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Surfactin Blocks NO Production in Lipopolysaccharide-activated Macrophages by Inhibiting NF-κB Activation

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Surfactin is a natural biosurfactant derived from *Bacillus subtilis* and has various biological activities such as anticancer, antiplatelet, and anti-inflammatory effects. In this study, the inhibitory mechanism of surfactin in NO production from macrophages was examined. Surfactin downregulated LPS-induced NO production in RAW264.7 cells and primary macrophages with IC_{50} values of 31.6 and 22.4 μM, respectively. Immunoblotting analysis showed that surfactin strongly blocked the phosphorylation of IKK and IκBα and the nuclear translocation of NF-κB (p65). Therefore, these data suggest that surfactin may act as a bacterium-derived anti-inflammatory agent with anti-NF-κB activity.

Keywords: Surfactin, *Bacillus subtilis*, nitric oxide, anti-inflammatory effect, lipopolysaccharide, NF-κB

Macrophages play a central role in managing many different immunopathological phenomena during inflammation, such as overproduction of inflammatory mediators [nitric oxide (NO) and prostaglandin E_2 (PGE_2)], generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 [18]. These inflammatory events require upregulated activity of the intracellular signaling machinery including mitogen-activated protein kinases (MAPKs) such as p38, extracellular signal-regulated kinase [ERK], and C-Jun N-terminal kinase [JNK], non-receptor type tyrosine kinases (such as Syk, JAK-2, and Src), and phosphoinositide-3-kinase (PI3K)/Akt. The activation of these signaling molecules is eventually linked to the activation of transcription factors such as nuclear factor (NF)-κB (p50/p65) and activator protein (AP)-1 [19]. In particular, NF-κB-mediated pro-inflammatory gene expression requires IκB phosphorylation by IκB kinase (IKK), a protein substrate of MAPKs or Akt, degradation of IκB by the proteasome pathway, and translocation of free NF-κB (p50/p65) dimer [7].

Surfactin isolated from *Bacillus subtilis* strain is a cyclic lipopeptide with a molecular mass of 1,036 Da [2]. It is one of the most powerful biosurfactants, playing various biological roles such as inhibition of fibrin clot formation, suppression of bacterial growth, diminishment of cancer cell proliferation, decrease of cholesterol level, relief of inflammatory symptoms, and downregulation of platelet aggregation [12, 15, 17]. The mechanism of how this macrolide lipopeptide can modulate numerous biological activities is not yet known. In this study, therefore, LPS-activated primary macrophages and macrophage-like RAW264.7 cells were employed to explore the regulatory role of surfactin on the activation of intracellular signaling cascades and transcription factor NF-κB.

Materials and Methods

Materials

Surfactin, N^G-monomethyl-L-arginine (N-MMA), and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Wortmannin, LY294002, SB203580, U0126, SP600125, and BAY11-7082 were obtained from Calbiochem (La Jolla, CA, U.S.A.). Cynaropicrin was a gift from Prof. Jung Jee Hyung (Pusan National University, Pusan, Korea) [3]. Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, U.S.A.). RAW264.7 and HEK293 cells were purchased from the American Tissue Culture Center (Rockville, MD, U.S.A.). Luciferase constructs containing NF-κB binding promoters were gifts from Prof. Chung Hae Young (Pusan National University, Pusan, Korea). All other

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chemicals were of Sigma grade. Phospho- or nonphospho-antibodies to Akt, ERK, p38, JNK, IκBα, and β-actin were purchased from Cell Signaling (Beverly, MA, U.S.A.). The primers (Bioneer, Seoul, Korea) used in this experiment are indicated as follows: TNF-α (forward [F]-5'-TTGACCTCAGCGCTGAGTTG-3' and reverse [R]-5'-CCTGTAGCCCACGTCGTAGC-3'); IL-1β (F-5'-CAGGATGAGGACATGAGCACC-3' and R-5'-CTCTGCAGACTCAAACTCCAC-3'); IL-6 (F-5'-GTACTCCAGAAGACCAGAGG-3' and R-5'-TGCTGGTGACAACCACGGCC-3'); iNOS (F-5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and R-5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'); and GAPDH (F-5'-CACTCACGGCAAATTCAACGGCAC-3' and R-5'-GACTCCACGACATACTCAGCAC-3').

