Thaliporphine selectively inhibits expression of the inducible, but not the constitutive, nitric oxide synthase

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Formation of nitrites/nitrates caused by lipopolysaccharide (LPS) in J774.2 macrophages was inhibited by thaliporphine, an aporphine derivative isolated from the plant *Neolitsea konishii* K. This inhibition of nitrite synthesis in LPS-stimulated macrophages by thaliporphine was similar to that by cycloheximide, N^G-methyl-L-arginine (MeArg) and dexamethasone. Thaliporphine, but not MeArg, inhibited expression of inducible NO synthase without directly affecting enzyme activity. However, thaliporphine did not inhibit nitrite production by NO synthase that had already been induced by prior exposure to LPS for which any possible further induction was inhibited by cycloheximide. In endothelial cells, nitrite formation induced by bradykinin (in the presence of 0.2 mM Ca^{2+}) was inhibited by MeArg. However, incubation of endothelial cells with dexamethasone, cycloheximide and thaliporphine did not affect this Ca^{2+}-dependent nitrite production. Thaliporphine (0.1–100 μM) dose-dependently inhibited nitrite accumulation in macrophages stimulated by interleukin-1β (IL-1β) whereas nitrite formation induced by tumour necrosis factor α was not inhibited. LPS-stimulated IL-1β synthesis in macrophages was significantly inhibited by thaliporphine, but thaliporphine had only minimal effect on LPS-stimulated IL-1β synthesis in endothelial cells. These results demonstrate that thaliporphine inhibits LPS induction of NO synthase expression, and that the mechanism of action of thaliporphine is via inhibition of LPS-stimulated IL-1β synthesis in macrophages. In anaesthetized rats subjected to LPS, pretreatment with thaliporphine partially restored the fall in mean arterial pressure and the vascular hyporeactivity to noradrenaline 3 h after LPS injection. In conclusion, thaliporphine selectively inhibited expression of inducible NO synthase, and may thus hold potential for the treatment of endotoxaemia.

INTRODUCTION

The demonstration of NO biosynthesis from the terminal guanidino nitrogen atom of L-arginine by vascular endothelial cells [1,2] has been followed by the finding of NO synthesis in macrophages, hepatocytes, neurons, neutrophils, vascular smooth-muscle cells and cardiac myocytes [3,4]. At least three isotypes of NO synthase have been cloned and they differ significantly in their sequences [5]. One is constitutive and is present in endothelial cells, requires Ca^{2+}, calmodulin, NADPH and tetrahydrobiopterin [5,6] and synthesizes NO as a transduction mechanism to regulate the activity of soluble guanylate cyclase. Another one is inducible and is expressed in response to endotoxin or cytokines in macrophages [7]. Furthermore, this inducible NO synthase does not require Ca^{2+} for activity but requires NADPH and tetrahydrobiopterin [8]. The NO synthesized by this enzyme in macrophages contributes to their cytotoxic activity against bacteria [9].

Endotoxin can initiate the sepsis syndrome [10] which is characterized by systemic vasodilatation. Bacterial lipopolysaccharide (LPS) is an endotoxin that releases cytokines, such as interleukin 1 (IL-1) and tumour necrosis factor (TNF); these contribute to the hypotension and lethality associated with endotoxaemia [11]. Interestingly, combinations of these cytokines induce an NO synthase in macrophages [12]. Induction of NO synthase, leading to excessive production of NO, has also been implicated in the endothelial cell damage *in vitro* brought about by prolonged incubation with endotoxin and cytokines [13]. Experimental and clinical studies suggest that excessive NO production has an important pathological role in the hypotension, hyporesponsiveness to vasoconstrictors and cardiovascular collapse associated with septic shock [14].

