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Pentoxifylline improves circulatory failure and survival in murine models of endotoxaemia

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Abstract

Pentoxifylline, a methylxanthine derivative, has been widely used to improve erythrocyte deformability and capillary blood circulation in patients with claudication and cerebrovascular disorders as well as in animals with sepsis. Here, we investigate the effects of pentoxifylline on the hypotension, vascular hyporeactivity to noradrenaline, release of tumour necrosis factor-α (TNF-α) and nitric oxide (NO), and inducible NO synthase protein expression in a rat model of circulatory shock induced by bacterial endotoxin (*Escherichia coli* lipopolysaccharide). In addition, we have evaluated the effect of pentoxifylline on the 36-h survival rate in a murine model of endotoxaemia. Male Wistar–Kyoto rats were anaesthetised and instrumented for the measurement of mean arterial pressure and heart rate. Injection of lipopolysaccharide (10 mg/kg, i.v.) resulted in a significant fall in mean arterial pressure and an increase of heart rate. In contrast, animals pretreated with pentoxifylline (3 mg/kg, i.v., at 30 min prior to lipopolysaccharide) maintained a significantly higher mean arterial pressure but showed no effect on the tachycardia when compared to rats given only lipopolysaccharide (lipopolysaccharide-rats). The pressor effect of noradrenaline (1 μg/kg, i.v.) was also significantly reduced after the treatment of rats with lipopolysaccharide. Similarly, rings of thoracic aorta obtained from lipopolysaccharide-rats showed a significant reduction in the contractile responses elicited by noradrenaline (1 μM). Pretreatment of lipopolysaccharide-rats with pentoxifylline partially, but significantly, prevented this lipopolysaccharide-induced hyporeactivity to noradrenaline in vivo and ex vivo. The injection of lipopolysaccharide resulted in bell-shape changes in plasma TNF-α level which reached a peak at 60 min, whereas the effect of lipopolysaccharide on the plasma level of nitrate (an indicator of NO formation) was increased in a time-dependent manner. This increase of both TNF-α and nitrate levels induced by lipopolysaccharide was significantly reduced in lipopolysaccharide-rats pretreated with pentoxifylline. Endotoxaemia for 240 min caused a significantly increased protein expression of inducible NO synthase in the lung. In lipopolysaccharide-rats pretreated with pentoxifylline, inducible NO synthase protein expression in lung homogenates was attenuated by 48 ± 5%. Treatment of conscious mice with a high dose of endotoxin (60 mg/kg, i.p.) resulted in a survival rate of only 10% at 36 h ($n = 20$). However, therapeutic application of pentoxifylline (3 mg/kg, i.p. at 0, 6, 15 and 24 h after lipopolysaccharide) increased the 36-h survival to 35% ($n = 20$). Thus, pentoxifylline protects against circulatory failure and improves survival in rodents with severe endotoxaemia. These effects may be due to inhibition of the release of TNF-α and of the induction of inducible NO synthase.

Keywords: Pentoxifylline; TNF-α (tumour necrosis factor-α); Nitric oxide (NO); Lipopolysaccharide; Mortality

1. Introduction

Pentoxifylline, a methylxanthine derivative which could inhibit phosphodiesterase, resulting in elevation of intracellular cyclic AMP, has had clinical use as an agent to improve peripheral circulation in intermittent claudication. Pentoxifylline has now been found to have a variety of pharmacological effects which could be of benefit in sepsis. In particular, it has been reported to decrease pulmonary injury and associated pulmonary neutrophil and protein sequestration in dog and guinea pig models of sepsis with endotoxin or *Escherichia coli* as the septic insult (Ishizaka et al., 1988; Welsh et al., 1988). The mechanism of this action of pentoxifylline is attributed to the inhibition of tumour necrosis factor-α (TNF-α) production (Strieter et al., 1988). As TNF-α has been implicated as a major mediator of many effects of the septic syndrome, the inhibitory effects of pentoxifylline may have beneficial results. It is believed that the mechanism for the suppression of TNF-α production by pentoxifylline involves generation of cyclic AMP. Also, treatment of

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isolated macrophages or mice with other cyclic AMP-elevating agents such as adenosine (Parmely et al., 1993), prostaglandin E (Kark et al., 1988), prostacyclin analogues (Crutchley et al., 1994) or phosphodiesterase inhibitors (Fischer et al., 1993) inhibits TNF-α production.

TNF-α is a 17 kDa protein produced by macrophages that appears to have a central role in the pathogenesis of Gram-negative shock. This is based on the fact that serum levels of TNF-α are induced early in endotoxaemia or Gram-negative sepsis models (Michie et al., 1988). In addition, injection of TNF-α to animals has been shown to induce all the characteristics of endotoxic shock, and TNF-α antisera or antibodies attenuate the lethality caused by sepsi or endotoxin (Beutler and Cerami, 1989; Thiemermann et al., 1993). The infection caused by Gram-negative bacteria is usually associated with fever, diarrhea, hypotension, vascular hyporeactivity to vasoconstrictor agents, myocardial dysfunction, maldistribution of organ blood flow, and in severe cases, may lead to disseminated intravascular coagulation and multiple organ failure (see Parker et al., 1987). These acute pathophysiological effects can also be elicited by killed bacteria. Their active principle was identified as a lipopolysaccharide and, because of its toxic properties, was termed endotoxin. There is growing evidence that the delayed hypotension induced by lipopolysaccharide is attributable to the overproduction of nitric oxide (NO), an important endogenous vasodilator (see Stoclet et al., 1993; Thiemermann, 1994). In addition to TNF-α, many cytokines have also been demonstrated to induce inducible NO synthase in experimental models of endotoxic shock (see Moncada et al., 1991; Thiemermann, 1994).

