

TERBUTALINE PREVENTS CIRCULATORY FAILURE AND MITIGATES MORTALITY IN RODENTS WITH ENDOTOXEMIA

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ABSTRACT—Septic shock is characterized by a decrease in systemic vascular resistance. Nevertheless, regional increases in vascular resistance can occur that may predispose mammals to organ dysfunction, including the acute respiratory distress syndrome. In the host infected by endotoxin (lipopolysaccharide, LPS), the expression and release of proinflammatory tumor necrosis factor- α (TNF α) rapidly increases, and this cytokine production is regulated by agents elevating cyclic AMP. In this report, we present evidence that terbutaline, a β_2 -agonist, inhibits TNF α production and enhances interleukin-10 (IL-10) release in the anesthetized rat treated with LPS. In addition, an overproduction of nitric oxide (NO, examined by its metabolites nitrite/nitrate) by inducible NO synthase (iNOS, examined by western blot analysis) is attenuated by pretreatment of LPS rats with terbutaline. Overall, pretreatment of rats with terbutaline attenuates the delayed hypotension and prevents vascular hyporeactivity to norepinephrine. In addition, pretreatment of mice with terbutaline also improves the survival in a model of severe endotoxemia. The infiltration of polymorphonuclear neutrophils into organs (e.g., lung and liver) from the surviving LPS mice treated with terbutaline was reduced almost to that seen in the normal controls. These findings suggest that the inhibition of TNF α and NO (via iNOS) production as well as the increment of IL-10 production contribute to the beneficial effect of terbutaline in animals with endotoxic shock.

KEYWORDS—Tumor necrosis factor- α , interleukin-10, nitric oxide, polymorphonuclear neutrophils, survival, lipopolysaccharide

INTRODUCTION

The acute respiratory distress syndrome (ARDS) often complicates the clinical course of patients with severe sepsis. Despite an advanced understanding of the pathophysiology, the mortality for severe sepsis-related ARDS remains between 40% and 70% (1). Usually, the sepsis induced by gram-negative bacteria is associated with hypotension, vascular hyporeactivity to vasoconstrictor agents, myocardial dysfunction, maldistribution of organ blood flow, and, in severe cases, may lead to disseminated intravascular coagulation, septic shock, and ARDS (2–4). These pathophysiological effects can also be observed by killed bacteria, since their active principle has been identified as endotoxin (lipopolysaccharide, LPS). Therefore, experimental endotoxemia has become a valuable experimental model for septicemia and has been studied extensively in laboratory animals. It is generally accepted that LPS acts via endogenous mediators, mainly produced by mononuclear phagocytes (5). Among these endogenous mediators, tumor necrosis factor- α (TNF α) seems to be of particular importance for endotoxic effects (6) since it has been shown to induce most characteristics for endotoxic shock and antisera or antibody against TNF α attenuated lethality or improved hemodynamic functions provoked by sepsis or endotoxin (7, 8). Interleukin-

10 (IL-10) inhibits LPS-induced production of TNF α by mononuclear cells *in vitro* and in mice *in vivo* (9–12) and reduces lethality of endotoxemic mice (11, 12). Production of IL-10 can therefore be considered to be part of a host-protective mechanism during endotoxemia. Delayed hypotension induced by LPS is mainly attributed to the overproduction of nitric oxide (NO), an important endogenous vasodilator, in this endotoxin model (13, 14). NO has now been recognized to be present in many tissues, acting as a ubiquitous intracellular signalling molecule in diverse mammalian cells. For instance, in the coronary vessels NO plays an important role in the regulation of vascular tone and myocardial blood flow both *in vivo* and *in vitro* (15, 16). The overproduction of NO is, however, suggested to be responsible for the vascular relaxation and hypotension seen in states of sepsis and endotoxemia (17). It has been established that NO is synthesized from L-arginine via three isoforms expressed either constitutively (neuronal NOS, endothelial NOS) or following stimulation by cytokines (inducible NOS, iNOS) (17, 18). The induction of iNOS has been identified in endotoxin- and cytokine-treated macrophages, hepatocytes, endothelial cells or myocardium (17), and, in some organs, from animals treated with endotoxin (14).

Terbutaline is a β_2 -selective agonist. It has been used clinically for the long-term treatment of obstructive airway diseases and for treatment of acute bronchospasm (19). Recent studies also have demonstrated that terbutaline improves hemodynamics and gas exchange as well as survival rate in animals with

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endotoxic shock (20, 21). However, these studies did not reveal the cellular mechanisms associated with the beneficial effect of terbutaline in sepsis. Stimulation of β -adrenergic receptors has been shown to inhibit the release of inflammatory mediators (e.g., TNF α) from monocytes or macrophages (22, 23) and to up-regulate LPS-induced IL-10 production in human blood (24). In this study, we examined whether a decrease in the production of cytokines or NO in sepsis is linked to the beneficial effect of terbutaline in animals with endotoxemia. Our results demonstrate that terbutaline inhibits TNF α and NO (via iNOS) production but increases IL-10 production, and thus, mitigates the development of detrimental effects of endotoxin (including circulatory failure and mortality) in mice. These results indicate that terbutaline has beneficial effects that may be considered in the treatment of patients with sepsis.

MATERIALS AND METHODS

In vivo experiments

Ten-week-old male Wistar-Kyoto (WKY) rats, 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 Center. 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 the use of experimental animals. Rats were anesthetized by intraperitoneal injection of urethane (1.2 g/kg). The trachea was cannulated to facilitate respiration and environmental temperature was maintained at 24°C with an air-conditioning system. The right carotid artery was cannulated and connected to a pressure transducer (P23ID, Statham, Oxnard, CA) for the measurement of phasic blood pressure and mean arterial blood pressure (MAP) as well as heart rate (HR), which were displayed on a Gould model TA5000 polygraph recorder (Gould Inc., Valley View, OH). The left jugular vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min.

