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Inhibition of lipopolysaccharide-induced bowel erythrocyte extravasation in rats, and of mesenteric hypoperfusion in dogs, by phosphodiesterase inhibitors

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Abstract

Sepsis is intricately associated with mesenteric ischemia. The remote complications of mesenteric ischemia are essentially those of sepsis, whether as a cause or as a consequence. Experimental endotoxic shock induces bowel hypoperfusion, erythrocyte extravasation and intestinal necrosis. The effects of pentoxifylline, rolipram and denbufylline, three phosphodiesterase inhibitors, were studied on endotoxin-induced bowel erythrocyte extravasation and intestinal and renal hypoperfusion, in conscious rats and anaesthetized dogs, respectively. Two hours after lipopolysaccharide i.v. injection in rats, erythrocyte extravasation was evident throughout the intestinal musculature and mucosa, apparently without affecting lungs, heart, kidneys, liver or pancreas. Pretreatment with the non-selective phosphodiesterase inhibitor, pentoxifylline, or selective phosphodiesterase IV inhibitors such as denbufylline or rolipram reduced intestinal haemoconcentration. In the anaesthetized dog, pentoxifylline and denbufylline both inhibited the *E. coli* lipopolysaccharide-induced mesenteric blood flow fall, without affecting renal blood flow or cardiac index. In conclusion, phosphodiesterase inhibitors protected from intestinal damage and bowel hypoperfusion after lipopolysaccharide challenge. This action may thus play a role in the protective effects against endotoxin-induced lethal toxicity previously described for phosphodiesterase inhibitors.

Keywords: Intestinal erythrocyte extravasation; Lipopolysaccharide; Phosphodiesterase inhibitor

1. Introduction

Severe endotoxemia induces a wide spectrum of changes in the gastrointestinal tract, including inflammation, disruption of the basal mucosa, perturbations in intestinal myoelectric activity (Hewett and Roth, 1993; Wallace et al., 1995), and a fall in mesenteric blood flow (Whitworth et al., 1989). By using different experimental models of intestinal ischemia, such as mechanical occlusion of mesenteric arteries (Sibbons and Spitz, 1992), hypoxia (Caplan et al., 1990) or platelet-activating factor (PAF) administration (Xiamong et al., 1995), it is possible to produce intestinal erythrocyte extravasation and necrosis. These lesions are similar to those observed in necrotizing enterocolitis in humans, where a thromboembolic sudden occlusion of large mesenteric vessels seems to be the trigger of intestinal ischemic necrosis. A synergistic effect between PAF and lipopolysaccharide on intestinal hypoxia has been already described in rats (Caplan et al., 1992). Immediately after i.v. infusion of *E. coli* lipopolysaccharide in anaesthetized dogs, a model that closely mimics endotoxic shock, mesenteric blood flow decreases dramatically, and bowel hypoperfusion occurs. It has been suggested that hypoxia due to intestinal hypoperfusion can vary from no damage to irreversible transmural bowel necrosis, depending on factors such as severity, duration, tissue maturity and others (Bulkley et al., 1985). The mechanism leading to lipopolysaccharide-induced mesenteric hypoperfusion is not well understood, but several studies indicate that PAF (Caplan et al., 1990), thromboxane A_2 (Temple et al., 1986) and other inflammatory mediators play a role as endogenous bowel vasoconstrictors. Other findings suggest a defensive role of constitutive nitric oxide (NO) by modulation of local blood flow and attenuation of platelet agregation after lipopolysaccharide challenge, but paradoxically low doses of endotoxin in

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vivo can induce a NO synthase in rats which is associated with microvascular injury in the jejunum and the colon (László et al., 1994).

It has also been reported that the release of endogenous tumor necrosis factor (TNFα) contributes to the vascular changes required for the extravasation of erythrocytes in the small intestinal mucosa (Pellón et al., 1994). Moreover, primary mesenteric ischemia is associated with a greatly increased risk of secondary sepsis because necrotic bowel areas and transmural ulceration can be permanent focuses of peritoneal sepsis. For those reasons, the maintenance of bowel integrity in septic shock may be of relevance and has been the target of some therapeutic approaches.

