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Andrographolide suppresses the expression of inducible nitric oxide synthase in macrophage and restores the vasoconstriction in rat aorta treated with lipopolysaccharide

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1 We investigated whether andrographolide, a diterpenoid lactone found at *Andrographis paniculata*, influences the induction of the inducible nitric oxide synthase (iNOS) in RAW264.7 cells activated by bacterial endotoxin (LPS), as well as in the rats with endotoxic shock and in aortic rings treated with LPS.

2 Incubation of RAW264.7 cells with andrographolide (1 to 50 μM) inhibited the LPS (1 μg ml^{-1})-induced nitrite accumulation in concentration- and time-dependent manners. Maximum inhibition was observed when andrographolide was added together with LPS and decreased progressively as the interval between andrographolide and LPS was increased to 20 h.

3 Western blot analysis demonstrated that iNOS expression was markedly attenuated in the presence of andrographolide for 6–24 h, suggesting that andrographolide inhibited iNOS protein induction.

4 Thoracic aorta incubation with LPS (300 ng ml^{-1}) for 5 h *in vitro* exhibited a significant decrease in the maximal contractile response to phenylephrine (10^{-9}–10^{-5} M). Andrographolide (30 μM) restored the contractile response to control level.

5 In anaesthetized rats, LPS (10 mg kg^{-1}, i.v.) caused a fall in mean arterial blood pressure (MAP) from 116±4 to 77±5 mmHg. The pressor effect of phenylephrine (10 μg ml^{-1}, i.v.) was also significantly reduced at 30, 60, 120 and 180 min after LPS injection. In contrast, animals pretreated with andrographolide (1 mg kg^{-1}, i.v., 20 min prior to LPS) maintained a significantly higher MAP when compared to LPS-rats given with vehicle. Administration of andrographolide 60 min after LPS caused a increase in MAP and significantly reversed the reduction of the pressor response to phenylephrine.

6 Our results indicated that andrographolide inhibits nitrite synthesis by suppressing expression of iNOS protein *in vitro*. And, this inhibition of iNOS synthesis may contribute to the beneficial haemodynamic effects of andrographolide in endotoxic shock.

Keywords: Andrographolide; nitric oxide; inducible nitric oxide synthase; lipopolysaccharide; vascular hyporeactivity; endotoxic shock

Introduction

Immunological stimuli including cytokines and endotoxin cause the expression on an inducible isoform of nitric oxide synthase (iNOS) which, once expressed, produces large amounts of nitric oxide (NO) (Kilbourn & Griffith, 1992). There is now good evidence that circulatory shock of various etiologies is associated with delayed induction of iNOS activity (e.g., macrophages, vascular smooth muscle, hepatocytes, and cardiac myocytes) (Wu & Thiemermann, 1996). In 1990, several groups independently discovered that an enhanced formation of endogenous NO contributes to hypotension and vascular hyporesponsiveness to vasoconstrictor agents in various animal models of septic shock (Julou-Schaeffer *et al*., 1990; Rees *et al*., 1990; Kilbourn *et al*., 1990; Thiemermann & Vane, 1990). In addition to shock, an enhanced formation of NO following the induction of iNOS also occurs in local (e.g., polyarthritis, osteoarthritis) or systemic inflammatory disorders, diabetes, arteriosclerosis, and other diseases (Stefanovic-Racic *et al*., 1993; Connor *et al*., 1995; Wu & Thiemermann, 1996). Thus, it has been argued that inhibition of NO formation may have therapeutic benefit in patients with septic shock or inflammatory diseases.

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One potential source for novel iNOS inhibitors is the diverse area of natural products. *Andrographis paniculata*, which has been utilized in traditional Chinese medicine for the treatment of bacteria infection and inflammatory diseases (e.g., rheumatoid arthritis). Recently, under large scale screening experiments, we found that andrographolide (3-[2-[Decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naph-thalenyl] ethylidene] dihydro-4-hydroxy-2(3H)-furanone) (Figure 1), a bicyclic diterpenoid lactone found in leaves of *Andrographis paniculata* (Lu *et al*., 1981), displays NO synthesis inhibitory effect. Here, we investigate the mechanism of action of andrographolide on the induction of iNOS caused by LPS in cultured macrophages, and examined its effect on LPS-induced vascular hypocontractility and on the haemodynamic parameters in endotoxaemic rats.

Methods

Cell culture

The murine macrophage cell line RAW264.7 (American Type Culture Collection ATCC, TIB 71, Rockville, MD U.S.A.) was cultured in DMEM containing 10% heat-inactivated fetal calf

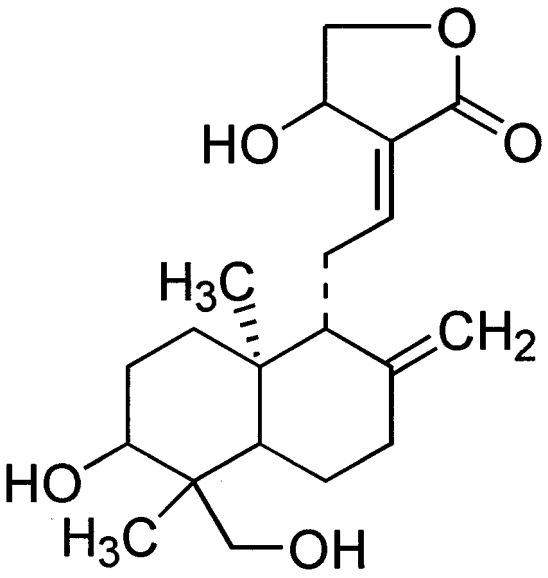

Figure 1 Structural formula of andrographolide.

serum, 100 U/ml penicillin and 100 μg ml^{-1} streptomycin as previously described (Chiou *et al*., 1997). Cells were grown at 37°C and with 5% CO_2 in fully humidified air and used for experiments between passage 5 and 20.

