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APT070 (Mirococept), a membrane-localised complement inhibitor, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury

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1 Activation of the complement system has been shown to play a major role in the mediation of reperfusion injury. Here, we assessed the effects of APT070 (Mirococept), a novel membrane-localised complement inhibitor based on a recombinant fragment of soluble CR1, on the local, remote and systemic injuries following intestinal ischaemia and reperfusion (I/R) in the rat.

2 In a model of mild I/R injury (30 min of ischaemia and 30 min of reperfusion), APT070 dose-dependently (1–10 mg kg^{-1}) inhibited the increase in vascular permeability of and neutrophil influx into intestine and lungs. Maximal inhibition occurred at 10 mg kg^{-1}.

3 Following severe I/R injury (120 min of ischaemia and 120 min of reperfusion), APT070 (10 mg kg^{-1}) markedly prevented neutrophil influx and the increase in vascular permeability both in the intestine and the lungs.

4 APT070 also effectively suppressed the increase of tissue (intestine and lungs) and serum concentrations of TNF-α and IL-6, but not those of IL-1β or IL-10. There was no significant reduction of mortality in the APT070 group.

5 In conclusion, treatment with the membrane-targeted complement inhibitor APT070 significantly reduced the hyperinflammatory response after mild and severe ischaemia and reperfusion injury (I/RI) in rats. APT070 may be effective in therapeutic indications involving gut I/RI.
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Abbreviations: I/RI, ischaemia and reperfusion injury; MPO, myeloperoxidase; sCR1, soluble complement receptor 1; SMA, superior mesenteric artery

Introduction

Intestinal ischaemia and reperfusion injury (I/RI) can occur to varying degrees during a number of clinically relevant situations, such as trauma accompanied by major blood loss, aortic aneurysm surgery, necrotic enterocolitis and cardiogenic shock (Wada *et al*., 2001). I/RI of tissues, especially of the intestine, has been shown to cause a significant inflammatory response (Welborn *et al*., 2000; Chan *et al*., 2003; Arumugam *et al*., 2004). Complement activation is a very early part of this process, as demonstrated by mechanistic studies, clinical investigations and animal models using complement inhibitors (Hill *et al*., 1992; Xiao *et al*., 1997a, b; Fosse *et al*., 1998; Fruchterman *et al*., 1998; Heller *et al*., 1999; Wada *et al*., 2001; Zhao *et al*., 2002). Local and remote damage are caused by the formation of the membrane attack complex (MAC) and through neutrophil activation *via* C3a and C5a as well as through cytokine signalling. Crosstalk between the complement cascade and proinflammatory cytokines – such as IL-6 and TNF-α – has been shown (Deboer & Clark, 1992; Hecke *et al*., 1997; Blatteis *et al*., 2004; Riedemann *et al*., 2004), and sublytic MAC concentrations have been demonstrated to be responsible for the synthesis and release of further mediators of inflammation, such as arachidonic acid metabolites (Mollnes & Fosse, 1994) and von Willebrand's factor (Fruchterman *et al*., 1998). Activated neutrophils have been identified as mediators of both local and remote organ injury after ischaemia and reperfusion of hind limbs (Kyriakides *et al*., 1999; Merchant *et al*., 2003), liver (Jaeschke *et al*., 1990), intestine (Xiao *et al*., 1997a, b; Souza *et al*., 2000a, b), kidney (Weight *et al*., 1996) and myocardium (Baxter, 2002; Kohtani *et al*., 2002). Complement inhibition could therefore be an attractive therapeutic opportunity for procedures involving I/RI, especially if early intervention is possible.

APT070 (Mirococept) is a complement inhibitor currently under development for treatment of rheumatoid arthritis and I/RI. It consists of the first three short consensus domains of human complement receptor 1, manufactured in recombinant bacteria and modified with a membrane-targeting amphiphilic peptide based on the naturally occurring membrane-bound myristoyl-electrostatic switch peptide (Smith & Smith, 2001; Smith, 2002). Derivatisation of a payload with such a peptide increases the affinity for cell surfaces, and as complement activation takes place predominantly on surfaces, APT070 showed significantly higher activity than the underivatised

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protein in *in vitro* assays of complement inhibition (Smith, 2002).