Adenosine (Firestein et al., 1994) and tyrosine (Novogrodsky et al., 1994) kinase inhibitors, PAF receptor antagonists (Xiamong et al., 1995), dual inhibitors of lipoxygenase (Schade et al., 1994) and pentoxifylline (Schade, 1990; Schönharting and Schade, 1989), are known to reduce mortality in mouse models of septic shock, but less is known about their properties on earlier symptoms of septic shock. Moreover, phosphodiesterase IV inhibitors are known to protect from lipopolysaccharide-induced hypothermia and lipopolysaccharide-induced increase of TNFα plasma levels (Ochalski et al., 1993), or liver (Fischer et al., 1993) and lung (Ishizaka et al., 1988) injury in animal models of gram-negative sepsis. It is also well known that phosphodiesterase inhibitors (i.e theophylline, papaverine, pentoxifylline and amrinone) are splanchnic vasodilators, but there are no data about such an effect on experimental models of septic shock. The purpose of the present study was to compare the activity of pentoxifylline, a non-selective phosphodiesterase inhibitor (Ward and Clissold, 1987), with that of rolipram and denbufylline, two well known selective phosphodiesterase IV inhibitors (Nicholson et al., 1989), on lipopolysaccharide-induced bowel erythrocyte extravasation and hypoperfusion in rats and dogs.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–250 g were obtained from Interfauna Ibérica (Barcelona, Spain) and were housed in rack-mounted cages and fed standard laboratory chow and water at libitum. Adult beagle dogs of both sexes obtained from Biocentre (Barcelona, Spain) and weighing between 11 and 14 kg were used. All experimental procedures described in this paper were previously notified to the regulatory authorities, and the guidelines recently approved by the Catalonia Parliament were strictly followed.

2.2. Endotoxic shock in rats

Male rats were randomized and deprived of food, but not water, for 18 h prior to an experiment. Lipopolysaccharide (40 mg/kg) or saline was injected as a bolus through the caudal vein, and 2 h later the rats were killed by cervical dislocation and the abdomen was incised along the midline. The whole intestine was excised and observed macroscopically. A complete postmortem examination, including sampling of abdominal and intrathoracic organs (i.e. heart, lungs, kidneys, pancreas, liver, and adrenal glands) for histological studies (see below), was performed. A time course study of lipopolysaccharide effects on bowel integrity up to the animal's death, using non-treated animals, was run in parallel. Rolipram (10 and 30 mg/kg), pentoxifylline (5, 10 and 50 mg/kg) and denbufillyne (0.1, 1 and 3 mg/kg) were administered thrice i.p., according to a preventive (−30, 0 and 60 min) or a curative (30, 60 and 90 min) design, considering the time when lipopolysaccharide was administered as zero time. Macroscopic intestinal hyperaemia was scored as absent (0), mild (1), moderate (2) or severe (3) by an observer unaware of the study design.

2.3. Extraction and quantitation of haemoglobin from intestinal tissue

Intestinal segments of about 2 cm were routinely collected from each animal, and the haemoglobin content was determined colorimetrically by the cyanomethaemoglobin method (Tavares de Lima et al., 1992). Briefly, fragments of ileum were excised and minced in 2 ml of Drabkin's solution. After 24 h at room temperature in the dark, the tissue was removed, the sample was centrifuged and the optical density of the supernatant was determined at 546 nm. The concentration of haemoglobin was calculated by comparison with a standard curve.

2.4. Histological studies

Ileum segments of about 2 cm from each rat were preserved in 10% buffer formalin, wax embedded, cut at a nominal thickeness of 5 μm and stained with haematoxylin eosin. Sections were analyzed by a pathologist unaware of the experimental protocol, and microscopic erythrocyte extravasation was assessed as absent (0), erythrocytes present only inside vessels; mild (1), erythrocytes present in the muscularis layers and vessels; moderate (2), erythrocytes present mainly in the muscularis and submucosa layers; and severe (3), erythrocytes spread through all muscularis, submucosa and mucosa layers.