After recording baseline hemodynamic parameters, animals were given norepinephrine [NE, 1 μ g/kg intravenously (i.v.)], and 10 min later animals received vehicle (saline) or *Escherichia coli* LPS (10 mg/kg i.v.) and were monitored for 240 min. The pressor responses to NE were reassessed every hour after vehicle or LPS injection. Prior to (i.e., at time 0) and every hour after vehicle or LPS, 0.3–0.5 mL of blood was taken to measure the changes in TNF α , IL-10, and nitrate (an indication of NO). Any blood withdrawn was immediately replaced by the injection of an equal volume of saline (i.v.) in order to maintain the blood volume. To obtain a suitable dose of terbutaline to perform this study, the dose-response study was evaluated, with the results shown in Table 1. Terbutaline at 0.3 mg/kg only had a mild depressor effect but had maximal inhibition of TNF α production and, hence, this dose was administered i.v. 30 min prior to the injection of LPS. In a separate experiment, a group of rats was anesthetized, instrumented (as above), and treated with terbutaline (0.3 mg/kg i.v.) only. All hemodynamic and biochemical parameters were recorded for 4 h in all animal groups.

Measurement of TNF α and IL-10 in plasma levels

Blood samples (0.3 mL) for the measurement of TNF α level in the plasma were obtained at 0, 60, 120, 180, and 240 min after the injection of saline or LPS. At 60 and 240 min after the injection of saline or LPS, the volume of blood sample taken from the animals was 0.5 mL instead of 0.3 mL for performing the measurement of IL-10 in addition to TNF α . These samples were collected from a catheter placed in the carotid artery and were centrifuged at 7200 g for 3 min to obtain the plasma for measuring the levels of TNF α , IL-10, and nitrate (as described below). The plasma samples (100 μ L) were diluted 1:2, and TNF α was measured in duplicate with an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme Co., Cambridge, MA) as described previously (25), and the amounts of IL-10 in the plasma (100 μ L) were measured by ELISA kit (Endogen Inc., Boston, MA).

Determination of plasma nitrate

Fifty microliter plasma that was kept in -20°C freezer was thawed and de-proteinized by incubating them with 95% ethanol (4°C) for 30 min. The samples were subsequently centrifuged for an additional 5 min at 14,000 g. It is noted that the nitrate concentration in plasma depicted 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 μ L) were measured by adding a reducing agent (0.8% VCl₃ 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 was then drawn into the Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers Inc., Boulder, CO). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate (Sigma Chemical Co., St. Louis, MO).

Western blot analysis of iNOS protein expression

At 240 min after the injection of saline or LPS, lungs were obtained from sham-treated controls as well as from endotoxemic rats pretreated with vehicle or terbutaline and frozen at -70°C before assay. Frozen samples were homogenized on ice with a polytron PT MR 3000 homogenizer (Kinematic AG, Littau) in a buffer composed of (mmol/L): 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 supernatant 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 μ g of protein were reduced and separated on 7.5% SDS-PAGE gel using PhastSystem with PhastGel (Pharmacia Biotech). Separated proteins were electrophoretically transferred to nitrocellulose membranes using 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 iNOS antibody (Transduction Laboratories, Lexington, KY; 1:2000 dilution) in TBS containing 0.1% Tween-20 for 2 h. The membrane was washed and finally incubated with a 1:1000 dilution of anti-mouse IgG 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 plc., Buckinghamshire) and exposed to x-ray film for 2–3 min. The relative expression of iNOS protein in each tissue was quantified by densitometric scanning of the western blots using Image-pro plus software (Media Cybernetics, MD) as previously described (25).

Organ bath experiments

At 240 min after the injection of LPS, thoracic aortae were obtained from sham-treated controls as well as from endotoxemic rats pretreated with vehicle or terbutaline. 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 rings were mounted in 20-mL organ baths filled with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs' solution (pH 7.4) consisting of (mmol/L): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, NaHCO₃ 25 and glucose 11. Indomethacin (5.6 μ mol/L) was added to prevent the production of prostanooids. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA) and recorded on a MacLab Recording and Analysis System (ADInstruments Pty Ltd., Castle Hill, Australia). A tension of 2 g was applied, and the rings were equilibrated for 60 min, changing the Krebs' solution every 15 min. The lack of a relaxation to acetylcholine (1 μ mol/L) following pre-contraction of rings with NE (1 μ mol/L) was considered as evidence that the endothelium had been removed. The contractile response to NE (1 μ mol/L) was obtained in the absence or presence of the NOS inhibitor L-NAME (0.3 mmol/L for 20 min) in all experimental groups.

Survival studies

Survival studies were performed in ICR mice (28–35 g), whose stock originated from the Institute of Cancer Research of National Institute of Health in U.S.A. They were purchased from the National Animal Center (Taipei, R.O.C., Taiwan). LPS [60 mg/kg intraperitoneally (i.p.)] was injected in the presence of vehicle or drugs and survival was monitored every 6 h until 36 h. Different

groups of animals received vehicle (saline) together with LPS ($n = 20$) or LPS plus terbutaline (0.3 mg/kg at time 0 and 6 h after LPS, $n = 20$).

Histological studies

Lungs and livers were obtained from surviving mice in each group after the survival study and these tissues were fixed in Carson-Millonig's solution for histopathological examination as described previously (26). The fixed lung and liver tissues were dehydrated in graded ethanol and embedded in paraffin. Three-micron sections were stained with the hematoxylin and eosin reagent for light microscopy. In preliminary experiments, a striking pathological feature of the mice receiving LPS was a prominent infiltration of neutrophils in the organs studied. This histological alteration was quantitatively analyzed as an index on the severity of tissue injury. This index was a neutrophil infiltration index which was determined by counting the numbers of neutrophils in 10 randomly selected high power fields. The index was expressed as the mean of these 10 numbers \pm standard error of the mean (SEM)/high power field.