Animals and Preparation of Peritoneal Macrophages

C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from DAEHAN BIOLINK (Chungbuk, Korea). Mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied *ad libitum*. Studies were performed in accordance with guidelines established by the Kangwon University Institutional Animal Care and Use Committee. Peritoneal exudates were obtained from C57BL/6 male mice (7–8 weeks old, 17–21 g) by lavage 4 days after intraperitoneal injection of 1 ml of sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI, U.S.A.). Cells were washed and resuspended in RPMI 1640 containing 2% FBS, and plated in 100-mm tissues culture dishes for 4 h at 37°C in 5% CO_2 humidified atmosphere to prepare peritoneal macrophages.

Cell Culture

RAW264.7 and HEK293 cells were maintained in RPMI1640 or DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2.

Determination of NO Production

RAW264.7 cells (1×10^6 cells/ml) were preincubated with surfactin for 30 min and continuously activated with LPS (1 μg/ml) for 24 h [3]. Nitrite in culture supernatants was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μl samples of the medium for 10 min at room temperature. OD at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). A standard curve of NO was made with sodium nitrite. The detection limit of the assay was 0.5 μM.

RT-PCR

For evaluating mRNA expression levels of cytokine and iNOS, total RNA from the LPS-treated RAW264.7 cells (5×10^6 cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. Semiquantitative RT reactions were conducted using MuLV reverse transcriptase. The total RNA (1 μg) was incubated with oligo-dT15 for 5 min at 70°C and mixed with a 5× first-strand buffer, 10 mM dNTP, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37°C and for 60 min by maintaining for the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70°C, and the total RNA was removed by adding RNase H. The PCR reaction was conducted with the incubation mixture (2 μl cDNA, 4 μM 5' and 3' primers, a 10× buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 μM each of dNTP, 25 mM $MgCl_2$, and 1 unit of *Taq* polymerase [Promega, U.S.A.]). The following incubation conditions were used: a 45 sec denaturation at 94°C, an annealing for 45 sec between 55 and 60°C, an extension for 60 sec at 72°C, and a final extension of 7 min at 72°C.

Luciferase Reporter Gene Activity Assay

HEK293 cells (1×10^6 cells/ml) were transfected with 1 μg of plasmids with NF-κB-Luc as well as β-galactosidase by using the calcium phosphate method in a 12-well plate according to the manufacturer's protocol. Luciferase assays were performed using the Luciferase Assay System (Promega) [13]. Briefly, the transfected cells treated with surfactin in the presence of TNF-α (15 ng/ml) were lysed in the culture dishes with reporter lysis buffer. Lysates were centrifuged at maximum speed for 10 min in a microcentrifuge. Ten μl of the supernatant was incubated with 50 μl of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent (Thermo Labsystems Oy, Helsinki, Finland). The activity was normalized to β-galactosidase activity.

Preparation of Total and Nuclear Lysates, and Immunoblotting

For total lysate preparation, RAW264.7 cells (5×10^6 cells/ml) were washed 3 times in cold PBS containing 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM hydrogen peroxide) for 30 min with rotation at 4°C.

Nuclear lysates were prepared with a three-step procedure. RAW264.7 cells (5×10^6 cells/ml) were grown in a 100-mm dish to near-confluence. After treatment, cells were collected with a rubber policeman, washed with 1× PBS, and lysed in 500 μl of lysis buffer containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM 1,4-dithiothreitol (DTT) on ice for 4 min. Cell lysates were centrifuged at 14,000 rpm for 1 min in a microcentrifuge. In the second step, the pellet (the nuclei fraction) was washed once in washing buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at −80°C, and then thawed on ice and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear extract.

Total cell or nuclear lysates were then analyzed by immunoblotting. Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidenedifluoride (PVDF) membranes by electroblotting. The membranes were blocked for 60 min in Tris-buffered saline, containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20, at room temperature. The membrane was incubated for 60 min with the specific primary antibody at 4°C, washed 3 times with the same buffer, and further incubated for an additional 60 min with the HRP-conjugated secondary antibody. The total and phosphorylated levels of IκBα, p38, JNK, ERK, p85, Akt, Src, JAK-2, and β-actin were visualized by the ECL system (Amersham, Little Chalfont, Buckinghamshire, U.K.).

Statistical Analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. The data are expressed as means± standard errors (SEM), and the results are taken from at least three

independent experiments performed in triplicate. P values of 0.05 or less were considered to be statistically significant.

