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Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury

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1 Reperfusion of an ischaemic tissue is associated with an intense inflammatory response and inflammation-mediated tissue injury. Physalins, a group of substances with secosteroidal chemical structure, are found in *Physalis angulata* stems and leaves. Here, we assessed the effects of physalins on the local, remote and systemic injuries following intestinal ischaemia and reperfusion (I/R) in mice and compared with the effects of dexamethasone.

2 Following I/R injury, dexamethasone ($10\,mg\,kg^{-1}$) or physalin B or F markedly prevented neutrophil influx, the increase in vascular permeability in the intestine and the lungs. Maximal inhibition occurred at $20\,mg\,kg^{-1}$. Moreover, there was prevention of haemorrhage in the intestine of reperfused animals.

3 Dexamethasone or physalins effectively suppressed the increase in tissue (intestine and lungs) and serum concentrations of TNF-α. Interestingly, treatment with the compounds was associated with enhancement of IL-10.

4 The anti-inflammatory effects of dexamethasone or physalins were reversed by pretreatment with the corticoid receptor antagonist RU486 ($25\,mg\,kg^{-1}$). The drug compounds suppressed steady-state concentrations of corticosterone, but did not alter the reperfusion-associated increase in levels of corticosterone. The IL-10-enhancing effects of the drugs were not altered by RU486.

5 In conclusion, the *in vivo* anti-inflammatory actions of physalins, natural steroidal compounds, appear to be mostly due to the activation of glucocorticoid receptors. Compounds derived from these natural secosteroids may represent novel therapeutic options for the treatment of inflammatory diseases.
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Abbreviations: I/R, ischaemia and reperfusion; SMA, superior mesenteric artery; MPO, myeloperoxidase

Introduction

Glucocorticoid actions are mediated by the glucocorticoid receptor (GR), which is a member of a superfamily of ligand-inducible transcription regulators for steroids and thyroid hormones. Upon binding to its ligands, the GR alters both cellular metabolism and gene expression (Beato & Klug, 2000). These receptors may cause the suppression of immune-inflammatory responses, exerting their effects in a variety of mammalian cells, including macrophages and lymphocytes. Several effects of glucocorticoids are secondary to their capacity of inhibiting the production of mediators of inflammation, including prostaglandins, leukotrienes, acute-phase reactants and several cytokines (Almawi *et al*., 1996; 1998; Mori *et al*., 1997; Paul-Clark *et al*., 2000).

A group of substances with secosteroidal chemical structure, known as physalins (Figure 1), are found in *Physalis angulata* stems and leaves. This plant is widely distributed throughout tropical and subtropical regions of the world. Extracts or infusions from *P. angulata* have been used in popular medicine as a treatment for a variety of illnesses. Physalins have been described as having potent antimycobacteria and antitumoral effects (Chiang *et al*., 1992a, b; Lin *et al*., 1992). A more recent study has demonstrated that physalins may also have anti-inflammatory activities in macrophages, as assessed by a marked inhibitory action on NO production and prevention of the lethality associated with lipopolysaccaride injection in mice (Soares *et al*., 2003).

Reperfusion of an ischaemic tissue is associated with an intense inflammatory response characterized by local and systemic leucocyte activation and trafficking, endothelial

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barrier dysfunction in postcapillary venules, enhanced production of inflammatory mediators and lethality (Lefer & Lefer, 1996; Granger 1999; Carden & Granger 2000). For example, after intestinal ischaemia 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). As there are several studies demonstrating that glucocorticoids may effectively suppress I/R injury (for example, Duran & Dillon, 1989; Engelman *et al*., 1989; Hafezi-Moghadam *et al*., 2002) and physalins have a steroidal chemical structure, the objectives of the present study were three-fold: (i) to confirm the anti-inflammatory effects of glucocorticoids in a model of intestinal I/R injury; (ii) to evaluate the effects of two different physalins, physalins B and F, in the model; and (iii) to verify whether physalins act *via* GRs to exert their anti-inflammatory actions.

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.