Nitrite measurement

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of RAW264.7 macrophages as described previously (Chiou *et al*., 1997). Briefly, the cells were cultured in 96-well plates with 200 μl of culture medium until cells reached confluence (approximately 200,000 cells per well). In order to induce iNOS, freash culture medium containing LPS (1 μg ml^{-1}) was added. Nitrite accumulation in the medium was measured at 24 h after the application of LPS. To assay drug's effect on nitrite production, andrographolide (final concentration, 1 to 50 μM) was added together with LPS. Viability was assessed by the MTT assay. In order to elucidate whether the inhibition of nitric formation by andrographolide in RAW 264.7 macrophages activated with LPS is due to inhibition of iNOS induction or inhibition of iNOS activity, separate experiments were performed in which andrographolide (10 μM) was given either together with LPS or at 2, 4, 6, 8, 16 or 20 h after LPS.

Nitrite was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μl samples of medium. The optical density at 550 nm (OD550) was measured with a microplate reader. Nitrite concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrite prepared in culture medium.

Cell respiration

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Mosmann, 1983). Cells in 96-well plates were incubated (37°C) with MTT (5 mg ml^{-1} for 4 h). Culture medium was removed by aspiration and cells were solubilized in acid-SDS (100 μl) (Chiou *et al*., 1997). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{570} against OD_{630}.

Immunoblotting

Confluent monolayers of RAW264.7 cells in T25 culture flasks (10×10^{-6}) were incubated for 24 h with either fresh DMEM or DMEM containing LPS (1 μg ml^{-1}) alone or in combination with andrographolide (10 μM). Incubations were terminated by rapid aspiration of the cell supernatant followed by washing with ice-cold phosphate buffer saline (PBS) (Sigma Chemical Company, U.S.A.). Cells were lysed in PBS containing 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and 0.1% leupeptin. Lysates (40 μg protein per lane) were separated by SDS-PAGE on 7.5% polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked at 4°C for overnight in 100 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween-20, pH7.4 (STT) containing 5% milk. Membranes were subsequently probed with mouse monoclonal anti-iNOS antibody (1 : 2500 dilution in STT, Transduction Laboratories) for 2 h at room temperature. Blots were washed with STT (2×15 min) and incubated with horseradish peroxidase-conjugated rabbit anti-mouse lgG (1 : 5000, Amersham) for 2 h at room temperature. Following further washing in STT (3×15 min), immunoreactive bands were visualized using ECL detection system (Amersham).

Preparation of rat aortic rings

Thoracic aortae were obtained from male Sprague-Dawley rats (250–350 g). The vessels were cleared of adhering periadventitial fat and the thoracic aortae were cut into rings of 3–4 mm length. The rings were mounted in 5 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO2) Krebs' solution (pH7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Isometric force was measured with Grass FT03 type transducer (Grass Instruments, Quincy, MA, U.S.A.) and recorded on a Gould model RS3400 polygraph recorder. A tension of 2 g was applied and the rings were equilibrated for 60 min. The endothelium was removed by gently rubbing the intimal surface and was considered denuded when 3 μM of acetylcholine failed to relax the phenylephrine (0.1 μM-precontracted rings. Before each experiment, each vessel was contracted with a standard dose of KCl (60 mM) to provide a reference contraction and, therefore, to control for variability on contractile responsiveness between vessels. All results were therefore subsequently expressed as a percentage of the KCl-induced contraction.

Effect of andrographolide on endotoxin-induced vascular hypocontractility

Four aortic rings from each animals were suspended in the organ baths (37°C) and incubated for 5 h after initial determination of vascular responsiveness to KCl (60 mM). Two of the rings were incubated with LPS (300 ng ml^{-1}) in Krebs solution, and two other rings were incubated in Krebs solution alone. During the last 30 min of incubation, andrographolide (10 or 30 μM) was added to two of the baths, one containing LPS and one without. At the end of the 5-h incubation period, a cumulative concentration-response curve to phenylephrine (1 nM to 10 μM) was obtained for each of the rings.

Measurement of haemodynamic changes

Male Sprague-Dawly rats (250–350 g) were anaesthetized with sodium pentobarbital (40 mg kg^{-1}, i.p.). The trachea was cannulated and connected to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was cannulated to a pressure transducer (P23XL, Viggo-Spectramed, Statham, U.S.A.) for the measurement of mean arterial blood pressure (MAP) which were displayed on a GOULD model RS3400 polygraph recorder. The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 30 min. After recording baseline haemodynamic parameters, animals were treated with vehicle (10% dimethylsulphoxide) or andrographolide (1 mg kg^{-1}, i.v.), and 10 min later the pressor response to phenylephrine (PE 10 μg kg^{-1}, i.v.) was recorded. At 20 min after injection of PE, animals received *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) as a slow injection over 10 min and pressor responses to PE were reassessed at 30, 60, 120, and 180 min after LPS injection. Additionally, the hypotensive effects elicited by LPS was also monitered for 180 min in the presence of vehicle or andrographolide, respectively. In a separate set of experiments, at 60 min after the injection of LPS, vehicle or andrographolide was administered to the rat as a single bolus injection and the haemodynamic parameters were monitored for another 120 min.

