

Inhibition of Lipopolysaccharide Activity by a Bacterial Cyclic Lipopeptide Surfactin

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Abstract Compounds that inactivate lipopolysaccharide (LPS) activity have the potential of being new anti-inflammatory agents. Therefore, we searched among microbial secondary metabolites for compounds that inhibited LPS-stimulated adhesion between human umbilical vein endothelial cells (HUVEC) and HL-60 cells. By this screening, we found a cyclic lipopeptide surfactin from the culture broth of *Bacillus* sp. BML752-121F2 to be inhibitory. The addition of the surfactin prior to the LPS stimulation decreased HL-60 cell-HUVEC adhesion without showing any cytotoxicity. We confirmed that surfactin inhibited LPS-induced expression of ICAM-1 and VCAM-1 in HUVEC. It also inhibited the cellular adhesion induced by lipid A, the active component of LPS; but it did not inhibit TNF- α or IL-1 β -induced cell adhesion. Then, surfactin was shown to suppress the interaction of lipid A with LPS-binding protein (LBP) that mediates the transport of LPS to its receptors. Finally, surface plasmon resonance (SPR) analysis revealed the surfactin to interact reversibly with lipid A. Thus, this *Bacillus* surfactin was shown to be an inhibitor of LPS-induced signal transduction, directly interacting with LPS.

Keywords surfactin, lipopolysaccharide, lipid A, human umbilical vein endothelial cells, HL-60 cells, adhesion

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Introduction

Lipopolysaccharide (LPS) is one of the major constituents of the outer membrane of Gram-negative bacteria and is recognized as a key molecule in the pathogenesis of inflammatory syndromes associated with Gram-negative bacterial infections. Most of the pathological activities of LPS are attributed to its lipid A portion, which consists of a backbone of 2 phosphorylated glucosamine molecules acylated with fatty acids [1, 2].

Recent research has revealed the detailed mechanism by which LPS activates mammalian cells. Upon its release from bacterial cell walls into the host's blood circulation, LPS interacts with LPS-binding protein (LBP) present in the blood and transfers LPS to CD14, the primary receptor for LPS, which exists as a soluble form in the blood or as a glycosylphosphatidylinositol (GPI)-linked molecule on the surface of target cells. Next, LPS binds to Toll-like receptor (TLR) 4, the putative transmembrane receptor for LPS that activates nuclear factor- κ B (NF- κ B) signaling [3–5]. NF- κ B is known to play a critical role in the development of inflammatory responses by upregulating the expression of cell adhesion molecules and many inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukins (IL). Systemic production of these proinflammatory mediators causes the sepsis syndrome, which is clinically characterized by hypotension, fever, and respiratory dysfunction, and may lead to multi-organ failure and death [6, 7].

LPS can be recognized by monocytes, macrophages, neutrophils, and endothelial cells in mammals. Especially in endothelial cells, LPS induces adhesion molecule expression [8]. The most important of the adhesion

molecules involved in this process are intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [8, 9]. Without stimulation, only a small amount of ICAM-1 is present on the endothelial cell surface [10], and VCAM-1 and E-selectin are absent entirely [11, 12], but they are immediately expressed in response to extracellular inflammatory stimulation [13]. ICAM-1 interacts with the ligand CD11a/CD18 (LFA-1), which is expressed on neutrophils, lymphocytes, and monocytes; and VCAM-1, with the ligand VLA-4, which is expressed on lymphocytes. E-selectin mediates the adherence of neutrophils, T cells, eosinophils, and monocytes.

As described above, LPS has been implicated in the pathogenesis of Gram-negative bacterial infections and its attendant vascular complications. Because it triggers the sepsis syndrome [6, 7], inflammation of the bowels including Crohn's disease [14, 15], and periodontitis [16, 17], LPS is considered to be one of the most common and potent pathogenic factors of blood vessels. Therefore, searching for molecules that can inhibit LPS-induced endothelial cell dysfunction should contribute to the discovery of new anti-inflammatory agents. As microorganisms produce a variety of compounds of low-molecular weight and having unique structures, they are ideal sources of novel bioactive secondary metabolites. Thus, in the present study, we screened microbial secondary metabolites for compounds that could inhibit the adhesion of human myelocytic cell line HL-60 cells to LPS-stimulated human umbilical vein endothelial cells (HUVEC). As a result, we isolated a known cyclic lipopeptide, surfactin, from the culture broth of *Bacillus* sp. BML752-121F2, and found it to be inhibitory.