2.5. Mesenteric blood flow in dogs

The procedure described by Vincent was used (Vincent et al., 1988). Briefly, beagle dogs were anaesthetized with sodium pentobarbitone (35 mg/kg i.v. plus a permanent infusion of 6 mg/kg/h through the left cephalic vein). The animals were tracheally intubated and mechanically ventilated with room air (respiratory rate: 15/min, tidal

volume 10 ml/kg) by means of a Harvard pump (Model 607-A). Left carotid artery, left femoral vein and right cephalic vein were cannulated to measure systemic blood pressure (HP transducer, type # 1146-DPT-100), to administer lipopolysaccharide and saline (see below) and to administer the compounds, respectively. Furthermore, a thermodilution catheter (The Baltherm flow-directed thermal dilution catheter, model 73-6067, Rahway, NJ, USA; 110 cm length, 7F diameter) was inserted up to the pulmonary artery through the right jugular vein to measure cardiac output, and perivascular electromagnetic blood flow probes (Narco bio-systems, Houston, TX, USA) were implanted on the superior mesenteric and left renal arteries. Cardiac output was obtained using a Cardiotherm (model 500), connected to an ECG Synchronized Injector 500 (Columbus Instruments International Corporation, Columbus, OH, USA). Mesenteric and renal arterial blood flow were obtained using a Narcomatic electronic flowmeter (Narco Bio-Systems, Houston, TX, USA). Haemodynamic parameters were continuosly recorded on an 8-channel polygraph (7758-B Hewlett Packard, Palo Alto, CA, USA). After a 30 min stabilization period, a vehicle (control), pentoxifylline (250 μg/kg/min) or denbufillyne (2.5 μg/kg/min) infusion was started ($t = 0$) and maintained for 255 min. Fifteen minutes later ($t = 15$), a slow (5 min) intravenous injection of 3 mg/kg of *E. coli* lipopolysaccharide was performed. At $t = 45$ min, a saline infusion was started at a rate of 20 ml/kg/h during the first 30 min and 10 ml/kg/h during the next 3 h in order to increase plasma volume and improve venous return.

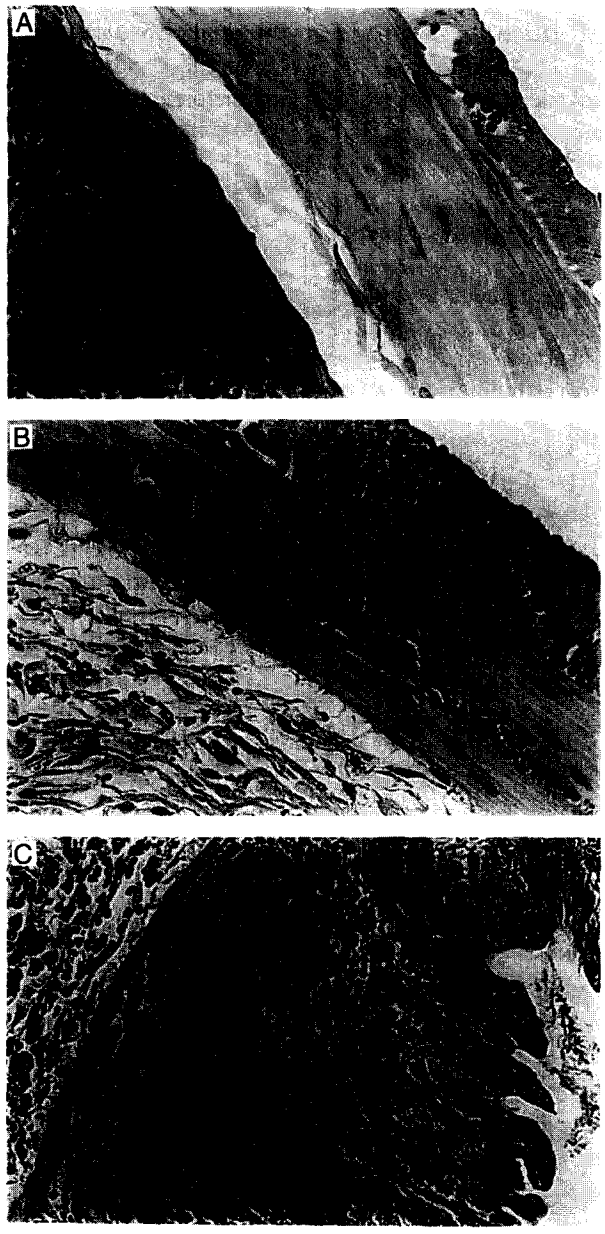

Fig. 1. Microphotograph of ileum sections from muscularis (A) of control rats (×400), (B) muscularis (×400) and (C) mucosa (×800), 2 h after administration of *E. coli* lipopolysaccharide. Note that several erythrocytes are inside a vessel in (A), (B) and (C) were scored as severe (see Methods).

