Korean J Physiol Pharmacol
Vol 5: 443-449, October, 2001

THI 52 Inhibits Inducible Nitric Oxide Synthase Gene Expression in RAW 264.7 Cells and Rat Lung Tissue by Lipopolysaccharide

Bog Kyu Lee, Min Kyu Park, Han Geuk Seo, Hye Sook Yun-Choi,[1] Duck Hyung Lee,[2] and Ki Churl Chang

Department of Pharmacology, College of Medicine, and Gyeongsang Institute of Health Sciences, Gyeongsang National University, Jinju 660-751, Korea, [1]Natural Product Research Institute, Seoul National University, Seoul, 460-110, Korea, [2]Department of Chemistry, College of Natural Sciences, Sogang University, Seoul 121-741, Korea

Previously we reported that THI 52 inhibits tumor necrosis factor (TNF)-α mRNA expression in mouse peritoneal macrophages exposed to LPS plus IFN-γ. In the present study, the effects of THI 52 on vascular reactivity *ex vivo*, and iNOS protein expression (rat lung) were investigated in LPS-treated rats. Treatment of THI 52 concentration-dependently reduced not only serum nitrite production but also the expression of iNOS protein in rat lung tissues. Thoracic aorta taken from LPS injected rat for 8 h *ex vivo* resulted in suppression of vasoconstrictor effects to phenylephrine (PE), which was restored by THI 52 (20 mg/kg) 30 min prior to LPS. When measured iNOS activity, treatment of THI 52 concentration-dependently reduced the enzyme activity in RAW 264.7 cells activated with LPS plus IFN-γ. Likewise, iNOS activity was significantly reduced in lung tissues taken those rats that were injected THI 52 prior to LPS injection compared with LPS injection alone. These results strongly suggest that THI 52 can suppress iNOS gene expression induced by LPS, and restore the vascular contractility to PE. Thus, THI 52, a new synthetic isoquinoline alkaloid, may be beneficial in inflammatory disorders where production of NO is excessed by iNOS expression.

Key Words: Nitric oxide, Inducible nitric oxide synthase, LPS, NF-κB, Septic shock

INTRODUCTION

The expression of inducible nitric oxide synthase (iNOS) and the production of large quantities of nitric oxide (NO) may contribute to the pathophysiology of endotoxaemia or sepsis, particularly the associated hypotension and hyporesponsiveness to vasoconstrictor stimuli (Thiemermann and Vane, 1990; Thiemermann et al, 1993). Inhibitors of nitric oxide synthase (NOS) can reverse or prevent the hypotension induced in animals by LPS, hemorrhage and anaphylatic shock. It is suggested that iNOS plays a crucial role in LPS-induced death. The selective inhibitors of iNOS activity and/or iNOS protein expression may be beneficial for the treatment of systemic inflammatory disorders. Tetrandrine, isoquinoline analog, anti-inflammatory agent, was reported to inhibit NF-kB activation in rat alveolar macrophages by lipopolysaccharide (LPS) (Chen et al, 1997). A series of isoquinoline alkaloids significantly suppressed NO production in murine peritoneal macrophages when stimulated with LPS, and they attenuated the LPS-induced hepatitis by suppression of tumor necrosis factor (TNF) production in mice (Kondo et al, 1993a; Kondo et al, 1993b).

The LPS-induced hepatitis and TNF production in mice (Kondo et al, 1993a) and NO production in macrophages (Kondo et al, 1993b) were suppressed by other isoquinolines. We also recently reported that

Corresponding to: Correspondence to: Ki Churl Chang, Department of Pharmacology, College of Medicine, Gyeongsang National University, Jinju 660-751, Korea. (Tel) +82-55-751-8741, (Fax) +82-55-759-0609, (E-mail) kcchang@nongae.gsnu.ac.kr.

