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Inhibition of tumour necrosis factor and reversal of endotoxin-induced shock by U-83836E, a 'second generation' lazaroid in rats

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1 Antioxidants can exert protective effects in endotoxic shock by either a reduction of the oxidant damage or attenuation of Tumour Necrosis Factor (TNF-α) production.

2 Lazaroids are a family of compounds that inhibit lipid peroxidation. Besides, they can also reduce TNF-α. U-83836E is a new lazaroid lacking the glucocorticoid ring.

3 Aim of our study was to investigate the effect of U-83836E on TNF-α production either *in vivo* or *in vitro*. Endotoxic shock was produced in male rats by a single intravenous (i.v.) injection of 20 mg kg^{-1} of S. enteritidis lipopolysaccharide (LPS). LPS administration reduced survival rate (0% survival, 72 h after endotoxin administration), decreased mean arterial blood pressure, increased serum and macrophage TNF-α and enhanced plasma malonylaldehyde (MAL) levels. Furthermore aortic rings from shocked rats showed a marked hyporeactivity to phenylephrine (PE 1 nM – 10 μM).

4 Treatment with U-83836E (7.5, 15 and 30 mg kg^{-1}, i.v.) 5 min after endotoxin challenge significantly protected against LPS induced lethality (90% survival rate and 80% survival rate 24 h and 72 h after LPS injection respectively, following the highest dose of the drug), reduced hypotension, blunted plasma MAL, decreased serum and macrophage TNF-α and restored the hyporeactivity of aortic rings to control values. *In vitro* LPS stimulation (50 μg ml^{-1} for 4 h) significantly increased cytokine production in macrophages (MΦ) harvested from untreated normal rats. Pretreatment with pertussis toxin (PT; 0.1, 1 and 10 ng ml^{-1} 4 h before LPS) significantly increased TNF-α production. PT effects on these LPS responses were correlated with a PT mediated ADP ribosylation of a 41 kDa protein. U-83836E (50 μM) reduced, in a dose dependent manner, LPS induced TNF-α production and inhibited the PT effects on cytokine production and on ADP ribosylation of the protein.

5 Our data suggest that lazaroids may affect the early events associated with LPS receptor mediated activation of a G protein in LPS induced TNF-α production. These molecular events may explain, at least in part, the *in vivo* inhibition of cytokine production and reversal of endotoxic shock.

Keywords: Lazaroids; endotoxic shock; TNF-α inhibition; U-83836E; lipid peroxidation; isolated rat aorta; macrophages; pertussis toxin

Introduction

Tumour Necrosis Factor (TNF-α) is a pleiotropic cytokine produced mainly by mononuclear phagocytes in response to endotoxin or other products derived from bacteria viruses or parasites (Vilcek & Lee, 1991). During recent years growing interest has accumulated regarding this cytokine. In fact it has been demonstrated that TNF-α plays an important role in the pathogenesis of endotoxic shock (Cerami, 1992; Tracey *et al*., 1989). Infusion of recombinant TNF-α in experimental animals mimics some features of endotoxic shock including pulmonary hypertension, systemic hypotension, hypoxemia, gastric lesions and lung injury (Johnson *et al*., 1989; Stephens *et al*., 1988; Tracey *et al*., 1987). Because TNF-α may mediate many of the pathological sequelae of endotoxic shock, methods to inhibit the cytokine production or its activity are the object of much research.

Dexamethasone has been used to decrease TNF-α production, an effect that results largely from a blockade of the translation depression of TNF-α mRNA (Beutler *et al*., 1986; Han *et al*., 1990). It has been suggested that certain methylxanthine derivatives and dibutyryl cyclic adenosine monophosphate, may diminish TNF-α production *in vitro* (Strieter *et al*., 1988; Endres *et al*., 1991). Furthermore, agents capable of reducing the synthesis of this cytokine display protective effects in endotoxic shock (Lechner *et al*., 1993; Altavilla *et al*., 1995). Besides TNF-α, it has been suggested that oxygen derived free radicals are involved in the pathogenesis of endotoxic shock (Morgan *et al*., 1988). Indeed inhibition of lipid peroxidation improves survival rate of endotoxemic rats (Kunimoto *et al*., 1987).

