British Journal of Pharmacology (1999) 128, 357–364
http://www.stockton-press.co.uk/bjp

Prevention of the expression of inducible nitric oxide synthase by a novel positive inotropic agent, YS 49, in rat vascular smooth muscle and RAW 264.7 macrophages

[1,2]Young Jin Kang, [1]Eui Bon Koo, [1,2]Young Soo Lee, [3]Hye Sook Yun-Choi & *,[1,2]Ki Churl Chang

[1]Department of Pharmacology, College of Medicine, Gyeongsang National University, Chinju, 660-280, Korea; [2]Cardiovascular Research Institute, College of Medicine, Gyeongsang National University, Chinju, 660-280, Korea and [3]Natural Product Research Institute, Seoul National University, Seoul, 460-100, Korea

1 The effects of a novel positive inotropic isoquinoline compound, YS 49, on NO production and iNOS protein expression were investigated in cultured rat aortic vascular smooth muscle cells (RAVSMC) and RAW 264.7 cells exposed to lipopolysaccharide (LPS) plus interferon-γ (IFN-γ). In addition, the effects of YS 49 on vascular hyporeactivity *in vitro* and *ex vivo*, and on survival rate (mice) and serum NOx (rat) levels, were also investigated in LPS-treated animals.

2 Pre- or co-treatment of YS 49 with LPS plus IFN-γ, concentration-dependently reduced NO production in RAVSMC and RAW 264.7 cells (IC_{50} values, 22 and 30 μM, respectively). Although the inhibitory effect on NO production was reduced when YS 49 was applied 2 and 4 h after cytokine in RAW 264.7 cells, it was still statistically significant ($P<0.05$).

3 YS 49 reduced iNOS mRNA expression in LPS-treated rat aorta *in vitro*, an effect which was associated with restoration of contractility to the vasoconstrictor, phenylephrine (PE), and reduction in L-arginine-induced relaxation.

4 Serum NOx levels were significantly ($P<0.01$) reduced by YS 49 (5 mg kg^{-1}, i.p.) in LPS-treated rats (10 mg kg^{-1}, i.p.). Administration of YS 49 (10 and 20 mg kg^{-1}) 30 min prior to LPS (10 mg kg^{-1}) also significantly ($P<0.01$) increased the subsequent survival rates in mice.

5 Finally, expression of iNOS protein induced by LPS plus IFN-γ in RAVSMC and RAW 264.7 cells was suppressed by YS 49, in a concentration-dependent manner.

6 These data strongly suggest that YS 49 suppresses iNOS gene expression induced by LPS and/or cytokines in RAVSMC and RAW 264.7 cells at the transcriptional level. YS 49 could therefore be beneficial in septic shock and other diseases associated with iNOS over-expression.

Keywords: Nitric oxide; vascular smooth muscle; murine macrophage; endotoxin shock; inducible nitric oxide synthase; lipopolysaccharide; tetrahydroisoquinoline; cytokine

Abbreviations: ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; IL-10, interleukin-10; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PE, phenylephrine; PVDF, polyvinyliden difluoride; RAVSMC, rat aortic vascular smooth muscle cells; SDS, sodium dodecyl sulphate; SSC, sodium chloride/sodium citrate; TNF-α, tumour necrosis factor-α; TXA_2, thromboxane A_2; U44619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_2\alpha$

Introduction

Sepsis and the systemic inflammatory response syndrome are characterized by peripheral vasodilatation and myocardial depression. An enhanced formation of nitric oxide (NO) in response to lipopolysaccharide (LPS) is an important promoter of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxaemia (Thiemermann & Vane, 1990; Parratt, 1989), and systemic inflammatory response syndrome in humans (Sakurai *et al*., 1995; McInnes *et al*., 1996). Selective inhibitors of inducible nitric oxide synthase (iNOS) activity and/or of iNOS expression may be beneficial in the treatment of systemic inflammation and septic shock. Isoquinoline alkaloids are folk remedies that have been used as cardiotonic and anti-inflammatory agents in oriental countries (Deng, 1990). For example, tetrandrine, a benzylisoquinoline analogue, is a traditional anti-rheumatic agent, that has been shown to inhibit the activation of nuclear factor-kappa B (NF-κB) and NF-κB-dependent reporter gene expression in rat alveolar macrophages exposed to LPS (Chen *et al*., 1997). Higenamine, a tetrahydroisoquinoline analogue, inhibits iNOS mRNA expression induced by LPS in the rat aorta (Kang *et al*., 1997). Other isoquinoline series have also been shown to suppress LPS-induced hepatitis and tumour necrosis factor-α (TNF-α) production in mice (Kondo *et al*., 1993a) and NO production in macrophages (Kondo *et al*., 1993b). In addition, the recently synthesized isoquinoline analogue, HMN-1180, inhibits glutamate stimulated NO production in the human neuroblastoma cell line, SK-N-MC (Nishio *et al*., 1998). Thus, the isoquinoline moiety may be able to inhibit iNOS expression and/or iNOS activity. We previously reported that YS 49, an isoquinoline compound (Figure 1), has a strong positive inotropic action in rat and rabbit isolated hearts through activation of cardiac β-adrenoceptors (Lee *et al*., 1994). Interestingly, current evidence suggests that many classical pharmacological agents, such as ligands of α-adrenergic

