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Suppression of tumor necrosis factor-α and inducible nitric oxide synthase gene expression by THI 52, a new synthetic naphthyl-benzylisoquinoline alkaloid

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Abstract

The effects of THI 52 (1-naphthylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) on (a) inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α) expression in RAW 264.7 cells stimulated by lipopolysaccharide (LPS)/interferon gamma (IFN-γ), (b) plasma nitrate concentration as well as iNOS protein expression (lung) *in vivo* in LPS-treated rats, and (c) the restoration of vascular contractility to vasoconstrictor agents in LPS-treated vessels *in vitro* were investigated. THI 52 concentration-dependently reduced not only nitric oxide (NO) production (IC_{50} value, 12.5 μM) but also the expression of TNF-α and iNOS mRNA in RAW 264.7 cells. Incubation of rat endothelium-denuded thoracic aorta with LPS (300 ng/mL) *in vitro* for 8 hr resulted in the suppression of vasoconstrictor effects to phenylephrine (PE), effects that were restored by co-incubation with THI 52. Administration of THI 52 (10 and 20 mg/kg, i.p.) 30 min before injection of LPS (10 mg/kg, i.p.) resulted in a significant reduction of the expression of iNOS protein in rat lung tissue and in the plasma nitrite/nitrate (NOx) level. Addition of THI 52-treated macrophage-conditioned medium to a TNF-sensitive L929 fibroblast cell line (CCL1) increased cell viability, depending on the concentration of THI 52. Finally, THI 52 inhibited the activation of nuclear factor κB (NF-κB) by inhibition of IκB degradation through the prevention of IκB phosphorylation. Collectively, these results strongly suggest that THI 52 suppresses both TNF-α and iNOS gene expression by inhibiting NF-κB. Thus, THI 52, a new synthetic isoquinoline alkaloid, may be beneficial in inflammatory disorders where the overproduction of NO and TNF-α is a matter of concern.

Keywords: Nitric oxide; Inducible nitric oxide synthase; LPS; NF-κB; TNF-α; Septic shock

1. Introduction

The expression of iNOS and the production of large quantities of NO may contribute to the pathophysiology of endotoxemia or sepsis, in which the associated hypotension and hyporesponsiveness to vasoconstrictor stimuli are noted [1–3]. Moreover, null mutant iNOS mice are resistant to the hypotension and death caused by LPS [4,5]. Thus, it is suggested that iNOS plays a crucial role in LPS-induced death. Many iNOS inhibitors have been reported to be beneficial in endotoxemic conditions. Isoquinoline alkaloids are of special interest because of their pharmacological actions on inflammation and related disorders [6,7]. Traditionally, isoquinoline alkaloids such as tetrandrine have been used for several decades for the treatment of silicosis and arthritis, two disease states associated with considerable inflammatory mediator release [6,7].

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Abbreviations: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NO, nitric oxide; PE, phenylephrine; TNF, tumor necrosis factor.

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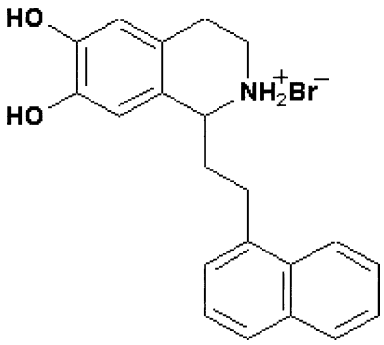

Fig. 1. Chemical structure of THI 52.

Although the mechanisms of action of isoquinoline chemicals on anti-inflammatory and anti-rheumatoid action are not clear, they are related to inhibition of NF-κB [8] or suppression of TNF production [9]. Recently, we reported that the benzylisoquinoline alkaloids higenamine and YS 49 inhibit LPS- and cytokine-induced iNOS mRNA and protein expression in the rat aorta and RAW 264.7 cells [10,11]. Because activation of NF-κB is essential for inducing inflammatory gene expression in many cells, it seemed reasonable to investigate whether isoquinoline compounds inhibit NF-κB. In the hope of developing an active and effective anti-inflammatory drug with an isoquinoline backbone, we synthesized several isoquinoline analogs [12]. Thus, the purpose of the present study was to investigate whether THI 52 (1-naphthylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; Fig. 1), a newly synthesized isoquinoline alkaloid, inhibits NF-κB, which is related to the inhibition of iNOS and TNF-α expression in RAW 264.7 cells stimulated by LPS/IFN-γ. (The procedure for the total synthesis of THI 52 is now in preparation and will be published elsewhere.) We provide evidence in LPS-treated RAW 264.7 cells that THI 52 inhibits the translocation of NF-κB from the cytosol to the nucleus by inhibiting the degradation of IκB via the prevention of IκB phosphorylation and that this is responsible for the inhibition of TNF-α and the iNOS gene expression.