Materials

Bacterial lipopolysaccharide (*E. coli* serotype 0111 : B4), penicillin, streptomycin, EDTA, Triton-X 100, phenylmethylsulfonyl fluoride, Tween-20, leupeptin, acetylcholine chloride, and phenylephrine hydrochloride were obtained from Sigma Chemical Company (St. Louis, U.S.A.). Andrographolide was purchased from Aldrich (Milwaukee, U.S.A.) and was dissolved in dimethylsulphoxide then further dilute with saline or PBS. All other drugs were dissolved in saline or distilled water.

Statistical evaluation

All values in the figures and text are expressed as mean $\pm$ s.e.mean of n observations, where n represents the number of animals, aortic vessels or plates (3 well in each plate) studied. All results for the vascular reactivity experiments were expressed as percentage of the contractile response to KCl (60 mM). Statistical analysis of the effect of andrographolide on the contractile responsiveness of the vessels was performed with one-way analysis of variance (ANOVA). The other assay data were compared with paired Student's t-tests. A P-value less than 0.05 was considered to be statistically significant.

Results

Andrographolide reduces the nitrite production in LPS-activated macrophages

Activation of RAW264.7 macrophages with LPS (1 μg ml^{-1}) resulted in a significant increase in nitrite concentration in the cell supernatant from 2.9 ± 0.2 μM (control cells treated with vehicle, but not LPS) to 31.8 ± 0.7 μM at 24 h after addition of LPS. Andrographolide in the absence of LPS did not alter the basal nitrite accumulation, even at a concentration of 50 μM. Coincubation of macrophages with andrographolide (1, 3, 10, 30 and 50 μM) and LPS resulted in a dose-dependent reduction of nitrite accumulation to 25.9 ± 0.9, 19.4 ± 0.4, 10.6 ± 0.6, 3.4 ± 0.2, and 2.1 ± 0.2 μM, respectively (Figure 2). However, the andrographolide solvent DMSO (0.001 to 0.05%, equivalent to the concentration present with 1 to 50 μM andrographolide) had no effect on nitrite production (data not shown). Inhibition was greater than 80% at 30 μM andrographolide. Based on both trypan blue uptake experiments and the MTT test, this level of andrographolide was not toxic to macrophages during a 24 h incubation in 10% serum. Concentrations exceeding 30 μM were associated with a small degree of cytotoxicity, so in subsequent experiments the maximum concentration of andrographolide was 30 μM.

We found the ability of andrographolide to inhibit nitrite production of was not altered significantly in conditions of limited nitrite induction. LPS concentrations ranging from 0.1 to 100 μg ml^{-1} were added to macrophages together with andrographolide. As shown in Figure 3a, andrographolide (1, 10 and 30 μM) treatment significantly attenuate the LPS-induced concentration-response curves with suppressed its maximal response. The effect of andrographolide (10 μM, approx. EC_{50}) ranged from 39.8 ± 3.6% inhibition at the highest concentrations of LPS to 33.4 ± 5.8 at the lowest concentration (Figure 3b). Significant inhibition by 10 μM andrographolide was observed at any concentration of LPS. These indicating no reversal by LPS of inhibition caused by andrographolide. A similar effect was observed when the concentration of IFN-γ was varied (Figure 4)

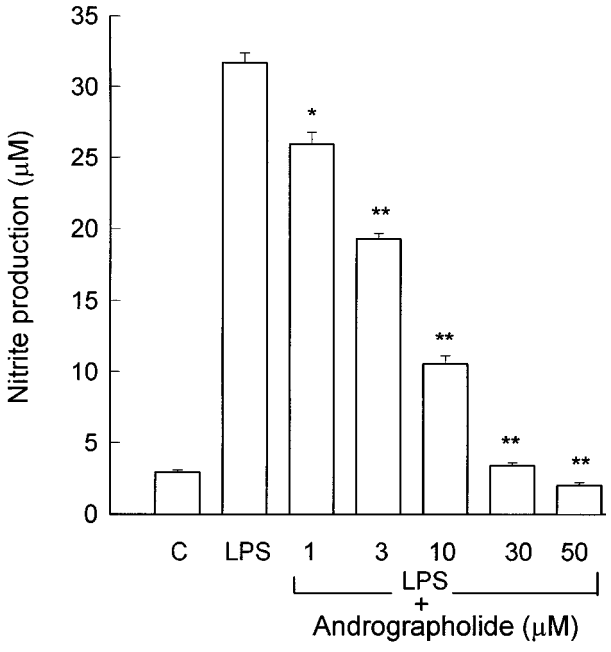

Figure 2 Andrographolide causes a dose-dependent inhibition of nitrite formation caused by *E. coli* lipopolysaccharide (LPS). Nitrite formation was measured in RAW264.7 macrophages which were cultured in 96-well plates to confluence and incubated with LPS (1 μg ml^{-1} for 24 h). Depicted is nitrite formation by RAW264.7 macrophages incubated with culture medium alone (control, C; $n=5$) for 24 h or cells treated with LPS alone (LPS; $n=6$) or with LPS plus treatment with andrographolide (1 to 50 μM; $n=7$-9). Data are expressed as mean $\pm$ s.e.mean of n observations. $^{*}P<0.05$ represents significant difference between cells subjected to LPS with and without andrographolide.