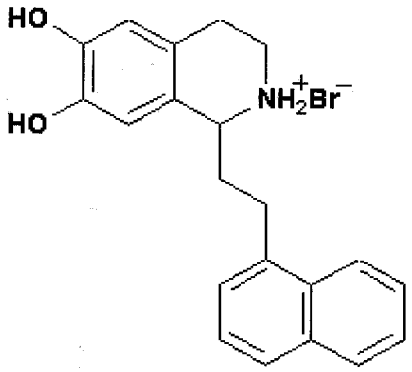

Fig. 1. Chemical structure of THI 52.

THI 52 (Fig. 1) inhibited the expression of TNF-α in mouse peritoneal macrophages activated with LPS plus interferon (IFN)-γ. It is generally accepted that TNF-α is the primary cytokine playing an important role in the induction of iNOS by LPS (Busse and Mulsch, 1990).

Therefore, we investigated whether THI 52 reduces NO production and iNOS protein expression when LPS was challenged to animals from which plasma nitrite concentrations, and iNOS activity, and vascular reactivity were compared. We provide the evidence that THI 52 inhibits iNOS gene expression and restores vascular contractility to vasopressor agent, phenylephrine (PE), and reduces serum nitrite levels.

METHODS

Materials

Lipopolysaccharide (E. Coli; serotype 0128:B12), indomethacin, phenylephrine, HCl, sulphanilamide, N-[1-naphthyl]ethyleneamine, sodium chloride, leupeptin, pepstatin A, phenylmethylsulfonylfluoride, and dithiothreitol were from Sigma (St. Louis, MO. USA). U46619 (9,11-dideoxy-11a, 9α-epoxymethanoprostaglandin $F_{2\alpha}$). iNOS antibody was from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase labeled goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories INC (West Grove, USA). ECL western blotting detection reagent was from Amersham (Buckinghamshire, U.K.).

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATTC, Rockville, MD, USA). The cells were grown in RPMI-1640 medium supplemented with 25 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulphoneic acid (HEPES), 100 u/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated fetal calf serum.

Cell stimulation

RAW 264.7 cells were plated at a density of 1×10^7 cells per 100 mm dish. The cells were rinsed with fresh medium and stimulated with LPS (10 ng/ml) plus IFN-γ (10 u/ml) in the presence or absence of different concentrations of THI 52 ($1\sim30\,\mu$M) simultaneously. THI 52 was dissolved in sterile distilled water and was filtered through a 0.2 μm filter.

Assay for nitrite production

NO was measured as its stable oxidative metabolites, nitrite, as described previously (Kang et al, 1999a). At the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Assay for iNOS protein expression

iNOS protein was analyzed by immunoblotting with the anti-iNOS antibody as described previously (Kang et al, 1999a). Briefly, the lung tissues were homogenized in a buffer containing 50 mM Tris/Cl, pH 7.5, 1 mM EDTA, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol and sonicated. The homogenates were then centrifuged at $7,500\times g$ for 15 min 4 times, and the supernatants were subjected to SDS-PAGE (7.5% gel) using the buffer system of Laemmli (1970). The separated proteins were electrophoretically transferred to PVDF membranes, and the membrane was incubated with anti-iNOS antibody for 2 h followed by peroxidase-labeled goat anti-rabbit IgG for 1 h. Antigen-antibody complexes were detected using ECL Western blotting detection reagents (A-

mersham) according to the manufacturer's instruction.

Plasma nitrite/nitrate measurement

Rats were divided into four groups: (i) LPS (10 mg/kg, i.p., n=4), (ii) LPS plus THI 52 (15 and 20 mg/kg, i.p, n=4), (iii) saline (i.p., n=3), and (iv) THI 52 (20 mg/kg, i.p., n=3). When used THI 52 was given 30 min prior to LPS. Eight hours after LPS treatment a whole blood sample was withdrawn by cardiac puncture after pentobarbital anesthesia.

The plasma nitrate 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 (compositon, mM): KH_2PO_4 50, NADPH 0.6, FAD 5 and nitrate reductase 10 units/ml, pH 7.5, for 30 min at 37°C. Subsequently culture medium was mixed with an equal volume of the Griess reagent (mixture of 1 part of 1% sulfanilamide in 5% phosphoric acid and 1 part of 0.1% naphthylethylenediamine dihydrochloride in water) and incubated at room temperature for 10 min. The absorbance at 550 nm of the mixture was determined by an ELISA plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA), using sodium nitrite as standard.

