies demonstrating an important role of neuropeptides and the tachykinin NK$_1$ receptor for neutrophil activation and/or recruitment in different models of inflammation (e.g. Ahluwalia *et al.*, 1998; Cao *et al.*, 2000; Medeiros *et al.*, 2001). However, this still leaves an open question as to whether tachykinin NK$_1$ receptors on the neutrophil or on an intermediate cell type (e.g. mast cell) (Maggi, 1997) mediate the action of locally-released neuropeptides. In addition to blocking neutrophil influx, tachykinin NK$_1$ receptor antagonists also prevented the release of TNF-α. This is in agreement with other studies demonstrating similar effects of these antagonists on LPS-induced systemic cytokine release (Dickerson *et al.*, 1998). As inhibition of the pro-inflammatory cytokine TNF-α is accompanied by abrogation of I/R injuries in our model (Souza *et al.*, 2001), this inhibitory effect could underline the marked suppressive effects of tachykinin NK$_1$ receptor antagonists in our I/R injury model. However, local, but not systemic, TNF-α release is dependent on the influx of neutrophils (Souza *et al.*, 2000b) suggesting that the suppression of the migration of that cell type may be a more dominant action in our model.

Substance P acting on tachykinin NK$_1$ receptors of macrophages markedly augmented LPS-induced production of the anti-inflammatory cytokine IL-10 (Ho *et al.*, 1996). Since IL-10 may be induced in reperfused tissue and may modulate reaction to I/R injury (Frangogiannis *et al.*, 2000), we measured the concentrations of this cytokine following severe I/R injury in control and SR140333-treated animals. There was a significant elevation of IL-10 concentrations in the serum and lung, but not intestine, following reperfusion of the ischaemic SMA. However, treatment with SR140333 markedly inhibited the elevations in IL-10 concentrations. These results demonstrate that an increase in IL-10 does not explain the inhibitory effects of tachykinin NK$_1$ receptor antagonists and are in agreement with the dependency of IL-10 production on local TNF-α release (Souza *et al.*, 2001).

Sensory nerves are a major source of tachykinin NK$_1$ receptor-acting neuropeptides, especially substance P (Holzer, 1991; Quartara & Maggi, 1998). In addition, substance P may be released by leukocytes, such as macrophages, and endothelial cells (reviewed by Maggi, 1997). To investigate the role of sensory nerves as a source of neuropeptides in our system, animals were pretreated neonatally with capsaicin (Holzer, 1991). At the dose used, capsaicin markedly suppressed the neuropeptide-dependent behavioural response to formalin injection and also effectively suppressed the injuries following mild intestinal I/R. These data suggest that sensory nerves are responsible for the majority of neuropeptides released during I/R injury. Indeed, there is evidence demonstrating the activation of cardiac vagal nerve chemosensitive endings in rats undergoing I/R (Ustinova & Schultz, 1994). Our data do not allow any conclusions in respect to

the molecular mechanisms responsible for the activation of sensory nerves and subsequent neuropeptide release and action on tachykinin NK$_1$ receptors. However, there are several possibilities which could account for the activation of sensory nerves during I/R. Previous studies have demonstrated that sensory nerves release tachykinins in response to ischaemia or low pH (reviewed in Maggi, 1995). In addition, locally released prostaglandins may participate in sensory nerve activation during ischaemia, but not during reperfusion (Ustinova & Schultz, 1994). Finally, bradykinin and leukotriene release during I/R injury may account for sensory nerve activation in this setting (Longhurst & Dittman, 1987; Souza *et al.*, 2000a; 2001; Montuschi *et al.*, 2000).

In addition to our present findings, previous studies from our group have shown that antagonism of PAF or LTB$_4$ receptors inhibited the development of I/R injury in rats (Souza *et al.*, 2000a, b). Moreover, we have recently shown that anti-TNF-α antibodies prevented both injury and lethality following severe I/R injury (Souza *et al.*, 2001). This leaves a question as to how these different mediators interact in our system to mediate I/R injury. Our working hypothesis is that with the onset of reperfusion, there is a marked local release of oxygen-derived metabolites which activate different tissue cells (including endothelial cells, macrophages, mast cells and nerve endings) to release inflammatory mediators (e.g. TNF-α, PAF) which will start the recruitment of neutrophils. This early neutrophil recruitment appears to be PAF- (Souza *et al.*, 2000a) and TNF-α-dependent (Souza *et al.*, 2001) whereas LTB$_4$ plays a greater role in the activation of neutrophils (Souza *et al.*, 2000b). One possibility to explain the importance of tachykinin NK$_1$ receptor activation in the system is that the activation of these receptors by neuronal-derived neuropeptides may underlie the initial release of lipid mediators. Alternatively, NK$_1$ receptor activation may occur later and be necessary for the amplification of the inflammatory response after the onset of reperfusion, i.e. after a first wave of incoming leukocytes (specially neutrophils) and released mediators, neuropeptide release and NK$_1$ receptor activation may facilitate further TNF-α release, leukocyte influx and mediator release. The released mediators and recruited leukocytes may then, in cooperation, achieve sufficient intensity to cause tissue injury and lethality.

In conclusion, our data demonstrate that the post-ischaemic blockade of tachykinin NK$_1$ receptors markedly inhibited the local and remote injuries, but not lethality, following reperfusion of the SMA in rats. Neuropeptides, possibly substance P, released from sensory nerves appear to account for the activation of these tachykinin NK$_1$ receptors. Altogether our results suggest that antagonists of the tachykinin NK$_1$ receptor may be useful adjuncts in the treatment of the injuries which occur following reperfusion of an ishcemic vascular territory.

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