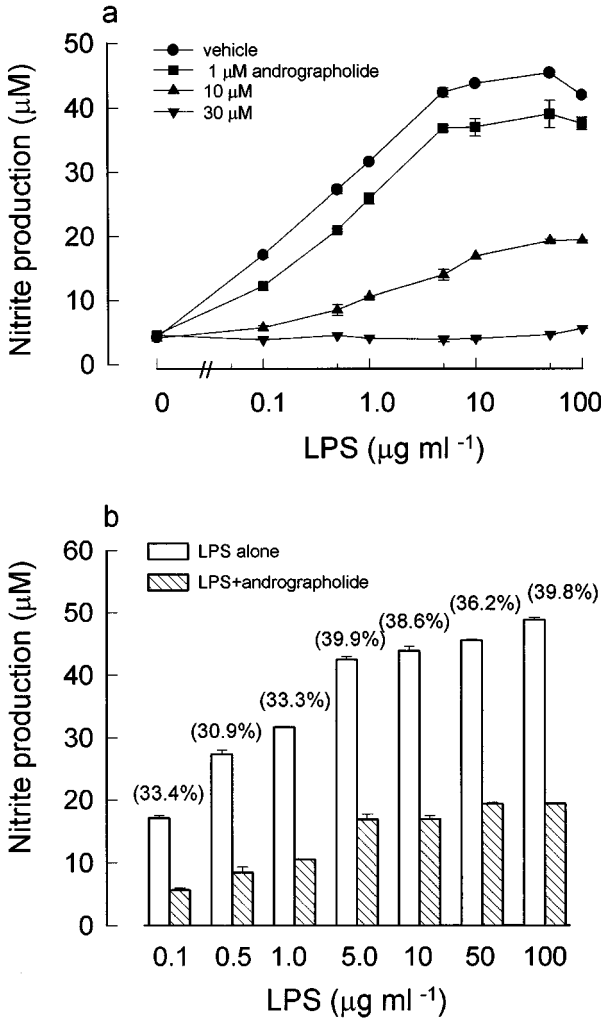

Figure 3 Effects of variation of andrographolide and *E. coli* lipopolysaccharide (LPS) concentrations on inhibition of nitrite formation. Depicted in (a) is nitrite formation by RAW264.7 macrophages coincubated either with vehicle ($n=5$) or various concentrations of andrographolide (1, 10, and 30 μM, $n=6$-9) and LPS (0.1, 0.5, 1, 5, 10, 50 and 100 μg ml^{-1}). Depicted in (b) shows the ability of andrographolide (10 μM, $n=7$) to inhibit nitrite formation is not altered in conditions of limited nitrite induction caused by fixed dose of LPS. Data within brackets represent the residue nitrite formation after andrographolide treatment when calculated as percentage of LPS groups ($n=7$). Data are expressed as mean$\pm$s.e.mean of n observations.

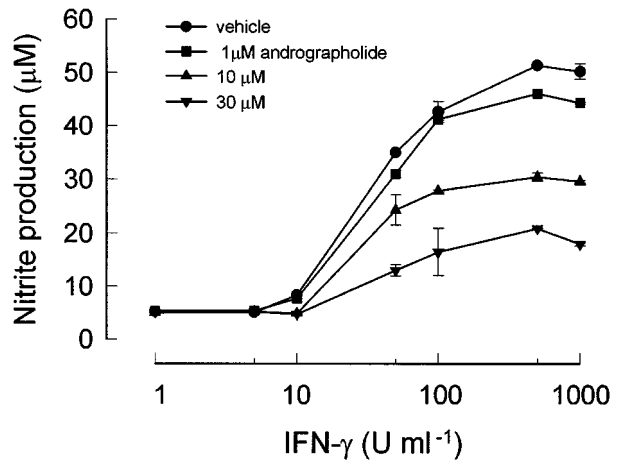

Figure 4 Effects of variation of andrographolide and interferon-γ (IFN-γ) concentrations on inhibition of nitrite formation. Depicted is nitrite formation by RAW264.7 macrophages coincubated either with vehicle ($n=6$) or various concentrations of andrographolide (1, 10 and 30 μM, $n=8$-9) and IFN-γ (1, 5, 10, 50, 100, 500 and 1000 U ml^{-1}). Data are expressed as mean$\pm$s.e.mean of n observations.

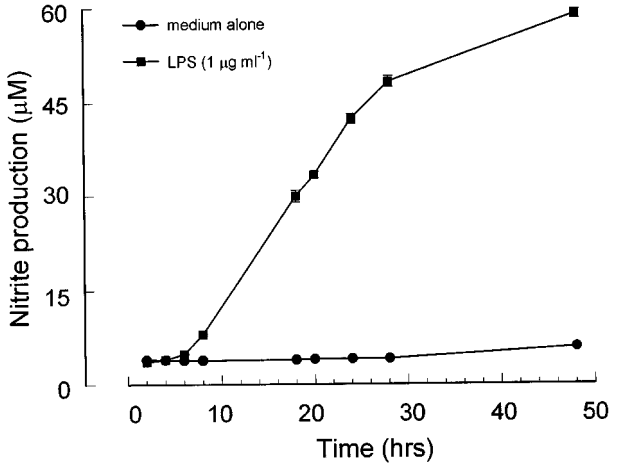

Figure 5 *E. coli lipopolysaccharide* (LPS) causes a time-dependent accumulation of nitrite formation in culture medium supernatant. Nitrite formation was measured in RAW264.7 macrophages which were cultured in 96-well plates and incubated with culture medium alone ($n=6$) or with LPS (1 μg ml^{-1}, $n=6$) for 2 to 48 h. Data are expressed as mean$\pm$s.e.mean of n observations.