Ischaemia and reperfusion

Mice were anaesthetized with urethane (1400 mg kg^{-1}, intraperitoneally) and laparotomy was performed. The superior mesenteric artery (SMA) was isolated and ischaemia was induced by totally occluding the SMA for 60 min. Reperfusion was allowed to occur for 30 min (I60R30) when mice were killed and tissues and blood obtained for the analysis of several parameters (described below). This time of reperfusion (30 min) was based on previous experiments showing the presence of significant tissue injury without high mortality rates. Sham-operated animals were used as controls. Physalins (0.2–20 mg kg^{-1}) or vehicle (DMSO 1% in sterile saline) was administered (subcutaneously (s.c.)) 45 min before reperfusion; dexamethasone or vehicle (saline) was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 or vehicle (DMSO 10% in sterile saline) were administered 15 min prior to dexamethasone or physalins.

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 described previously (Souza *et al*., 2000a). Evans blue (20 mg kg^{-1}) was administered intravenously (1 ml kg^{-1}) *via* a tail vein 2 min prior to reperfusion of the ischaemic 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 the Evans blue content was extracted using 1 ml of formamide (24 h at room temperature) and quantified 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 in μg of Evans blue per 100 mg of dry 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 (MPO) as described below.

MPO concentrations

The extent of neutrophil accumulation in the intestine and right lung tissue was measured by assaying MPO activity, as described previously (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 at 450 nm using tetramethylbenzidine. Results were expressed as the neutrophil index that denotes the activity of MPO correspondent to that of a given number of casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of haemoglobin concentrations

The determination of haemoglobin concentrations in tissue was used as an index of tissue haemorrhage. After washing the intestines to remove excess blood, a sample of approximately 100 mg of duodenum was excised and homogenized 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 × *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/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, U.S.A.). 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 all, 100 mg of duodenum or lung of sham-operated or reperfused animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing antiproteases (0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween-20. The samples were then centrifuged of 10 min at 3000 × *g* and the supernatant immediately used for ELISA assays at a 1 : 3 dilution in PBS.

Detection of corticosterone

The radioimmunoassay for corticosterone was conducted in duplicate, using an antibody obtained from Sigma (St Louis, MO, U.S.A.) and (^{3}H)-corticosterone from New England Nuclear (Boston, MA, U.S.A.). The method was adapted from Sarnyai *et al*. (1992). Briefly, 20 μl of plasma was diluted 50 times with 0.01 M PBS and placed in a water bath at 75°C for 1 h to heat inactivate the corticosterone plasma globulin.

A measure of 100 μl of a solution of antibody and (^{3}H)-corticosterone (10,000–20,000 c.p.m. ml^{-1}) was added to each sample, which was then mixed and incubated overnight at 4°C. Dextran-coated charcoal was used to adsorb free steroid after incubation. The tubes were centrifuged at 2000 × *g* for 15 min at 4°C, the supernatant from each tube was transferred to scintillation vials and the radioactivity was quantified by liquid scintillation spectrometry. Standard curves were constructed using 10, 25, 50, 100, 250, 500, 750, 1000 and 2000 pg 100 μl^{-1} of corticosterone (Sigma, St Louis, U.S.A.). Inter- and intra-assay variation was 4.0 and 7.2%, respectively.

Drugs and reagents

The following drugs were obtained from Sigma (St Louis, MO, U.S.A.): urethane, Evans blue, hexadecyltrimethylammonium bromide and RU486. Physalins B and F (Figure 1) were obtained from extraction of the stems of *P. angulata* L. following the procedure described in the patents BRPI 9904635 and US 10/403.003. The purity of the compounds was greater than 97%.

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 followed by Student–Newman–Keuls *post hoc* analysis. Results with a $P<0.05$ were considered significant.

Results

Dexamethasone inhibits intestinal I/R injury in mice

The first series of experiments was performed with the aim of confirming the role of glucocorticoids in a model of intestinal I/R in mouse. In this model, in addition to the severe local (intestine) and remote (lung) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx, haemorrhage and release of cytokines, there are clear signs of systemic inflammation, as unveiled by circulating levels of TNF-α. At the dose of 10 mg kg^{-1}, but not at 4 mg kg^{-1}, dexamethasone treatment almost completely prevented reperfusion-induced tissue injury, as assessed by the increase in vascular permeability (Figure 2), neutrophil accumulation (Figure 3) and haemorrhage (Figure 4). Similarly, the reperfusion-induced increases in serum and tissue TNF-α concentrations were markedly suppressed by dexamethasone treatment (Figure 5). In contrast, treatment with dexamethasone enhanced the reperfusion-associated release of IL-10 in the intestine and lungs (Figure 6).