Statistical analysis

All values in the figures and text are expressed as mean \pm SEM of n observations, where n represents the number of animals studied. Statistical evaluation was performed by using analysis of variance (ANOVA) followed by a multiple comparison test (Scheffé's test), except for the determination of iNOS protein expression, the IL-10 level and the neutrophil infiltration index which were analyzed by unpaired Student's *t* test. The chi-square test was used for determining the significant differences in the survival rate between control and drug-treated groups. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Plasma TNF α , IL-10, and nitrate

A preliminary study was performed to select a suitable dose of terbutaline to inhibit TNF α production. Table 1 shows that terbutaline inhibited the TNF α production in plasma in a dose-dependent manner with a maximal inhibition at 0.3 mg/kg. In addition, this dose did not cause a prolong hypotension. Thus, this dose was chosen for the following studies.

The basal plasma levels of TNF α , IL-10, and nitrate were not significantly different between any of the experimental groups studied. The injection of LPS caused a significant increase in the plasma levels of TNF α , which reached a peak at 1 h after LPS injection and subsequently decreased slowly (Fig. 1A), whereas LPS caused a slight peak elevation of IL-10 plasma concentration at 60 min (Fig. 1B) and returned to near baseline levels at 240 min (unpublished data). In addition, the administration of LPS caused a time-dependent elevation in the plasma level of nitrate, which reached a 25-fold increase in 240 min (Fig. 1C). In the sham-treated group, no significant

TABLE 1. Dose-response of terbutaline on the plasma TNF α level at 1 h after LPS injection and on blood pressure change in magnitude (Δ BP) and duration (D)

Terbutaline (mg/kg)	TNF α (pg/mL) $n = 4$	Δ BP (mmHg) $n = 5$	D (min) $n = 5$
0	2020 \pm 329	0	0
0.03	1622 \pm 216	15 \pm 2*	11 \pm 2*
0.1	819 \pm 122*	21 \pm 3*	18 \pm 3*
0.3	351 \pm 67*	32 \pm 2*	28 \pm 3*
1.0	366 \pm 58*	38 \pm 4*	70 \pm 8*

**P* < 0.05 represents significant differences when compared with the absence of terbutaline. The plasma TNF α level was measured at 1 h after rats were injected with LPS (10 mg/kg i.v.) and terbutaline was administered at 30 min prior to LPS injection. The Δ BP represents the depressor effect of terbutaline.

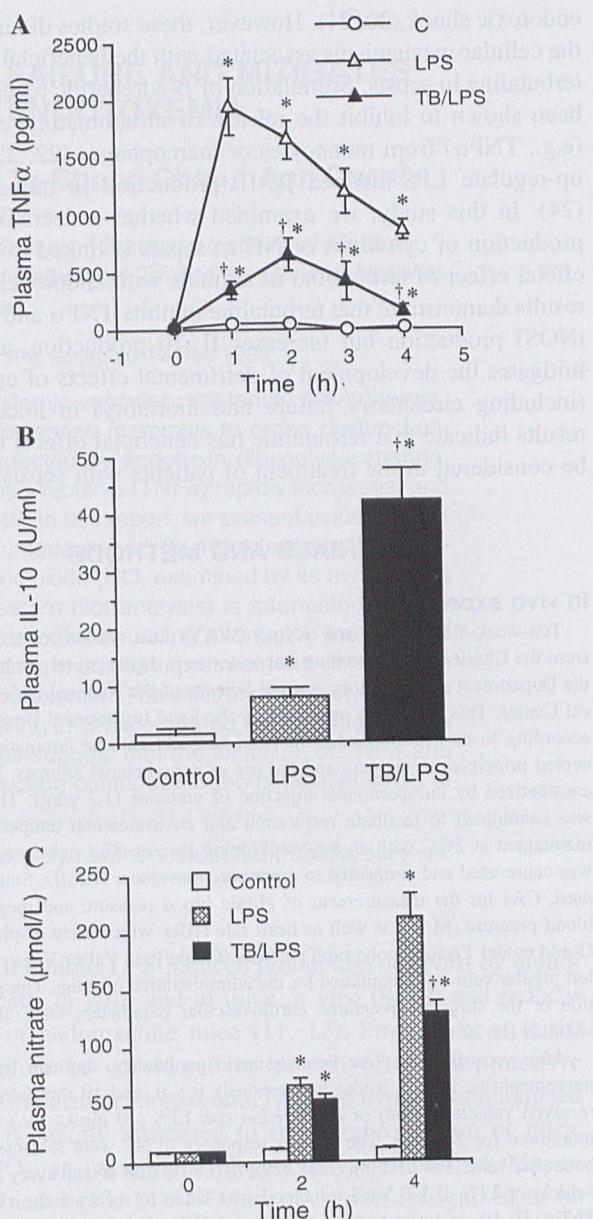


FIG. 1. Effects of terbutaline on plasma levels of TNF α , IL-10, and nitrate in rats treated with endotoxin. Depicted are the changes in plasma TNF α (A), IL-10 (B), and nitrate levels (C) during the experimental period in different groups of animals which received injections of vehicle (Control; $n = 4$ –8), vehicle plus LPS (LPS; 10 mg/kg; $n = 8$ –16), or terbutaline (0.3 mg/kg, at 30 min prior to LPS) plus LPS (TB/LPS; $n = 8$ –16). Note that each value of TNF α is the mean of duplicate plasma samples from the same animal. Data are expressed as mean \pm SEM of n animals. **P* < 0.05 represents significant differences when compared with the control group. †*P* < 0.05 represents significant differences between endotoxemic rats pretreated with and without terbutaline.

increase in TNF α or IL-10 was detectable during the experimental period, indicating that the surgical procedure alone did not cause an increase in plasma TNF α or IL-10 levels. There was also no change in plasma level of nitrate in the sham-treated group during the experimental period.

Pretreatment of rats with terbutaline significantly inhibited the LPS-induced increase in TNF α level in plasma and shifted the time course for production of TNF α to the right (Fig. 1A), whereas the IL-10 concentration at 60 min after LPS was

significantly enhanced by terbutaline (Fig. 1B). In addition, the late increase in plasma nitrate caused by endotoxemia was also significantly reduced in LPS rats pretreated with terbutaline (Fig. 1C). However, injection of normal control rats with terbutaline alone had no significant effects on the TNF α , IL-10, or nitrate levels in plasma (unpublished data).