In this work, we investigated whether APT070 could attenuate the hyperinflammatory response caused by gut I/RI. In particular, we investigated the effect of APT070 administration prior to reperfusion on neutrophil infiltration, cytokine levels and vascular leakage into gut and lungs.

Methods

Animals

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

Ischaemia and reperfusion injury

Rats were anaesthetised with urethane (1400 mg kg^{-1}, i.p.) and laparotomy was performed. This procedure was sufficient to keep the animals under anaesthesia until the end of the experiment. 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 injury) or 120 (severe injury) 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 were used as controls for the reperfusion-induced injury, and, at the end of the experiment, animals were killed by cervical dislocation. Inflammatory parameters were assessed only in animals that were alive at 120 min after reperfusion. For measuring percentage of surviving rats, reperfusion was re-established, and rats were monitored for indicated time periods.

Initial experiments were carried out in the mild reperfusion injury model to examine the dose-dependent effects of APT070 (1 to 10 mg kg^{-1}). In these experiments, APT070 was administered i.v. 15 min prior to the reperfusion of the SMA. We then tested the effects of the administration of APT070 (10 mg kg^{-1}, i.v., 15 min prior to reperfusion) in the severe reperfusion injury model. An equivalent volume of APT070 vehicle, solution of phosphate-buffered saline (PBS) containing mannitol 50 mg ml^{-1} and arginine 0.2 mg ml^{-1} was used for comparison in both the mild and severe model.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability (Souza *et al*., 2000a). 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. At 30 (in the mild injury model) or 120 min (in the severe injury model) after reperfusion, 5 cm-fragments of the first portion of the intestine were cut open and allowed to dry in a Petri dish for 24 h at 37°C. The dry weight of the tissue was determined 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 optical density (OD) of the extract 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 in μg per 100 mg of tissue. The right ventricle was flushed with 20 ml of 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 (MPO) as described below.

MPO concentrations

The extent of neutrophil accumulation in fragment of intestines (different from Evans Blue fragment) and the flushed right lung tissue was measured by assaying MPO activity, as previously described (Souza *et al*., 2002b). Results were expressed as Neutrophil index that denotes activity of MPO related with casein-elicited murine peritoneal neutrophils processed in the same way.

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 levels

The levels of haemoglobin in tissues were used as an index of tissue haemorrhage. Tissues were carefully washed with excess saline to remove blood attached to the intestinal epithelia or serosa. No attempt was made to perfuse the vessels with saline as no obvious hyperaemia was present. After washing, a sample of approximately 100 mg of intestine was removed and homogenised in Drabkin's colour 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 haemoglobin.

Measurement of cytokine levels in serum, intestine and lungs

TNF-α, IL-1β, IL-6 and IL-10 levels were measured in serum, intestine and lung of animals using ELISA techniques previously described (Hagan *et al*., 1993; Rees *et al*., 1999a, b; Francischi *et al*., 2000). 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 : 3 dilution in PBS. In total, 100 mg of intestine

or lung of sham-operated and reperfused animals were homogenised in 1 ml of PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing antiproteinases (0.1 mM PMSF, 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 dilution in PBS. ELISA plates (Nunc MaxiSorb) were coated with a sheep anti-rat TNF-α/IL-1β/IL-6 or IL-10 polyclonal antibodies (1–2 $\mu g\,ml^{-1}$) 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 $pg\,ml^{-1}$.

Reagents

The following reagents were obtained from Sigma (U.S.A.): urethane, Evans blue, hexadecyltrimethylammonium bromide, 3,3,5,5, tetramethyl-benzidine. APT070 (Mirococept) consists of the first three short consensus domains of human complement receptor 1, manufactured in recombinant bacteria and modified with a membrane-targeting amphiphilic peptide based on the naturally occurring membrane-bound myristoyl-electrostatic switch peptide (Smith & Smith, 2001; Smith, 2002). The vehicle for APT070 was a solution of PBS containing mannitol 50 $mg\,ml^{-1}$ and arginine 0.2 $mg\,ml^{-1}$.

Statistical analysis

Results are shown as the mean ± s.e.m. Differences were evaluated by using analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* analysis. Results with a $P<0.05$ were considered significant. For survival curves, differences between groups at different time points were compared using Fisher's exact test and considered significant when $P<0.05$.

