Pinosylvin Suppresses LPS-stimulated Inducible Nitric Oxide Synthase Expression via the MyD88-independent, but TRIF-dependent Downregulation of IRF-3 Signaling Pathway in Mouse Macrophage Cells

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Key Words

Pinosylvin • iNOS • TRIF • IRF3 • STAT1

Abstract

Since inhibitors of inducible nitric oxide synthase (iNOS) have been considered as potential anti-inflammatory and cancer chemopreventive agents, we have evaluated the inhibitory effects on the production of nitric oxide (NO) in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells with natural and synthetic compounds. Pinosylvin (3,5-dihydroxy-*trans*-stilbene), a stilbenoid mainly found in heartwood of *Pinus sylvestris*, exhibited the inhibition of iNOS protein and mRNA expression. The plausible mechanisms of pinosylvin on the suppression of iNOS gene expression were found to be associated with the downregulation of interferon regulatory factor 3 (IRF-3) and interferon- β (IFN- β) expression, which are related to Toll/IL-1 receptor domain-containing adapter inducing interferon- β (TRIF)-mediated signaling. Decreased IFN- β expression suppressed a phosphorylation of JAK kinase, and subsequently, the phosphorylation of signal transducer and activator of transcription-1, one

of the iNOS transcriptional activators, was inhibited by pinosylvin. In addition, the suppression of poly(I:C)-induced iNOS expression, and the attenuation of iNOS expression under the IRF-3 gene knock-down condition also confirmed that pinosylvin affects TRIF pathway. These findings demonstrate that the suppression of LPS-induced iNOS expression by pinosylvin is associated with the regulation of MyD88-independent, but TRIF-mediated signaling pathway.

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Introduction

Nitric oxide (NO), a product of nitric oxide synthase (NOS), has been reported to mediate various physiological processes including vasorelaxation in cardiovascular system [1], protection from injuries by luminal toxins and microbial invasion in gastrointestinal system [2], modulation of synaptic function as neurotransmitter and promotion of neural plasticity in nervous system [3], and defense from invading pathogens in innate immune system [4].

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There are three isoforms of NOS identified to date including constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS), and inducible NOS (iNOS). Especially, iNOS is involved in the production of the excessive amount of NO which may result in diverse diseases including asthma, arthritis, multiple sclerosis, colitis, psoriasis, neurodegenerative disorders, and tumor development, or serious medical conditions such as transplant rejection of septic shock [4]. iNOS expression is induced by various stimuli including interferon- γ (IFN- γ), interleukin-1 (IL-1), lipopolysaccharide (LPS), and tumor necrosis factor- α (TNF- α) [5]. LPS, an endotoxin localized in the outer membrane of Gram-negative bacteria, can evoke the innate immune response and subsequent inflammatory responses in macrophages [6]. As the first step of stimulation, LPS induces the activation of receptor complex composed of Toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD-2). Subsequently, activated TLR4 transfers a signal through two major downstream signaling pathways including myeloid differentiation factor 88 (MyD88)-dependent and Toll/IL-1 receptor domain-containing adapter inducing interferon- β (TRIF)-dependent pathways. In MyD88-dependent pathway, phosphorylation of mitogen-activated protein kinases (MAPKs) results in the activation of transcriptional activators of iNOS, including activating protein-1 (AP-1), cAMP-responsive element binding protein (CREB), and CCAAT-enhancer box binding protein (C/EBP) [6]. On the other hand, MyD88-independent pathway induces phosphorylation and activation of interferon regulatory factor 3 (IRF3). In turn, activated IRF3 binds to the corresponding DNA binding site of promoter region and enhances the transcription of type I IFNs (IFN- α and - β). Sequentially, IFN- β interacts with transmembrane protein Janus kinase (JAK), and eventually causes phosphorylation and activation of signal transducer and activator of transcription-1 (STAT-1), another transcriptional activator of iNOS. In concord with these two signaling pathways, iNOS is expressed as one of the inflammatory mediators [7].

Various classes of compounds from natural or synthetic sources have been identified as inhibitors of iNOS in LPS-stimulated RAW 264.7 cells. Especially, various stilbenoids such as resveratrol, piceatannol, pholidotols, and rhapontigenin have been discovered to exhibit the inhibitory activities on NO production [8-11]. Based on these previous reports, we assumed that one of natural stilbenoids, pinosylvin (3,5-dihydroxy-*trans*-stilbene), might have inhibitory activities on NO

Fig. 1. Chemical structure of pinosylvin.

production. Pinosylvin is a naturally occurring stilbenoid mainly found in heartwoods and leaves of *Pinus* species as a phytoalexin against environmental stresses such as fungal attacks, ultraviolet light, ozone or dessication [12]. Pinosylvin has been reported to have algicidal [13], anti-feedant [14], anti-fungal [15], anti-neoplastic [16], nematocidal [17], and antioxidant effect [18].

