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Role of PAF receptors during intestinal ischemia and reperfusion injury. A comparative study between PAF receptor-deficient mice and PAF receptor antagonist treatment

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1 The reperfusion of ischemic tissues may be associated with local and systemic inflammation that prevents the full benefit of blood flow restoration. The present study aimed to confirm a role for platelet-activating factor receptor(s) (PAFR) during ischemia and reperfusion injury by using genetically modified mice deficient in the PAFR ($PAFR^{-/-}$ mice) and to evaluate comparatively the effectiveness of pharmacological treatment using the PAFR antagonist UK-74,505 (modipafant).

2 The reperfusion of the ischemic superior mesenteric artery (SMA) induced marked local (intestine) and remote (lungs) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx and intestinal hemorrhage and in the production of TNF-α. There was also a systemic inflammatory response, as shown by the increase in serum TNF-α concentrations and marked reperfusion-associated lethality.

3 After reperfusion of the ischemic SMA, $PAFR^{-/-}$ mice had little tissue or systemic inflammation and lethality was delayed, but not prevented, in these mice. Interestingly, the reperfusion-associated increases in tissue concentrations of IL-10 were significantly greater in $PAFR^{-/-}$ than wild-type mice.

4 Pretreatment with PAFR antagonist UK-74,505 ($1\,mg\,kg^{-1}$) markedly prevented tissue injury, as assessed by the increase in vascular permeability, neutrophil accumulation, hemorrhage and TNF-α concentrations in the intestine and lungs. In contrast, UK-74,505 failed to affect reperfusion-associated lethality and increases in serum TNF-α when used at $1\,mg\,kg^{-1}$.

5 Reperfusion-associated lethality and increase in serum TNF-α were only affected when a supra-maximal dose of the antagonist was used ($10\,mg\,kg^{-1}$). At this dose, UK-74,505 also induced a marked enhancement of reperfusion-associated increases in tissue concentrations of IL-10. However, at the same dose, UK-74,505 failed to prevent reperfusion-associated lethality in $PAFR^{-/-}$ mice any further.

6 The present studies using genetically modified animals and a receptor antagonist firmly establish a role of PAFR activation for the local, remote and systemic inflammatory injury and lethality which follows reperfusion of the ischemic SMA in mice. Moreover, it is suggested that high doses of PAFR antagonists need to be used if the real efficacy of these compounds is to be tested clinically.
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Abbreviations: I/R, ischemia and reperfusion; MPO, myeloperoxidase; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor(s); $PAFR^{-/-}$ mice, platelet-activating factor receptor-deficient mice; SMA, superior mesenteric artery

Introduction

A major goal in the treatment of ischemia of a vascular territory is to restore blood flow to normal values, that is, to 'reperfuse' the ischemic vascular bed (Carden & Granger, 2000). However, reperfusion of ischemic tissues is associated with local and systemic leukocyte activation and trafficking, endothelial barrier dysfunction in postcapillary venules, enhanced production of inflammatory mediators and great lethality (Lefer & Lefer, 1996; Granger, 1999; Carden & Granger, 2000). For example, after intestinal ischemia and reperfusion (I/R), there is marked intestinal and pulmonary injury that may also be accompanied by a systemic inflammatory response syndrome and significant lethality (Souza *et al*., 2000b; 2001; 2002). Among the mediators of the inflammatory cascade released and thought to be important for the reperfusion-associated injury is platelet-activation factor (PAF) (Kubes *et al*., 1990a, b; Montrucchio *et al*., 2000; Souza *et al*., 2000b).

PAF is a natural phospholipid that under normal physiological conditions is minimally expressed. However, during acute inflammation or under conditions of oxidative stress, as occurs during I/R injury, PAF is released by neutrophils and/

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or monocytes and expressed at the outer leaflet of endothelial cells (Montrucchio *et al*., 2000). Once released, activation of PAF receptors (PAFR) results in diverse biological activities associated with acute inflammation, including neutrophil activation and chemotaxis, alterations in vascular permeability and platelet activation, all of which may contribute to the clinical manifestation of I/R injury (Montrucchio *et al*., 2000).

We have recently described the effects of the treatment with the PAFR antagonists UK-74,505 and WEB-2086 in models of neutrophil-dependent mild and severe I/R injury in rats (Souza *et al*., 2000b). Our results demonstrated that treatment with the PAFR antagonists prevented the increases in vascular permeability, neutrophil recruitment and TNF-α production (Souza *et al*., 2000b). Furthermore, activation of PAFR has been shown to play an important pathophysiological role in models of I/R injury in several other vascular territories, including the heart, gut, kidney and lung (Canale *et al*., 1994; Carter *et al*., 1996; Riera *et al*., 1997; Qayumi *et al*., 1998; Morgan *et al*., 1999; Kecskemeti & Balogh, 2000; Kim *et al*., 2000; Sun *et al*., 2001; 2002). The objectives of the present study were two-fold: (i) to confirm a role for PAFR during I/R injury by using genetically modified mice deficient in the PAFR (PAFR$^{-/-}$ mice); and (ii) to evaluate comparatively the effectiveness of pharmacological treatment using the PAFR antagonist UK-74,505 and the genetic PAFR deficiency.

