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European Journal of Pharmacology 494 (2004) 241–249

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# Lung microvascular permeability and neutrophil recruitment are differently regulated by nitric oxide in a rat model of intestinal ischemia–reperfusion

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Received 4 December 2003; received in revised form 7 April 2004; accepted 30 April 2004

**Abstract**

We investigated the effect of two inhibitors of nitric oxide (NO) synthesis, $N_w$-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine, on lung inflammation caused by intestinal ischemia/reperfusion in rats. Relative to the sham-operated rats, intestinal ischemia/reperfusion (ischemia: 45 min; reperfusion: 30 min, 2 and 4 h) induced neutrophil recruitment (increased myeloperoxidase activity) and increased microvascular permeability (Evans blue dye extravasation) in the lungs and increased tumor necrosis factor (TNF) levels in the serum (L-929 cytotoxicity assay). L-NAME given before the ischemia exacerbated neutrophil accumulation, plasma extravasation, serum TNF and caused death of the animals, which was prevented by concomitant injection of L-arginine. Lung and systemic effects of intestinal ischemia/reperfusion were not modified when L-NAME was given just before reperfusion. Treatment with aminoguanidine inhibited plasma extravasation without affecting the other parameters evaluated. Dexamethasone reduced all the parameters. Our results indicate that during intestinal ischemia/reperfusion both constitutive and inducible NO synthases are called to exert a differential modulatory effect on lung inflammation and that maintenance of adequate levels of NO during ischemia is essential for the animals survival.



*Keywords:* Intestinal ischemia/reperfusion; Lung inflammation; Neutrophil accumulation; Adult respiratory distress syndrome; Vascular permeability; NO (Nitric oxide)

## 1. Introduction

Abdominal trauma causing intestinal ischemia/reperfusion may induce remote organ injury where the lung is the first organ affected (Ito et al., 2003). Intestinal ischemia/reperfusion is also associated to induction of systemic inflammatory response, a fact that may indicate a casual link between mediators released during systemic inflammation and the pulmonary dysfunction in adult respiratory distress syndrome (Van Soeren et al., 2000). Data obtained from experimental models of adult respiratory distress syndrome showed that this syndrome is characterised by pulmonary neutrophil accumulation and increased microvascular leakage (Pelosi et al., 2000; Desai, 2002). It was shown that neutrophil–endothelial cell adhesion may be a rate-limiting step in the pathogenesis of pulmonary injury induced by intestinal ischemia/reperfusion (Kuzu et al., 2002). The mechanisms that regulate neutrophil accumulation in the lungs and increased microvascular permeability as well as those that exert protective effects against the severity of such lung dysfunction are yet unclear. Many inflammatory mediators are released during intestinal is-

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doi:10.1016/j.ejphar.2004.04.048

chemia/reperfusion, including tumor necrosis factor (TNF) and nitric oxide (NO), both potential candidates to mediate the pulmonary dysfunction elicited by intestinal ischemia/reperfusion (Yamamoto et al., 2001; Koksoy et al., 2001; Zhou et al., 2003).

The systemic inflammatory response following intestinal ischemia/reperfusion is also mediated by NO. This molecule seems to mediate hypotension, vascular hyporesponsiveness to vasoconstrictor agents, mucosal intestinal injury, and may contribute to lung microvascular injury (Squadrito et al., 1994, 1997; Wang et al., 2001).

Ward et al. (2000) suggested that upon intestinal ischemia/reperfusion, unclear mechanisms are triggered in order to reduce constitutive NO synthase activity (cNOS) and in parallel, increase inducible NO synthase (iNOS), mediating therefore, the increased microvascular injury of intestine and lung. Thus, unbalanced levels of constitutive and inducible NOS activity during intestinal ischemia/reperfusion are suggested to be the main cause of lung dysfunction. Data concerning the pharmacological effects of nitric oxide inhibitors on the lung dysfunction in experimental adult respiratory distress syndrome elicited by intestinal ischemia/reperfusion are fragmentary. In the present study we compared the effect of two inhibitors of NO synthesis, $N_w$-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine, on lung inflammation and on the systemic inflammatory response elicited by intestinal ischemia/reperfusion in rats.

## 2. Material and methods

### 2.1. Animal

Male Wistar rats weighing 180–200 g from our Departmental animal facilities were used. The animals were housed in temperature (21–23 °C) and humidity (45–65%) and artificially lighted rooms on a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) with free access to food and water. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the Institute of Biomedical Sciences, University of São Paulo; these guidelines are similar to those of Canadian Council of Animal Care, Ca.

### 2.2. Intestinal ischemia/reperfusion rat model

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.). The superior mesenteric artery was exposed through a midline abdominal incision and occluded using a microsurgical clip ("Vascu-statt" no. 1001–531, Scalan Internat, MN, USA). After 45 min of arterial occlusion, the clip was removed and intestinal perfusion established. The animals were killed 30 min, 2 and 4 h later, still under anaesthesia by exsanguination via the abdominal aorta. The control group consisted of rats submitted to the same surgical procedures including mesenteric artery dissection but not submitted to the arterial occlusion (sham-operated). An additional group of nonmanipulated rats was added to obtain normal values of the variables studied.

### 2.3. Pulmonary myeloperoxidase activity

Neutrophil recruitment to the lung was assessed by measuring the myeloperoxidase activity in lung tissue. Samples of lung were obtained from rats killed 30 min, 2 and 4 h after intestinal reperfusion (see above) and processed as described by Goldblum et al. (1985) and Warren et al. (1990). The thorax was cut open and the lungs perfused with saline heparinized (5 IU/ml) with phosphate-buffered saline solution (PBS, pH 7.0) via the pulmonary artery (Tavares de Lima et al., 1992). The whole lung was then homogenized using a Brinkmann Tissue Homogenizer (Polytron®) (1 × 40 s, at a setting 30, using 3 ml/g of PBS containing 0.5% of hexadecyl-trimethylammonium bromide and 5 mM EDTA, pH 6.0). The homogenized samples were sonicated (Vibra Cell-Sonics Materials®) for 3 × 2 min at 40 Hz and then centrifuged at 37,000 × *g* for 15 min. Lung myeloperoxidase activity was measured in the supernatant by the method described by Henson et al. (1978). Briefly, the samples of lung homogenates were incubated with $H_2O_2$ and *ortho*-dianisidine and after 15 min the reaction was stopped by the addition of $NaNO_3$ (1%) and the absorbance determined at 460 nm.