Effect of andrographolide on nitrite production induced by IFN-γ

RAW264.7 macrophages nitrite can be induced not only by LPS but also by IFN-γ. As shown in Figure 4, nitrite production was induced by increasing concentrations of IFN-γ (1, 5, 10, 50, 100, 500, and 1000 U ml^{-1}). To determine if andrographolide inhibits cytokine-mediated NO synthesis, RAW 264.7 macrophages were cultured with IFN-γ, in the presence of absence of various concentrations (1, 10 and 30 M) of andrographolide. Results showed IFN-γ induced increased concentrations of nitrite, and its effect was inhibited by andrographolide concentration-dependently.

Timing of andrographolie treatment

Macrophage iNOS mRNA is induced by LPS, nitrite appears at about 6 h and the rate of its production remains almost constant for at least 48 h (Moncada *et al*., 1991). This is consistent with our finding (Figure 5). Therefore, the time period for addition of inhibitors of iNOS mRNA expression should be within 6 h after addition of LPS. To test this conclusion, actinomycin D was added to cell cultures together with LPS at times 0, 2, 4, 6 and 8 h. Culture supernatants were collected after 24 h of incubation. Maximal suppression was observed when actinomycin D was added at the beginning of the cell culture (Figure 6a). When actinomycin D was added later, the extent of suppression progressively decreased from the time 0 value of 81.8$\pm$1.2% suppression (5.9$\pm$0.4 μM nitrite) to a 6-h value of 16.7$\pm$2.8% (27.0$\pm$0.9 μM nitrite). There was no significant inhibition when actinomycin D was added later than 8 h after initiation of culture. These results are in agreement with the idea that LPS induces a progressively increase in NOS mRNA until it reaches a plateau value 4–6 h after addition of the stimulus; actinomycin D can block at any time during the period of transcription, but has no effect on the

NOS that has been transcribed prior to the addition of actinomycin D.

Time course analysis also revealed that the inhibition caused by andrographolide was critically dependent on the time of addition of this compound. The inhibition by andrographolide (10 μM; approx. EC_{50}) on the nitrite formation became progressively weaker when andrographolide was added to the cells at 0, 2, 4, 8, 16 or 20 h after LPS activation (percentage of inhibition were 70.5 ± 2.0, 63.8 ± 2.3, 52.7 ± 1.3, 41.3 ± 1.0, 16.4 ± 2.0, and $9.4 \pm 5.4\%$, respectively) (Figure 6b). Maximum inhibition was observed when andrographolide was added to the culture medium at the time of activation of macrophages together with LPS. When andrographolide was added 16 h after activation, the inhibition was less effective. In contrast to actinomycin D, andrographolide significantly suppressed LPS-induced NO production when added up to 8 h. Even 16-20 hr after exposure of RAW264.7 cells to LPS, andrographolide still caused a partial inhibition of the accumulation of nitrite. The kinetic inhibition of NO synthesis by andrographolide was obviously different from that of actinomycin D. This suggested that andrographoide may directly or indirectly interfere iNOS enzyme synthesis or enzyme activity rather than iNOS gene induction.

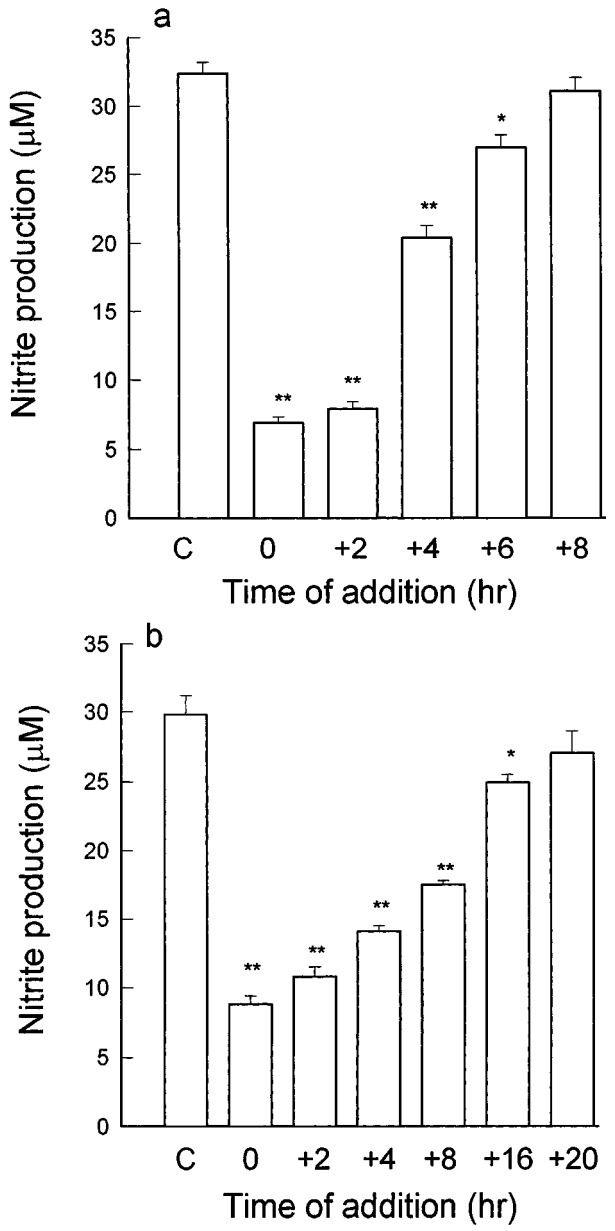

Figure 6 The inhibition by actinomycin D and andrographolide of the nitrite formation produced by *E. coli lipopolysaccharide* (LPS) are time-dependent. Nitrite formation was significantly increased in RAW264.6 macrophages incubated with LPS (control, C: 1 μg ml^{-1} for 24 h). In separate experiments, actinomycin D (a: 0.1 μg ml^{-1}) or andrographolide (b: 10 μM) was given either together with LPS (time 0), or at 2, 4, 6, 8, 16 or 20 h after LPS ($n = 7$-9 at each time point). Data are expressed as mean $\pm$ s.e.mean of n observations. $^{*}P < 0.05$ respresents significant difference between cells treated with LPS with and without actinomycin D or andrographolide.