Methods

Animals

Male C57BL/6 mice (8–10 weeks) 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. PAFR$^{-/-}$ mice were generated as previously described (Ishii *et al*., 1998) and intercrossed for at least seven generations to establish the C57BL/6 strain.

Ischemia and reperfusion

Mice were anesthetized with urethane (140 mg kg^{-1}, i.p.) and laparotomy was performed. The superior mesenteric artery (SMA) was isolated and ischemia was induced by totally occluding the SMA for 60 min. For measuring the percentage of surviving mice, reperfusion was re-established, and mice were monitored for indicated time periods. For the other parameters, reperfusion was allowed to occur for 30 min (I60R30) when mice were killed. This time of reperfusion (30 min) was chosen based on the presence of significant tissue injury without unduly high mortality rates. Sham-operated animals were used as controls. The treatment with UK-74,505 or vehicle was administrated (i.v.) 10 min before reperfusion.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability, as previously described (Saria & Lundberg, 1983; Souza *et al*., 2000a). Evans blue (20 mg kg^{-1}) was administered i.v. (1 ml kg^{-1}) *via* a tail vein 2 min prior to reperfusion of the ischemic artery. At 30 min after reperfusion, a segment of the duodenum (approximately 3 cm) was 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 1 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 right ventricle was flushed with 10 ml of phosphate-buffered saline (PBS) 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 (MPO) concentrations

The extent of neutrophil accumulation in the intestine and right lung tissue was measured by assaying myeloperoxidase (MPO) activity, as previously described (Kuebler *et al*., 1996; Souza *et al*., 2002). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone I/R injury were removed and snap frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for MPO activity by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine. Results were expressed as the neutrophil infiltration. An index unit denotes the MPO activity present in 10^5 casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of hemoglobin concentrations

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing the intestines to remove excess blood, a sample of approximately 100 mg of duodenum was removed and homogenized in Drabkin's color reagent according to the instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). 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 hemoglobin.

Measurement of cytokine/chemokine concentrations in serum, intestine and lungs

The concentration of TNF-α and IL-10 in samples was measured in serum and tissue of animals using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis). 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 analyzed at a 1 : 3 dilution in PBS. Duodenum (100 mg) or lung of sham-operated and reperfused animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM de $NaPO_4$) containing antiproteases (0.1 mM phenylmethylsulfonyl 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 $\times$ *g* and the supernatant immediately used for ELISA assays at a 1 : 3 dilution in PBS.

Drugs and reagents

The following drugs were obtained from Sigma (U.S.A.): urethane, Evans blue, hexadecyltrimethylammonium bromide. The PAF receptor antagonist UK-74,505 (modipafant) was a gift of Dr J. Parry (Pfizer, Sandwich, U.K.) (Alabaster *et al*., 1991). UK-74,505 was dissolved in 0.1 M HCl and further diluted 10-fold in saline just prior to use.

Statistical analysis

Results are shown as means ± s.e.m. Percent inhibition was calculated by subtracting the background values obtained in sham-operated animals. Differences were compared by using analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* analysis. Results with $P<0.05$ were considered significant.

Results

Intestinal I/R injury and lethality in wild-type and PAFR$^{-/-}$ mice

The reperfusion of the ischemic SMA artery induced marked local (intestine) and remote (lungs) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx and intestinal hemorrhage observed in wild-type mice (Figure 1). In contrast, there was a marked and almost complete inhibition of tissue injury in PAFR$^{-/-}$ mice submitted to the same experimental conditions (Figure 1).