Treatment with the steroid receptor antagonist RU486 reversed the protective effects of dexamethasone on reperfusion-induced in vascular permeability (Figure 2), neutrophil accumulation (Figure 3), haemorrhage (Figure 4) and TNF-α

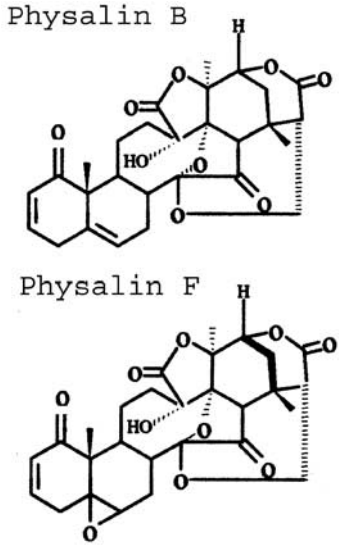

Figure 1 Chemical structure of physalins B and F.

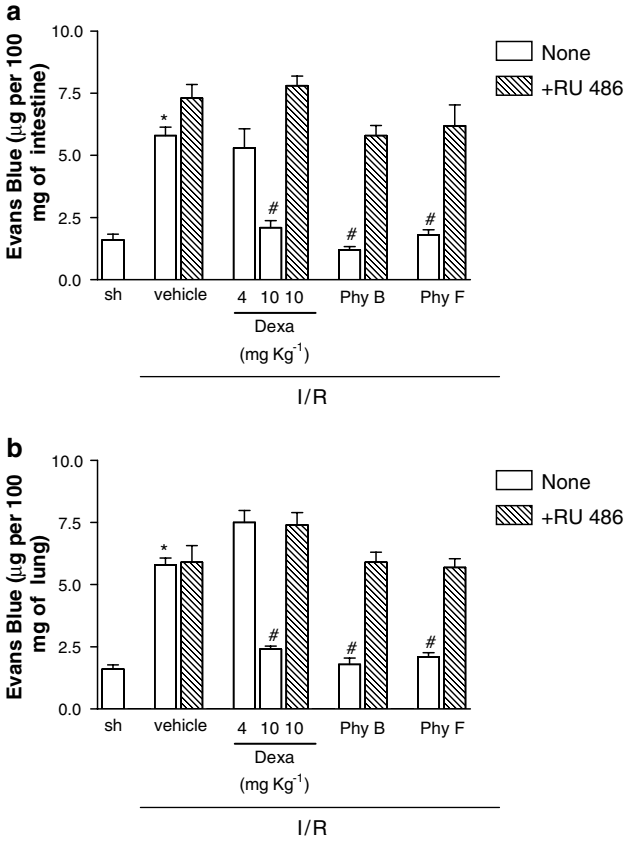

Figure 2 Dexamethasone or physalins prevent the increase of vascular permeability in mice submitted to I/R. This effect was reversed by treatment with RU486. Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. Changes in vascular permeability in the intestine (a) and lungs (b) were evaluated by measuring the extravasation of Evans Blue (μg per 100 mg of tissue). Physalins (20 mg kg^{-1}) or vehicle was administered (s.c.) 45 min before reperfusion, and dexamethasone (4 or 10 mg kg^{-1}) or vehicle was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 (25 mg kg^{-1}) or vehicle were administered 15 min prior to dexamethasone or physalins. Data are shown as the mean ± s.e.m. of five to six mice in each group. $^*P<0.01$ when comparing to the sham-operated group and $^{\#}P< 0.01$ when comparing to vehicle mice submitted to I/R.