The 21-aminosteroids are a family of compounds that potently inhibit lipid peroxidation (McCall *et al*., 1987) and protect against circulatory shock (Squadrito *et al*., 1995). It has been suggested that the 21 aminosteroid tirilazad mesylate reduces TNF-α generation and protects against endotoxic shock in neonatal calves (Semrad *et al*., 1993). However the study did not give conclusive informations about the mechanisms underlying the inhibitory effect on the cytokine production. Tirilazad mesylate belongs to the first generation lazaroids that are related to methylprednisolone and have a 21-aminosteroid structure. Therefore the

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tirilazad – induced inhibition on cytokine generation may be likely due to the aminosteroid ring. In contrast U-83836E (−)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pirymidinyl)-1-piperazinyl]-methyl]-3,4-dihydro-2,5,7,8,-tetramethyl-2H-1-benzopyran-6-ol, dihydrochloride, the active enantiomer of the racemate U-78517F, is one of the second generation lazaroids that are based on a non steroid structure characterized by a ring portion of α-tocopherol binded to various groups. The aim of the present study was to assess the possible ability of this drug to inhibit TNF-α generation either *in vivo* or *in vitro*.

Methods

Endotoxic shock procedure

Male Sprague-Dawley rats (250 – 300 g), obtained from Harlam Nossam S.r.l. (Comezzano, Milano, Italy), fed on standard diet and with tap water *ad libitum*, were used. Environmental conditions were standardized, including a room temperature of 22 ± 2°C and 12 h artificial lighting. Endotoxic shock was induced by administering a single intravenous (i.v.) dose of 20 mg kg^{-1} of S. enteritidis endotoxin. Control rats received an equal volume of vehicle (0.9% NaCl). Five minutes after endotoxin injection, control rats received an i.v. bolus of 0.9% NaCl (1 ml kg^{-1}) while treated rats were injected with U-83836E (7.5, 15 and 30 mg kg^{-1}) as an i.v. bolus. Survival rate was evaluated for 72 h after endotoxin administration.

Arterial blood pressure measurements

A second group of rats was used to monitor blood pressure. Briefly, the animals were anaesthetized with urethane (1.3 g kg^{-1}) and a cannula (PE 50) was inserted into the left common carotid artery and connected to a pressure transducer. The arterial blood pressure, was monitored for 6 h and displayed on a polygraph (Ugo Basile, Varese, Italy). Basal value of mean arterial blood pressure (MAP) ranged between 80 and 100 mmHg. These rats were subjected to the same experimental protocol as described above.

Macrophage isolation and culture

Peritoneal macrophages were obtained from control normal rats by washing the abdominal cavity with RPMI 1640. The cells were centrifuged twice and resuspended in the same medium at a concentration 1×10^{-6} ml^{-1}. Peritoneal macrophages were obtained after 2 h adhesion to plastic Petri dishes (Nunc, Denmark) at 37°C. The homogeneity and the viability of macrophages were greater than 98% as determined by differential staining and trypan blue exclusion. The cells were pretreated for 4 h with pertussis toxin (0.1, 1 and 10 ng ml^{-1}) and then stimulated for an additional 4 h with 50 μg ml^{-1} of S. enteritidis LPS. Macrophage culture supernatants were collected and frozen at −20°C until the measurement of TNF-α. In some experiments U-83836E (50 μM) was incubated together with pertussis toxin.

ADP-ribosylation reaction

The macrophage membranes were prepared as previously described (Rizzoli *et al*., 1986). A total of 20 μg $sample^{-1}$ of membrane was used in the ADP-ribosylation reaction. The volume of each sample was 60 μl. Pertussin toxin was activated by incubation of 50 mg ml^{-1} of the toxin in 25 mM potassium phosphate, pH 7.5, and 20 mM dithiothreitol at 37°C for 30 min. ADP-ribosylation (Schleifer *et al*., 1982) was carried out at 37°C for 1.5 h in the presence of 10 μg ml^{-1} activated pertussis toxin, 100 nM potassium phosphate, pH 7.5, 10 nM thymidine, 100 μM guanosine triphosphate, 1 mM ATP, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 μM NAD and 100 μCi ml^{-1} [α-^{32}P]NAD. The final reaction volume was 100 μl. The reaction was stopped with 1 ml 10% ice-cold TCA. Samples were incubated on ice for 30 min, centrifuged at 16,000 g at 4°C for 20 min to precipitate the proteins, applied to 11% SDS – PAGE, and the gels autoradiographed. In some experiments U-83836E (50 μM) was incubated together with pertussis toxin.

