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Sp1 is an essential transcription factor for LPS-induced tissue factor expression in THP-1 monocytic cells, and nobiletin represses the expression through inhibition of NF-κB, AP-1, and Sp1 activation

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ABSTRACT

Nobiletin is a citrus polymethoxylated flavonoid extracted from *Citrus depressa*, and has several reported biological effects. In this study, we investigated the effect of nobiletin on bacterial lipopolysaccharide (LPS)-induced expression of tissue factor (TF), a trigger protein for the blood coagulation cascade, and studied the possible mechanism of TF transcriptional regulation. THP-1 monocytic cells stimulated with LPS showed an increased expression of both TF protein and mRNA levels. However, pretreatment with nobiletin resulted in inhibition of LPS-induced expression of both TF protein and mRNA in a dose-dependent manner. Electrophoretic mobility shift assays revealed that binding of nuclear proteins from LPS-stimulated THP-1 cells to the NF-κB or AP-1 binding motif was increased as compared to non-stimulated control cells. Such increased binding activities were significantly reduced by pretreatment with nobiletin. Binding activity of nuclear proteins to the Sp1 binding motif was observed irrespective of LPS stimulation, but Sp1 activation was inhibited by nobiletin treatment of the cells. Treatment of THP-1 cells with Sp1-specific small interfering RNA (Sp1 siRNA) abolished the ability of LPS to induce TF activity. A similar reduction in the level of TF mRNA was also observed upon treatment of cells with Sp1 siRNA. These studies reveal that constitutive Sp1 activation is an essential event for transcriptional activation of TF, and nobiletin prevents LPS-induced TF expression by inhibiting NF-κB, AP-1, and Sp1 activation.

1. Introduction

Septic shock is induced by infection with pathogenic bacteria or fungi and associated with a high mortality [1]. In Gram-negative sepsis, lipopolysaccharide (LPS, endotoxin) released from Gram-negative bacteria activates monocytes, endothelial cells, and epithelial cells and triggers an inflammaroty response that is orchestrated by monocytes [2]. As an additional characteristic feature of Gram-negative sepsis, the procoagulant molecule tissue factor (TF) is inducibly

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expressed by circulating monocytes, vascular endothelial cells, and epithelial cells in the lungs and kidneys through stimulation of those cells with LPS [3,4]. TF is a membrane glycoprotein receptor that specifically binds to coagulation factor VII and VIIa. TF binding with factor VII induces conformational change in VII and subsequent auto-activation of VII to VIIa. TF also serves as a specific cofactor for VIIa-dependent activation of factors IX and X, leading to the advance of the blood coagulation cascade [3]. The TF gene promoter contains a variety of transcription factor binding sites, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), sequence-specific protein 1 (Sp1), and early growth response gene-1 (Egr-1). Other than LPS, TF expression is also stimulated by external signals, such as inflammatory cytokines (tumor necrosis factor-α [TNF-α] and interleukin-1β [IL-1β]), growth factors, verotoxin, and oxidized low-density lipoprotein [3,5–7]. TF is not expressed or is expressed at very low activity on the surface of monocytes and endothelial cells under normal circumstances [8]. Therefore, normal vascular monocytes and endothelial cells are relatively inert with respect to coagulation. Aberrant TF expression is likely to be associated with problems relating to intravascular fibrin deposition, such as thromboembolic disorders and disseminated intravascular coagulation (DIC), and contributes to multiorgan failure [9–11]. Previous work demonstrated that cyclic AMP, retinoic acid, and curcumin, a major chemical component of turmeric, inhibited TF gene transcription. Furthermore, TF activity increased in endothelial cells exposed to TNF-α, verotoxin, or LPS *in vitro* [7,12–15]. Additionally, administration of anti-TF antibodies reduced mortality in baboon and mouse models of septic shock [16]. Administration of other coagulation inhibitors, such as antithrombin III, protein C, and TF pathway inhibitor, in various animal models of sepsis also reduced mortality [17,18]. Therefore, it is widely accepted that TF expression plays a pivotal role for vascular thrombosis in inflammatory disorders such as septic shock. Furthermore, a medicinal compound to control TF expression in monocytes and endothelial cells is expected to prevent septic shock with thrombomotic disorder and to reduce mortality.

Recently, it was reported that nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), a citrus polymethoxy flavonoid, exerts anti-inflammatory by inhibiting IL-1- or LPS-induced production of PGE2. PGE2 is an inflammatory mediator in rheumatoid arthritis and osteoarthritis in human synovial cells, through selective down-regulation of inducible cyclooxygenase-2 (COX-2), and by inhibiting promatrix metalloproteinase (proMMP)-1, -3, and -9 [19,20]. The expression of COX-2 and proMMPs is regulated by the transcriptional activation of their genes, the promoter sequences of which contain putative binding sites for NF-κB, and AP-1 [21-23]. The promoter activity of MMP-9 is induced by NF-κB and/or AP-1 [24], whereas transcriptional activation of MMP-1 and -3 are induced by activation of AP-1 and/or polyomavirus enhancer A-binding protein-3 (PEA3) [25]. Therefore, the results imply that the anti-inflammatory activity of nobiletin might be derived from inhibition of NF-κB and/or AP-1 activation.

The present work was undertaken to evaluate whether LPS-induced TF expression in THP-1 monocytic cells is attenuated by nobiletin pretreatment, and to understand a possible mechanism of polymethoxy flavonoid-induced control of TF transcription. Nobiletin inhibited LPS-induced TF expression in cells in a dose-dependent manner in parallel with its mRNA levels. The present work indicates that Sp1 activation is essential for maximum transcriptional activation of LPS-induced TF expression, and nobiletin attenuates LPS-induced TF expression not only by inhibiting LPS-induced NF-κB and AP-1 activation, but also by basal activation of Sp1.