Measurement of iNOS enzyme activity

Samples were homogenized in 5 volumes of 20 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA. Thereafter, homogenates were centrifuged (10,000× g) for 10 min at 4°C and the supernatant used for iNOS activity. Activity of iNOS was determined using a method based on the conversion of ^{3}H-L-citrulline with minor modification. In these experiments, 50 μL of a sample was incubated at 37°C in the presence of 50 mM Tris-HCl, pH 7.8, 0.5 mM NADPH, 5 μM tetrahydrobiopterin, and 12 μM L-[2,3,4,5-^{3}H]arginine (118 mCi/mmol) in a total volume of 200 μl. The reaction was stopped by the addition of 1 ml of Dowex 50W equilibrated in the same buffer. The samples were then centrifuged and the concentration of L-[^{3}H]citrulline was determined in the supernatant by liquid scintillation counting. Protein was measured by the procedure of Bradford.

Ex vivo vascular reactivity

Sprague Dawley rats (male, 250~300 g weight) were intraperitonially injected with (i) LPS (10 mg/kg n=4), (ii) THI 52 (20 mg/kg, n=4) 30 min prior to LPS, (iii) saline (n=4) or (iv) THI 52 (20 mg/kg, n=4). Eight hours after injection the thoracic aortas were taken under pentobarbital anesthesia. The aortas were cleared of adhering periadventitial fat and cut into rings of 3~4 mm width. Endothelium was removed by gently rubbing the intimal surface with a wooden stick as reported previously (Chang et al, 1993). The rings were mounted in organ bath (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, USA). A tension of 1 g was applied and the rings were equilibrated for 60 min, changing the Krebs' solution every 20 min (Chang et al, 1993). Indomethacin (10 μM) was used to prevent the production of cyclooxygenase metabolites that are predominantly vasoconstrictors in this experimental setting (Wu, 1994) Concentration-response curves PE (1 nM~10 μM) were obtained.

Statistical evaluations

Data are expressed as mean±SEM of results obtained from number (n) of animals used. Differences between data sets were assessed by one way analysis of variance (ANOVA) followed by Dunnett's test. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

THI 52 inhibits NO production in RAW 264.7 cells

Nitrite was accumulated time-dependent manner up to 24 hr. When measured at 18 h time point, nitrite levels from control media was 5±0.8 μM, which was increased 63.5±2.6 μM by LPS+IFN-γ. Pretreatment of THI 52, concentration-dependently, decreased the nitrite (Fig. 2A). The concentration of 50% inhibition of NO production (IC_{50}) was 32 μM. As tested with dexamethasone to compare the relative potency of THI 52, 30 μM THI 52 showed almost the same effect as 10 μM dexamethasone in reducing the

