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Effect of a BLT receptor antagonist in a model of severe ischemia and reperfusion injury in the rat

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Abstract

Pharmacological strategies which limit neutrophil recruitment may also limit the damage induced by the reperfusion of an ischemic vascular territory. In the present study, we have investigated the effects of the BLT receptor antagonist, CP-105,696 ((+)-1-(3*S*,4*R*)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid), on the local, remote and systemic inflammatory changes observed during severe intestinal ischemia (120 min) and reperfusion (120 min) injury. The post-ischemic treatment with CP-105,696 (3 mg/kg) virtually abolished the increase in vascular permeability, but not neutrophil accumulation, in the intestine and lungs. CP-105,696 partially inhibited the reperfusion-induced neutropenia, but failed to affect intestinal haemorrhage or lethality. CP-105,696 had no inhibitory effect on the local and systemic increases in the concentrations of tumour necrosis factor (TNF-α), interleukin-1β and interleukin-10, but markedly suppressed interleukin-6. Overall, our results show that activation of BLT receptor plays a minor role in the local, remote and systemic injuries following severe ischemia and reperfusion in rats.

Keywords: Ischemia–reperfusion; Neutrophil; Oedema; Artery, mesenteric; Inflammation; Leukotriene B_4

1. Introduction

Ischaemia and reperfusion injury is characterised by a series of inflammatory events, including cell recruitment, changes in vascular tone and enhanced vascular permeability to plasma proteins. Severe intestinal ischemia and reperfusion injury is accompanied not only by an acute local inflammatory response, but also by significant pulmonary injury and systemic inflammatory changes (Yao et al., 1997; Souza et al., 2000b). These local, remote and systemic responses are initiated during the ischemic period and driven by the recruitment and activation of neutrophils, which accumulate in tissues during reperfusion (Lefer and Lefer, 1996; Willerson, 1997). In this regard, pharmacological strategies which limit neutrophil recruitment also limit the damage induced by the reperfusion process (Willerson, 1997; Souza et al., 2000a,b).

Leukotriene (LT)B_4 is one of the inflammatory mediators known to activate neutrophils and induce their recruitment *in vivo* (Salmon and Garland, 1991; Marleau et al., 1999). Thus, leukotriene B_4 can stimulate neutrophil chemotaxis, aggregation, respiratory burst, degranulation and adhesion to vascular endothelial cells (Powell et al., 1996). We have recently described the effects of the treatment with BLT receptor antagonists, CP-105,696 and LY-255,283, in a model of neutrophil-dependent mild ischemia and reperfusion injury (Souza et al., 2000a). In this model, the leukotriene B_4 receptor antagonists effectively inhibited the changes in vascular permeability, but failed to alter significantly the recruitment of neutrophils to most affected tissues (Souza et al., 2000a).

More recently, we showed that there was substantial leukotriene B_4 production in intestinal tissues following severe ischemia and reperfusion injury of the superior mesenteric artery (Souza et al., 2001). In this severe model, in addition to the increase of vascular permeability and neutrophil influx observed in the mild model, there was marked intestinal haemorrhage and necrosis, blood leucopoenia, local and sys-

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temic pro-inflammatory cytokine release and approximately 60% lethality within 2 h (Souza et al., 2001). In the present study, we have investigated whether the locally produced leukotriene B_4 plays a physiopathological role in the local, remote and systemic inflammatory changes observed during severe intestinal ischemia and reperfusion injury.

2. Material and methods

2.1. Animals

Male Wistar rats (200–220 g) obtained from the Bioscience unit of Instituto de Ciências Biológicas were housed under standard conditions and had free access to commercial chow and water. All procedures described here had prior approval from the local animal ethics committee.

2.2. Ischemia and reperfusion injury

Rats were anaesthetized with urethane (140 mg/kg, i.p.) and laparotomy was performed. The superior mesenteric artery was isolated and ischemia was induced by totally occluding the superior mesenteric artery for 120 min. After ischemia, reperfusion was initiated by removal of the occlusion. Animals made ischemic for 120 min were allowed to reperfuse for 120 min (severe ischemia and reperfusion). The duration of ischemia and reperfusion was based upon previous experiments (Souza et al., 2000a,b) and was optimal for severe reperfusion injuries. Sham-operated animals or animals only made ischemic (i.e. without reperfusion) were used as controls for the reperfusion-induced injury. CP-105,696 was administered i.v. 5 min prior to the reperfusion at the dose of 3 mg/kg. At this dose, the drug maximally suppressed the changes in vascular permeability after mild ischemia and reperfusion injury in rats (Souza et al., 2000a). CP-105,696 had no significant effects on basal parameters (data not shown).