In our previous study, we reported that pinosylvin and its analogs have the inhibitory effects on the production of inflammatory mediator prostaglandin E_2 (PGE $_2$) in LPS-stimulated RAW 264.7 mouse macrophage cells [19]. Recent study also showed that pinosylvin inhibited the NF- κ B activation in RAW 264.7 cells [20].

In the present study, we further explored the precise mechanisms of actions in the regulation of iNOS expression by pinosylvin in LPS-stimulated RAW 264.7 cells. Herein, we report the suppression of iNOS expression by pinosylvin is associated with the downregulation of IFN- β /STAT1 via TRIF-dependent signaling pathway.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and antibiotics-antimycotics (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B) were purchased from Invitrogen Co. (Carlsbad, CA, USA). Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), β -actin, and polyinosinic-polycytidylic acid [poly (I:C)] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Oligonucleotides for PCR and EMSA were synthesized by Bioneer Corp. (Daejeon, Korea). Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, rabbit polyclonal anti-iNOS antibody, and rabbit polyclonal anti-JAK1 antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against ERK1/2, p-ERK1/2, p38, p-p38, SAPK/JNK, p-SAPK/JNK, p-IRF3, IRF3, p-JAK1, STAT1, and p-STAT1 were purchased from Cell Signaling Technology (Beverly, MA, USA). A 21-mer oligonucleotide sequence Silencer® Pre-designed

Target gene	Sequences		Product size (bp)
iNOS	Sense	5'-ATG TCC GAA GCA AAC ATC AC-3'	401
	Antisense	5'-TAA TGT CCA GGA AGT AGG TG-3'	
IFN- β	Sense	5'-GAG TTA CAC TGC CTT TGC C-3'	430
	Antisense	5'-GAT TCA CTA CCA GTC CCA GA-3'	
β -Actin	Sense	5'-TGT GAT GGT GGG AAT GGG TCA G-3'	514
	Antisense	5'-TTT GAT GTC ACG CAC GAT TTC C-3'	

Table 1. Sequences of mouse gene specific primers used in PCR

siRNA for mouse Irf3 (GeneBank accession #; NM_016849, siRNA ID #; 184586) was obtained from Ambion (Austin, TX, USA). *TransIT-TKO* Reagent was from Mirus Bio LLC (Madison, WI, USA). Pinosylvin was synthesized as described previously [19] and the structure is shown in Fig. 1.

Cell culture

RAW 264.7 mouse macrophage cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics-antimycotics (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B) at 37°C in 5% CO₂ humidified air.

Measurement of nitric oxide

Nitric oxide (NO) has a short half-life and subsequently is oxidized to stable nitrite. Therefore, the nitrite level was measured to estimate the NO level. RAW 264.7 cells were plated in 24-well culture plates (5 x 10⁵ cells/ml), and incubated for 24 h. Cells were washed twice with PBS and pre-incubated with phenol red-free medium containing various concentrations of pinosylvin (10~40 μ M) for 15 min followed by 1 μ g/ml of LPS treatment for 20 h. The amount of nitrite released in the cultured media was measured by the Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide in 5% H₃PO₄ and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride solution]. Serially diluted sodium nitrite was used as a standard and the absorbance was measured at 540 nm. To evaluate the cytotoxic effect of pinosylvin on RAW 264.7 cells in the same condition, MTT assay was performed. Briefly, after the Griess reaction, MTT solution (final concentration of 500 μ g/ml) was added to each well and further incubated for 4 h. The formazan crystals produced by mitochondrial dehydrogenase were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm. % Survival was determined by comparison with the control group.