Results and Discussion

We previously reported that surfactin inhibited NO production in LPS-activated RAW264.7 cells [11, 12]. Therefore, in this study, the NO inhibitory effect of this compound and its inhibitory mechanism were evaluated using peritoneal macrophages and macrophage-like RAW264.7 cells under LPS stimulation. In agreement with previous data [11, 12], surfactin remarkably downregulated NO production by activated primary and cancerous macrophages with IC_{50} values of 31.6 and 22.4 μM, without affecting cell viability (data not shown). The inhibitory potency of this compound on NO production was comparable to other naturally occurring or synthetic inhibitors such as cordycepin [14], and higher than those of savinin, pentoxifylline, theophylline, and dbcAMP [4, 5]. N-MMA, a control drug, also strongly suppressed LPS-induced NO production with an IC_{50} value of 192 μM.

To address whether the inhibition of NO production by surfactin occurred at the transcriptional level, iNOS mRNA levels were determined. Interestingly, surfactin suppressed the expression of iNOS mRNA up to 75%, suggesting an inhibition at the transcriptional level. In contrast, the mRNA levels of other cytokines such as TNF-α, IL-1β, and IL-6 were not dramatically altered under the present conditions (Fig. 1B). Instead, the compound induced basal expression of these cytokines (Fig. 1B). Although this induction effect of surfactin in this study cannot exactly be explained, it is highly possible that surfactin alone might have immunostimulatory ability due to its structural property derived from bacterial membrane. With stimuli, however, surfactin seems to act as an antagonist of inflammatory responses. The exact mechanism between inductive effect and suppressive activity should be evaluated in future experiments. Nonetheless, surfactin has previously been reported to block serum TNF-α levels and the mRNA expression of cytokines (IL-1β and IL-6) and chemokines (MCP-1 and MIP-1α) induced by LPS and *Mycoplasma hyopneumoniae* at a higher concentration (approximately 200 μM) [11, 12]. These results suggest that surfactin may regulate a common pathway involved in modulating pro-inflammatory responses at the transcriptional level.

Based on previous studies that LPS activates a series of transcriptional factors including NF-κB activation for expressing inflammatory genes [7, 9], we first investigated a possibility whether NF-κB is a target of surfactin, using LPS-treated RAW264.7 cells. As shown in Fig. 2A, surfactin strongly suppressed the phosphorylaion of IκBα and its degradation at 5 and 15 min. Thus, the proteasome pathway-mediated disappearance of IκBα due to LPS-induced