2.3. Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability (De Matos et al., 1999; Souza et al., 2000a). Evans blue (20 mg/kg) was administered i.v. (1 ml/kg) via a femoral vein 2 min prior to reperfusion of the ischemic artery. Two hours 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 enzyme-linked immunosorbent assay (ELISA) plate reader. Results are presented as the amount of Evans blue per μg per 100 mg of tissue. 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.

2.4. Myeloperoxidase concentrations

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; Souza et al., 2000a). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone ischemia and reperfusion 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 (0.1 M NaCl, 0.02 M $NaPO_4$, 0.015 M NaEDTA), 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 NaCl 1.6% and glucose 5%). After a further centrifugation, the pellet was resuspended in 0.05M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide and re-homogenized. One-milliliter aliquots of the suspension were transferred into 1.5-ml Eppendorf tubes and subjected to 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 and lung were diluted prior to assay. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density (O.D.) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results are expressed as total number of neutrophils by comparing the O.D. of tissue supernatant with the O.D. 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 O.D. was obtained by processing purified neutrophils as above and assaying for myeloperoxidase activity.

2.5. Determination of the concentrations of circulating leukocytes

The total numbers of circulating leukocytes and neutrophils were evaluated in blood samples obtained via a cannula in a femoral artery. Samples were collected just prior to reperfusion (time 0) 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 were obtained by evaluating the percentage of each leukocyte on blood films stained with May–Grunwald–Giemsa.

2.6. Measurement of haemoglobin concentrations

The determination of the concentrations of haemoglobin in tissue was used as an index of tissue haemorrhage. After

