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Regulation of lipopolysaccharide-induced inducible nitric oxide synthase expression through nuclear factor-κB pathway and IFN-β/Tyk2/JAK2-STAT-1 signaling cascades by 2-naphtylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (THI 53), a new synthetic isoquinoline alkaloid.

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Running title: Signal pathways of INOS inhibition by THI 53

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemoluminescence; ERK, extracellular regulated kinase; FBS, fetal bovine serum; IFN, interferon; iNOS, inducible nitric oxide synthase; JAK, Janus protein tyrosine kinase; JNK, c-Jun *N*-terminal kinase; LPS,

lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; NO, nitric oxide; SDS, sodium dodecyl sulfate; STATs, signal transducers and activators of transcriptions; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline/Tween 20; THI 53, 2-naphtylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; TNF, tumor necrosis factor

ABSTRACT

The effects of THI 53 (2-naphtylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), on NO production and iNOS protein induction by LPS were investigated in RAW 264.7 cells and mice. In cells, THI 53 concentration-dependently reduced NO production and iNOS protein induction by LPS. In addition, THI 53 inhibited NO production and iNOS protein induction in LPS-treated mice. LPS-mediated iNOS protein induction was inhibited significantly by the specific tyrosine kinase inhibitor AG126 as well as THI 53. In addition, a JNK inhibitor (SP600125), but not an ERK inhibitor (PD98029) or a p38 inhibitor (SB203580), reduced iNOS protein level induced by LPS. Moreover, a Janus kinase 2 (JAK2) inhibitor AG490 dose-dependently prevented LPS-mediated iNOS protein induction. LPS activated phosphorylations of tyrosine kinases, especially Tyk2 and STAT-1; these were reduced by THI 53. LPS also phosphorylated the JNK pathway; however, this was unaffected by THI 53. Interestingly, a JNK inhibitor (SP600125) and another tyrosine kinase inhibitor (genistein) significantly inhibited STAT-1 phosphorylation, suggesting that LPS-activated JNK pathway and a tyrosine kinase pathway (especially Tyk2) may link to the STAT-1 pathway, which is involved in iNOS induction. However, THI 53 regulates LPS-mediated iNOS protein induction by affecting the Tyk2/JAK2-STAT-1 pathway, not the JNK pathway. The inhibition THI 53 on LPS-induced NO production was recovered by a tyrosine phosphatase inhibitor

(Na_3VO_4), which supports that THI 53 inhibits the LPS-induced inflammatory response through regulation of tyrosine kinase pathways. THI 53 also inhibited LPS-mediated IFN-β production and NF-κB activation. Thus, THI 53 may regulate LPS-mediated inflammatory response through both the NF-κB and IFN-β/Tyk2/JAK2-STAT-1 pathways.

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 endotoxemia or sepsis (Thiemermann and Vane, 1990). Moreover, mice carrying the null mutant gene for iNOS are resistant to the hypotension and death caused by lipopolysaccharide (LPS) treatment (Wei et al., 1995; MacMicking et al., 1995). Thus, iNOS might play a central role in LPS-induced death. Many iNOS inhibitors have been reported to be beneficial in endotoxemic conditions. Previously, we reported that isoquinoline analogs inhibit iNOS mRNA and protein expression in rat aorta and RAW 264.7 cells, induced by LPS and cytokines (Tainlin et al., 1982; Kang et al., 1992; Chen et al., 1997). Therefore, isoquinoline alkaloids are of special interest for their pharmacological actions on inflammation and related disorders. In fact, isoquinoline alkaloids such as tetrandrine and higenamine have been used for several decades for the treatment of silicosis and arthritis, two disease states associated with considerable inflammatory mediator release (Kondo et al., 1993; Kang et al., 1999a). The anti-inflammatory and antirheumatoid activities of isoquinoline chemicals might be associated with inhibition of the transcription agent nuclear factor kappa B (NF-κB) (Chen et al., 1997; Kang et al., 1999b) or suppression of the production of tumor necrosis factor (TNF) (Tainlin et al., 1982). However, the exact mechanism is not yet clear.

The induction of iNOS expression involves the activation of multiple signal transduction pathways, including mitogen-activated protein kinases (MAPK) such as p38, ERK1/2 or JNK, NF-κB, PI3 kinase, and Janus tyrosine kinase (JAK)-signal transducers and activators of transcription (STATs) (Dell'Albani et al., 2001; Liu et al., 2001; Tan et al., 2002). Both in vitro and in vivo studies with tyrosine kinase inhibitors have shown that activation of tyrosine kinases is necessary for a number of the biological responses to LPS, including activation of JNK (Dong et al., 1993; Novogrodsky et al., 1994; Hambleton et al., 1996). Moreover, tyrosine kinase 2 (Tyk2) is essential for LPS-induced endotoxin shock (Kamezaki et al., 2004). Tyk2 belongs to the JAK family, and the best-known substrate for these factors is the family of STAT proteins (Rane and Reddy, 2000; Ihle, 1995; 2001). The members of the JAK family, JAK1, JAK2, JAK3 and Tyk2, act as important protein tyrosine kinases (PTKs) (O'Shea et al., 2002; Schindler, 2002). JAK2 is reported to be involved in the LPS-induced expression of iNOS in skin-derived dendritic cells (Cruz et al., 2001); however, little is known about the molecular mechanisms by which JAK2 transduces the LPS-induced signals to downstream molecules to activate proinflammatory genes. Because higenamine (Kang et al., 1999a) and related isoquinolines (Kang et al., 1999b) effectively reduce iNOS gene expression in RAW 264.7 cells and smooth muscle cells by inhibition of NF-κB, we speculate that the isoquinoline molecular backbone may be important for the inhibition of

NF-κB. In this process, phosphorylation of IκBα is vulnerable, hindering translocation of p65 from the cytosol to the nucleus (Kang et al., 2003). Indeed, it is possible to affect the LPS-activated PTKs pathway and its signaling cascade including MAPK or JAK/STATs by isoquinolines. Therefore, the purpose of the present study was to determine whether THI 53 (Fig. 1), a newly synthesized isoquinoline alkaloid, inhibits NO production as well as iNOS expression in RAW 264.7 murine macrophage cells and in mice stimulated by LPS. In addition, we aimed to determine the molecular mechanism by which THI 53 inhibits iNOS induction by LPS in RAW264.7 cells. The procedure for the total synthesis of THI 53 is now being prepared for publication in a relevant journal.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Gibco BRL (Rockville, MD). Anti-iNOS antibody was from Transduction Laboratories (Lexington, KY), and anti-p-Tyk2, anti-p-JNK, and anti-p-STAT-1 antibodies were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase labeled goat anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). AG490, AG126, SB203580, and PD98059 were from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) western blotting detection reagent was from Amersham (Buckinghamshire, UK). All other chemicals, including LPS (*E. Coli* serotype 0128:B12), SP600125 and genistein, were from Sigma-Aldrich. (St. Louis, MO).

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

Cell viability. Cell viability was determined colorimetrically using the MTT assay. Cells at the exponential phase were seeded at 1×10^4 cells/well in 24-well plates. After different treatments, 20 μl of 5 mg/ml MTT solution was added to each well (0.1 mg/well)

and incubated for 4 h, the supernatants were aspirated and the formazan crystals in each well were dissolved in 200 μl dimethyl sulfoxide (DMSO) for 30 minutes at 37 °C, and optical density at 570 nm was read on a Microplate Reader (Bio-Rad, Hercules, CA).

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 (1 μg/ml) in the presence or absence of different concentrations of THI 53 (1–30 μM) simultaneously. THI 53 was dissolved in sterile distilled water and sterilized via a 0.2 μm filter.

Assay for nitrite production. Nitric oxide was measured as its stable oxidative metabolite, nitrite (NOx), as described by 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). Light absorbance was measured at 550 nm, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Western blot analysis. The cells were harvested and lysed with buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and protease inhibitors. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL). To detect iNOS, 20 μg of the total protein was electrophoresed on a 10% polyacrylamide gel, and to detect phosphor-Tyk2, STAT-1 or JNK,

30 μg of the total protein was electrophoresed on a 12% polyacrylamide gel. The gels were transferred to polyvinylidene difluoride (PVDF) membranes by semidry electrophoretic transfer at 15 V for 60–75 min. The PVDF membranes were blocked overnight at 4 °C in 5% bovine serum albumin (BSA). The cells were incubated with primary antibodies diluted 1:500 in Tris-buffered saline/Tween 20 (TBS-T) containing 5% BSA for 2 h and then incubated with the secondary antibody at room temperature for 1 h. Anti-rabbit IgG was used as the secondary antibody (1:5000 dilution in TBST containing 1% BSA). The signals were detected by ECL (Amersham, Piscataway, NJ).

Quantitative mouse IFN-β immunoassay. The quantity of IFN-β secreted into the culture medium was analyzed using a commercially available mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit (catalogue number 42400-1, R&D Systems, Minneapolis, MN), according to the manufacturer's manual. Both the samples and standards were assayed in parallel.

Transfection. Transient transfections with NF-κB-luciferase constructs were as described by Kim et al. (2006) using Lipofectin (Gibco-BRL, Rockville, MD). Briefly, 5×10^5 cells were plated on 60 mm plates the day before transfection and grown to about 70% confluence. Cells were transfected with empty vector (pGL3 and/or pcDNA3), or 1 μg of NF-κB-luciferase + 0.5 μg of pRL-TK-luciferase. Transfections were allowed to proceed for 4 h.