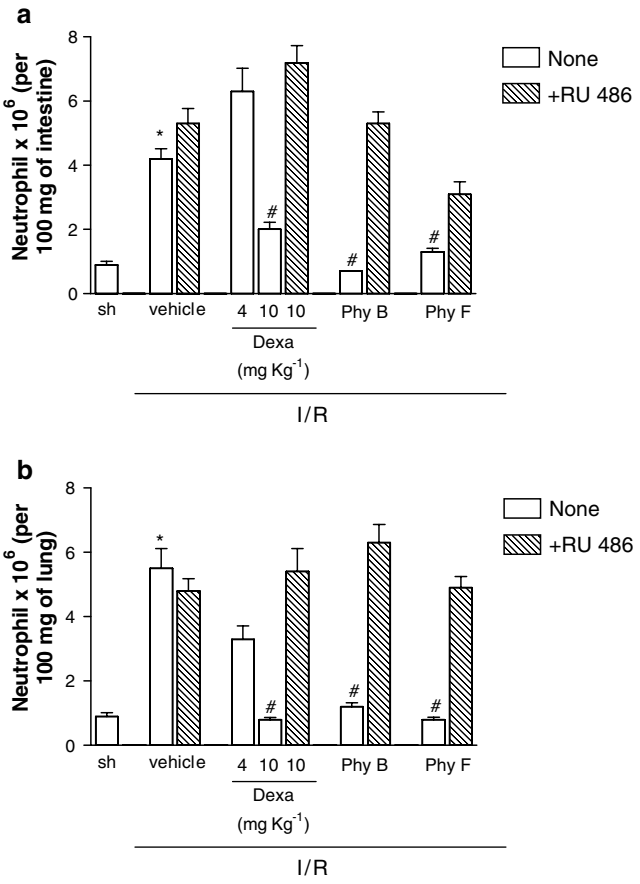

Figure 3 Dexamethasone or physalins prevent the increase of neutrophil recruitment in mice submitted to I/R. This effect was reversed by treatment with RU486. Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. Neutrophil infiltration was determined by measurement of intestinal (a) and pulmonary (b) MPO activity. Physalins (20 mg kg^{-1}) or vehicle was administered (s.c.) 45 min before reperfusion, and dexamethasone (4 or 10 mg kg^{-1}) or vehicle was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 (25 mg kg^{-1}) or vehicle were administered 15 min prior to dexamethasone or physalins. Data are shown as the mean ± s.e.m. of five to six mice in each group. $^{*}P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle mice submitted to I/R.

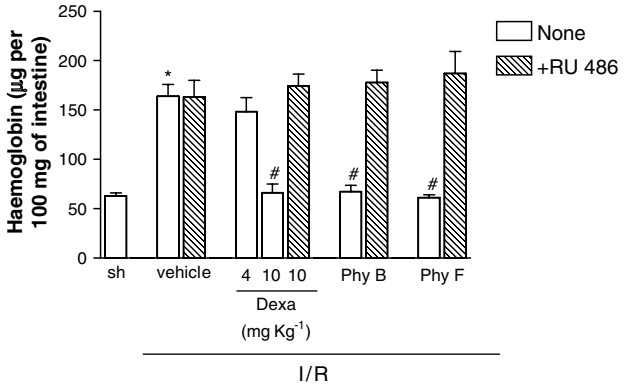

Figure 4 Dexamethasone or physalins prevent the increase of haemorrhage in mice submitted to I/R. This effect was reversed by treatment with RU486. Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. Haemorrhage in the intestine was evaluated by measuring the concentration of haemoglobin in 100 mg of tissue. Physalins (20 mg kg^{-1}) or vehicle were administered (s.c.) 45 min before reperfusion, and dexamethasone (4 or 10 mg kg^{-1}) or vehicle was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 (25 mg kg^{-1}) or vehicle were administered 15 min prior to dexamethasone or physalins. Data are shown as the mean ± s.e.m. of five to six mice in each group. $^{*}P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle mice submitted to I/R.

production (Figure 5). Surprisingly, RU486 had no effect on the enhancement of IL-10 concentration induced by dexamethasone treatment (Figure 6).