2. Materials and methods

2.1. Materials

LPS (*Escherichia coli*; serotype 0128:B12), indomethacin, PE, HCl, sulfanilamide, *N*-[1-naphthyl]ethyleneamine, sodium chloride, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and DTT were purchased from the Sigma Chemical Co. iNOS antibody was obtained from the Transduction Laboratories. IκBα and p-IκBα antibody were from NEB., Inc. Horseradish peroxidase labeled goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories Inc. ECL western blotting detection reagent was obtained from Amersham Biosciences.

2.2. Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATTC). The cells were grown in RPMI-1640 medium supplemented with 25 mM HEPES, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated FBS at 37° in 5% CO_2 under a humidified atmosphere.

2.3. Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan [13]. After stimulating the cells with LPS (1 μg/mL) for 24 hr in 24-well plates in the presence of THI 52 (0–100 μM, 37°), cells were treated with MTT (0.1 mg/mL) for 3.5 hr. Culture medium was removed by aspiration, and cells were solubilized in DMSO. Changes in absorbance at a wavelength of 570 nm (OD_{570}) were measured using a microplate reader (Bio-Rad model 550).

2.4. Cell stimulation

RAW 264.7 cells were plated at a density of 1×10^7 cells/100 mm dish. The cells were rinsed with fresh medium and stimulated with LPS (1 μg/mL) in the presence or absence of different concentrations of THI 52 (10–100 μM). THI 52 was dissolved in sterile distilled water and was filtered through a 0.2 μm filter.

2.5. Mouse peritoneal macrophage-conditioned medium

Thioglycollate (40 mg/kg) was injected into specific pathogen-free Balb/c mice (male, 20 ± 3 g) at 5 to 8 weeks of age to activate peritoneal macrophages. Peritoneal macrophages (2×10^6 cells/mL) were cultured in RPMI-1640 medium supplemented with 25 mM HEPES, 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37° in a humidified incubator. Cells were dispensed onto 60-mm culture dishes, incubated for 2 hr, and then treated with LPS (100 ng/mL) in the absence or presence of different concentrations of THI 52 (1–100 μM) for 24 hr. Aliquots of this medium were used for testing TNF-α toxicity in CCL1 cells, a TNF-sensitive L929 fibroblast cell line (see below).

2.6. TNF-α toxicity test

TNF-α activity was measured using macrophage-conditioned medium, in which CCL1 cells were cultured and cell viability was determined. Briefly, confluent monolayers of L929 cells were established by incubating fibroblast suspensions (4×10^5 cells/mL in M199 medium, pH 7.4, supplemented with 2% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) in 96-well plates for 24 hr

(37°, 5% CO_2). The supernatants were discarded, and the fibroblast monolayers were covered with fresh M199 medium containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5 mg/mL of actinomycin D (pH 7.4). Aliquots of macrophage-conditioned medium were added to the wells in serial dilution, with the final concentration of THI 52 in the range of 0.1 to 100 μM. The L929 cells were then incubated for a second 24-hr period (37°, 5% CO_2), at which time they were fixed and stained with 5% crystal violet. Dye uptake was determined at 590 nm using a microplate reader (model 550; Bio-Rad Laboratories). Each assay included a blank consisting of actinomycin D alone and internal standards of recombinant murine TNF-α. All samples were assayed in duplicate.

2.7. Assay for nitrite production

NO was measured as its stable oxidative metabolite, nitrite, as described previously [10,14]. 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.