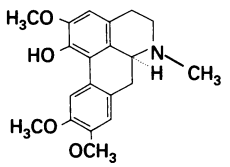

Figure 1 Chemical structure of thaliporphine

Medicinal plants have been used as traditional remedies in oriental countries for hundreds of years. In a large-scale screening test, we found many aporphine compounds that had effects on the cardiovascular system [15,16]. Recently, we found that thaliporphine, an aporphine derivative (Figure 1) isolated from the plant *Neolitsea konishii* K, exhibits vasoconstricting activity in rat aorta [17] and protects against cardiovascular failure, prolonging survival time in a model of endotoxin shock. In this present paper, we investigate the effects of thaliporphine on the expression of inducible NO synthase by LPS in cultured macrophages and on constitutive NO synthase in cultured endothelial cells.

MATERIALS AND METHODS

Materials

Thaliporphine (Figure 1) was isolated from the plant *Neolitsea konishii* K, and its purity (> 99%) was confirmed by n.m.r., mass, i.r. and proton spectrophotometry [18]. LPS

Abbreviations used: LPS, lipopolysaccharide; IL-1β, interleukin 1β; TNFα, tumour necrosis factor α; MeArg, N^G-methyl-L-arginine.

(*Escherichia coli*; serotype no. 0127:B8), calmodulin, cycloheximide, N^G-methyl-L-arginine (MeArg), NADPH, dexamethasone, sulphanilamide, naphthylethylenediamine, sodium nitrite, dithiothreitol, FAD, FMN, diltiazem, verapamil, Bay K 8644 [methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate], noradrenaline, AG 50W-X8 cation-exchange resin, tetrahydrobiopterin and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[2,3,4,5-^{3}H]arginine hydrochloride was from Amersham International (Amersham, Bucks., U.K.).

Cell cultures

The murine macrophage cell line (J774.2) and bovine pulmonary artery endothelial cells were obtained from the American Type Culture Collection. Cells were maintained in continuous culture in T75 tissue culture flasks in Dulbecco's modified Eagle's medium containing 0.4 mM L-arginine and supplemented with 4 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% fetal calf serum. Cells were harvested by using 0.25% trypsin/4 mM EDTA and passaged every 3–6 days by dilution of the cell suspension 1:3 in fresh medium.

Measurement of nitrite production as an assay of NO synthesis

Nitrite production was measured in the supernatant of endothelial cells or J774.2 macrophages as described previously [19]. Briefly, the cells were seeded into 96-well plates (10^5 cells/well) with 200 μl of culture medium until cells reached confluence. The effects of thaliporphine on the constitutive or inducible NO synthase were investigated in endothelial cells and J774.2 macrophages respectively. Ca^{2+}-dependent release of nitrite was determined in endothelial cells incubated with bradykinin (1–100 nM) for 30 s at 37 °C in culture medium containing indomethacin (10 μM) and 0.2 mM Ca^{2+}. To assess the effects of thaliporphine, dexamethasone, cycloheximide or MeArg on Ca^{2+}-dependent nitrite production, these agents were added simultaneously with bradykinin to the 0.2 mM Ca^{2+}-containing medium. To induce Ca^{2+}-independent NO synthase in J774.2 macrophages, *E. coli* LPS (1 μg/ml) was added for 24 h to Ca^{2+}-free culture medium containing 1 mM EGTA. To assess the effects of thaliporphine (0.1–100 μM), cycloheximide (10 μM), MeArg (10 μM) or dexamethasone (10 μM) on nitrite production, these agents were added simultaneously with LPS to the Ca^{2+}-free culture medium for 24 h. To determine whether thaliporphine affects the activity of induced NO synthase, thaliporphine and cycloheximide (10 μM) were added to J774.2 macrophages that had been treated with LPS (1 μg/ml for 24 h), and nitrite production was measured after a further 24 h. After a specified incubation time, a portion (100 μl) of the medium was removed, mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) and then incubated at room temperature for 10 min. A_{550} was read using a Multiskan II plate reader (Titertek). Nitrite concentration in the medium was determined with $NaNO_2$ as standard. To determine the effect of thaliporphine on nitrate synthesis, in analysis of macrophages that contain both nitrate- and nitrite-producing species, one portion was passed through a cadmium column [consisting of high-pressure Teflon tubing, about 25 cm in length (3.2 mm outer diameter, 2.7 mm inner diameter) packed with copper-plated cadmium filings] yielding a composite response for nitrates and nitrites, and another portion bypassed the cadmium column, yielding a response for nitrite alone; the nitrate content of the sample was calculated by difference.