A recent study has further demonstrated that pentoxifylline suppresses cytokine-induced NO production via inhibition of the expression of inducible NO synthase mRNA in macrophages (Trajkovic et al., 1997). However, there is no in vivo evidence that pentoxifylline has similar inhibitory effects in animals with endotoxaemia. Therefore, we examined the hypothesis that pentoxifylline inhibits the release of TNF-α, which in turn may exert beneficial effects (e.g., reducing NO overproduction, preventing inducible NO synthase expression) on circulatory failure (e.g., vascular hyporeactivity to vasoconstrictor agents and delayed hypotension) in anaesthetised rats treated with endotoxin. In addition, we evaluated the effect of pentoxifylline on survival rate in a murine model of endotoxaemia.

2. Methods

2.1. Endotoxic shock

Male Wistar–Kyoto rats (230–300 g) whose stock originated from the Charles River Breeding Laboratories in Japan, were purchased from the Department of Laboratory Animal Science of the National Defense Medical Centre. This study was approved by the local Institutional Review Board according to the recommendations from Helsinki and the internationally accepted principles in the care and use of experimental animals. The rats were anaesthetised by intraperitoneal injection of urethane (1.2 g/kg). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (Harvard Apparatus, South Natick, MA, USA). The right carotid artery was cannulated and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, USA) for the measurement of phasic and mean arterial pressure and heart rate which were displayed on a Gould model TA5000 polygraph recorder (Gould, Valley View, OH, USA). The left jugular vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 20 min. After recording of the baseline haemodynamic parameters, the animals were given saline or pentoxifylline (3 mg/kg, i.v.). At 30 min after injection of vehicle (saline) or pentoxifylline, the animals received saline or *E. coli* lipopolysaccharide (10 mg/kg, i.v.) and were monitored for 240 min. The pressor responses to noradrenaline (1 μg/kg, i.v.) were reassessed at 10 min prior to vehicle or lipopolysaccharide and at every hour after vehicle or lipopolysaccharide injection. Prior to (i.e., at time 0) and at every hour after vehicle or lipopolysaccharide, 0.3 ml of blood was taken to measure the changes in plasma levels of TNF-α and nitrate (an indicator of NO formation). Any blood withdrawn was immediately replaced by the injection of an equal volume of saline.

2.2. Organ bath experiments

At 240 min after the injection of lipopolysaccharide, thoracic aortae were taken from sham-operated controls as well as from endotoxemic rats pretreated with vehicle or pentoxifylline. The vessels were cleared of adhering periadventitial fat and the thoracic aortae were cut into rings of 3–4 mm width. The endothelium was removed by gently rubbing the intimal surface. The lack of relaxation in response to acetylcholine (1 μM) following pre-contraction of rings with noradrenaline (1 μM) was considered as evidence that the endothelium had been removed. The rings were mounted in 20-ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Kreb's solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. Indomethacin (5.6 μM) was added to prevent the production of prostanoids. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA, USA) and recorded on a MacLab Recording and Analysis System (ADInstruments, Castle Hill, Australia). A tension of 2 g was applied and the rings were equilibrated for 60 min, with changing of the Krebs' solution every 15 min. The contractile response to noradrenaline (1 μM) was obtained in all four experimental groups.

2.3. Measurement of plasma levels of TNF-α and nitrate

Blood samples (0.3 ml) for the measurement of TNF-α and nitrate levels in the plasma were taken at 0, 60, 120, 180 and 240 min after the injection of saline or lipopolysaccharide. These samples were collected from a catheter placed in the carotid artery and were centrifuged at 7200 × *g* for 3 min in order to obtain plasma for measuring the levels of TNF-α and nitrate. The plasma samples (100 μl) were diluted 1:2 and TNF-α was measured in duplicate with an enzyme-linked immunoadsorbent assay (ELISA) kit (Genzyme, Cambridge, MA, USA) as previously described (Wu et al., 1996). Another 50 μl of plasma was de-proteinised by incubation with 95% ethanol (4°C) for 30 min. These samples were subsequently centrifuged for a further 5 min at 14,000 × *g*. It is noted that the nitrate concentration in plasma mentioned in the study is actually the total nitrite and nitrate concentration in plasma. In this method, nitrate is reduced to NO via nitrite. The amounts of nitrate in the plasma (2 μl) were measured by adding a reducing agent (0.8% VCl_3 in 1N HCl) to the purge vessel to convert nitrate to NO which was stripped from the plasma by using a helium purge gas. The NO is then drawn into the Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate as previously described (Wu and Yen, 1997).