2.8. Analysis of iNOS and TNF-α mRNA expression

Total RNA was extracted as described previously [10]. A 15-μg sample of total RNA per lane was subjected to electrophoresis on 1% agarose gels containing formaldehyde and transferred to nylon filters. The filters were then hybridized with a random-primed ^{32}P-labeled iNOS cDNA probe in rapid hybridization solution (Quikhyb) at 68° for 1 hr. The sequence of the sense primer for iNOS was 5′-TGGACCAGTATAAGGCAAGC-3′ and the antisense primer was 5′-GCTCTGGATGAGCCTATATTG-3′. The probe for TNF-α was an oligonucleotide (5′-TTGACCTCAGCGCTGAGTTCCCCCTTCTCAGCTGGAAGACT-3′) designed from the murine TNF-α mRNA. The hybridized filter was subsequently washed twice for 15 min at room temperature with 2 × SSC (0.3 mM sodium chloride/3 mM sodium citrate)/0.1% SDS and then washed twice for 15 min at 42° with 0.2 × SSC/0.1% SDS. The filter was then exposed to an x-ray film, and was subsequently stripped and rehybridized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9. Assay for iNOS protein expression

iNOS protein was analyzed by immunoblotting with anti-iNOS antibody as described previously [10]. 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 then were sonicated. The homogenates were centrifuged at 7500 *g* for 15 min (4 times) at 4°, and the supernatants were subjected to SDS–PAGE (7.5% gel) using the buffer system of Laemmli [15]. The separated proteins were electrophoretically transferred to PVDF membranes, and the membranes were incubated with anti-iNOS antibody at 4° overnight followed by peroxidase-labeled goat anti-rabbit IgG for 1 hr. Antigen–antibody complexes were detected using ECL western blotting detection reagents (Amersham) according to the instructions of the manufacturer.

2.10. In vitro vascular contractility

As described by Kang *et al.* [11], endothelium-denuded thoracic aortic rings (3- to 4-mm width) were prepared from healthy rats under pentobarbital anesthesia. To determine the effects of THI 52 on iNOS induction *in vitro*, the rings were divided into groups and treated as follows: (i) LPS (300 ng/mL) (N = 24 rings from 4 rats), (ii) THI 52 (1 μM) with LPS (N = 16 rings from 4 rats), (iii) THI 52 (10 μM) with LPS (N = 16 rings from 4 rats), and (iv) control (Krebs solution) (N = 20 rings from 3 rats). The tissues were incubated at 37°, 95% O_2–5% CO_2 for 8 hr. After completion of the incubation, isometric tension experiments were determined as described above.

2.11. Plasma nitrite/nitrate measurement

Rats were divided into four groups: (i) LPS (10 mg/kg, i.p., N = 4), (ii) LPS plus THI 52 (10 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). THI 52 was given via an i.p. route 30 min prior to LPS injection. Eight hours after LPS treatment, a whole blood sample was withdrawn by cardiac puncture after pentobarbital anesthesia. The plasma nitrite/nitrate concentration was determined by reducing the nitrate enzymatically, using nitrate reductase from the *Aspergillus* species. Briefly, plasma samples were diluted 1:10 with normal saline and incubated with assay buffer: 50 mM KH_2PO_4, 0.6 mM NADPH, 5 mM FAD, and 10 U/mL of nitrate reductase, pH 7.5, for 30 min at 37°. 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 of the mixture at 550 nm was determined using an ELISA plate reader (model 550; Bio-Rad Laboratories), with sodium nitrite as the standard.

2.12. EMSA

RAW 264.7 cells were plated at a density of 1×10^7/100 mm dish. The cells were rinsed with fresh medium

and preincubated with THI 52 for 1 hr before addition of LPS plus IFN-γ. The cells were washed three times with cold PBS and harvested by scraping into 5 mL of PBS, and then pelleted at 1000 *g* for 5 min at 4°. The pellets were resuspended in 1 mL of ice-cold lysis buffer (10 mM Tris–Cl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL of leupeptin, pepstatin, and aprotinin) and incubated for 15 min on ice with occasional vortexing. After centrifugation at 3000 *g* for 5 min at 4°, nuclei were washed with 1 mL of wash buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 5 μg/mL of leupeptin, pepstatin, and aprotinin); 30–50 μL of hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 5 μg/mL of leupeptin, pepstatin, and aprotinin) was added to the nuclear pellets and the samples were incubated on ice for 40 min with constant shaking. Nuclear proteins were isolated by centrifugation at 10,000 *g* for 30 min at 4°. The protein concentration in aliquots of the nuclear fractions was determined by the Bradford assay. The nuclear extracts (5 μg) were stored at −70° until used for EMSA. Five micrograms of nuclear protein and 1 μg of poly(dI-dC) per reaction were incubated for 15 min at room temperature with the NF-κB consensus sequence (Santa Cruz Biotechnology), labeled at its 3′-end with ^{32}P. The sequence of the oligonucleotide κB probe was 5′-CAAAACAGGGGCTTTCCCTCCTCA-3′. After the binding reaction, samples were analyzed by electrophoresis on a 6% native polyacrylamide gel that was run in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA), pH 8.0, dried, and then subjected to autoradiography.

