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The lazaroid, U-74389G, inhibits inducible nitric oxide synthase activity, reverses vascular failure and protects against endotoxin shock

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Abstract

The aim of our study was to investigate the effect of the 21-aminosteroid U-74389G [21-< 4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl-pregna-1,4,9,(11) triene-3,20-dione(*z*)-2-butenedionate] on the *l*-arginine-nitric oxide (NO) pathway in a rat model of endotoxin shock. Endotoxin shock was produced in male rats by a single intravenous (i.v.) injection of 20 mg/kg of Salmonella Enteritidis lipopolysaccharide (LPS). Rats were treated with U-74389G (7.5, 15 and 30 mg/kg i.v.) or vehicle (1 ml/kg i.v.) 5 min after endotoxin challenge. Lipopolysaccharide administration reduced survival rate (0%, 72 h after endotoxin administration) decreased mean arterial blood pressure, enhanced plasma concentration of bilirubin and alanine aminotransferase and increased plasma nitrite concentrations. Lipopolysaccharide injection also increased the activity of inducible NO synthase in the liver and in the aorta. Furthermore aortic rings from shocked rats showed a marked hyporeactivity to phenylephrine (1 nM–10 μM). In addition lipopolysaccharide (50 μg/ml for 4 h) in vitro stimulation significantly increased nitrite production in peritoneal macrophages harvested from normal rats. Treatment with U-74389G (15 and 30 mg/kg i.v., 5 min after endotoxin challenge) significantly protected against lipopolysaccharide-induced lethality (90% survival rate 24 h and 80% 72 h after lipopolysaccharide injection, respectively, following the highest dose of the drug), reduced hypotension, ameliorated liver function, decreased plasma nitrite levels, restored the hyporeactivity of aortic rings to their control values and inhibited the activity of inducible NO synthase in the liver and in the aorta. Finally, U-74389G in vitro (12.5, 25 and 50 μM) significantly inhibited nitrite production in endotoxin stimulated peritoneal macrophages. The data suggest that U-74389G may exert beneficial effects in an experimental model of septic shock by inhibiting the activity of the inducible NO synthase.

Keywords: Lazaroids; Endotoxin shock; Nitric oxide (NO) synthase, inducible

1. Introduction

There is evidence to suggest that, among the various mediators implicated in endotoxemia, nitric oxide (NO), together with tumor necrosis factor and oxygen-derived free radicals, is involved in the pathogenesis of endotoxin shock (Morgan et al., 1987; Thiemermann and Vane, 1990). The L-arginine/NO pathway also plays an important role in other forms of circulatory shock such as haemorrhagic and splanchnic artery occlusion shock (Wright et al., 1992; Squadrito et al., 1994; Szabò and Thiemermann, 1994).

As far as endotoxin shock is concerned, the administration of bacterial lipopolysaccharide to experimental animals induces severe metabolic and physiological disturbances which lead to shock, vascular failure and death by multi-organ system failure. These pathological sequelae are induced by endotoxin which triggers the release of a large number of mediators, including cytokines, arachidonic acid metabolites, reactive oxygen metabolites and more specifically NO from several cell types.

In the last few years, there has been increasing interest in the physiological and pathophysiological roles of NO in sepsis and experimental endotoxemia.

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NO is produced by three different synthases: the inducible NO synthase, the constitutive brain NO synthase, and the constitutive NO synthase in endothelial cells.

Controversial results have been reported regarding the positive effects of increasing or decreasing NO production during endotoxic shock (Wang et al., 1994; Gundersen et al., 1997).