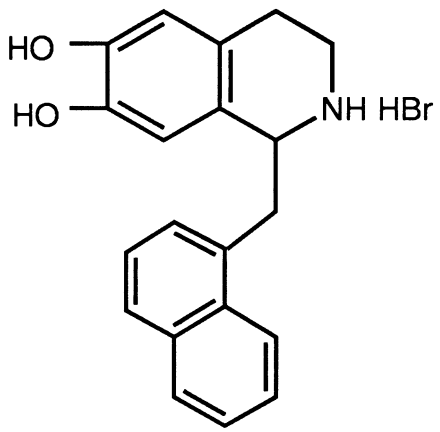
*Author for correspondence at: Department of Pharmacology, College of Medicine, Gyeongsang National University, 92 Chilamdong, Chinju, 660-280, Korea;
E-mail: kcchang@nongae.gsnu.ac.kr

(Ignatowski & Spengler, 1995), β-adrenergic (Severn *et al*., 1992; Szabo *et al*., 1997), dopamine (Hasko *et al*., 1996) and adenosine receptors (Bouma *et al*., 1994), can influence the production of pro- and anti-inflammatory cytokines in response to endotoxin (bacterial LPS). These influences can in turn modulate iNOS gene expression. For example, isoproterenol has been reported to exert marked anti-inflammatory effects by suppressing LPS-induced TNF-α and NO production in rodents (Szabo *et al*., 1997). Similarly, epinephrine increases LPS-induced interleukin-10 (IL-10) release by a combined effect on α and β adrenergic receptors in human whole blood (van der Poll *et al*., 1996). Furthermore, combined treatment with the NOS inhibitor, N^G-nitro L-arginine (L-NNA), and the positive inotropic agent, dobutamine, produces marked additional improvements in cardiovascular parameters in endotoxaemia (Kilbourn *et al*., 1994). Here, we present evidence that YS 49, an isoquinoline alkaloid with positive inotropic action, inhibits iNOS expression induced by LPS and/or cytokines in vascular smooth muscle and in RAW 264.7 cells. This effect appears to be exerted at the transcriptional level, and could well be beneficial in septic shock or inflammatory disease, in which iNOS is implicated as a central causative mediator.

Methods

Cell culture

RAVSMC were isolated from rat thoracic aorta by enzymatic dissociation and cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 u ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. Cells were passaged twice a week by harvesting with trypsin/EDTA and seeding at a 1 : 5 ratio in 75 cm^2 flasks. For experiments, cells between passage levels 9 and 15 were seeded into dishes (2×10^4 cells cm^{-2}), fed every other day, and used at confluence (6–7 days). RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and were grown in RPMI-1640 medium supplemented with 25 mM HEPES, 100 u ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 10% heat-inactivated foetal calf serum.



YS 49

Figure 1 Chemical structure of YS 49.

Cell stimulation

Confluent RAVSMC were cultured in serum-free DMEM for 24 h, washed with serum-free DMEM, and exposed for 18 h to LPS (300 ng ml^{-1}) plus IFN-γ (10 u ml^{-1}) in the presence or absence of YS 49 (10, 30 and 100 μM). Similarly, RAW 264.7 cells were also incubated with LPS (10 ng ml^{-1}) plus IFN-γ (10 u ml^{-1}) for 18 h in the presence or YS 49 (1, 10 and 100 μM). YS 49 was dissolved in sterile distilled water, filtered through a 0.2 mm filter, and administered 1 h prior to, or simultaneously, or 2, 4, 8 or 16 h after LPS plus IFN-γ.

Assay for nitrite production

NO was measured as its stable oxidative metabolite, nitrite. At the end of the incubation for 18 h, 500 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured and the nitrite concentration was determined using a curve calibrated with sodium nitrite standards.

Ex vivo *vascular reactivity*

Male Sprague Dawley rats (250–300 g weight) were injected (i.p.) with (i) LPS (10 mg kg^{-1}, $n=3$), (ii) YS 49 (5 mg kg^{-1}, $n=5$) 30 min prior to LPS (10 mg kg^{-1}), (iii) saline ($n=3$) or (iv) YS 49 (5 mg kg^{-1}, $n=3$). Eight hours later animals were anaesthetized with pentobarbital sodium (i.p.) and the thoracic aortas removed. The aortas were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width. Endothelium was removed by gentle rubbing of the intimal surface with a wooden stick (Chang *et al*., 1993a). Rings were then mounted in organ baths (5 ml) filled with Krebs' solution (pH 7.4) consisting of (mM); NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11 and EDTA 0.03. Isometric force was measured with force transducer (FT 03, Grass Instrument, U.S.A.). A tension of 1 g was applied and the rings were equilibrated for 60 min, with the Krebs' solution being changed every 20 min. Indomethacin (10 μM) was included in the Krebs' buffer to prevent the production of cyclo-oxygenase metabolites. Concentration-response curves to phenylephrine (PE, 1 nM–10 μM) were obtained. For relaxation studies, rings were contracted submaximally with U46619 (20 nM). Once a stable contraction was obtained, L-arginine (1, 3, 10, 30 and 100 μM) was introduced cumulatively.

In vitro *vascular reactivity*

As described above, endothelium-denuded thoracic aortic ring preparations (3–4 mm width) were prepared from untreated rats. To investigate the effects of YS 49 on iNOS induction *in vitro*, the rings were divided into groups; (i) plus LPS (300 ng ml^{-1}, $n=3$), (ii) different concentrations of YS 49 (10, 30 and 100 μM) plus LPS (300 ng ml^{-1}, $n=3$), and (iii) control (Krebs' solution, $n=3$). The tissues were incubated with the LPS and/or drug combination for 8 h at 37°C. At the end of the incubation period, isometric responses were determined as described above.