2.13. Western blot for IκB and IκB phosphorylation

RAW 264.7 cells were plated in 60-mm diameter culture dishes at a density of 5×10^6 cells and allowed to adhere overnight. Thereafter, fresh medium was added, and the cells were treated with various concentrations of THI 52 (10, 50, and 100 μM) for 1 hr before the addition of LPS (1 μg/mL). After incubation, the cells were scraped off into 50 mM Tris–HCl (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/mL of aprotinin, 5 μg/mL of antipain, 5 μg/mL of pepstatin A, and 5 μg/mL of leupeptin) and sonicated. Proteins were resolved by SDS–PAGE and transferred onto PVDF membranes. The membranes were blocked for 1 hr in TTBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk and incubated for 1.5 hr in primary antisera (anti-rabbit IκB, 1:1000; anti-rabbit p-IκB, 1:1000) containing 1% nonfat dry milk. The blots were then washed four times with TTBS (5 min/wash) and incubated for 45 min at room temperature in horseradish peroxidase-conjugated anti-rabbit secondary antibody at a dilution of 1:7000. The blots were washed three times in TTBS at room temperature. IκB and p-IκB proteins were detected by using ECL reagent.

2.14. Statistical evaluations

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

3. Results

3.1. Effects of THI 52 on NO production

In cultured RAW 264.7 cells, nitrite increased time dependently until 24 hr (data not shown). Co-treatment of LPS-stimulated cells with THI 52 concentration-dependently decreased nitrite levels (Fig. 2A). The THI 52 concentration producing a 50% inhibition of NO (IC_{50}) was 12.5 μM. There was no effect on cell viability up to 100 μM THI 52, the highest concentration used (Fig. 2B).

3.2. Effects of THI 52 on iNOS mRNA expression

To determine if the reduced production of NO by THI 52 was caused by the inhibition of iNOS expression, northern blot analysis was performed. THI 52 concentra-

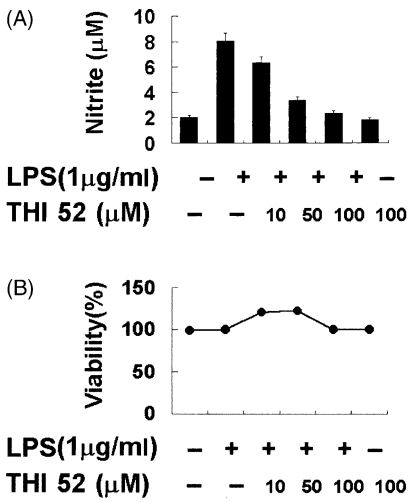

Fig. 2. Effects of THI 52 on NO production (A) and cell viability (B) in RAW 264.7 cells activated with LPS (1 μg/mL). RAW 264.7 cells were stimulated with LPS for 24 hr in the presence of different concentrations of THI 52 (10, 50, and 100 μM). Aliquots (100 μL) of the culture medium were mixed with an equal volume of Griess reagent. The absorbance at 570 nm was measured, and the nitrite concentration was determined using a curve calibrated to sodium nitrite standards. For the viability test, the medium of the treated cells was replaced with fresh medium, and they were incubated further for 3.5 hr in the presence of MTT. Data represent the means ± SEM of triple determinations.