It has been suggested that NO derived from endothelial NO synthase plays a protective role in endotoxin-induced gastrointestinal damage; on the other hand a reduced formation of NO in the endothelium can induce vasoconstriction, microvascular thrombosis and death (Hutcheson et al., 1990). Nitric oxide produced in macrophages after the induction of inducible NO synthase by lipopolysaccharide (or other agents) may have an important role in the antimicrobial activity of activated macrophages (Nathan, 1992), but the increased production of NO by inducible NO synthase is responsible for some pathological features (hypotension, myocardial dysfunction and a decreased response to vasoconstrictors) of endotoxic shock (Nava et al., 1992). Thus, it has been suggested that modulation of NO produced by inducible NO synthase could be beneficial in experimental models of sepsis.

The 21-aminosteroid, U74389G [21-< 4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl-pregna-1,4,9,(11)-triene-3,20-dione(*z*)-2-butenedionate] has been shown to inhibit lipid peroxidation (McCall et al., 1987). Moreover, it has been demonstrated that the inhibition of lipid peroxidation improves the survival rate of endotoxemic rats and sheeps (Kunimoto et al., 1987; Semrad et al., 1993; Remmers et al., 1996).

In a rat model of splanchnic artery occlusion shock (Squadrito et al., 1995) and during ischemia and reperfusion of rat myocardium (Campo et al., 1996), U-74389G has been reported to have anti-shock, endothelial and cardiac protective actions.

Thus, our study was carried out to further evaluate the effects of this compound on the L-arginine-nitric oxide (NO) pathway in a rat model of endotoxin shock. U-74389G was found to reduce the pathological sequelae associated with endotoxemia by inhibiting inducible nitric oxide activity.

2. Materials and methods

2.1. Endotoxin shock procedure

Male Sprague–Dawley rats (200–250 g) fed on a standard diet and with water ad libitum, were used. Environmental conditions were standardized, including a room temperature of 22 ± 2°C and 12 h artificial lighting. The experiments were approved by the Ethical Committee of the University of Messina. Salmonella enteritidis lipopolysaccharide was dissolved in sterile 0.9% NaCl at a concentration of 20 mg/ml. Endotoxin shock was induced, under light anaesthesia with ether, by administering a single i.v. (tail vein) dose of 20 mg/kg of LPS. Control rats received an equal volume of vehicle (0.9% NaCl).

2.2. Survival evaluation

Five minutes after LPS injection, the control rats were injected with an i.v. bolus of 0.9% NaCl (1 ml/kg) while the treated rats received U-74389G (7.5, 15 and 30 mg/kg) as an i.v bolus through a previously implanted intrajugular catheter. Survival rate was evaluated for 72 h after endotoxin administration.

2.3. Arterial blood pressure

A second group of rats was used to monitor blood pressure. Briefly, the animals were anaesthetized with urethane (1.3 g/kg) and a cannula (PE 50) was inserted into the left common carotid artery and the arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure, monitored for 5 h, was displayed on channels of a polygraph. Arterial blood pressure is reported as mean arterial pressure in mmHg. These rats were treated as previously described: 5 min after LPS injection the control rats were injected with an i.v. bolus of 0.9% NaCl (1 ml/kg) while the treated rats received U-74389G (7.5, 15 and 30 mg/kg) as an i.v bolus.

2.4. Plasma concentration of bilirubin and alanine aminotransferase

A third group of rats (control and U-74389G treated rats) was used to measure plasma bilirubin, alanine aminotransferase, and nitrite as well as inducible NO synthase activity and in vitro vascular reactivity. In these animals a cannula was inserted into the right jugular vein and blood (1 ml) was collected 5 h following LPS administration to study liver function (bilirubin) and the intracellular marker enzyme alanine aminotransferase. The EDTA-treated plasma concentration of bilirubin and ALT were evaluated with commercially available kits.