Analysis of iNOS mRNA

After completion of the *in vitro* tension study, tissues were quickly frozen with a clamp precooled in liquid nitrogen. Total

RNA was then extracted from the tissues by homogenization. A sample 15 μg of total RNA per lane was subjected to electrophoresis on 1.2% agarose gels containing formaldehyde and transferred to nylon filters. The filter was then hybridized with a random-primed ^{32}P-labelled murine macrophage iNOS cDNA probe (from 826 to 1560 bp) in rapid hybridization solution (Quickhyb; Stratagene, CA, U.S.A.) at 68°C for 1 h. The hybridized filter was subsequently washed twice for 15 min at room temperature with 2 × SSC (sodium chloride/sodium citrate)/0.1% SDS (sodium dodecyl sulphate) and then twice for 15 min at 42°C with 0.2 × SSC/0.1% SDS. The filter was then exposed to an X-ray film. The filter was subsequently stripped and rehybridized with a ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

Assay for iNOS protein

iNOS protein was analysed by immunoblotting with an anti-iNOS antibody. Briefly, cells were lysed in a buffer containing (mM): Tris/HCl 50, pH 7.5, EDTA 1, leupeptin 1, pepstatin A 1, phenylmethylsulphonyl fluoride 0.1 and dithiothreitol 1 and sonicated. The homogenates were then centrifuged at 7500 × *g* for 15 min at 4°C, and the supernatants were subjected to SDS–PAGE (7.5% gel). The separated proteins were electrophoretically transferred to polyvinyliden difluoride (PVDF) membranes, and the membranes incubated overnight at 4°C with anti-iNOS antibody, followed by horse radish peroxidase-labelled goat anti-rabbit IgG for 90 min. Antigen-antibody complexes were detected using ECL Western blotting detection reagents (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instruction.

Survival studies

ICR mice (26–30 g), whose stock originated from the Institute of Cancer Research of the National Institute of Health (Bethesda, MD, U.S.A.) were purchased from DaeHan Animal Care (Umsung, Korea). *E. coli* LPS (20 mg kg^{-1}, i.p.) was injected in the presence of YS 49 (10 mg kg^{-1}, 20 mg kg^{-1}) or vehicle and survival monitored every 6 h until 48 h. YS 49 was administered 30 min prior to LPS.

Serum nitrite/nitrate measurement

Rats were divided into four groups. (i) LPS (10 mg kg^{-1}), i.p., $n=4$), (ii) LPS plus YS 49 (5 mg kg^{-1}, i.p., $n=5$), (iii) saline ($n=2$), and (iv) YS 49 ($n=3$). When used YS 49 was given 90 min prior to LPS. Eight hours after LPS treatment a whole blood sample was withdrawn by cardiac puncture after pentobarbital (i.p.) anaesthesia. The plasma nitrite concentration was determined by reducing the nitrate enzymatically, using nitrate reductase from *Aspergillus* species. Briefly, plasma samples were diluted 1 : 10 with distilled water and incubated with assay buffer (composition, mM): KH_2PO_4 50, NADPH 0.6, FAD 5 and nitrate reductase 10 u ml^{-1}, pH 7.5, for 30 min at 37°C. A standard curve for nitrate was constructed by incubation of nitrate (1–100 μM) with assay buffer. The resultant nitrite concentrations were determined with Griess reagent using sodium nitrite as standard.

Materials

DMEM, foetal calf serum, penicillin, streptomycin and glutamine were supplied by Gibco BRL (Gaithersburg, MD, U.S.A.). Lipopolysaccharide (*E. coli*; serotype 0128:B12), indomethacin, phenylephrine HCl, sulphanilamide, N-[1-naphthyl]ethylenediamine, sodium chloride, leupeptin, pepstatin A, phenylmethylsulphonyl fluoride, dithiothreitol and sodium citrate were from Sigma (St. Louis, MO, U.S.A.). U44619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_2\alpha$) was from Biomol (Plymouth, PA, U.S.A.). iNOS cDNA was kindly provided by Dr H.T. Chung (Wonkwang University, Korea). iNOS antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Horseradish peroxidase-labelled goat anti-rabbit IgG was purchased from Pierce (Rockford, IL, U.S.A.). ECL Western blotting detection reagents was from Amersham (Buckinghamshire, U.K.). YS 49 was obtained from the Natural Product Research Institute, Seoul, Korea.

Statistical evaluations

Data are expressed as mean ± s.e.mean of three observations. Differences between data sets were assessed by one way analysis of variance followed by Dunnett's test. A level of $P<0.05$ was accepted as statistically significant.

Results

Effects of YS 49 on NO production in RAVSMC and RAW 264.7

In cultured RAVSMC, LPS plus IFN-γ caused a time-dependent increase nitrite production, that was significant after 6 h, peaked at 18 h, and then declined when checked every 3 h until 24 h (data not shown). In contrast, in RAW 264.7 cells, nitrite levels time-dependently increased until 24 h (data not shown). Therefore, unless otherwise indicated, data are at the 18 h time point. YS 49 concentration-dependently inhibited the accumulation of nitrite in both RAVSMC and RAW 264.7 macrophages, with IC_{50} values of 22 and 30 μM, respectively (Figure 2). Similar results were obtained when YS 49 was applied either 1 h before or simultaneously with LPS plus IFN-γ. Addition of YS 49 either 2 or 4 h after the