The transfected cells were washed with 4 ml of 1 × phosphate buffered saline (PBS, pH 7.4) and then stimulated with 1 μg/ml LPS. The cells were cultured in serum-free DMEM until harvested. Luciferase activity was normalized using a pRL-TK-luciferase activity (*Renilla* luciferase activity) for each sample.

Luciferase assay. *A*fter experimental treatments, cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega, Madison, WI) and assayed for luciferase activity using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer's protocol. All transfections were done in triplicate. The data are presented as a ratio between Firefly and *Renilla* luciferase activity.

Plasma nitrite/nitrate measurement. Mice (ICR strain, 22-25 g, male) were divided into four groups: (i) LPS (10 mg/kg, i.p., n = 4), (ii) LPS plus THI 53 (20 mg/kg, i.p., n = 4), (iii) saline (i.p., n = 4), and (iv) THI 53 (20 mg/kg, i.p., n = 4). THI 53 was administered 30 min before LPS injection. Eight hours after LPS treatment a whole blood sample was taken by cardiac puncture after anesthetizing the mice with pentobarbital. 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 (composition in mM): KH_2PO_4 50, NADPH 0.6, FAD 5 and nitrate reductase 10 U/ml, pH 7.5, for 30 min at 37 °C. Subsequently, culture

medium was mixed with an equal volume of 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 using a microplate reader. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (NIH publication No. 85-23, revised 1996) and were treated ethically. The protocol was approved in advance by the Animal Research Committee of the Gyeongsang National University, Korea.

iNOS protein detection in LPS-treated mice. 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 *g* for 15 min four times, and the supernatants were subjected to SDS–PAGE (7.5% gels). The separated proteins were transferred electrophoretically to PVDF membranes, and the membrane was incubated with anti-iNOS antibody complexes that were detected using ECL western blotting detection reagents according to the manufacturer's instructions.

Statistical evaluations. Data are expressed as the mean ± SEM of results obtained from the number (n) of animals used. Differences between data sets were assessed by one-

way analysis of variance (ANOVA) followed by Newman-Keuls tests. $P < 0.05$ was accepted as statistically significant.

Results

Effects of THI 53 on LPS-mediated NO production and iNOS protein induction in RAW264.7 cell lines. Previously, nitrite increased time dependently and peaked 18 h after LPS treatment in cultured RAW 264.7 cells (Kang et al., 1999a). Thus, we treated the cells with LPS in the presence of THI 53 (5, 10, 20, 30 μM) for 18 h. The accumulated nitrite was 3.8 ± 1.07 μM in control media, which increased to 19.5 ± 1.24 μM following LPS treatment for 18 h. Cotreatment of THI 53 decreased the nitrites concentration dependently (Fig. 2A). Western blot analysis was performed to determine if the reduced production of NO by THI 53 was caused by the inhibition of iNOS expression. THI 53 decreased LPS-mediated iNOS protein production in a concentration-dependent manner (Fig. 2B). More than 80% of cells were viable at treatments of up to 20 μM THI 53 in the presence of LPS.

Effects of THI 53 on NO production and iNOS protein induction in LPS-treated mice. To confirm further the effect of THI 53 on NO production and iNOS protein induction in vivo, we examined plasma NOx levels and iNOS protein levels in lung tissues of mice injected with LPS (10 mg/kg) with or without THI 53 (20 mg/kg, i.p.). Eight hours after LPS injection, the plasma NOx was elevated to 38 ± 3.5 μM, which was decreased significantly to 19 ± 1.8 μM by treatment with 20 mg/kg of THI 53. Because lung tissues are known to express iNOS protein abundantly when LPS is injected in rats and other animals, we

investigated whether iNOS protein expression was reduced by THI 53 in lung tissues from LPS-treated mice. As expected, the iNOS protein in lung tissue increased markedly after LPS challenge, whereas THI 53 (20 mg/kg) significantly reduced this. Treatment with saline or THI 53 alone (20 mg/kg, i.p.) had no effect on NOx production or iNOS protein expression (Fig. 3).

The differential involvement of MAPK and Janus tyrosine kinase 2 (JAK2) pathways in the iNOS induction by LPS. As mentioned in the introduction, the most extensively investigated intracellular signaling cascades involved in proinflammatory responses such as iNOS expression are the MAPK pathway and JAK/STATs pathway (including Tyk2). Therefore, we first confirmed the role of MAPK or JAK2 on LPS-mediated iNOS induction to clarify the action of THI 53 on the anti-inflammatory response. LPS-mediated iNOS protein induction was significantly inhibited by the specific tyrosine kinase inhibitor AG126 as well as by THI 53 (Fig. 4A). In addition, the JNK inhibitor SP600125 reduced the iNOS protein level induced by LPS, but the ERK inhibitor PD98059 or the p38 inhibitor SB203580 did not affect this (Fig. 4B). Moreover, the JAK2 inhibitor AG490 prevented LPS-mediated iNOS protein induction in a dose-dependent manner.

Effects of THI 53 on the tyrosine kinase pathway and JNK pathway involved in LPS-mediated iNOS expression. Treatment with various kinase inhibitors or with THI 53

showed that increased iNOS protein production induced by LPS occurs via JNK of the MAPK, tyrosine kinase and JAK2 pathways. This suggested that THI 53 may affect LPS-activated kinase pathways. Thus, we next examined whether THI 53 inhibited LPS-mediated phosphorylation of tyrosine kinase, especially Tyk2. Phosphorylation of Tyk2 peaked 60 min after LPS treatment, and this was reduced by treatment with THI 53 (20 μM) (Fig. 5A). In addition, phosphorylation of STAT-1 was first detected 2 h after LPS treatment, peaked at 4 and 8 h, and then decreased (Fig. 5B). The level of phosphorylated STAT-1 was also reduced by THI 53 (20 μM). In addition, JNK was phosphorylated and peaked first at 1 h but again much more strongly at 16 h after LPS challenge (Fig. 5C). When we examined if THI 53 inhibited LPS-phosphorylated JNK at 1 h, we found that THI 53 failed to inhibit JNK phosphorylation by LPS: instead it was enhanced prominently (Fig. 5C).

Next, we aimed to investigate the LPS-related signaling cascade between tyrosine kinase, JNK and JAK-STAT pathways. JNK inhibitor (SP600125) and another tyrosine kinase inhibitor (genistein) inhibited STAT-1 phosphorylation significantly, suggesting that the LPS-activated JNK and tyrosine kinase pathways (especially Tyk2) may link to the STAT-1 pathway (Fig. 5D). Moreover, THI 53 and AG126 (a tyrosine kinase inhibitor) effectively inhibited LPS-induced NO production (Fig. 6A). This effect was counteracted by the tyrosine phosphatase inhibitor Na_3VO_4 in a dose-dependent manner (Fig. 6B, C). This result supports

the hypothesis that the inhibitory effects of THI 53 on LPS-mediated inflammatory response are regulated via tyrosine kinase pathways.

Phosphorylated STAT-1 is involved in iNOS expression. Because JAK/STAT pathway is in the downstream of IFN-β production and THI53 inhibited STAT-1 phosphorylation in our results, finally, we'd like to confirm if THI 53 inhibits iNOS expression by LPS through the regulation of IFN-β/JAK-2-STAT-1 pathway. Thus, we investigated the effect of THI 53 on the induction of IFN-β by LPS. As expected, LPS induced IFN-β production in a time dependent manner, started to increase prominently at 2 h and showed dramatic increase of IFN-β production at 4 h of LPS treatment, which was efficiently inhibited by treatment of THI 53 (Fig. 7).

Effect of THI 53 on the activation of NF-κB by LPS. LPS activates IκB kinase and MAP kinases, which result in the activation of NF-κB and AP-1. The inducibility of iNOS depends upon NF-κB (Xie et al., 1994). Therefore, we investigated the effect of THI 53 on NF-κB activation by LPS. As expected, THI 53 appeared to inhibit NF-κB activation by LPS, when determined by western blot analysis and luciferase assay (Fig. 8A, B). However, this inhibitory effect of NF-κB activation by THI 53 was not pronounced, which means that the LPS-mediated inflammatory response may be mediated through both the NF-κB and Tyk2/JAK2-STAT-1 pathways.

Discussion

We demonstrated here that THI 53 reduced NO production and iNOS protein induction concentration dependently in LPS-treated RAW 264.7 cells. 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 (Kilbourn et al., 1990). Therefore, selective inhibitors of iNOS with various chemical structures have been used as tools for the investigation of LPS-induced sepsis in animals. Some isoquinoline alkaloids inhibit NO production and iNOS induction in vascular smooth muscle cells and RAW 264.7 cells activated with LPS and/or IFN-γ (Kang et al., 1999a,b). We have confirmed that THI 52 inhibits both TNF-α and iNOS mRNA expression in RAW 264.7 cells activated with LPS via regulation of NF-κB activation (Kang et al., 2003). In the present study, we showed that THI 53, a newly synthesized isoquinoline alkaloid and a structural analog of THI 52, blocked NO production and iNOS protein induction in RAW264.7 macrophage cells and in lung tissues from mice following LPS stimulation. In addition, THI 53 inhibited LPS-mediated phosphorylations of Tyk2 and STAT-1, which are involved in iNOS expression. Furthermore, we demonstrated that LPS-phosphorylated JNK was also involved in iNOS induction; however, THI 53 did not inhibit the LPS-activated JNK pathway. Phosphorylated JNK peaked twice following LPS treatment, once at 1 h and later at

16 h, whereas phosphorylated STAT-1 peaked at 4–8 h. Therefore, we presume that LPS may activate Tyk2 and JNK, followed by activation of JAK2/STAT-1 pathways; thereafter, JNK is phosphorylated again by activated STAT-1 (Fig. 9).