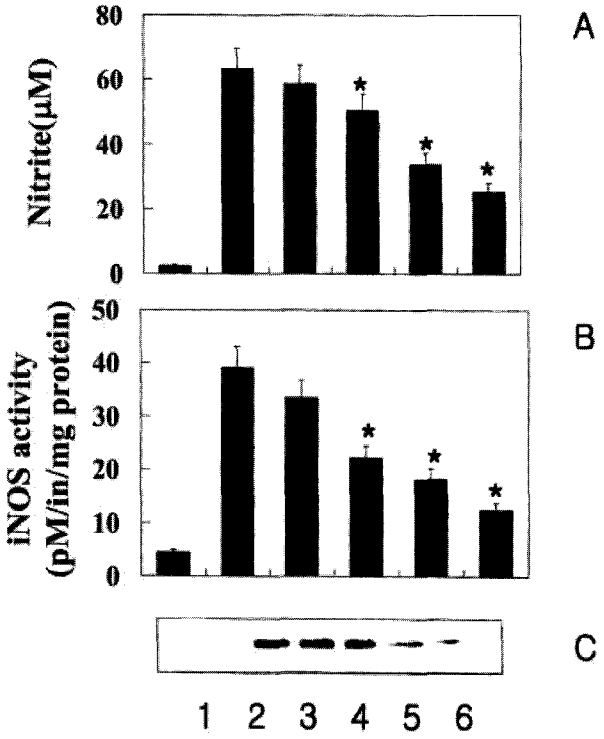

Fig. 2. Effects of THI 52 on NO production (A), iNOS activity (B) and iNOS protein expression (C) in RAW 264.7 cells activated with LPS plus IFN-γ. RAW 264.7 cells were incubated for 12 h without or with different concentrations of THI 52 (10, 20 and 30 μM) after addition of LPS (10 ng/ml) plus IFN-γ (10 u/ml). Aliquots (100 μl) of the culture medium was mixed with an equal volume of Griess reagent. The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards. Arabic numbers indicate that 1; control, 2, LPS plus IFN-γ, 3; THI 52 (10 μM), 4, THI 52 (20 μM), 5; THI 52 (30 μM) and 6, dexamethasone 10 μM. Data represent the mean $\pm$ SEM of triple determinations. *, significantly different from LPS plus IFN-γ group at $P < 0.05$.

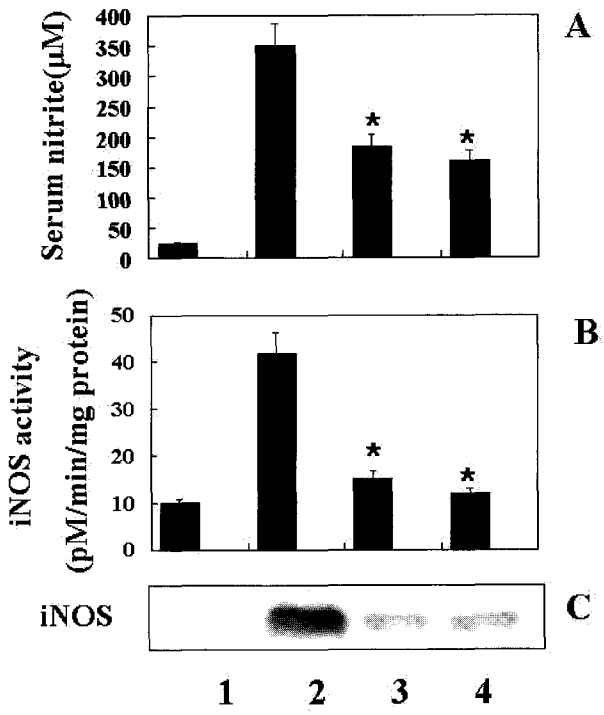

Fig. 3. Concentration-dependent inhibition of serum nitrites (A) and iNOS activity (B) and iNOS protein expression (C) by THI 52 in rats treated with LPS. THI 52 was administered via i.p 30 min prior to injection of LPS. Eight hours later nitrite and iNOS protein were analyzed. Arabic numbers indicate that 1; saline group, 2; LPS group, 3; THI 52 (15 mg/kg) plus LPS group and 4; THI 52 (20 mg/kg) plus LPS group. Data express mean SEM of determinations from a representative experiment performed at least three separate times with comparable results. *, significantly different from LPS group at $P < 0.05$.

production of NO.

Effects of THI 52 on iNOS expression and activity

To know the effect of THI 52 on iNOS expression, Western analysis was performed after 18 h incubated cells. To investigate the reduced production of NO in the medium was due to iNOS protein expression, iNOS protein expression was examined. As shown in Fig. 2C THI 52 significantly reduced the protein expression in a concentration-dependent manner. To further confirm as to whether reduced expression of iNOS protein by THI 52 diminishes the iNOS activity, the iNOS activity was measured. As shown in Fig. 2B, THI 52 significantly reduced iNOS activity. For comparison we used dexamethasone, which was significantly reduced both iNOS protein expression and iNOS enzyme activity. To investigate the inhibitory effect of THI 52 on iNOS expression in vivo, we injected LPS with or without THI 52. Eight hours later, we sacrificed the rats and blood and lung were collected as described in method section. As shown in Fig. 3A, serum nitrite level was significantly increased by injection of LPS. In contrast, treatment with THI 52 significantly reduced the serum nitrite concentration (Fig. 3A). However, THI 52 by itself did not influence the serum level of nitrite (data not