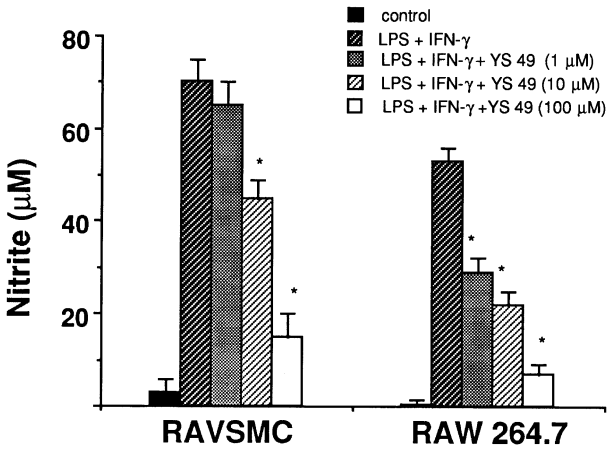


Figure 2 Concentration-dependent inhibition of production of nitrite by YS 49 in RAVSMC and RAW 264.7 cells exposed to LPS + INF-γ. LPS (300 ng ml^{-1} for RAVSMC and 10 ng $^{-1}$ for RAW 264.7 macrophages) + INF-γ (10 u ml^{-1}) were added simultaneously (RAW 264.7 cells) or 1 h before (RAVSMC) YS 49 (1–100 μM), and then incubated for further 18 h. Nitrite was measured by the Griess reaction in 0.5 ml of conditioned medium. Each value represents the mean ± s.e.mean of triplicate determinations from a representative experiment performed three separate times with comparable results. *Significantly different from all other groups at $P<0.05$.

treatment of the cells with IPS plus IFN-γ still significantly inhibited the nitrite production, although the inhibitory effect was decreased (Figure 3). Addition of YS 49 8 h after LPS treatment failed to produce a statistically significant effect ($P>0.05$). Similarly, no inhibitory effect was observed when YS 49 was applied 16 h after.

Effects of YS 49 on LPS-induced vascular reactivity ex vivo

Aortas from LPS (10 mg kg^{-1})-treated rats had significantly depressed contractile responses to PE *ex vivo*, an effect which was prevented by YS 49 (5 mg kg^{-1}) pretreatment *in vivo*. The maximum contractile force to 10 μM PE was 0.97 ± 0.43 g ($n=3$) and 3.5 ± 0.59 g ($n=5$) in LPS-treated and YS 49 plus LPS-treated groups, respectively (Figure 4a). YS 49 (5 mg kg^{-1}) did not affect the contractile ability to PE in saline treated groups (maximum contraction to 10 μM PE, 2.32 ± 0.53 g, $n=3$, and 2.32 ± 0.47 g, $n=3$, in saline and YS 49 treated rings, respectively). To confirm the diminished contraction was due to induction of iNOS, the pro-relaxant effects of L-arginine was studied in vessels preconstricted with U46619. L-arginine, but not D-arginine, significantly ($P<0.01$) relaxed aortas from rats treated with LPS ($n=3$) but not those from rats treated with YS 49 plus LPS ($n=4$). Aortas from rats treated with saline ($n=3$) or YS 49 ($n=3$) were not relaxed by L-arginine (Figure 4b).

Effects of YS 49 on iNOS mRNA expression and LPS-induced vascular reactivity in vitro

Incubation with LPS (300 ng ml^{-1}, $n=3$) for 8 h *in vitro* significantly depressed the contraction of rat aortas to PE (1 nM – 10 μM). Co-administration of YS 49 with LPS ($n=3$) prevented the development of this hypocontractile state. Incubation of aortas in Krebs' solution alone for 8 h also caused some reduction in contractility (maximum contraction to 10 μM PE: *in vitro*, 1.72 ± 0.42 g; *ex vivo*, 2.32 ± 0.47 g). (Figure 5). This depression in contractility was associated with expression of mRNA for iNOS in control rings. However, in accordance with the contraction study, iNOS mRNA expression was further increased in LPS treated rings. This latter expression was decreased concentration-dependently by YS 49 (Figure 6).

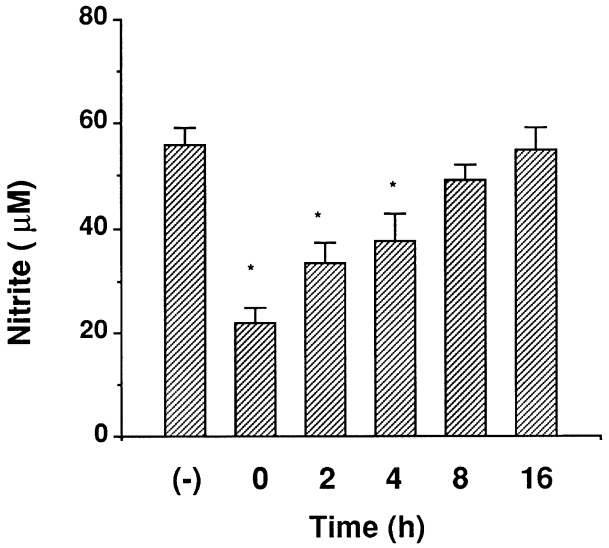


Figure 3 The time-dependent effects of YS 49 on NO production in RAW 264.7 cells stimulated with LPS + INFγ. Cells were incubated with LPS (10 ng ml^{-1}) + INF-γ (10 u ml^{-1}), together with YS 49 (30 μM) added at the indicated times, for a total of 18 h. Nitrite was measured by the Griess reagent in 0.5 ml of the conditioned medium. Each value represents the mean $\pm$ s.e.mean of triplicate determinations from a representative experiment performed three separate times with comparable results. *Significantly different from all other groups at $P<0.05$.