Table 1
Haemoglobin accumulation (mg·g^{-1} tissue) in intestinal sections, 2 h after lipopolysaccharide challenge in rats

	Control	*E. coli* lip.-treated rats
Duodenum	4.75 ± 0.59 (8)	5.43 ± 0.81 (7)
Distal ileum	3.36 ± 0.61 (9)	9.26 ± 1.05 [a] (8)
Ascendent colon	2.42 ± 0.57 (7)	5.95 ± 0.49 [a] (7)
Rectum	3.81 ± 0.71 (7)	4.01 ± 0.68 (7)

lip. = lipopolysaccharide. Values are means ± S.E.M. (7–9). [a] $P < 0.01$ Student's *t*-test vs. control.

2.6. Compounds

E. coli lipopolysaccharide (strain 0127:B8) was from Difco Laboratories, Detroit, MI, USA. Pentoxifylline and Drabkin's solution were from Sigma Chem. Co., St. Louis, Missouri, USA. Rolipram and denbufylline were synthetised at Almirall Laboratories, Barcelona, Spain. Sodium pentobarbitone was from Grinsted Products, Denmark. All compounds were dissolved fresh daily in saline.

2.7. Statistics

A paired or unpaired Student's *t*-test was used, and differences between groups were considered significant when $P < 0.05$. A chi-square Mantel-Haenszel test of macroscopic categories was applied to the data in Table 2.

3. Results

3.1. Macroscopic and histological examination of lipopolysaccharide-induced bowel erythrocyte extravasation

Thirty minutes after lipopolysaccharide injection (40 mg/kg) to the rats, a marked bowel reddening could be

Table 2
Macroscopic observation of lipopolysaccharide-induced intestinal erythrocyte extravasation in rats, scored as absent, mild, moderate or severe

Treatment	Dose (3×)	Score			
	(mg/kg i.p.)	Severe	Moderate	Mild	Absent
Saline		12	1	0	0
POF	5 [a]	3	2	1	0
	10 [b]	3	3	2	0
	50 [c]	0	0	9	2
RLP	10 [a]	2	0	1	1
	30 [c]	0	0	3	1
DBF	0.1	4	1	1	0
	1 [c]	0	3	3	0
	3 [c]	0	1	4	4

Values are numbers of rats. POF, pentoxifylline; RLP, rolipram; DBF, denbufylline. Chi-square Mantel-Haenszel test (global P value < 0.05 for each drug versus saline). [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$.

observed macroscopically. The intensity and the area affected increased with time. Histological examination showed that erythrocyte extravasation progressed from the jejunum to the distal colon. Only 3 h later the stomach and rectum were variably affected. With time, this erythrocyte extravasation changed to bowel necrosis, and the animals died from 12 to 24 h later. Two hours after lipopolysaccharide treatment, no erythrocyte accumulation was detected in heart, pancreas, lungs or kidneys. Only the intestine showed a severe and diffuse intramuscular and intramucosal haemorrhage compared with vehicle-treated animals. Lipopolysaccharide-induced erythrocyte extravasation was clearly observed in the mucosa, submucosa and muscularis layers of ileum sections, but no necrotic signals were seen at this time (see Fig. 1).

3.2. Effects of phosphodiesterase IV inhibitors on lipopolysaccharide-induced ileum hyperaemia and haemoglobin accumulation in rats

As seen in Table 1, 2 h after lipopolysaccharide challenge, total haemoglobin was significantly increased in the ileum and the ascending colon, while the rectum was not yet affected. Phosphodiesterase inhibitors administered i.p. according to a preventive protocol, produced dose-dependent protection against lipopolysaccharide-induced intestinal hyperaemia (Table 2), and a significant reduction of haemoglobin accumulation in ileum sections (Fig. 2). The order of potency in our studies in rats was denbufylline > rolipram > pentoxifylline. None of the compounds tested was able to inhibit significantly lipopolysaccharide-induced erythrocyte extravasation when they were given more than 30 min after lipopolysaccharide (data not shown).