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 (Nathan, 1987). LPS is mainly triggered and regulated by a series of signaling pathways including the NF-κB transcription factor and MAPKs (Marczin et al., 1993; Paul et al., 1995). There is one report that LPS activates MAP kinases to induce COX-2 but not iNOS in RAW 264.7 cells (Paul et al., 1999). In contrast, many chemicals inhibit iNOS induction by blocking MAPKs in macrophages activated by LPS (Lee et al., 2000; Chakravortty et al., 2001). In the present study, we found that the specific JNK inhibitor SP600125 effectively reduced LPS-induced iNOS protein levels; however, the specific ERK1/2 inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 did not inhibit iNOS induction by LPS. This suggests a differential role of MAPK in LPS-mediated iNOS induction even though THI 53 did not inhibit the JNK pathway activated by LPS.

LPS is the major active agent in the pathogenesis of endotoxin-mediated shock. The binding of LPS to toll-like receptor 4 (TLR4) leads to the activation of monocytes and

macrophages, which then release cytokines and NO. LPS activates both MyD88-dependent and MyD88-independent pathways, each of which leads to the activation of MAPKs and NF-κB. In addition to the signaling cascade downstream of TLR4 (Akira, 2003), IFN-β and IFN-γ are also involved in sensitivity to LPS. Deficiencies in the IFN-β (Karaghiosoff et al., 2003) or IFN-γ receptors (Car et al., 1994) result in resistance to high dose LPS challenge. Kamezaki et al. (2004) reported that the LPS-induced activations of NF-κB and AP-1 were unaffected by the absence of Tyk2, and that IFN-β and γ signals activated by LPS challenge were severely affected in Tyk2-deficient mice. Tyk2 is a member of the JAK family and has been demonstrated to play a restricted role in IFN-α/β signaling and to have an important role in IL-12 signaling (Karaghiosoff et al., 2000; Shimoda et al., 2000). Some IFN-α/β-induced biological activities, such as inhibition of the growth of bone marrow progenitor cells, and NO production from macrophages after LPS stimulation, were abrogated in the absence of Tyk2. In addition, it has been reported the role of STAT-1 in the induction by LPS of the iNOS gene in mouse macrophages (Gao et al., 1998). From these reports, we speculate that there may be two pathways related to LPS challenge: one via NF-κB and the other via the link between Tyk2 and IFN. Our results also showed that THI 53 effectively inhibited LPS-induced iNOS expression, LPS-activated Tyk2 and STAT-1 phosphorylation, but its inhibitory effect on LPS-activated NF-κB was relatively weak. In fact, Fig. 7 showed that LPS

treatment induced IFN-β production in a time dependent manner, which is efficiently inhibited by treatment of THI 53. This may be because the LPS-mediated inflammatory response is mediated through not only the NF-κB pathway but also the IFN-β/Tyk2/JAK-2-STAT-1 pathway. Our result is supported by Jung et al. (2005), where TLR4 agonists such as LPS induced NF-κB activation and IFN-β production in microglia, and neutralizing Ab against IFN-β attenuated TLR4-mediated microglial apoptosis. IFN-β alone, however, did not induce a significant cell death.

Okugawa et al. (2003) reported that JAK2 regulated phosphorylation of JNK by LPS in RAW264.7 cells, and we showed that inhibition of JNK pathway with the specific JNK inhibitor SP600125 reduced phosphorylated STAT-1. Furthermore, we found that LPS activated the JNK and Tyk2-JAK2/STAT-1 pathways; consequently, phosphorylated STAT-1 reactivates JNK. The major function of the JAK family kinases is considered to be activation of STAT. Thus, from these reports, we can conclude that LPS-induced activation of JAK2 protein tyrosine kinase pathway in macrophages is central to mediation of inflammation through Tyk2/JAK2-STAT-1. In addition, we wish to highlight the role of JNK in LPS-induced inflammation by activating STAT-1 phosphorylation or by affecting other transcription factors such as AP-1, even though JNK is not a point where THI 53 acts to inhibit the LPS-mediated iNOS induction. We conclude that the ability of THI 53 to suppress

NO production and iNOS expression by LPS is via regulation of the Tyk2 pathway linked to JAK2-STAT-1, and via NF-κB activated by LPS. Thus, THI 53 is highly likely to be therapeutic in conditions where upregulation of iNOS is the main cause of health problems, such as septic shock.

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Footnotes

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Legends for Figures

Fig. 1. The structure of the isoquinoline compound THI 53: 2-naphtylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline.

Fig. 2. Effects of THI 53 on NO production and iNOS protein level (A), and cell viability (B) in RAW 264.7 cells activated with LPS (1 μg/ml). (A) RAW264.7 cells were stimulated with LPS for 18 h in the presence of different concentrations of THI 53 (5, 10, 20 and 30 μM). After treatment of LPS with and without THI 53 for 18 h, 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. Treatment with THI 53 reduced LPS-induced iNOS protein levels in RAW 264.7 cells. (B) Cell viability was determined by MTT assay as described in the methodology. Data represent the mean ± SEM of triple determinations. One-way analysis of variance was used to compare group means, followed with Newman–Keuls tests (significance compared with the control, ** $P < 0.01$; significance compared with LPS, † $P < 0.05$ or ‡ $P < 0.01$).

Fig. 3. Effect of THI 53 on plasma NOx levels and iNOS induction in LPS-treated mice. Mice were pretreated with 20 mg/kg of THI 53 for 30 min. Thereafter, mice were activated

by LPS (10 mg/kg) for 8 h, and plasma NOx (A) or iNOS protein levels in the lung tissues were measured following euthanasia. Data represent the mean ± SEM of three experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls tests (significance compared with the control, ** $P < 0.01$; significance compared with LPS, $^{‡}P < 0.01$).

Fig. 4. Signaling pathway involved in LPS-induced iNOS expression in RAW264.7 cells. RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 18 h after 1 h pretreatment with various kinase inhibitors, and iNOS protein levels from cell lysate were detected by western blotting. (A) AG126, a tyrosine kinase inhibitor given together with THI 53 reduced the iNOS level induced by LPS. (B) SP600125, a specific JNK inhibitor, significantly inhibited LPS-mediated iNOS expression, but an ERK1/2 inhibitor PD98059 or the p38 inhibitor SB203580 did not. (C) AG490, a Janus kinase 2 (JAK2) inhibitor, also blocked LPS-induced iNOS expression. The results were confirmed by two independent experiments.

Fig. 5. Inhibition of THI 53 on LPS-mediated Tyk2 and STAT-1 phosphorylation. Cell lysates were extracted from cells treated with LPS at the indicated times, and western blot analysis was performed using antibodies to phosphorylated Tyk2 and Tyk2 (A), to phosphorylated

STAT-1 and STAT-1 (B), or to phosphorylated JNK and JNK (C). Pretreatment with THI 53 (20 μM) prevented activation of the LPS-induced Tyk2 or STAT-1 pathway; this did not prevent JNK phosphorylation by LPS but enhanced it. (D) To investigate the involvement of tyrosine kinase or JNK in the STAT-1 pathway by LPS, cells were treated with a tyrosine kinase inhibitor (genistein; GT) or a JNK inhibitor (SP600125). The experiments were repeated twice.

Fig. 6. The inhibitor of tyrosine phosphatase, Na_3VO_4, reversed the inhibition of NO production caused by THI 53 or by the tyrosine kinase inhibitor AG126 in dose-dependent manners. (A) THI 53 (20 μM) or AG126 (20 μM), a tyrosine kinase inhibitor, reduced NO production in LPS-treated RAW 264.7 cells. Cells were pretreated with the tyrosine phosphatase inhibitor Na_3VO_4 (1 μM or 5 μM for 1 h) and were then stimulated with LPS. The tyrosine phosphatase inhibitor reversed the inhibition of LPS-induced NO production caused by THI 53 (B) or AG126 (C). Data represent the mean ± SEM of triple determinations. One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls test (significance compared with the control, ** $P < 0.01$; significance compared with LPS, † $P < 0.05$ or ‡ $P < 0.01$; significance compared with THI 53 or AGS126, § $P < 0.05$ or §§ $P < 0.01$).

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Fig. 7. Effects of THI 53 on LPS-induced IFN-β production in RAW 264.7 cells. ELISA was performed using the supernatant from the LPS-treated cells in a time-dependent manner. Before LPS treatment, cells were pretreated with THI 53 (20 μM) for 1 h. The data are presented as means ± SEM of three independent experiments. One-way analysis of variance was used for comparisons of the multiple group means followed by Newman–Keuls test (significance compared to the control, ** $P < 0.01$; significance compared with LPS, $^{\dagger} P < 0.05$ or $^{\ddagger} P < 0.01$).