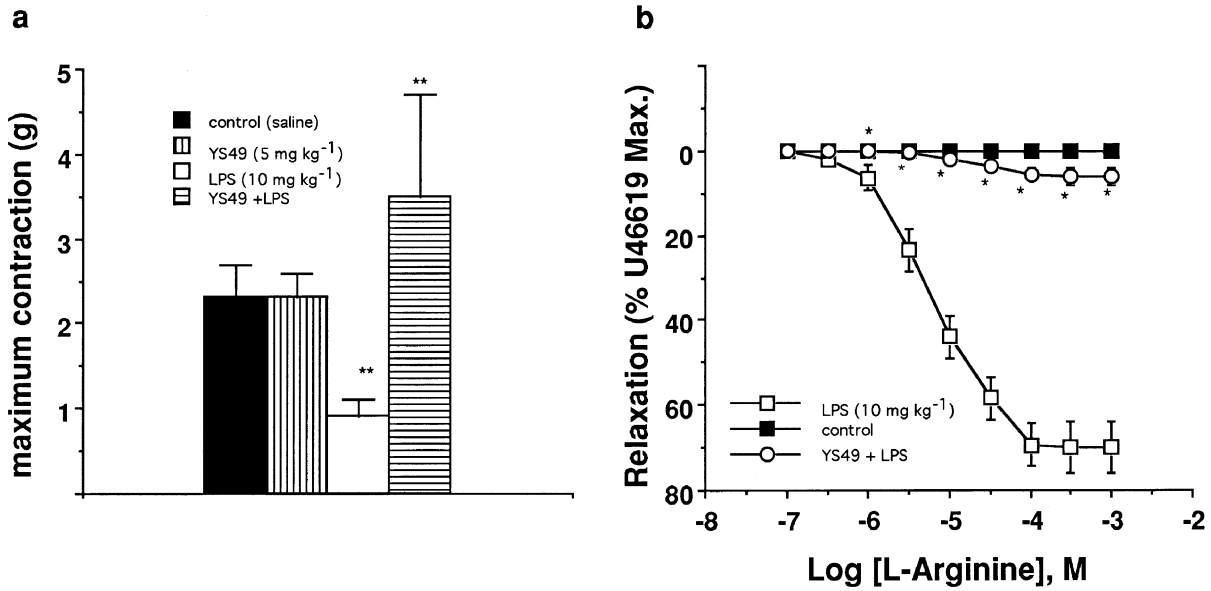


Figure 4 Preventive effect of YS 49 on LPS-induced vascular hyporeactivity *ex vivo*. Rats were injected (i.p.) with either LPS (10 mg kg^{-1}), YS 49 (5 mg kg^{-1}) + LPS, YS 49 (5 mg kg^{-1}) or saline, and then sacrificed 8 h after injection. Thoracic aortas were removed and isometric responses recorded. Maximum contractile forces (g) of rat thoracic aorta exposed to 10 μM PE are shown (a) The contractile response to PE in aortas taken from LPS-treated rats was significantly ($P<0.01$) lower than controls, an effect that was reversed by treatment with YS 49 ($P<0.01$). (b) L-arginine, concentration-dependently relaxed aortas taken from LPS-treated rats. This response was almost entirely absent in aortas taken from YS 49 + LPS-treated rats. Aortas from control rats did not relax in response to L-arginine. All aortas were contracted with U 46619. Values are expressed as mean $\pm$ s.e.mean of three to five separate experiments. * and **Significantly different from corresponding controls at $P<0.05$ and $P<0.01$, respectively.

Effects of YS 49 on iNOS protein expression

Exposure of RAW 264.7 macrophages to LPS plus IFN-γ for 18 h stimulated the expression of a 130 kDa protein which was recognized by the iNOS antibody (Figure 7a). This expression of iNOS protein was significantly inhibited in a concentration-dependent manner by treatment of the cells with YS 49. For comparison, we used dexamethasone (0.1 μM), and the other isoquinoline compounds higenamine (10 μM) and tetrandrine (10 μM). Higenamine effectively inhibited the expression of iNOS protein in RAW 264.7 cells, as shown in LPS-treated rat aorta (Kang *et al.*, 1997). Tetrandrine also inhibited the expression of the protein, but was least potent among the three isoquinoline derivatives. In RAVSMC, like RAW 264.7 macrophages, co-administration of YS 49, concentration-dependently inhibited the expression of iNOS protein induced by LPS plus IFN-γ (Figure 7b).

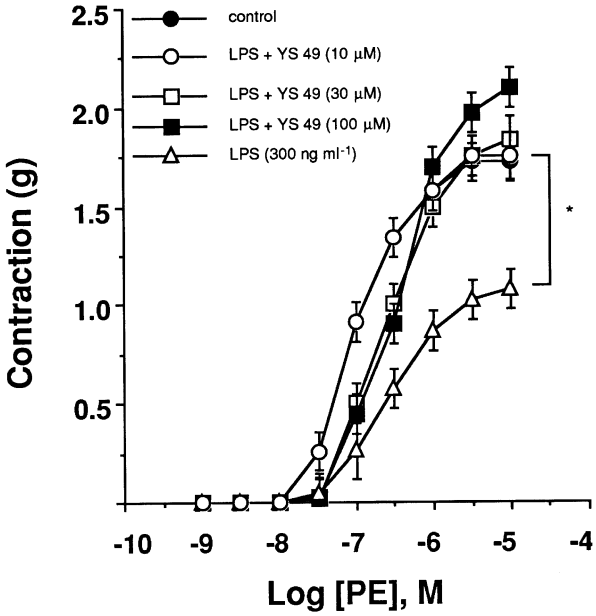


Figure 5 Concentration-response curves to PE in endothelium-denuded aortic rings. Rings were incubated either with Krebs' solution (control), LPS (300 ng ml^{-1}), or different concentration of YS 49 + LPS for 8 h. Values are expressed as contractile force in grams and are means ± s.e.mean of three to five separate experiments. *Significantly different from all other groups at $P<0.05$.