Western blot analysis

RAW 264.7 cells (5 x 10⁵ cells/ml in 60 mm dish) were treated with various concentrations of pinosylvin and LPS (1 μ g/ml) for the indicated times. Cells were washed with PBS and lysed in boiling 2x loading buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol, 2 mM sodium ortho-vanadate). Equal amounts of protein (30 ~ 40 μ g) were electrophoresed on

10% or 12% SDS-polyacrylamide gel for 3 h and electro-transferred to PVDF membrane. The membranes were blocked with 5% skimmed milk in phosphate buffered saline with 0.1% Tween 20 (PBST) for 1 h at room temperature. Membranes were then incubated with specific primary antibodies for 3 h and washed with PBST three times. After washing, membranes were incubated with the corresponding HRP-conjugated antibodies. β -Actin was used as an internal standard. The blot was detected by West Save® HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) using LAS-3000 Imager (Fuji Film Corp., Tokyo, Japan). Band intensity was measured using ImageJ software from the National Institutes of Health, and presented as a fold change in comparison with control after the normalization to an internal standard β -actin.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RAW 264.7 cells were treated with 1 μ g/ml LPS and pinosylvin for the indicated time. Total RNA was extracted using Trizol® (Sigma, St. Louis, MI, USA) according to Chomczynski and Sacchi method [21]. RNA (1 μ g) was reverse transcribed using reverse transcriptase system (Promega, Madison, WI, USA) and produced cDNA was amplified by Go Taq™ DNA polymerase system (Promega) with the corresponding oligonucleotides (Table 1) according to the manufacturer's instruction. β -Actin was used as an internal standard. PCR products were resolved on 2% agarose gel electrophoresis, and DNA bands were visualized by SYBR-Gold (Molecular probes, Eugene, OR, USA) staining, and images were captured using Alpha Imager® (Alpha Innotech Corp., USA). Primers used in this study were detailed in Table 1.

Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)

To evaluate the effect of pinosylvin on DNA binding of transcription factors, EMSA was performed as previously described [22]. The oligonucleotide sequences of STAT-1 α in iNOS is as following: 5'-CAT GTT ATG CAT ATT CCT GTA AGT G-3', RAW 264.7 cells were pretreated with pinosylvin (5~40 μ M) for 15 min and 1 μ g/ml LPS was added and incubated for 4 h. Cells were harvested with ice-cold PBS and centrifuged at 2,500 rpm for 4 min. Pellets were washed once with PBS and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM

Fig. 2. Inhibitory effect of pinosylvin on NO production and iNOS expression. (A) Inhibitory effect of pinosylvin on NO production. RAW 264.7 cells (5×10^5 cells/ml) were incubated in 24 well plate for 24 h and then treated with LPS (1 μg/ml) and test compounds. After incubation for 20 h, the NO was determined by Griess reaction. (B) Inhibitory effect of pinosylvin on iNOS protein expression. RAW 264.7 cells (5×10^5 cells/ml) were incubated in 60 mm dish for 24 h, and then treated with LPS (1 μg/ml) and pinosylvin for 12 h. The level of iNOS protein expression was examined by Western blot analysis. The number under each band indicates the relative band intensity presented as a fold change in comparison with control after the normalization to β-actin. (C) Inhibitory effect of pinosylvin on iNOS mRNA expression. RAW 264.7 cells (5×10^5 cells/ml) were incubated in 60 mm dish for 24 h, and then treated with pinosylvin for 15 min. After incubation, LPS (1 μg/ml) was treated for an additional 4 h. Cells were lysed, and total RNA (1 μg) was used in the RT-PCR. PCR products were separated on a 2% agarose gel, stained with SYBR Gold, and visualized under UV illumination. The illustration is a representative of three independent experiments. (D) Significant suppression on iNOS mRNA expression by pinosylvin (40 μM). The intensity of each band in RT-PCR data was measured and demonstrated as numeric values. After normalization to β-actin, fold-change relative to LPS-treated control was presented (n = 3). An asterisk (*) indicates a significant difference with *P* values less than 0.05.

PMSF, 0.2% Nonidet P-40) and incubated on ice for 5 min. Nuclear fractions were collected by centrifugation at 2,500 rpm for 4 min at 4°C and washed with lysis buffer (without NP-40). The resultant pellets were resuspended in nuclear extract buffer [20 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol] and centrifuged at 15,000 rpm for 30 min at 4°C. Supernatant containing nuclear proteins was stored at -70°C until analyzed. Protein content was determined by the Bradford method. For electrophoretic mobility shift assay (EMSA), each nuclear extract (5 μg) was bound with the 50,000 cpm of [³²P] dATP-labeled double-stranded oligonucleotide probes in a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 0.5 mM DTT, 10% glycerol and poly(dI-dC) and incubated at room temperature for 25 min. The mixtures were separated on 5% polyacrylamide gel and vacuum dried at 80°C for 2 h and exposed to cold radiography film for about 12 h at -70°C with intensifying screens. Specificity of DNA binding was examined by competition with unlabeled oligonucleotide.