2. Materials and methods

2.1. Materials

Nobiletin was isolated from the juice of *Citrus depressa* Hayata (*Rutaceae*), as described previously [20]. Reagents were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan), unless otherwise indicated. Poly (dI-dC), oligonucleotides for κB-like (TF-κB), proximal AP-1 (proximal TF-AP-1) and distal AP-1 (distal TF-AP-1) binding sites in TF promoter region, and prototypic Sp1 and Egr-1 binding oligonucleotides were obtained from Amersham Pharmacia Biotech AB (Tokyo Japan). RPMI 1640 medium and Dulbecco's phosphate-buffered saline (−) (PBS(−)) were obtained from Nissui Pharm. Co. (Tokyo, Japan). Penicillin, streptomycin, 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide, rabbit brain thromboplastin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). [γ^{32}P]ATP was from NEN Research Products (Tokyo, Japan). A monoclonal antibody against human tissue factor (TF9-10H10) was obtained from American Diagnostica Inc. (Greenwich, CT). A monoclonal antibody against human β-actin (AC-15) was from Sigma Chemical Co. (St. Louis, MO). DharmaFECT 2 transfection reagent was purchased from GE Healthcare Ltd. (Amersham Place, UK). Sp1 small interfering RNA was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primers for real-time reverse transcription polymerase chain reaction (RT-PCR) were purchased from Sigma Genosys (Tokyo, Japan).

2.2. Culture of THP-1 cells

THP-1 cells (3×10^6 cells/35-mm dish) obtained from American Type Culture Collection (Rockville, MD) were washed three times with cold PBS(−), and incubated with fresh culture medium for 3–4 days with daily medium changes before use in assays. Cell survival and viability were determined by a tetrazolium-based MTT colorimetric assay described previously [7,26].

2.3. Measurement of TF procoagulant activity

After incubation of THP-1 cells (24-well plates, 6.5×10^5 cells/well) with or without LPS in culture medium, the cells were washed with PBS(−) and scrape-harvested into 300 μL of 30 mM Tris–HCl (pH 7.4). The cells were frozen in liquid nitrogen and thawed in a water bath at 37 °C, three times. The cell lysate was assayed in a one-stage clotting assay for TF procoagulant activity and one unit of activity was defined as a clotting time of 20 s in a standard assay with normal human

plasma, as described previously [27]. The coagulant activity observed reflects TF activity, since procoagulant activity was not detected from monocytes when factor VII- or X-deficient plasma was used instead of normal plasma.

2.4. Electrophoretic mobility shift assay (EMSA)

The following oligonucleotides were end-labeled with [γ^{32}P]ATP using polynucleotide kinase: TF-κB, 5′-AGGGTCCCG-GAGTTTCCTACCGGGA-3′; distal TF-AP-1, 5′-TCGGTGGCGCG-GTTGAATCACTGG-3′; proximal TF-AP-1, 5′-GGTGAGTCATCC-CTTGCAGGGTCC-3′; a prototypic Sp1, 5′-ATTCGATCGGGGC-GGGGCGAGC-3′; a prototypic Egr-1, 5′-CCCGGCGCGGGGGC-GATTTCGAGTCA-3′. THP-1 monocytic cells (3×10^6/plate) were exposed to various concentrations of LPS for 2 h. The nuclear extracts were prepared as described by Schreiber et al. [28] and a previous paper [7]. Protein concentrations of nuclear extracts were determined by the Bradford method (Pierce Chemical Co., Rochford, IL, USA) [29]. The nuclear extract (2.0 μg) was incubated in 10 μL of 89 mM Tris/89 mM borate/1 mM EDTA (pH 8.0) containing 0.05% NP-40 and 10% glycerol, 2 μg of poly (dI-dC) and 20,000 cpm of [^{32}P]-end-labeled double-stranded oligonucleotide probe for 30 min at 25 °C. For detection of Sp1 and Egr-1, BSA was added to the incubation mixture to prevent nonspecific binding. After incubation, NF-κB- and AP-1-oligonucleotide complexes were separated from free oligonucleotides by electophoresis in 4% polyacrylamide gels (acrylamide:bis-acrylamide 30:1) with 1 × TBE buffer (89 mM Tris/89 mM borate/1 mM EDTA running buffer, pH 8.0) at 4 °C. Sp1- and Egr-1-oligonucleotide complexes were separated with 0.5 × TBE buffer. The gels were dried and radiolabeled bands were visualized by BAS 5000 Bio-Imaging Analyser (Fuji Photo Film Co., Ltd., Tokyo, Japan) or exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) for 3 h at −80 °C. Loading efficiency was controlled by normalization to the lower nonspecific band on the EMSA gels.

2.5. Transfection of small interfering RNA

Cells were treated with small interfering RNAs (siRNAs) according to the instructions provided with the Dharma*FECT* 2 transfection reagent (GE Healthcare Ltd., Amersham Place, UK) with slight modifications. THP-1 cells (1×10^5) were treated with 0.1 nmol of siRNA in RPMI 1640 medium