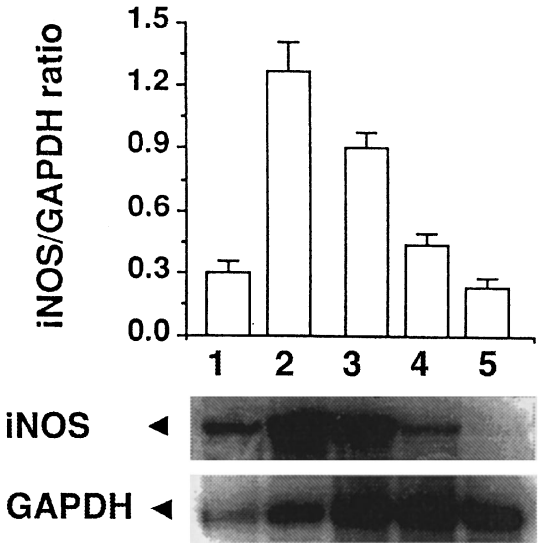


Figure 6 Effects of YS 49 on iNOS mRNA expression in rat aortic smooth muscle incubated with LPS (300 ng ml^{-1}). Aortic rings were incubated for 8 h at 37°C with: 1, Krebs' solution; 2, LPS; 3, YS 49 (10 μM) + LPS; 4, YS 49 (30 μM) + LPS; and 5, YS 49 (100 μM) + LPS. After completion of incubation, isometric force to PE was measured and then iNOS mRNA expression analysed in the same tissues. Upper panel: densitometric analysis of the gel photograph. Representative experiment from at least three. Lower panel: mRNA for iNOS and GAPDH.

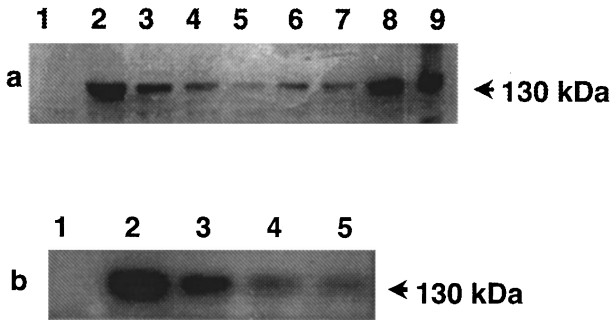


Figure 7 Effects of YS 49 and other related chemicals on iNOS protein expression in LPS + INF-γ stimulated RAW 264.7 macrophages and RAVSMC. (a) Exposure of RAW 264.7 macrophages to LPS (10 ng ml^{-1}) + INF-γ (10 u ml^{-1}) for 18 h resulted in the expression of a 130 kDa protein. This expression was significantly inhibited by treatment of the cells with YS 49, tetrandrine, higenamine or dexamethasone. Lane 1, control; Lane 2, LPS + INF-γ; Lane 3, YS 49 (10 μM) with LPS + INF-γ; Lane 4, YS 49 (30 μM) with LPS + INF-γ; Lane 5, YS 49 (100 μM) with LPS + INF-γ; Lane 6, dexamethasone (0.1 μM) with LPS + INF-γ; Lane 7, higenamine (10 μM) with LPS + INF-γ; Lane 8, tetrandrine (10 μM) with LPS + INF-γ; Lane 9, iNOS protein. (b) In RAVSMC, exposure to LPS (300 ng ml^{-1}) + INF-γ (10 u ml^{-1}) for 18 h also led to the expression of the 130 kDa protein, which was inhibited concentration-dependently by YS 49. Lane 1, control; Lane 2, LPS + INF-γ; Lane 3, YS 49 (10 μM) with LPS + INF-γ; Lane 4, YS 49 (30 μM) with LPS + INF-γ; Lane 5, YS 49 (100 μM) with LPS + INF-γ. This immunoblot is representative of three separate experiments.

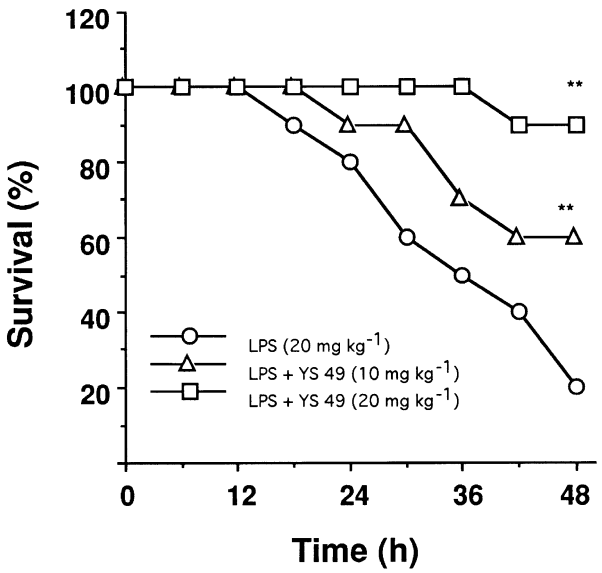


Figure 8 Effects of YS 49 on the survival rate of LPS-treated mice. Each group consisted of 20 animals. Saline was injected (i.p.) in control groups and LPS (20 mg kg^{-1}, i.p.) in LPS-treated groups. Two different doses of YS 49 (10 and 20 mg kg^{-1}) were injected (i.p.) 30 min before the LPS-injection. Survival was monitored every 6 h up to 48 h. **Represents significantly different at $P<0.01$.