2.5. Inducible NO synthase-activity assay

The animals were killed with an overdose of anaesthetic and tissues (collected 5 h after LPS challenge) were first rinsed with saline solution and then frozen. The tissues were homogenized on ice in Tris buffer (50 mM, Tris–HCl, pH 7.4, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates, as previously described (Southan et al., 1995). In some experiments, homogenates of tissues collected from untreated endotoxin shocked rats were incubated with U-74389G (50 μM) for 1 h. To determine Ca^{2+}-independent inducible NO synthase activ-

ity in the homogenates, production of [^{3}H]L-citrulline from [^{3}H]L-arginine (10 μM, 5 kBq per tube) was measured in the presence of 1 mM NADPH, 30 nM calmodulin, 5 μM tetrahydrobiopterin, 2 mM EGTA for 20 min at room temperature. The reactions were stopped by adding 1 ml of ice-cold HEPES buffer pH 5.5, containing 2 mM EDTA and 2 mM EGTA. After separation by means of Dowex 50 W (sodium form), [^{3}H]L-citrulline activity was measured by scintillation counting (Bekman analytical instruments Milan, Italy). Experiments performed in the absence of NADPH measured the extent of [^{3}H]L-citrulline formation independent of inducible NO synthase activity.

2.6. Macrophage culture

Peritoneal macrophages were harvested from control normal rats by washing the abdominal cavity with RPMI 1640. As previously reported (Altavilla et al., 1995) the cells were centrifuged twice and resuspended in the same medium at a concentration of 1×10^6/ml. Peritoneal macrophages were obtained after 2 h adhesion to plastic Petri dishes (Nunc, Denmark), at 37°C in an atmosphere of 5% CO_2 in air. The homogeneity and the viability of macrophages were greater than 98% as determined by differential staining and trypan blue exclusion.

In order to study the effects of U-74389G on the inducible nitric oxide (iNOS) activity, peritoneal macrophages (1×10^6/ml) were stimulated for 24 h with 50 μg/ml of S. enteritidis LPS. U-74389G (12.5, 25 and 50 μM) was added to the medium 6 h after the induction of iNOS with LPS. At this time there is no detectable increase in the concentration of nitrite, and agents such as glucocorticoids (that inhibit the induction but not the activity of iNOS) have no effect on subsequent nitrite production (Szabò et al., 1994). Macrophage culture supernatants were collected after 24 h and nitrite concentrations were determined as described below.

2.7. Nitrite measurement

Nitric oxide release was determined spectrophotometrically by measuring both nitrite and nitrate in plasma. Blood (0.7 ml) was drawn at different time points (0, 1 h, 2 h, 3 h 4 h and 5 h following LPS challenge). The blood removed was replaced by saline solution (NaCl 0.9%, at a ratio of 1:2).

Nitrate was stoichometrically reduced to nitrite by incubation of the sample (100 μl plasma) for 2 h at 37°C, in the presence of 0.1 unit/ml nitrate oxidoreductase, 120 μM NADPH and 5 μM FAD (flavinadenine dinucleotide) in a final volume of 103 μl in 1.5-ml microtubes. After nitrate had been reduced to nitrite, NADPH which interfered with the subsequent nitrite determination was oxidized with 10 units/ml of L-lactic dehydrogenase and 10 mM sodium pyruvate for 30 min at 37°C in a final volume of 114 μl. Nitrite concentration in plasma or macrophage supernatants was assayed in a standard Griess reaction (Ding et al., 1988). Briefly, 100 μl of plasma (or supernatant) was incubated with an equal volume of Griess reagent (sulphanilamide 1% and naphthylethylenediamine 0.1% in 5% H_3PO4) at room temperature for 10 min. The absorbance was determined at 540 nm, using a microtiter plate reader (340 ATTC, SLT-Labinstruments, Austria) and 96-well plates. Nitrite concentrations were calculated by comparison with a standard curve with sodium nitrite ($NaNO_2$: 1.25–100 μM) with control baseline plasma (or supernatant) as a blank.