Immunoassay for tumor necrosis factor-α activity

Factor-Test-X™ Rat TNF-α ELISA Kit (Genzyme, Cambridge, MA, U.S.A.) was used to measure TNF-α levels in serum, collected 0, 2 h, 4 h and 6 h following endotoxin challenge (20 mg kg^{-1}, i.v.) in the second group of animals and in peritoneal macrophage supernatants. For the *in vitro* studies macrophages collected from untreated endotoxic and sham shocked rats were incubated for 3 h either with RPMI or several doses of U-83836E (12, 25, 50 μM). The assay sensitivity was 15 pg/ml.

Plasma MAL analysis

Assessment of the MAL levels was carried out in plasma samples to give an indication of lipid peroxidation in the cellular membranes. Samples (0.2 ml) of arterial blood were drawn from the carotid catheter at 0, 2 h, 4 h and 6 h following endotoxin injection (20 mg kg^{-1}, i.v.). The blood was collected in polyethylene tubes to which had been added 10 μl of heparin solution (1000 iu). The plasma samples, obtained after centrifugation at 3000 g for 10 min at 4°C, were frozen at −70°C until the analysis. The assay was carried out using a high performance liquid chromatography (HPLC) technique, as previously described (Ceconi *et al*., 1991). Briefly, each sample of plasma was diluted with an equal volume of acetonitrile to precipitate proteins. The resulting suspension was vortex-mixed for 30 s and centrifuged at 3000 g for 5 min; the clear supernatant was injected into the HPLC. For separation of MAL a reverse-phase, ion-pair chromatography technique was used. HPLC analysis was performed on an Altex C_{18} 5 μm reversed-phase column (0.45 × 15 cm) (Beckman Instruments Inc., Berkeley, CA, U.S.A.) with a mobile phase of 10 mM Na_2HPO_4, 2.5 mM myristyltrimethylammonium bromide, 25% acetonitrile, and corrected with orthophosphoric acid until the pH was 6.7. The flow rate was 1.5 ml min^{-1} at ambient temperature, the injected volume was 50 μl and the variable wave length detector (mod 165, Beckman Instruments Inc., San Ramon, U.S.A.) was fixed at 267 nm. The HPLC instrument was the Beckman Solvent Delivery System (Beckman Instruments Inc., San Ramon, U.S.A.). A calibration chromatogram of an accurately prepared standard MAL solution was also run every time for peak identification and quantitation. Standard MAL was prepared by acid hydrolysis of malonylaldehyde-bisdimethyl acetal. In these conditions the detection limit of MAL in the plasma samples was 100 pmol ml^{-1}.

Isolated aortic rings

Thoracic aortae were removed 3 h after LPS challenge (20 mg kg^{-1}) and placed in cold Krebs' solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, Na HCO_3 25.0 and glucose 11.7;

then aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. Rings were then placed under 1 g of tension in an organ bath containing 10 ml of Krebs' solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs' solution at 15–20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the presence or absence of endothelium was assessed by administering acetylcholine (ACh, 100 nM). Concentration-response curves were obtained by cumulative concentrations of PE (1 nM – 10 μM) to intact or endothelium denuded aortic rings.

Materials

Phenylephrine hydrochloride, acetylcholine, indomethacin, dithiothreitol, thymidine, [α-^{32}P]NAD, heparin, pertussis toxin and S. enteritidis endotoxin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. U-83836E was a kind gift from Upjohn S.p.A Italy. Myristyltrimethylammonium bromide and malonylaldehydebisdimethyl acetal were from Janssen Chemical Beerse, Belgium.

Statistical analysis

Data are expressed as means ± s.e.mean and were analysed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. A probability of less than 0.05 was selected as criterion for statistical significance. For survival data, statistical analysis was done with the Kaplan-Meier test.