2.4. Western blot analysis of inducible NO synthase protein expression

Lungs were taken from rats treated with or not treated with lipopolysaccharide and homogenized on ice with an polytron PT MR 3000 homogenizer (Kinematic, Littau) in a buffer composed of (mM): Tris–HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12, and phenylmethylsulphonyl fluoride 1 (pH 7.4). The homogenized tissues were centrifuged at 10,000 × *g* for 30 min and the supernant was stored at −70°C until further analysis. Aliquots of tissue homogenates were used for protein assay (Bio-Rad protein assay reagent) and Western blot analysis. Tissue homogenates containing 10 μg protein were denatured and separated on 7.5% sodium dioctyl sulphate/polyacrylamide minigels using PhastSystem with PhastGel (Pharmacia Biotech). Separated proteins were transferred electrophoretically to nitrocellulose membranes using a PharmTransfer Semi-Dry transfer kit (Pharmacia Biotech). The membranes were blocked with 1% bovine serum albumin in Tris buffer solution (TBS) containing 0.1% Tween-20 for 2 h and then incubated with rabbit and anti-rat inducible NO synthase antibody (Transduction Laboratories, Lexington, KY, USA; 1:2000 dilution) in TBS containing 0.1% Tween-20 for 2 h. The membranes were washed and finally incubated with a 1:1000 dilution of anti-mouse immunoglobulin G conjugated to horseradish peroxidase antibody for 2 h. After successive washes as before, the immunocomplexes were developed, using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham International, Buckinghamshire) and exposed to X-ray film for 2–3 min. The relative expression of inducible NO synthase protein in each tissue was quantified by densitometric scanning of the Western blots using Image-pro plus software (Media Cybernetics, MD, USA) as previously described (Wu et al., 1996).

2.5. Survival studies

Survival studies were performed with mice (28–35 g), whose stock originated from the Institute of Cancer Research of National Institute of Health, USA, were purchased from the National Animal Centre (Taipei, Taiwan, R.O.C.). Lipopolysaccharide (60 mg/kg, i.p.) was injected in the presence of vehicle or drugs and survival was monitored every 6 up to 36 h. Different groups of animals received vehicle (saline) together with lipopolysaccharide ($n = 20$) or lipopolysaccharide plus pentoxifylline (3 mg/kg, i.p. at 0, 6, 15 and 24 h after lipopolysaccharide, $n = 20$).

2.6. Drugs

Acetylcholine chloride, bacterial lipopolysaccharide (*E. coli* serotype 0127:B8), noradrenaline bitartrate, pentoxifylline, sodium nitrate and urethane were purchased from Sigma (St. Louis, MO, USA). Vanadium chloride (VCl_3) was obtained from Aldrich Chemical (Milwaukee, WI, USA). All solution were made in saline or distilled water, except for VCl_3 which was dissolved in 1 N HCl.

2.7. Statistical analysis

All values in the figures and text are expressed as means ± S.E.M. of *n* observations, where *n* represents the number of animals studied. Statistical evaluation was performed with an analysis of variance (ANOVA) followed by a multiple comparison test (Scheffe's test), except for the inducible NO synthase expression which was analysed by unpaired Student's *t*-test. The chi-square test was used for determining the significance of differences in survival rate between control and drug-treated groups. A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of pentoxifylline on the cardiovascular changes caused by lipopolysaccharide in the anaesthetised rat

The baseline values for mean arterial pressure (Fig. 1a) and heart rate (Fig. 1b) of the vehicle- and pentoxifylline-pretreated animal groups were not significantly different