Purification of macrophage NO synthase by affinity chromatography

J774.2 macrophages were sonicated with a Soniprep (6 × 5 s; 100 W) (MSE Scientific Instruments, Crawley, Sussex, U.K.) in ice-cold sucrose-free Hepes buffer containing proteinase inhibitors (antipain, leupeptin, pepstatin, chymostatin; 10 μg/ml each). Cytosol obtained by centrifugation (1 h; 100000 *g*) was stored at −70 °C. Protein was determined by using the Bradford reagent (Bio-Rad) [20] with BSA as standard. Cytosolic fractions (5 mg of protein) were loaded on an ADP–Sepharose column (5 mm × 100 mm), which was subsequently washed with low-, high (0.6 M NaCl included)- and low-ionic-strength buffer [40 mM Tris/HCl (pH 7.4)/5 mM L-arginine/3 mM dithiothreitol/2 μM FAD/2 μM FMN/2 μM tetrahydrobiopterin/10% (v/v) glycerol]. NO synthase was then eluted with 5 ml of 8 mM NADPH. This procedure yielded a 20-fold-purified enzyme (determined by the increase in specific enzyme activity) with 20% recovery.

Measurement of NO synthase activity by determining L-[^{3}H]citrulline formation

NO synthase activity was measured as the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline. NO synthase preparations were incubated with L-[^{3}H]arginine (0.05 μCi), NADPH (1 mM), L-citrulline (0.1 mM) and tetrahydrobiopterin (5 μM) in the presence or absence of thaliporphine or MeArg for 20 min at 37 °C in Hepes buffer (pH 7.5). Samples (2 ml) were applied to columns (1 cm diameter) containing 1 ml of Dowex-AG 50W-X8, Na^+ form (prepared from the H^+ form), that had been pre-equilibrated with 20 mM sodium acetate, pH 5.5, containing 1 mM L-citrulline, 2 mM EDTA and 0.2 mM EGTA (stop buffer). The eluate (2 ml) was collected into a liquid-scintillation vial. Columns were eluted with 2 ml of water and the eluates collected in another vial. Aquasol-2 (5 ml) was added to each vial and radioactivity was counted in a Beckman LS 5000. The reaction was stopped by dilution with 1 ml of ice-cold Hepes buffer (pH 5.5) containing EGTA (2 mM). Reaction mixtures were applied to AG 50W-X8 (100–200 mesh) columns and eluted L-[^{3}H]citrulline activity was measured in a liquid-scintillation counter (Beckman LS 5000). NO synthase activity was further characterized by incubation with EGTA (2 mM) to determine the dependence of the enzyme activity on Ca^{2+}. Ca^{2+}-dependent activity under control conditions was taken as constitutive NO synthase, and that not inhibited by EGTA was assumed to be a Ca^{2+}-independent inducible isoform of NO synthase [21,22].

Assay of IL-1β

To assess the effect of thaliporphine on the synthesis of IL-1β, thaliporphine (100 μM) and LPS were simultaneously added to macrophages or endothelial cells for 24 h. IL-1β was assayed in supernatants from macrophages or endothelial cell cultures by using the IL-1β e.l.i.s.a. kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.). This assay was linear for IL-1β concentrations of 200–2000 pg/ml.

Haemodynamic measurements

Male Wistar rats (300–350 g; Biological Research Laboratories, Fullinsdorf, Switzerland) were anaesthetized with sodium pento-