Fig. 8. Effects of THI 53 on LPS-induced NF-κB activation. (A) Cells were pretreated with THI 53 (5, 10 or 20 μM) for 1 h and then treated with LPS (1 μg/ml) for 1 h. After treatment, nuclear or cytoplasmic fractions were extracted and NF-κB (p65) protein level was determined by western blot analysis as described in the methodology. The data are from two independent experiments. (B) Cells were transfected with empty vector or 1 μg of NF-κB-luciferase plus 0.5 μg of pRL-TK-luciferase. Cells were allowed to recover for 24 h and then treated with 1 μg/ml of LPS with/without THI 53 (5, 10 or 20 μMl). Cells were harvested 1 h after treatment. Luciferase activities are presented as fold activation relative to that of the untreated cells. Results are presented as the mean ± SEM of three independent experiments.

One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls test (significance compared with the control, * $P < 0.05$; significance compared to the LPS, $^{\dagger} P < 0.05$).

Fig. 9. Possible mechanisms by which THI 53 inhibits LPS-mediated inflammatory responses such as iNOS induction or NO production. LPS activates Tyk2 and JNK pathways, which are linked to the STAT-1 and NF-κB pathways, resulting in iNOS induction. THI 53 can affect the IFN-β/Tyk2/JAK2-STAT-1 and NF-κB pathways but not the JNK pathway.

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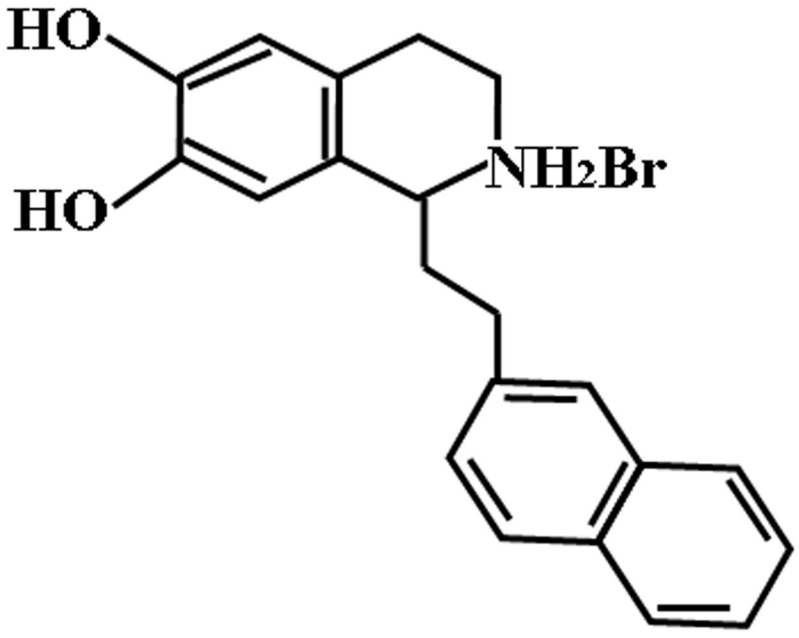

THI53

Figure 1

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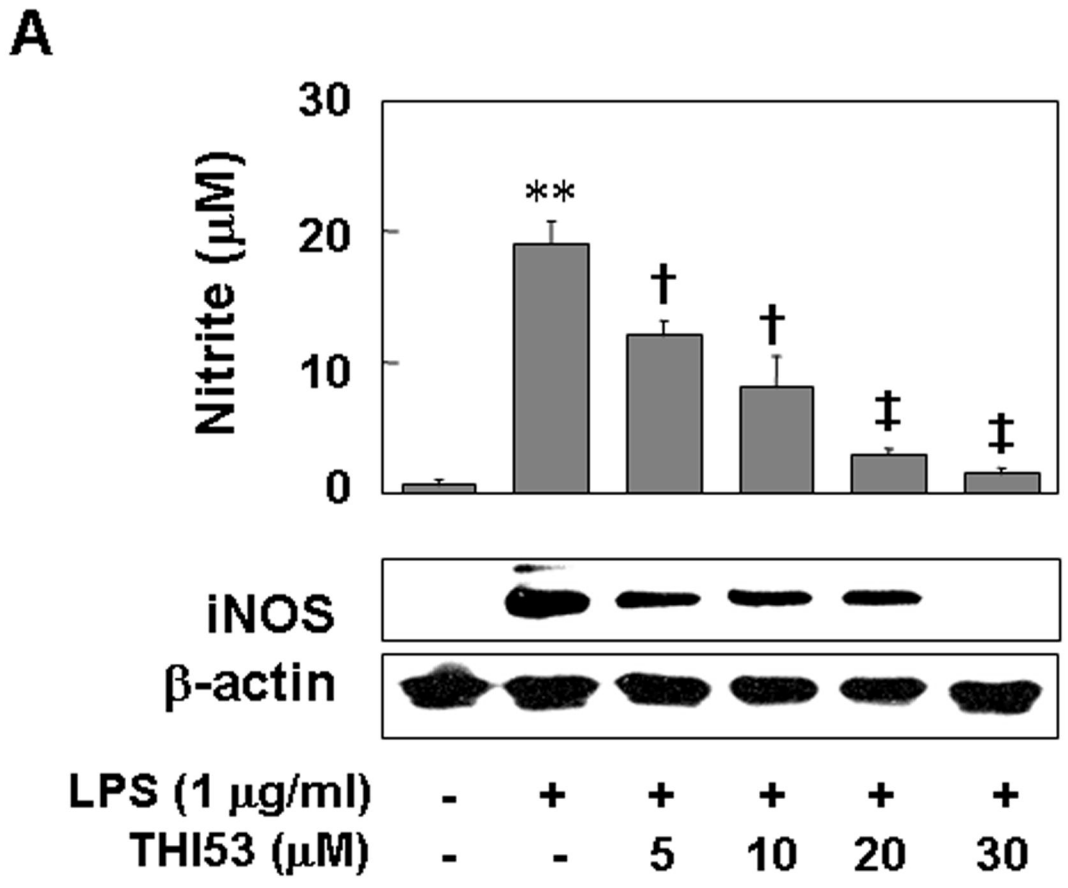