siRNA transfection

A 21-mer oligonucleotide sequence Silencer® Pre-designed siRNA for mouse IRF3 (GeneBank accession #; NM_016849, siRNA ID #; 184586) was purchased from Ambion (Austin, TX, USA). TransIT-TKO Reagent (Mirus Bio LLC, Madison, WI, USA) was used for the transient transfection of siRNAs according to the manufacturer's instruction. After 48 h of transfection, cells were pretreated with pinosylvin followed by LPS treatment for 18 h. Cells were lysed and performed for Western blot analysis.

Statistical analysis

All experiments were repeated at least three times. Data are expressed as means ± standard deviation (SD) for the indicated number of independently performed experiments. Student's *t*-test (SigmaStat 3.1, Systat software Inc.) was used for the determination of statistical significance. The difference was considered to be statistically significant when *P* < 0.05.

Fig. 3. Effect of pinosylvin on LPS-induced MAPK activation in cultured RAW 264.7 cells. (A) Effect of pinosylvin on the activation or nuclear translocation of transcription factors. Cells were pre-incubated with pinosylvin for 15 min, and then stimulated with LPS for 1 h. Cell lysates were subjected to Western blot analysis. (B) Time course of phosphorylations of MAPKs after LPS exposure. Cells were stimulated with LPS (1 μg/ml) for indicated time points, and then examined the protein levels of phosphorylated MAPKs using Western blot analysis. (C) Effect of pinosylvin on the phosphorylations of MAPKs. After pretreatment of pinosylvin (10, 20, 40 μM) for 15 min, cells were exposed to LPS (1 μg/ml) for 35 min. Cells were lysed and levels of MAPKs were determined using Western blot analysis. β-Actin was used as an internal standard.

Results

Inhibitory effect of pinosylvin on LPS-induced NO production

The effect of pinosylvin on LPS-stimulated NO production was evaluated in RAW 264.7 mouse macrophage cells. Cells were treated with LPS (1 μg/ml) in the absence or presence of pinosylvin for 20 h. The accumulation of NO in the media was determined by Griess reaction. As shown in Fig. 2A, the basal level of NO in culture media after 20 h incubation without LPS-treatment was 1.38 ± 0.31 μM. However, the stimulation of RAW 264.7 cells by LPS (1 μg/ml) resulted in the markedly accumulation of NO in the media up to 29.20 ± 1.12 μM, which is approximately 20-fold induction compared to the basal level. In this condition, pinosylvin significantly attenuated NO production in a concentration-dependent manner with an IC_{50} value of 39.9 μM. An IC_{50} value of a known positive control L-NMMA (a non-selective inhibitor of NOS) was 30.7 μM in the same assay. No significant cell cytotoxicity was observed in all concentrations tested of pinosylvin as determined by MTT assay (>95% of cell survival), suggesting that the inhibitory effect of NO production was not mediated by its cytotoxicity.

Suppressive effects of pinosylvin on iNOS protein and mRNA expression in LPS-stimulated macrophage cells

To further elucidate the mechanism of action on the inhibition of NO production by pinosylvin, we examined the effects on the iNOS protein and mRNA expressions. RAW 264.7 cells were incubated with LPS (1 μg/ml) in the presence or absence of various concentrations of pinosylvin. After 4 h and 12 h incubation, total RNA and protein were isolated for RT-PCR and Western blot analysis, respectively. As shown in Fig. 2B, iNOS protein expression in LPS-stimulated RAW 264.7 cells was markedly increased (band intensity: 1.00) compared to non-stimulated control cells (band intensity: 0.00). However, the co-treatment with pinosylvin attenuated the iNOS protein expression in a concentration-dependent manner (10 μM: 0.44; 20 μM: 0.18; 40 μM: 0.07). In addition, the effect of pinosylvin on the iNOS mRNA expression was also investigated by RT-PCR analysis. Treatment with LPS (1 μg/ml) for 4 h augmented the iNOS mRNA expression, but the pretreatment of pinosylvin for 15 min dose-dependently suppressed LPS-induced iNOS mRNA expression (Fig. 2C). Especially, it showed a significant difference between LPS-treated control and pinosylvin (40 μM)-treated group (Fig. 2D).