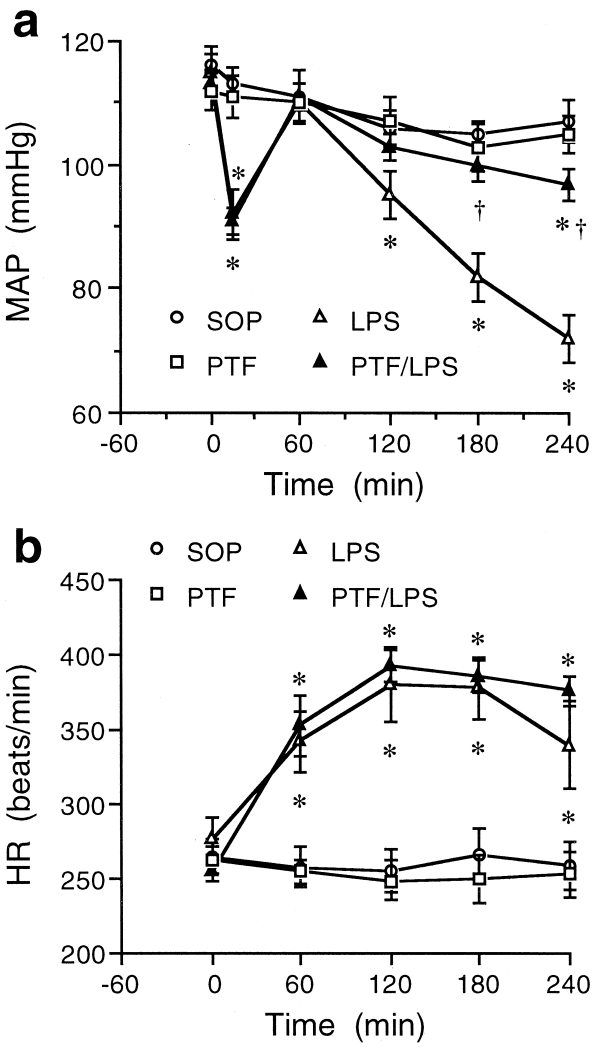

Fig. 1. Pentoxifylline (PTF) ameliorates the delayed hypotension in the anaesthetised rat treated with endotoxin. The changes in (a) mean arterial pressure (MAP) and (b) heart rate (HR) in rats receiving an injection of vehicle (sham-operated rats, SOP; $n = 10$), PTF alone (3 mg/kg, i.v., PTF; $n = 5$), or in rats treated with *E. coli* lipopolysaccharide (LPS, 10 mg/kg, i.v. at time 0) are shown. Different LPS-treated groups of animals were pretreated with vehicle (saline, LPS; $n = 16$) or PTF (3 mg/kg, i.v. at 30 min prior to LPS, PTF/LPS; $n = 16$). Data are expressed as means ± S.E.M. for the number (n) of animals studied. $^{*}P < 0.05$ represents significant differences when compared to SOP at the same time point. $^{\dagger}P < 0.05$ represents significant differences between treated and untreated LPS-rats.

between groups. Administration of lipopolysaccharide (10 mg/kg, i.v.) caused a rapid fall in mean arterial pressure within 15 min. Thereafter, mean arterial pressure returned to the pre-lipopolysaccharide value at 60 min. At 120 min after lipopolysaccharide, there was a continuous further fall in mean arterial pressure at 120–240 min (Fig. 1a). In the sham-operated group, there was no significant change of mean arterial pressure during the experimental period. Fig. 1b shows that endotoxaemia for 240 min was associated with a significant increase in heart rate, whereas in the sham-operated group, there was no significant change of heart rate during the experimental period. Pretreatment of rats with pentoxifylline (3 mg/kg, i.v.) did not exert a significant effect on mean arterial pressure (prior to injection of lipopolysaccharide). Pretreatment of lipopolysaccharide-rats with pentoxifylline attenuated the delayed hypotension (i.e., after 120 min). Thus, the mean arterial pressure of lipopolysaccharide-rats pretreated with pentoxifylline was significantly higher than that of the respective lipopolysaccharide control group at 120–240 min (Fig. 1a). As for the change in heart rate, administration of pentoxifylline had no significant effect on the tachycardia induced by lipopolysaccharide (Fig. 1b). Injection of normal control animals with pentoxifylline alone had no significant effects on mean arterial pressure (Fig. 1a) or heart rate (Fig. 1b) during the observed experimental period.

3.2. Effects of pentoxifylline on the lipopolysaccharide-induced vascular hyporeactivity to noradrenaline in vivo and ex vivo

The mean baseline values for the pressor responses to noradrenaline (1 μg/kg, i.v.) were not significantly different between any of the experimental groups studied. Injection of lipopolysaccharide resulted in a substantial, time-dependent attenuation of the pressor responses elicited by noradrenaline (Fig. 2a), whereas injection of vehicle rather than lipopolysaccharide had no significant effect on the noradrenaline-induced pressor responses during the 240 min experimental period. In addition, endotoxaemia for 240 min was also associated with the vascular hyporeactivity to noradrenaline (1 μM) ex vivo (Fig. 2b). Pretreatment of lipopolysaccharide-rats with pentoxifylline enhanced the pressor responses to noradrenaline (Fig. 2a). Thus, the pressor responses to noradrenaline at 1–4 h in lipopolysaccharide-rats pretreated with pentoxifylline were significantly greater than those in animals treated with lipopolysaccharide alone ($P < 0.05$; Fig. 2a). The similar attenuation of vascular hyporeactivity to noradrenaline was also observed in thoracic aorta rings obtained from endotoxaemic rats pretreated with pentoxifylline when compared to rings from the lipopolysaccharide control group (Fig. 2b). Injection of normal control animals with pentoxifylline alone had no significant effects on the pressor response to noradrenaline during the observed experimental period (Fig. 2a) and the vascular reactivity to noradrenaline ex vivo (Fig. 2b).