### 2.4. Pulmonary microvascular leakage

Pulmonary vascular permeability was assessed by Evans blue dye extravasation as described by Sirois et al. (1988). In brief, Evans blue dye (25 mg/kg) was given intravenously to rats 5 min before the animals were killed. At different times after reperfusion commenced (30 min, 2 and 4 h), the rats were killed, the lungs perfused as described above and two samples of lung parenchyma removed. Both were weighed and then one was placed in formamide (4 ml/g wet weight) at 20 °C for 24 h and the other was put to dry in oven (60 °C) till constant weight. The concentration of Evans blue dye extracted in formamide was determined by spectrophotometry at a wavelength of 620 nm using standard dilution of Evans blue in formamide (0.3–100 μg/ml). The dry/wet ratio of each lung sample was determined (index of edema) and used in the final calculation of Evans blue extravasation which was expressed as μg Evans blue/g dry weight. The expression of the results as a function of dry weight of tissue avoided underevaluation of changes due to edema.

### 2.5. Quantification of tumor necrosis factor (TNF) activity

TNF activity was quantified in the rat serum by bioassay using L-929 cells based on the method described by Flick and Gifford (1984). After intestinal reperfusion, samples of blood were collected from the abdominal aorta. The samples

were centrifuged (170 × *g*, 10 min) and the serum obtained stored at − 70 °C. TNF activity was assayed by the addition of 50 μl of serum, serially diluted (twofold dilutions) into 96-well plates, with L-929 cells ($2.5 \times 10^6$ cells/well), in presence of actinomycin D (final concentration 5 μg/ml). The plates were incubated overnight. The degree of lyses of L-929 cells was assessed by staining with crystal violet (0.05% in methanol 10%) for 15 min, followed by rinsing of the plates with distilled water and drying. Methanol (10%) was then added to each well to dissolve the stain, and absorbance was read at 620 nm on a Microplates Reader (Bio-Tek Instruments). The TNF titre (units/ml) was defined as the reciprocal of the dilution that induces 50% of lyses of L-929 cells. The data were homogenized using a probit analysis.

### 2.6. Pharmacological treatments

Groups of rats were treated 60 min before induction of intestinal ischemia with the NOS inhibitors, aminoguanidine (15, 50 and 150 mg/kg, i.v.) or L-NAME (5, 10 and 30 mg/kg, i.v.). In a parallel study, L-arginine (300 mg/kg, i.p.) was given together with L-NAME. In another group of rats, the L-NAME (30 mg/kg, i.v.) was given immediately after induction of intestinal ischemia. Another group of rats was treated with dexamethasone (2 mg/kg, i.v.), 60 min before intestinal ischemia. A group of 2 h sham-operated rats was treated with L-NAME (30 mg/kg, i.v.), aminoguanidine (50 mg/kg, i.v.) or dexamethasone (2 mg/kg, i.v.) 60 min before gut manipulation.

### 2.7. Statistical analysis

Data are expressed as mean ± standard error of mean (S.E.M.). Comparisons between the groups were made by *t*-test after analysis of variance (GraphPad Software V. 2.01, Graphpad Instat™). Values of $P < 0.05$ were considered significant.

## 3. Results

### 3.1. Acute lung injury induced by intestinal ischemia/reperfusion

The level of myeloperoxidase activity in pulmonary tissue was taken as a marker of neutrophil sequestration into the lungs after intestinal ischemia/reperfusion. As can be seen in Fig. 1, a significant increase in lung myeloperoxidase activity was observed at 2 and 4 h of reperfusion following ischemia compared to the sham-operated animals. Levels of lung myeloperoxidase activity of sham-operated rats were significantly increased compared to those of nonmanipulated (Basal levels) but only at 2 h after manipulation.

The protocol used in this study of 45 min intestinal ischemia followed by reperfusion caused increased pulmonary microvascular leakage, assessed by Evans blue dye extravasation. Fig. 2 shows the extravasation, measured over a period of 5 min before the indicated times. A significant extravasation was observed already at 30 min of reperfusion and was still present after 4 h. In sham-operated rats, the levels of Evans blue dye extravasation were similar to the basal levels measured in the lungs of nonmanipulated rats.

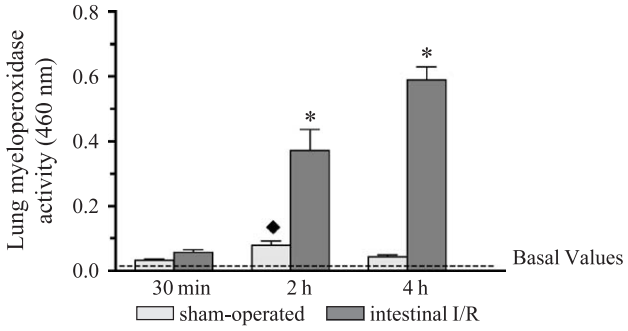


Fig. 1. Lung myeloperoxidase activity following intestinal ischemia/reperfusion (I/R). Groups of rats were submitted or not (sham-operated) to intestinal ischemia (45 min) and at different times after reperfusion (30 min, 2 and 4 h) the myeloperoxidase activity was measured in lung homogenates. Basal values were obtained from nonmanipulated rats. Data are expressed as mean ± S.E.M. from 10 experiments. *$P < 0.05$ comparing the experimental versus sham-operated group; ◆$P < 0.05$ comparing sham-operated versus basal.

### 3.2. Systemic effects of intestinal ischemia/reperfusion

Fig. 3 shows that following intestinal ischemia/reperfusion, TNF activity was markedly increased in serum after 2 and 4 h, with the peak value at 2 h. The sham-operated rats also showed a small increase in TNF activity at 2 h in comparison to the nonmanipulated rats (basal levels), but these values were significantly lower than those of the intestinal ischemia/reperfusion group.