3.3. Effects on mesenteric and renal blood flow in dogs

Administration of 3 mg/kg i.v. of *E. coli* lipopolysaccharide, induced a dramatic fall in systemic blood pressure, cardiac index and renal and mesenteric blood flow. Saline resuscitation elicited a marked recovery in cardiac index, and a more moderate effect on systemic blood pressure and on renal and mesenteric blood flow. Pentoxifylline, infused at 250 μg/kg/min, produced a rapid recovery of mesenteric blood flow, reaching the basal values 3 h after endotoxin administration. The effect of pentoxifylline on renal blood flow followed the same pattern but to a lesser extent. However, mean blood pressure, heart rate and cardiac index were not improved by pentoxifylline.

In the same study, denbufylline, at a dose 100 times lower than pentoxifylline, caused a clear recovery of basal mesenteric blood flow values even during the first hour. The effects on renal blood flow were moderate and statistically non-significant. The effects on blood pressure, heart rate and cardiac index were no different from those in saline group (see Fig. 3).

4. Discussion

Septic shock is a major cause of death among patients in intensive care units (Parrillo, 1993), and is commonly defined as sepsis accompanied by hypotension, perfusion abnormalities, which may include lactic acidosis, oliguria or an acute alteration of mental status and dysfunctions in the blood coagulation/fibrinolytic system (Benjamin and Iberti, 1991). Perfusion abnormalities and ischemic damage are known to play an important role in multiorgan failure, which greatly compromises life expectation in these patients. Moreover, sepsis appears intricately associated with mesenteric ischemia (Benjamin et al., 1993).

Our histological observations showed that, after a lethal lipopolysaccharide injection to rats, an important erythrocyte accumulation occurs in the small intestine. We have no data regarding the haemodynamic effects of lipopolysaccharide in our study in rats, but results found by Whitworth et al. (1989) have demonstrated a progressive

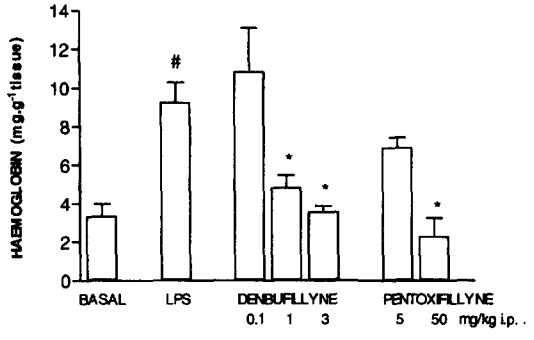

Fig. 2. Effects of denbufylline 0.1, 1 and 3 mg/kg i.p. and pentoxifylline 5 and 50 mg/kg i.p. on *E. coli* lipopolysaccharide-induced ileum hyperaemia. Data represent the means ± S.E.M. of six to nine rats. #,* Significant differences ($P < 0.01$, Student's t-test) vs. control group and hyperaemic group, respectively.