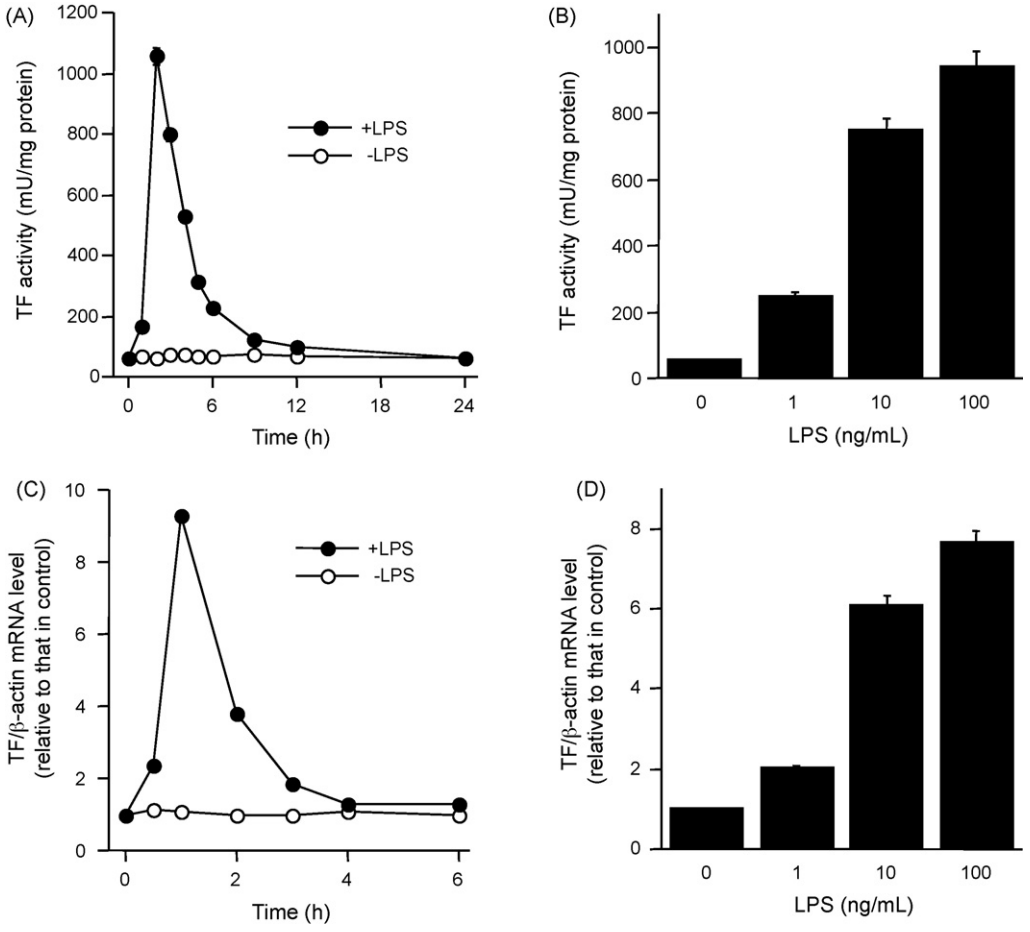

Fig. 1 – Enhancement of TF activity and the mRNA levels in LPS-treated THP-1 cells. (A and C) THP-1 cells were incubated with LPS (100 ng/mL) for the indicated times. (B and D) THP-1 cells were incubated with LPS at the indicated concentrations for 2 h. After incubation, the TF procoagulant activity in the cell lysates was measured by a one-stage clotting assay described in Section 2 (A and B). The TF mRNA levels were measured by quantitative real-time PCR described in Section 2 (C and D). The data are presented as means ± S.D. of the results of three independent experiments.

supplemented with 10% FCS in the presence of the Dharma-FECT 2 transfection reagent. After a 48 h incubation at 37 °C, the cells were used for experiments.

2.6. Western blotting analysis

Cells were washed twice with PBS and lysed in lysis buffer [20 mM Tris–HCl (pH 7.4), 0.15 M NaCl, and 15 mM octyl-β-D-glucopyranoside] at 37 °C for 15 min. A cell lysate containing 30 μg of protein was fractionated by SDS-PAGE and then proteins were transferred to a PVDF membrane. The membrane was first rinsed with TBST [20 mM Tris–HCl (pH 7.4), 0.15 M NaCl, and 0.05% Tween 20] and then blocked with 2% (w/v) skimmed milk and 3% BSA in TBST for 1 h at room temperature. The blocked membrane was subsequently probed for 2 h at room temperature with a 1:1000 dilution of first antibodies in 1:10 dilution of blocking buffer. After the membrane had been washed three times with TBST, it was incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibodies against mouse IgG. After the membrane had been washed with TBST, bands of protein on the membrane were visualized with an ECL Western blotting detection kit (PerkinElmer Life Sciences, Inc., Boston, MA).

2.7. Analysis of TF mRNA by real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from THP-1 cells (5×10^5/well) was extracted with ISOGEN (Nippongene, Toyama, Japan) and reverse transcribed with the SuperScript II preamplification system (Invitrogen, Carlsbad, CA). The sequences of the oligonucleotides used in PCR reactions were as follows: TF-forward 5′-CACTAAGTCAGGAGATTGG-3′, TF-reverse 5′-CTCCAGGTAAGGTGTGAAC-3′, β-actin-forward 5′-CACTAAGTCAGGA-