### 3.3. Pharmacological studies: effect of NO synthase inhibitors and dexamethasone

Groups of rats were treated with dexamethasone (2 mg/kg, i.v.), 60 min before ischemia induction. After 2 h of reperfusion, the myeloperoxidase activity (A) and Evans blue dye extravasation (B) were determined in lung homogenates and TNF activity was measured in the serum (C). Groups of sham-operated rats were similarly treated.

Fig. 4A shows that the increased pulmonary myeloperoxidase activity caused by intestinal ischemia/reperfusion was significantly reduced (about 50%) by pretreatment with dexamethasone. Sham-operated rats were not affected by this treatment (nontreated: 0.095 ± 0.01; treated: 0.072 ± 0.01). Dexamethasone also reduced (about 20%) the increased plasma extravasation in the lung induced by intestinal ischemia/reperfusion (Fig. 4B). The values of Evans blue dye extravasation obtained in sham-operated rats did not significantly change after this treatment (nontreated:

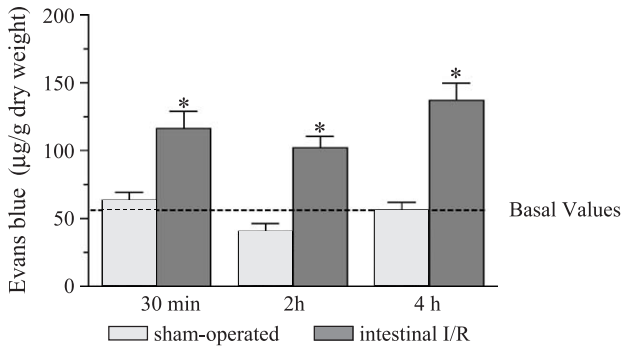


Fig. 2. Lung vascular permeability following intestinal ischemia/reperfusion (I/R). Groups of rats were submitted or not (sham-operated) to intestinal ischemia (45 min) and after different times of reperfusion (30 min, 2 and 4 h) the Evans Blue dye was measured in fragments of lung parenchyma. The dye was injected 5 min before the indicated times. Basal values were obtained from nonmanipulated rats. Data are expressed as mean ± S.E.M. from 10 experiments. *$P < 0.05$ comparing the experimental versus sham-operated group.

35.37 ± 4.5; treated: 30.69 ± 3.0). The levels of TNF after dexamethasone treatment were reduced about 30% in rats subjected to intestinal ischemia/reperfusion (Fig. 4C).

The same protocol of treatment was used for NO inhibitors, L-NAME and aminoguanidine. Fig. 5A shows the effect of these treatments on myeloperoxidase activity and Evans blue extravasation in pulmonary tissue and serum TNF levels measured at 2 h after reperfusion. L-NAME treatment (Fig. 5A) caused a dose-dependent exacerbation of lung myeloperoxidase activity (by about twofold relative to control group, at 10 mg/kg and threefold at 30 mg/kg), whereas aminoguanidine did not affect the increased lung myeloperoxidase activity induced by intestinal ischemia/reperfusion (Fig. 5B). Treatment with L-NAME (30 mg/kg, i.v.) or aminoguanidine (50 mg/kg, i.v.) did not modify the lung myeloperoxidase activity in the sham-operated rats (nontreated: 0.095 ± 0.01; L-NAME: 0.083 ± 0.01; amino-

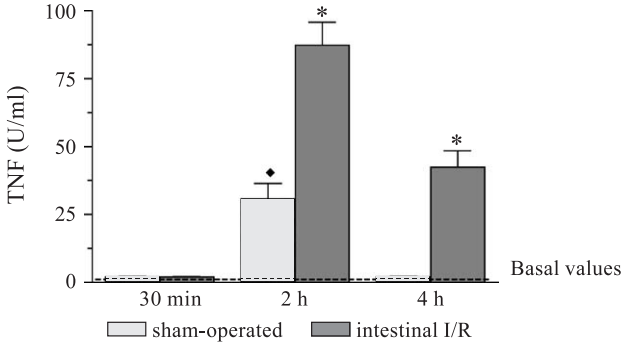


Fig. 3. Serum TNF levels following intestinal ischemia/reperfusion (I/R). Groups of rats were submitted or not (sham-operated) to intestinal ischemia (45 min) and after different times of reperfusion (30 min, 2 h and 4 h) the TNF activity was evaluated in the serum. Basal values were obtained from nonmanipulated rats. Data are expressed as mean ± S.E.M. from 10 to 15 experiments. *$P < 0.05$ comparing the experimental versus sham-operated group; ◆$P < 0.05$ comparing sham-operated versus basal group.