2.8. Isolated aortic rings

Thoracic aortae from control and U-74389G treated rats were removed 5 h after LPS injection and placed in cold Krebs' solution of the following composition (mM): NaCl 118.4, KCl 4. 7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, Na HCO_3 25.0 and glucose 11.7. Then the aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. The rings were then placed under 1 g of tension in an organ bath containing 10 ml of Krebs' solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs' solution at 15–20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (100 nM). When the contraction was stable, the functional integrity of endothelium was assessed by a relaxant response to acetylcholine (100 nM; 50% relaxation). Relaxation of the rings was calculated as a percentage of contractile force. Concentration-response curves were obtained by cumulative concentrations of phenylephrine (1 nM–10 μM), added to intact or endothelium denuded aortic rings. The results (mean ± S.D.) are expressed as g of tension/mg of tissue. In some experiments aortic rings were obtained from untreated rats 5 h following LPS injection and then were incubated in vitro with U-74389G (50 μM) for 1 h.

2.9. Drugs

NADPH, nitrate oxidoreductase (EC 1.6.6.2; aspergillus species), FAD: flavinadenine dinucleotide, L-lactic dehydrogenase (EC 1.1.1.27; type XI; from rabbit muscle), phenylephrine hydrochloride, acetylcholine, indomethacin and *Salmonella* enteritidis lipopolysaccharide (trichloroacetic acid extraction) were obtained from Sigma Chemical, St. Louis, MO. U-74389G was a kind gift from Upjohn Italy.

2.10. Statistical analysis

The data are expressed as means ± S.D. and were analyzed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. A probability error of less than 0.05 was selected as criterion for statistical significance. For survival data, statistical analysis was done with Fisher's exact probability test.

3. Results

3.1. Survival rate

Table 1 shows the ratio of animals surviving in each group to the total number of animals throughout the experimental period. All control rats survived the entire period of the study. The endotoxin treated group had 1 and 0 survivors out of 10 within 24 and 72 h, respectively. U-74389G, administered 5 min after LPS injection, significantly protected against endotoxin induced lethality. The endotoxin shocked group treated with 30 mg/kg of U-74389G had 9 survivors and 8 survivors out of 10 after 24 and 72 h, respectively (Table 1). This highest dose was the most effective.

3.2. Arterial blood pressure and heart rate

Rats injected with endotoxin showed a sharp and long-lasting decrease in mean arterial blood pressure (Fig. 1a). Heart rate was also significantly decreased 5 h following LPS injection (Sham shock + vehicle = 322 ± 16 beats/min; Sham shock + U-74389G = 298 ± 23 beats/min; LPS + vehicle 198 ± 18 beats/min; $P < 0.001$) U-74389G (5 min after LPS challenge) significantly inhibited the sustained decrease in MAP (Fig. 1a) and heart rate (234 ± 21 beats/min and 265 ± 24 beats/min following the U-74389G doses of 15 mg/kg and 30 mg/kg, respectively. $P < 0.05$ vs. LPS + vehicle).

Table 1
Effects of U-74389G on survival rate in rats subjected to endotoxin shock (LPS)

Treatment	Hours after LPS				
	3	6	12	24	72
Control + vehicle (1 ml/kg)	10/10	10/10	10/10	10/10	10/10
Control + U-74389G (30 mg/kg)	10/10	10/10	10/10	10/10	10/10
LPS + vehicle (1 ml/kg)	10/10	5/10[a]	2/10[a]	1/10[a]	0/10[a]
LPS + U-74389G (7.5 mg/kg)	10/10	6/10	4/10	3/10	2/10
LPS + U-74389G (15 mg/kg)	10/10	8/10	8/10[b]	6/10[b]	5/10[b]
LPS + U-74389G (30 mg/kg)	10/10	10/10[b]	10/10[c]	9/10[c]	8/10[c]

The statistical analysis was performed using Fishers's probability test. Animals received U-74389G or its vehicle (0.9% NaCl), 5 min after endotoxin challenge.
[a] $P < 0.01$ vs. controls; [b] $P < 0.05$ vs. LPS + vehicle; [c] $P < 0.01$ vs. LPS + vehicle.