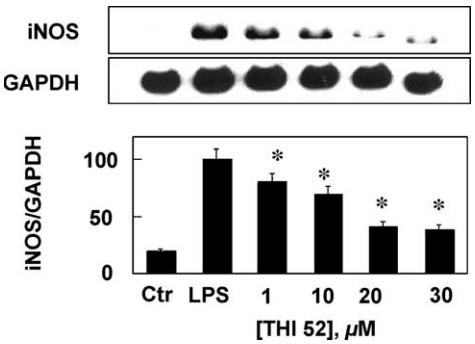

Fig. 3. Concentration-dependent inhibition of the expression of iNOS mRNA by THI 52 in RAW 264.7 cells activated with LPS plus IFN-γ. RAW 264.7 cells were incubated for 8 hr after the addition of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with or without different concentrations of THI 52 (1–30 μM). The total RNA was extracted and subjected to assay by northern blotting. Upper panels: northern blots of expressed iNOS and GAPDH mRNA. Lower panel: relative densitometric intensities of expressed iNOS mRNA and GADPH mRNA. Data are expressed as means ± SEM of determinations from a representative experiment performed at least three separate times with comparable results. Key: (*) significantly different from the LPS plus IFN-γ group at $P < 0.05$.

tion-dependently decreased the expression of iNOS mRNA (Fig. 3). For example, at 1 μM, THI 52 reduced iNOS mRNA expression stimulated with LPS/IFN-γ about 20%; a further decrease (up to 60%) was seen at 30 μM THI 52.

3.3. Effects of THI 52 on TNF-α mRNA expression

When cells were treated with LPS/IFN-γ, TNF-α mRNA was expressed as early as 30 min after exposure and reached a maximum at 2.5 hr (data not shown). TNF-α mRNA expression was concentration-dependently decreased by THI 52, as shown by northern blot analysis data measured at the 2.5 hr time-point (Fig. 4A). To determine whether the reduction in TNF-α mRNA expression induced by THI 52 was functionally related to the reduction of TNF-α toxicity, viability was measured using macrophage-conditioned medium in a TNF-sensitive L929 fibroblast cell line (CCL1). As shown in Fig. 4B, the viability of CCL1 cells increased as the THI 52 concentration was increased in the conditioned medium.

3.4. Effects of THI 52 on LPS-induced vascular reactivity in vitro

To examine the functional role of the inhibitory effect of THI 52 on iNOS expression, we measured vascular contractility *in vitro*. Fig. 5A shows a typical physiological recording of vascular contractility to PE (10 nM–10 μM) in aortas treated with LPS (300 ng/mL) in the absence or presence of THI 52 (1 or 10 μM). Co-incubation of THI 52 with LPS prevented the LPS-induced hypocontractile response to PE. For example, the maximum contractile force to PE (10 μM) was 0.53 ± 0.04 g (N = 4) in the LPS-

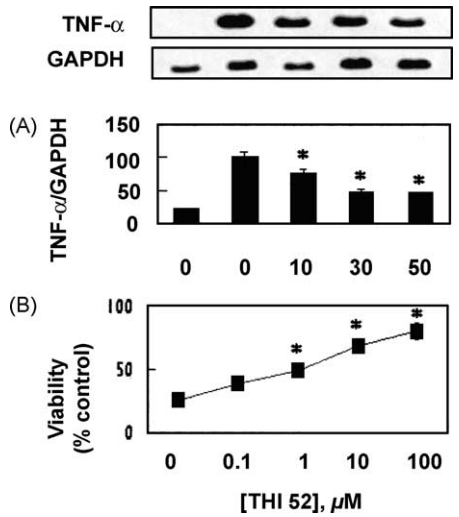

Fig. 4. Effects of THI 52 on inhibition of the expression of TNF-α mRNA (A) in RAW 264.7 cells activated with LPS plus IFN-γ and on TNF-α toxicity (B) in CCL1 cells. RAW 264.7 cells were incubated for 10 hr after the addition of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with or without different concentrations of THI 52 (10–50 μM). The total RNA was extracted and subjected to northern blotting. Upper panel: northern blots showing the expression level of TNF-α mRNA. Lower panel: relative densitometric intensities of expressed TNF-α and GADPH mRNA. In macrophage-conditioned medium, CCL1 cells, a TNF-sensitive L929 fibroblast cell line, exposed to 0.1 to 100 μM THI 52 were cultured and viability was measured after 24 hr. Data are expressed as means ± SEM of determinations from a representative experiment performed at least three separate times with comparable results. Key: (*) significantly different from the LPS plus IFN-γ group at $P < 0.05$.

treated group and 1.38 ± 0.25 g (N = 4) in the THI 52 (10 μM) + LPS-treated group. The concentration–response curves to PE indicate that there was a significant difference ($P < 0.05$) between the two (Fig. 5B).