Results

Dose-dependent effects of APT070 in a model of mild I/R injury

A significant reduction of vascular leakage could be observed at doses as low as 1 $mg\,kg^{-1}$ (lungs) or 3 $mg\,kg^{-1}$ (intestine) (Figure 1). At 10 $mg\,kg^{-1}$, vascular leakage and MPO levels were greatly reduced by APT070. Indeed, levels found in the latter animals were not statistically different from sham-operated controls ($P>0.05$). The potency of APT070 to prevent vascular leakage and neutrophil influx in intestine was comparable to that in the lung (Figure 1).

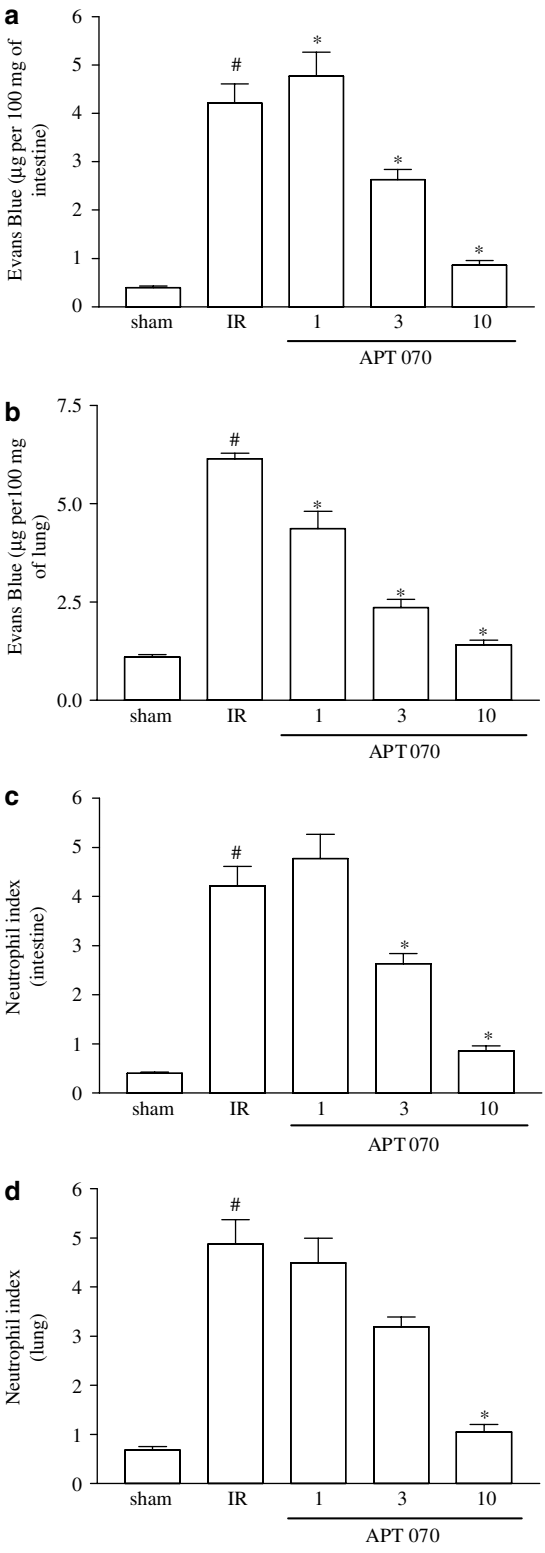

Figure 1 Dose-dependent effects of the treatment with APT070 on the increase in vascular permeability and recruitment of neutrophils in the intestine and lungs following mild ischaemia (30 min) and reperfusion (30 min) injury of the superior mesenteric artery. Changes in vascular permeability in the (a) intestine and (b) lungs were assessed by evaluating the extravasation of Evans blue dye. Neutrophil recruitment in the (c) intestine and (d) lungs was assessed by evaluating tissue levels of myeloperoxidase. APT070 (1–10 $mg\,kg^{-1}$) was given i.v. 15 min prior to reperfusion. Control animals (I/R) received vehicle. Results are shown as μg Evans blue per 100 mg of tissue or the Neutrophil index and are the mean ± s.e.m. of at least 5–6 animals in each group. # for $P<0.01$ when compared to sham-operated animals and * for $P<0.05$ when compared to mild I/R animals.