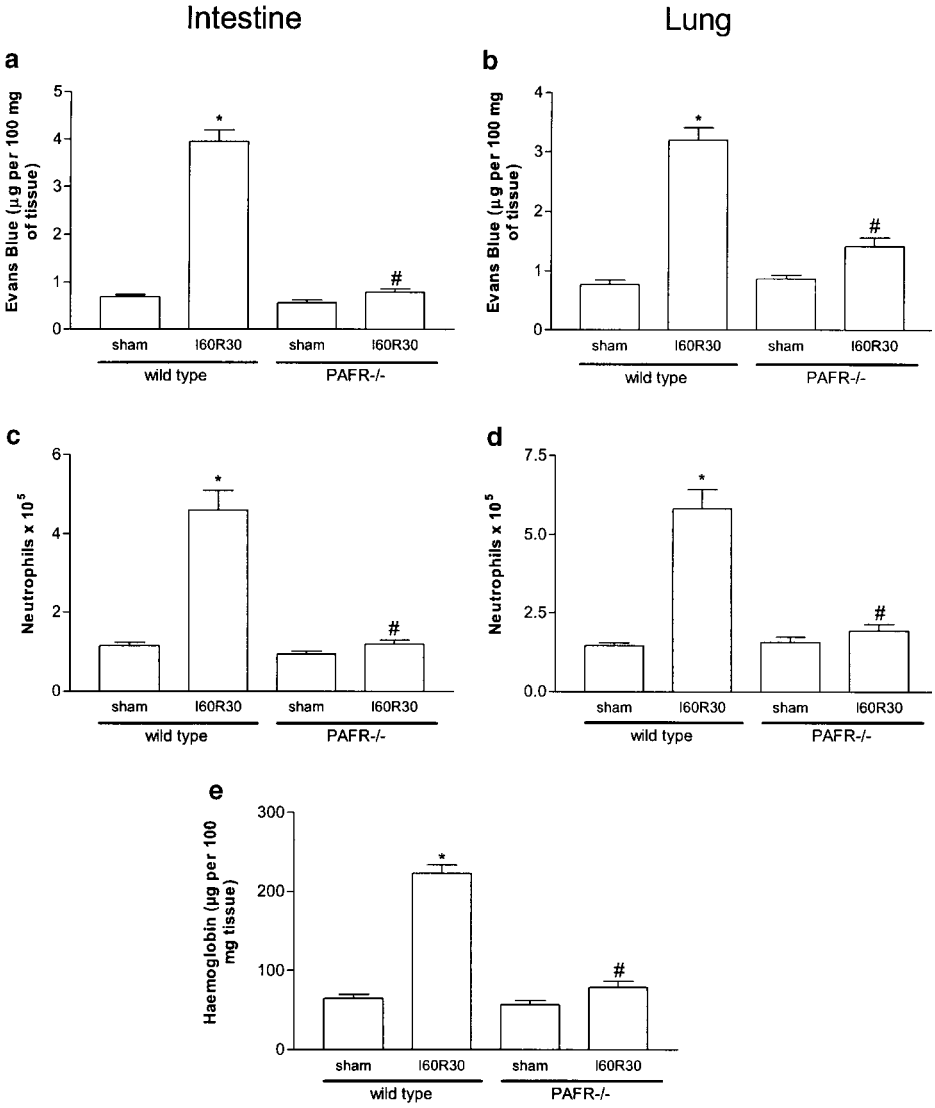

Figure 1 Tissue injury in wild-type (WT) and in PAFR-deficient mice (PAFR$^{-/-}$) submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. Changes in vascular permeability in the intestine (a) and lungs (b) were evaluated by measuring the extravasation of Evans Blue (μg per 100 mg of tissue). Neutrophil infiltration was determined by measurement of intestinal (c) and pulmonary (d) MPO activity. Hemorrhage in the intestine (e) was evaluated by measuring the concentration of hemoglobin in 100 mg of tissue. Data are shown as the mean ± s.e.m. of five to six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with WT mice submitted to I/R.

In addition to the local and remote inflammatory changes described above, there was a marked increase in the concentrations of TNF-α in the intestine, lungs and serum of wild-type reperfused mice (Figure 2). Again, the increases in the concentration of this cytokine were markedly suppressed in PAFR$^{-/-}$ mice submitted to intestinal I/R (Figure 2). Following reperfusion of the ischemic SMA, the concentrations of the anti-inflammatory cytokine IL-10 were much greater in the intestine and lungs of reperfused PAFR$^{-/-}$ mice than in the tissues of their wild-type controls (Figure 3).

In addition to the observed suppression of tissue and systemic inflammation, there was a significant delay in reperfusion-induced lethality in PAFR$^{-/-}$ (Figure 4). However, delay in lethality was not accompanied by prevention of lethality, as all animals were dead after 90 min of reperfusion (Figure 4).

Effects of the treatment with UK-74,505 on intestinal ischemia and reperfusion injury and lethality

As tissue and systemic inflammation was suppressed and lethality delayed in PAFR$^{-/-}$ mice, it was of interest to examine whether similar effects could be observed after treatment with a PAF receptor antagonist. To this end, UK-74,505, a selective and potent PAFR antagonist, was used at a