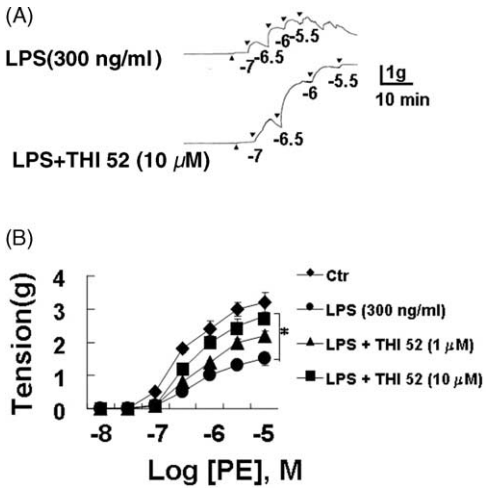

Fig. 5. Effects of THI 52 on LPS-induced vascular contractility by a vasoconstrictor in rat aorta *in vitro*. (A) A representative tracing of PE-induced contractions in rat thoracic aortas that had been incubated with either LPS (300 ng/mL) or LPS + THI 52 (10 μM) for 8 hr *in vitro*. Arabic numbers indicate log molar concentration. (B) Concentration–response curves of PE-induced contractions in control, LPS, and LPS + THI 52 (1 and 10 μM). Data represent the means ± SEM of triple determinations. Key: (*) significantly different from all other groups at $P < 0.05$.

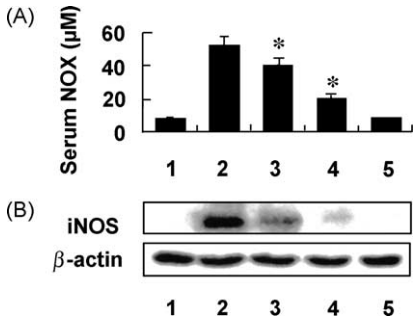

Fig. 6. Effects of THI 52 on plasma nitrite/nitrate levels and iNOS protein expression in lung tissues from rats injected with LPS. (A) Blood samples were collected by cardiac puncture before the rats were killed for the iNOS protein expression experiment; the samples were centrifuged, and the serum fraction was analyzed to measure nitrite/nitrate (NOx) concentrations. Values are expressed as means ± SEM of four separate experiments. Key: (*) significantly different from the LPS-treated group at $P < 0.05$. (B) Pretreatment with THI 52 significantly reduced LPS-induced iNOS protein expression in lung tissues. THI 52 alone had no effect. Lane 1, control; lane 2, LPS (10 mg/kg) alone; lane 3, LPS plus 10 mg/kg of THI 52; lane 4, LPS plus 20 mg/kg of THI 52; and lane 5, THI 52 (20 mg/kg) alone.

3.5. Effects of THI 52 on iNOS protein expression and serum nitrite/nitrate (NOx) in LPS-treated rats

To further confirm the effect of THI 52 on iNOS expression *in vivo*, we examined plasma NOx concentrations and iNOS protein expression in lung tissues of rats injected with LPS (10 mg/kg) with or without THI 52 (10 or 20 mg/kg, i.p.). As shown in Fig. 6A, the concentration of NOx in the plasma after treatment with saline and THI 52 alone (20 mg/kg, i.p.) was 7.72 ± 1.07 and 7.81 ± 1.04 μM, respectively ($N = 4$). Eight hours after LPS injection (10 mg/kg, i.p.), the plasma NOx was elevated to 52.0 ± 6 μM, which was decreased significantly ($P < 0.05$) to 39.8 ± 5.1 and 19.7 ± 2.8 μM by treatment with 10 and 20 mg/kg of THI 52, respectively ($N = 4$). Lung tissues are known to express iNOS protein by LPS in rats and other animals. As expected, the iNOS protein markedly increased in lung tissues after an LPS challenge. However, THI 52 (10 mg/kg) significantly reduced the iNOS protein expression induced by LPS. A higher concentration of THI 52 (20 mg/kg), in particular, almost completely abolished the increased iNOS protein expression by LPS (Fig. 6B).