Lung iNOS expression

An iNOS protein expression was undetectable in lung homogenates obtained from sham-treated rats, whereas a significant induction of iNOS protein was observed in lung homogenates of rats treated with LPS for 4 h (Fig. 2). Pretreatment of rats with terbutaline significantly reduced the induction of iNOS in the lung, which was caused by LPS challenge (Fig. 2).

Hemodynamic parameters

As shown in Figure 3A, the mean baseline values for MAP were between 115 ± 3 to 118 ± 4 mmHg in all animal groups studied, and there was no significant difference between groups. The injection of LPS resulted in a rapid decrease in MAP from 115 ± 3 to 92 ± 4 mmHg within 15 min and a

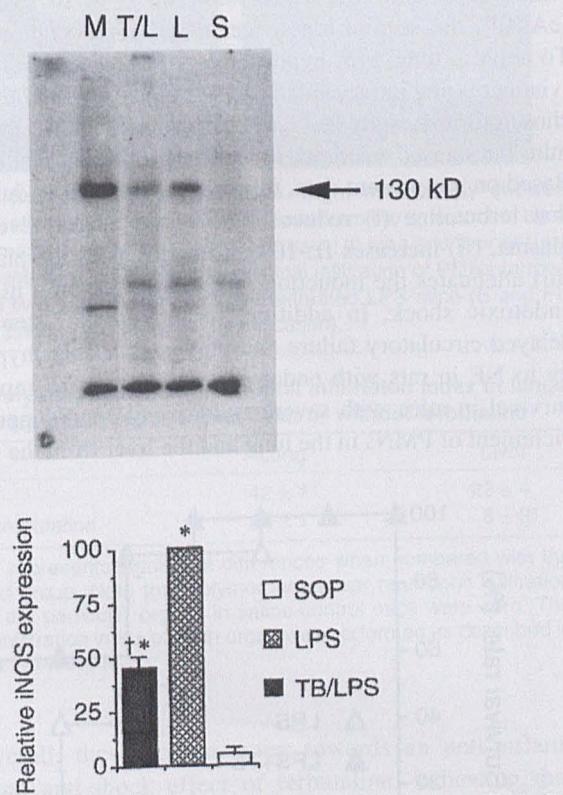


Fig. 2. Effects of terbutaline on the expression of inducible nitric oxide synthase (iNOS) in lungs from rats with endotoxic shock. This figure depicts a typical display of iNOS protein expression (upper figure) and the statistical analysis of changes of iNOS protein expression in the lung of rats injected with vehicle (S or SOP; n = 4), vehicle plus LPS (L or LPS; 10 mg/kg; n = 6), or terbutaline (0.3 mg/kg, at 30 min prior to LPS) plus LPS (T/L or TB/LPS; n = 6). Note that the left lane in the upper figure is the iNOS marker (M). Data are expressed as mean \pm SEM of n animals. *P < 0.05 represents significant differences when compared with the control group (SOP). †P < 0.05 represents significant differences between endotoxemic rats pretreated with and without terbutaline.

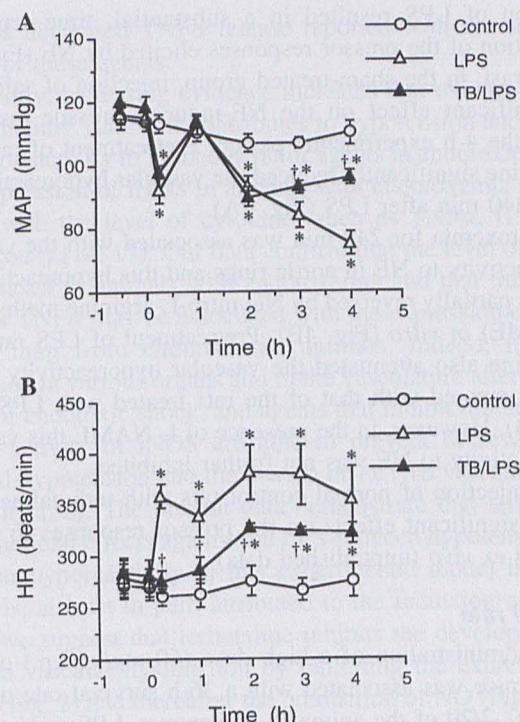


Fig. 3. Effects of terbutaline on mean arterial blood pressure (MAP) and heart rate (HR) in rats treated with endotoxin. Depicted are the changes in MAP (A) and HR (B) during the experimental period in different groups of animals which received injections of vehicle (Control; n = 8), vehicle plus lipopolysaccharide (LPS; 10 mg/kg; n = 16), or terbutaline (0.3 mg/kg, at 30 min prior to LPS) plus LPS (TB/LPS; n = 16). Data are expressed as mean \pm SEM of n animals. *P < 0.05 represents significant differences when compared with the control group. †P < 0.05 represents significant differences between endotoxemic rats pretreated with and without terbutaline.

continuous further decrease in MAP at 120–240 min (e.g., 75 ± 4 mmHg at 240 min). In the sham-treated group, there was no significant change of MAP during the experimental period (i.e., from 114 ± 3 mmHg at time 0 to 110 ± 4 mmHg at 240 min). The injection of terbutaline (0.3 mg/kg, i.v.) caused a transient decrease of MAP (maximal decrease was 29 ± 3 mmHg), but the MAP returned to pre-injection level within 30 min. Pretreatment of LPS rats with terbutaline had no significant effect on the early hypotension but significantly prevented the delayed fall in MAP.

As results shown in Figure 3B, the baseline mean values for HR were between 269 ± 12 and 281 ± 15 beats/min in all animal groups studied, and there was no significant difference between groups. Figure 3B demonstrates that administration of LPS caused a significant increase in HR during the experimental period (i.e., from 274 ± 14 beats/min at time 0 to 353 ± 33 beats/min at 240 min).

The injection of normal control rats with terbutaline alone caused only a transient hypotension, which was accompanied by a reflex tachycardia lasting 30 min (unpublished data).