Effects of APT070 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, leucopoenia, increase in the levels of cytokine in tissue and blood and significant lethality (Souza *et al*., 2000b).

For the experiments evaluating the role of APT070 during severe I/R injury, the drug was used at a dose shown to be the maximally inhibitory in the mild I/R injury model (10 mg kg^{-1}). Post ischaemic treatment with APT070 markedly suppressed the increase in vascular permeability and neutrophil recruitment in the intestine and in the lung following severe I/R injury (Figure 2). Treatment with APT070 also suppressed the intestinal increase of haemoglobin, a marker of tissue haemorrhage (Figure 2).

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). The concentration of circulating neutrophils at 120 min of ischaemia was similar and markedly greater in both APT070 and vehicle-treated than sham-operated animals (Figure 3). This is consistent with the administration of APT070 at the end of the ischaemic period. In vehicle-treated animals, reperfusion of the ischaemic SMA induced a rapid fall of circulating neutrophils to levels observed in sham-operated

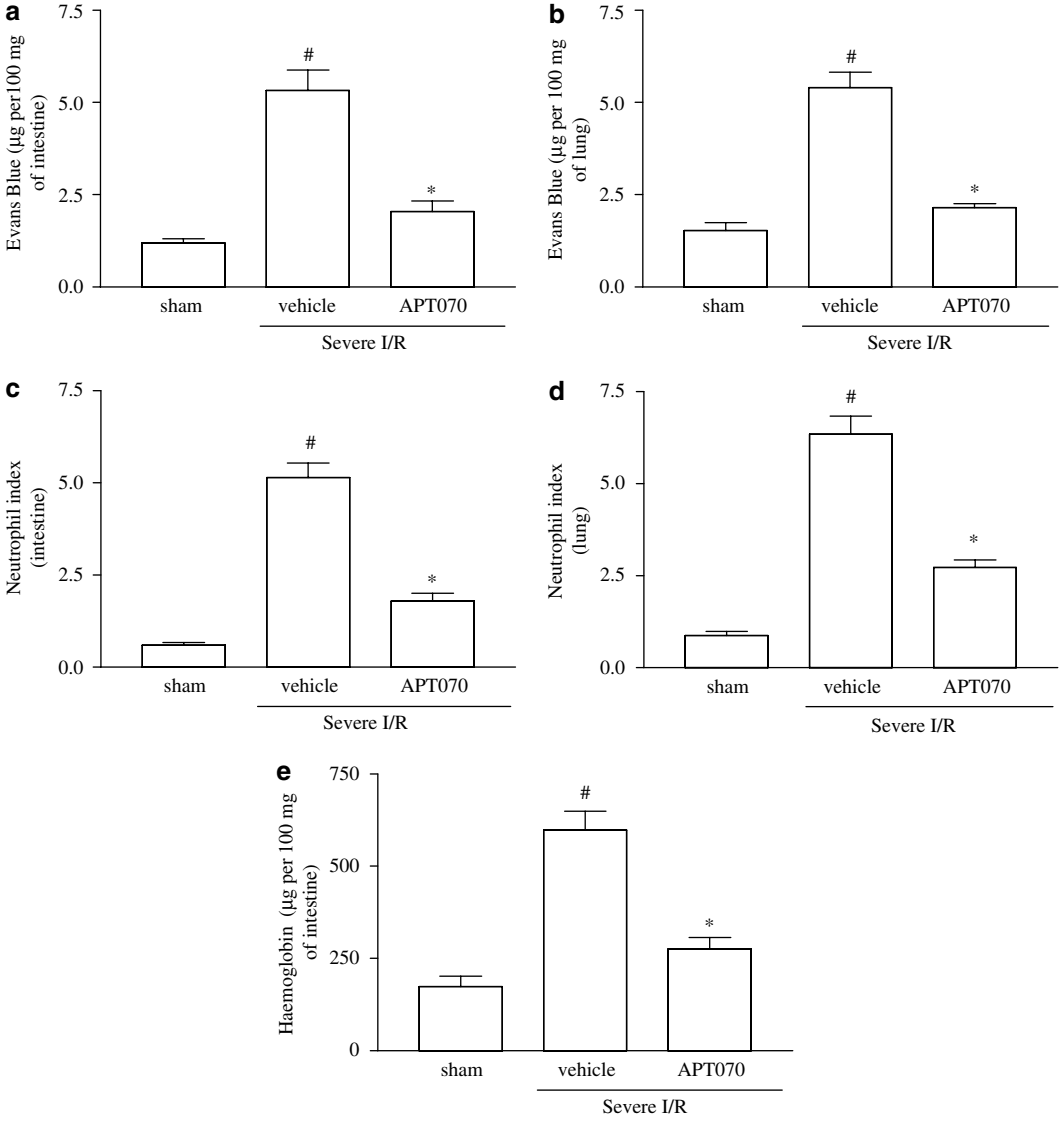

Figure 2 Effects of the treatment with APT070 on the increase in vascular permeability and recruitment of neutrophils in the intestine and lung and haemorrhage observed in the intestine following severe ischaemia (120 min) and reperfusion (120 min) injury of the superior mesenteric artery. Changes in vascular permeability in the (a) intestine and (b) lungs were assessed by evaluating the extravasation of Evans blue dye. Neutrophil recruitment in the (c) intestine and (d) lungs was assessed by evaluating tissue levels of myeloperoxidase. (e) Tissue haemorrhage was assessed by evaluating tissue levels of haemoglobin. APT070 (10.0 mg kg^{-1}) was given i.v. 15 min prior to