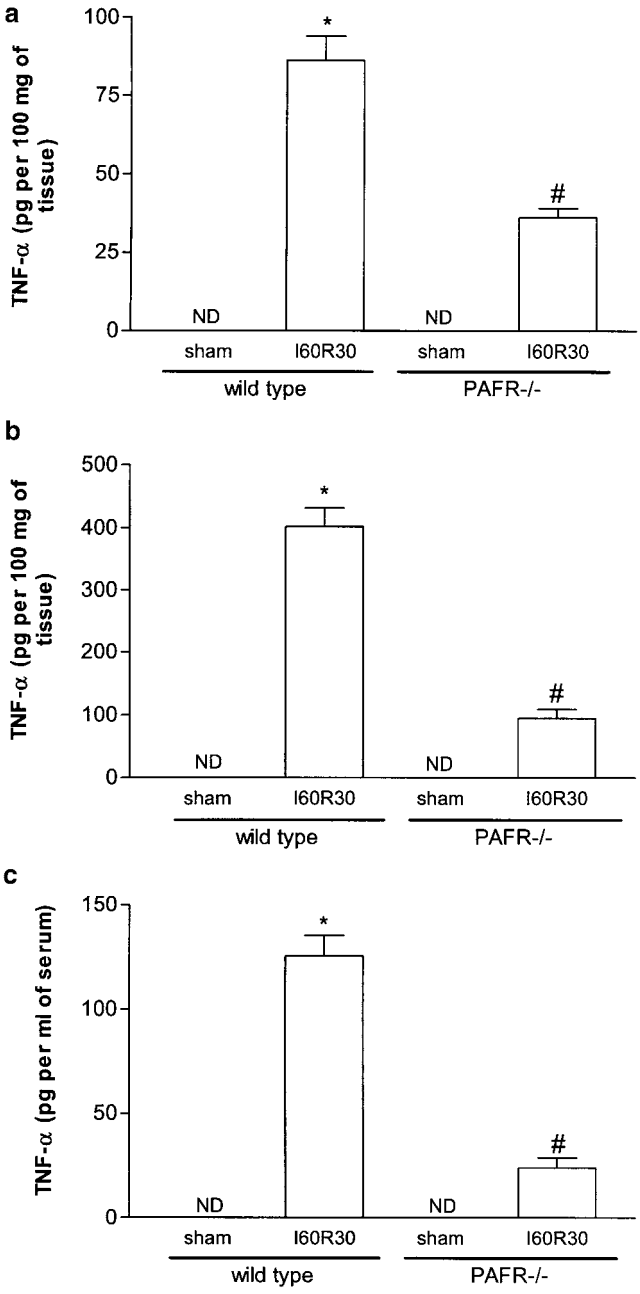

Figure 2 Concentration of TNF-α in the intestine, lungs and serum of WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. The concentrations of TNF-α in intestine (a), lungs (b) and serum (c) were measured by ELISA. Results are shown as picogram of TNF-α per 100 mg of tissue or as picogram TNF-α per milliliter of serum and represent the mean±s.e.m. of five to six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with WT mice submitted to I/R.

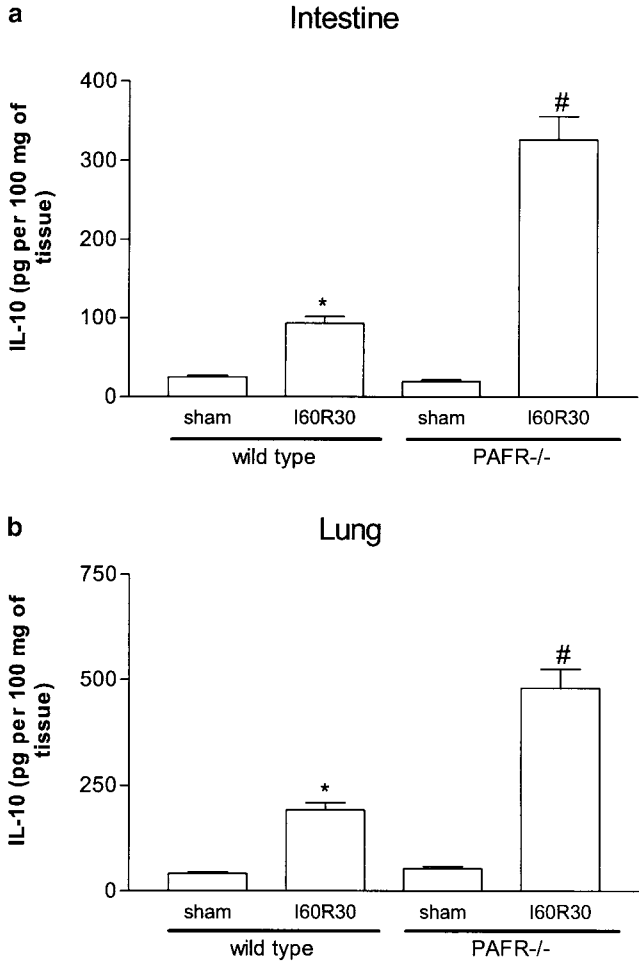

Figure 3 Concentration of IL-10 in the intestine and lungs WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. The concentrations of IL-10 in intestine (a) and lungs (b) were measured by ELISA. Results are shown as picogram of IL-10 per 100 mg of tissue and represent the mean±s.e.m. of five to six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with wild-type mice submitted to I/R.