Andrographolide attenuates the levels of iNOS protein in macrophages stimulated with LPS

Because of the greater inhibition by andrographolide when added 0–16 h after LPS stimulation, it was reasonable to expect an effect on induction of the protein for iNOS. To determine whether the inhibitory effect of andrographolide was due to the inhibition of iNOS expression, Western blot analysis was carried out on whole cell lysates using a monoclonal antibody for mouse macrophage iNOS. As shown in Figure 7, cell stimulated with 1 μg ml^{-1} LPS alone expressed a time -dependent increasing in iNOS protein (at 6, 12, 18 and 24 h, respectively). When LPS and andrographolide (10 μM) were added together, LPS-induced expression of iNOS protein was markedly attenuated.

Andrographolide attenuates the LPS-induced vascular hyporeactivity to phenylephrine in vitro

Exposure of rat aorta to phenylephrine (0.3 μM) causes a steady-state tonic contraction maintained at least for 25 min. During phenylephrine-induced sustained contraction andrographolide did not produce any significant relaxation, however, relaxation were caused both by acetylcholine and nitroglycerin in endothelium-intact and -denuded preparations, respectively (data not shown). Increasing concentrations of phenylephrine (10^{-9}–10^{-5} M) caused a concentration-related vasoconstriction in endothelium -denuded rings (Figure. 8, filled circles). Incubation with Krebs solution alone for 5 h did not significantly change the contractile response to phenylephrine. By contrast, incubation of rat aorta with LPS (300 ng ml^{-1}) for 5 h caused a right shift of the concentration-response curve to phenylephrine (Figure 8, filled squares). The maximal constriction (T_{max}) to phenylephrine was also significantly attenuated by incubation with LPS (133 ± 14 and

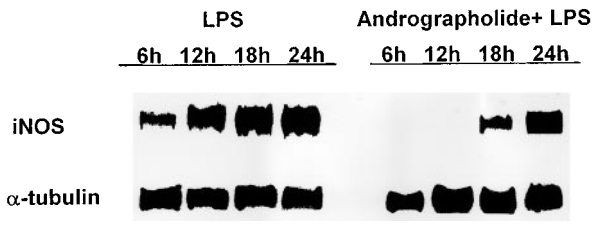

Figure 7 Treatment of RAW264.7 macrophages with andrographolide inhibits the inducible nitric oxide synthase (iNOS) protein expression produced by *E. coli lipopolysaccharide* (LPS). Immunoblot was measured in cell lysates obtained from LPS (1 μg ml^{-1}) treatment or together with LPS and andrographolide (10 μM) treatment for 6, 12, 18, and 24 h, respectively. Depicted are protein bands after blotted against iNOS antibody (upper bands, 130 kDa) or α-tubulin antibody (lower band, 47 kDa).

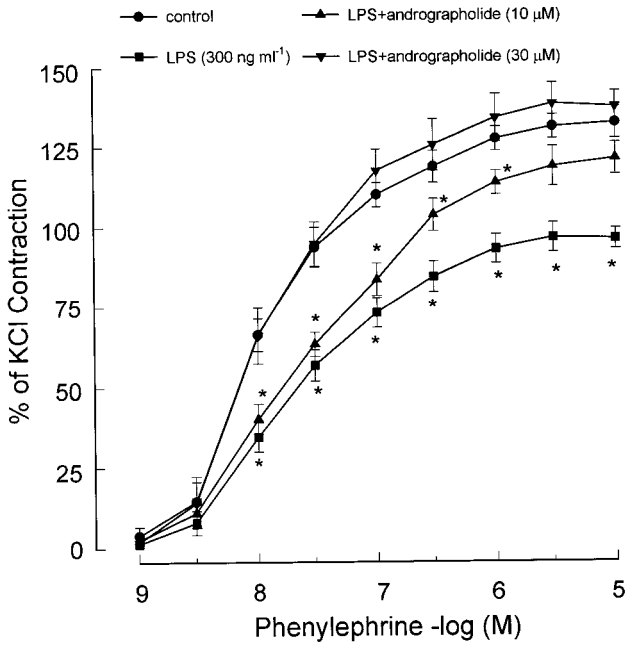

Figure 8 The vascular hyporeactivity to phenylephrine caused by *E. coli* lipopolysaccharide (LPS) is attenuated by treatment of aortic rings with andrographolide. Depicted is the phenylephrine (10^{-9}–10^{-5} M) concentration-response curves of control rat aortic rings ($n=6$) and rings exposed to LPS alone (300 ng ml^{-1}, $n=6$), or LPS combined with andrographolide (10 and 30 μM, $n=9$ and 7 respectively). Values are expressed as a percentage of maximal KCl (60 mM) response and are mean$\pm$s.e.mean of n observations. $^{*}P<0.05$ represents significant differences when compared to control rings.