Results

Survival rate

Figure 1 shows the animals surviving in each group throughout the experimental period. U-83836E, administered curatively, significantly protected against endotoxic shock. The effect was dose-dependent and the endotoxic group given with the highest dose of U-83836E (30 mg kg^{-1}, i.v.) showed the maximum effect (Figure 1). Therefore, we chose the 30 mg kg^{-1} dose as the most effective and we employed it in the functional studies.

Arterial blood pressure

Rats injected with endotoxin experienced a sharp and long-lasting decrease in mean arterial blood pressure. U-83836E (30 mg kg^{-1}, 5 min after endotoxin challenge) significantly reverted the sustained decrease in MAP (Figure 2).

Modulation of LPS-induced Serum TNF-α production and macrophage TNF-α release ex vivo

The basal values of TNF-α were <15 pg ml^{-1} in the serum and in the supernatants of peritoneal macrophages of control

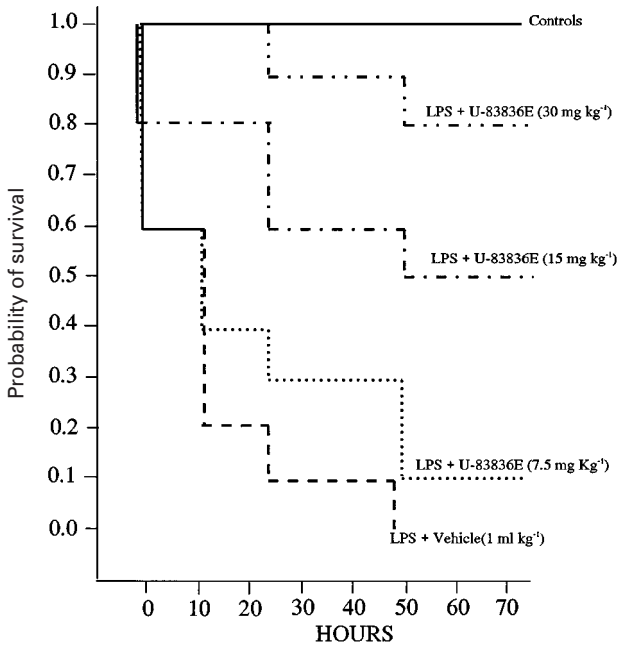

Figure 1 Effects of U-83836E (7.5, 15 and 30 mg kg^{-1}, 5 min after endotoxin challenge) on LPS-induced lethal toxicity in rats.

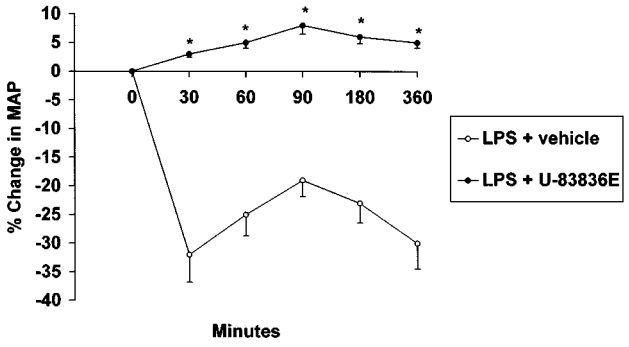

Figure 2 Effects of vehicle (open circles; 1 ml kg^{-1} of a 0.9% NaCl solution, 5 min after endotoxin challenge) or U-83836E (closed circles; 30 mg kg^{-1}, 5 min after endotoxin challenge) on mean arterial blood pressure in rats subjected to endotoxic shock. Each value represents the mean ± s.e.mean of six experiments. *$P<0.05$ vs vehicle-treated rats.

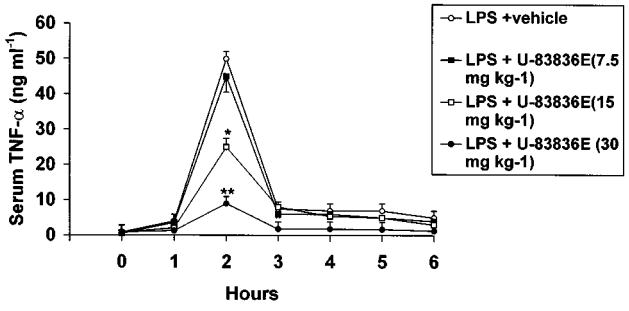

Figure 3 Time course of TNF-α appearance in the serum of vehicle (open circles; 1 ml kg^{-1} of a 0.9% NaCl solution, 5 min after endotoxin challenge) or U-83836E (7.5 mg kg^{-1} closed squares; 15 mg kg^{-1} open squares; 30 mg kg^{-1} closed squares, 5 min after endotoxin challenge) treated endotoxic shocked rats. All values are the means ± s.e.mean of six experiments. *$P<0.01$ vs vehicle treated rats.