dose of 1 mg kg^{-1} that has been previously shown to block PAFR effectively in the mouse or rat (Miotla *et al*., 1998; Borges *et al*., 2000; Souza *et al*., 2000b; Klein *et al*., 2002). The treatment with UK-74,505 10 min prior to the reperfusion virtually abolished the increases in vascular permeability and influx of neutrophils in the intestine and lungs following intestinal I/R (Table 1). The reperfusion-induced intestinal hemorrhage, as assessed by extravasation of hemoglobin, was abrogated in UK-74,505-treated animals (Table 1).

Interestingly, although the increase in reperfusion-induced tissue concentrations of TNF-α was markedly inhibited by UK-74,505 used at 1 mg kg^{-1}, this dose of the PAFR antagonist had no significant effect on serum concentrations of TNF-α (Figure 5b). Note that the concentrations of TNF-α in the intestine and lungs of UK-74,505-treated mice were equivalent to the concentrations found in reperfused PAFR$^{-/-}$ mice (compare Figure 2 and Table 1). At this dose, UK-74,505 also failed to enhance significantly the increases in IL-10 production in the lungs and intestine following reperfusion of the ischemic SMA (Figure 6). Our previous studies have shown a strong correlation between serum, but not tissue, concentrations of TNF-α and lethality (Souza *et al*., 2001; 2002). Consistently with these results, treatment of mice with 1 mg kg^{-1} of UK-74,505 had no effect on the lethality that followed reperfusion of the ischemic mesenteric artery (Figure 5a).

The lack of effects of UK-74,505 on reperfusion-associated increase in serum concentrations of TNF-α and lethality was unexpected in the face of the results obtained in PAFR$^{-/-}$ mice. Although the dose of UK-74,505 used has been previously to block PAFR effectively in several *in vivo* systems (Miotla *et al*., 1998; Borges *et al*., 2000; Souza *et al*., 2000b; Klein *et al*., 2002) and effectively blocked tissue injury in our model, we carried out a series of experiments using a supra-maximal dose of the antagonist (10 mg kg^{-1}) reasoning that maximal occupation of the receptor by the antagonist might be necessary to prevent reperfusion-associated lethality. At the higher dose of UK-74,505 used, there was a marked suppression of the reperfusion-induced increase in the serum concentrations of TNF-α (Figure 5b). More importantly, the inhibition of TNF-α was associated with a delay and partial prevention of reperfusion-associated lethality (Figure 5a). Akin to the results observed in PAFR$^{-/-}$ mice, treatment with the higher dose of UK-74,505 markedly enhanced the increases in IL-10 production in the intestine and lung of reperfused mice (Figure 6). To exclude an action of UK-74,505 (10 mg kg^{-1}) outside its effects on the PAFR, PAFR$^{-/-}$ mice were treated with the drug prior to experiments evaluating reperfusion-associated lethality. As seen in Figure 4, the treatment of PAFR$^{-/-}$ with UK-74505 (10 mg kg^{-1}) had no further effect on lethality in addition to that of the PAFR$^{-/-}$ phenotype.

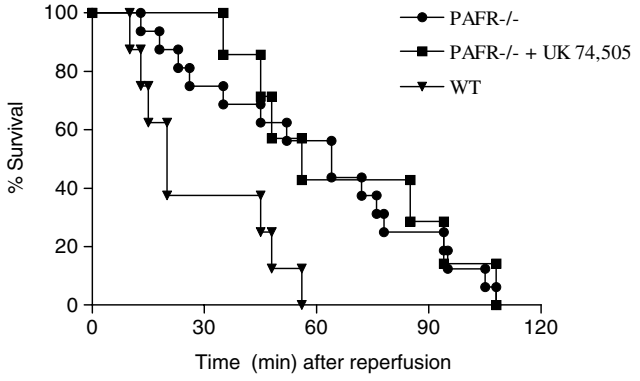

Figure 4 Survival curves of WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. Mice ($n=10$ in each group) were anesthetized and submitted to ischemia of the SMA for 60 min. Vehicle or UK-74,505 (10 mg kg^{-1}) was administered i.v. 10 min prior to reperfusion. Tissue perfusion was then re-established and survival was monitored. The survival curve of PAFR$^{-/-}$ mice was significantly ($P<0.05$) different from that of WT mice.