Fig. 4. Inhibitory effect of pinosylvin on the phosphorylation of IRF3 (A), induction of IFN β mRNA expression (B), and the activation of Jak/STAT (C) in cultured RAW 264.7 cells. RAW 264.7 cells were pretreated with various concentration of pinosylvin for 15 min, and then stimulated with LPS for 1 h 30 min (A, Western Blot), or for 3 h 30 min (B: RT-PCR, C: Western blot). β -Actin was used as an internal standard.

Fig. 5. Inhibitory effect of pinosylvin on DNA binding activity of STAT1 α in LPS-stimulated RAW 264.7 cells as measured by EMSA. RAW 264.7 cells (5×10^5 cells/ml) were incubated in 100 mm dish for 24 h, and then pretreated with pinosylvin for 15 min. After incubation, LPS (1 μ g/ml) was treated for an additional 4 h. Cells were lysed and nuclear extracts were prepared, and 5 μ g of nuclear extracts were used in the EMSA. No specific band was detected in lane 1 (STAT-1 α probe alone). Nuclear fractions interacted with STAT-1 α probe showed specific bands (the indicated arrow). LPS-treated sample (lane 6) showed the up-regulation in DNA-binding activity (2.0-fold) in comparison with LPS-untreated sample (lane 2). Pre-treatment with pinosylvin (40 μ M) blocked LPS-induced STAT-1 α DNA-binding activity (lane 4). Competition study was performed by the addition of excess unlabeled oligonucleotide (lane 7) using the same fraction as lane 6.

These results suggest that the inhibition of NO production by pinosylvin might be correlated with the suppressive effect of iNOS protein and mRNA expression.

Effect of pinosylvin on MyD88-dependent pathways (AP-1 and MAPK signaling pathway)

In order to further explore the detailed mechanisms of actions in LPS-stimulated iNOS expression, we examined the MyD88-dependent signaling pathway by LPS. In the regulation of iNOS expression, one of transcription factors, activator protein-1 (AP-1) which is composed of c-Fos and c-Jun subunits is considered

to be playing a role in the MyD88-mediated signaling pathway. Therefore, we determined whether pinosylvin affects the activation of AP-1 component by LPS treatment. LPS treatment for 1 h increased the translocation of c-Fos into nucleus and the level of phosphorylated c-Jun, however, these events were not suppressed by the co-treatment of pinosylvin (Fig. 3A). Similarly, the activated ATF-2 (phosphorylated ATF-2), a

Fig. 6. Correlation between the inhibitory effect of pinosylvin on iNOS expression and TRIF pathway. (A) The concentration-dependent up-regulation of iNOS protein expression by poly(I:C) in RAW 264.7 cells. RAW 264.7 cells were treated the indicated concentrations of poly(I:C) for 12 h, and protein level of iNOS was determined by Western blot. (B) Inhibitory effect of pinosylvin on poly(I:C)-induced iNOS expression. RAW 264.7 cells were pretreated with various concentration of pinosylvin for 15 min, and then stimulated with poly(I:C) for 12 h. The protein level of iNOS was examined using Western blot. (C) Effect of pinosylvin under the condition of siIRF3 transient knock-down. RAW 264.7 cells were transiently transfected with siIRF3 for 48 h. Then pinosylvin and LPS were treated for additional 12 h. The protein levels of iNOS and IRF3 were evaluated by Western blot. β-Actin or GAPDH were used as an internal standard.

trans-acting element of CREB, by LPS treatment, was not affected by pinosylvin (Fig. 3A). These results suggest that the suppression of iNOS gene expression by pinosylvin might not be associated with the AP-1 and ATF-2 transcriptional factors. Next, we investigated the effect on the activation of upstream signaling molecules, MAPKs. Primarily, to determine the maximum phosphorylation time point, cell lysates were collected after LPS treatment for 20, 35, or 120 min. As a result, phosphorylated forms of ERK1/2, p38, and JNK1/2 were shown in the maximum level at 35 min (Fig. 3B). Based on the result, pinosylvin was pre-incubated for 15 min followed by the treatment of LPS for 35 min, and then analyzed the expression levels of MAPKs. As illustrated in Fig. 3C, the levels of phosphorylated MAPKs were not changed by pinosylvin. Taken together, data demonstrate that the suppression of LPS-stimulated iNOS expression by pinosylvin might be the MyD88-independent signaling pathway.