Effects of the treatment with physalins on intestinal I/R injury

Next, we investigated the effects of physalins, extracted from *P. angulata*, in the model of intestinal reperfusion injury. Previous studies have suggested that a dose of 20 mg kg^{-1} was optimal to reduce lipopolysaccharide-induced TNF-α increase (Soares *et al*., 2003). Initial experiments were conducted with physalin B to find an ideal dose of the drug to use in our experimental conditions. This drug induced a dose-dependent inhibition of the reperfusion-associated neutrophil accumulation that was maximal at 20 mg kg^{-1} (sham, 0.6 ± 0.04 neutrophils × 10^6 per 100 mg of intestine; I/R, 4.2 ± 0.3; I/R plus physalin B 0.2 mg kg^{-1}, 4.5 ± 0.6; I/R plus physalin B 2 mg kg^{-1}, 2.6 ± 0.3; I/R plus physalin B 20 mg kg^{-1}, 1.3 ± 0.1, $n=4$). The compound dose-dependently inhibited the reperfusion-induced increase of TNF-α in the intestine, lungs and serum (Table 1). Other parameters, including plasma extravasation and haemorrhage, were also dose-dependently inhibited by physalin B with maximal inhibition at 20 mg kg^{-1} (data not shown). Owing the more limited availability of physalin F and similar effectiveness when compared to physalin B (Soares *et al*., 2003), the drug was not used in dose–response experiments. All further experiments were conducted using a dose of 20 mg kg^{-1} of either drug.

The treatment of animals with physalins B or F markedly inhibited both the increase in vascular permeability (Figure 2) and the recruitment of neutrophils (Figure 3) in the intestine and lungs following reperfusion of the ischaemic SMA. Treatment with physalins also abolished the increase of haemoglobin, a marker of intestinal haemorrhage (Figure 4). Physalins significantly inhibited the elevations of TNF-α in the intestine, lung and serum (Figure 5). Interestingly, pretreatment with physalin was accompanied by an enhancement in reperfusion-induced IL-10 production (Figure 6).

As clearly observed from the experiments above, both physalins and dexamethasone had similar quantitative and qualitative protective effects in the model of intestinal I/R. The next step was to verify whether physalins would act *via* the GR in the model. Pretreatment with RU486 completely reversed the protective effects of physalins on the reperfusion-induced increases in vascular permeability (Figure 2), neutrophil influx (Figure 3), haemorrhage (Figure 4) and TNF-α production (Figure 5). In contrast and similarly to the case of dexamethasone-treated animals, RU486 failed to affect the enhancing effects of physalin on the reperfusion-induced IL-10 production (Figure 6).