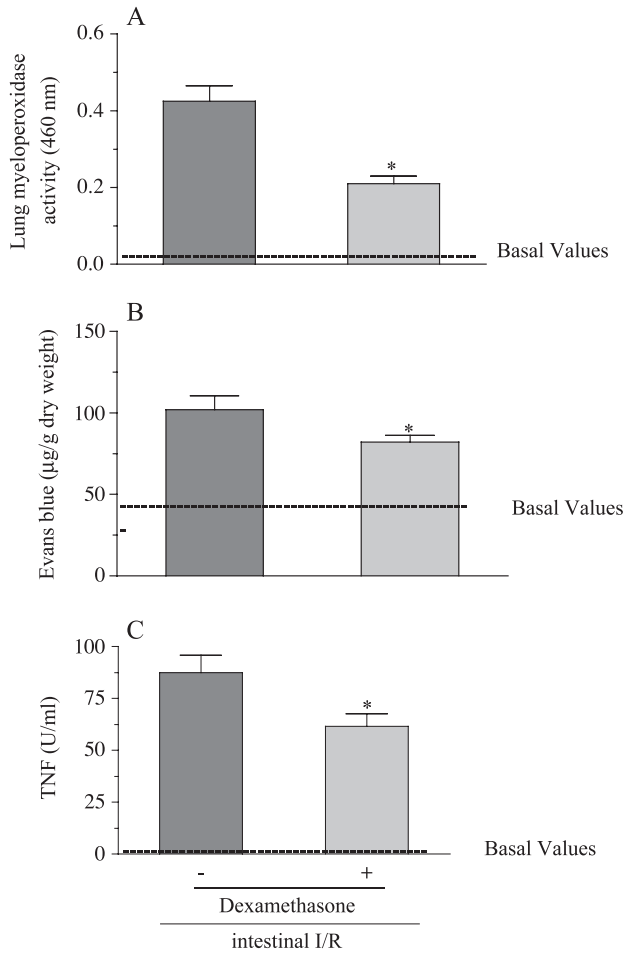


Fig. 4. Effect of dexamethasone on the increased lung myeloperoxidase activity (A) and microvascular permeability (B) and on serum TNF levels (C) following ischemia/reperfusion (I/R). Groups of rats were treated or not with dexamethasone (2 mg/kg, i.v.) 60 min before induction of intestinal ischemia (45 min). The above parameters were evaluated 2 h after the reperfusion. Data are expressed as mean ± S.E.M. from 5 to 10 experiments. *$P < 0.05$ comparing the dexamethasone-treated with the nontreated group.

guanidine: 0.100 ± 0.017). The Evans blue dye extravasation was exacerbated (about 50% increase) already with the dose of 5 mg/kg of L-NAME and remained elevated at doses of 10 and 30 mg/kg (Fig. 5C). Contrarily, aminoguanidine inhibited the increased vascular permeability which returned to basal levels with the three doses used (Fig. 5D). Pulmonary microvascular permeability levels in sham-operated rats was not affected by L-NAME (30 mg/kg) or aminoguanidine (50 mg/kg) treatments (nontreated: 35.37 ± 4.5; L-NAME: 40.4 ± 1.1; aminoguanidine: 35.5 ± 3.5).

Fig. 5E shows that L-NAME treatment increased the levels of TNF. This effect was observed with the higher dose only. Aminoguanidine treatment failed to modulate the levels of TNF at all doses utilized (Fig. 5F).

We then compared the effect of L-NAME given before ischemia with L-NAME given immediately before the reperfusion. Fig. 6A shows that L-NAME given before

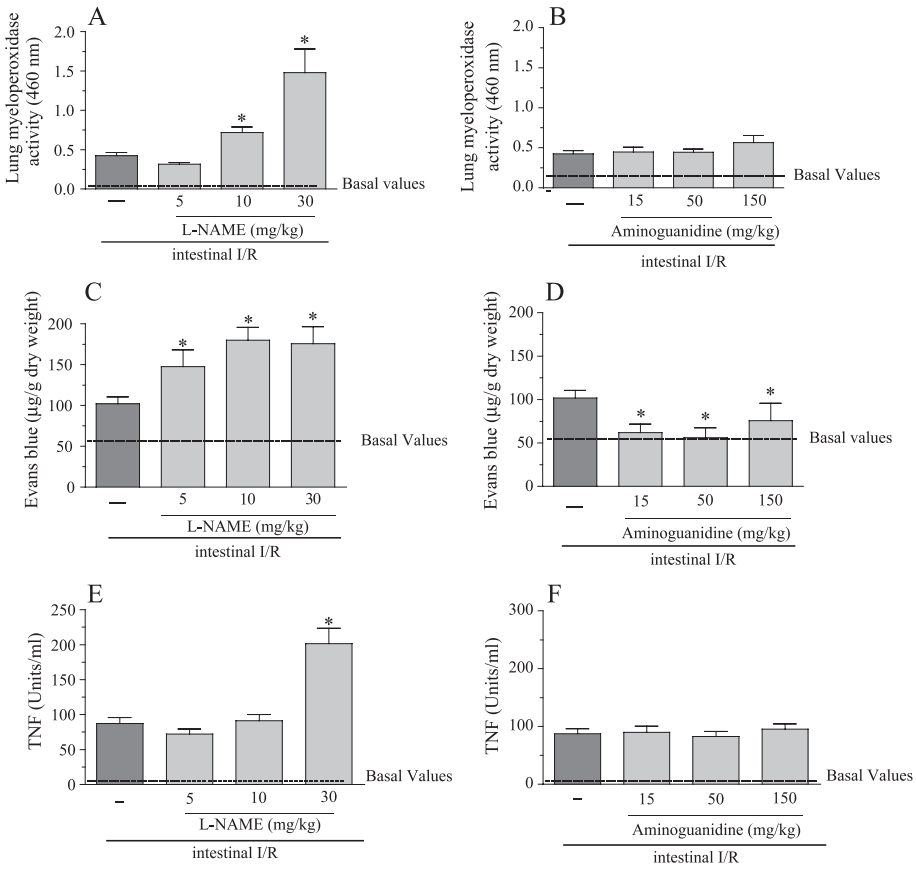


Fig. 5. Effect of inhibitors of NO synthase on lung myeloperoxidase activity (A and B) and microvascular permeability (C and D), and on serum TNF levels (E and F) following intestinal ischemia/reperfusion (I/R). Groups of rats were treated or not with L-NAME (5, 10 and 30 mg/kg, i.v.) or aminoguanidine (15, 50 and 150 mg/kg, i. v.) 60 min before induction of intestinal ischemia (45 min). The above parameters were evaluated 2 h after reperfusion. Data are expressed as mean ± S.E.M. from 10 to 15 experiments. *$P < 0.05$ comparing the treated groups with the nontreated groups.

reperfusion did not change the levels of lung myeloperoxidase activity in contrast to the exacerbated myeloperoxidase activity found in the rats treated with L-NAME before induction of intestinal ischemia. A similar pattern was observed with microvascular permeability (Fig. 6B) and serum TNF activity (Fig. 6C).

The rats submitted to intestinal ischemia/reperfusion all survived till the end of the experimental protocol (45 min of ischemia followed by 2 h of reperfusion). However, pretreatment with L-NAME (Fig. 7) before induction of intestinal ischemia/reperfusion caused the death of the rats in a manner dependent on the doses of L-NAME used. Mortality was completely reversed by administration of L-arginine given together with L-NAME. Animals treated with L-NAME after reperfusion or with aminoguanidine, all survived after the intestinal ischemia/reperfusion.