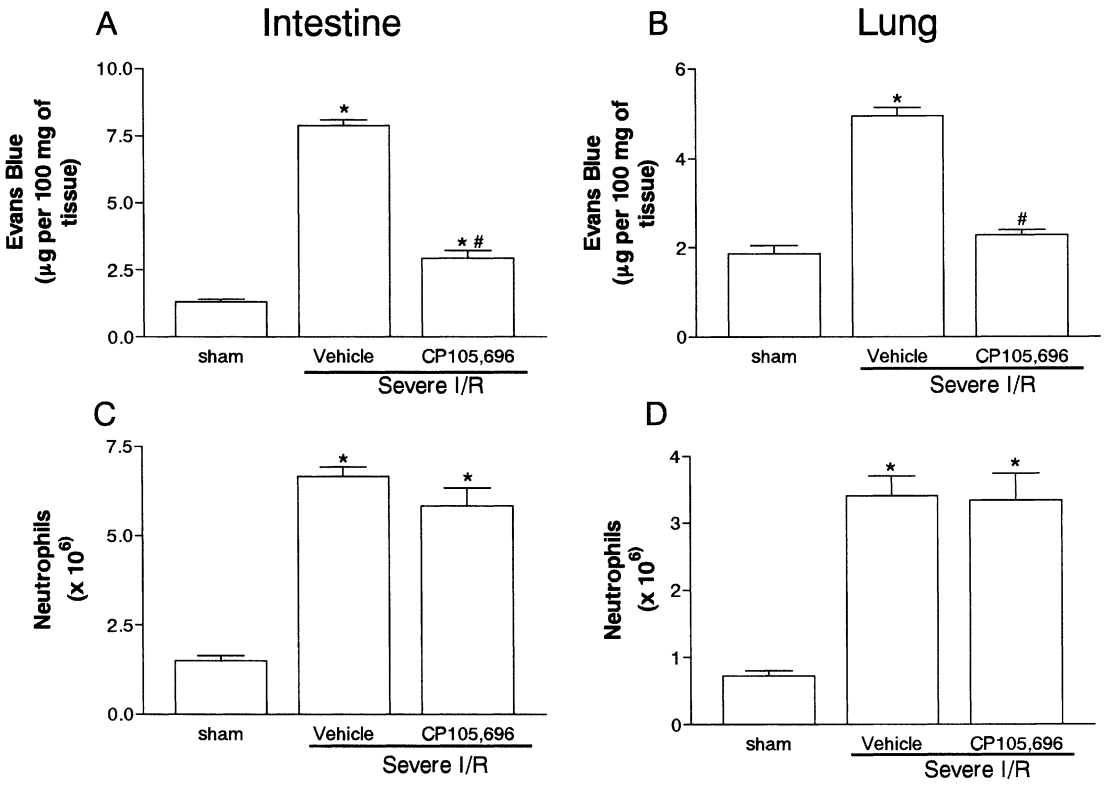

Fig. 1. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the increase in vascular permeability and neutrophil recruitment in the intestine (A,C) and lungs (B,D) following severe ischaemia and reperfusion of the superior mesenteric artery. Changes in vascular permeability (A,B) were assessed by evaluating the extravasation of Evans blue dye and neutrophil accumulation (C,D) by evaluating the tissue levels of myeloperoxidase. CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. Results are shown as μg Evans blue per 100 mg of tissue or as number of neutrophils per 100 mg of tissue and are the mean ± S.E.M of five animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.01$ when compared to vehicle-treated animals.

washing and perfusing the intestines to remove excess blood in the intravascular space, a sample of approximately 100 mg of duodenum was removed and homogenised in Drabkin's colour reagent according to instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 min at 3000 × *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.

2.7. Measurement of cytokine concentrations in serum, intestine and lungs

Tumour necrosis factor (TNF-α), interleukin-1β, interleukin-6 and interleukin-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 phosphate-buffered saline (PBS). One hundred milligrams of duodenum or lung of sham-operated and reperfused animals were

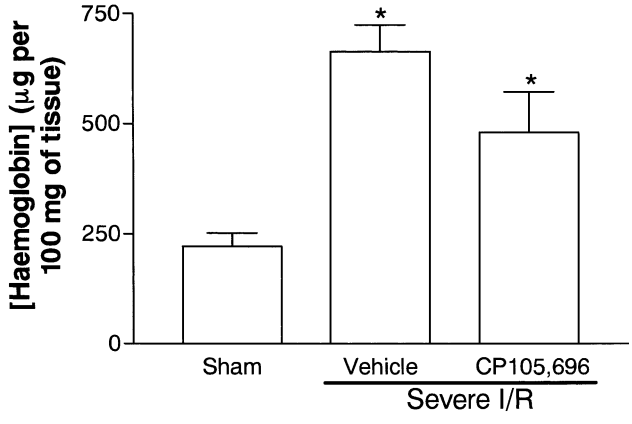

Fig. 2. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the increase in tissue haemorrhage in the intestine following severe ischaemia and reperfusion of the superior mesenteric artery in rats. Tissue haemorrhage was assessed by evaluating the tissue levels of haemoglobin. CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. Results are shown as μg Evans blue per 100 mg of tissue or as number of neutrophils per 100 mg of tissue and are the mean ± S.E.M of five animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.01$ when compared to vehicle-treated animals.

homogenised in 1 ml of PBS (0.4 m NaCl and 10 mM $NaPO_4$) containing anti-proteases (0.1 mM phenylmethil-sulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000×*g* and the supernatant immediately used for ELISA assays at a 1:5