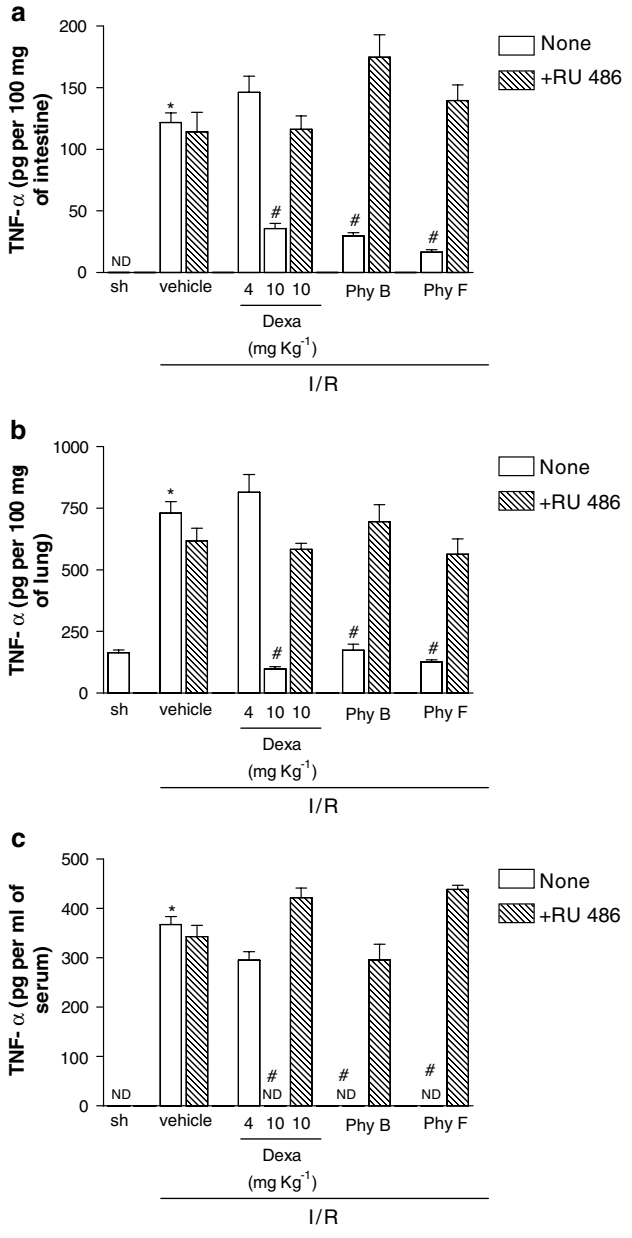

Figure 5 Dexamethasone or physalins prevent the increase of TNF-α in tissue and serum of mice submitted to I/R. This effect was reversed by treatment with RU486. Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. The concentrations of TNF-α in intestine (a), lungs (b) and serum (c) were measured by ELISA. Results are shown as pg of TNF-α per 100 mg of tissue or as pg TNF-α per ml of serum. Physalin B (20 mg kg^{-1}), physalin F (20 mg kg^{-1}) or vehicle was administered (s.c.) 45 min before reperfusion, and dexamethasone (4 or 10 mg kg^{-1}) or vehicle was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 (25 mg kg^{-1}) or vehicle were administered 15 min prior to dexamethasone or physalins. Data are shown as the mean ± s.e.m. of five to six mice in each group. *$P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle mice submitted to I/R.

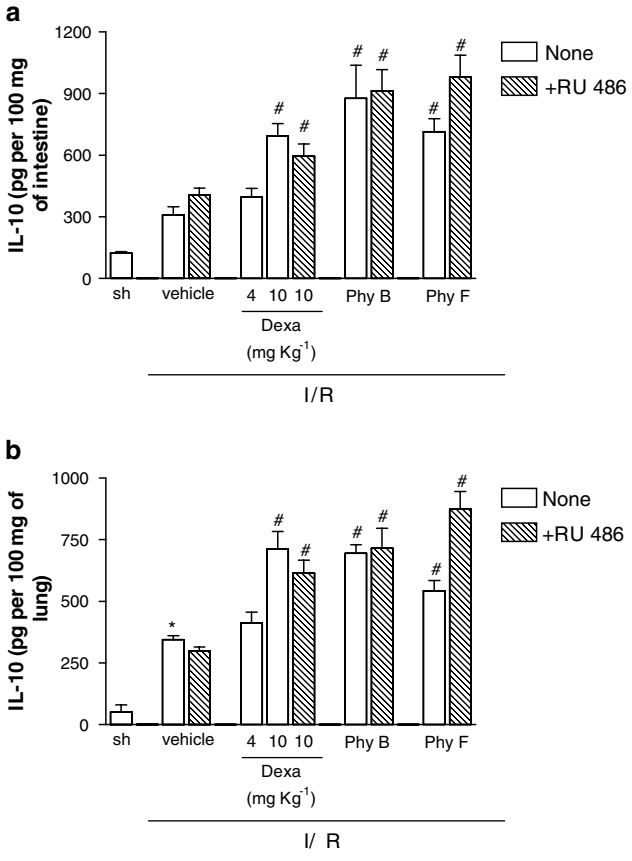

Figure 6 Dexamethasone or physalins enhance the reperfusion-induced release of IL-10 in tissue of mice. This effect was not reversed by treatment with RU486. Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. The concentrations of IL-10 in the intestine (a) and lungs (b) were measured by ELISA. Physalins (20 mg kg^{-1}) or vehicle was administered (s.c.) 45 min before reperfusion, and dexamethasone (4 or 10 mg kg^{-1}) or vehicle was administered (s.c.) 75 min before reperfusion. In some experiments, RU486 (25 mg kg^{-1}) or vehicle was administered 15 min prior to dexamethasone or physalins. Data are shown as the mean ± s.e.m. of five to six mice in each group. *$P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle mice submitted to I/R.