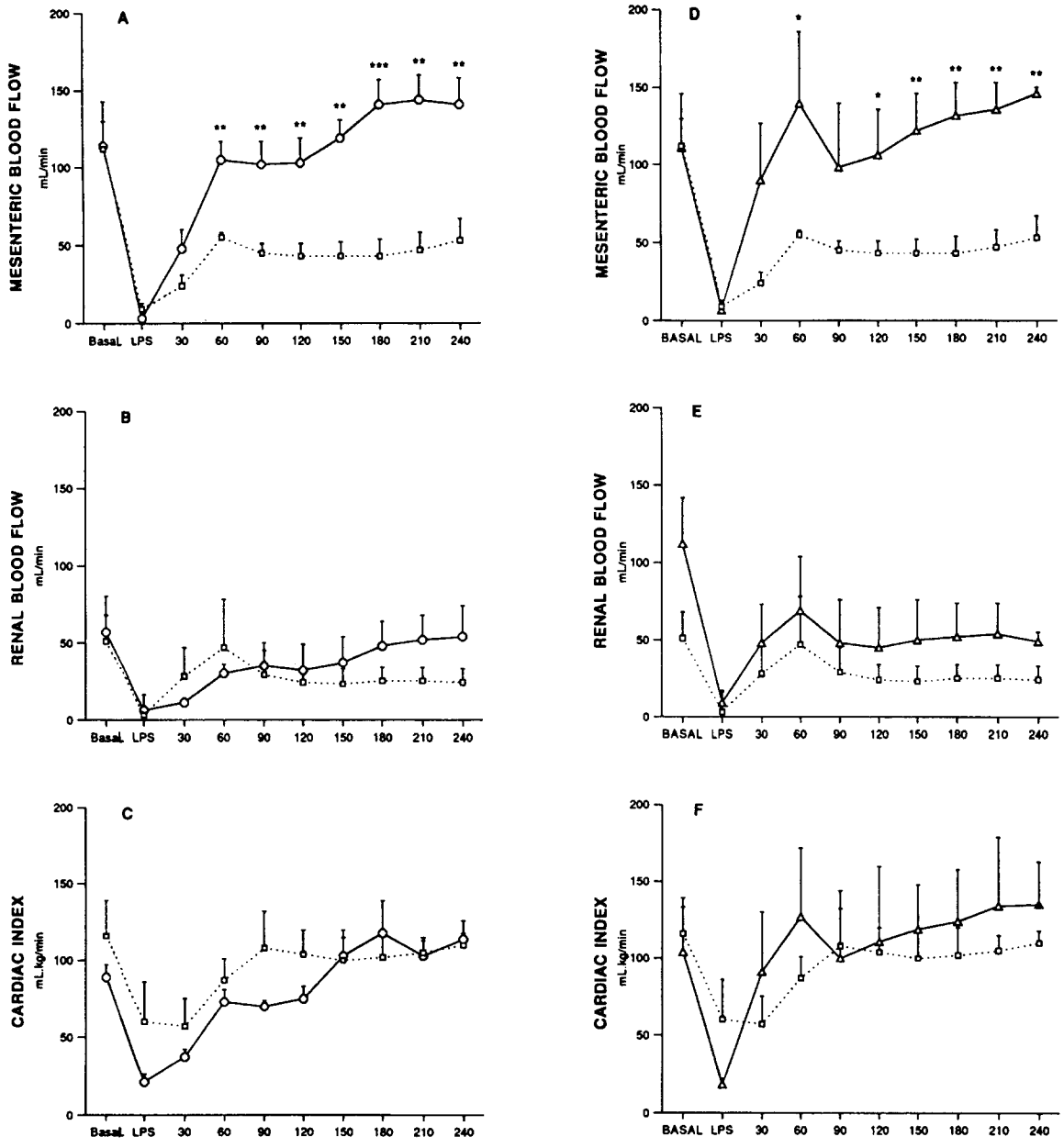

Fig. 3. Evolution of mesenteric blood flow (A), renal blood flow (B), and cardiac index (C), during endotoxic shock in pentoxifylline (○), denbufylline (△) and saline-treated dogs (□). Lipopolysaccharide represents the maximum effect attained after the administration of 3 mg/kg of *E. coli* lipopolysaccharide. Time is expressed in min, considering when the administration of lipopolysaccharide starts as zero. Each point is the mean ± S.E.M. for five animals. [a] $P < 0.05$ and [b] $P < 0.01$ Student's *t*-test vs. vehicle.

and profound vasoconstriction of the third-order arterioles that terminate as central villous arterioles, after live *E. coli* infusion in a rat model of hyperdynamic shock. These authors did not measure haemoconcentration, but it has been reported that endotoxins like lipopolysaccharide induce microcirculatory disorders through endothelial disruption and subsequent release of vasoactive mediators (Balis et al., 1978). As a result of these microcirculatory changes it is very likely that haemoconcentration can occur previous to erythrocyte extravasation. Therefore, the measurement of haemoglobin content in our study should be related to the possible pathological events additional to erythrocyte extravasation.