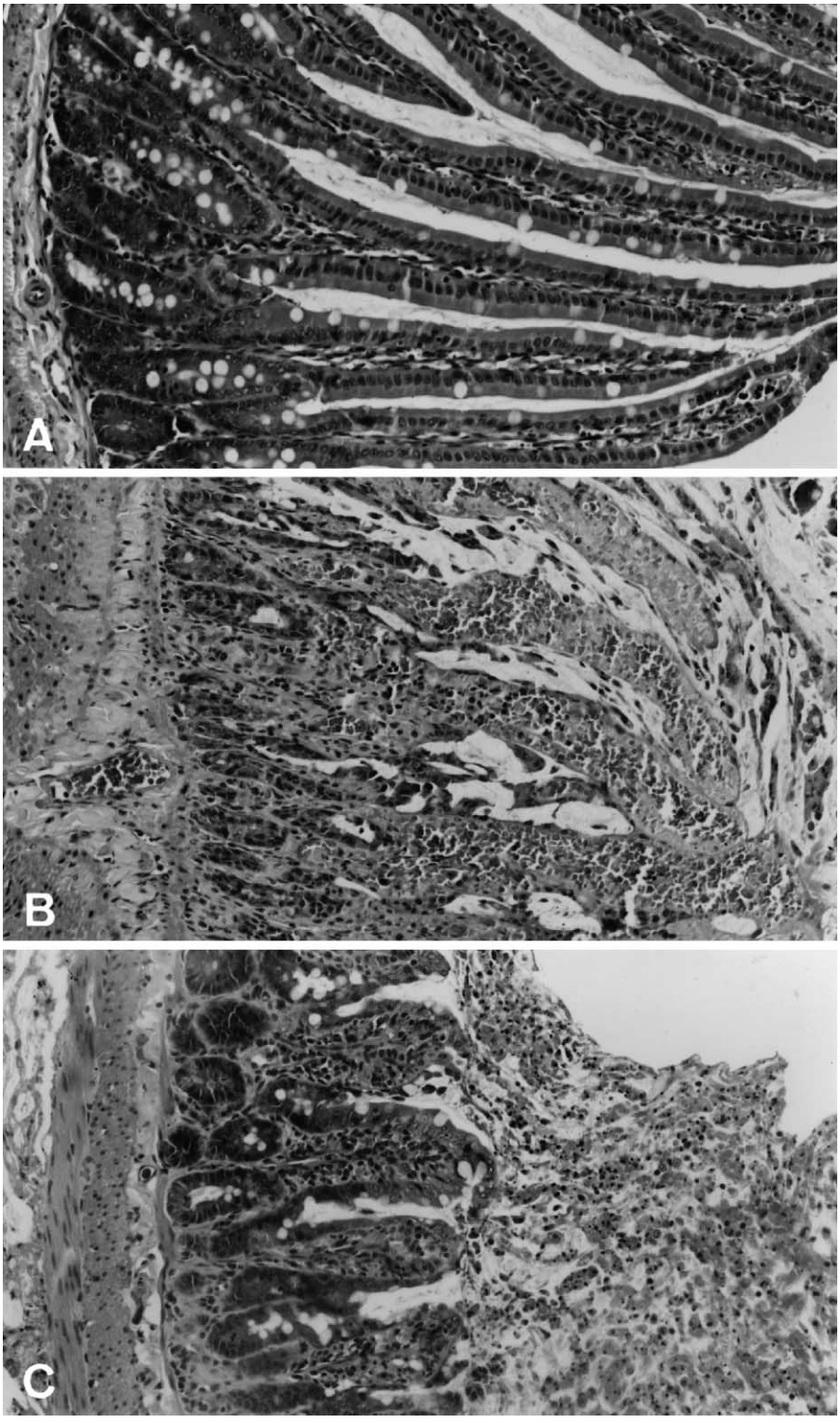

Fig. 3. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the histopathological changes in the intestine following severe ischaemia and reperfusion of the superior mesenteric artery in rats. CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. In (A), note the normal tissue histology in sham-operated animals. After severe ischemia and reperfusion injury, note the extensive oedema, haemorrhage and necrosis (B). In CP-105,696-treated animals (C), there was also significant tissue injury, but necrosis never reached the cripts.

dilution in PBS. ELISA plates (Nunc MaxiSorb) were coated with sheep anti-rat TNF-α/interleukin-1β/interleukin-6 or interleukin-10 polyclonal antibodies (1–2 μg/ml) overnight. The plates were washed thrice and then blocked with 1% bovine serum albumin. After further washes, 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 all of the assays had a sensitivity of 16 pg/ml.

2.8. Histology

Sections of duodenum were obtained from similar areas of the small intestine from representative animals in each of the treatment groups. The tissue was fixed in 10% formalin, embedded in paraffin and 4-μm-thick section cuts. The sections were stained with haematoxylin and eosin and examined under a light microscope. Lungs were inflated with 2 ml of 10% buffered formalin, removed from the animals and embedded and sectioned as above.