Further experiments were carried out to confirm that the anti-inflammatory actions of physalins were due to an action on steroid receptors rather than secondary to an enhancement of endogenous release of steroids. First, we assessed whether

Table 1 Physalin B dose-dependently inhibited TNF-α concentration in the intestine, lung and serum

	Intestine	*Lung*	*Serum*
Sham	ND	50 ± 4	ND
Vehicle	102 ± 18*	704 ± 82*	310 ± 39*
Phy 0.2	66 ± 7	796 ± 120	195 ± 19
Phy 2.0	38 ± 7$^{\#}$	442 ± 90$^{\#}$	88 ± 10$^{\#}$
Phy 20	22 ± 2$^{\#}$	90 ± 12$^{\#}$	ND$^{\#}$

Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. The concentrations of TNF-α in the intestine, lungs and serum were measured by ELISA. Results are shown as pg of TNF-α per 100 mg of tissue or as pg TNF-α per ml of serum. Physalin B (0.2–20 mg kg^{-1}) or vehicle was administered (s.c.) 45 min before reperfusion. ND = not detectable. *$P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle mice submitted to I/R.

Table 2 Physalins did not alter the reperfusion-induced increase in systemic corticosterone levels in mice

Group	*Corticosterone* (pg 100 µl^{-1} of serum)
Sham	12±0.5
I/R	
Vehicle	26±1.6*
Physalin B	27±1.8*
Physalin F	24±0.8*

Mice were sham operated or submitted to 60 min of ischaemia and 30 min of reperfusion of the SMA. The concentration of corticosterone was measured by radioimmunoassay. Physalins (20 mg kg^{-1}) or vehicle were administered (s.c.) 45 min before reperfusion. Data are shown as the mean±s.e.m. of four mice in each group. *$P<0.01$ when compared to the sham-operated group.

physalins could interfere with systemic corticosterone concentration under steady-state conditions. Our results demonstrated that physalin B diminished steady-state concentrations of corticosterone to a similar degree to that of dexamethasone (vehicle: 12±0.5 pg 100 µl^{-1} of serum; dexa 10 mg kg^{-1}: 1.2±0.2; physalin B 20 mg kg^{-1}: 2.6±0.7, $n=4$ animals in each group, $P<0.05$ when compared to vehicle). Secondly, we assessed the ability of physalins to interfere with the reperfusion-induced increase in endogenous corticosterone levels. There was a significant enhancement of corticosterone after I/R and treatment with physalins failed to modify the reperfusion-induced increase in corticosterone concentrations (Table 2).

Discussion

Ischaemia underlies many of the most important cardiovascular diseases, including myocardial infarction, thrombotic stroke, embolic vascular occlusions and peripheral vascular insufficiency (Willerson, 1997; Carden & Granger, 2000). The restoration of blood flow to an ischaemic vascular bed, that is, reperfusion, is a major therapeutic objective after ischaemia 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 (Willerson, 1997). 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.

It is increasingly recognized that glucocorticoids are potent inhibitors of injury induced by I/R (Duran & Dillon, 1989; Engelman *et al*., 1989; Hafezi-Moghadam *et al*., 2002). The current view is that glucocorticoids act through cytoplasmic receptors that control the transcription of certain genes encoding regulatory proteins (Beato *et al*., 1989; Barnes, 1998; Mckay & Cidlowski, 1999). By activating these GRs, glucocorticoids may affect reperfusion injury in a multitude of ways, including inhibition of leucocyte recruitment (Perretti & Flower, 1993; Pitzalis *et al*., 1997), reduction of vascular permeability (Evans & Whittle, 2003; Romero *et al*., 2003; Su *et al*., 2004), inhibition of formation of cytokines or other mediators (Almawi *et al*., 1998; Turesin *et al*., 2003) and modulation of enzyme systems involved in inflammation (Koehler *et al*., 1990; Kleinert *et al*., 1996; Wallerath *et al*., 2004). Potential mechanisms may involve modulation of neutrophil and endothelial function (Korompilias *et al*., 1996; Tjandra *et al*., 1996; Takahira *et al*., 2001), inhibition of formation of arachidonic acid products (Turesin *et al*., 2003) and attenuation of lipid peroxidation of biological membranes through membrane stabilization and scavenging of toxic-free radicals (Korompilias *et al*., 1996; Marumo *et al*., 1998). Here, treatment with dexamethasone totally suppressed the increase in vascular permeability, neutrophil influx and haemorrhage induced by reperfusion of the ischaemic SMA. In addition to inhibiting the above-mentioned parameters, dexamethasone effectively suppressed reperfusion-induced increases in the concentration of TNF-α in tissues. As both neutrophil influx and TNF-α production are essential for reperfusion injury (Souza *et al*., 2000b; 2001; 2002), the protective effect of dexamethasone in the system may be secondary to inhibition of these two parameters.