barbitone (40 mg/kg; intraperitoneally). The left carotid artery and the femoral vein were cannulated for blood-pressure measurement and intravenous injection respectively. The arterial cannula was connected via a Statham pressure transducer to a Grass model 7 polygraph (Grass Instruments Co., Quincy, MA, U.S.A.) for arterial-pressure measurement. Heart rate was monitored on a tachograph (Grass Instruments Co.) triggered by a standard Lead II electrocardiograph. After a 30 min stabilization period, the basal values of blood pressure and heart rate were recorded. To assess the effects of thaliporphine on LPS-induced hypotensive shock, after the end of the stabilization period, rats were treated with vehicle or thaliporphine (20 μg/kg; intravenously) for 20 min and then with LPS (10 mg/kg; intravenously) for 3 h. To assess the effects of thaliporphine on LPS-induced hyporesponsiveness to vasoconstrictor, rats were treated with vehicle or thaliporphine for 20 min and then LPS (10 mg/kg; intravenously) as a slow injection over 2 min, and pressor responses to noradrenaline (1 μg/kg; intravenously) were reassessed before and at 1, 2 and 3 h after injection of LPS.

Data analysis

The results are expressed as means ± S.E.M. A one-way analysis of variance was performed for multiple comparison, and if there was significant variation between treatment groups, then the mean values for an antagonist were compared with those for the control by using Student's t test; P values of less than 0.05 were considered to be statistically significant.

RESULTS

Effects of thaliporphine on Ca^{2+}-independent nitrite production in macrophages

In J774.2 macrophages activated with LPS (1 μg/ml) for up to 2 h, there was no detectable nitrite formation in the absence of Ca^{2+}, but significant Ca^{2+}-independent formation of nitrite occurred in macrophages activated with LPS for 24 h (540 ± 40 nmol/24 h per 10^6 cells) (Figure 2a). Thaliporphine (0.1–100 μM) dose-dependently inhibited nitrite accumulation in LPS-stimulated macrophages (Figure 2b). The IC_{50} value for thaliporphine inhibition of nitrite production in LPS-stimulated macrophages was 1.0 ± 0.1 μM. In macrophages activated with LPS for 24 h, there was significant formation of nitrite and nitrate (540 ± 40 and 1242 ± 79 nmol/24 h per 10^6 cells respectively). The ratio nitrite/nitrate was 1:2.3. Thaliporphine (30 μM) completely inhibited nitrate accumulation in LPS-stimulated macrophages. This result demonstrates that the effect of thaliporphine was not diversion of nitrite to nitrate.

In macrophages in which NO synthase had already been induced by prior exposure to LPS and any possible further induction was blocked by cycloheximide (10 μM), thaliporphine (0.1–100 μM) did not inhibit nitrite production (Figure 2c).

At 10 μM, dexamethasone (an NO synthase induction inhibitor), cycloheximide (a protein synthesis inhibitor) and MeArg (an L-arginine analogue, NO-formation inhibitor) all significantly inhibited Ca^{2+}-independent production of nitrite in LPS-stimulated macrophages (results not shown).

Effects of thaliporphine on Ca^{2+}-dependent nitrite production in endothelial cells

In the presence of 0.2 mM Ca^{2+}, unstimulated endothelial cells produce a small, but significant, amount of nitrite (3 ± 1 nmol of nitrite/10^6 cells in Ca^{2+}-free conditions and 15 ± 4 nmol/10^6 cells in the presence of 0.2 mM Ca^{2+}). Nitrite production was significantly increased to 80 ± 4 nmol/10^6 cells when endothelial cells were stimulated with 100 nM bradykinin for 30 s (Figure 3). After a further 48 h of culture, basal and bradykinin-stimulated nitrite formation were not significantly different from these values (n = 4). In the absence of Ca^{2+}, no basal or bradykinin-stimulated nitrite formation was detected either initially or after a further 48 h of culture in medium alone. Thaliporphine (100 μM) did not inhibit nitrite production of basal and bradykinin-stimulated endothelial cells (Figure 3). Again, incubation of endothelial cells with dexamethasone and cycloheximide (10 μM) did not affect bradykinin-stimulated Ca^{2+}-dependent nitrite production (results not shown). However, both Ca^{2+}-independent and Ca^{2+}-dependent nitrite production were inhibited by MeArg (10 μM).