3.3. Effects of pentoxifylline on the plasma TNF-α and nitrate levels induced by endotoxaemia

The basal plasma levels of TNF-α and nitrate were not significantly different between any of the experimental groups studied. The injection of lipopolysaccharide resulted in bell-shape changes in the plasma levels of TNF-α which reached a peak at 1 h after lipopolysaccharide

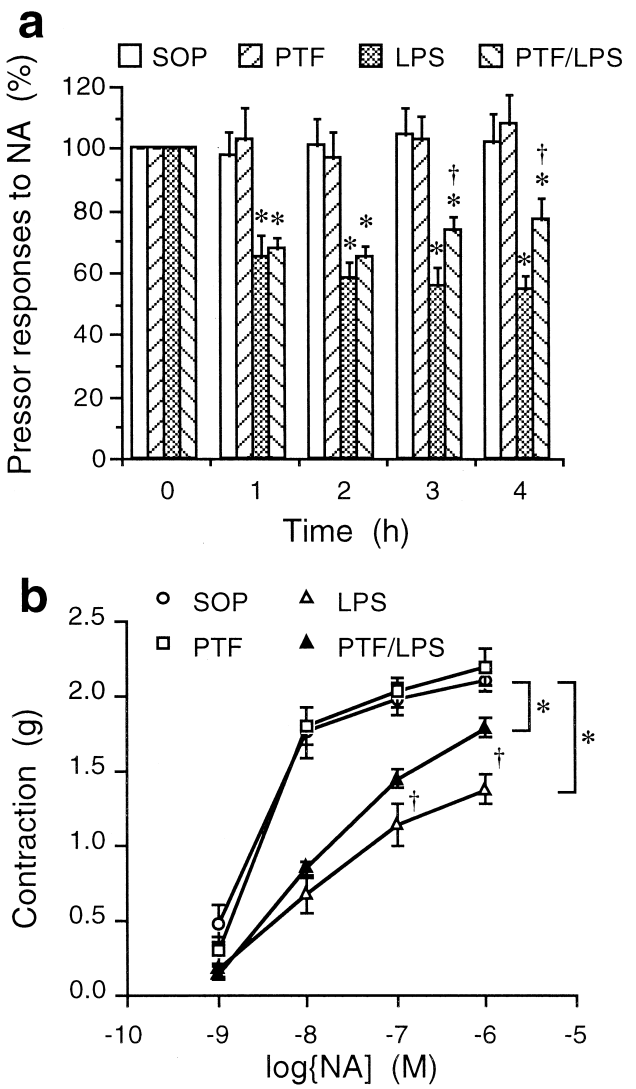

Fig. 2. Pentoxifylline (PTF) attenuates the vascular hyporeactivity to noradrenaline (NA) (a) in vivo and (b) ex vivo in rats with endotoxic shock. The changes in (a) pressor responses to NA (1 μg/kg, i.v.) in rats that received an injection of vehicle (sham-operated rats, SOP; $n = 10$), PTF alone (3 mg/kg, i.v., PTF; $n = 5$), or in rats treated with *E. coli* LPS (10 mg/kg, i.v. at time 0) are shown. Different LPS-treated groups of animals were pretreated with vehicle (saline, LPS; $n = 16$) or PTF (3 mg/kg, i.v. at 30 min prior to LPS, PTF/LPS; $n = 16$) and (b) NA (1 μM)-induced contraction in endothelium-denuded aorta rings obtained from above groups. Data are expressed as means ± S.E.M. for the number (n) of animals studied. $^{*}P < 0.05$ represents significant differences when compared to SOP at the same time point. $^{\dagger}P < 0.05$ represents significant differences between treated and untreated LPS-rats.

injection and subsequently decreased slowly (Fig. 3a), whereas the injection of lipopolysaccharide caused a time-dependent increase in the plasma level of nitrate, and endotoxaemia for 240 min was associated with a 24-fold rise ($P < 0.05$; Fig. 3b). In the sham-operated group, no significant amounts of TNF-α were detectable during the experimental period, indicating that the surgical procedure alone did not result in a significant rise in plasma TNF-α. In addition, in the sham-operated group, there was no significant change in the plasma nitrate level during the experimental period. Pretreatment of lipopolysaccharide-rats with pentoxifylline significantly decreased the TNF-α and nitrate levels in plasma. In other words, the peak value of plasma TNF-α was significantly reduced and the curve for the plasma TNF-α level was shifted to the right (i.e., from 1 to 2 h) in lipopolysaccharide-rats pretreated with pentoxifylline (Fig. 3a). Similarly, pretreatment of lipo-