Survival rates

The administration of LPS (20 mg kg^{-1}, i.p.) to ICR mice resulted in a 48 h survival rate of only 20% (i.e., 4/20 of animals). In contrast, pretreatment with YS 49 led to a survival rate of 65% (10 mg kg^{-1}, i.p.) and 80% (20 mg kg^{-1}, i.p.) 48 h after LPS injection (Figure 8). Survival in saline- and YS 49-injected groups was 100% after 48 h.

Plasma nitrite/nitrate levels

The serum nitrite/nitrate levels of rats 8 h after injection of LPS (10 mg kg^{-1}, i.p.) was 86 ± 7 μM. This was decreased to 34 ± 5 μM by YS 49 (5 mg kg^{-1}) given 90 min prior to LPS. For comparison, the NOx levels in saline and YS 49 treated animals were 5.2 ± 1.3 and 4.8 ± 1.7 μM, respectively (Figure 9).

Discussion

Here, we clearly demonstrate that YS 49, concentration-dependently, reduces the production of NO and the expression of iNOS protein in LPS and IFN-γ treated RAVSMC and RAW 264.7 macrophages, as well as the increase in mRNA and iNOS expression caused by LPS in rat aortas *in vitro*. Furthermore, YS 49 prevents LPS-induced vascular hyporeactivity *in vitro* and *ex vivo*.

Although YS 49 alone had no effects, it inhibited the LPS and/or IFN-γ-induced nitrite production in a concentration-dependent manner. This inhibitory effect was reduced when YS 49 was added several hours after the cytokines, suggesting that the inhibition of nitrite production caused by YS 49 is due to inhibition in induction of iNOS. Indeed, YS 49 suppressed LPS- or IFN-γ-induced increases in iNOS mRNA and protein levels. These results indicate that YS 49, a novel isoquinoline compound, inhibits cytokine-induced NO production by blocking iNOS expression. This accounts for the ability of YS 49 to prevent vascular hyporeactivity produced by LPS and/or cytokine. These results are in accord with those of others that have shown exposure of various cells to endotoxin alone or together with pro-inflammatory cytokines, such as IFN-γ, leads to the induction of a calcium-independent NO synthase (Busse & Mulsch, 1990; Deakin *et al*., 1995).

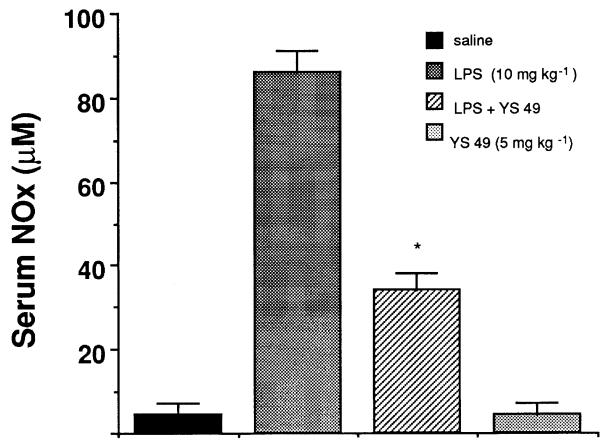


Figure 9 Effects of YS 49 on NOx production in rat plasma 8 h after injection of LPS (10 mg kg^{-1}, i.p.). Blood was collected by cardiac puncture using a 21 G injection needle. Samples were centrifuged and serum fraction was analysed for its content of nitrite/nitrate. As control, saline and YS 49 were injected (i.p.) in separate animals. Data represent mean $\pm$ s.e.mean of three separate experiments. *Significantly different at $P<0.05$.

LPS play a pivotal role in triggering the development of both clinical and laboratory manifestations of gram-negative septicaemia, such as impaired responsiveness to vasoconstrictor agents (Julou-Schaeffer *et al*., 1990; Chang *et al*., 1993b) and sympathetic nerve stimulation (Gray *et al*., 1990). Indeed, blood vessels isolated from animals given endotoxin *in vivo* (Pomerantz *et al*., 1982) and *in vitro* have been shown to express iNOS (Deakin *et al*., 1995), which is believed to be responsible for the diminished vasoconstrictor responsiveness of the vascular smooth muscle. The results of the present study confirm that contractile responses to PE are impaired in aortas from either rats treated *in vivo* with LPS or incubated with LPS *in vitro*. Furthermore, this impairment is associated with induction of iNOS enzyme. Incubation rat aortas with 300 ng ml^{-1} LPS for 8 h has been shown to increase cyclic GMP and to induce relaxation by L-arginine (Moritoki *et al*., 1996). We found similarly that incubation with LPS (300 ng ml^{-1}) for 8 h induced iNOS mRNA expression in rat aortas *in vitro*, which accounts for vascular hyporeactivity to vasoconstricting agent such as PE. In fact, iNOS is a unique eucaryotic enzyme, which is a calmodulin-containing cytochrome p450-like hematoprotein that combines reductase and oxygenase domains and carries out a 5-electron oxidation of L-arginine to produce the free radical, NO. NO promotes relaxation of the smooth muscle *via* activation of guanylate cyclase (Schmidt & Walter, 1994). We also examined the induction of NO synthase in vascular tissues by relaxation studies in which U46619, a thromboxane A_2 (TXA_2) mimetic, was used as a vasoconstrictor, because circulating TXA_2 has been reported to be increased in endotoxic shock (Cook *et al*., 1980) and has been used as a vasoconstrictor to investigate the altered responses of the vasculature in endotoxic models (Vallance *et al*., 1989).