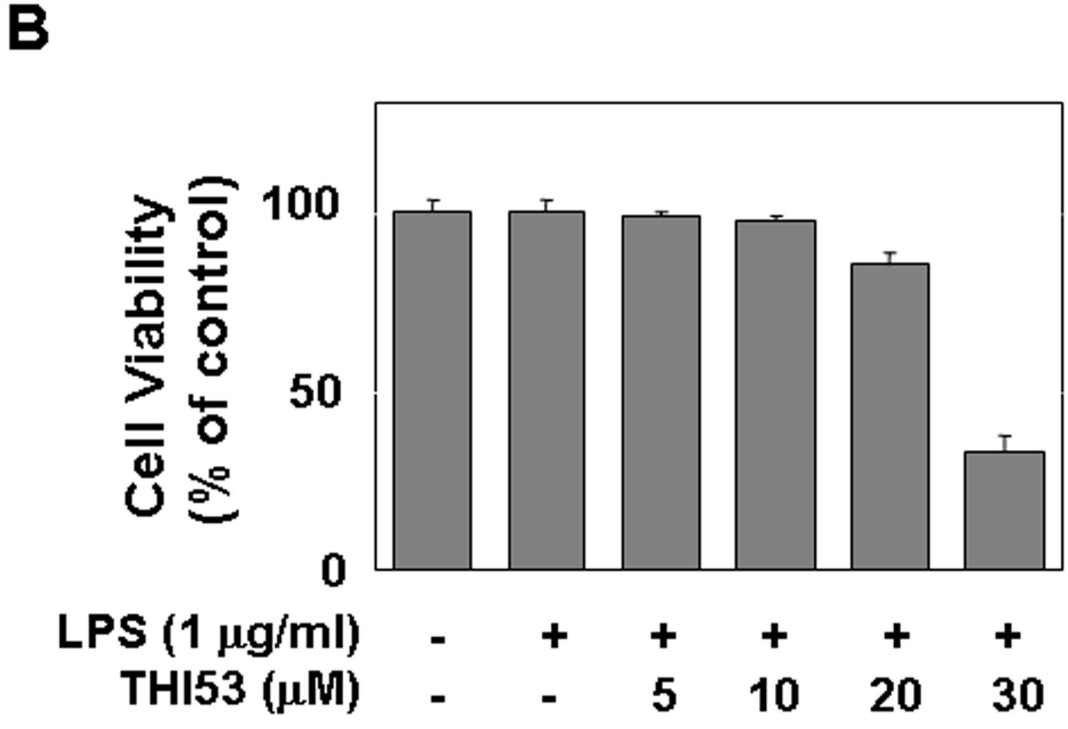

Figure 2

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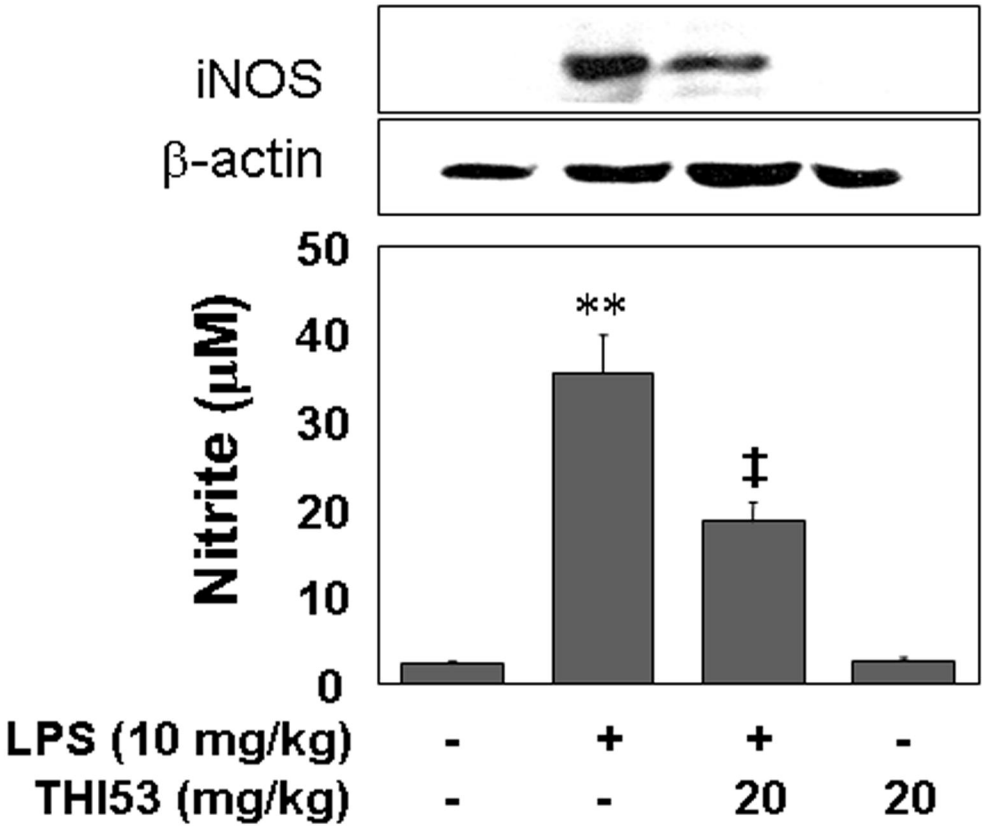

Figure 3

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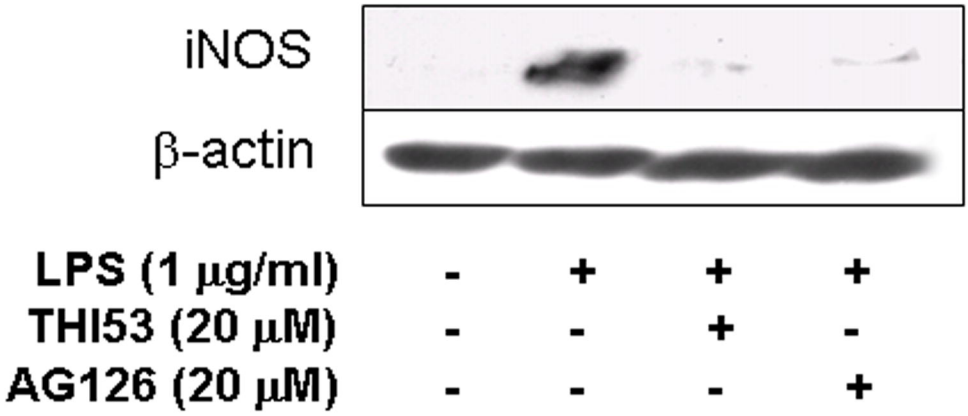

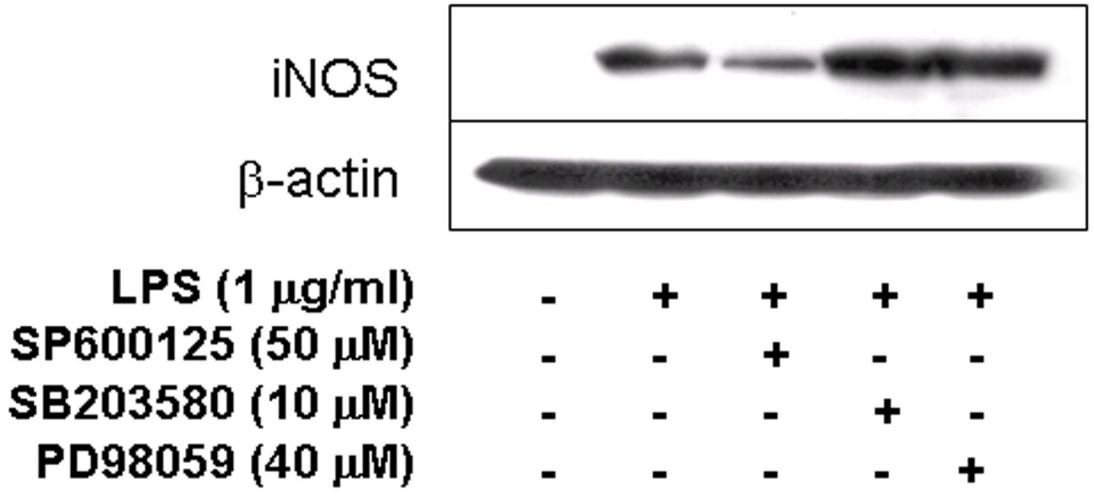

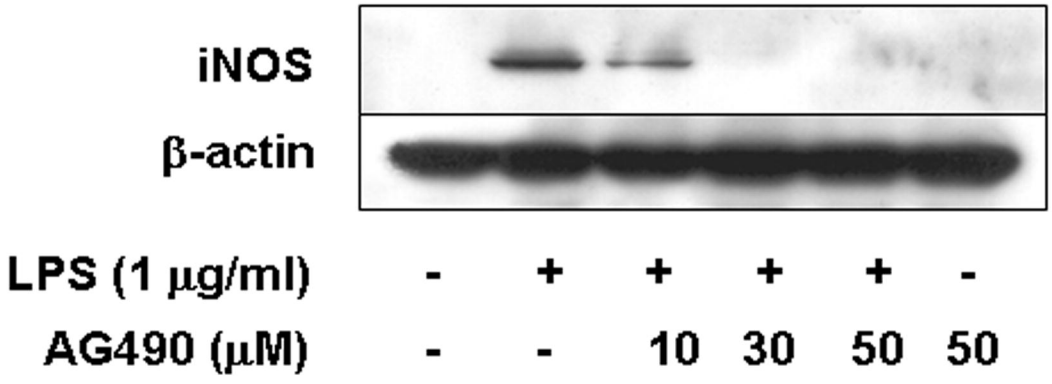

Figure 4

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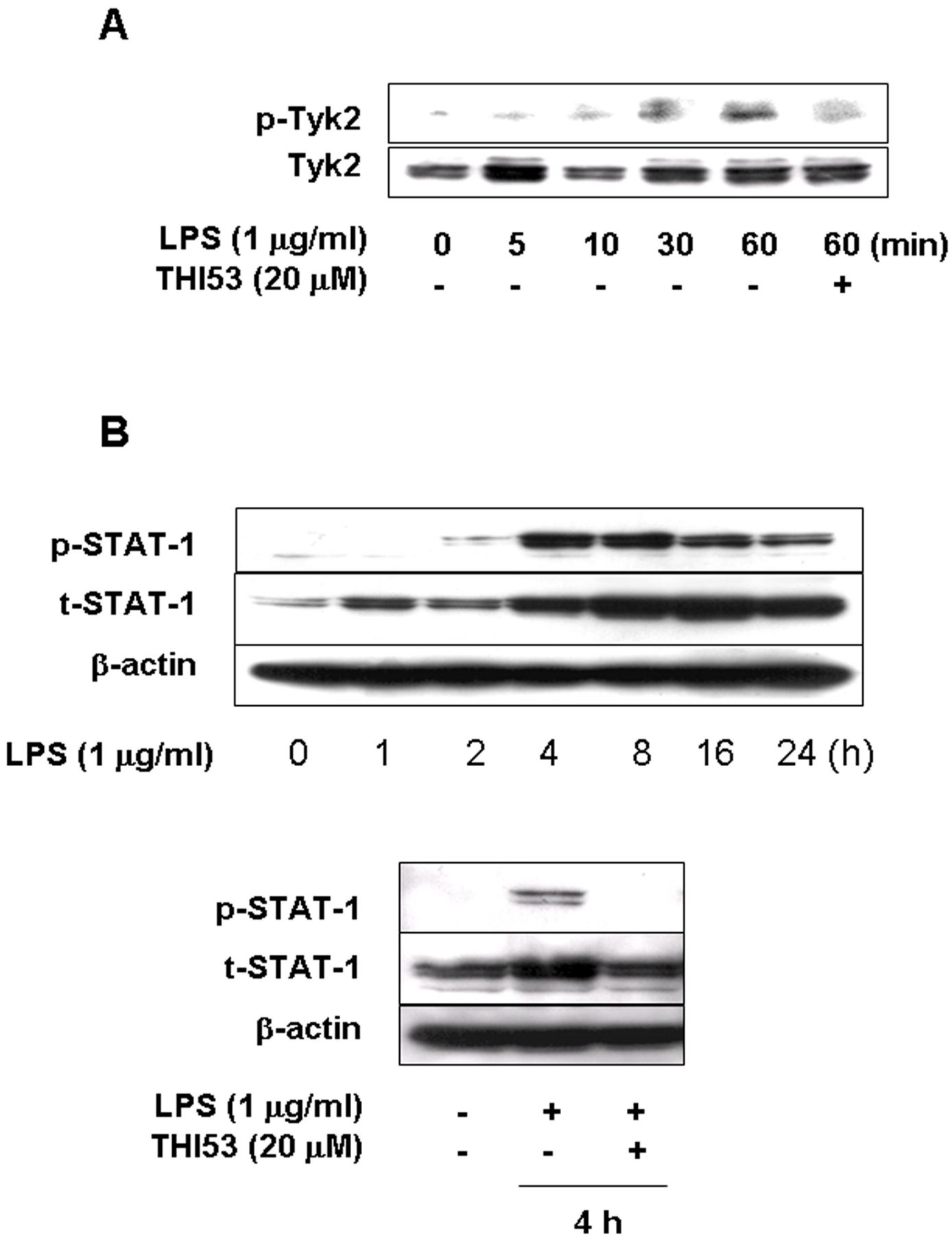