To investigate the role of the Ca^{2+} channel and the specificity of thaliporphine in NO synthase induction, we looked at the

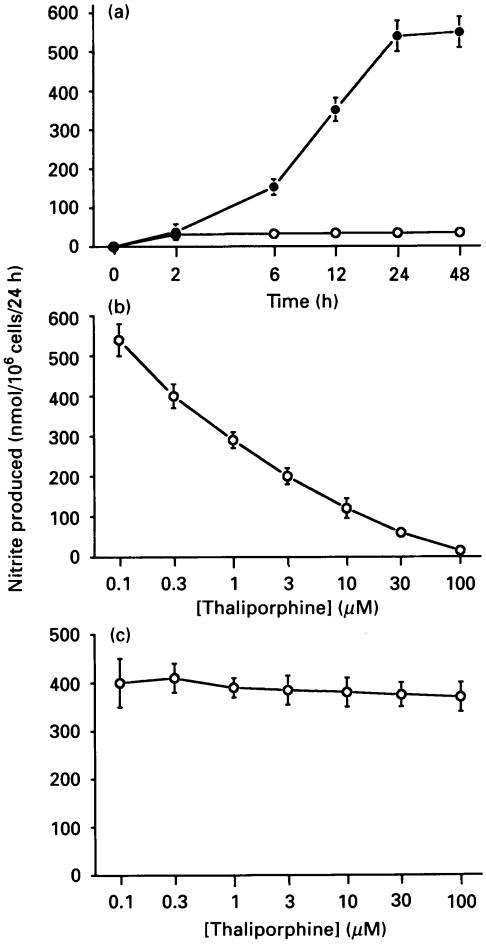

Figure 2 Inhibition of Ca^{2+}-independent nitrite production by thaliporphine

(**a**) Time-course of Ca^{2+}-independent LPS-stimulated (1 μg/ml; ●) and non-stimulated (○) nitrite production in macrophages in Ca^{2+}-free medium. (**b**) Dose-dependent inhibition by thaliporphine of nitrite accumulation in Ca^{2+}-free culture medium over 24 h in J774.2 macrophages stimulated with LPS (1 μg/ml). (**c**) J774.2 macrophages that had been preinduced by LPS (1 μg/ml) to synthesize NO and in which further NO synthase induction was blocked by cycloheximide (10 μM). Thaliporphine (0.1–100 μM) was added and nitrite production was measured after a further 24 h. Nitrite accumulation in the medium was determined using the Griess reaction. Data are expressed as means ± S.E.M. (n = 6).

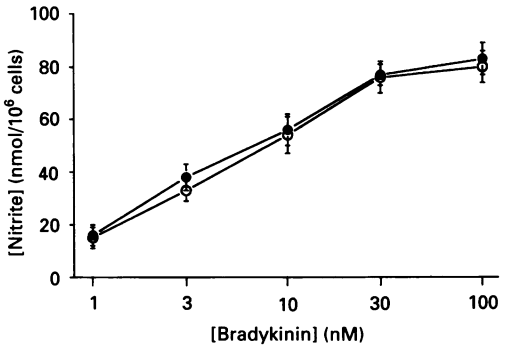

Figure 3 Effects of thaliporphine on Ca^{2+}-dependent nitrite production

To induce Ca^{2+}-dependent nitrite production in endothelial cells, bradykinin (1–100 nM) was added for 30 s in 0.2 mM Ca^{2+}-containing medium (○). Thaliporphine (100 μM) was added simultaneously with bradykinin (1–100 nM) plus 0.2 mM Ca^{2+} (●) for 30 s in endothelial cells. Nitrite accumulation in the medium was determined using the Griess reaction. Data are expressed as means ± S.E.M. ($n = 3$).