shown). In order to know this reduced serum level of nitrite was due to reduced expression of iNOS protein, we performed Western analysis from the lung tissues. The iNOS protein was significantly diminished by THI 52 (Fig. 3C). To further confirm that THI 52 reduces iNOS protein expression challenged by LPS, we measured the iNOS activity from the same preparation that used in Western analysis. As shown in Fig. 2B, THI 52 significantly reduced the enzyme activity. This indicates that the reduced serum nitrite concentration by THI 52 was the result of diminished expression of iNOS protein.

Vascular reactivity

Aortas from LPS (10 mg/kg)-treated rats caused a significant depression of the contractile ability to PE *ex vivo*, an effect that was prevented by THI 52 (20 mg/kg) pretreatment *in vivo*. As shown in Fig. 4, PE-induced contraction was significantly reduced in LPS-treated groups. However, in those aortas taken from THI 52 plus LPS treated groups, the contractile force to PE was significantly higher compared with those of LPS-treated rats. For example, contractile force to 1 μM PE was 1.2±0.01 g (n=4) in the LPS-treated group, while 2.7±0.17 g (n=4) in THI 52 (20 mg/kg) plus LPS-treated groups, respectively. While THI 52 (20 mg/kg) alone did not affect the contractile ability to PE as compared with saline treated groups, in which the maximum contraction to 10 μM PE was 2.98±0.53 g (n=3) and 2.84±0.47 g (n=3) in saline and THI 52 treated rings, respectively (data not shown).

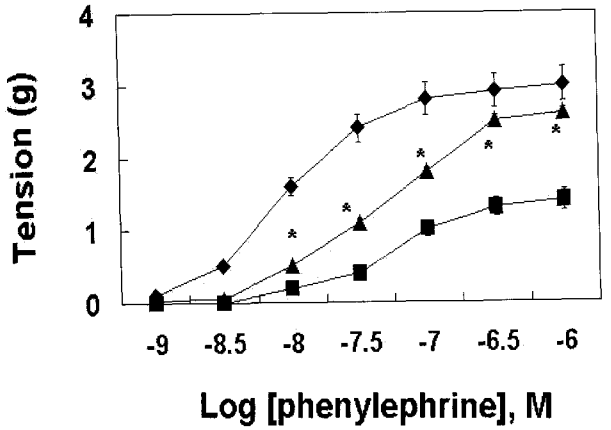

Fig. 4. Effects of THI 52 on LPS-induced vascular hyporeactivity *ex vivo*. Rats were injected (i.p.) with either saline (◆), LPS (10 mg/kg, ■), THI 52 (20 mg/kg) plus LPS (▲), and then sacrificed 8 h after injection. Thoracic aortas were removed and isometric tension was recorded. Comparison of the concentration response curves of PE-induced concentration in aortas taken LPS-treated and LPS plus THI 52-treated rats. Contractile force to PE (10 nM~10 μM) in aortas taken from LPS-treated rats was significantly lower than controls. Contractile force to PE in aortas taken from THI 52+LPS-treated rats was significantly ($P<0.05$) greater than LPS-treated ones. Values are expressed as means± SEM of contractile forces in grams. *Significantly different from all other groups at $P<0.05$.

DISCUSSION

Bacterial LPS (endotoxin) and a number of cytokines including IFN-γ induce an isoform of NO synthase, iNOS, in macrophages, resulting in NO formation which destroys not only bacterial pathogens and tumor cells (Marletta et al, 1990) but modulate local cytotoxicity, edema formation and leukocyte traffic in the pathophysiology of inflammatory disorders (Middleton et al, 1993). Because some analogs of THI compounds are known to inhibit iNOS expression in rat vascular smooth muscles and RAW 264.7 cells (Kang et al, 1999a, 1999b), we want to get more information on the inhibition of iNOS by using 1-naphthyl methyl substituted THI analog. What's the effect on iNOS inhibitory action if structural modification of THI? Is it enhance or decrease the activity? From this kind of information, we would like to design a novel therapeutic agent for iNOS inhibitor.