3.6. Inhibition of activation of NF-κB by THI 52

To investigate the mechanism of the inhibition of iNOS mRNA expression by THI 52, we observed the appearance of the NF-κB–DNA complex in nuclear extracts of RAW cells challenged with LPS plus IFN-γ for 60 min. As shown in Fig. 7, LPS plus IFN-γ caused a significant increase in the level of the NF-κB–DNA complex, which was decreased concentration-dependently by THI 52 treatment.

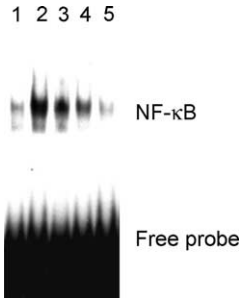

Fig. 7. Concentration-dependent inhibitory action of NF-κB–DNA complex formation by THI 52. Cells were stimulated with LPS plus IFN-γ for 60 min in the absence or presence of THI 52. Nuclear extracts from these cells were obtained and combined with a labeled NF-κB oligonucleotide probe. Lane 1, control; lane 2, LPS/IFN-γ; lane 3, LPS/IFN-γ + 1 μM THI 52; lane 4, LPS/IFN-γ + 10 μM THI 52; and lane 5, LPS/IFN-γ + 100 μM THI 52. Experiments were performed at least three separate times with comparable results.

3.7. Inhibition of degradation of IκB by THI 52

The effect of THI 52 on IκB phosphorylation was investigated to determine a possible mechanism(s) by which THI 52 inhibits the activation of NF-κB. As shown in Fig. 8A, THI 52 concentration-dependently inhibited the translocation of NF-κB (p65) from the cytosol to the nucleus. Next, we investigated whether the THI 52 inhibition of the translocation of NF-κB was due to the phosphorylation state of IκB and, consequently, its degradation. As shown in Fig. 8B, THI 52 concentration-dependently

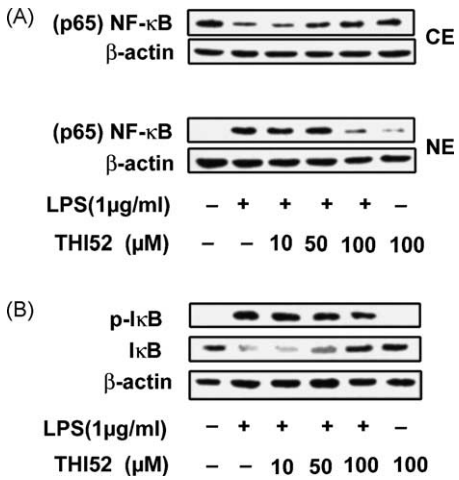

Fig. 8. Inhibitory effects of THI 52 on NF-κB activation. (A) Concentration-dependent inhibition of the translocation of NF-κB (p65) by THI 52. Cells were stimulated with LPS (1 μg/mL) for 60 min in the presence of THI 52 (0–100 μM). Cytosolic extract (CE) and nuclear extract (NE) were isolated and investigated as to whether THI 52 inhibited NF-κB translocation. (B) Concentration-dependent inhibition of phosphorylation of IκB (p-IκB) and consequent decreased degradation of IκB by THI 52. To investigate whether THI 52 inhibits the degradation of IκB (Ser 32) by affecting IκB phosphorylation, p-IκB and IκB proteins were transferred to PVDF membranes for western blot analysis. IκB and p-IκB proteins were detected by using the ECL reagent. Experiments were performed at least three separate times with comparable results.

inhibited the phosphorylation of IκB, which resulted in the reduction of degradation of IκB. Consequently, the activation of NF-κB was prevented by THI 52.