## 4. Discussion

In this study, we have analysed the acute lung injury that followed intestinal ischemia/reperfusion. The reason for the study is that pulmonary dysfunction such as adult respiratory distress syndrome is commonly associated with intestinal ischemia/reperfusion (Blaisdell et al., 1966; Van Soeren et al., 2000). This model of adult respiratory distress syndrome is characterised by increased microvascular leakage and pulmonary neutrophil infiltration (Hewett and Roth, 1993; Ashbaugh et al., 1967), systemic release of many inflammatory mediators, including tumor necrosis factor (TNF-α) and nitric oxide (NO) (Klosterhalfen and Bhardwaj, 1998; Pugin et al., 1995; Yi and Ulich, 1992), cardiovascular shock, followed in many cases by multiple organ failure, and death (Mayeux, 1997).

Adult respiratory distress syndrome-like syndrome can be experimentally induced by intestinal ischemia/reperfusion (Welling, 1996; Desai, 2002). In this study, the experimental design used consisted of 45 min of superior mesenteric artery occlusion (ischemia) followed by 30 min, 2 or 4 h of reperfusion, a protocol similar to that described by Squadrito et al. (1997). The increased pulmonary myeloperoxidase activity we observed is consistent with the presence of neutrophils in the lung (Sun et al., 1999; Takayama et al., 2001). However, we are unable to say if the increased myeloperoxidase levels are due to neutrophils sequestration in pulmonary microcirculation or

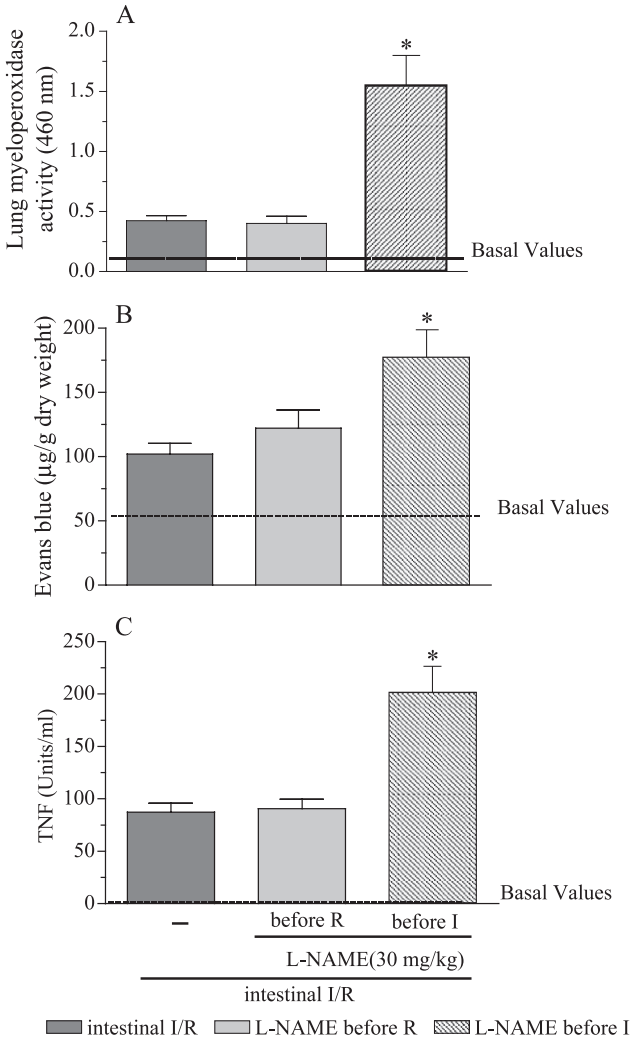


Fig. 6. Effect of the time of administration of L-NAME on the increased lung myeloperoxidase activity (A) and microvascular permeability (B) and on the serum TNF levels (C) following intestinal ischemia/reperfusion (I/R). Groups of rats were treated with L-NAME (30 mg/kg, i.v.) 60 min before induction of the intestinal ischemia (I) or just before reperfusion (R). The above parameters were evaluated 2 h after the reperfusion. Data are expressed as mean ± S.E.M. from 10 experiments. *$P < 0.05$ comparing the treated groups with the nontreated group.

its migration into the lung tissue or airways. Evans blue dye extravasation measured under the conditions employed in this study is indicative of increased vascular permeability and positively correlates with edema development (Iglesias et al., 1998). Moreover, the increased levels of serum TNF is indicative of systemic inflammatory response.

Although NO is among the wide spectrum of mediators released during intestinal ischemia/reperfusion, the effect of NO on lung inflammation induced by intestinal ischemia/reperfusion remains controversial (Ialenti et al., 1992; Miura et al., 1996; Kubes and Granger, 1992; Guidot et al., 1995). In this study, we showed that inhibition of NO synthesis by L-NAME, a compound inhibiting both the constitutive and inducible NO synthases (Chen et al., 2000), exacerbated the lung inflammation (as assessed by plasma extravasation and neutrophil infiltration) caused by intestinal ischemia/reperfusion. The lung myeloperoxidase activity was exacerbated with 10 and 30 mg/kg, whereas the microvascular permeability was exacerbated also with the dose of 5 mg/kg. These results are suggestive of a differential sensibility of these two pathological events to the effects of NO generated by intestinal ischemia/reperfusion, pulmonary plasma extravasation being more sensitive to NO effects than neutrophil recruitment. Tavaf-Motamen et al. (1998), using a more selective inhibitor of the constitutive NOS, the compound $N_w$-nitro-L-arginine (LNNA, Linas et al., 1997), in a similar model of intestinal ischemia/reperfuson reported an accelerated establishment of plasma extravasation that was accounted to neutrophil activation in the systemic compartment. Turnage et al. (1998) reported that lung microvascular leakage induced by intestinal ischemia/reperfusion is potentiated by L-NAME.