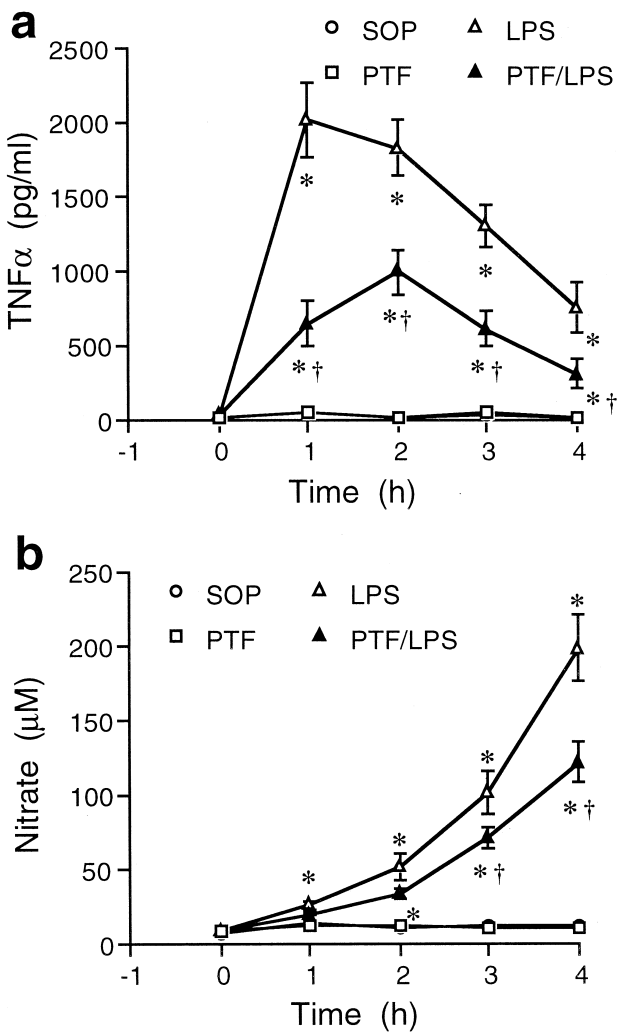

Fig. 3. Pentoxifylline (PTF) reduces the levels of (a) tumour necrosis factor-α (TNF-α) and (b) nitrate in plasma from rats treated with endotoxin. The changes of TNF-α and nitrate levels in plasma of rats that received an injection of vehicle (sham-operated rats, SOP; $n = 4$ for TNF-α, $n = 8$ for nitrate), PTF alone (3 mg/kg, i.v., PTF; $n = 3$ for TNF-α, $n = 5$ for nitrate), or in rats treated with *E. coli* LPS (10 mg/kg, i.v. at time 0) are shown. Different LPS-treated groups of animals were pretreated with vehicle (saline, LPS; $n = 6$ for TNF-α, $n = 16$ for nitrate) or pentoxifylline (3 mg/kg, i.v. at 30 min prior to LPS, PTF/LPS; $n = 6$ for TNF-α, $n = 16$ for nitrate). Note that each value of TNF-α is the mean of duplicate plasma samples from the same animal. Data are expressed as means ± S.E.M. for the number (n) of animals studied. $^{*}P < 0.05$ represents significant differences when compared to SOP at the same time point. $^{\dagger}P < 0.05$ represents significant differences between treated and untreated LPS-rats. Please note that open circles are hidden behind open squares.

polysaccharide-rats with pentoxifylline also significantly prevented the plasma nitrate production and shifted the curve for plasma nitrate levels to the right (Fig. 3b). Injection of normal control animals with pentoxifylline alone had no significant effects on the plasma TNF-α (Fig. 3a) and nitrate levels (Fig. 3b) during the observed experimental period.

3.4. Effects of pentoxifylline on the expression of inducible NO synthase in lungs obtained from rats treated with lipopolysaccharide

Fig. 4 shows that inducible NO synthase protein expression was undetectable in lung homogenates obtained from sham-operated rats, whereas a significant induction of inducible NO synthase protein was observed in lung homogenates of rats treated with lipopolysaccharide for 4 h. Pretreatment of rats with pentoxifylline significantly prevented the induction of inducible NO synthase in the lung, which was induced by lipopolysaccharide challenge. In contrast, injection of normal control animals with pentoxifylline alone had no effects on the inducible NO synthase protein expression.