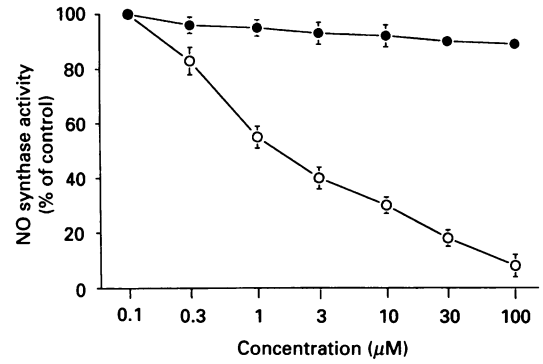

Figure 4 Effects of thaliporphine on NO synthase activity

ADP–Sepharose-purified cytosolic macrophage NO synthase was preincubated with thaliporphine (●) or MeArg (○) for 20 min at 37 °C in Hepes buffer. NO synthase activity was measured by following L-[^{3}H]citrulline formation from L-[^{3}H]arginine. Data are expressed as means ± S.E.M. ($n = 4$).

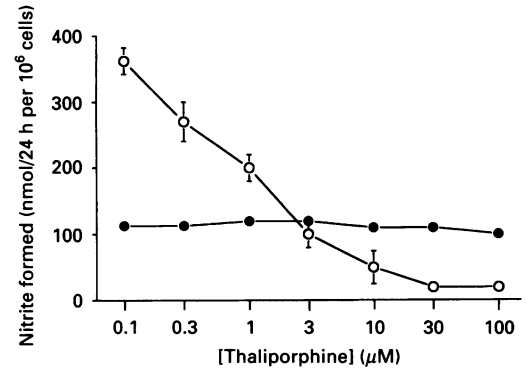

Figure 5 Effects of thaliporphine on IL-1β- and TNFα-induced nitrite production

Macrophages were incubated with IL-1β (30 units/ml; ○) or TNFα (0.1 μg/ml; ●) for 24 h. Thaliporphine (0.1–100 μM) was added simultaneously with IL-1β or TNFα. Nitrite release from unstimulated cells was 14.2 ± 9.0 nmol/24 h per 10^6 cells and that from cells treated with IL-1β or TNFα was 362.5 ± 19.8 and 113.3 ± 12.3 nmol/24 h per 10^6 cells, respectively. Data are expressed as means ± S.E.M. ($n = 4$).

Table 1 Effects of thaliporphine on LPS-induced IL-1β synthesis

J774.2 macrophages or bovine endothelial cells were incubated in the absence (control) or presence of 1 μg/ml LPS for 24 h. In some experiments, thaliporphine (100 μM) was added simultaneously with LPS for 24 h. IL-1β was quantified by using an IL-1β e.l.i.s.a. kit. Values are expressed as means ± S.E.M. ($n = 5$). *$P < 0.001$ compared with the respective LPS-treated level.

Cell types	Treatment	[IL-1β] (pg/ml)
Macrophage	Control	400 ± 33
	LPS	18306 ± 186
	Thaliporphine + LPS	1809 ± 110*
Endothelial cell	Control	448 ± 15
	LPS	3007 ± 57
	Thaliporphine + LPS	2840 ± 57

effects of Bay K 8644 (a Ca^{2+}-channel activator), A23187 (a Ca^{2+} ionophore), verapamil and diltiazem (both Ca^{2+}-channel antagonists) and LPS-stimulated nitrite production. None of these compounds altered LPS-stimulated Ca^{2+}-independent nitrite production.

Effects of thaliporphine on NO synthase activity

To elucidate whether thaliporphine directly inhibits NO synthase activity in macrophages, we assessed this activity by following conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline. Thaliporphine did not affect NO synthase activity except at the highest concentration tested (100 μM) which caused a 16 ± 3% inhibition. In contrast, MeArg (0.1–100 μM) elicited a dose-dependent inhibition of NO synthase activity (Figure 4).

Effects of thaliporphine on IL-1β- and TNFα-induced nitrite production in macrophages

Nitrite release from unstimulated cells was 14.2 ± 9.0 nmol/24 h per 10^6 cells and that from cells treated with IL-1β (30 units/ml) and TNFα (0.1 μg/ml) for 24 h was 362.5 ± 19.8 and 113.3 ± 12.3 nmol/24 h per 10^6 cells respectively. Thaliporphine dose-dependently inhibited nitrite accumulation in IL-1β-stimulated macrophages but not in TNFα-stimulated cells (Figure 5).