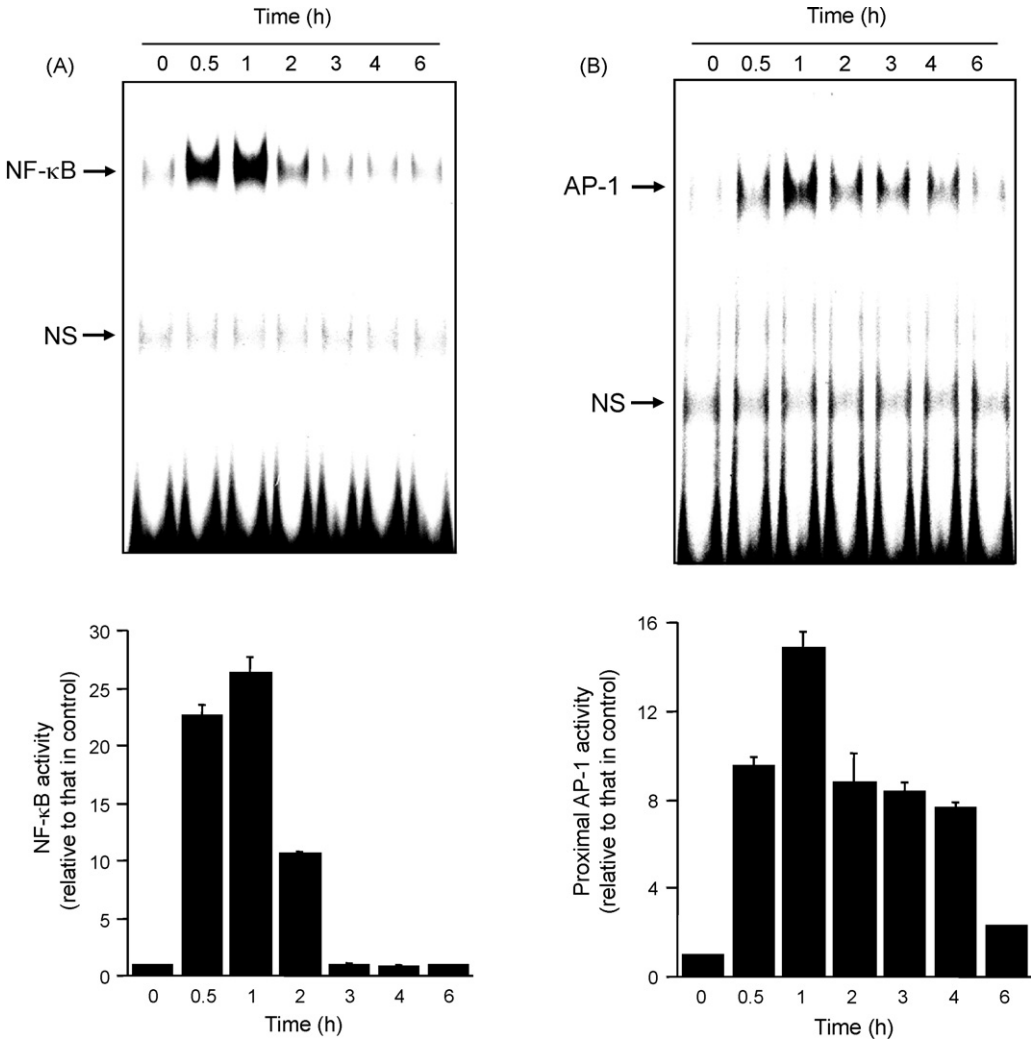

Fig. 2 – Enhancement of TF-κB, proximal AP-1, Sp1, and Egr-1 activation in LPS-treated THP-1 cells. After incubation of THP-1 cells with LPS (100 ng/mL) for the indicated times, the nucleus proteins were subjected to EMSA with NF-κB motif of TF (A), proximal AP-1 motif of TF (B), consensus Sp1-oligonucleotide (C), and consensus Egr-1-oligonucleotide (D) as described in Section 2. For the determination of complex, the corresponding area in the gel was cut and the radioactivity was measured. The data are presented as means ± S.D. of the results of three independent experiments. NS indicates nonspecific binding reaction.

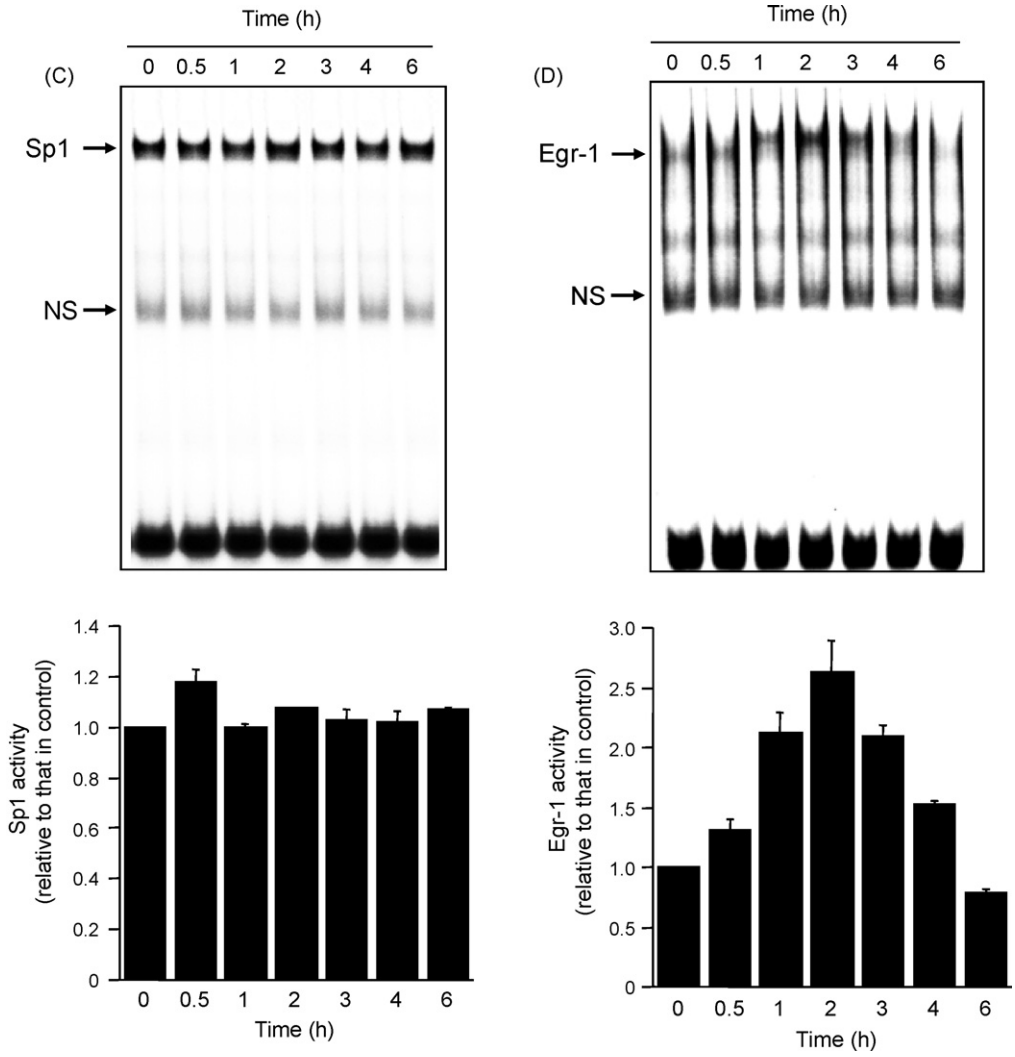

Fig. 2. (Continued).

GATTGG-3′, β-actin-reverse 5′-CTCCAGGTAAGGTGTGAAC-3′. PCR reactions were performed using qPCR™ Mastermix Plus for SYBR™ Green I (Eurogentic SA, Belgium), and assayed on the ABI 7300 Detector (Applied Biosystems). The transcript number of β-actin was quantified, and each sample was normalized on the basis of β-actin content.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation. Results were analyzed using Student's *t*-test, and statistical significance for all comparisons was assigned at $P < 0.05$.

3. Results

3.1. Changes in TF activity and mRNA levels in THP-1 cells upon treatment with LPS

The effect of LPS on TF activity and mRNA levels in THP-1 monocytic cells was examined (Fig. 1). TF activity in cells rapidly increased and showed a 17-fold increase over the control 2 h after LPS stimulation (Fig. 1A). A dose-dependent increase in TF activity was also observed 2 h after LPS stimulation (Fig. 1B). The mRNA level was increased about 9-fold 1 h after LPS stimulation relative to that in untreated control cells (Fig. 1C). A dose-dependent increase in TF mRNA levels was also observed following treatment with LPS (Fig. 1D).