Figure 5-1

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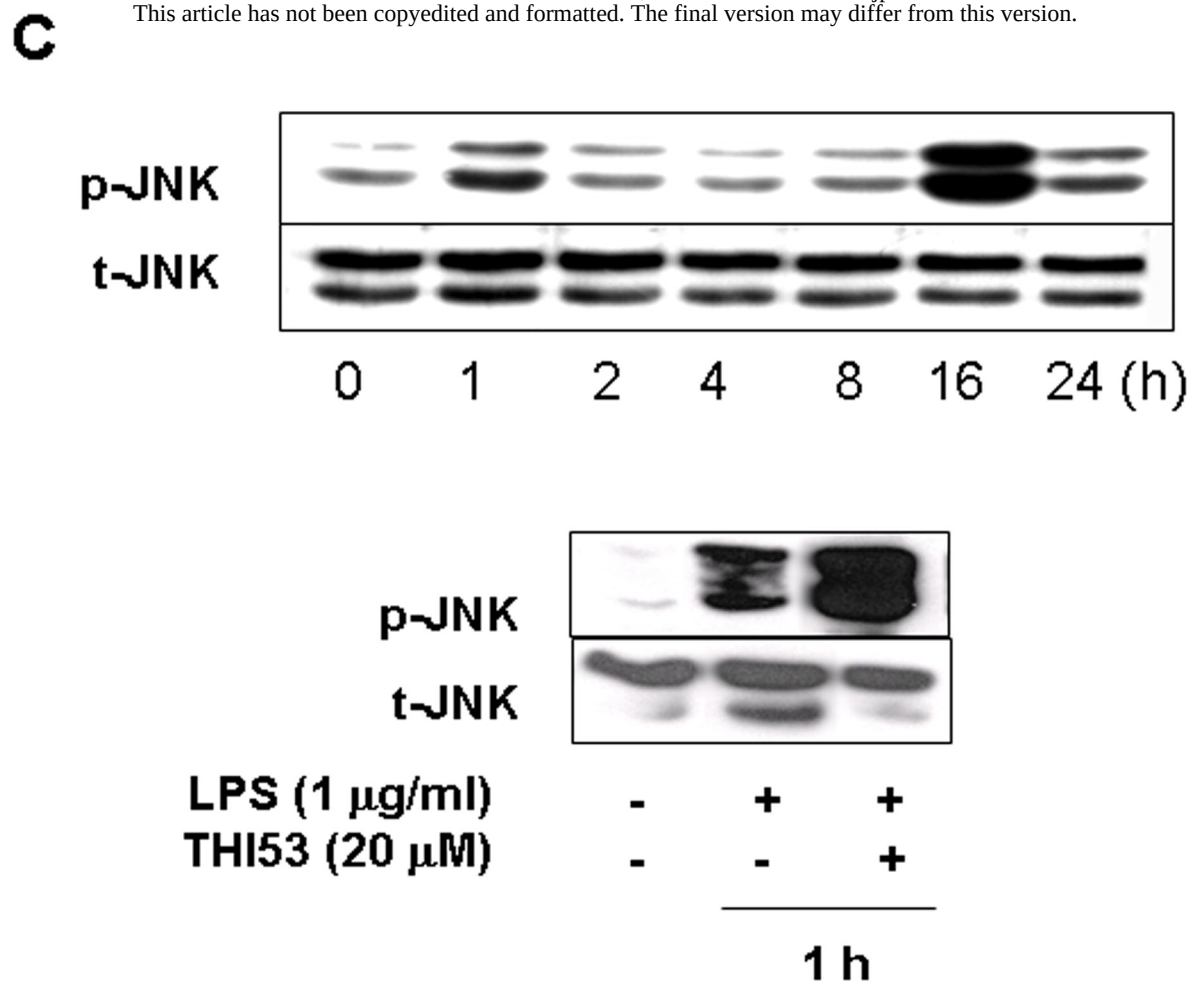

Figure 5-2

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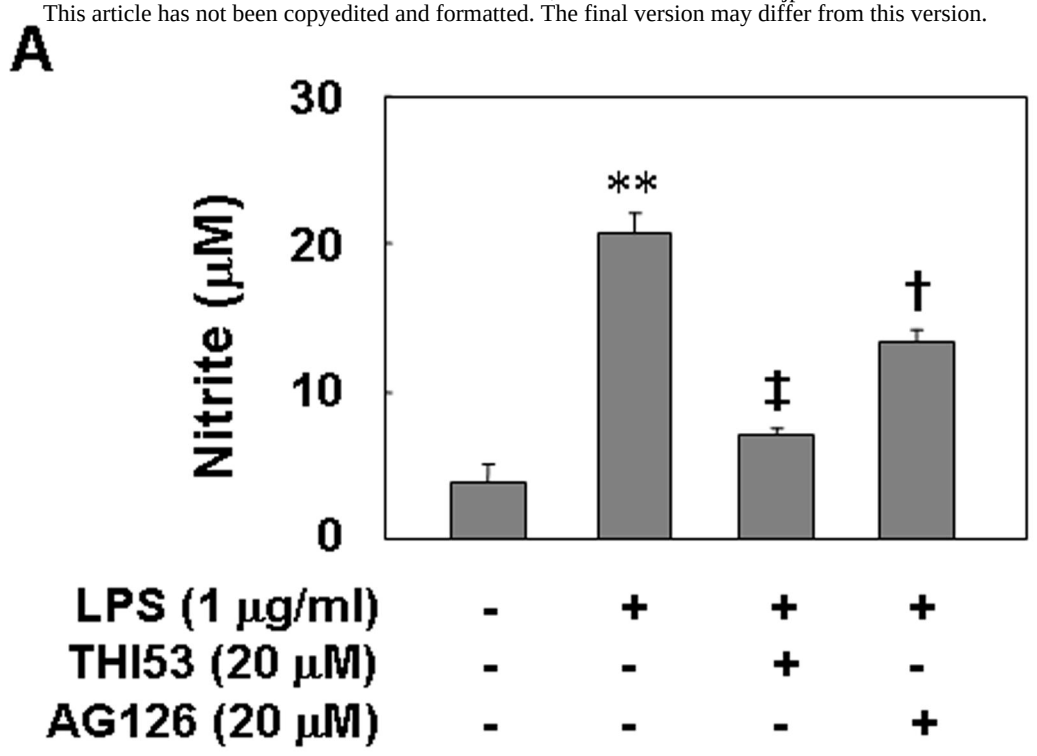