Effects of thaliporphine on IL-1β synthesis

In macrophages, stimulation with LPS (1 μg/ml) for 24 h produced an approx. 45-fold increase in IL-1β synthesis, which was significantly inhibited by pretreatment with thaliporphine (100 μM) (Table 1). In contrast, although less IL-1β was synthesized in LPS-stimulated endothelial cells, thaliporphine had only a minimal effect on IL-1β synthesis. Thaliporphine (100 μM) also did not significantly affect the TNFα synthesis in LPS-treated macrophages (results not shown).

Thaliporphine prevents the delayed hypotension and vascular hyporeactivity to noradrenaline in endotoxic shock

At the end of the 30 min stabilization period, mean values for mean arterial pressure ranged from 120 ± 8 to 126 ± 6 mmHg and for heart rate from 389 ± 25 to 402 ± 7 beats per min. Administration of LPS (10 mg/kg; intravenously) induced a fall in mean arterial pressure from 126 ± 4 to 76 ± 5 mmHg ($P < 0.001$; $n = 8$) within 5 min. At 2 h after LPS injection, mean arterial pressure

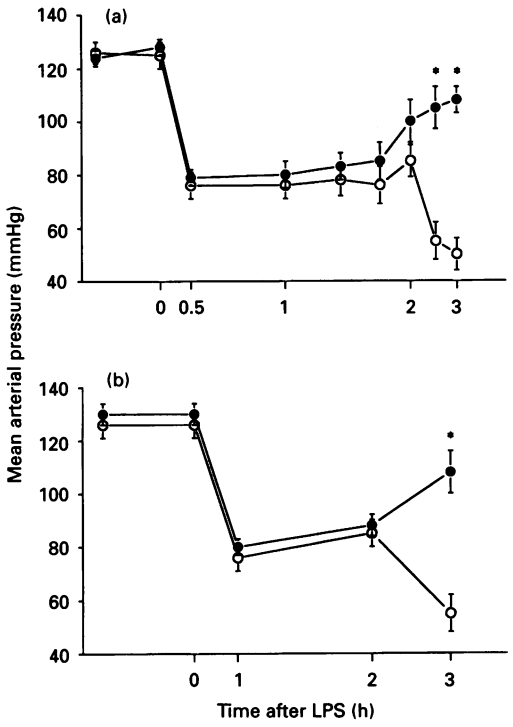

Figure 6 Thaliporphine prevents the delayed hypotension and vascular hyporeactivity to noradrenaline in endotoxic shock

Depicted are the changes in mean arterial pressure (**a**) and pressor responses to noradrenaline (1 μg/kg; intravenously) (**b**) in rats treated with LPS (10 mg/kg; intravenously). Groups of rats were pretreated with either vehicle (○; $n = 8$) or thaliporphine (20 μg/kg; intravenously) (●; $n = 8$). LPS was administered at time 0. Data are expressed as means ± S.E.M. *$P < 0.001$ represent significant differences compared with control at the same time point.

had partially recovered to 85 ± 7 mmHg, but thereafter, there was a significant further fall to 55 ± 7 mmHg at 3 h (Figure 6a). LPS treatment significantly attenuated the pressor response to noradrenaline (1 μg/kg) at 1, 2 and 3 h after LPS administration (Figure 6b). Rats pretreated with thaliporphine (20 μg/kg; intravenously) and subjected to LPS maintained significantly higher mean arterial pressure at 3 h compared with LPS-treated controls (105 ± 8 mmHg compared with 55 ± 7 mmHg; $P < 0.001$) (Figure 6a). In thaliporphine-pretreated rats, the pressor responses to noradrenaline 3 h after LPS treatment returned to 108 ± 5 mmHg, whereas there was a significant fall in mean arterial pressure to 58 ± 6 mmHg in LPS-treated control rats (Figure 6b).

DISCUSSION

The present results demonstrate that thaliporphine selectively inhibits expression of inducible NO synthase by LPS in J774.2 macrophages.