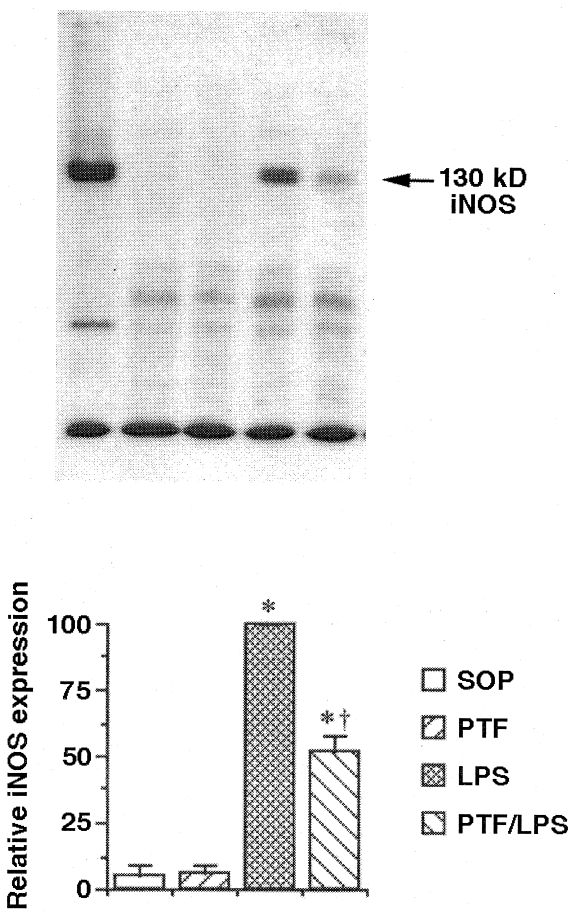

Fig. 4. Pentoxifylline (PTF) prevents the expression of inducible nitric oxide synthase (iNOS) in lungs from rats with endotoxic shock. A typical trace is shown for iNOS protein expression (upper figure) and the statistical analysis of changes of iNOS protein expression in the lung of rats that received an injection of vehicle (sham-operated rats, SOP or S; $n = 4$), PTF alone (3 mg/kg, i.v., PTF; $n = 3$), or in rats treated with *E. coli* LPS (10 mg/kg, i.v. at time 0) (lower figure). Different LPS-treated groups of animals were pretreated with vehicle (LPS or L; $n = 4$) or PTF (PTF/LPS or P, 3 mg/kg, i.v. at 30 min prior to LPS; $n = 4$). Note that the left lane in the upper figure is the iNOS marker (M). Data are expressed as means ± S.E.M. for the number (n) of animals studied. $^{*}P < 0.05$ represents significant differences when compared to SOP at the same time point. $^{\dagger}P < 0.05$ represents significant differences between treated and untreated LPS-rats.

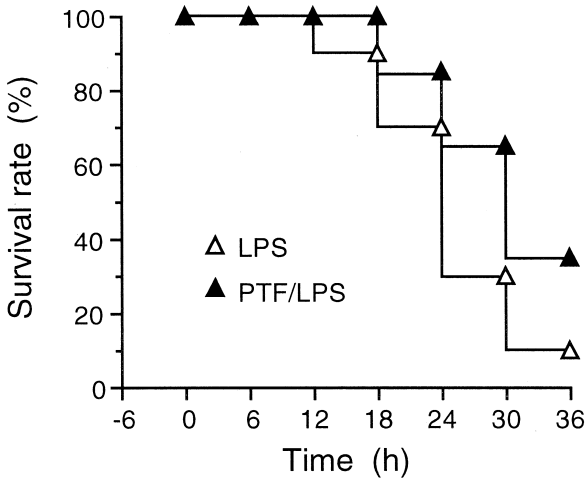

Fig. 5. Effects of pentoxifylline (PTF) on the survival rate of mice treated with endotoxin. Depicted are the changes in survival during the experimental period of different groups of ICR mice which received intraperitoneal injection of *E. coli* LPS (60 mg/kg; solid triangles; $n = 20$) or PTF (3 mg/kg; at time 0, 6, 15 and 24 h after LPS, $n = 20$) plus LPS. Data are expressed as percentage of mice surviving at the observed time point.

3.5. Effects of pentoxifylline on the survival rate of mice treated with lipopolysaccharide

The administration of a high dose (60 mg/kg, i.p.) of lipopolysaccharide to mice was associated with a 36-h survival rate of only 10% of the animals. In contrast, lipopolysaccharide-mice treated with pentoxifylline (3 mg/kg, i.p. at 0, 6, 15 and 24 h after lipopolysaccharide, $n = 20$) had a higher survival rate, 35%, at 36 h (Fig. 5).

4. Discussion

In our preliminary studies, we did a dose-dependent study of pentoxifylline (0.3–10 mg/kg), and results demonstrated that 3 mg/kg pentoxifylline produced the maximal pharmacological effects on the suppression of TNF-α production in rats treated with LPS (unpublished observations). Thus, we examined the beneficial effects of pentoxifylline on animals treated with endotoxin by using the dose, 3 mg/kg. The results demonstrate that pentoxifylline significantly prevents the delayed hypotension and the vascular hyporeactivity to noradrenaline, reduces the

formation of plasma TNF-α and nitrate, and attenuates the expression of inducible NO synthase in the anaesthetized rats treated with endotoxin. In addition to causing beneficial haemodynamic effects in a rat model of endotoxic shock, pentoxifylline also improves survival in a conscious-mouse model of severe endotoxaemia. Thus, pentoxifylline has beneficial effects and improves survival in rodents with endotoxaemia or endotoxic shock. This protective effect of pentoxifylline may be mediated by the inhibition of the release of TNF-α and of the induction of inducible NO synthase induced by lipopolysaccharide.

Growing evidence shows that administration of lipopolysaccharide reproduces many of cardiovascular features of septic shock, including the hypotension and the persistent loss of vascular tone, which is often unresponsive to vasoconstrictors (e.g., noradrenaline in this study) and is associated with a high mortality (see Parratt, 1989). The suggestion that endotoxaemia is associated with an overproduction of NO, presumably via inducible NO synthase, is based on findings, using electron paramagnetic resonance, of a time-dependent increase of nitrosylated hemoglobin in mice and rats with endotoxic shock (Wang et al., 1991; Kosaka et al., 1992a). Indeed, we showed that injection of rats with lipopolysaccharide causes a time-dependent increase of nitrate (an indicator of NO formation) in the plasma and expression of inducible NO synthase protein in the lung. This enhanced formation of NO (i) contributes to the hypotension caused by endotoxin in anaesthetised animals (Kilbourn et al., 1990; Thiemermann and Vane, 1990) and (ii) accounts for the vascular hyporeactivity to vasoconstrictors including noradrenaline, phenylephrine and calcium (Fleming et al., 1990; Gray et al., 1990; Rees et al., 1990), as intravenous infusion of the NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), or in vitro treatment of blood vessels with NO synthase inhibitors (e.g., L-NMMA) can improve the vascular hyporesponsiveness (Szabo et al., 1993). Thus, in endotoxic shock, the excessive production of NO stimulated by lipopolysaccharide contributes to the development of profound hypotension and hyporesponsiveness to exogenous vasoconstrictors. Indeed, pentoxifylline significantly improved these haemodynamic changes, an effect which was accompanied by the reduction of plasma nitrate levels in this study. Therefore, we suggest that the mechanism of these actions of pentoxifylline is associated with inhibition of the overproduction of NO by inducible NO synthase (examined by Western blot) in rats treated with endotoxin.