When we used aminoguanidine, reported as being more selective for inducible NOS (Griffiths et al., 1993; Wang et al., 2001), we observed that it did not affect neutrophil infiltration and it effectively abolished Evans blue dye extravasation.

Taken together, these results suggest that (a) endogenous NO can have proinflammatory or antiinflammatory effects; (b) that different aspects of inflammation, such as microvascular permeability and leukocyte infiltration, respond differently to NO. The proinflammatory and antiinflammatory effects of NO are probably also correlated with the activity of the different isoforms, as suggested by results of Tavaf-Motamen et al. (1998) with the constitutive endothelial NOS playing an antiinflammatory role and inducible NOS, as implied by our results, contributing relatively little to neutrophil recruitment but more to the increased microvascular permeability. An increased level of constitutive NO synthase activity could be elicited by intestinal ischemia/reperfusion process as increased shear stress and hypoxia, characteristic of intestinal ischemia/reperfusion are known

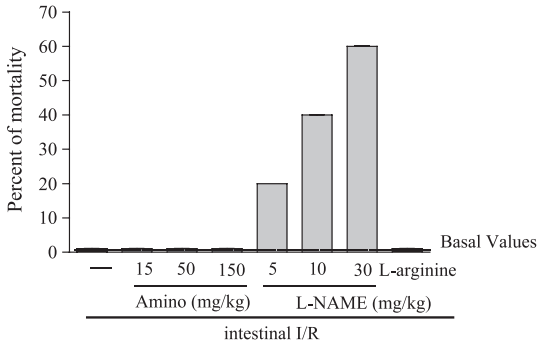


Fig. 7. Effect of pretreatment with NO synthase inhibitors on the survival to intestinal ischemia/reperfusion (I/R). Groups of rats were treated or not with L-NAME (5, 10 and 30 mg/kg, i.v.), aminoguanidine (15, 50 and 150 mg/kg, i.v.) or L-NAME (30 mg/kg, i.v.) plus L-arginine (300 mg/kg, iv.) 60 min before intestinal ischemia. Mortality was observed till 2 h after the reperfusion. Data are expressed as the mean percentage of mortality from 10 to 18 experiments.

to stimulate endothelial NOS (Ward et al., 2000). This stimulation of constitutive NOS may be seen as an attempt to protect the circulation against the deleterious effects of intestinal ischemia.

According to Tavaf-Motamen et al. (1998), an increased activation of circulating neutrophils is required to induce elevated pulmonary microvascular permeability following intestinal ischemia/reperfusion. Activation of inducible NOS providing high levels of NO in an oxidative milieu such as that generated by activated neutrophils adhered to endothelium would thus have proinflammatory effect. This concept is compatible with our results with aminoguanidine since it significantly reduced the Evans blue extravasation, although it did not affect the increased lung myeloperoxidase activity following intestinal ischemia/reperfusion.

The apparent separation between pulmonary vascular permeability and neutrophil accumulation observed with aminoguanidine was also observed with dexamethasone treatment. Here the lung neutrophils (myeloperoxidase activity) were drastically reduced (50%) but there was only a small effect on dye extravasation (19%). Thus, a causal link between increased microvascular permeability and neutrophil extravasation seems not to be likely, in this model at least. These results would also imply that dexamethasone is affecting systems other than those inhibited by aminoguanidine as the profiles of antiinflammatory effects were clearly distinct. The effects of L-NAME, by contrast, showed no distinction between neutrophil accumulation and microvascular permeability, increasing both variables. It would seem that a total absence of endogenous NO is strongly deleterious to vascular integrity and indeed to survival.

Moreover, extravasation of pulmonary Evans blue dye occurred at similar levels when analysed 30 min, 2 or 4 h after reperfusion (see Fig. 2). In this context, at 30 min, when pulmonary extravasation was already at maximal levels, the lung myeloperoxidase activity was close to basal levels (see Fig. 1). At 2 and 4 h after reperfusion when the levels of myeloperoxidase activity were highly increased the amount of dye extravased remained similar to 30 min. Thus, these data suggest that the mechanisms that regulate the lung plasma extravasation induced by the intestinal ischemia/reperfusion are triggered early and are maintained throughout the period of reperfusion.

Treatment of sham-operated rats with NOS inhibitors or with dexamethasone did not significantly modify lung myeloperoxidase activity and pulmonary microvascular permeability. This indicates that the increased levels of lung myeloperoxidase activity observed in sham-operated rats at 2 h after reperfusion (see Fig. 1) are not mediated by NO-generating systems or by corticoid-sensitive mediators. One speculation that might be of clinical relevance is that the manipulation of the gut as occurs in exploratory surgery might be a factor contributing to pulmonary inflammation.

The inhibition of both, constitutive and inducible isoforms of NOS, by L-NAME (10 and 30 mg/kg) treatment in rats subjected to intestinal ischemia/reperfusion, caused an exacerbated lung inflammation, concomitant to high mortality rate; a fact that was not observed when rats were treated with aminoguanidine at all doses utilized. Administration of L-arginine to the rats pretreated with L-NAME reversed the L-NAME-induced mortality. Therefore, the constitutive lung NOS isoform seems to play a fundamental role to counteracting the effects of intestinal ischemia/reperfusion. In contrast, the NOS susceptible to inhibition of aminoguanidine, likely exerts a proinflammatory effect.

Ward et al. (2000) suggested that the reduction of constitutive NOS activity during the reperfusion is associated to the onset of pulmonary and intestinal microvascular permeability. In our model, in contrast, the inhibition of both isoforms during the reperfusion did not exacerbate lung myeloperoxidase activity, Evans blue dye extravasation and TNF levels (see Fig. 5) and did not cause animals death as occurred when L-NAME was given before the intestinal ischemia.