rats. In endotoxic-shocked rats serum TNF-α progressively rises upon endotoxin administration and reaches the maximum increase 2 h after endotoxin challenge (Figure 3). At this time TNF-α was significantly increased in the supernatants of peritoneal macrophages collected from endotoxic shocked rats (6 ± 0.5 ng ml^{-1}). The administration of U-83836E, given curatively, to endotoxic-shocked rats, decreased TNF-α appearance in serum (Figure 3) and reduced the cytokine levels in macrophage supernatants (1.9 ± 0.3 ng ml^{-1}).

Plasma malonylaldehyde levels

Analysis of plasma malonyladehyde (MAL) was carried out to evaluate damage by free radicals on biological membranes 0, 2 h, 4 h and 6 h following endotoxin challenge. In plasma obtained at time 0, low levels of MAL were observed in each group. In contrast endotoxin administration significantly increased plasma levels of MAL (Figure 4). The maximum increase was observed 2 h after LPS challenge. Administration of U-83836E reduced this increase in MAL (Figure 4).

Contractile response to phenylephrine

Figure 5 shows the contractile force induced by phenylephrine (PE; 1 nM – 10 μM) in aortic rings from sham or endotoxic shocked rats. Endotoxic shock markedly decreased the contractile response to PE in aortae with (results not shown) or without endothelium (Figure 5). The administration of U-83836E (30 mg kg^{-1}) did not modify the aortic reactivity of sham rats. In contrast, administration of U-83836E, at the same dose, significantly improved responsiveness to PE in aortae from endotoxic shocked rats (Figure 5).

Macrophage TNF-α release in vitro

In order to investigate whether U-83836E interferes with TNF-α release by macrophages, we primed macrophages *in vitro* with LPS (50 μg ml^{-1} for 4 h). LPS markedly increased TNF-α production (Figure 6). U-83836E (12, 25 and 50 μM) reduced, in a dose-dependent manner, TNF-α release by macrophages (Figure 6).

Effects of pertussis toxin on LPS-induced TNF-α release

Pertussis toxin (PT) pretreatment (0.1, 1 or 10 ng ml^{-1}) produced a higher increase in LPS-induced TNF-α release (Figure 7), while PT alone did not modify the basal levels of macrophage (< 15 pg ml^{-1}). U-83836E (50 μM) incubated together with PT markedly decreased the PT effects on cytokine production (Figure 7).

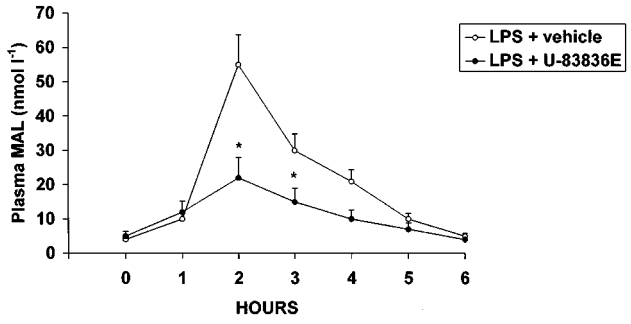

Figure 4 Time course of MAL appearance in the plasma of vehicle (open circles, 1 ml kg^{-1} of a 0.9% NaCl solution, 5 min after endotoxin challenge) or U-83836E (closed circles; 30 mg kg^{-1}, 5 min after endotoxin challenge) treated endotoxic shocked rats. All values are the means ± s.e.mean of six experiments. *$P<0.01$ vs vehicle treated rats.