Although it is not known why lipopolysaccharide produces bowel hypoperfusion in vivo, it is accepted that several endogenous inflammatory cell mediators and vasoactive hormones, which are usually increased after septic shock, could act as local vasocontrictors in this vascular bed (Brackett et al., 1985). These lipopolysaccharide-induced intestinal changes were initially detected as early as 30 min after lipopolysaccharide administration, as a disruption in the mucosa layer; but with time, erythrocyte

extravasation, ulceration and a necrotic process could also be observed. Differences between intestinal areas could be explained by a different pattern of irrigation. There is still some controversy as to whether mesenteric hypoperfusion may or may not be sufficient to account by itself for the effects of lipopolysaccharide mucosal injury, because the small intestine has the ability to maintain oxygen consumption during low flow states (Bulkley et al., 1985; Fink, 1993). It is possible, however, that hypoperfusion together with direct lipopolysaccharide actions on the endothelium, mitochondrial oxidative phosphorylation or/and cytokine and cell mediator release, could explain the deleterious effects observed. Under normal conditions, the intestinal wall acts as a formidable barrier to the passage of lipopolysaccharide from the intestinal tract to the bloodstream, but disruption of this barrier can lead to systemic endotoxemia. Moreover, the liver plays an important role in clearing (detoxification) lipopolysaccharide from the circulation; because of its location with respect to the intestinal tract, the liver is specially suited for protecting the systemic circulation from gut-derived lipopolysaccharide (Hewett and Roth, 1993).

In the dog, endovenous perfusion of lipopolysaccharide induced a dramatic fall in mesenteric blood flow in saline-treated animals. The cardiac index also diminished, but was rapidly restored by a saline infusion (see Fig. 3). The relevance of this action is highlighted by the fact that mesenteric ischemia secondary to sepsis and septic shock, often heralds the syndrome of multisystem organ failure (Fry et al., 1980).

In our experiments in rats, pentoxifylline, denbufylline and rolipram protected from intestinal erythrocyte extravasation at doses that are known to prevent mortality in mice (Rice et al., 1994). In the same way, in anaesthetized dogs, pentoxifylline and denbufylline reversed the lipopolysaccharide-induced fall in mesenteric blood flow without affecting significantly either renal blood flow or cardiac index compared with saline resuscitation.

The phosphodiesterase inhibitory effect, a property showed by all the compounds tested in this study, implies an increase in intracellular cAMP levels, an action related to inhibition of TNFα production and release (Strieter et al., 1988), and it has been described that compounds that elevate intracellular levels of cAMP, as do phosphodiesterase inhibitors, are known to be potent inhibitors of PAF receptor gene expression (Rola-Pleszczynski et al., 1994). Moreover, pentoxifylline inhibits the production of TNFα mRNA in mouse peritoneal macrophages (Noel et al., 1990), in vivo TNFα production in a piglet model of streptococcal infection (Gibson et al., 1991), and TNFα formation in human volunteers (Schade and Zabel, 1994). These actions have suggested that pentoxifylline could be useful for the treatment of septic shock (Waxman, 1990) and respiratory distress syndrome (Turner et al., 1993). It was shown that amrinone, a selective phosphodiesterase III inhibitor, in spite of protecting from lipopolysaccharide-induced lethality in mice (Giroir and Beutler, 1992) and enhancing cardiac output, causes peripheral vasodilation, an effect that would be hazardous in the treatment of shock (Fretschner et al., 1992). Thus, selective phosphodiesterase IV inhibitors seem to be more suitable when the maintenance of perfusion homeostasis is essential.

We have studied pentoxifylline and denbufylline, using either a preventive or a curative protocol, and significant protection was seen only if the compounds were administered before or just after lipopolysaccharide challenge. It should be taken into account that experiments in rats have demonstrated that, as early as 10 min after the onset of intestinal ischemia, there is a partial loss of villous tips (Robinson and Antonioli, 1966), indicating that intestinal disturbances associated with hypoperfusion have a rapid onset.

Phosphodiesterase IV inhibitors are the object of a vast array of animal and clinical studies regarding their effects on cytokine production. Since TNFα and PAF both induce intestinal changes, and play a central role in the cascade of events after endotoxemia, selective and potent phosphodiesterase IV inhibitors with less adverse effects, rather than non-selective ones, could be a promising therapy in this field.

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