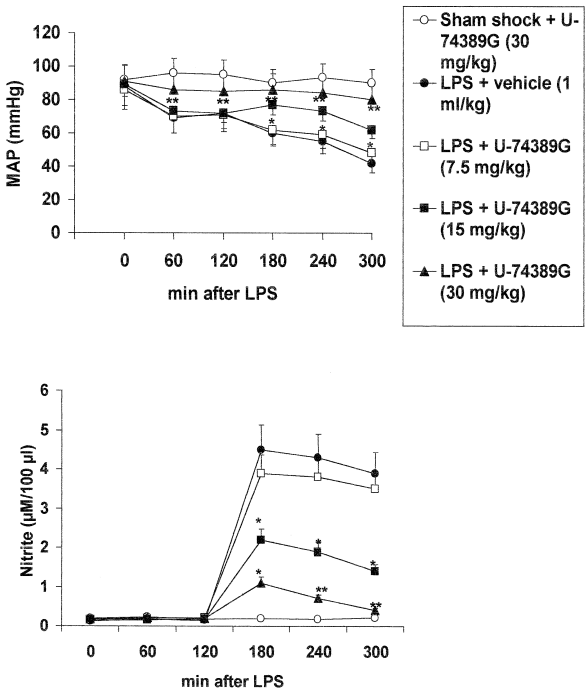

Fig. 1. Effects of vehicle (1 ml/kg of a 0.9% NaCl solution, 5 min after endotoxin challenge) or U-74389G (7.5, 15 and 30 mg/kg, 5 min after endotoxin challenge) on mean arterial blood pressure (a) and plasma nitrite concentration (b) in rats subjected to endotoxin shock (LPS). Each value represents the mean ± S.D. of six experiments. * $P < 0.05$ vs. vehicle-treated rats. * * $P < 0.01$ vs. vehicle-treated rats.

3.3. Plasma nitrite

In control rats treated either with vehicle or U-74389G, plasma nitrite levels (Fig. 1b) were very low (0.11 ± 0.06 μM/100 μl and 0.10 ± 0.05 μM/100 μl, respectively). In endotoxin-shocked rats plasma concentrations of nitrite rose 3 h after endotoxin injection (4.2 ± 0.5 μM/100 μl). The administration of U-74389G (Fig. 1b) to endotoxin-shocked rats decreased nitrite appearance in plasma. Following the highest dose of U-74389G, the plasma nitrite levels were 0.10 ± 0.04 μM/100 μl (5 h after LPS).

3.4. Plasma concentration of bilirubin and alanine aminotransferase

Endotoxin shock was accompanied by marked liver dysfunction. In fact, 5 h after LPS injection, there was a marked increase in the plasma concentration of bilirubin and alanine aminotransferase (Table 2) The administration of U-74389G (15 and 30 mg/kg) blunted the increase in blood levels of bilirubin and alanine aminotransferase.

Table 2
Effect of U-74389G on the plasma concentration of alanine aminotransferase (ALT) and bilirubin and on iNOS activity in rats subjected to endotoxin induced shock

Treatment	ALT (iu/ml)	Bilirubin (μM)
Control + vehicle (1 ml/kg)	0.06 ± 0.01	3.2 ± 1.2
Control + U-74389G (30 mg/kg)	0.02 ± 0.01	2.5 ± 0.9
LPS + vehicle (1 ml/kg)	0.9 ± 0.1[a]	14.4 ± 2.3[a]
LPS + U-74389G (7.5 mg/kg)	0.7 ± 0.1	12.7 ± 3.4
LPS + U-74389G (15 mg/kg)	0.3 ± 0.09[b]	6.2 ± 2.1[b]
LPS + U-74389G (30 mg/kg)	0.09 ± 0.05[c]	3.9 ± 1.6[c]