reperfusion. Control animals received vehicle. Results are shown as μg Evans blue per 100 mg of tissue or the Neutrophil index and are the mean $\pm$ s.e.m. of 5–6 animals in each group. # for $P<0.01$ when compared to sham-operated animals and * for $P<0.05$ when compared to vehicle I/R animals.

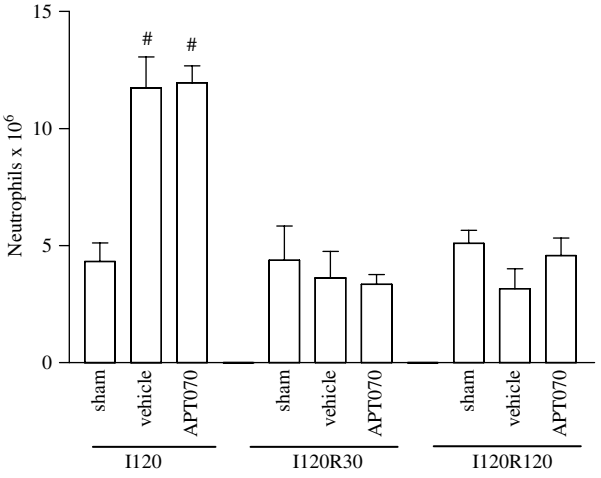

Figure 3 Effects of the treatment with APT070 on the concentration of circulating neutrophils following severe ischaemia (120 min) and reperfusion (120 min) of the superior mesenteric artery. Neutrophils were counted in May–Grunwald–Giensa-stained blood smears just prior to (I120 R0) and at 30 (I120 R30) and 120 (I120 R120) min after reperfusion. APT070 (10.0 mg kg^{-1}) was given i.v. 15 min prior to reperfusion. Control animals received vehicle. Results are shown as the number of neutrophils $\times 10^6$ per μl of blood and are the mean $\pm$ s.e.m. of six animals. * for $P<0.01$ when compared to sham-operated animals.

animals. Pretreatment with APT070 did not reverse the neutropaenia that occurred 30 or 120 min after reperfusion (Figure 3).

The levels of proinflammatory cytokines – IL-1β, IL-6 and TNF-α – and of the anti-inflammatory cytokine IL-10 are markedly elevated in serum and tissues after severe I/R injury (Figure 4, Table 1) (Souza *et al*., 2000b). Postischaemic treatment with APT070 significantly inhibited the elevations of TNF-α in tissue and serum after severe I/R injury (Figure 4a, c and e). APT070 had no significant effects on the reperfusion-induced changes in the concentrations of IL-1β and IL-10 in serum or tissues (Table 1).

Our previous studies have shown that severe reperfusion injury is accompanied by significant TNF-α-dependent lethality, reaching 60% in most experiments (Souza *et al*., 2001). Treatment with APT070 failed to alter the reperfusion-induced lethality significantly (Figure 5).