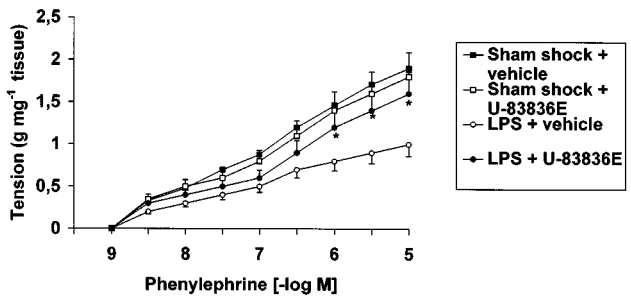

Figure 5 Contractile response to cumulative concentrations of phenylephrine in aortic rings without endothelium from sham shocked rats treated with vehicle (closed squares) and U-83836E (open squares) or endotoxic shocked rats treated with vehicle (open circles; 1 ml kg^{-1} of 0.9% NaCl solution) or U-83836E (closed circles; 30 mg kg^{-1}, 5 min after endotoxin challenge). Each value represents the mean ± s.e.mean of six experiments. *$P<0.05$ vs vehicle treated rats.

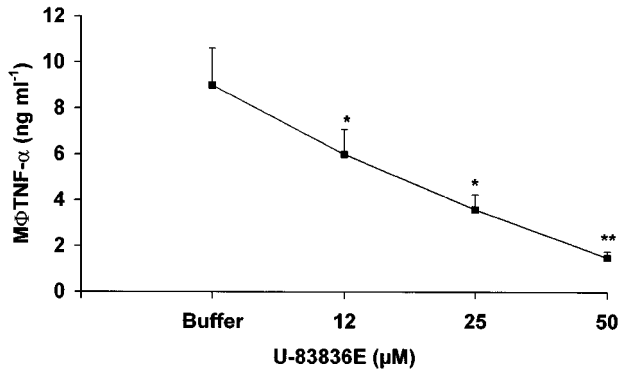

Figure 6 *In vitro* effect of U-83836E (12, 25 and 50 μM) on TNF-α release by macrophages primed with LPS (50 μg ml^{-1} for 4 h). Each point represents the mean ± s.e.mean of six experiments. *$P<0.05$ vs buffer, **$P<0.001$ vs buffer.

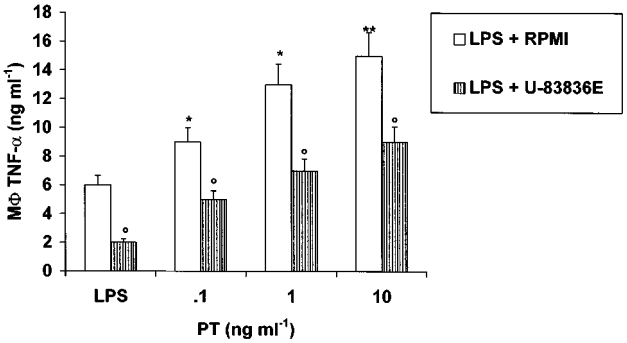

Figure 7 Effects of pertussis toxin pretreatment (PT; 0.1, 1 or 10 ng ml^{-1}) on LPS (50 μg ml^{-1} for 4 h) induced TNF-α release in peritoneal macrophages incubated together with RPMI (open bars) or U-83836E (shaded bars; 50 μM). Bar heights represent the mean ± s.e.mean of six experiments °$P<0.01$ vs RPMI, *$P<0.05$ vs LPS without PT, **$P<0.01$ vs LPS without PT.

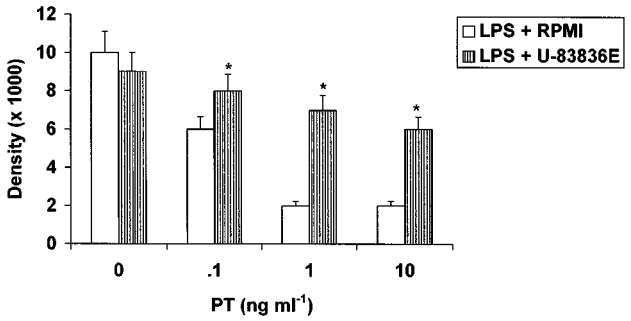

Figure 8 Effects of pertussis toxin (PT; 0.1, 1 or 10 ng ml^{-1}) on ADP-ribosylation (density) of a 41 kDa protein in peritoneal macrophages incubated together with RMPI (open bars) or U-83836E (shaded bars; 50 μM). Each value represents the mean± s.e.mean of six experiments. *$P<0.01$ vs RPMI.