One important additional finding was the ability of the pretreatment with dexamethasone to enhance the concentrations of IL-10, an anti-inflammatory cytokine, in tissue following severe reperfusion-associated injury. This result is consistent with previous studies demonstrating that dexamethasone is an effective inducer of IL-10 production (Tabardel *et al*., 1996; Gayo *et al*., 1998; Mozo *et al*., 2004). We have previously shown that IL-10 was a major protective endogenous cytokine during I/R injury (Souza *et al*., 2003a) and strategies that diminish reperfusion injury are associated with increased IL-10 production (Souza *et al*., 2003b, c; 2004). Therefore, the ability of dexamethasone to enhance IL-10 production may contribute towards the protective effects afforded by dexamethasone in the system.

Physalins are secosteroids purified from *P. angulata* L. extracts with anti-inflammatory properties (Soares *et al*., 2003). Here, we showed that physalins B and F inhibited the reperfusion-induced increase in vascular permeability, recruitment of neutrophils in the intestine and lung and intestinal hemorrhagic. In addition, physalins decreased TNF-α concentration, delayed and prevented lethality and enhanced IL-10 concentration in tissues. Overall, these effects were remarkably similar to the effects observed after treatment with dexamethasone.

To confirm whether physalins were acting *via* GRs, we used the steroid receptor antagonist RU486 and evaluated the effects of physalins on corticosterone levels after reperfusion and under steady-state conditions. The dose of RU486 (25 mg kg^{-1}) used was based on previous studies in witch the antagonist was active in preventing the effects of dexamethasone (Tiao *et al*., 1996; Su *et al*., 2004). Interestingly, treatment with RU486 alone had little effect on the reperfusion-induced injury, indicating that in our model endogenous glucocorticoids do not have a protective effect during intestinal I/R. However, pretreatment with RU486 completely reversed the effects of dexamethasone, suggesting that RU486 was effective at blocking steroid receptors in our system at the dose used. Consistent with an action of physalins on steroid receptors, RU486 also prevented the protective effects exerted by physalins in the I/R model. An alternative possibility was that physalins induced a further release of endogenous corticosterone that could potentially mediate their anti-inflammatory

effects. However, our results did not favour the latter hypothesis as physalins did not modify the release of endogenous corticosterone after I/R. To confirm that physalins could potentially interact with GRs, physalin B or dexamethasone were given to naïve animals and corticosterone levels evaluated 90 min later. Both compounds greatly diminished adrenocorticotropic hormone (ACTH)- and GR-dependent steady-state levels of corticosterone. Altogether, the findings above suggest that physalins are indeed acting *via* GRs. Further experiments evaluating the binding of physalins to GRs are being conducted to confirm this possibility.

Although RU486 prevented the protective effects of the two classes of drugs, the enhancement of the induction of IL-10 was not reversed by RU486. The latter result was intriguing and suggests that IL-10 enhancement induced by dexamethasone or by physalins was not dependent on their action on corticoid receptors. This result was in contrast of a previous study that demonstrated that IL-10 induced by dexamethasone is reversed by RU486 in human monocytes (Mozo *et al*., 2004). Studies aimed at the investigation of other intracellular mechanisms shared by dexamethasone and physalins are currently being carried out.

In summary, our results confirm that glucocorticoids, acting *via* steroid receptors, are effective in preventing inflammatory injury and lethality associated with intestinal I/R. Moreover, natural secosteroids physalins have actions similar to those of dexamethasone that are equally susceptible to the receptor antagonist RU486. Altogether, these results suggest that the *in vivo* anti-inflammatory actions of physalins are mostly due to activation of GRs. Natural steroid-derived compounds may represent novel therapeutic options for the treatment of inflammatory diseases.

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