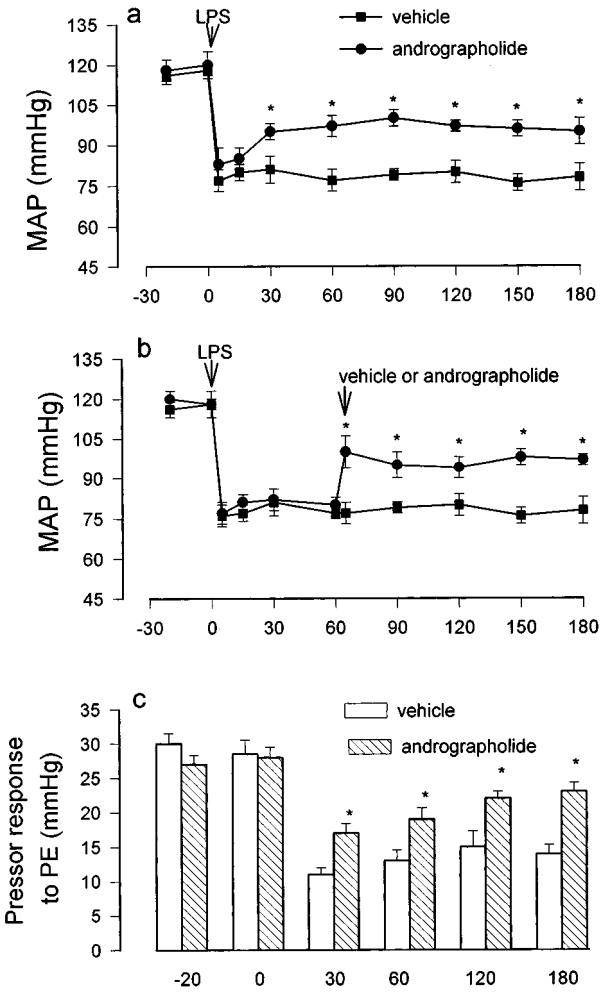

Figure 9 Andrographolide ameliorates the hypotension and vascular pressor response to phenylephrine (PE) in endotoxic shock in the anaesthetized rat. Depicted are the changes in (a, b) mean arterial pressure (MAP) and (c) pressor responses to PE (10 μg kg^{-1}) in rats treated with *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v. at time 0). Different groups of animals were pretreated either with vehicle ($n=6$-8) or with andrographolide ($n=6$-9) 20 min prior to LPS (1 mg kg^{-1}, i.v., for a and c), or 60 min after LPS (for b). Data are expressed as mean$\pm$s.e.mean of n observations. $^{*}P<0.05$ represents significant differences when compared to control at the same time point.

92$\pm$8% for control and LPS-treated vessels, respectively, relative to KCl response). Coincubation of vessels with LPS and andrographolide restored significantly the contractile response to phenylephrine. As shown in Figure 8, treatment of the LPS-vessels with 10 μM andrographolide caused an apparently reverse of vascular hyporeactivity to phenylephrine (filled triangles). However, coincubation of vessels with LPS and 30 μM andrographolide resulted in a contraction (filled reversed triangles, T_{max} 135$\pm$15%, relative to KCl response) that was not different from control vessels.

Andrographolide ameliorates the haemodynamic effects of LPS in rats

Baseline values for MAP and HR of the vehicle- and andrographolide-pretreated animal groups were 116$\pm$4 and 118$\pm$5 mmHg, and 406$\pm$15 and 410$\pm$17 beats min^{-1}, respectively. LPS (10 mg kg^{-1}, i.v.) infusion produced a rapid (within 10 min) and prolonged (over the 180 min observation period) fall in MAP (from 116$\pm$4 mmHg to 77$\pm$5 mmHg at 60 min) (Figure 9a, filled squares) and a decrease in the pressor responses to PE (10 μg ml^{-1}, i.v.) elicited 30, 60, 120 and 180 min after the onset of LPS infusion (Figure 9c, open columns). However, endotoxaemia for 180 min was not associated with a significant change in HR (time 0: 406$\pm$15, time 180 min: 413$\pm$21 beats min^{-1}). Andrographolide (1 mg kg^{-1}, i.v., 20 min prior to the onset of LPS infusion), which by itself did not affect basal MAP nor reactivity to PE in control groups, significantly attenuated the fall in MAP and the pressor response to PE (10 μg kg^{-1}, i.v.) observed after LPS infusion. Figure 9a showed LPS-rats that had been pretreated with andrographolide maintained significantly higher MAP values (filled circles) when compared with LPS-rats treated with vehicle (filled squares) during 180 min period. The pressor responses to PE seen at 30, 60, 120 and 180 min after LPS were also significantly greater in LPS-rats treated with andrographolide (hatched columns) when compared with vehicle treatment (open columns) (Figure 9c). Additionally, administration of andrographolide (1 mg kg^{-1}, i.v.) at 60 min after the onset of endotoxaemia resulted in a rapid (within 5 min) and sustained increase in MAP (Figure 9b). Thus, the MAP of LPS-rats treated with andrographolide was significantly higher than in the LPS-control rats at 65, 90, 120, 150 and 180 min. Andrographolide did not alter the heart rate significantly, and the heart rate of LPS-rats treated with andrographolide was not different from the heart rate in vehicle-treated LPS-rats (the former 376$\pm$29 beats min^{-1}, the later: 406$\pm$15 beats min^{-1}, $P<0.05$, $n=6$).