We showed the presence of TNF in the blood of rats submitted to intestinal ischemia/reperfusion confirming the development of systemic inflammation following intestinal ischemia/reperfusion, corroborating previous findings (Koksoy et al., 2001). It was shown that TNF released during intestinal ischemia/reperfusion contributes to the recruitment of neutrophils to the lung (Caty et al., 1990; Koksoy et al., 2001). In our study, pretreatment of the rats with L-NAME increased the levels of TNF whereas pretreatment with aminoguanidine had no effect. These findings would indicate that during intestinal ischemia/reperfusion, NO regulates the induction of TNF. Studies by Rahat et al. (2001), demonstrated that NO increases the levels of mRNA for TNF in the lungs of rats submitted to intestinal ischemia/reperfusion, suggesting a role for NO on regulating TNF transcription. It is also possible that the increased lung inflammation induced by L-NAME would induce TNF from additional cell sources.

In our experiments, we did not find association between serum TNF levels and Evans blue extravasation. As observed in Fig 2, intestinal ischemia/reperfusion induced plasma extravasation as early as 30 min after reperfusion and at this time serum TNF levels were undetectable which suggests that other mediators are responsible for the increased lung vascular permeability. Using a similar model of lung inflammation, Ishii et al. (2000) demonstrated that in bronchoalveolar lavage of rats subjected to the intestinal ischemia/reperfusion, the increased levels of cytokine-induced neutrophil chemoattractant (CINC-1) accounts for increased pulmonary vascular permeability. Nevertheless, in adult respiratory distress syndrome the involvement of a wide range of mediators has been suggested in a recent review by Bhatia and Moochhala (2004).

In conclusion, the results presented here indicate that during intestinal ischemia/reperfusion both NOS isoforms, constitutive and inducible are called to exert a differential modulatory effect. Activation of constitutive NOS has a

protective effect whereas activation of inducible NOS is responsible for the pulmonary dysfunction. This can be viewed as an adaptative response of the organism to the stress generated by the intestinal ischemia/reperfusion which modulates the vascular tone and the accumulation and activation of neutrophils in the lung microvasculature. Our studies also suggest that maintenance of adequate levels of NO during ischemia is essential for the survival. Finally, systemic administration of L-arginine could be used as coadjuvant therapy in intestinal ischemia/reperfusion patients.

## Acknowledgements

We are indebted to Dr. Y.S. Bahkle from Imperial College, London for his relevant involvement in the discussion and careful revision of this manuscript. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). G. Cavriani is recipient from a CAPES fellowship. W. Tavares de Lima is a fellow researcher from Conselho Nacional de Pesquisa e Desenvolvimento (CNPq).

## References

Ashbaugh, D.G., Bigelow, D.B., Petty, T.L., Levine, B.E., 1967. Acute respiratory distress syndrome in adults. Lancet, 319–323.

Bhatia, M., Moochhala, S., 2004. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. J. Pathol. 202, 145–156.

Blaisdell, F.W., Lim, R.C., Amberg, J.R., Choy, S.H., Hall, A.D., Thomas, A.N., 1966. Pulmonary microembolism. A cause of morbidity and death after major vascular surgery. Arch. Surg. 93, 776–786.

Caty, M.G., Guice, K.S., Oldham, K.T., Remick, D.G., Kunkel, S.I., 1990. Evidence for tumor necrosis factor-induced pulmonary microvascular injury after intestinal ischemia–reperfusion injury. Ann. Surg. 212, 694–700.

Chen, J.C., Chen, H.M., Shyr, M.H., Fan, L.L., Chi, T.Y., Chi, C.P., Chen, M.F., 2000. Selective inhibition of inducible nitric oxide in ischemia–reperfusion of rat small intestine. J. Formos Med. Assoc. 99, 213–218.

Desai, S.R., 2002. Acute respiratory distress syndrome: imaging of the injured lung. Clin. Radiol. 57, 8–17.

Flick, D.A., Gifford, G.A., 1984. Comparison of in vitro cell cytotoxic assay factor. J. Immunol. Methods 68, 167–175.

Goldblum, S.E., Kuei-Meng, W., Jay, M., 1985. Lung myeloperoxidase as a measure of pulmonary leukostasis in rabbits. J. Appl. Physiol. 59, 1978–1985.

Griffiths, M.J., Messent, M., MacAllister, R.J., Evans, T.W., 1993. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br. J. Pharmacol. 110, 963–968.

Guidot, D.M., Repine, M.J., Hybertson, B.M., Repine, J.E., 1995. Inhaled nitric oxide prevents neutrophil-mediated, oxygen radical-dependent leak in isolated rat lung. Am. J. Physiol. 269, L2-5.

Henson, P.M., Zanolari, B., Schwartzman, N.A., Hong, S.R., 1978. Intracellular control of human neutrophil secretion. C5a-induced stimulus-specific desesitization and the effects of cytochalasin B. J. Immunol. 121, 851–855.

Hewett, J.A., Roth, R.A., 1993. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol. Rev. 45, 382–411.

Ialenti, A., Ignarro, A., Moncada, S., Di Rosa, M., 1992. Modulation of acute inflammation by endogenous nitric oxide. Eur. J. Pharmacol. 211, 177–182.

Iglesias, J.L., LaNoue, J.L., Rogers, T.E., Inman, L., Turnage, R.H., 1998. Physiologic basis of pulmonary edema during intestinal ischemia reperfusion. J. Surg. Res. 80, 156–163.

Ishii, H., Ishibashi, M., Takayama, M., Nishida, T., Yoshida, M., 2000. The role of cytokines-induced neutrophil chemoatractant-1 in neutrophil mediated remote lung injury after intestinal ischemia/reperfusion in rat. Respirology 5, 325–331.

Ito, K., Ozaka, H., Kojima, N., Miura, M., 2003. Pharmacological preconditioning protects lung injury induced by intestinal ischemia/reperfusion in rat. Shock 19, 462–468.

Klosterhalfen, B., Bhardwaj, R.S., 1998. Septic shock. Gen. Pharmacol. 31, 25–32.

Koksoy, C., Kuzu, M.A., Kuzu, I., Ergun, H., Gurhan, I., 2001. Role of tumor necrosis factor in lung injury caused by intestinal ischaemia–reperfusion. Br. J. Surg. 88, 464–468.