ADP-ribosylation

Macrophages were preincubated for 4 h with the same doses of pertussis toxin as shown in Figure 8. Then the macrophage plasma membranes were purified and PT-mediated ADP-ribosylation was carried out in the presence of [^{32}P]NAD. ADP-ribosylation of a 41 kDa proteins(s) (as shown by the decrease in density) was reduced in a dose-dependent manner by pertussis toxin treatment (Figure 8), which correlates very well with the dose dependence of the PT effects on the LPS-induced macrophages responses (Figure 8). These results implicate a pertussis sensitive G-protein as potentially involved in modulation of LPS-induced TNF-α production. U-83836E markedly blunted the reduction in ADP-ribosilation induced by pertussis toxin (Figure 8).

Discussion

It has been suggested that endotoxin acts via endogenous mediators, mainly produced by mononuclear phagocytes (Nathan, 1987). Among these mediators, TNF-α plays a key role in endotoxin effects. In fact many of the pathophysiological sequelae of endotoxic shock may be reproduced in experimental animals by TNF-α administration.

Systemic administration of recombinant human TNF-α to experimental animals produces hypotension, alterations in glucose homeostasis, hemoconcentration, metabolic acidosis, intravascular thrombosis, intestinal hypoperfusion, generation of other vasoactive substances and death (Tracey *et al*., 1987). Moreover exogenous recombinant TNF-α, administrated systemically, produces a decrease in vascular responsiveness to contractile agents (Takahashi *et al*., 1992) that is abated by inhibition of nitric oxide (NO) production (Kilbourne *et al*., 1990). Furthermore it has been demonstrated that passive immunization against TNF-α has a strong protective effects in sepsis models in which lethal doses of endotoxin or living bacteria are administered intravenously (Jesmok *et al*., 1992; Emerson *et al*., 1992). In addition, several studies have reported a survival benefit in these models when anti-TNF treatment is given 30 min after endotoxin or bacterial challenge (Silva *et al*., 1990). However, other investigations have focused attention on the fact that neutralization of TNF-α may not be of benefit or even harmful in some, less acute, forms of sepsis. Anti-TNF-α is not effective in protecting rodents from lethal peritonitis induced by either cecal ligation and puncture or by intraperitoneal injection of living bacteria (Eskandari *et al*., 1992; Zanetti *et al*., 1992). Furthermore, it has been shown that there is no clear relationship between the levels of TNF-α produced by endotoxin and the endotoxin-induced mortality. However these findings do not negate the possibility that TNF-α has a role in endotoxin shock. Currently available data indicate that TNF-α plays a permissive role in inducing the release of other factors (i.e. interleukin 1 and 6, adhesion molecules) that are relevant to shock. Indeed TNF-α orchestrates inflammatory reactions early in the course of infection explaining why neutralization of TNF-α is not always of benefit.

The present data indicate that administration of the second generation lazaroid U-83836E, given curatively 5 min after endotoxin challenge, was able to dramatically improve survival rate, to restore mean arterial blood pressure and vascular responsiveness to phenylephrine. Since U-83836E reduced the serum levels of TNF-α, it can be speculated that the beneficial effects of the compound in this model of experimental shock may be due, at least in part to inhibition of this inflammatory cytokine. The beneficial effect of U-83836E in our model of shock, in terms of survival rate and reduction in cytokine levels, was greater than that of first generation marketed lazaroid tirilazad mesylate (Semrad *et al*., 1993). This finding suggests that U-83836E behaves as stronger inhibitor of TNF-α in comparison to tirilazad mesylate.

Indeed the lazaroid U-83836E restored the vascular failure and the hyporeactivity of the vasculature to vasoconstrictor agents, phenomena that represent an important aspect in the pathophysiology of circulatory shock. It has been proposed that the L-arginine/NO pathway plays an important role in the pathogenesis of vascular failure during circulatory shock. In fact the production of large amount of NO by the inducible isoform of nitric oxide synthase (iNOS) contributes to the vascular decompensation and to the hyporeactivity of the vasculature to vasoconstrictor agents observed in several experimental models of circulatory shock, including endotoxic shock (Szabo' & Thiemermann, 1994).