In addition, the mechanism of these actions of pentoxifylline involves, at least in part, the inhibition of TNF-α release (see Zabel et al., 1993). The inhibition of TNF-α release by pentoxifylline, a methylxanthine derivative, is due to an elevation of cyclic AMP levels by inhibition of phosphodiesterase IV activity (Strieter et al., 1988; Semmler et al., 1993). Here, we confirmed results of previous studies which showed that pentoxifylline has beneficial effects in animal models of septic shock (see Mandell, 1995). The latter results suggest that TNF-α plays a central role in the pathogenesis of Gram-negative shock, as serum levels of TNF-α are induced early in endotoxaemia or Gram-negative sepsis models (Michie et al., 1988). Indeed, pentoxifylline has been reported to decrease the serum levels of TNF-α in humans after an intravenous dose of endotoxin, but not to alter other endotoxin-induced and TNF-α-associated physiological changes such as tachycardia or fever, nor to diminish serum levels of interleukin-6 (Zabel et al., 1989). In the present study, we obtained similar findings with pentoxifylline in an endotoxic shock model, including inhibition of TNF-α in the plasma, amelioration of delayed hypotension, improvement of vascular responsiveness to vasoconstrictors, and no effects on tachycardia. However, it is important to note that macrophage and vascular smooth muscle cell inducible NO synthase is activated by bacterial lipopolysaccharide and several cytokines including TNF-α (Stuehr and Marletta, 1985; Busse and Mulsch, 1990). These cytokines, when given either alone or together, cause the induction of inducible NO synthase in various cells in vitro (see Nathan, 1992) or an enhanced formation of NO in rats in vivo (Kosaka et al., 1992b). In addition, a monoclonal antibody for TNF-α significantly attenuates the hyporeactivity to noradrenaline in aorta rings ex vivo, and a NO synthase inhibitor does not further enhance the contractions to noradrenaline in these preparations in vitro, indicating that TNF-α contributes to the induction of inducible NO synthase in the aorta (Thiemermann et al., 1993). Thus, the inhibition of inducible NO synthase induction by pentoxifylline may result from the reduction of TNF-α production.

Due to concerns that recent studies have demonstrated that selective inhibitors of inducible NO synthase only prevent the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin (Wray et al., 1998), the more directly relevant need, is to examine survival in animal models with endotoxaemia. How can we reconcile the relatively modest effect of pentoxifylline on survival with the marked reduction of plasma TNF-α levels in vivo? In addition to TNF-α, many cytokines and mediators play an important role in the pathophysiology of septic shock; these mediators include interleukins, granulocyte-colony stimulating factor, macrophage-colony stimulating factor, as well as certain chemokines and adhesion molecules (see Sessler et al., 1996). Thus, the reduction of TNF-α levels may not be the only factor needed to improve survival in animals with endotoxaemia.

One could argue that pentoxifylline almost entirely suppressed the delayed fall in blood pressure with no significant effect on the increase in heart rate and a moderate effect on vascular hyporeactivity to noradrenaline caused by lipopolysaccharide. Physiologically, the changes in blood pressure are the sum of total peripheral resistance times cardiac output, which is linked to heart rate. Since the heart rate was not significantly altered by

pentoxifylline in the endotoxaemic rats, one would expect an increase in total peripheral resistance. However, our results demonstrated that the vascular hyporeactivity to noradrenaline induced by lipopolysaccharide was only mildly ameliorated by pretreatment of rats with pentoxifylline. It has been shown that pentoxifylline produces endothelium-dependent (Berkenboom et al., 1991) and -independent (Kaputlu and Sadan, 1994) vasodilatation in the aorta. Therefore, the vasodilatation induced by pentoxifylline may counteract the pressor responses to noradrenaline. We suggest that the inhibitory effect of pentoxifylline in blood vessels may improve the blood supply to the tissues or organs, which may lead to an increase in survival rate in animals with endotoxaemia.

In summary, pentoxifylline suppresses the release of TNF-α and prevents the induction of inducible NO synthase in the anaesthetised rat treated with lipopolysaccharide. Thus, pentoxifylline improves the delayed circulatory failure caused by endotoxin. In addition, the pentoxifylline-induced increased survival of animals with sepsis may also be associated with the reduction of TNF-α production and of inducible NO synthase expression. We suggest that the mechanism underlying the inhibition by pentoxifylline of the release of TNF-α involves the elevation of intracellular cyclic AMP levels. Inhibition of the expression of inducible NO synthase in vivo is, at least in part, due to the reduction of the release of TNF-α.

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