Vascular reactivity to NE in vivo and ex vivo

The mean baseline values for the pressor responses to NE ranged from 28 ± 3 to 33 ± 3 mmHg, and there was no significant difference between the experimental groups studied.

Injection of LPS resulted in a substantial, time-dependent attenuation of the pressor responses elicited by NE (Fig. 4A). In contrast, in the sham-treated group, injection of saline had no significant effect on the NE-induced pressor responses during the 4-h experimental period. Pretreatment of rats with terbutaline significantly reduced the vascular hyporeactivity to NE at 240 min after LPS (Fig. 4A).

Endotoxemia for 240 min was associated with the vascular hyporeactivity to NE in aortic rings and this hyporeactivity to NE was partially reversed by N^{ω} -nitro-L-arginine methyl ester (L-NAME) *in vitro* (Fig. 4B). Pretreatment of LPS rats with terbutaline also attenuated the vascular hyporeactivity to NE when compared with that of the rats treated with LPS alone (Fig. 4B). However, in the presence of L-NAME this vascular hyporeactivity to NE was not further inhibited.

The injection of normal control rats with terbutaline alone had no significant effects on the pressor responses to NE *in vivo* and *ex vivo* (unpublished data).

Survival rate

The administration of a high dose (60 mg/kg i.p.) of LPS to ICR mice was associated with a 36-h survival rate of only 10% (i.e., 2/20 of the animals). In contrast, LPS mice treated with terbutaline (0.3 mg/kg i.p. at time 0 and 6 h after LPS, n

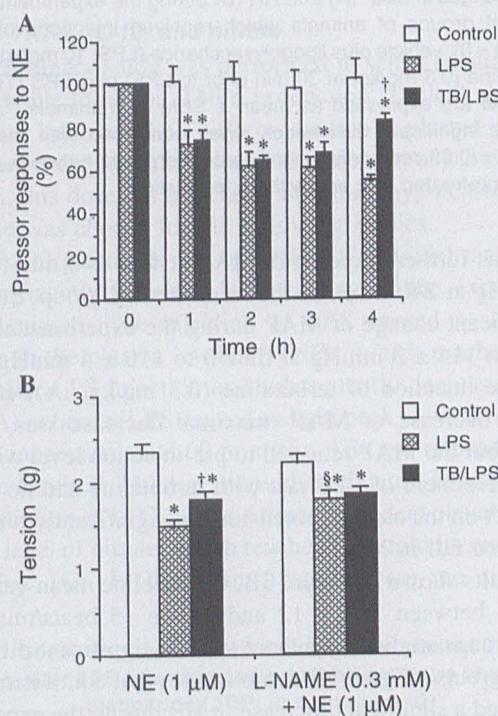


FIG. 4. Effects of terbutaline on pressor responses to NE *in vivo* or *ex vivo* in rats treated with endotoxin. Depicted are the changes in pressor responses to NE in different groups of animals and contraction induced by NE in the absence or presence of NOS inhibitor L-NAME in aortae from different groups of animals, which received injections of vehicle (Control; n = 8), vehicle plus LPS (LPS; 10 mg/kg; n = 16), or terbutaline (0.3 mg/kg, 30 min prior to LPS) plus LPS (TB/LPS; n = 16). Data are expressed as mean \pm SEM of n animals. *P < 0.05 represents significant differences when compared with the control group. †P < 0.05 represents significant differences between endotoxemic rats pretreated with and without terbutaline. \$P < 0.05 represents significant differences between with and without L-NAME in the same group.

= 20) had a survival rate of 60% at 36 h (Fig. 5). Thus, terbutaline significantly increased the survival rate of animals injected with LPS. In a time-control group (i.e., saline instead of LPS) there was no mortality within 36 h (n = 6, unpublished data).

Polymorphonuclear neutrophils (PMNs) filtration

In the time-control group, light microscopy did not show any infiltration of PMNs in lungs or livers (Fig. 6A and 6B). In contrast, 36 h after the injection of mice with LPS there was an overt infiltration of PMNs in both lungs and livers (Fig. 6C and 6D; Table 2). In addition, all of the LPS-treated mice showed marked interstitial edema and/or congestion diffusely in lungs and livers. In mice treated with terbutaline, these pathological changes were focal in distribution and mild in intensity (Fig. 6E and 6F; Table 2).

DISCUSSION

Terbutaline is a potent, selective β_2 -agonist with very low affinity for α -adrenoceptors. Occupation of β_2 -adrenoceptor by terbutaline results in a conformational change that leads to G protein activation. This in turn activates adenylate cyclase, which results in the conversion of ATP to cyclic AMP (cAMP), the second messenger of β_2 -adrenoceptor function. To avoid a long-term hypotensive effect raised by terbutaline (via increasing intracellular cAMP), the dose of terbutaline was chosen for this study that caused a hypotension for less than 30 min, but caused maximal reduction in TNF α level (Table 1). Based on our present data, this study is the first to demonstrate that terbutaline (i) reduces TNF α and nitrate levels in the plasma, (ii) increases IL-10 concentrations in the plasma, and (iii) attenuates the induction of iNOS in the lung in rats with endotoxic shock. In addition, terbutaline (i) attenuates the delayed circulatory failure and prevents vascular hyporeactivity to NE in rats with endotoxic shock and (ii) improves the survival in mice with severe endotoxemia and suppresses the increment of PMNs in the lung and the liver from the surviving

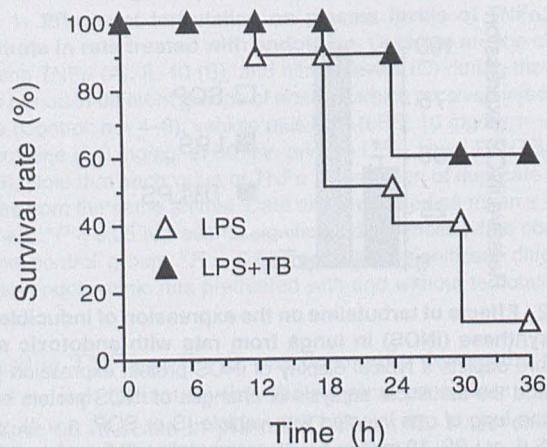


FIG. 5. Effects of terbutaline on the survival rate in mice treated with endotoxin. Depicted are the changes of survival during the experimental period in different groups of ICR mice which received intraperitoneal injection of LPS (LPS; 60 mg/kg; n = 20) or terbutaline (LPS + TB; 0.3 mg/kg; at time 0 and 6 h after LPS, n = 20) plus LPS. Data are expressed as percentage of mice survived at the observed time point.