Discussion

The restoration of blood flow to an ischemic vascular bed, that is, reperfusion, is a major therapeutic objective after ischemia of an organ or tissue. However, reperfusion may be accompanied by significant local and systemic inflammatory injury, limiting the potential benefits of blood flow restoration. Thus, understanding the pathophysiology of the inflammation that occurs after reperfusion may be useful in the development of novel therapeutic strategies that limit the injury caused by the reperfusion process. Here, the role of PAFR in I/R injury was investigated using mice with a targeted deletion of the PAF receptor gene (Ishii *et al*., 1998) and the PAFR antagonist UK-74,505 (Alabaster *et al*., 1991).

Table 1 Effects of the postischemic treatment with the PAF receptor antagonist, UK-74,505 (1 mg kg^{-1}) on the tissue injury in the intestine and lungs of mice submitted to ischemia and reperfusion of the superior mesenteric artery (SMA)

	Intestine			*Lung*		
	Sham	*Vehicle*	*UK*	*Sham*	*Vehicle*	*UK*
Evans Blue	0.7±0.1	4.0±0.2	1.3±0.1	0.8±0.1	3.2±0.2	1.1±0.1
MPO	1.2±0.1	4.6±0.5	1.6±0.1	1.5±0.1	5.8±0.6	2.1±0.2
Hemoglobin	64.6±5.3	223±10.5	79.6±6.7	—	—	—
TNF	ND	86.2±8.0	38.7±4.0	ND	401.3±31	162.5±18.3

Changes in vascular permeability were evaluated by measuring the extravasation of Evans Blue (μg per 100 mg of tissue), neutrophil infiltration was determined by measurement of tissue myeloperoxidase activity, hemorrhage by measuring the concentration of hemoglobin in 100 mg of tissue and concentrations of TNF-α in intestine and lungs by ELISA. UK-74,505 (1 mg kg^{-1}) was given i.v. 5 min prior to reperfusion and control animals received vehicle. Results are shown as picogram of TNF-α per 100 mg of tissue and represent the mean±s.e.m. of six mice in each group. *$P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle-treated mice submitted to I/R.

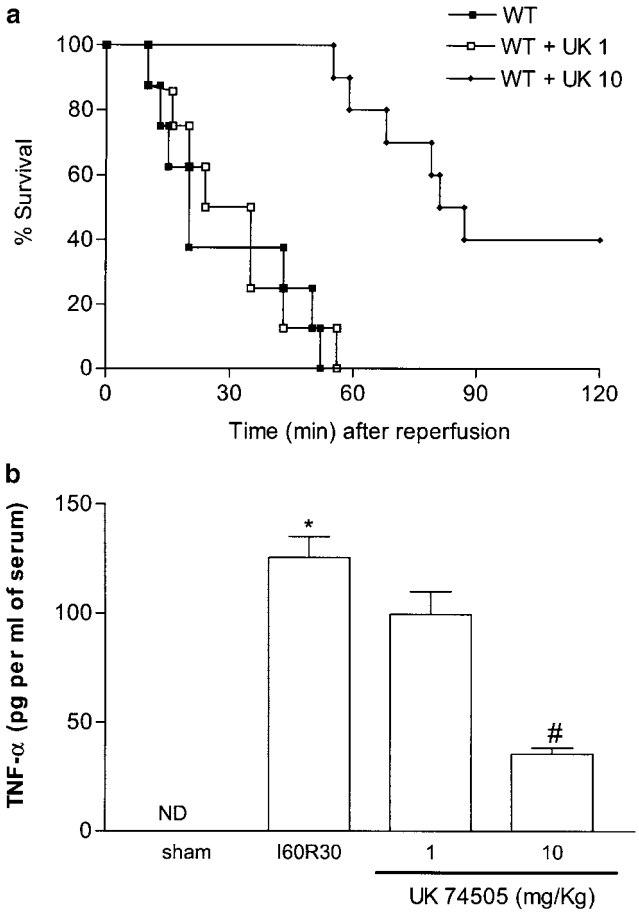

Figure 5 Effects of postischemic treatment with the PAF receptor antagonist UK-74,505 on the (a), survival and (b) serum concentration of TNF-α in mice submitted to ischemia and reperfusion of the SMA. In (a) mice treated with UK-74,505 (1 or 10 mg kg^{-1}) or vehicle ($n=10$ in each group) were anesthetized and submitted to ischemia of the SMA for 60 min. Tissue perfusion was then re-established and survival was monitored. The survival curve of UK-74,505-treated mice (10 mg kg^{-1}) was significantly ($P<0.05$) different from that of vehicle-treated mice. In (b), mice were sham-operated or submitted to 60 min of ischemia of the SMA and reperfusion was allowed for 30 min. Concentrations of TNF-α serum were measured by ELISA. Results are shown as picogram of TNF-α per milliliter of serum and represent the mean ± s.e.m. of six mice in each group. *$P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with vehicle-treated mice submitted to I/R.