3.2. Activation of transcription factors in THP-1 cells treated with LPS

To confirm the mechanism by which LPS induces transcriptional activation of the TF gene in THP-1 cells, we performed electrophoretic mobility shift assays in nondenaturing polyacrylamide gels. These EMSA evaluated the binding of nuclear proteins from THP-1 cells stimulated with LPS to oligonucleotides containing the TF motifs for NF-κB or AP-1 binding and consensus motifs for Sp1 or Egr-1 binding. In LPS-stimulated cells, the binding activity of nuclear proteins to the NF-κB or proximal AP-1-oligonucleotide was increased as compared to that of nuclear proteins from the control cells and reached a maximum at 1 h after LPS stimulation (Fig. 2A and B). In this

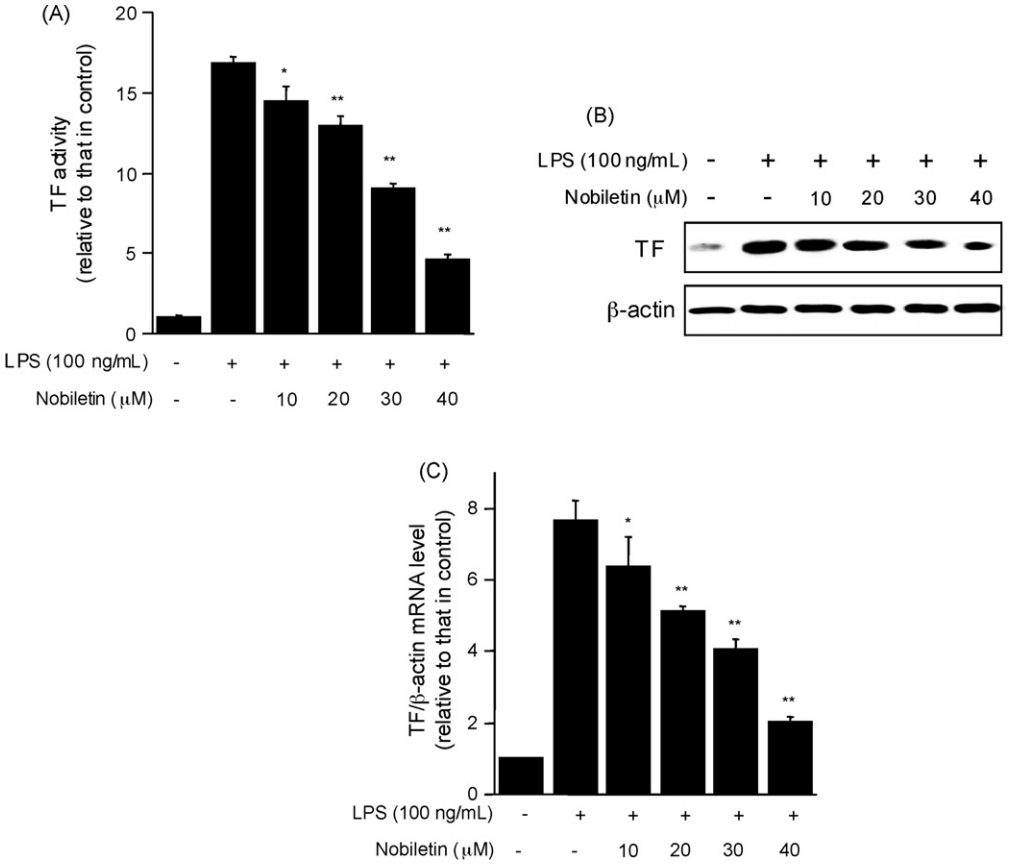

Fig. 3 – Inhibitory effects of nobiletin on TF expression induced by LPS. THP-1 cells were pretreated with nobiletin at various concentration for 2 h and then stimulated with LPS (100 ng/mL) for 2 h (A and B) or 1 h (C). After LPS stimulation, the TF procoagulant activity (A) or the protein levels (B) in the cell lysates were measured by a one-stage clotting assay or Western blotting described in Section 2, respectively. The TF mRNA levels were measured by quantitative real-time PCR described in Section 2 (C). The data are presented as means ± S.D. of the results of three independent experiments. (*) and (**) Data with statistical significance against LPS treatment. *$P < 0.05$; **$P < 0.01$.

experiment, same intensity of nonspecific bands was observed in each lane on the gel, indicating that same amounts of nuclear proteins were applied on each lane. Binding of nuclear proteins to the distal AP-1-oligonucleotide was not observed (data not shown). Binding of nuclear proteins to the Sp1-oligonucleotide was observed constitutively and unchanged by treatment with LPS (Fig. 2C). Although the maximum increase in binding activity of nuclear proteins to the Egr-1-oligonucleotide was observed at 2 h after LPS stimulation, the activity was later than that for TF mRNA expression levels.

3.3. *Inhibitory effect of nobiletin on activation of transcription factors by LPS*

The effect of nobiletin on LPS-induced TF expression and on activation of transcription factors was examined. Pretreatment of the cells with nobiletin suppressed LPS-induced increase in TF activity (Fig. 3A) and antigen levels (Fig. 3B) in a concentration-dependent manner. Furthermore, nobiletin dose-dependently suppressed LPS-induced up-regulation of TF mRNA levels (Fig. 3C). As shown in Fig. 4A and B, the maximal binding of nuclear proteins to NF-κB or proximal AP-1-oligonucleotides in LPS-treated THP-1 cells was also inhibited by treatment with nobiletin and the effect was dose-dependent. The inhibitory effects of nobiletin on binding activity were observed from lower concentrations, such as 10 and 20 μM. Interestingly, although an increase in Sp1 binding activity was not observed following treatment with LPS, nobiletin inhibited the basal binding activity of nuclear proteins to the consensus Sp1- oligonucleotide (Fig. 4C). A similar inhibitory effect of nobiletin on Sp1 binding activity was also observed in THP-1 cells treated with nobiletin alone (data not shown). An approximate 2-fold increase in the binding activity of Egr-1 observed in the cells treated with LPS was not inhibited by treatment with lower concentrations (10 and 20 μM) of nobiletin and was slightly inhibited by higher concentrations of nobiletin (30 and 40 μM) (Fig. 4D). Therefore, these results indicate that nobiletin not only inhibited NF-κB and AP-1 activation, but also inhibited basal activation of Sp1.