2.9. Drugs and reagents

The following drugs were obtained from Sigma (USA): urethane, Evans blue, hexadecyltrimethylammonium bromide. The leukotriene B_4 receptor antagonist CP-105,696, (+)-1-(3*S*,4*R*)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid, was a kind gift of Pfizer (Groton, USA). CP-105,696 was dissolved in dimethylsulfoxide (DMSO) and further diluted in PBS just prior to administration (final concentration of DMSO was 5%).

2.10. Statistical analysis

Results are shown as means ± S.E.M. Percent inhibition was calculated by subtracting the background concentrations 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.

3. Results

3.1. Effects of the treatment with CP-105,696 on the local and remote tissue injuries in a model of severe ischemia and reperfusion injury

The treatment with CP-105,696 5 min prior to the reperfusion virtually abolished the increase in vascular permeability in the intestine and lungs following severe ischemia and reperfusion injury (Fig. 1A,B). In contrast, the leukotriene B_4 receptor antagonist had no inhibitory effect on the accumulation of neutrophils in the intestine and lungs of reperfused animals (Fig. 1C,D). Moreover, intestinal haemorrhage, as assessed by the extravasation of haemoglobin, was not significantly inhibited in CP-105,696-treated animals (Fig. 2). The histopathological analysis of sections of the intestine revealed significant oedema, haemorrhage and necrosis in the mucosa after ischemia and reperfusion injury (Fig. 3). Whereas in CP-105,696-treated animals the necrosis was limited to the surface of the epithelium (Fig. 3B), in control animals significant necrosis was observed in the base of the crypts and, at times, in the muscularis mucosa (Fig. 3C).

3.2. Effects of the treatment with CP-105,696 on the concentrations of circulating neutrophils in a model of severe ischemia and reperfusion injury

In agreement with our previous studies (Souza et al., 2000b), prolonged ischemia of superior mesenteric artery was accompanied by significant neutrophilia, which dropped rapidly and persistently to below background concentrations after reperfusion (Fig. 4). The neutrophilia prior to reperfusion was not different in the two experimental groups (Fig. 4), as treatments were administered at the end of the ischemic period. Post-ischemic treatment with CP-105,696

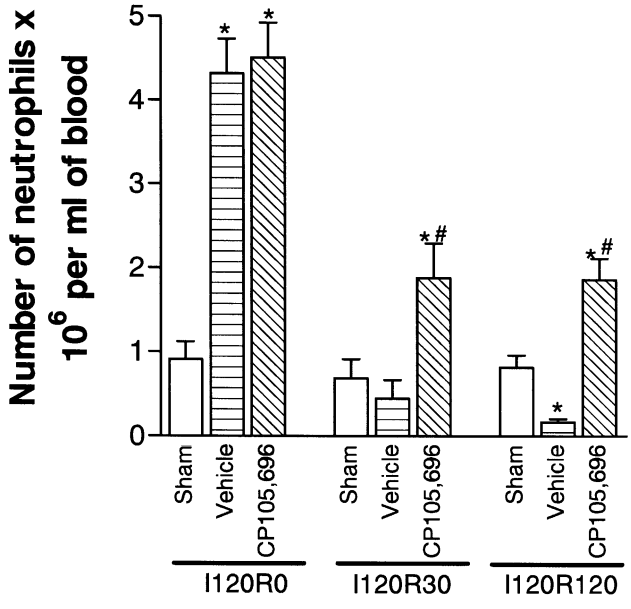

Fig. 4. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the concentration of circulating neutrophils following reperfusion of the ischemic superior mesenteric artery in rats. CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. The number of neutrophils was evaluated just prior to (time 0) and 30 and 120 after reperfusion. Results are show as the concentration of neutrophils $\times 10^6$ per ml of blood and are the mean ± S.E.M of five animals in each group. $*P < 0.01$ when compared to sham-operated animals and $\#P < 0.01$ when compared to vehicle-treated animals.

partially reversed the neutropenia at 30 and 120 min after the onset of reperfusion (Fig. 4).