A number of proinflammtory cytokine and more specifically TNF-α induce the expression of iNOS in vasculative smooth muscle cells (Busse & Mulsch, 1990). Therefore agents able to inhibit the pleiotropic cytokine are expected to protect against the vascular failure that occurs during shock. In agreement with this hypothesis, U-83836E restored the vascular reactivity to phenylephrine, an effect that results from the inhibition of TNF-α induced induction of iNOS in the vasculature. Previous data have already indicated that lazaroids reduce the serum levels of TNF-α in different models of circulatory shock (Semrad *et al*., 1993; Squadrito *et al*., 1995). However the mechanism(s) underlying this inhibitory activity are not completely understood.

Previous work has indicated a major role for the NF-κB transcription factor in the induction of the TNF-α gene by endotoxin (Collart *et al*., 1990). Furthermore the generation of cytoplasmatic reactive oxygen intermediates (ROI) and particularly OH has been reported to be a crucial event in the activation of NF-κB (Schreck *et al*., 1992a, 1992b). Therefore it has been proposed that antioxidants, by quenching cytoplasmatic ROI, may in turn block NF-κB activation and finally inhibit TNF-α production. However this scheme cannot be easily applied to U-83836E, a second generation lazaroid that stabilizes cell membrane, inhibits lipid peroxidation but does not enter the cell (McCall *et al*., 1987). Furthermore in order to investigate whether lipid peroxidation might contribute to TNF-α production during endotoxic shock, we measured the time course of both serum TNF-α and plasma MAL following endotoxin challenge. If lipid

peroxidation had a role in cytokine production, the peak of plasma MAL would occur earlier than the peak of serum TNF-α. In contrast our results clearly indicate that the peak of both MAL and TNF-α occurred at 2 h hours after LPS administration, thus also ruling out the hypothesis that TNF-α reduction by U-83836E may be due to inhibition of lipid peroxidation. However in considering the relationship between cytokine reduction and lipid peroxidation, the presence of preformed TNF-α into the cell should be taken also into account. Several reports have suggested that G-proteins may be involved in some cellular responses of macrophages to LPS (Matsunaga *et al*., 1990). Pertussis toxin, which can ADP-ribosylate the α subunits of certain types of G-proteins (i.e. G_0 and G_i) (Stryer & Bourne, 1986) has been used as a specific inhibitor to uncouple the G protein-mediated signal transduction.

It has been suggested that pertussis toxin increased LPS induced TNF-α production by macrophages (Zhang & Morrison, 1993), thus suggesting that a pertussis toxin sensitive G-protein mediated signal transduction is involved in LPS dependent macrophage production of TNF-α. In agreement with these data, our results show that pertussis toxin significantly increased LPS-induced cytokine production in peritoneal macrophages harvested from normal rats. As previously indicated, pertussis toxin can irreversibly ADP-ribosylate G-protein α subunits. Therefore, pretreatment of the intact macrophages with pertussis toxin should result in reduction of available substrates for subsequent ADP-ribosylation by pertussis toxin with purified macrophage plasma membrane. If a pertussis toxin sensitive G-protein were to be involved in regulation of LPS-induced TNF-α production, it would be expected that the reduction of ADP-rybosylation substrates as a consequence of pertussis pretreatment should parallel the observed effects of pertussis toxin on the LPS-induced macrophage response. In agreement with this hypothesis we found a good relationship between ADP-ribosylation inhibition and the dose dependence of the pertussis toxin effects on the LPS-induced macrophage responses. Three kinds of pertussis toxin sensitive G_i proteins, G_{i1}, G_{i2}, and G_{i3} have been cloned (Linder & Gilman, 1991; Daniel-Issakani *et al*., 1989). However we do not know which kind of G_i is involved in our experimental conditions.

U-83836E reduced LPS-induced TNF-α production and inhibited the pertussis toxin effects on cytokine production and on ADP ribosylation of the protein, thus suggesting that the lazaroid may alter the functionality of this membrane associated G-protein. Thus U-83836E effect could be the consequence of its ability to interfere with membrane fluidity, as previously reported for this class of compounds. In conclusion our data suggest that the second generation lazaroid U-83836E may affect the early event associated with LPS mediated activation of G protein in LPS induced TNF-α production. These molecular events may explain, at least in part, the *in vivo* inhibition of cytokine production and reversal of endotoxic shock.

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