Treatment	iNOS activity, pmol of mg of L-citrulline × 30 min × mg of Protein	
	Aorta	Liver
Control + vehicle (1 ml/kg)	4.2 ± 1.2	5.2 ± 0.5
Control + U-74389G (30 mg/kg)	3.9 ± 1.6	5.5 ± 0.9
LPS + vehicle (1 ml/kg)	45.2 ± 5.3[a]	65.5 ± 4.3[a]
LPS + U-74389G (7.5 mg/kg)	35.3 ± 4.7	52.7 ± 3.4
LPS + U-74389G (15 mg/kg)	18.5 ± 5.9[b]	28.5 ± 6.1[b]
LPS + U-74389G (30 mg/kg)	9.6 ± 5.3[c]	10.6 ± 2.6[c]
LPS + U-74389G (50 μM)	8.4 ± 3.3[c]	7.7 ± 1.9[c]

Each point represents the mean ± S.D. of six experiments.
Blood and tissues were collected 5 h following endotoxin challenge.
[a] $P < 0.001$ vs. controls; [b] $P < 0.05$ vs. LPS + vehicle; [c] $P < 0.001$ vs. LPS + vehicle.

3.5. Inducible NO synthase activity

LPS administration induced a sharp increase in the activity of the inducible isoform of nitric oxide synthase. Table 2 shows that a marked increase in inducible NO synthase activity was observed in the aorta and in the liver of rats challenged with LPS. Treatment with U-74389G (15 and 30 mg/kg) significantly decreased inducible NO synthase activity in both liver and aorta. In another set of experiments, tissue homogenates, collected from untreated LPS shocked rats, were incubated for 1 h with U-74389G. The lazaroid was able to reduce inducible NO synthase activity (Table 2) also under these experimental conditions.

In order to further investigate whether U-74389G interferes with inducible NO synthase, we stimulated macrophages in vitro with lipopolysaccharide (LPS 50 μg/ml for 24 h). LPS significantly increased nitrite levels (Table 3). U-74389G (12.5, 25 and 50 μM) added 6 h after endotoxin reduced, in a dose-dependent manner, the nitrite concentrations in the supernatants of LPS-primed macrophages (Table 3).

Table 3
Effect of U-74389G on nitrite concentrations in the medium of peritoneal macrophages activated with LPS

Treatment	Nitrite (μM)
Control + vehicle (1 ml)	2.4 ± 0.4
LPS (50 μg/ml)	25.3 ± 3.1[a]
LPS + U-74389G (12.5 μM)	15.2 ± 1.5[b]
LPS + U-74389G (25 μM)	9.3 ± 1.1[b]
LPS + U-74389G (50 μM)	7.5 ± 1.3[c]

Data are expressed as means ± S.D. for 6 wells from 4 experiments.
Macrophages were stimulated for 24 h with lipopolysaccharide (LPS), U-74389G was added 6 h after LPS.
[a] $P < 0.05$ vs. control; [b] $P < 0.05$ vs. LPS; [c] $P < 0.01$ vs. LPS.

3.6. Contractile response to phenylephrine

Fig. 2 shows the contractile response to phenylephrine (1 nM–10 μM) of aortic rings collected from control or endotoxin shocked rats. Endotoxin shock markedly decreased the responsiveness to phenylephrine in aortae with (results not shown) or without endothelium (Fig. 2). The administration of U-74389G (30 mg/kg) did not modify the aortic reactivity of control rats. In contrast, U-74389G treatment significantly improved the impaired contractile response to phenylephrine in endotoxin shocked rats (Fig. 2).