Discussion

Complement activation is part of the first line of defence against invading organisms, a humoral cascade leading to very early immune system activation and direct attack through the formation of the porous MACs. In the context of autoimmune diseases, complement likewise constitutes the first line of hyperinflammation and might thus contribute to the development of detrimental reactions at a very early stage (Fosse *et al*., 1998; Fruchterman *et al*., 1998; Arumugam *et al*., 2004). Complement inhibition is therefore an especially promising therapeutic approach in indications where the inhibitor can be administered in the early phase of hyperinflammation.

The complement inhibitor APT070, being derived from the first three short consensus repeats ('site 1') of human CR1, has been shown to be a strong inhibitor at the levels of the C3 and C5 convertases, especially following activation mediated by the classical pathway (Mossakowska *et al*., 1999). Previous studies have shown that I/RI of the viscera involves mainly the classical or lectin pathway of complement activation (Woodcock *et al*., 2000; Fiane *et al*., 2003). Use of APT070 has the potential to attenuate the hyperinflammatory response through reduction of local damage caused by MAC as well as reduction of neutrophil activation directly through inhibition of C3a/C5a release and indirectly through inhibition of the complement-mediated release of cytokines. The importance of neutrophils in the mediation of intestinal I/RI has been demonstrated in a number of studies in rats (Ma *et al*., 1993; Lefer *et al*., 1996; Omata *et al*., 1997; Ritter *et al*., 1998; Souza *et al*., 2000a, b; Onai *et al*., 2003). In this study, we investigated whether treatment with APT070 prior to reperfusion resulted in improvement of the hyperinflammatory response and tissue injury.

Although APT070 was not able to reverse the drop in the number of circulating neutrophils in this model, it significantly reduced the tissue infiltration into gut and lung and the associated vascular leakage. This indicates that the main therapeutic effect in this model was on the neutrophil emigration rather than the mobilisation. Following aortic aneurysm surgery, it has been hypothesised that it is the pool of neutrophils already residing in the lung vasculature that is responsible for causing damage (Brown *et al*., 2003), and it is possible that this pool is being mobilised by complement activation in this model.

Inhibition of the complement cascade by treatment with APT070 resulted in significantly decreased concentration of at least two proinflammatory cytokines, TNF-α and IL-6. This is further evidence for the action of the complement cascade as a very early or potentially first step in the mediation of I/RI. The existence of amplification loops between complement and cytokines has been demonstrated previously (Deboer & Clark, 1992; Hecke *et al*., 1997; Blatteis *et al*., 2004; Riedemann *et al*., 2004). Interestingly, complement inhibition did not have an effect on IL-1 levels in serum or tissues. Additionally, APT070 did not alter the concentrations of IL-10 in lung following severe reperfusion-associated injury. We have previously shown that IL-10 was a major protective endogenous cytokine during ischaemia and reperfusion injury in rats and that IL-1β was a major force driving IL-10 production (Souza *et al*., 2003a). Thus, it is clear that inhibition of complement activation inhibited reperfusion injury by a mechanism other than IL-10 production. In APT070-treated animals, there was a marked inhibition of both TNF-α production and neutrophil infiltration, which is in agreement with the existence of an amplification loop between TNF-α production and neutrophil infiltration (Souza *et al*., 2000b; 2001). The shown ability of APT070 to modulate both neutrophil influx and tissue TNF-α production could be contributing to the beneficial effects of the drug in the system.

Our previous studies have suggested that the concentration of TNF-α in serum was a predictor of mortality following severe intestinal I/RI. In support of the latter possibility, strategies that prevented the elevation of TNF-α in serum were associated with protection from lethality (Souza *et al*., 2001; 2002a), whereas those associated with no or only partial inhibition of TNF-α provided no protection (Souza *et al*., 2002b; 2003b). Moreover, usage of anti-TNF-α antibodies or experiments in TNFR1 (p55)-deficient mice clearly demonstrate a role for TNF-α in mediating lethality in the system