Kubes, P., Granger, D.N., 1992. Nitric oxide modulates microvascular permeability. Am. J. Physiol. 262, H611.

Kuzu, M.A., Koksoy, C., Kuzu, I., Gurhan, I., Ergun, H., Demirpence, E., 2002. Role of integrins and intracellular adhesion molecule-1 in lung injury after intestinal ischemia–reperfusion. Am. J. Surg. 183, 70–74.

Linas, S., Whittenburg, D., Repine, J.E., 1997. Nitric oxide prevents neutrophil-mediated acute renal failure. Am. J. Physiol. 272, F48–F54.

Mayeux, P.R., 1997. Pathobiology of lipopolysaccharide. J. Toxicol. Environ. Health 51, 415–435.

Miura, M., Ichinose, M., Kageyama, N., Tomaki, M., Takahashi, T., Ishikawa, J., Ohuchi, Y., Oyake, T., Endoh, N., Shirato, K., 1996. Endogenous nitric oxide modifies antigen-induced microvascular leakage in sensitized guinea pig airways. J. Allergy Clin. Immunol. 98, 144–151.

Pelosi, P., Aspesi, M., Franchi, D., Colombo, G., Gamberoni, C., Caironi, P., Bottino, N., 2000. Mechanical ventilation in acute respiratory distress syndrome. New trends. AACN Clin. Issues 11, 179–197.

Pugin, J., Ulevitch, R.J., Tobias, P.S., 1995. Tumor necrosis factor-alpha and interleukin-1 beta mediate human endothelial cell activation in blood at low endotoxin concentrations. Inflammation 45, 49–55.

Rahat, M.A., Lahat, N., Smollar, J., Brod, V., Kinarty, A., Bitterman, H., 2001. Divergent effects of ischemia/reperfusion and nitric oxide donor on TNFalpha mRNA accumulation in rat organs. Shock 15, 312–317.

Sirois, M.G., Jancar, S., Braquet, P., Plante, G.E., Sirois, P., 1988. PAF increases vascular permeability in selected tissues: effect of BN-52021 and L-655,240. Prostaglandins 36, 631–644.

Squadrito, F., Calapai, G., Altavilla, D., Cucinotta, D., Zingarelli, B., Arcoraci, V., Campo, G.M., Caputi, A.P., 1994. Central serotoninergic system involvement in the anorexia induced by NG-nitro-L-arginine, an inhibitor of nitric oxide synthase. Eur. J. Pharmacol. 255, 51–55.

Squadrito, F., Altavilla, D., Squadrito, G., Campo, G.M., Ioculano, M., Ammedolia, L., Arlotta, M., Saitta, A., Caputi, A.P., 1997. The effects of recombinant human granulocyte-colony stimulating factor on vascular dysfunction and splanchnic ischaemia–reperfusion injury. Br. J. Pharmacol. 120, 333–339.

Sun, Z., Wang, X., Lasson, A., Borjesson, A., Leveau, P., Haraldsen, P., Andersson, R., 1999. Roles of platelet-activating factor, interleukin-1 beta and interleukin-6 in intestinal barrier dysfunction induced by mesenteric arterial ischemia and reperfusion. J. Surg. Res. 87, 90–100.

Takayama, M., Ishibashi, M., Ishii, H., Kuraki, T., Nishida, T., Yoshida, M., 2001. Effects of neutrophil elastase inhibitor (ONO-5046) on lung injury after intestinal ischemia–reperfusion. J. Appl. Physiol. 91, 1800–1807.

Tavaf-Motamen, H., Miner, T.J., Starnes, B.W., Shea-Donohue, T., 1998. Nitric oxide mediates acute lung injury by modulation of inflammation. J. Surg. Res. 78, 137–142.

Tavares de Lima, W., Sirois, P., Jancar, S., 1992. Immune-complex alveolitis in the rats: evidence for platelet activating factor and leukotrienes as mediators of the vascular lesions. Eur. J. Pharmacol. 213, 63–70.

Turnage, R.H., Wright, J.K., Iglesias, J., LaNoue, J.L., Nguyen, H., Myers, S., 1998. Intestinal reperfusion-induced pulmonary edema is related to

increased pulmonary inducible nitric oxide synthase activity. Surgery 124, 457–462.

Van Soeren, M.H., Diehl-Jones, W.L., Maykut, R.J., Haddara, W.M., 2000. Pathophysiology and implications for treatment of acute respiratory distress syndrome. AACN Clin. Issues 11, 179–197.

Wang, Y., Xu, H., Mizoguchi, K., Oe, M., Maeta, H., 2001. Intestinal ischemia induces late preconditioning against myocardial infarction: a role for inducible nitric oxide synthase. Cardiovasc. Res. 49, 391–398.

Ward, D.T., Lawson, S.A., Gallagher, C.M., Conner, W.C., Shea-Donohue, T., 2000. Sustained nitric oxide production via L-arginine administration amelioratres effects of intestinal ischemia–reperfusion. J. Surg. Res. 89, 13–19.

Warren, J.S., Yabroff, K.R., Mandel, D.M., Johnson, K.J., Ward, P.A., 1990. Role of $O2^-$ in neutrophil recruitment into sites of dermal and pulmonary vasculitis. Free Radic. Biol. Med. 8, 163–172.

Welling, K.L., 1996. Acute respiratory distress syndrome. Pathogenesis and therapy of acute pulmonary failure. Ugeskr. Laeger 158, 3593–3600.

Yamamoto, S., Tanabe, M., Wakabayashi, G., Shimazu, M., Matsumoto, K., Kitajima, M., 2001. The role of tumor necrosis factor-alpha and interleukin-1beta in ischemia–reperfusion injury of the rat small intestine. J. Surg. Res. 99, 134–141.

Yi, E.S., Ulich, T.R., 1992. Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. Am. J. Pathol. 140, 659–663.

Zhou, J.L., Jin, G.H., Yi, Y.L., Zhang, J.L., Huang, X.L., 2003. Role of nitric oxide and peroxynitrite anion in lung injury induced by intestinal ischemia–reperfusion in rats. World J. Gastroenterol. 9, 1318–1322.