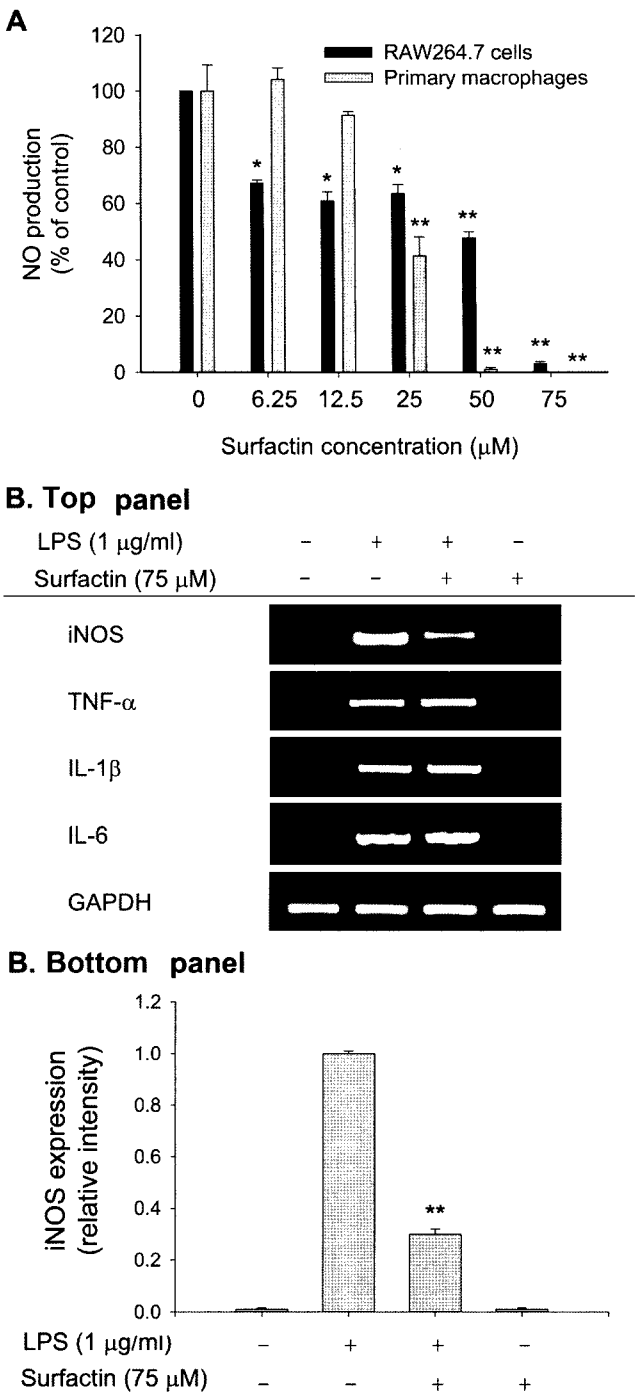

Fig. 1. The effect of surfactin on NO production and iNOS gene expression in LPS-activated RAW264.7 cells.

A. RAW264.7 cells (1×10^6 cells/ml) were treated with surfactin in the presence or absence of LPS (1 μg/ml) for 24 h. Supernatants were collected, and nitrite (NO) concentration in the supernatants was determined by Griess reagent, as described in Materials and Methods. **B.** Top panel: RAW264.7 cells (5×10^6 cells/ml) were incubated with surfactin in the presence or absence of LPS (1 μg/ml) for 6 h. The mRNA levels of iNOS and cytokines were determined by semiquantitative RT-PCR. Bottom panel: the band intensity was calculated by a band calculation program (Quant) using the GAPDH level. The results show one experiment out of three. *, $p<0.05$ and **, $p<0.01$ compared with the control group.

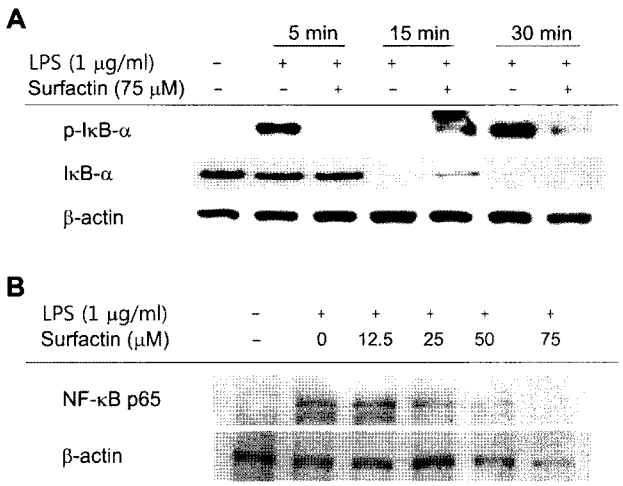

Fig. 2. The effect of surfactin on NF-κB activation.
A. RAW264.7 cells (5×10^6 cells/ml) pretreated with surfactin were stimulated in the absence or presence of LPS (1 μg/ml) at the indicated time points. After immunoblotting, the phosphorylation or total levels of IκBα and β-actin were identified by phospho-specific or total protein antibodies. **B**. RAW264.7 cells (5×10^6 cells/ml) pretreated with surfactin were stimulated in the absence or presence of LPS (1 μg/ml) for 30 min. After preparation of nuclear lysates, the translocation level of p65 was examined by immunoblotting analysis as described in Materials and Methods.

phosphorylation was remarkably prevented by surfactin treatment: LPS-induced IκBα phosphorylation was clearly suppressed at an early time point. In concordance with the results, surfactin dose-dependently diminished the nuclear translocation level of p65 (Fig. 2B), demonstrated by immunoblotting analysis with nuclear extracts. To check whether this inhibitory effect is signal dependent, the luciferase assay was employed using NF-κB-Luc-transfected HEK293 cells under PMA or TNF-α treatment, as reported previously