3.4. *Involvement of Sp1 in the regulation of TF expression*

Nobiletin strongly inhibited the basal binding activity of nuclear proteins to the consensus Sp1-oligonucleotide. To

elucidate the mechanism of nobiletin-induced down-regulation of Sp1 activity in the transcriptional regulation of the TF gene, we investigated the effects of Sp1-specific siRNA on LPS-induced TF mRNA expression. As shown in Fig. 5A, treatment of THP-1 cells with Sp1-specific siRNA (Sp1 siRNA) significantly suppressed the expression of Sp1, as compared to cells treated with non-silencing siRNA (NS siRNA) or in untreated control cells. Levels of β-actin in THP-1 cells were unaffected by treatment with Sp1-specific siRNA under the same experimental conditions. The effect of Sp1 knockdown on the binding of nuclear proteins from THP-1 cells treated with LPS to NF-κB, proximal AP-1, Sp1, or Egr-1 was examined by using these cells. Sp1-specific siRNA had no silencing effect on the binding activity of NF-κB, proximal AP-1 or Egr-1 (Fig. 5B). Furthermore, when THP-1 cells that had been treated with Sp1-specific siRNA were exposed to 100 ng/mL LPS for 1 h, significant inhibition of TF activity was observed as compared to cells exposed to LPS after treatment with non-silencing siRNA or in untreated control cells (Fig. 5C). LPS-induced up-regulation of TF mRNA levels was also prevented in THP-1 cells after treatment with Sp1-specific siRNA, as compared to cells that had been treated with non-silencing siRNA (Fig. 5D). These observations indicate that basal Sp1 expression is important for LPS-induced TF activity and the mRNA expression.

3.5. *Nobiletin inhibits nuclear localization of Sp1*

To investigate the details of the mechanism of the inhibition of Sp1 activity by nobiletin, we examined the effect of nobiletin on nuclear localization of Sp1 by subcellular fractionation and Western blotting analysis. As shown in Fig. 6, when THP-1 cells that had been pretreated with nobiletin were exposed to LPS, the extent of Sp1 protein detected in nuclear fraction decreased with increasing nobiletin in a dose-dependent manner. However, the amount of Sp1 protein in the whole cell lysate was unaffected by treatment with nobiletin under the same experimental conditions.

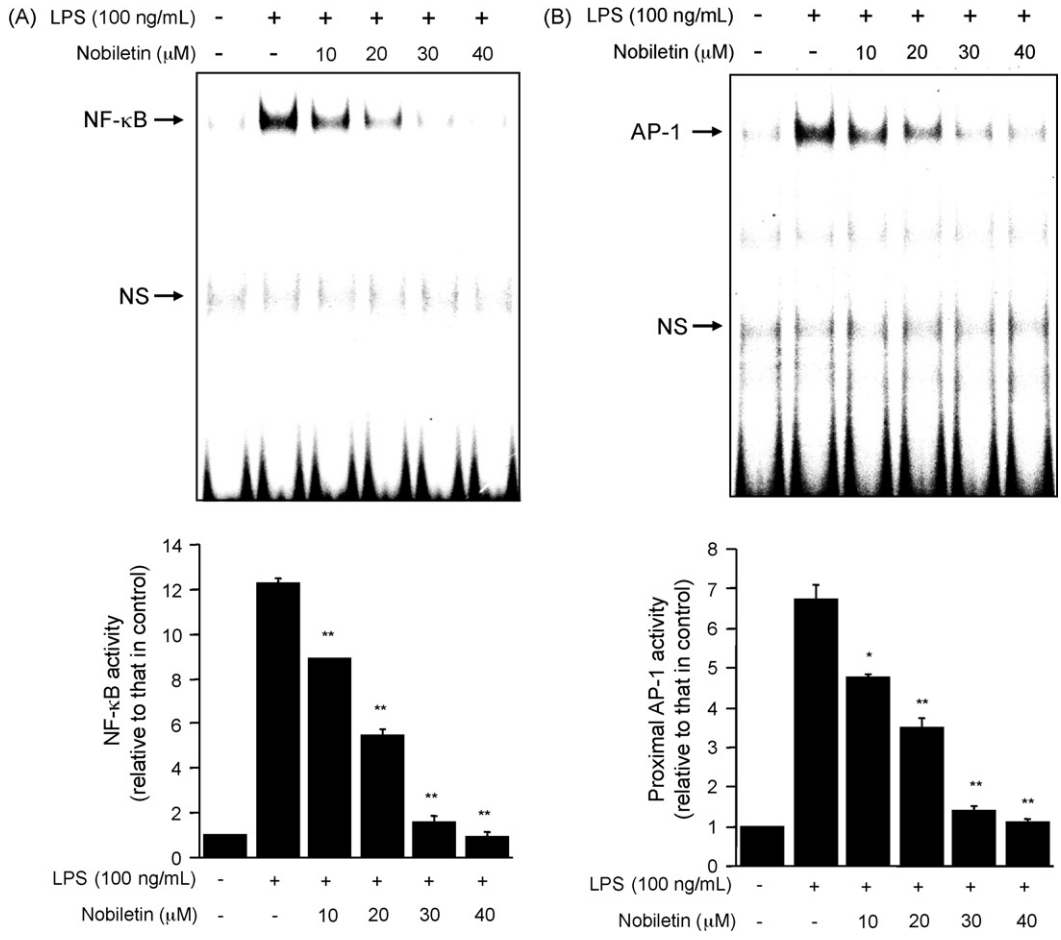

Fig. 4 – Inhibitory effect of nobiletin on LPS-Induced TF-κB, proximal AP-1, Sp1, and Egr-1 activation. THP-1 cells were pretreated with nobiletin at various concentrations for 2 h and then stimulated with LPS (100 ng/mL) for 1 h. After LPS stimulation, the nucleus proteins were subjected to EMSA with NF-κB motif of TF (A), proximal AP-1 motif of TF (B), consensus Sp1-oligonucleotide (C), and consensus Egr-1-oligonucleotide (D) as described in Section 2. For the determination of complex, the corresponding area in the gel was cut and the radioactivity was measured. NS indicates nonspecific binding reaction. The data are presented as means ± S.D. of the results of three independent experiments. (*) and (**) Data with statistical significance against LPS treatment. *$P < 0.05$; **$P < 0.01$.