Although L-arginine, but not D-arginine relaxed in a concentration-dependent manner both LPS and YS 49 plus LPS-treated rings, there was a statistically significant difference between these two groups. Of particular interest was the observation that iNOS mRNA was expressed in those aortas that were not incubated with LPS *in vitro*. The possible explanation for this may be contamination with LPS during incubation, since NO synthase has been reported to be induced in the rat aorta at concentrations of LPS as low as 10 ng ml^{-1} that could well be present in the physiological salt solution (Busse & Mulsch, 1990). Another explanation may be a constitutive expression of iNOS in rat aorta (Akyurek *et al*., 1996; Bishop-Bailey *et al*., 1997), such that after prolonged incubation in physiological solutions vascular preparations can show relaxation to L-arginine (Schini *et al*., 1990; Wood *et al*., 1990). In the present study, we found that incubated in Krebs' solution alone for 8 h, also had depressed contractions in response to PE when compared to responses of control *ex vivo* tissues. Furthermore, after completion of contraction studies with PE, administration of L-arginine to the organ baths produced relaxation (data not shown), indicating that NO is involved in this relaxation. Although it is not clear whether the expression of iNOS mRNA in control preparations was due to contamination with LPS or constitutive expression, it is quite certain that iNOS expression in LPS-treated rat aortas renders the vessel not only hyporeactive to PE but also reactive to the prorelaxant effects of L-arginine. Whatever the reason for iNOS mRNA expression in control aortas might be, in vessels also incubated with LPS there was a further increase in expression of iNOS mRNA. It appears that this expression is within smooth muscle cells rather than

endothelial cells for endothelium-denuded preparations were used in this experiment. From the results of Northern as well as Western blot analysis, YS 49 clearly reduced iNOS mRNA and iNOS protein expression in rat vascular smooth muscle and RAW 264.7 macrophages, indicating an action at the transcriptional level.

Although administration of inotropic agents alone did not reverse endotoxin-mediated hypotension in humans (Vincent *et al*., 1990), endotoxin-mediated myocardial depression has been shown to be reversed by combination treatment with L-NNA and dobutamine in experimental animals (Kilbourn *et al*., 1994). Our finding that YS 49 prevents the expression of iNOS raises the following issues: (1) Because the EC_{50} for the positive inotropic action of YS 49 in isolated rat atria was 9.3 μM (Lee *et al*., 1994) and the IC_{50} to inhibit production of NO by LPS was $\sim$30 μM in the present study, agents like YS 49 that have a positive inotropic activity along with the ability to inhibit iNOS protein expression could be beneficial in a condition where myocardial contractility is likely to be depressed due to an overexpression of iNOS, such as is seen in septic shock (Parrat, 1989) or inflammatory cardiac diseases and (2) Agents such as YS 49, which inhibit the expression of iNOS, may be useful in diseases associated with an ongoing local or systemic inflammatory response in which an enhanced formation of NO by iNOS has been reported to be a central pathophysiologic mediator. YS 49 is also effective *in vivo*, as shown in plasma NOx and survival experiments. At the present time, the precise mechanism by which YS 49 prevents iNOS induction is not known. Because suppression of vascular contractility function by LPS requires *de novo* synthesis of protein (McKenna, 1990), the ability of YS 49 to inhibit iNOS induction may be its main mechanism of action. Alternatively, just because direct exposure to LPS induces NO synthase in vascular smooth muscle this does not preclude the intervention of cytokines produced by circulating and/or vascular cells in the induction process. Thus, a possible immunomodulating action through β-adrenoceptors can not be ruled out. In fact, previous research has established that stimulation of β-adrenoceptor inhibits LPS-induced TNF-α production (Monastra & Secchi, 1993; Ignatowski & Spengler, 1995), and some β-receptor agonists have been reported to attenuate the release of TNF-α after administration of LPS to mice (Severn *et al*., 1992; Monastra & Secchi, 1993; Sekut *et al*., 1995). Furthermore, TNF-α, released after LPS treatment, represents a key factor leading to expression of iNOS in macrophages and smooth muscle cells (Busse & Mulsch, 1990). Indeed, anti-TNF-α antibodies can partially inhibit iNOS expression induced by LPS in macrophages or in vascular smooth muscles. Moreover, TNF-α is able to activate of NF-κB (Hohmann *et al*., 1990), which is an important transcription factor involved in the control of iNOS gene expression (Xie, 1997).

In our experiments, tetrandrine and higenamine, which are isoquinoline compounds, also reduced the expression of iNOS protein in RAW 264.7 cells activated with LPS plus IFN-γ. Tetrandrine has also been reported to inhibit the activation of NF-κB and NF-κB-dependent reporter gene expression in rat alveolar macrophages caused by LPS (Chen *et al*., 1997). Therefore, further studies are needed to investigate whether YS 49 may also inhibit TNF-α production as well as NF-κB activation. In this regard, our further studies will focus on whether the isoquinoline moiety may have an inhibitory action of iNOS gene expression.

In conclusion, it is quite clear from both our functional and molecular studies that the ability of YS 49 to suppress iNOS gene expression in the rat thoracic aorta is responsible for the prevention of the LPS-induced vascular hyporeactivity. Thus, the present data demonstrate that YS 49 may be beneficial against the LPS-induced vascular hyporeactivity and may be useful in inflammatory diseases in which enhanced formation of NO, clearly driven by iNOS, is a main causative factor. Furthermore, YS 49 may have additional beneficial effects in cardiac depressive states associated with septic shock or other cardiac inflammatory diseases due to its strong positive inotropic effect (Lee *et al*., 1994) at concentrations that also inhibit iNOS expression.

This study was supported by Korea Science and Engineering Foundation grants (96-0403-1701-3).

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(Received March 11, 1999
Accepted June 22, 1999)