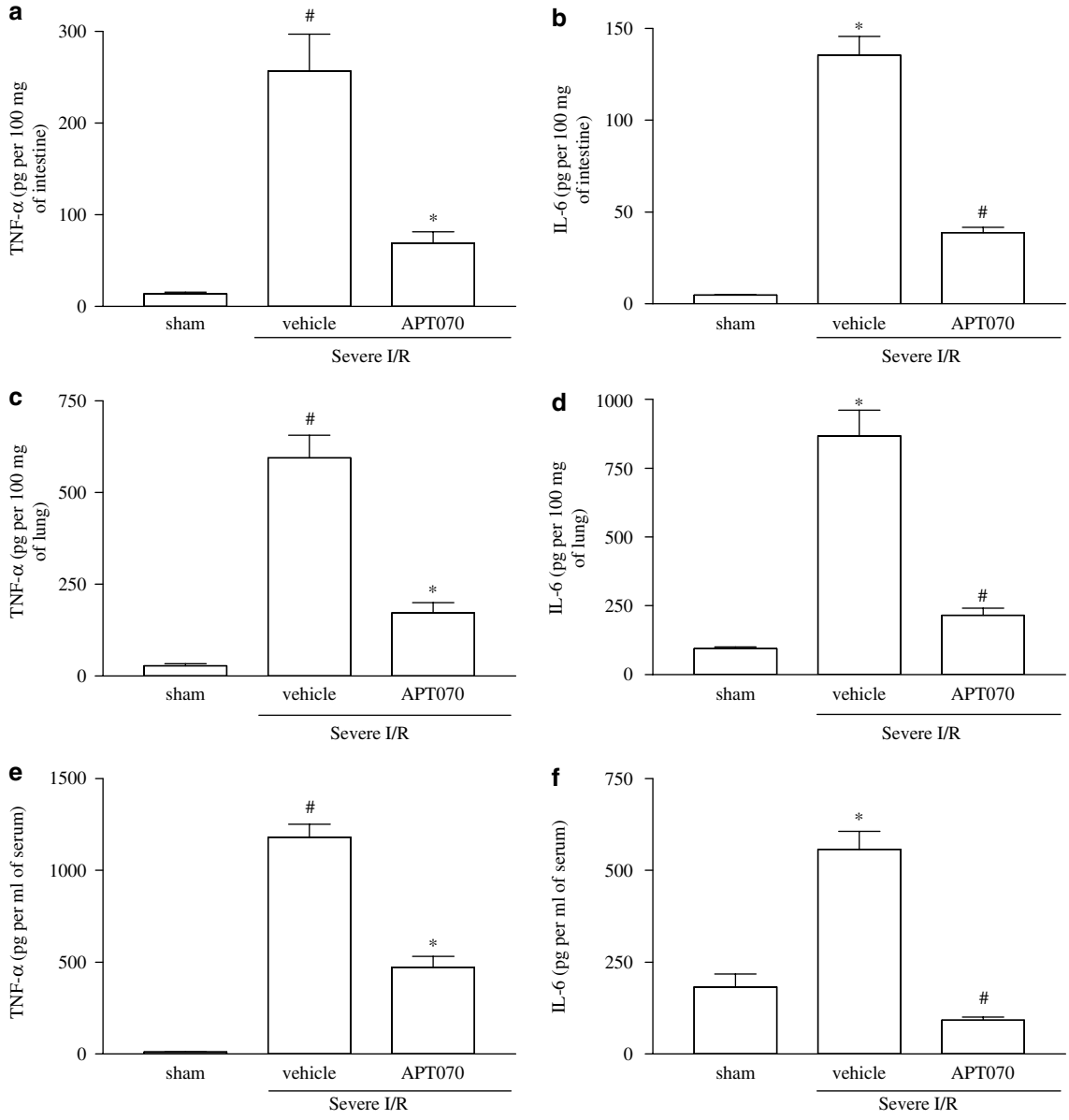

Figure 4 Effects of the treatment with the APT070 on the concentrations of TNF-α and IL-6 in the intestine, lung and serum following severe ischaemia (120 min) and reperfusion (120 min) of the superior mesenteric artery. The concentration of TNF-α (a, c, e) and IL-6 (b, d, f) were assessed in the intestine (a, b), lung (c, d) and serum (e, f) by using specific ELISA. APT070 (10.0 mg kg^{-1}) was given i.v. 15 min prior to reperfusion. Control animals received vehicle. Results are shown as pg TNF-α or IL-6 per ml of serum or as pg TNF-α or IL-6 per 100 mg of tissue and are the mean ± s.e.m. of 5–6 animals in each group. * for $P<0.01$ when compared to sham-operated animals and # for $P<0.05$ when compared to severe I/R animals.