4. Discussion

Because NO is massively generated from iNOS in endothelial and vascular smooth muscle cells several hours after a challenge with LPS, and is known as an important pathologic mediator of endotoxin shock [16], inhibitors of iNOS with various chemical structures are being developed. Previously, we reported that isoquinoline alkaloids such as higenamine inhibit NO production and iNOS induction in vascular smooth muscle cells and RAW 264.7 cells activated with LPS/IFN-γ [10,11]. In the present study, we clearly demonstrated that THI 52 concentration-dependently reduced the production of NO and the expression of iNOS mRNA in RAW 264.7 cells treated with LPS plus IFN-γ. Most importantly, THI 52 restored the vascular contractility to the vasoconstrictor, PE, in rat aortas treated with LPS *in vitro*. These results underscore the importance of the beneficial effect of THI 52 in cases of endotoxemia, since the major problem of endotoxemia in human and experimental animal studies is a rapid lowering of blood pressure (shock) and impaired responsiveness to vasoconstrictor agents (vasoplegia). In fact, LPS plays a pivotal role in triggering the development of both clinical and laboratory manifestations of Gram-negative septicemia, such as impaired responsiveness to vasoconstrictor agents [1,17,18]. Indeed, blood vessels isolated from animals given endotoxin *in vivo* [19] and *in vitro* have been shown to express an iNOS isoform [10], which is believed to be responsible for the diminished vasoconstrictor responsiveness of vascular smooth muscle cells. We confirmed further that incubation for 8 hr with 300 ng/mL of LPS is sufficient for induction of iNOS activity in the vasculature *in vitro* [10]. Although we did not measure expression of the iNOS protein in the rat aorta *in vitro* experiment, we believe that THI 52 can reduce iNOS protein expression in the aorta as it did in lung tissues and in RAW 264.7 cells in the present study. Additionally, we believe that the treatment with THI 52 accounts for the restoration of contractility to PE in LPS-treated vessels. The inducibility of iNOS by LPS has been already shown to be dependent upon the transcription factor NF-κB [20]. The expression of murine macrophage iNOS is regulated largely by transcription activation [21]. The presence of NF-κB-binding sequences in the iNOS promoter [22] and the requirement for nuclear translocation of NF-κB in iNOS induction [23] are necessities for NF-κB activation in iNOS gene expression. Activation of NF-κB, in turn, is regarded as a pivotal event for the development of a variety of proinflammatory mediators such as TNF-α and interleukin-1β. Indeed, chemicals to inhibit the activation of NF-κB, such as calpain inhibitor I [24] or pyrrolidine dithiocarbamate (PDTC) [25], are reported to be beneficial in LPS-induced shock in experimental animal studies. Here, we provide evidence that THI 52 concentration-dependently inhibited the translocation of NF-κB by inhibiting IκB degradation, which results from the prevention of IκB phosphorylation. The concentration of THI 52 necessary for the inhibition of LPS-induced NF-κB activation was similar to that needed to inhibit NO production and iNOS mRNA expression. It seems quite reasonable to speculate that THI 52 may affect NF-κB inducing kinase or IκB kinase activity, thus inhibiting the phosphorylation of IκB, but this theory requires further investigation. Although the cellular and biochemical mechanisms leading to endotoxicity are not understood completely, it is generally accepted that LPS acts via endogenous mediators, mainly produced by mononuclear phagocytes [26]. Among these endogenous mediators, TNF-α seems to be of particular importance [27], since it has been shown to induce all the characteristics for endotoxic shock, and antisera or antibodies against TNF-α attenuate the lethality caused by sepsis or endotoxin [28,29]. There is abundant evidence that TNF-α is an important mediator of the shock and organ failure complicating Gram-negative sepsis [27]. The expression of the mouse TNF-α gene is also regulated by NF-κB [30], and the product of the TNF-α gene also causes the activation of NF-κB [31] as well as inducing the expression of the iNOS gene [32]. That the expression of the TNF-α gene in response to LPS is strongly inhibited by the NF-κB inhibitor PDTC has been reported [32]. These results support the hypothesis that inhibition of TNF-α mRNA expression by THI 52 can result from the inhibition of NF-κB, which is also related to the reduced TNF toxicity in CCL1 cells. In conclusion, we have investigated the effect of a new synthetic isoquinoline alkaloid, THI 52, on NO production, TNF-α mRNA, iNOS mRNA, and iNOS protein expression *in vitro* and *in vivo*. The results show that THI 52 inhibited NO production and reduced the expression of TNF-α and iNOS. All of these effects are related to the ability of THI 52 to inhibit NF-κB. Therefore, these effects may be beneficial in endotoxemic conditions. Besides NF-κB's inhibitory action, other actions such as the antiplatelet-aggregating action of THI 52[1] may be additionally beneficial in cases of endotoxemia. The exact mechanism(s) responsible for the inhibition of NF-κB by THI 52 is now under investigation.

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