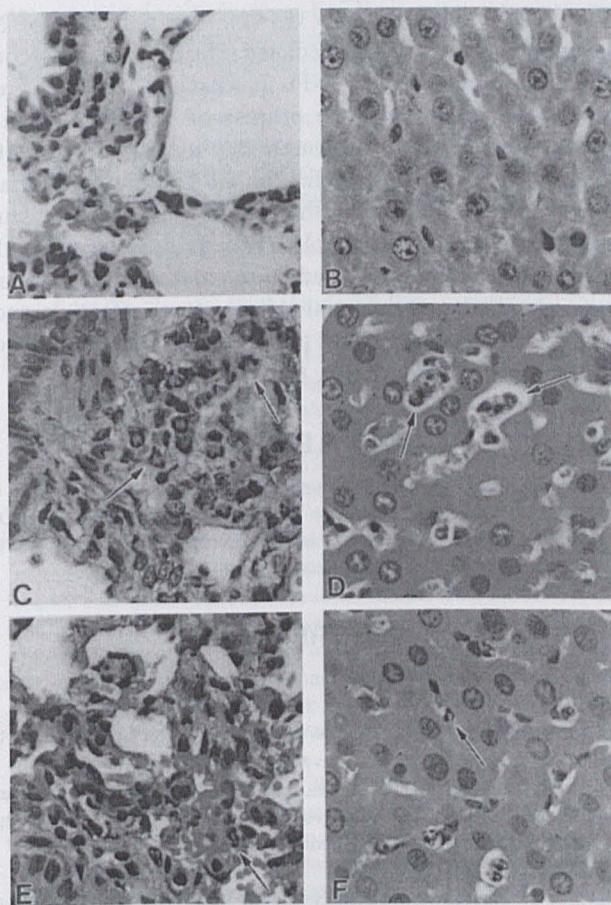


FIG. 6. Histopathological studies. Light microscopy showed morphologically normal lung and liver tissues from the time-control mice (A and B), marked infiltration of PMNs (arrows) in lung and liver tissues from LPS mice (C and D), and only minimal infiltration of PMNs (arrow) in lung and liver tissues from terbutaline-treated LPS mice (E and F). H&E stain. Each, $\times 400$ (original magnification).

TABLE 2. Polymorphonuclear neutrophil infiltration index in lungs and livers of LPS mice treated with or without terbutaline

	Lung	Liver
LPS	42 \pm 7	22 \pm 4
LPS + terbutaline	14 \pm 1*	8 \pm 2*

* $P < 0.001$ represents significant differences when compared with the LPS-treated group. Note that polymorphonuclear neutrophil infiltration indices for the particular organs in saline-control mice were zero. The neutrophil infiltration index of each organ was performed as described in Materials and Methods.

mice. Overall, these actions point towards an anti-inflammatory and anti-shock effect of terbutaline, indicating that terbutaline has beneficial effects in animals with endotoxemia.

The suppression of LPS-induced TNF α plasma levels by terbutaline and the parallel increase in IL-10 is not unique: similar effects were observed with prostaglandin E₂ (27), with epinephrine or isoproterenol (24, 28), and with adenosine receptor agonists (29). The inhibition of TNF α production by all of these agents has been linked to their stimulating effect on adenylate cyclase, leading to an increase in intracellular cAMP levels. Thus, it is generally assumed that increased intracellular cAMP levels are responsible for both increased IL-10 produc-

tion and decreased TNF α release reported with the administration of these agents.

Recently, increasing evidence indicates that overproduction of NO mainly via iNOS contributes to hypotension and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock. The expression of iNOS in animals with endotoxemia is associated with the level of cytokines such as TNF α , IL-1, and interferon- γ (14, 17). Our data confirm that the level of TNF α is increased in animals with endotoxemia and that this increment of TNF α may be associated with the expression of iNOS in the lung from endotoxicemic animals. Indeed, iNOS is expressed in various organs and in the vasculature after several hours of endotoxic shock, and agents that inhibit the induction or the activity of iNOS are able to prevent or reverse the delayed hypotension and the *in vivo* or *ex vivo* vascular hyporeactivity (30). The present data demonstrate that terbutaline has beneficial effects against the LPS-induced hypotension and vascular hyporeactivity in this experimental model in which injury is, at least in part, attributed to the induction of iNOS. Thus, we suggest that terbutaline inhibits the development of delayed vascular dysfunction by inhibiting the expression of iNOS (Fig. 2) and thereafter the production of NO (Fig. 1C) in this model. Inhibition of iNOS activity with L-NAME in the same model resulted in partial recovery from the endotoxin-induced vascular hyporeactivity, while L-NAME did not alter contraction in aortae from terbutaline-pretreated LPS rats (Fig. 4B). The non-additive effects of terbutaline *in vivo* and L-NAME *in vitro* further supports the hypothesis that the protection against the suppression of vascular hyporeactivity by terbutaline (in this study) or isoproterenol (28) is due to inhibition of the expression of iNOS. Thus, we suggest that the inhibition of iNOS expression by terbutaline may result from the reduction of TNF α (Fig. 1A) and/or the increase of IL-10 (Fig. 1B). It has been shown that IL-10 inhibits LPS-induced production of TNF α by mononuclear cells *in vitro* and in mice *in vivo* (9–12) and reduces lethality of endotoxicemic mice (11, 12). Therefore, production of IL-10 can be considered to be part of a host-protective mechanism during endotoxemia, although a recent study suggests that IL-12 may be more important than IL-10 in the regulation of inflammatory mediators (31). The enhancement of IL-10 production by β -adrenoceptor stimulation presumably involves adenylate cyclase, since agents that are known to increase intracellular cAMP levels augment LPS-induced IL-10 synthesis in murine peritoneal macrophages (32), human whole blood (33), and rats (29; this study). However, a recent study indicates that the enhancement of IL-8 instead of IL-10 is one of the pathways via which β_2 -adrenergic agonists influence inflammatory responses (34).