In endothelium denuded aortic rings from untreated shocked rats U-74389G (50 μM) added for 1 h in the organ bath restored phenylephrine sensitivity to control value (Fig. 3), while it did not modify the contractile

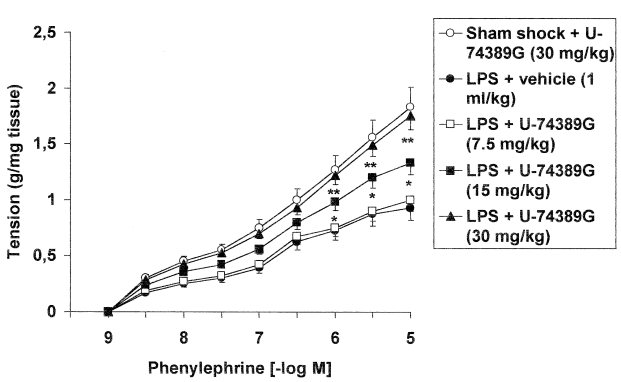

Fig. 2. Contractile response to cumulative concentrations of phenylephrine in aortic rings without endothelium from control or endotoxin shocked rats treated with vehicle (1 ml/kg of 0.9% NaCl solution) or U-74389G (7.5, 15 and 30 mg/kg, 5 min after endotoxin challenge). Each value represents the mean ± S.D. of six experiments. * $P < 0.05$ vs. vehicle-treated rats. * * $P < 0.01$ vs. vehicle-treated rats.

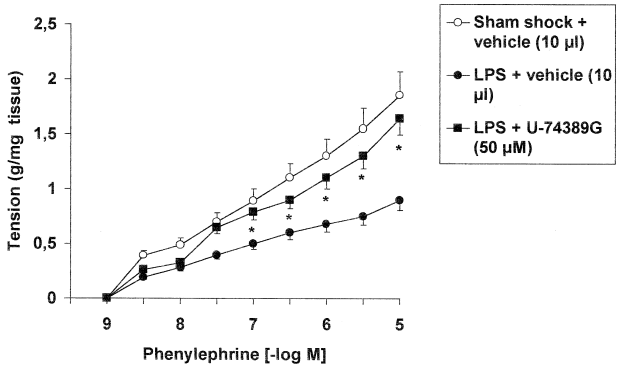

Fig. 3. Effects of U-74389G (50 μM) on contractile response to cumulative concentrations of phenylephrine in aortic rings without endothelium from sham or endotoxin (LPS)-shocked rats. U-74389G was added to the bath 1 h before phenylephrine. $^{*}P < 0.01$ vs. LPS + vehicle.

response to phenylephrine of aortic rings collected from sham rats (Fig. 3).

4. Discussion

Our data confirmed that U-74389G has beneficial effects in experimental shock, as previously shown (Squadrito et al., 1995; Remmers et al., 1996) and furthermore add new information regarding the underlying mechanism of action.

We found that U-74389G administration was able to increase the resistance of rats to the pathophysiological consequences of endotoxin shock. The effect was marked in terms of survival rate, protection of liver function and improvement in vascular dysfunction.

The latter was improved by U-74389G treatment: the lazaroid injected 5 min after endotoxin challenge produced a marked increase in blood pressure, and aortic rings of U-74389G treated rats exhibited a greater contractile response to phenylephrine. Together, these findings suggest that U-74389G was able to reverse vascular dysfunction that occurs during septic shock.

The mechanisms underlying the circulatory failure that occurs during shock have been the subject of intense investigation.

It has been proposed that the L-arginine/NO pathway plays an important role in the pathogenesis of vascular dysfunction during circulatory shock. NO generated by constitutive endothelial NO synthase contributes to the regulation of local and systemic vascular resistance, distribution of blood flow and oxygen delivery, sodium balance and arterial blood pressure (Vane et al., 1990; Vane, 1994).

Impaired NO synthesis may lead to pathological vasoconstriction, to tissue ischaemia with organ dysfunction, and to the genesis or perpetuation of hypertension (Dinerman et al., 1993).

Increased NO production following the induction of a distinct isoform of NO synthase (inducible NO synthase) by endotoxin, interleukin 1β and other cytokines in several cell types, including macrophages and smooth muscle cells, has been shown to play an important role in the pathogenesis of inflammation and circulatory shock (Nathan, 1992).