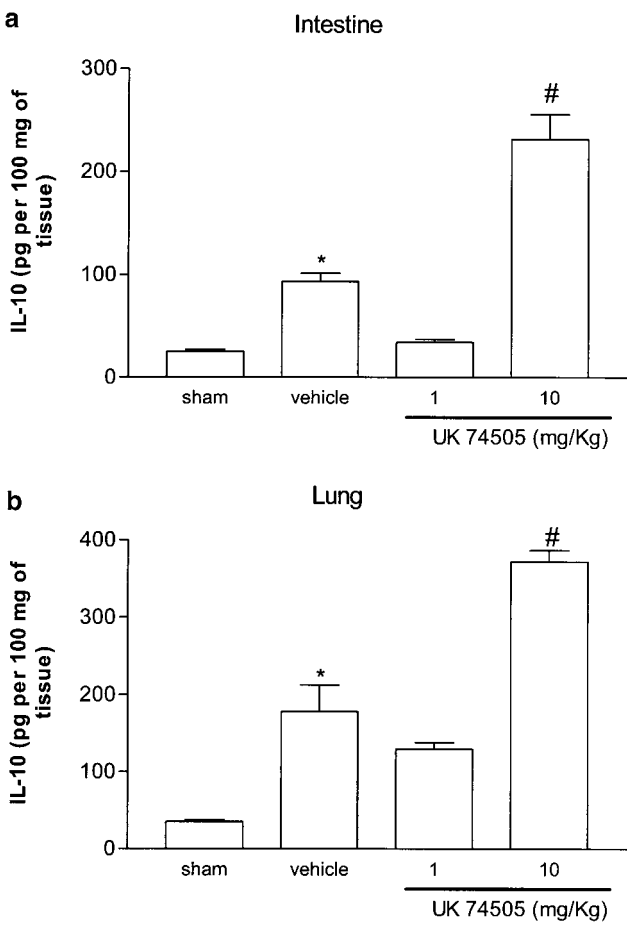

Figure 6 Effects of postischemic treatment with the PAF receptor antagonist UK-74,505 on the concentrations of IL-10 (a) intestine and (b) lungs in mice submitted to ischemia and reperfusion of the SMA. Mice were sham-operated or submitted to 60 min of ischemia of the SMA and reperfusion was allowed for 30 min. UK-74,505 (1 or 10 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animal received vehicle. Concentrations of IL-10 in the intestine (a) and lungs (b) were measured by ELISA. Results are shown as pg of IL-10 per 100 mg of tissue and represent the mean ± s.e.m. of six mice in each group. *$P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when compared with vehicle-treated mice submitted to I/R.

PAFR$^{-/-}$ mice have been shown to have a normal response to LPS administration, but were extremely resistant to antigen-induced systemic anaphylaxis (Ishii *et al*., 1998) and to hydrochloric acid aspiration-induced lung injury (Nagase *et al*., 1999). In addition to playing a role during inflammatory tissue injury caused by different stimuli, we have recently shown that PAFR$^{-/-}$ mice were more susceptible to *Klebsiella pneumoniae* pulmonary infection (Soares *et al*., 2002) and to *Trypanosoma cruzi* infection (Talvani *et al*., 2003). Of interest, the protective role of PAFR during infection appeared to be largely related to the relevance of this receptor in mediating phagocytosis of the bacteria or the parasites (Soares *et al*., 2002; Talvani *et al*., 2003). In the present study, we show that the inflammatory injury that follows intestinal I/R is markedly inhibited in PAFR$^{-/-}$ mice when compared to their wild-type controls. Thus, there was no increase in vascular permeability, neutrophil accumulation and hemorrhage in the intestine and lung of reperfused PAFR$^{-/-}$ mice. In PAFR$^{-/-}$ mice, the reperfusion-associated increases in serum concentration of TNF-α was significantly suppressed and this was associated with an increase in the concentrations of IL-10. Additionally, there was a significant delay in reperfusion-associated lethality in PAFR$^{-/-}$ mice. These results strongly corroborate the role of PAFR during I/R tissue injury and, to the best of our knowledge, are the first demonstration that the PAFR plays a relevant role for reperfusion-associated lethality.

Several studies, including one of our own, have shown that blockade of PAFR with receptor antagonists blocks the inflammatory injuries that occur following I/R of several vascular beds (Canale *et al*., 1994; Carter *et al*., 1996; Riera *et al*., 1997; Qayumi *et al*., 1998; Morgan *et al*., 1999; Kecskemeti & Balogh, 2000; Kim *et al*., 2000; Souza *et al*., 2000b; Sun *et al*., 2001; 2002). It was, thus, of interest to