Effect of pinosylvin on JAK/STAT signaling pathway

To further elucidate the possibility of MyD88-independent mechanism in the regulation of iNOS by pinosylvin, we next examined the signaling molecules in the TRIF-mediated pathway. LPS (1 μg/ml) treatment

for 1 h 30 min drastically increased the activation of IRF3 (phosphorylated IRF3), however, pinosylvin suppressed the expression level of phosphorylation of IRF3 (Fig. 4A), which, in turn, down-regulated IFN-β mRNA expression, one of downstream effectors of IRF3 (Fig. 4B). IFN-β functions as an inflammatory mediator and transfers the signal through Jak receptors. The activation of Jak1 (Tyr1022/Tyr1023) and Stat1 (Tyr701) was also attenuated by pinosylvin. However, the phosphorylation of Stat1 (Ser727) which is known to be controlled by active p-p38 was not affected by pinosylvin (Fig. 4C).

Suppressive effects of pinosylvin on STAT-1 α-DNA binding activity

To further confirm the involvement of STAT1 by pinosylvin in the regulation of iNOS expression, EMSA was performed to analyze DNA binding activity of a transcription factor STAT-1α which exists in promoter region of iNOS. As shown in Fig. 5, LPS treatment for 4 h (lane 6) caused an increase of STAT-1α-DNA binding, but the pretreatment of pinosylvin (40 μM) for 15 min prior to LPS treatment (lane 4) clearly suppressed STAT-1α-DNA binding activity. The addition of excessive amount of specific unlabeled consensus oligonucleotide (200-fold higher concentration) completely

prevented the band shifts, demonstrating the specificity of the protein-DNA interaction.

Effect of pinosylvin on Poly(I:C)-induced iNOS expression

Poly(I:C) is known to activate TLR3 which specifically mediates TRIF-dependent pathway in the induction of iNOS [23]. To further elucidate the involvement of TRIF-dependent signaling by pinosylvin, RAW 264.7 cells were incubated with poly(I:C) (10 µg/ml) for 12 h after pretreatment with various concentrations of pinosylvin for 15 min. As shown in Fig. 6A, poly(I:C) dose-dependently up-regulated the iNOS protein expression. However, the treatment of pinosylvin with poly(I:C) (10 µg/ml) suppressed the iNOS protein expression in a concentration-dependent manner (Fig. 6B). In addition, to further confirm the involvement of IRF3, we determined the iNOS protein expression under knock-down of IRF3 using siRNA treatment. Knock-down of IRF3 resulted in the abrogation of pinosylvin-mediated iNOS down-regulation in LPS-treated cells (Fig. 6C). These results further support the involvement of MyD88-independent pathway in the suppression of iNOS expression by pinosylvin in LPS-stimulated RAW 264.7 cells.

Discussion

Accumulating evidences suggest that the overproduction of nitric oxide (NO) is highly associated with the pathophysiological conditions including inflammation, and the expression of inducible nitric oxide synthase (iNOS) plays a key role in the overproduction of NO in activated macrophages [4]. Targeting NO production and iNOS expression, therefore, might be considerable points in the development of anti-inflammatory agents. In our program to procure chemical entities in the modulation of inflammation, we found that natural stilbenoids and their synthetic analogs exhibited anti-inflammatory potential with the inhibition of prostaglandin E_2 (PGE₂) [19, 24]. Although natural stilbenoids showed an inhibitory effect on the NO production, the detailed mechanisms of actions remained unclear. Therefore, in the present study, we elucidated the plausible mechanisms by pinosylvin in the regulation of iNOS expression through signaling pathways, especially, TRIF-mediated signalings. In accordance with the previous report [20], pinosylvin exhibited the inhibitory effect on NO production without cytotoxicity