Table 1 Effects of the treatment with the APT070 on the concentrations of IL-1β and IL-10 in the intestine, lung and serum following severe ischemia (120 min) and reperfusion (120 min) of the superior mesenteric artery

	IL-1β			*IL-10*		
	Intestine	*Lung*	*Serum*	*Intestine*	*Lung*	*Serum*
Sham	92 ± 9	203 ± 35	23 ± 4	141 ± 21	48 ± 3	261 ± 24
IR + Vehicle	150 ± 27[a]	1219 ± 96[a]	128 ± 14[a]	321 ± 46[a]	400 ± 21[a]	1816 ± 152[a]
IR + APT070	131 ± 24	1404 ± 126	149 ± 22	286 ± 53	436 ± 76	1352 ± 220

[a]For $P<0.05$ when compared to sham-operated animal.
IL-1β and IL-10 were assessed by using specific ELISA. APT070 (10.0 mg kg^{-1}) was given i.v. 15 min before reperfusion. Control animals received vehicle. Results are shown as pg cytokine per ml of serum or as pg cytokine per 100 mg of tissue and are the mean ± s.e.m. of 5–6 animals in each group.

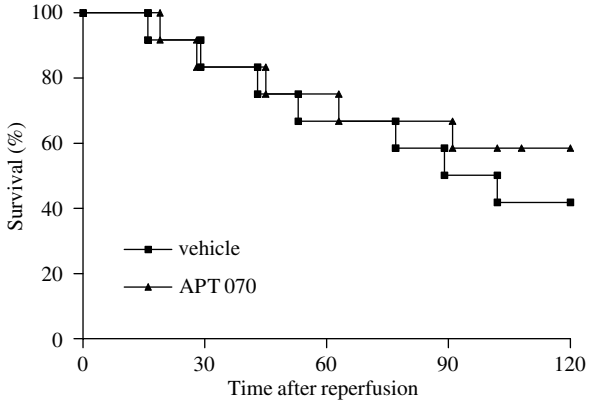

Figure 5 Effects of the treatment with APT070 on the lethality following severe ischaemia and reperfusion of the superior mesenteric artery. APT070 (10.0 mg kg^{-1}) was given i.v. 15 min prior to reperfusion. Control animals received vehicle. Survival was monitored as indicated and animals were killed after 120 min.

(Souza *et al*., 2002a). In APT070-treated animals, there was only a partial, albeit significant, reduction of the concentration of serum TNF-α. However, the partial suppression of the production of TNF-α was insufficient for modify reperfusion-induced lethality. It is likely that greater suppression of TNF-α is necessary for the lethality to be prevented. A study conducted by Hill *et al*. (1992) previously demonstrated a degree of efficacy of full-length sCR1 in a milder model of gut I/RI. Following 1 h of ischaemia and 3 h of reperfusion, sCR1 administered 15 min prior to reperfusion was successful in ameliorating some of the tissue injuries, although it did not seem to have any effect on neutrophil infiltration, in contrast to our findings with the modified sCR1 fragment APT070. Of note, sCR1 significantly improved mortality in the previous study; however, this might be due to the fact that the sCR1 experiment analysed 5-day mortality (compared to the probably more acute 2 h end point) following a less severe ischaemic insult of 1 h (compared to 2 h).

In conclusion, this study demonstrates that complement inhibition was effective in improving local and remote tissue damage and the systemic inflammatory response in a model of severe gut I/RI. Although the benefits did not translate into a statistically significant improvement of acute mortality, the fact that both cytokine levels and tissue damage parameters were considerably improved indicates that complement inhibition in indications associated with gut I/RI – such as haemorrhagic shock and aortic aneurysm surgery – might be effective in reducing the hyperinflammatory response that leads to downstream single and multiple organ failure and, thus, contribute significantly to nonacute mortality.

As gut ischaemia is one of the major components during traumatic haemorrhagic shock, the inhibition of haemorrhage in the form of vascular leakage could also pose an additional, more immediate benefit supporting resuscitation efforts and reducing the amount of resuscitation fluid required to stabilise the patient. Apart from a faster normalisation of blood parameters, the administration of large volumes of blood or blood replacement fluids has been correlated with negative outcome, possibly through additional immune modulation (Malone *et al*., 2003), and therefore a reduction in the amount of fluid administered might prove beneficial.

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