3.3. Effects of the treatment CP-105,696 on the lethality and cytokine concentrations following severe ischemia and reperfusion injury

Cytokine concentrations were measured in serum and tissues at the end of the reperfusion period. CP-105,696 treatment had no inhibitory effects on the ischemia and reperfusion-induced elevation in TNF-α concentrations in serum, intestine and lungs (Fig. 5A,B,C). Similarly, treatment with CP-105,696 had no significant effect on the increases in interleukin-10 or interleukin-1β concentrations in serum or in tissue (data not show). In contrast, the elevations in interleukin-6 concentrations in serum and tissues were markedly inhibited by post-ischemic treatment with CP-105,696 (Fig. 5D,E,F).

Animals began to die from around 30 min after reperfusion and approximately 60% were dead by 120 min (Fig. 6).

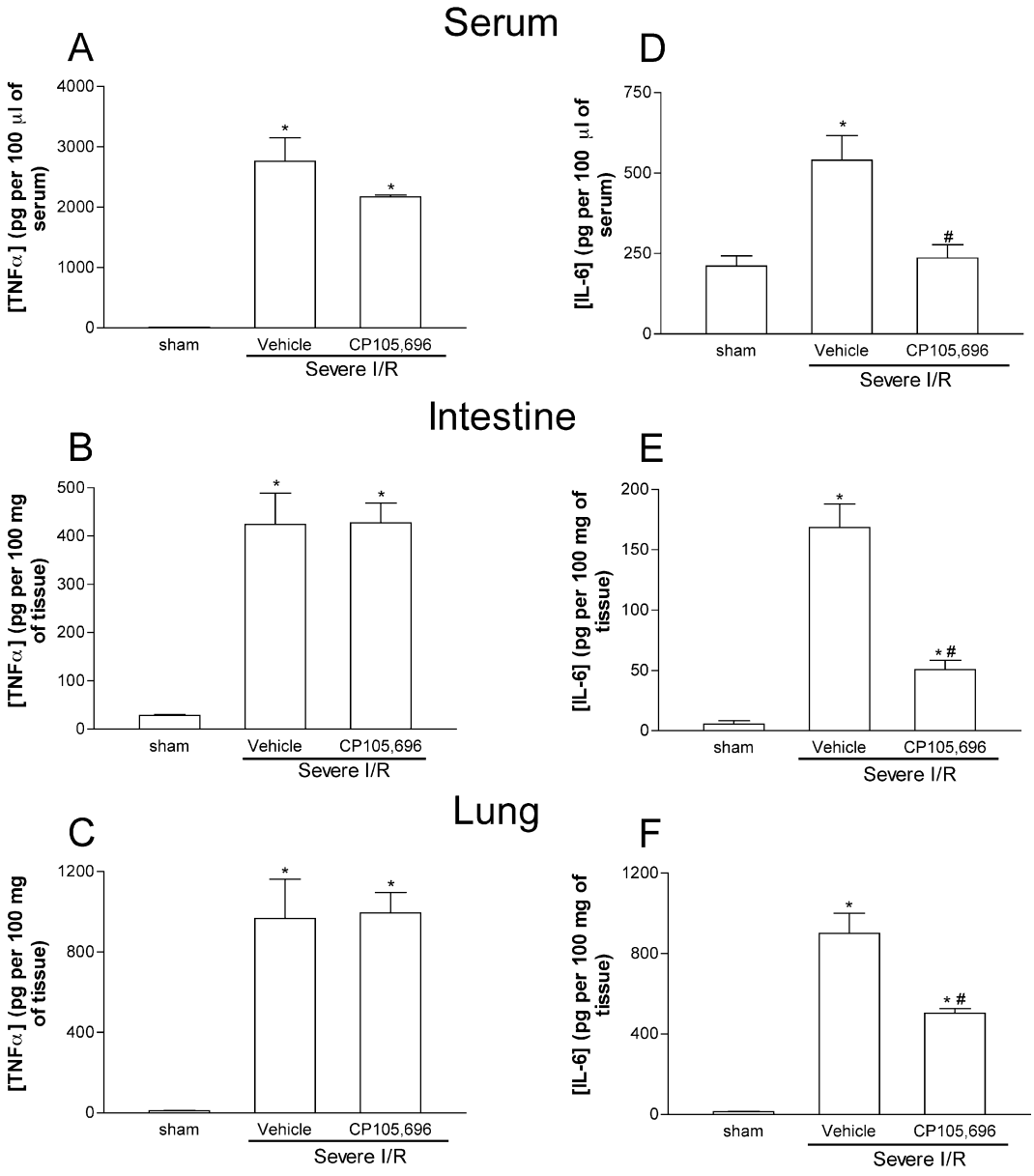

Fig. 5. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the concentration of TNF-α and IL-6 in serum, intestine and lungs following severe ischaemia and reperfusion of the superior mesenteric artery in rats. CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. TNF-α (A,B,C) or IL-6 (D,E,F) were measured using specific ELISA. Results are shown as pg of cytokine per ml of plasma or as pg cytokine per 100 mg of tissue and are the mean ± S.E.M of five animals in each group. *$P < 0.01$ when compared to sham-operated animals and #$P < 0.01$ when compared to vehicle-treated animals.

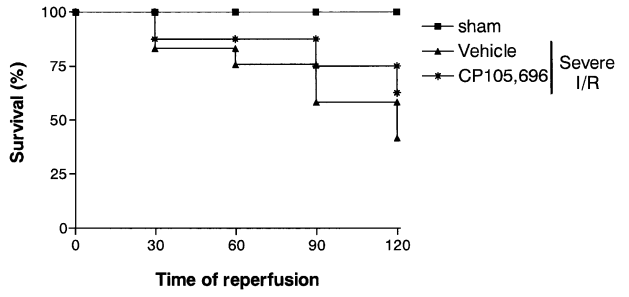

Fig. 6. Effects of the post-ischaemic treatment with the leukotriene B_4 receptor antagonist, CP-105,696, on the lethality which follows reperfusion of the ischemic superior mesenteric artery vascular bed in rats (severe I/R). CP-105,696 (3 mg/kg, i.v.) was given 5 min prior to reperfusion and controls animal received drug vehicle. The time of lethality after reperfusion was noted. No deaths occurred in sham-operated animals ($n = 5$) and there were no significant differences between reperfused animals treated with CP-105,696 ($n = 8$) or vehicle ($n = 13$).

Under the same conditions, sham-operated animals did not die. Treatment with CP-105,696 had no significant effect on lethality following severe ischemia and reperfusion injury (Fig. 6).

4. Discussion