in LPS-stimulated RAW 264.7 cells. The inhibitory effect of NO production by pinosylvin was also associated with the suppression of iNOS protein and mRNA expression. It is known that iNOS gene expression is regulated by several transcription factors such as NF-κB, IRF-1, AP-1, cAMP-responsive element binding protein (CREB), CCAAT-enhancer box binding protein (C/EBP), and signal transducer and activator of transcription-1α (STAT-1α) [25]. Because Lee et al. reported the inhibitory effects of pinosylvin on NF-κB transcriptional activity [20], in this study, therefore, we examined the effect of pinosylvin on MAPKs in MyD88-dependent pathway. Pinosylvin, however, merely affected the nuclear translocation of c-Fos, phosphorylation of c-Jun and ATF-2 and the upstream MAPKs. This result suggests that the regulation of iNOS by pinosylvin might not be highly associated with the MyD88-dependent pathway. Based on this information, we next investigated whether pinosylvin might affect to the TRIF-mediated signaling in the regulation of iNOS expression. The suppressive effects of pinosylvin on phosphorylation of IRF3, IFNβ mRNA expression and the consequent phosphorylations of Jak and Stat1 demonstrated that pinosylvin might affect the TRIF-mediated signaling pathway, and these might be one of the major contributions in the regulation of iNOS expression. Further studies was designed to confirm the TRIF-mediated pathway using poly(I:C). Poly(I:C) specifically binds and activates TLR3 which has only TRIF-mediated signaling pathway in the regulation of iNOS. Pinosylvin suppressed poly(I:C)-induced iNOS protein expression, indicating the possible involvement of TRIF-mediated pathway. Additional study with siRNA of IRF3 also showed the attenuation of iNOS expression by pinosylvin, suggesting that the regulation of iNOS expression by pinosylvin in LPS-stimulated macrophages is highly correlated with the TRIF-dependent pathway. Indeed, various naturally occurring compounds including curcumin [26], isoliquiritigenin [27], auranofin [28], luteolin [29], 6-shogaol [30], (-)-epigallocatechin-3-gallate [31] demonstrated their anti-inflammatory activities with the MyD88-independent mechanisms. Similarly, in the present study, we showed that the regulation of iNOS expression by pinosylvin might be the MyD88-independent, but TRIF-dependent modulation of IRF3-mediated signaling pathway.

In addition, the activation of IRF3 is known to be occurred through phosphorylation at specific serine sites by two kinases including TNF-receptor-associated factor-family member-associated NF-κB activator-

binding kinase 1 (TBK1) and I κ B kinase ϵ (IKK ϵ) [32, 33]. Although the suppression of iNOS expression by pinosylvin is associated with the TRIF-dependent modulation of IRF3-mediated signaling pathway, further study is warranted to clarify whether pinosylvin inhibits phosphorylation of IRF3 through affecting the upstream kinases such as TBK1 and IKK ϵ or directly binding to the phosphorylation sites of IRF3.

In conclusion, we demonstrated that pinosylvin possesses the inhibitory activities of NO production and iNOS expression via the MyD88-independent, but

TRIF-dependent down-regulation of IRF3 signaling pathway, and this might be associated with the anti-inflammatory activity.