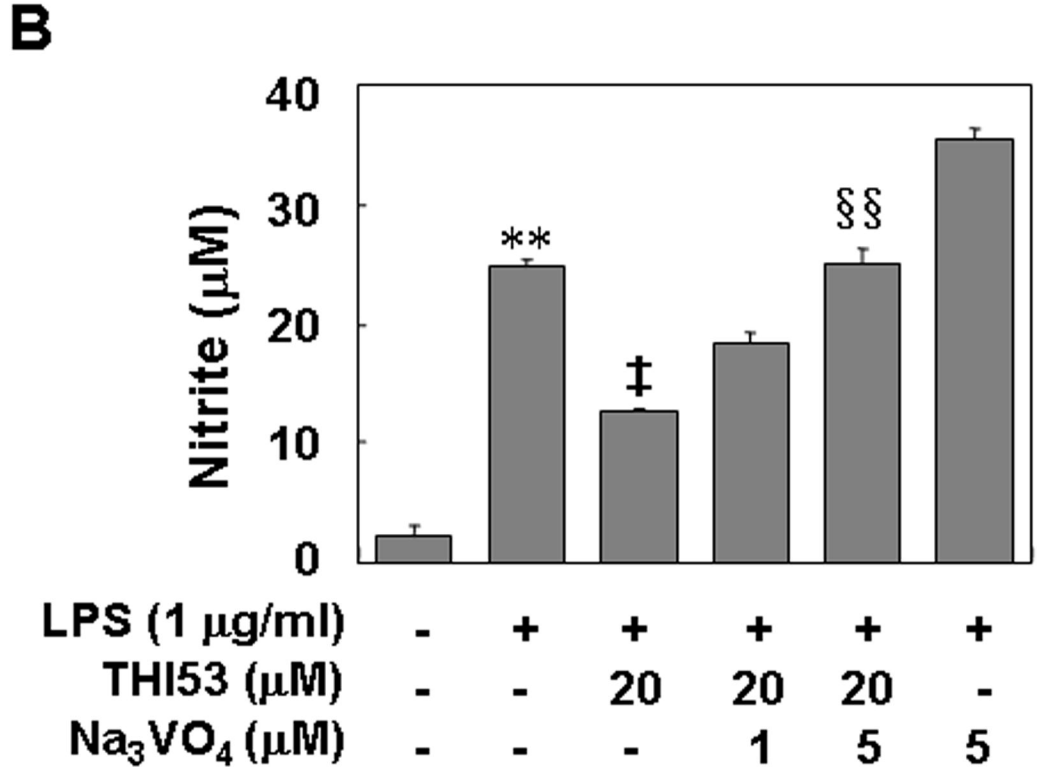

Figure 6-1

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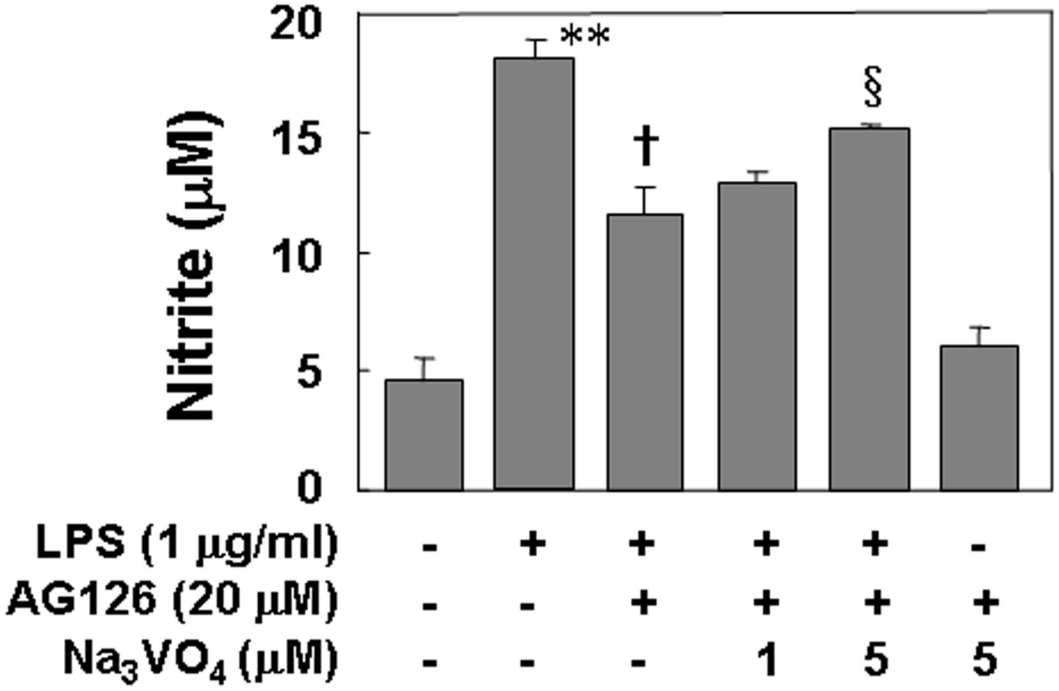

Figure 6-2

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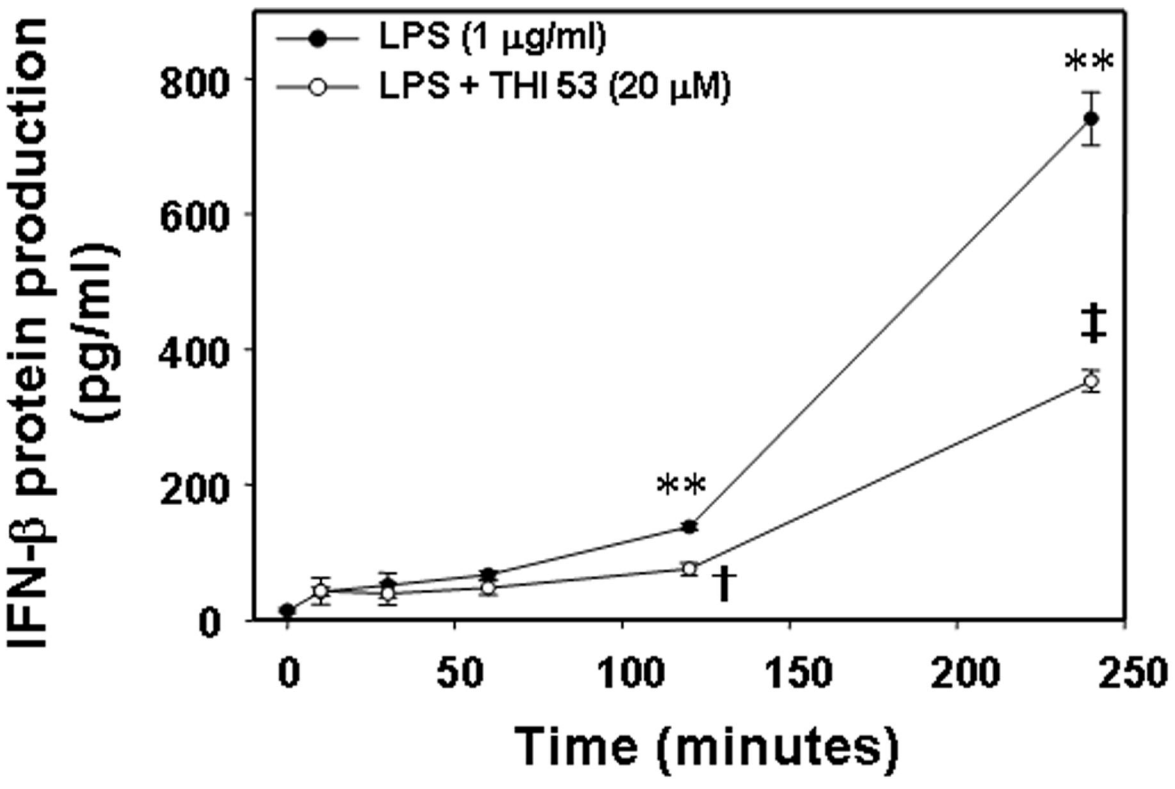

Figure 7

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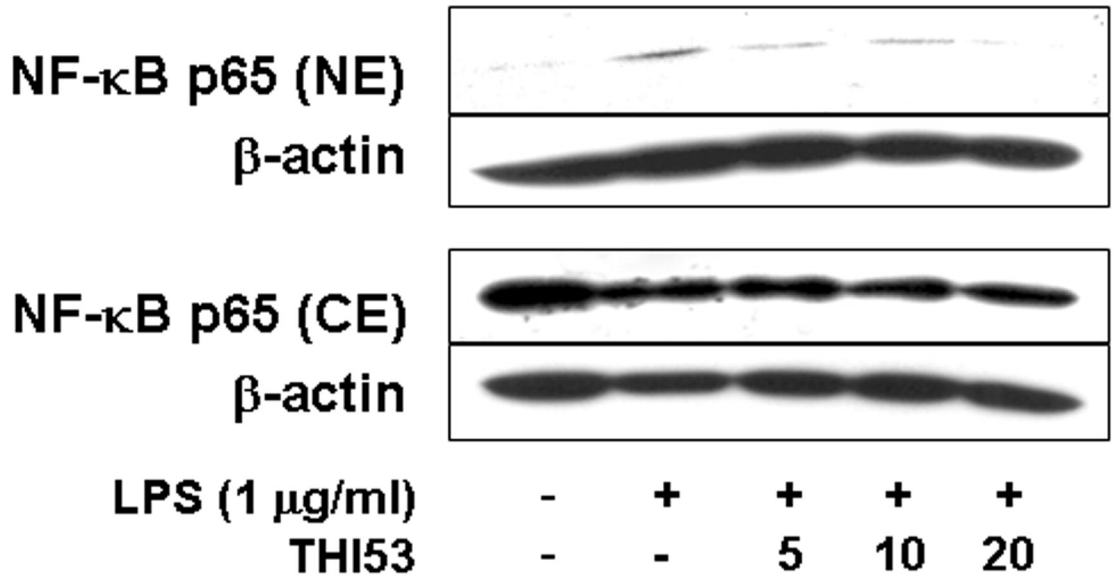