The reperfusion of an ischemic tissue is followed by an acute inflammatory response characterized by local vasodilatation, increased vascular permeability and the formation of exudates containing plasma proteins and inflammatory cells. Leukotriene B_4 is a potent inflammatory mediator shown to be released during ischemia and reperfusion injury (Souza et al., 2001) and known to be an effective inducer of neutrophil recruitment and activation (Powell et al., 1996). In this study, we have investigated the effects of the administration of a BLT receptor antagonist, CP-105,696, in our model of severe ischemia and reperfusion injury (Souza et al., 2000b, 2001). Treatment with CP 105,696 after the onset of ischemia and just prior to reperfusion inhibited the increase in vascular permeability in the intestine and lung. These results are in agreement with previous experiments in a mild model of ischemia and reperfusion injury (Souza et al., 2000a) and are consistent with the *ex-vivo* production of leukotriene B_4 by intestinal explants of animals submitted to ischemia and reperfusion of the superior mesenteric artery (Stojadinovic et al., 1999). Thus, it is clear that locally produced leukotriene B_4 participates in the cascade of events leading to increase in vascular permeability and edema formation in the intestine and lungs after ischemia and reperfusion injury.

Previous studies have suggested a role of 5-lipoxigenase-derived products, most likely leukotriene B_4, for the migration of neutrophils to sites of ischemia and reperfusion injury in mice (Chiang et al., 1999; Noiri et al., 2000). Here, the post-ischemic treatment with CP-105,696 had no significant effects on the accumulation of neutrophils in the intestine or lungs of reperfused animals. In agreement with the lack of effect of CP-105,696 on the recruitment of neutrophils following ischemia and reperfusion injury, the drug only partially inhibited the leukopenia that accompanied the onset reperfusion. This in contrast to our previous studies in a mild model of ischemia and reperfusion injury in which CP-105,696 suppressed neutrophil recruitment into the intestine, but not the lungs, of reperfused animals (Souza et al., 2000b). Moreover, Karasawa et al. (1991) also showed decreased ileal recruitment of neutrophils following pre-treatment of rats with a leukotriene B_4 receptor antagonist in a model of ischemia and reperfusion of the splanchnic artery. From the discussion above, it is clear that, in different models of ischemia and reperfusion injury (mild × severe), BLT receptors play greater or lesser roles in the recruitment of neutrophils into different tissues (intestine × lungs) of different species (mice × rats).