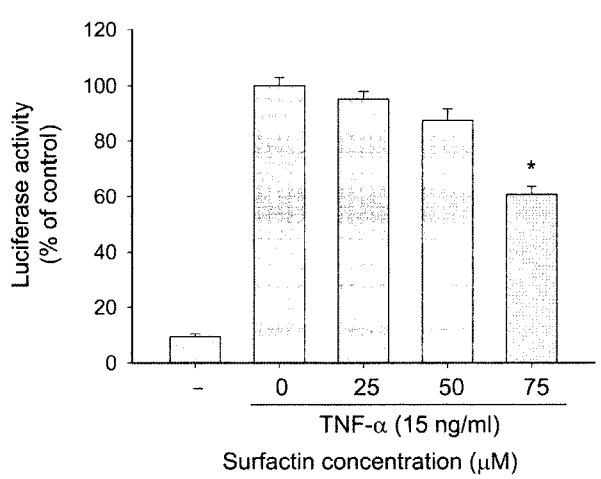

Fig. 3. The effect of surfactin on NF-κB reporter gene assay.
HEK293 cells transfected with plasmid constructs including NF-κB-Luc (1 μg/ml) as well as β-gal (0.5 μg/ml) were treated with surfactin in the presence or absence of PMA (10 ng/ml) or TNF-α (15 ng/ml) for 6 h. Luciferase activity was determined by a luminometer as described in Materials and Methods.

[13]. Interestingly, the upregulation of NF-κB-dependent luciferase activity by PMA treatment was not diminished by surfactin (data not shown). The TNF-α-induced enhancement of luciferase activity was also partially downregulated (up to 43%) with 75 μM surfactin (Fig. 3). These results suggest that surfactin can directly block neither the binding of NF-κB to promoter sites nor the common pathways (*eg.*, IκBα phosphorylation and degradation of IκBα) found in NF-κB activation signaling. Thus, the finding that surfactin blocked the phosphorylation of IKKβ and IκBα at 5 min seems to imply that the target of surfactin may be one of the upstream signaling enzymes activated by LPS exposure, as reported previously [15].

Because of extensive studies on the signaling cascade for NF-κB activation by microbial products, understanding of the molecular mechanism involved in NF-κB activation has been greatly improved. Several major pathways are currently considered to be relevant to the activation of NF-κB. Signaling cascades composed of PI3K and Akt or MAPKs (ERK, p38, and JNK) are an examples of the pathways involved in activating IKK, phosphorylating IκBα, and translocating NF-κB (p50/p65) [8]. Although many different kinds of natural and synthetic compounds have been reported to negatively regulate NF-κB activation pathways [10], not many inhibitory targets of these compounds to suppress NF-κB activation have exactly been proven. In order to obtain further information on whether surfactin was able to modulate the upstream signaling events, MAPK and PI3K/

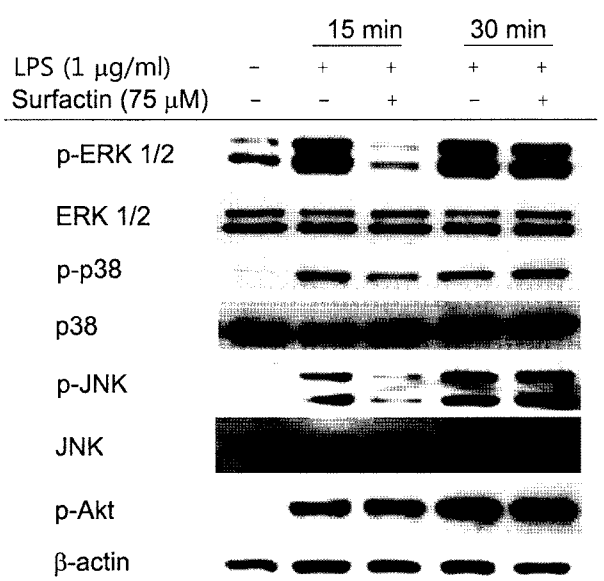

Fig. 4. The effect of surfactin on the upregulation of upstream signaling pathways for NF-κB activation.
RAW264.7 cells (5×10^6 cells/ml) were stimulated with surfactin in the presence or absence of LPS (1 μg/ml). After immunoblotting, the total or phosphoprotein levels of Akt, ERK, p38, JNK, and β-actin were identified by their total protein- or phospho-specific antibodies. The results show one experiment out of three.