Figure 8-1

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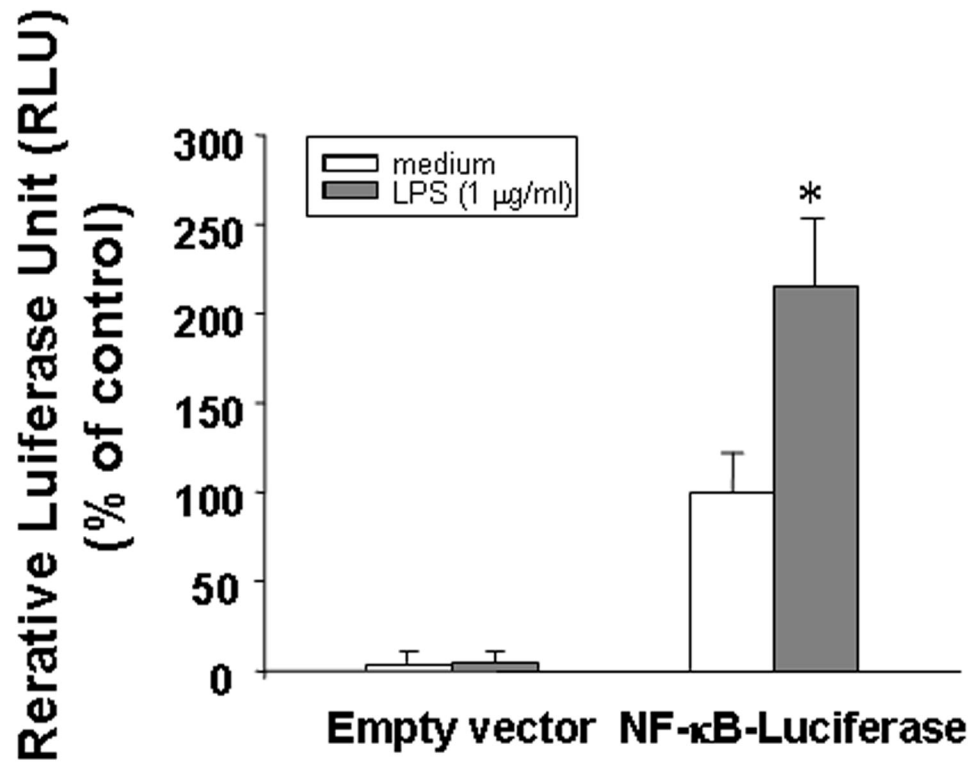

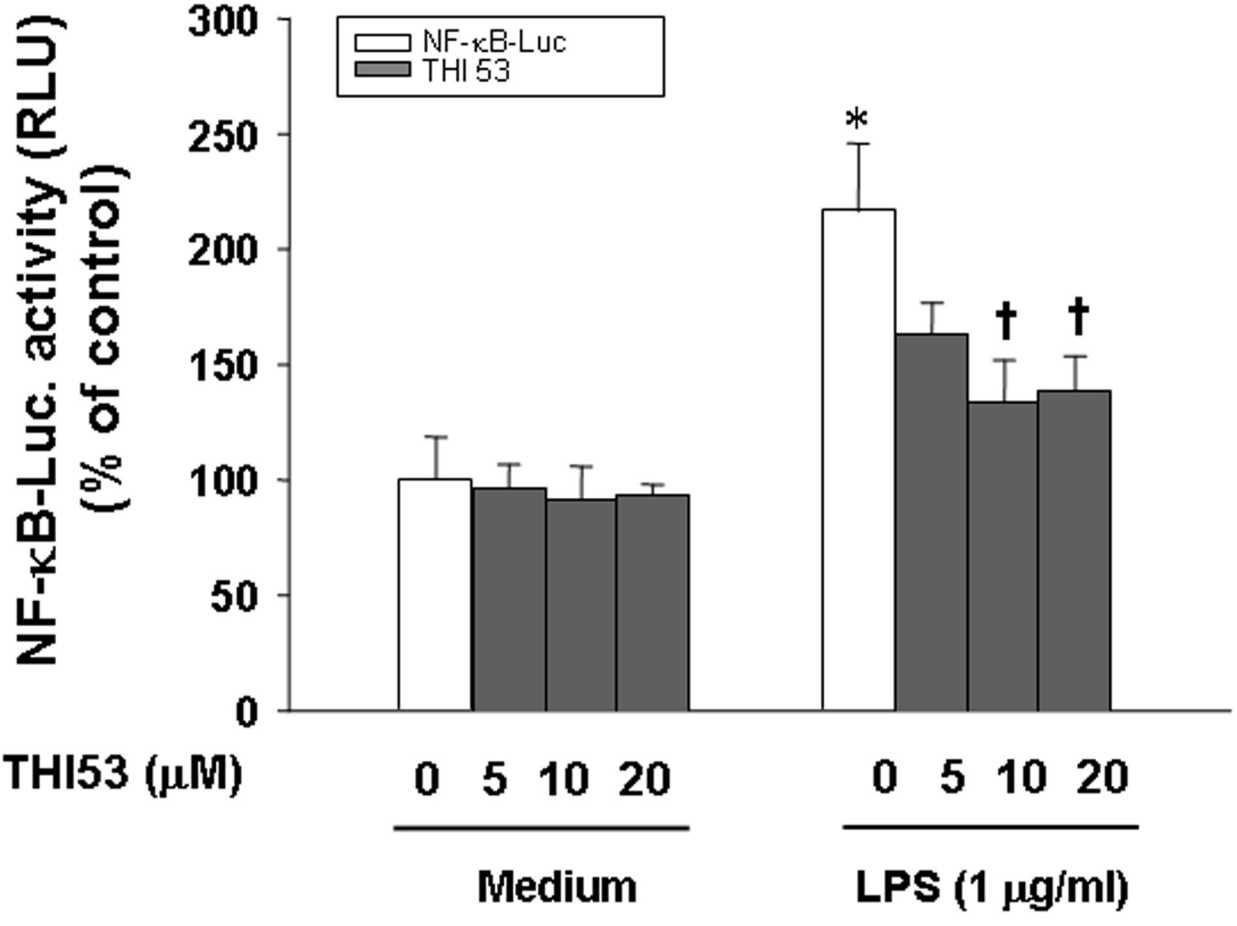

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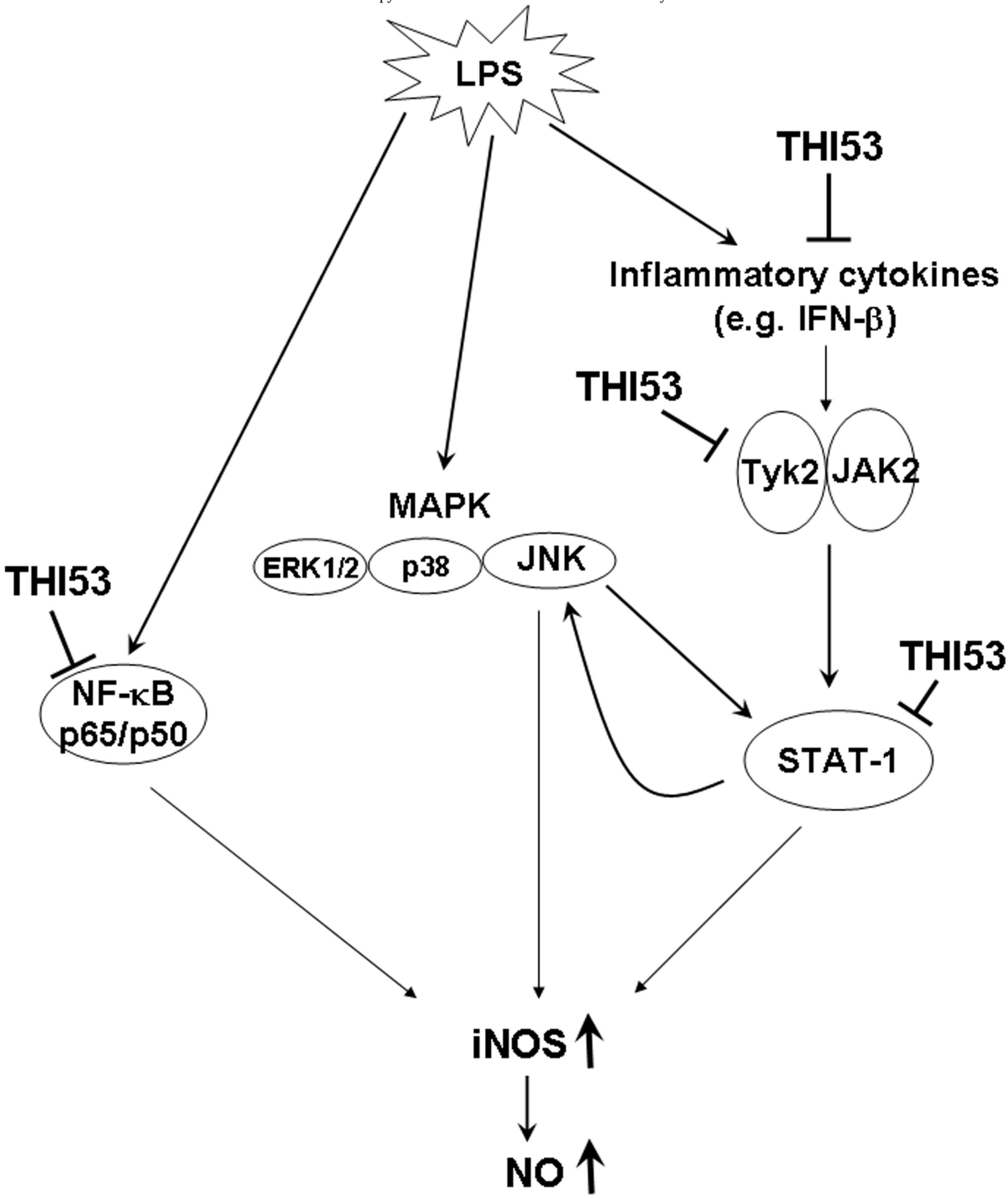

Figure 9