Neutrophils also play a major role in the development of haemorrhage, necrosis, epithelial shedding and other marked histopathological changes observed in the intestine following severe ischemia and reperfusion injury (Souza et al., 2001). Post-ischemic treatment with CP-105,696 had little effect on the local increase in haemoglobin content, an index of tissue haemorrhage, observed after severe ischemia and reperfusion injury. Moreover, CP-105,696 only partially inhibited tissue injury as assessed by light microscopy. Thus, the inability of CP-105,696 to inhibit the recruitment of neutrophils into sites of injury was also reflected in the inability of the drug to prevent tissue damage. However, the same dose of CP-105,696 effectively prevented oedema formation and IL-6 production (see below) in the model of severe ischemia and reperusion injury and neutrophil accumulation and oedema formation in the intestine after mild injury, suggesting the dose of the drug used was sufficient to block BLT receptors effectively.

Previous studies have shown that some bioactivities of leukotriene B_4 may involve inflammatory cytokines (Rola-Pleszczynski and Stankova, 1992) and connections have been established between the 5-lipoxygenase pathway and TNF-α (Bureau et al., 1997), an essential mediator of lesions induced by ischemia and reperfusion injury (Souza et al., 2001). Indeed, leukotriene B_4 may enhance TNF-α production by monocytes/macrophages and i.v. injection of endotoxin induces the appearance of TNFα and of leukotrienes in the rat heart and lung (Tanaka et al., 1994). However, in our experimental model, CP-105,696 failed to affect TNF-α synthesis. These data are in agreement with our previous results showing that infiltration of neutrophils is essential for the local production of TNF-α (Souza et al., 2000b). Since CP-105,696 failed to affect neutrophil influx, it is not surprising that the compound failed to alter local and systemic TNF-α production. Moreover, since TNF-α appears to be the major cytokine underlying lethality after severe ischemia and reperfusion injury, the lack of effect of CP-105,696 on TNFα concentration agrees with the lack of effect of the drug on overall lethality in the present experiments.

In addition to TNF-α, other studies have shown that concentrations of interleukin-1β are elevated and may have a pathophysiological role following intestinal ischemia and reperfusion injury (e.g. Seekamp et al., 1993; Yao et al., 1997). Further, interleukin-10 may be induced in reperfused tissue and may modulate reaction to injury (Frangogiannis et al., 2000). Pretreatment with CP-105,696 had no inhibitory effects on concentrations of interleukin-1β and interleukin-10. Thus, the inhibitory effects of CP-105,696 on reperfusion-induced increase in vascular permeability cannot be ascribed to inhibition of interleukin-1β release or increased interleukin-10 production.

In contrast to the lack of effects of CP-105,696 on the increases in the concentrations of TNF-α and interleukin-1β, the drug markedly inhibited the changes in interleukin-6 concentrations in serum and tissues of animals submitted to severe ischemia and reperfusion injury. Leukotriene B_4 has been shown to stimulate the production of bioactive and immunoreative interleukin-6 by monocytes in vitro (Rola-Pleszczynski and Stankova, 1992). Thus, it is possible that the leukotriene B_4 produced during the reperfusion of an ischemic tissue may be acting on mononuclear cells to stimulate the production of interleukin-6. Whatever the cell type responsible for interleukin-6 production in our model, it is clear from our results that endogenous production of leukotriene B_4 and action on BLT receptors underlie a great proportion of the interleukin-6 released during ischemia and reperfusion injury in rats.

Here we describe the inhibitory effects of the potent and specific leukotriene B_4 receptor antagonist, CP-105,696, on the neutrophil-dependent reperfusion injuries following severe ischemia of the superior mesenteric artery in rats. CP-105,696 protected only against the reperfusion-induced increases in vascular permeability and in the increased concentrations of interleukin-6. Overall, our results show that activation of leukotriene B_4 receptor plays a minor role in the local, remote and systemic injuries following severe ischemia and reperfusion in the rat.

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