Akt pathways were selected to evaluate their involvement. The LPS-induced phosphorylation of p38, JNK, and ERK at 15 min was markedly suppressed by this compound, whereas the phosphorylation of these proteins at 30 min was not diminished (Fig. 4). In contrast, however, the phosphorylation of Akt (Fig. 4) and PI3K (p85, a regulatory subunit of PI3K) (data not shown) was not decreased by surfactin treatment. In agreement with this result, the activation-related phosphorylation pattern of other nonreceptor-type tyrosine kinases (such as Syk, JAK-2, and Src), which are responsible for activating PI3K [1], was not altered by surfactin (data not shown). Therefore, these results suggest that surfactin-mediated inhibition of IKK/IκBα phosphorylation may be due to blocking the activity of either MAPK or Akt itself, but not PI3K/other upstream tyrosine kinases.

Although MAPK and Akt have been known to participate in modulating IKK/IκBα pathway, the involvement of these enzymes in inflammatory responses currently remains controversial: LY294002, a PI3K inhibitor, upregulated NF-κB activation, whereas MAPK inhibitors did not block NO production [6]. Therefore, to elucidate what target(s) of surfactin mediates the inhibition of NO production, specific inhibitors to ERK (U0126), JNK (SP600125), p38 (SB203580), PI3K/Akt (LY294002 and wortmannin), and IKK/IκBα (cynaropicrin and BAY11-7082) were employed. Interestingly, three MAPK inhibitors showed marginal inhibitory effects

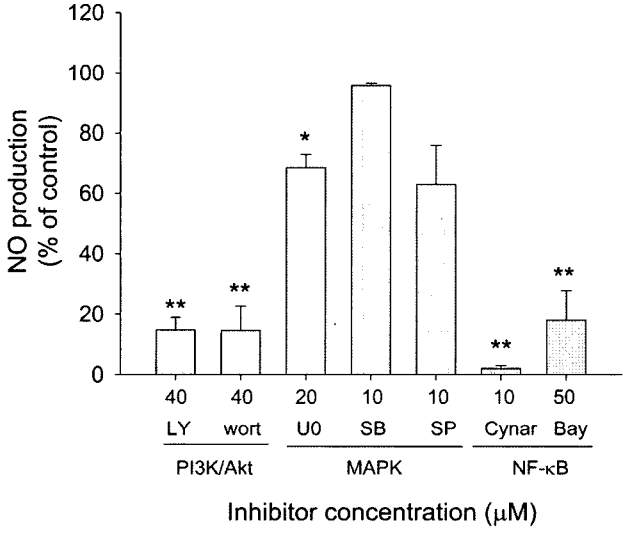

Fig. 5. The effect of various signaling inhibitors on the production of NO in LPS-activated macrophages.
RAW264.7 cells (1×10^6 cells/ml) were pretreated with various enzyme inhibitors [wort (wortmannin, 25 μM, a PI3K inhibitor)], LY (LY294002, 25 μM, a PI3K inhibitor), U0126 (50 μM, an ERK inhibitor), SB203580 (25 μM, a p38 inhibitor), and SP600125 (25 μM, a JNK inhibitor)], and various NF-κB inhibitors [SN50 (50 μM), a cell-permeable NF-κB inhibitor], cynaropicrin (20 μM, a sesquiterpene lactone), and Bay 11–7082 (10 μM, IKK inhibitor)] in the presence or absence of LPS (1 μg/ml) for 24 h. Supernatants were collected, and nitrite (NO) concentration in the supernatants was determined by Griess reagent, as described in Materials and Methods. $^*p<0.05$ and $^{**}p<0.01$ compared with the control group.

(up to 10–35%) on NO production (Fig. 5), and they strongly suppressed the production of PGE_2 and TNF-α under the same conditions (data not shown). Unlike MAPK inhibitors, PI3K/Akt and IKK inhibitors displayed strong NO inhibitory effect as reported previously [16], implying that these pathways may play a critical role in upregulating iNOS expression and NO production, and may act as a target of surfactin inhibition.

In summary, surfactin was able to downregulate LPS-induced NO production in RAW264.7 cells and primary macrophages. Surfactin inhibition seemed to be mediated by a series of intracellular signaling cascades composed of Akt and IKK/IκBα, but not MAPKs for NF-κB activation, thus suggesting that surfactin may act as a bacterium-derived anti-inflammatory agent with anti-NF-κB activity.

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