Therefore, the one of the aims of the present study was to know whether newly synthesized THI analog, THI 52, shows inhibitory action on the production of NO or iNOS activity when RAW 264.7 cells were activated by LPS and IFN-γ. A major finding of the present study is that THI 52, concentration-dependently, reduced NO production in RAW 264.7 cells when activated with LPS and IFN-γ, in which the IC_{50} was 32 μM. We confirmed that THI series of compound related to THI 52 and YS 49 can inhibit iNOS expression in RAW 264.7 cells when activated by LPS+IFN-γ. The inhibition of nitrite accumulation by THI 52 is correlated with a reduction of iNOS protein expression. From the Western analysis, iNOS protein expression is reduced concentration-dependently by THI 52. When tested the iNOS enzyme

activity, THI 52 also decreased the enzyme activity, concentration-dependent manner. This conclusion was based on the result of the reduced expression of iNOS by THI 52. From MTT test, at higher concentration of THI 52, such as 100 μM, showed cytotoxic action (data not shown), which indicates that elongation of carbon side chain may increase the toxicity, while iNOS inhibitory action increases. For example the IC_{50} for reducing NO production of higenamine was 53 μM, YS 49 was 30 μM, in contrast THI 52 was 32 μM. At the present time, the precise mechanism by which THI 52 prevents iNOS induction is not known. Previously we reported that YS 49 and higenamine prevented iNOS expression by inhibiting the activation of NF-kB in RAW 264.7 cells, which is accounted for the mechanism of these chemicals (Kang et al, 1999a; 1999b; Chen et al, 1997).

At the present experiment we do not test the effect of THI 52 on NF-kB activation, however, possible mechanism of action of this compound also involves in the inhibition of NF-kB activation for the following reasons: TNF-α, released after LPS treatment, represents a fundamental factor for iNOS expression in macrophages and smooth muscle cells (Busse & Mulsch, 1990). The presence of anti-TNF-α antibodies can partially inhibit the iNOS expression induced by LPS in macrophages or in vascular smooth muscles. Moreover, TNF-α is able to determine the activation of NF-kB (Hohmann et al, 1990), which is an important transcription factor involved in the control of iNOS gene expression (Xie, 1997). In septic shock, the release of cytokines that activate iNOS in cells such as macrophages and vascular smooth muscle is associated with extreme hypotension that has been reversed by NOS inhibitors in animal shock model of shock (Kilbourn & Belloni, 1990) and in humans (Petros et al, 1991). The effect of THI 52 on the inhibitory action of TNF-α (Jung et al, 2000) and iNOS expression in the present study may be beneficial for the treatment of sepsis or related. There is an abundant evidence that TNF-α is an important mediator of shock and organ failure complicating Gram-negative sepsis (Tracey et al, 1995). The expression of the mouse TNF-α gene is regulated by NF-kB (Langrehr, 1993), and the product of TNF-a gene also causes the activation of NF-kB (Vilcek, 1991) as well as inducing the expression of the iNOS gene (Sherman, 1993). Stamler et al (1992) demonstrated that expression of TNF-α gene in response to LPS is strongly inhibited by the NF-kB inhibitor PDTC. However, that THI 52 reduced TNF-α mRNA expression was reported previously (Jung et al, 2000). We believe that the ability of THI 52 to inhibit TNF-α (Jung et al, 2000) and iNOS protein expression was probably due to inhibition of in NF-kB activation.

Therefore further study as to whether THI 52 inhibits NF-kB activation is needed. In summary, we demonstrated the new THI compound, THI 52, reduced NO production and iNOS expression in RAW 264.7 cells when activated with LPS and IFN-γ. In addition, treatment of THI 52 resulted in reduction of serum nitrite levels, iNOS protein expression as well as iNOS activity in rat lung tissues that had been injected with LPS. We concluded that THI 52 might be beneficial in systemic inflammatory disorders and other diseases, in which enhanced NO is a causative factor by iNOS up-regulation.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Health 21 R & D Project, Ministry of Health and Welfare Republic of Korea (HWP-98-D-4-0045).