There is conflicting evidence on the role of cAMP and the effect of cAMP-elevating agents in the induction of iNOS. For instance, in some studies elevation in cAMP caused a reduction in the induction of iNOS (35, 36), in other studies elevation of cAMP caused induction of iNOS or enhancement of the induction of iNOS in response to additional stimulants (37, 38). It has been shown that the regulation of the induction of iNOS *in vivo* is cell-specific: while in certain cells and tissues cAMP up-regulates the induction of iNOS, in other cells, it may be inhibitory. Moreover, the time-course of the intervention may

be an important factor: the suppression of iNOS induction by cAMP elevating agents may require more prolonged pretreatment. Assuming that the regulation by terbutaline of iNOS induction *in vivo* is cell-specific, it is not surprising that overall effect of terbutaline on plasma nitrate levels are relatively modest. It is conceivable that NO production from non-vascular sources (macrophages, epithelial cells, etc.) importantly contribute to the whole body NO production. Indeed, our data show that the expression of iNOS in the lung is modestly suppressed by pretreatment of LPS rats with terbutaline.

Because in 80% of the bodies resident macrophages are localized in the liver and the lung (39), liver- or lung-derived TNF α may affect not only liver or lung, but also other major organs. A characteristic feature of sepsis-induced liver or lung injury (Fig. 6 and Table 2) is the activation and subsequent sequestration of PMNs in hepatic or pulmonary microvasculature with endothelial injury. This results from a highly regulated sequence of events involving chemical mediators and a complex series of cell surface adhesion of activated neutrophils to endothelium and subsequent damage of hepatic or pulmonary microvasculature. These events subsequently lead to the appearance of liver dysfunction or respiratory failure (e.g., ARDS), which may ultimately causes animals or patients to death. One could argue that the impairment of endothelial function by endotoxin and the suppression of iNOS expression by terbutaline may reduce the formation of NO, which combats the vasoconstriction observed in vascular beds in multiple vital organs. Thus, this may lead to further reduction of blood flow to these vital organs. However, the injection of terbutaline caused a sustained increase of pulse pressure in rats treated with endotoxin (unpublished data), suggesting a reduction of peripheral vascular resistance by terbutaline could be a beneficial effect on protection of vital organs against the impairment by endotoxin. Overall, terbutaline increases the blood flow (possibly via increasing intracellular cAMP levels) to multiple organs and inhibits the overproduction of NO (via prevention of iNOS expression). Thus, terbutaline protects against the circulatory failure and may preserve the organ function in animals with endotoxemia. The latter was supported by the survival study demonstrating that terbutaline significantly increased the survival rate in endotoxemic animals (20, this study). The administration of terbutaline could therefore be considered as a part of the treatment of patients with septic shock, in particular associated with ARDS, in clinics. This is based on the fact that this agent has been shown to have some beneficial effects in experimental endotoxic shock and in human ARDS (20, 40). However, it must be recognized that translational research from the encouraging experimental findings to the clinic will require extensive, carefully designed and well controlled clinical trials before the utility of this treatment can be determined (41).

In conclusion, the data presented here provide mechanistic evidence that terbutaline exerts marked anti-inflammatory effects by suppressing LPS-induced TNF α and NO production, while simultaneously increasing LPS-induced IL-10 production. Regarding the mechanism of action, several possibilities are suggested. Some of the effects may be related to the primary cAMP-increasing effect of the drug (effects on TNF α ,

IL-10, and possibly, IL-12) (31, 42). The reduction of NO production may be a cAMP-mediated effect, or may be secondary to the suppression of TNF α production, since TNF α is known to be involved in the process of iNOS induction in response to LPS (8). Although the mechanism and the sequence of effects of terbutaline on the LPS-induced inflammatory response require further studies, the present study coupled with other current observations (33) suggests that β -adrenoceptor agonists, in addition to their cardiovascular effects, also have multiple anti-inflammatory effects in endotoxemia, via modulation of the production of key inflammatory mediators (e.g., TNF α , IL-10, and NO in this study).

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REFERENCES

1. McLean JS, Byrick RJ: ARDS and sepsis—definitions and new therapy. *Can J Anesth* 40:585–590, 1993.
2. Altura BM, Lefer AM, Schumer W: *Handbook of Shock and Trauma. Vol. 1: Basic Science*. New York: Raven Press, 1983.
3. Parker MM, Shelhamer JH, Natanson C, Alling D, Parillo JE: Serial cardiovascular variables in survivors and non-survivors of human septic shock: heart rate as an early predictor of prognosis. *Crit Care Med* 15: 923–929, 1987.
4. Repine JE: Scientific perspectives on adult respiratory distress syndrome. *Lancet* 339:466–469, 1992.
5. Nathan CF: Secretory products of macrophages. *J Clin Invest* 79:319–326, 1987.
6. Tracey KJ, Cerami A: Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* 9:317–343, 1993.
7. Beutler B, Cerami A: The biology of cachectin/TNF α : a primary mediator of the host response. *Ann Rev Immunol* 7:625–655, 1989.
8. Thiemermann C, Wu CC, Szabo C, Perretti M, Vane JR: Role of tumor necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. *Br J Pharmacol* 110:177–182, 1993.
9. de Waal Malefyt, R, Abrams J, Bennett B, Fidgor CG, de Vries JE: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 174: 1209–1220, 1991.
10. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 147: 3815–3822, 1991.
11. Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenbeele P, Delvaux A, Fiers W, Goldman M, Velu T: Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J Exp Med* 177:547–550, 1993.
12. Howard M, Muchamuel T, Andrade S, Menon S: Interleukin 10 protects mice from lethal endotoxemia. *J Exp Med* 177:1205–1208, 1993.
13. Stoclet JC, Fleming I, Gray G, Julou-Schaeffer G, Schneider F, Schott C, Schott C, Parratt JR: Nitric oxide and endotoxemia. *Circulation* 87(Suppl V):V77–V80, 1993.
14. Thiemermann C: The role of the L-arginine: nitric oxide pathway in circulatory shock. *Adv Pharmacol* 28:45–79, 1994.
15. Amezcua JL, Palmer RM, de Souza BM, Moncada S: Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br J Pharmacol* 97:1119–1124, 1989.
16. Chu A, Chambers DE, Lin CC, Kuehl WD, Palmer RM, Moncada S: Effects of inhibition of nitric oxide formation on basal vasomotion and endothelium-dependent responses of the coronary arteries in awake dog. *J Clin Invest* 87:1964–1968, 1991.

17. Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109–142, 1991.
18. Nathan C, Xie QW: Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269:13725–13728, 1994.
19. Hoffman BB, Lefkowitz RJ: Catecholamines, sympathomimetic drugs, and adrenergic receptor antagonists. In Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (eds): *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th ed. New York: The McGraw-Hill Co. Inc., 1996, pp 199–248.
20. Sigurdsson GH, Christenson: Terbutaline increases survival after endotoxin shock. A comparative study of methyl prednisolone, isoprenaline and terbutaline in rats. *Microcirc Endothelium Lymphatics* 3:231–240, 1987.
21. Sigurdsson GH, Youssef HAE, Al-Mousawi M, Khourshid M, Christenson JT: Effects of the beta-2 receptor agonist terbutaline on hemodynamics and gas-exchange in endotoxin shock. *Acta Chir Scand* 155:369–375, 1989.
22. Severn A, Rapson NT, Hunter CA, Liew FY: Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists. *J Immunol* 148:3441–3445, 1992.
23. Taffet SM, Singhal KJ, Overholtzer JF, Shurtliff SA: Regulation of tumor necrosis factor expression in a macrophage-like cell line by lipopolysaccharide and cyclic AMP. *Cell Immunol* 120:291–300, 1989.
24. van der Poll T, Coyle SM, Barbosa K, Braxton CC, Lowry SF: Epinephrine inhibits tumor necrosis factor- α and potentiates interleukin 10 production during human endotoxemia. *J Clin Invest* 97:713–719, 1996.
25. Wu CC, Hong HJ, Chou TZ, Ding YA, Yen MH: Evidence for inducible nitric oxide synthase in spontaneously hypertensive rats. *Biochem Biophys Res Commun* 228:459–466, 1996.
26. Chen A, Chou WY, Ding SL, Shaio MF: Glomerular localization of nephritogenic protein complexes on a nonimmunological basis. *Lab Invest* 67:175–185, 1992.
27. Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T: Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E₂. *J Exp Med* 180:2365–2370, 1994.
28. Szabo C, Hasko G, Zingarelli B, Nemeth ZH, Salzman AL, Kvetan V, Pastores SM, Vizi ES: Isoproterenol regulates tumor necrosis factor, interleukin-10, interleukin-6 and nitric oxide production and protects against the development of vascular hyporeactivity in endotoxemia. *Immunology* 90:95–100, 1997.
29. Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES: Adenosine receptor agonists differentially regulate IL-10 and TNF production in endotoxemic mice. *J Immunol* 157:4634–4640, 1996.
30. Szabo C: Alterations in nitric oxide production in various forms of circulatory shock. *New Horizons* 3:2–32, 1995.
31. Hasko G, Szabo C, Nemeth ZH, Salzman AL, Vizi ES: Stimulation of β -adrenoceptors inhibits endotoxin-induced IL-12 production in normal and IL-10 deficient mice. *J Neuroimmunol* 88:57–61, 1998.
32. Kilbourn RG, Cromeens DM, Chelly FD, Griffith OW: N^G-methyl-L-arginine, an inhibitor of nitric oxide formation, acts synergistically with dobutamine to improve cardiovascular performance in endotoxemic dogs. *Crit Care Med* 22:1835–1840, 1994.
33. Pastores SM, Hasko G, Vizi ES, Kvetan V: The role of vasoactive drugs in the regulation of cytokine production in sepsis. *New Horizons* 4:252–264, 1996.
34. Kavelaars A, van de Pol M, Zijlstra J, Heijnen CJ: β_2 -Adrenergic activation enhances interleukin-8 production by human monocytes. *J Neuroimmunol* 77:211–216, 1997.
35. Bulut V, Severn A, Liew FY: Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP. *Biochem Biophys Res Commun* 195:1134–1138, 1993.
36. Feinstein DL, Galea E, Reis DJ: Norepinephrine suppresses inducible nitric oxide synthase activity in rat astroglial cultures. *J Neurochem* 60:1945–1948, 1993.
37. Imai T, Hirata Y, Kanno K, Marumo F: Induction of nitric oxide synthase by cyclic AMP in rat vascular smooth muscle cells. *J Clin Invest* 93:543–549, 1994.
38. Koide M, Kawahara Y, Nakayama I, Tsuda T, Yokoyama M: Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. Synergism with the induction elicited by inflammatory cytokines. *J Biol Chem* 268:24959–24966, 1993.
39. Laskin DL: Nonparenchymal cells and hepatotoxicity. *Semin Liver Dis* 10:293–304, 1990.
40. Basran GS, Byrne AJ, Hardy JG: Beta-2-adrenoceptor agonists as modulators of inflammatory oedema in the adult respiratory distress syndrome (ARDS) in man. *Chest* 86:293, 1984.
41. Szabo C: Simultaneous targeting or multiple pathways of shock: how promising, how novel? *Shock* 11:296–297, 1999.
42. Panina-Bordignon P, Mazzeo D, Di Lucia P, D'Ambrosio D, Lang R, Fabbri L, Self C, Sinigaglia F: β_2 -Agonists prevent Th1 development by selective inhibition of interleukin 12. *J Clin Invest* 100:1513–1519, 1997.