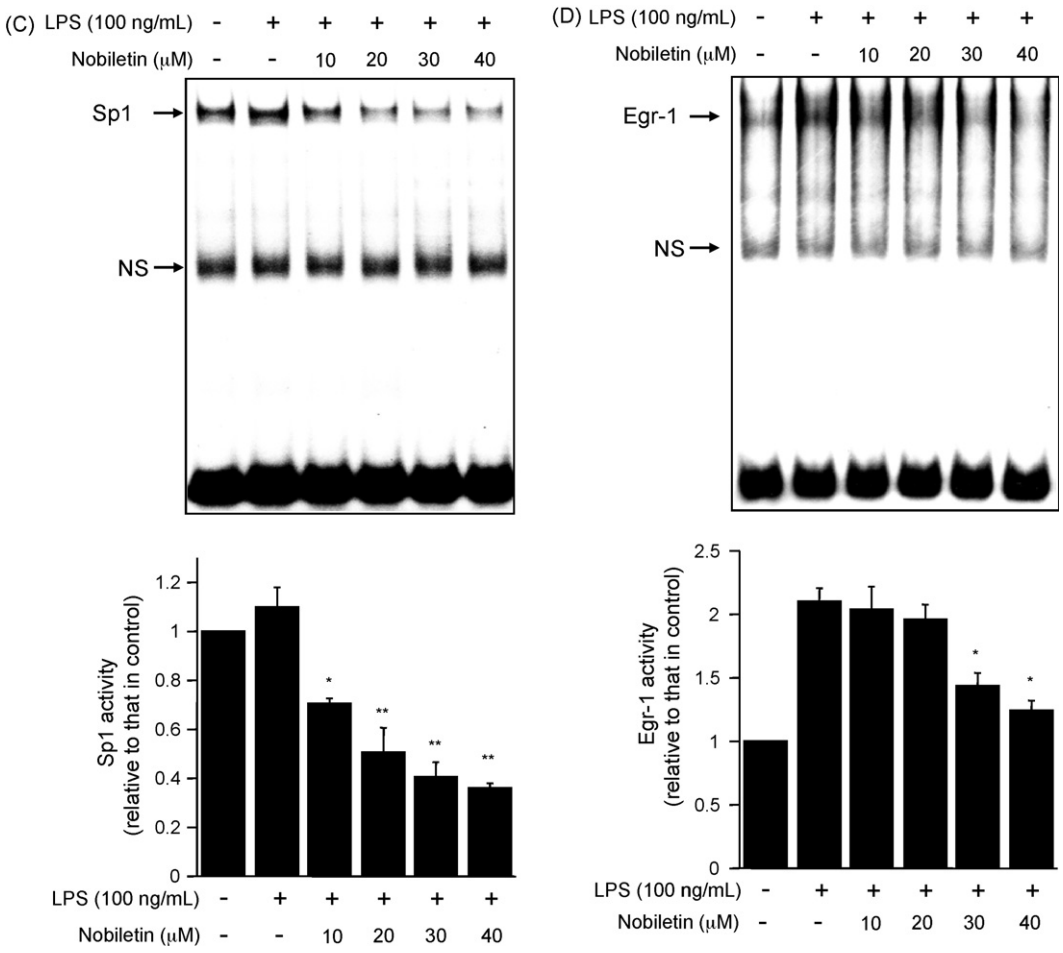

Fig. 4. (Continued).

4. Discussion

Tissue factor, a transmembrane glycoprotein, is an initiator of the coagulation protease cascade. The expression of TF mRNA is undetectable in unstimulated monocytes and normal vascular endothelial cells, but is increased by stimulation of these cells with TNF-α, phorbol ester 12-*O*-tetradecanoyl-13-phorbol-acetate (TPA), IL-1β, and bacterial endotoxin LPS [30-33]. The present study confirmed that LPS-induced TF upregulation in THP-1 monocytic cells and demonstrated that pretreatment with nobiletin, a polymethoxy flavonoid, inhibited LPS-induced TF activity and TF mRNA in a dose-dependent manner. The level of inhibition of TF activity induced by LPS correlated with the level of mRNA expression suggesting that the inhibitory action of nobiletin on TF activity was regulated at the transcriptional level. The present work focused on the molecular basis for transcriptional regulation of TF gene expression by nobiletin in THP-1 cells.

The human TF gene promoter contains binding motifs for NF-κB/Rel, AP-1, Sp1, and Egr-1 transcription factors [3,34]. Two AP-1 binding sites and an NF-κB binding site are present within a distal enhancer region (227–172 bp) of the TF promoter region and have been shown to regulate TF expression in endothelial and monocytic cells exposed to bacterial LPS or cytokines [3]. Disruption of the proximal AP-1 binding site and the NF-κB binding site abolishes LPS-induced TF promoter activity in human monocytic cells, indicating that these AP-1 and NF-κB sites are required for maximal activity of the human TF promoter [35]. The results of EMSA showed that the binding activities of nuclear proteins from THP-1 cells treated with LPS to the NF-κB or AP-1 sequence were significantly inhibited in the presence of nobiletin. Sp1 and Egr-1 are zinc finger transcription factors that share three GC-rich binding domains within the TF promoter [5,18]. Interestingly, although the level of constitutive activation of Sp1 did not change with treatment of cells with LPS, nobiletin also inhibited the binding activity of Sp1 to oligonucleotide containing the Sp1 motif. These findings are the first to show the possible involvement of Sp1 in nobiletin-induced biological activity, suggesting that inhibition of Sp1 activation by nobiletin might play a crucial role in regulation of TF expression. This hypothesis is supported by Sp1 knockdown by siRNA experiment. The extent of LPS-induced TF mRNA expression was inhibited in THP-1 cells that had Sp1 expression suppressed by siRNA, as compared to cells treated with non-silencing siRNA. Furthermore, exposure of THP-1 cells to nobiletin caused a decrease in the amount of Sp1 in the nuclear fraction, although the amount of Sp1 protein in the whole cell lysate was unaffected by treatment with nobiletin.