REFERENCES

Busse R, Mulsch. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett* 26: 87−90, 1990

Chang KC, Chong WS, Sohn DR, Kwon BH, Lee IJ, Kim CY, Yang JS, Joo JI. Endothelial potentiation of relaxation response to ascorbic acid in rat and guinea pig thoracic aorta. *Life Sci* 52: 37−42, 1993

Chen F, Sun S, Kuhn DC, Lu Y, Gaydos LJ, Shi X, Demers LM. Tetrandrine inhibits signal-induced NF-kappa B activation in rat alveolar macrophages. *Biochem Biophys Res Commun* 231: 99−102, 1997

Hohmann HP, Brockhaus M, Baeuerle PA, Remy R, Kolbeck R, van Loon AP. Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-kappa B. TNF alpha is not needed for induction of a biological effect via TNF receptors. *J Biol Chem* 265: 22409-22417, 1990

Jung TH, Lee YS, Kang YJ, Lee BK, Ko YS, Seo HG, Chung SY, Lee DH, Yun-Choi HS, and Chang KC. Inhibition of tumor necrosis factor-a mRNA expression by a limited series of tetrahydroisoquinoline in mouse

peritoneal macrophages. *Korean J Physiol Pharmmacol* 4: 325-331, 2000

Kang YJ, Koo EB, Lee YS, Yun-Choi HS, Chang KC. 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. *Br J Pharmacol* 128: 357−364, 1999a

Kang YJ, Lee YS, Lee GW, Lee DH, Ryu JC, Yun-Choi HS, Chang KC. Inhibition of activation of nuclear factor kappaB is responsible for inhibition of inducible nitric oxide synthase expression by THI 52, an active component of aconite root. *J Pharmacol Exp Ther* 291: 314−320, 1999b

Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J Natl Cancer Inst* 82: 772−776, 1990

Kondo Y, Takano F, Hojo H. Inhibitory effect of bisbenzylisoquinoline alkaloids on nitric oxide production in activated macrophages. *Biochem Pharmacol* 46: 1887−1892, 1993a

Kondo Y, Takano F, Hojo H. Suppression of lipopolysaccharide-induced fulminant hepatitis and tumor necrosis factor production by bisbenzylisoquinoline alkaloids in bacillus Calmette-Guerin-treated mice. *Biochem Pharmacol* 46: 1861−1863, 1993b

Marletta MA, Tayeh MA and Hevel JM. Unraveling the biological significance of nitric oxide. *Biofactors* 2: 219−225, 1990

Middleton SJ, Shorthouse M, Hunter JO. Relaxation of distal colonic circular smooth muscle by nitric oxide derived from human leucocytes. *Gut* 34: 814−817, 1993

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680−685, 1970

Langrehr JM, Muller AR, Hoffman RA, Simmons RL, Schraut WH, Neuhaus P. Nitric oxide synthesis during rejection of orthotopic small bowel allografts is not a strain-specific phenomenon. *Transplant Proc* 25: 2669, 1993

Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 338: 1557−1558, 1991

Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, Ignarro LJ. Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem Biophys Res Commun* 191: 1301−1308, 1993

Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science* 258: 1898-1902, 1992

Thiemermann C, Vane J. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur J Pharmacol* 182: 591−595, 1990

Thiemermann C, Wu CC, Szabo C, Perretti M, Vane JR. Role of tumor necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. *Br J Pharmacol* 110: 177−182, 1993

Tracey WR, Tse J, Carter G. Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. *J Pharmacol Exp Ther* 272: 1011−1015, 1995

Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J Biol Chem* 266: 7313−7316, 1991

Wu SL. The role of EDRF in the modulation of blood pressure and vascular reactivity in estrogen-treated rats. *Chin J Physiol* 37: 31−35, 1994

Xie QW. A novel lipopolysaccharide-response element contributes to induction of nitric oxide synthase. *J Biol Chem* 272: 14867−14872, 1997