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References

- Rubio AR, Morales-Segura MA: Nitric oxide, an iceberg in cardiovascular physiology: far beyond vessel tone control. *Arch Med Res* 2004;35:1-11.
- Wallace JL, Miller MJ: Nitric oxide in mucosal defense: a little goes a long way. *Gastroenterology* 2000;119:512-520.
- Contestabile A, Ciani E: Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. *Neurochem Int* 2004;45:903-914.
- Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, Darley-Usmar VM: Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* 1999;1411:385-400.
- Davis KL, Martin E, Turko IV, Murad F: Novel effects of nitric oxide. *Annu Rev Pharmacol Toxicol* 2001;41:203-236.
- Pålsson-McDermott EM, O'Neill LA: Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 2004;113:153-162.
- Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H: Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* 2003;100:171-194.
- Cho DI, Koo NY, Chung WJ, Kim TS, Ryu SY, Im SY, Kim KM: Effects of resveratrol-related hydroxystilbenes on the nitric oxide production in macrophage cells: structural requirements and mechanism of action. *Life Sci* 2002;71:2071-2082.
- Islam S, Hassan F, Mu MM, Ito H, Koide N, Mori I, Yoshida T, Yokochi T: Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)- κ B activation by inhibiting I κ B kinase (IKK). *Microbiol Immunol* 2004;48:729-736.
- Wang J, Matsuzaki K, Kitanaka S: Stilbene derivatives from *Pholidota chinensis* and their anti-inflammatory activity. *Chem Pharm Bull* 2006;54:1216-1218.
- Kageura T, Matsuda H, Morikawa T, Toguchida I, Harima S, Oda M, Yoshikawa M: Inhibitors from rhubarb on lipopolysaccharide-induced nitric oxide production in macrophages: structural requirements of stilbenes for the activity. *Bioorg Med Chem* 2001;9:1887-1893.
- Jorgensen E: The formation of pinosylvin and its monomethyl ether in the sapwood of *Pinus resinosa* ait. *Can J Botany* 1961;39:1765-1772.
- Sheers EH: *trans*-3,5-Dihydroxystilbene as algicide. US Patent 3, 1971;577:230.
- Sullivan TP, Crump DR, Wieser H, Dixon EA: Influence of the plant antifeedant, pinosylvin, on suppression of feeding by snowshoe hares. *J Chem Ecol* 1992;18:1151-1164.
- Lee SK, Lee HJ, Min HY, Park EJ, Lee KM, Ahn YH, Cho YJ, Pyee JH: Antibacterial and antifungal activity of pinosylvin, a constituent of pine. *Fitoterapia* 2005;76:258-260.
- Simard F, Legault J, Lavoie S, Mshvildadze V, Pichette A: Isolation and identification of cytotoxic compounds from the wood of *Pinus resinosa*. *Phytother Res* 2008;22:919-922.
- Suga T, Ohta S, Munesada K, Ide N, Kurokawa M, Shimizu M, Ohta E: Endogenous pine wood nematocidal substances in pines, *Pinus massoniana*, *P. strobus* and *P. palustris*. *Phytochemistry* 1993;33:1395-1401.
- Fang JG, Lu M, Chen ZH, Zhu HH, Li Y, Yang L, Wu LM, Liu ZL: Antioxidant effects of resveratrol and its analogues against the free-radical-induced peroxidation of linoleic acid in micelles. *Chemistry* 2002;8:4191-4198.
- Park EJ, Min HY, Ahn YH, Bae CM, Pyee JH, Lee SK: Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E2 production in lipopolysaccharide-induced mouse macrophage cells. *Bioorg Med Chem Lett* 2004;14:5895-5898.
- Lee J, Jung E, Lim J, Lee J, Hur S, Kim SS, Lim S, Hyun CG, Kim YS, Park D: Involvement of nuclear factor- κ B in the inhibition of pro-inflammatory mediators by pinosylvin. *Planta Med* 2006;72:801-806.
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.
- De Stefano D, Maiuri MC, Iovine B, Ialenti A, Bevilacqua MA, Carnuccio R: The role of NF- κ B, IRF-1, and STAT-1 transcription factors in the iNOS gene induction by gliadin and IFN- γ in RAW 264.7 macrophages. *J Mol Med* 2006;84:65-74.
- O'Neill LA: 'Fine tuning' TLR signaling. *Nat Immunol* 2008;9:459-461.

- 24 Lee SK, Min HY, Huh SK, Kim EY, Lee E, Song S, Kim S: Styrylheterocycles: a novel class of inhibitors on lipopolysaccharide-induced nitric oxide production. *Bioorg Med Chem Lett* 2003;13:3689-3692.
- 25 Kleinert H, Pautz A, Linker K, Schwarz PM: Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* 2004;500:255-266.
- 26 Youn HS, Saitoh SI, Miyake K, Hwang DH: Inhibition of homodimerization of Toll-like receptor 4 by curcumin. *Biochem Pharmacol* 2006;72:62-69.
- 27 McCoy CE, Carpenter S, Pålsson-McDermott EM, Gearing LJ, O'Neill LA, Park SJ, Song HY, Youn HS: Suppression of the TRIF-dependent signaling pathway of Toll-like receptors by isoliquiritigenin in RAW264.7 macrophages. *Mol Cells* 2009;28:365-368.
- 28 Park SJ, Lee AN, Youn HS: TBK1-targeted suppression of TRIF-dependent signaling pathway of toll-like receptor 3 by auranofin. *Arch Pharm Res* 2010;33:939-945.
- 29 Lee JK, Kim SY, Kim YS, Lee WH, Hwang DH, Lee JY: Suppression of the TRIF-dependent signaling pathway of Toll-like receptors by luteolin. *Biochem Pharmacol* 2009;77:1391-1400.
- 30 Park SJ, Lee MY, Son BS, Youn HS: TBK1-targeted suppression of TRIF-dependent signaling pathway of Toll-like receptors by 6-shogaol, an active component of ginger. *Biosci Biotechnol Biochem* 2009;73:1474-1478.
- 31 Youn HS, Lee JY, Saitoh SI, Miyake K, Kang KW, Choi YJ, Hwang DH: Suppression of MyD88- and TRIF-dependent signaling pathways of Toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. *Biochem Pharmacol* 2006;72:850-859.
- 32 Yoneyama M, Suhara W, Fujita T: Control of IRF-3 activation by phosphorylation. *J Interferon Cytokine Res* 2002;22:73-76.
- 33 Honda K, Taniguchi T: IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 2006;6:644-658.