Activation of macrophages with LPS induced, after a 2 h lag period, Ca^{2+}-independent production of NO, measured as nitrite. MeArg (an L-arginine analogue) completely inhibited nitrite accumulation in response to LPS, demonstrating that this nitrite originates from the L-arginine–NO pathway [23]. The enhanced NO formation in response to LPS is due to induction of NO synthase, as dexamethasone, an inhibitor of NO synthase induction [24], and cycloheximide, a protein synthesis inhibitor, both inhibited nitrite formation in macrophages. The expression, but not the activity, of the inducible enzyme was abolished by cycloheximide, showing that this enzyme is synthesized *de novo* after activation with LPS. Thaliporphine selectively inhibited Ca^{2+}-independent nitrite production in macrophages stimulated with LPS in a dose-dependent manner. It (30 μM) also completely inhibited nitrate accumulation in LPS-stimulated macrophages. This result demonstrates that the effect of thaliporphine was not to divert nitrite production to nitrate production. The lack of effect of thaliporphine on nitrite production in macrophages that had been preinduced to synthesize NO and in which further protein synthesis was inhibited by cycloheximide suggests that thaliporphine inhibits NO synthase induction, but not NO synthase activity. As with the J774.2 cells, the lack of direct effect of thaliporphine on NO synthase activity demonstrates that thaliporphine does not inhibit the activity of the enzyme, but attenuates its expression.

Vascular endothelial cells in culture release NO. This release, which can be enhanced by stimulation with bradykinin, is Ca^{2+}-dependent and inhibited by MeArg. These data, together with the finding of Ca^{2+}-dependent NO synthesis by the cytosol of these cells, confirm the existence of a constitutive NO synthase in the vascular endothelium [25,26]. However, thaliporphine (100 μM) did not inhibit nitrite production in basal and bradykinin-stimulated endothelial cells. These observations suggest significant selectivity, with thaliporphine inhibiting the inducible rather than the constitutive form of NO synthase.

Cytokines such as IL-1β and TNFα are secreted by macrophages in response to endotoxin and may be responsible for the severe hypotension and circulatory failure observed in septic shock [27,28]. IL-1β and TNFα are known to induce NO synthase in macrophages [11] and vascular smooth muscle [29]. Induction of NO synthase has an important role in endotoxic shock [30,31]. Thaliporphine dose-dependently inhibited nitrite accumulation in IL-1β-stimulated cells, but not in TNFα-activated cells. Moreover, pretreatment with thaliporphine significantly inhibited IL-1β synthesis in LPS-treated macrophages. These results demonstrate that thaliporphine inhibits LPS induction of NO synthase expression through inhibition of LPS-stimulated IL-1β synthesis (or release) by macrophages. It exerts its vasoactive effects in rat aorta mainly by promoting Ca^{2+} entry through voltage-dependent Ca^{2+} channels. However, it is unlikely that the inhibitory effect of thaliporphine on NO synthase induction is strictly dependent on Ca^{2+}-channel activation. Bay K 8644 (known to open Ca^{2+} channels) [32,33] and two Ca^{2+}-channel antagonists (verapamil and diltiazem) had less pronounced inhibitory effects.

The immediate and delayed hypotension and vascular hyporeactivity to vasoconstrictor agents observed in response to LPS in the model of endotoxaemia used in the present study are the result of enhanced NO formation which is triggered by two distinct mechanisms: activation of the constitutive NO synthase and induction of NO synthase [34]. The finding that thaliporphine had no effect on the immediate hypotension and hyporeactivity to noradrenaline in response to LPS (within 1 h), but significantly protected against the delayed secondary decrease in mean arterial pressure (3 h after LPS treatment) supports the hypothesis that the beneficial haemodynamic effects of thaliporphine demonstrated in this study are due to prevention of NO synthase induction. The prevention of NO synthase induction caused by dexamethasone [35,36] was similarly associated with a reduced fall in mean arterial pressure and restoration of the vascular hyporeactivity to noradrenaline 3 h after LPS injection [31].

In conclusion, thaliporphine selectively inhibits the expression of inducible NO synthase and may therefore have potential for

the treatment of endotoxaemia caused by inhibition of NO synthase expression.

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