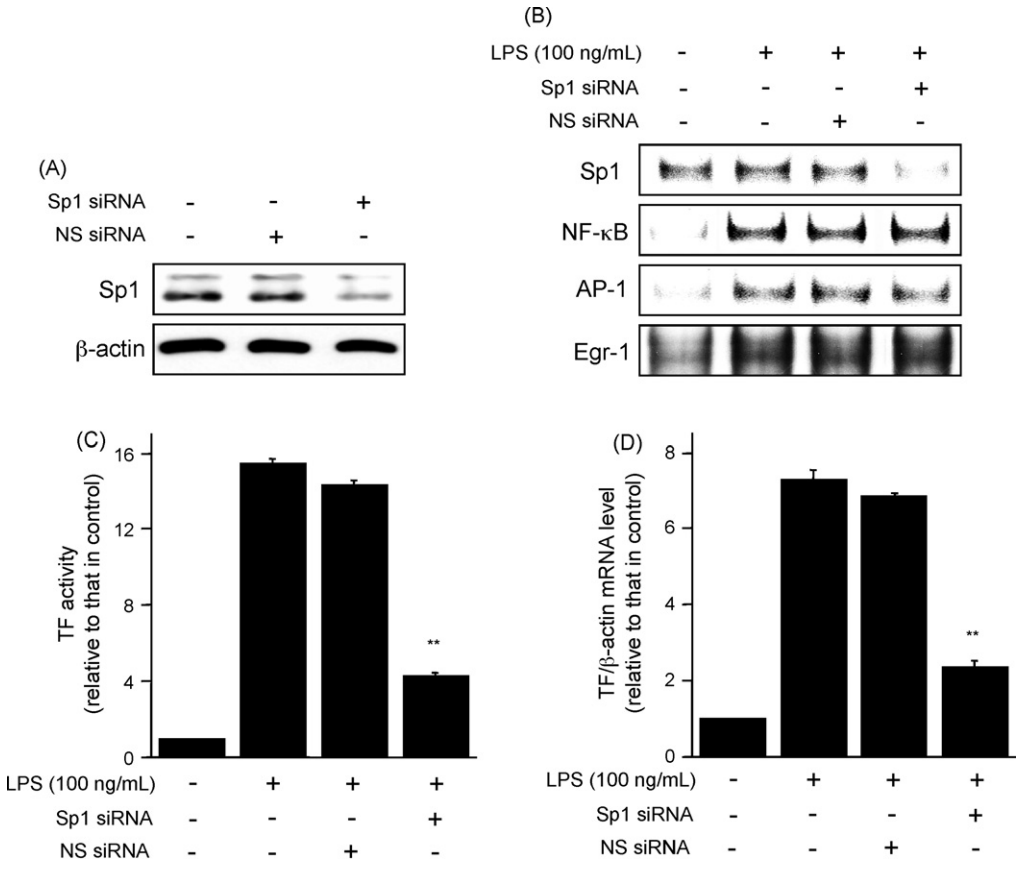

Fig. 5 – Involvement of Sp1 in the regulation of TF expression. (A) THP-1 cells were transfected with non-silencing siRNA (NS siRNA) and Sp1-specific siRNA (Sp1 siRNA) as described in Section 2. After transfection, the levels of expression of Sp1 and β-actin were analyzed by Western blotting. The results are typical of the results of three experiments that gave similar results. (B) After transfection of THP-1 cells with siRNAs, the cells were stimulated with LPS (100 ng/mL) for 1 h and the nucleus proteins were subjected to EMSA with NF-κB motif of TF, proximal AP-1 motif of TF, consensus Sp1-oligonucleotide, and consensus Egr-1-oligonucleotide. The results are typical of the results of three experiments that gave similar results. (C and D) After transfection of THP-1 cells with siRNAs, the cells were stimulated with LPS (100 ng/mL) for 2 h (C) or 1 h (D). The TF procoagulant activity in the cell lysates was measured by a one-stage clotting assay (C). The TF mRNA levels were measured by quantitative real-time PCR (D). The data are presented as means ± S.D. of the results of three independent experiments. (**) Data with statistical significance against LPS treatment with NS siRNA. $P < 0.01$.

These results suggest that nobiletin might prevent activation of Sp1 through inhibition of nuclear localization of Sp1.

Although the effect of nobiletin on signaling pathways involved in the regulation of NF-κB and AP-1 has not yet been revealed, recent reports have shown that nobiletin prevented LPS-induced phosphorylation of JNK and ERK. This would suggest that inhibition of JNK and ERK phosphorylation by nobiletin may participate in blocking AP-1 and NF-κB activation, respectively [36,37]. The present work indicated inhibition of constitutive activation of Sp1 by nobiletin, although the precise mechanisms responsible for the regulation of Sp1 activity by nobiletin are not known. There have been suggestions that phosphorylation of Sp1 increases its transcriptional activity [38]. So nobiletin might exert an inhibitory effect on Sp1 activity via direct inhibition of certain protein kinases involved in the regulation of Sp1 activity. Moreover, recent report described about Sp1 acting as an anchor protein to recruit transcription factor c-Jun for growth factor and/or phorbol ester-induced expression of several genes, suggesting the possible role of Sp1 in the regulation of JNK signal [39]. Thus it is also likely that nobiletin might indirectly participate in the regulation of JNK pathway through inhibition of Sp1 activation.

In conclusion, our results indicate that nobiletin suppresses LPS-induced TF activity and mRNA levels in THP-1 cells. Nobiletin not only effectively prevents NF-κB and AP-1 activation, but also reduces basal activation of Sp1. A siRNA-induced reduction in the level of expression of Sp1 inhibited LPS-induced expression of TF mRNA, suggesting that the inhibitory effect of nobiletin on the expression of TF mRNA is probably mediated by suppression of NF-κB, AP-1 and Sp1 activity. Further studies are required to clarify the details of the molecular mechanisms of action of nobiletin.

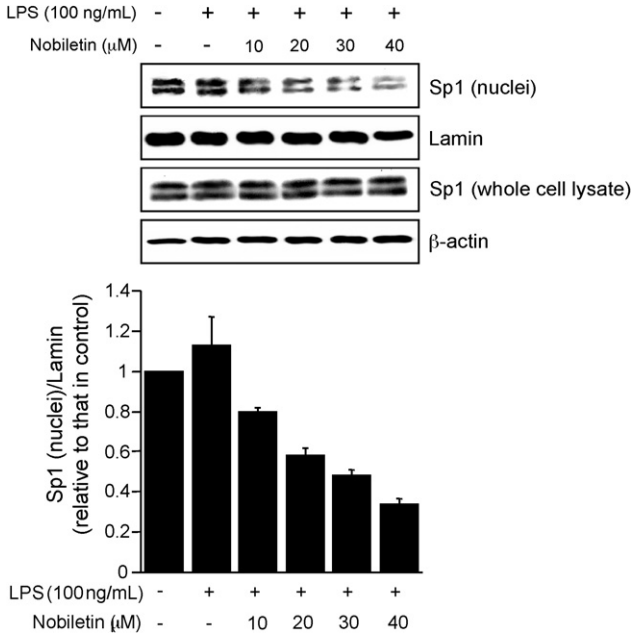

Fig. 6 – Inhibitory effects of nobiletin on nuclear localization of Sp1. THP-1 cells were pretreated with nobiletin at various concentrations for 2 h and then stimulated with LPS (100 ng/mL) for 1 h. After LPS stimulation, Sp1 protein levels in the nuclei or in the cell lysates were analyzed by Western blotting. The intensities of bands of Sp1 in nuclear fractions were quantified with the NIH Image program. The data are presented as means ± S.D. of the results of three independent experiments.

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