examine whether pharmacological antagonism of PAFR would prevent tissue injury and lethality, as observed in PAFR$^{-/-}$ mice. To this end, we used the long-acting and selective PAF receptor antagonist, UK-74,505 (Alabaster *et al*., 1991). In rats, UK-74,505 markedly blocked the severe injuries that followed prolonged I/R of the SMA (Souza *et al*., 2000b). Maximal inhibition occurred at the dose of 1 mg kg^{-1}. Similar to our previous studies in rats, postischemic treatment with UK-74,505 suppressed the increase in vascular permeability, neutrophil influx and hemorrhage induced by reperfusion of the ischemic SMA. In addition to inhibiting the above-mentioned parameters, UK-74,505 effectively suppressed reperfusion-induced increases in the concentration of TNF-α in tissues. Overall, these results are in agreement with other studies demonstrating a role for PAFR during I/R injury (Canale *et al*., 1994; Carter *et al*., 1996; Riera *et al*., 1997; Qayumi *et al*., 1998; Morgan *et al*., 1999; Kecskemeti & Balogh, 2000; Kim *et al*., 2000; Sun *et al*., 2001; 2002). In contrast, at the lower dose used, UK-74,505 failed to affect reperfusion-induced increase in serum concentrations of TNF-α and lethality. As neutrophil influx is essential for tissue production of TNF-α (Souza *et al*., 2000b; 2001), the inhibitory effects of UK-74,505 on tissue TNF-α concentrations may be secondary to its ability to suppress neutrophil influx. However, the inability of UK-74,505 to affect serum TNF-α concentrations and lethality was surprising, as we used a previously shown effective dose of the drug (in the present experiments, tissue injury was abolished) and PAFR$^{-/-}$ mice had diminished amounts of serum TNF-α and delayed lethality.

Although tissue injury was abolished by the lower dose of UK-74,505, it was possible that the mechanisms underlying serum TNF-α production and ensuing lethality relied on PAFR not totally inhibited by the lower dose of the drug. In support of this possibility, intracellular PAFR that are relevant for proinflammatory cytokine production have been recently demonstrated on neutrophils (Marrache *et al*., 2002). Thus, it is feasible that higher doses (and hence intracellular concentrations) of UK-74,505 are necessary to block this intracellular receptor. Alternatively and akin to previous studies demonstrating the effects of selectin inhibition during I/R injury, it is possible that a virtually complete blockade of PAFR is necessary if inhibition of systemic TNF-α and lethality is to occur (Kubes *et al*., 1995). Regardless of the underlying explanation for our results, they clearly showed that the use of a higher dose (10 mg kg^{-1}) of UK-74,505 was associated with effective inhibition of serum TNF-α release and delay and prevention of lethality. Interestingly and similar to results seen in PAFR$^{-/-}$ mice, the higher dose of UK-74,505 enhanced the reperfusion-associated increase in tissue concentrations of IL-10. Overall, these results demonstrate that a high dose of PAFR antagonists is needed if we are to delay and/or suppress lethality, in addition to suppressing tissue injury. Whether the need for the use of a high dose of UK-74,505 reflects inhibition of an intracellular PAFR is not known at present, but clearly deserves further investigation. Another relevant observation was that the high dose of UK-74,505 was more effective in preventing lethality than the genetic strategy, suggesting that compensatory mechanisms may be operative in PAFR$^{-/-}$ mice. One alternative explanation to our results was the possibility that UK-74,505 was having an effect distinct from its action on the PAFR. To address this possibility, we administered UK-74,505 (10 mg kg^{-1}) to PAFR$^{-/-}$ mice and evaluated reperfusion-induced lethality. There was no prevention of lethality in addition to that observed in PAFR$^{-/-}$ mice, suggesting that the effects of high-dose UK-74,505 were still related to the ability of the drug to block the PAFR and not because of nonspecific effects.

Finally, the inhibition of reperfusion-associated increases in serum TNF-α concentration and lethality was associated with an enhanced production of IL-10. Other studies have shown that the endogenous IL-10 produced in response to I/R injury may protect the tissue from excess injury (e.g. Frangogiannis *et al*., 2000; Zingarelli *et al*., 2001). Whether the IL-10 produced in response to PAFR blockade or in PAFR$^{-/-}$ mice is relevant for the suppressive effects observed is not known. Moreover, the observation that the enhancement of IL-10 production is only observed when a high dose of UK-74,505 or PAFR$^{-/-}$ mice are used is intriguing and may suggest that the activation of intracellular PAFR may play a role in controlling not only proinflammatory, but also anti-inflammatory, cytokines (Marrache *et al*., 2002). This possibility needs further investigation.

In conclusion, our studies using genetically modified animals and receptor antagonists firmly establish a role of PAFR activation for the local, remote and systemic inflammatory injury and lethality, which follows reperfusion of the ischemic SMA in mice. Whereas tissue injury was inhibited by a lower dose of the PAFR antagonist UK-74,505, lethality was only suppressed when a higher dose of the compound was used. These results suggest that high doses of PAFR antagonists need to be used if the real efficacy of these compounds is to be tested clinically.

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