The production of a large amount of NO by the inducible isoform of NO synthase contributes to the vascular decompensation and to the hyporeactivity of the vasculature to vasoconstrictor agents observed in several experimental models of circulatory shock, including septic shock (Szabò and Thiemermann, 1994).

LPS administration caused two-phase hypotension. It has been shown that the first component of endotoxin-induced hypotension is due to the release of eicosanoids, while the second one is caused by an overproduction of NO derived from increased activity of inducible NO synthase (Szabò and Thiemermann, 1994). In agreement with these data, we found enhanced plasma levels of nitrite 180 min following the injection of endotoxin. Inhibitors of eicosanoid biosynthesis prevent only the first component of endotoxin hypotension, but do not affect the second one. Lazaroids by stabilizing the cell membrane, also reduce eicosanoids (Semrad et al., 1993), which explains why U-74389G blunts the first phase of the LPS-induced decrease in blood pressure. The hypothesis that U-74389 may also inhibit constitutive NOS and in turn increase blood pressure in the first-phase hypotension can probably be ruled out. In fact, using the calcium-dependent conversion of L-arginine to L-citrulline in cell homogenates obtained from the scraped intimal surface of fresh bovine aortas as experimental model to investigate endothelial NO synthase, we found that U-74389G (50 μM) does not affect constitutive isoform of NO synthase (unpublished observations). However, the compound was also able to reverse the late phase of endotoxin-induced hypotension Furthermore our results show that aortic rings collected from rats subjected to LPS-induced shock and treated with U-74389G has a greater contractile response to phenylephrine than those from vehicle-treated rats. The effect seems to be a direct phenomenon, since it was observed even when the lazaroid was added in the organ bath containing aortic rings taken from untreated LPS-shocked rats.

Together, these findings would suggest that U-74389G might interfere with NO synthesis and therefore prompted us to explore more deeply the effect of U-74389G on the L-arginine/nitric oxide pathway.

LPS challenge produced a significant increase in iNOS activity in the liver and in aorta. Furthermore plasma nitrite levels were significantly increased in endotoxemic rats compared to those of shocked rats.

Treatment with U-74389G markedly reduced inducible NO synthase activity in both tissues and blunted the enhanced plasma levels of nitrite. This inhibition of NO production by the inducible isoform of iNOS caused a marked improvement in vascular dysfunction and in organ

failure. This latter was significantly affected, as evidenced by the reduced plasma concentration of bilirubin and alanine aminotransferase found in U-74389G-treated endotoxin shocked rats. Expression of iNOS activity in hepatocytes and Kuppfer cells results in a reduction of the synthesis of protein (Curran et al., 1991), prostaglandins and interleukin-6 (Stadler et al., 1993); and inhibition of mitochondrial respiration (Morris and Billiar, 1994), possibly due to the formation of peroxynitrite (Szabò and Salzman, 1995). Thus, we propose that the beneficial effects of NOS inhibitors on the liver dysfunction elicited by endotoxaemia are due to the prevention of the above-mentioned cytotoxic effects of NO.

Together, these results strongly support the idea that this lazaroid inhibits iNOS activity.

In order to confirm the mechanism by which U-74389G interferes with inducible NO synthase we performed experiments in macrophages activated with endotoxin. Our results suggest that the lazaroid reduces nitrite production, indicating that this compound may inhibit the activity of inducible nitric oxide synthase. This confirms the results obtained when U-74389G was added to endothelium denuded aortic rings produced from untreated rats subjected to LPS injection.

In conclusion, we have shown that the lazaroid, U-74389G, inhibits both in vivo and in vitro iNO synthase activity. This inhibition, at least in endotoxin shock in rats, enhances blood pressure, increases survival rate, improves vascular hypocontractility and reverses LPS-induced liver damage. Finally, these findings would suggest that inducible NO synthase inhibition may contribute, at least in part, to the acute protective effects of U-74389G during low-flow states, such as circulatory shock.

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