Discussion

The present experiments clearly show that andrographolide inhibits the nitrite formation caused by LPS in cultured RAW264.7 macrophages concentration-dependently. The inhibition was maximal if added together with LPS and decreased progressively as the interval between andrographolide and LPS was increased. In contrast to actinomycin D, a inhibitor of transcription, the inhibitory effect caused by andrographolide was less effective while added 2 h prior to LPS (data not shown) and still significantly suppressed LPS-induced nitrite accumulation when added up to 8 h as shown in Figure 5b. The kinetic inhibition of nitrite synthesis by andrographolide was obviously different from a agent which disrupts gene transcription. Additionally, it is worth to note that the inhibitory effect caused by andrographolide was long lasting for 18 h. Suggesting andrographolide acts more like a protein synthesis inhibitor. Immunoblots with monoclone antibody to murine macrophage iNOS also indicated a large inhibition by 10 μM andrographolide of iNOS expression. Together with these findings suggesting that andrographolide may interferes with the step in protein synthesis or processing. If protein synthesis can be considered a late event compared with message induction, the immunoblotting are consistent with timing assay. However, the influence on the induction of the iNOS mRNA and/or the activity of this enzyme can not be excluded entirely.

Treatment of rat thoracic aortic rings with LPS results in an attenuation of the vascular responsiveness to phenylephrine. We have shown that andrographolide resulting in a reversal of the endotoxin-induced hyporesponsiveness and even restores vascular responsiveness of LPS-treated vessels to control levels after 30 μM andrographolide treatment. The role of NO overproduction following iNOS induction in systemic hypotension and decreased response to vasoconstrictor agents after endotoxin treatment has been reported by many authors in *in vivo*, *ex vivo* or *in vitro* studies (Thiemermann, 1994). Dexamethasone, which inhibits the induction of iNOS (Paya *et al*., 1993; Rees *et al*., 1990), and cycloheximide, an inhibitor of protein synthesis, when coincubated with endotoxin, completely reversed the reduced response to noradrenaline. Moreover, the reversal by nitric oxide synthase inhibitors, N^G-methyl-L-arginine and aminoguanidine, of this reduced responses strongly suggest that enhanced nitric oxide production was responsible for endotoxin-induced vascular hyporesponsiveness (Joly *et al*., 1994; Kilbourn *et al*., 1990; Scott *et al*., 1996). Thus, the apparent reversal of vascular hyporeactivity by andrographolide may be, at least in part, due to inhibition of nitrite (iNOS) induction. This hypothesis is supported by our findings that andrographolide can inhibit induction of nitrite by destroying the expression of iNOS protein in cultured RAW264.7 macrophages activated with endotoxin, LPS.

In an attempt to evaluate the beneficial haemodynamic effect of andrographolide, we established an animal model of endotoxaemia by slow injection of LPS. Prolonged periods of endotoxaemia in the anaesthetized rat result in the induction of iNOS in several organs, and the consequent enhanced formation of NO by iNOS in the vascular smooth muscle contributes importantly to the delayed hypotension and the vascular hyporeactivity to vasoconstrictor agents (Szabo *et al*., 1993a). There is a good correlation between the degree of iNOS induction and the magnitude of the fall in blood pressure caused by endotoxaemia in the rat (Kilbourn, 1997; Kilbourn *et al*., 1997). Moreover, the beneficial haemodynamic effects in animal models of septic shock given by dexamethasone (Wright *et al*., 1992), PAF-receptor antagonists (Szabo *et al*., 1993c) or IL-1 receptor antagonists (Szabo *et al*., 1993b) are partly due to inhibition of iNOS induction. The present experiments showed that either pretreatment of andrographolide 20 min prior to LPS or at 60 min after LPS, reversed the hypotension and subsequently restored the pressor responses to phenylephrine. However, the increase in MAP caused by phenylephrine after LPS in rats treated with andrographolide was still significantly reduced as compared with control (before LPS). In fact, andrographolide restored vascular responsiveness of LPS-treated vessels to control levels *in vitro*, however, did not completely reverse the pressor response elicited by phenylephrine in LPS-rats *in vivo*. Our explanation is existing of other mediators than NO may contribute to the complexity of hypotension after endotoxaemia *in vivo*. However, it should be point out that a inhibition of iNOS induction by andrographolide is still sufficient to cause a substantial improvement of the haemodynamic alterations caused by endotoxin *in vivo*.

In conclusion, this study demonstrated that andrographolide (i) dose-dependently inhibited nitrite accumulation and iNOS protein expression in cultured RAW264.7 macrophages, (ii) attenuated the reduction of vasoconstrictor responses to phenylephrine (vascular hyporeactivity) in rat aortic rings treated with LPS, and therefore (iii) improved the haemodynamic parameter in rats with endotoxaemia. It seems that andrographolide may be useful in the alleviation of circulatory shock. However, clinical relevance of the present experiments is unknown. Although our data suggest that andrographolide may be acting at the molecular level to inhibit NO synthase induction, the precise mechanism by which this effect is mediated still remains to be established. Additionally, the present finding provides a mechanism by which the anti-inflammatory properties of this compound could be mediated. But further studies are required to clarify whether our observations have any clinical relevance particularly in chronic inflammation where the dominant inflammatory cells are macrophages and over-production of NO at the sites of inflammation can augment the inflammatory response (Boughton-Smith *et al*., 1993; McCartney-Francis *et al*., 1993; Vane *et al*., 1994; Weinberg *et al*., 1994; Salvemini *et al*., 1995).

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