Materials and Methods

Materials

HUVEC were purchased from Cell Systems (Lake Kirkland, WA). HL-60 cells were obtained from the Japanese Collection of Research Bioresources. Surfactin (surfactin C₁; from *Bacillus subtilis*), LPS (from *Escherichia coli* Serotype 055:B5), lipid A (1,4'-diphosphoryl form, from *Escherichia coli* F-583), and polymyxin B were purchased from Sigma Chemical Co. (St. Louis, MO). TNF- α was procured from Genzyme-Techne (Cambridge, MA); and IL-1 β , from Pepro Tech EC Ltd. (London, UK). Polyclonal antibodies against ICAM-1 and VCAM-1 came from Santa Cruz Biotechnology (Santa Cruz, CA); and monoclonal antibody against LBP (6G3), from HyCult Biotechnology (Uden, The Netherlands).

Cell Culture

HUVEC were cultured on type I collagen-coated dishes (Costar, Acton, MA) in MCDB-131 medium (Sigma) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and 10 ng/ml basic fibroblast growth factor (bFGF; Pepro Tech EC Ltd.). HL-60 cells, THP-1 cells, and Jurkat cells were cultured in RPMI1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS, 100 μ g/ml kanamycin, 100 units/ml penicillin G, 300 μ g/ml L-glutamine, and 2.25 mg/ml NaHCO₃.

Cell Adhesion Assay

Stimulant-induced adhesion between HUVEC and leukocytes was measured by the method described previously [18]. HUVEC were seeded at 4×10^4 cells/well in 48-well collagen-coated plates (Costar) and cultured overnight at 37°C in 5% CO₂. The cells were preincubated with several concentrations of surfactin for 2 hours, and then treated with the stimulant (1 μ g/ml LPS, 1 μ g/ml lipid A, 10 ng/ml TNF- α or 10 ng/ml IL-1 β) for 4 hours. Then, the medium was removed, and the wells were washed with PBS twice. Next, leukemic cells (HL-60, THP-1, or Jurkat) were added at 6×10^4 cells/well to the treated HUVEC monolayers. After 1 hour, nonadhering leukemic cells were removed by 3 washes with PBS. Then, the number of adherent cells in 1 microscopic field was counted.

Trypan Blue Dye Exclusion

HUVEC were seeded at 1.6×10^5 cells/well in 24-well collagen-coated plates (Costar) and cultured overnight. The cells were treated with various concentrations of surfactin for 24 hours in the presence of 1 μ g/ml LPS. Then, they were stained with trypan blue, after which the number of stained cells was counted.

Western Blotting Analysis

HUVEC (8×10^5 cells) were treated with LPS for the desired periods. Then, the cells were scraped off, and suspended in lysis buffer (20 mM Tris, pH 8.0, containing 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μ M Na₃VO₄, 1% NP-40, 1 μ g/ml leupeptin, 1 μ g/ml antipain, and 1 mM PMSF). The supernatants were combined with loading buffer (150 mM Tris, 30% glycerol, 3% bromophenol blue, 3% sodium *n*-dodecyl sulfate, 15% 2-mercaptoethanol), and electrophoresed in 9% polyacrylamide gels. The gels were electrophoretically transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ) at 4°C for 3 hours. The membranes were then blocked with 5% skim milk, and incubated with anti-ICAM-1 or anti-VCAM-1 antibody in TBS buffer (20 mM

Tris-HCl, pH 7.6, and 137 mM NaCl) at room temperature for 1 hour. The blotted membranes were next washed 6 times with 0.2% Tween 20 in TN buffer (50 mM Tris-HCl, pH 7.5, contains 137 mM NaCl) and incubated with horseradish-peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham Biosciences) for 1 hour. Immunoreactive proteins were visualized by using the ECL chemiluminescence system (Perkin Elmer Life Sciences, Boston, MA) and exposure to Fuji Medical X-ray film HR-H (Fuji Photo Film, Tokyo, Japan).

Assay for the Lipid A/LBP Interaction

Ninety-six-well microtiter plates (Immulon 2H; Dynex Technologies, Ashford, U.K.) were coated with lipid A, and the interaction of the immobilized-lipid A with LBP was measured as described previously [19, 20]. Briefly, lipid A at 5 $\mu\text{g}/\text{well}$ in 20 μl of ethanol was placed in the ninety-six-well microtiter plates, and the solvent was evaporated in ambient air. After the nonspecific binding had been blocked by incubating the wells for 30 minutes at 37°C with 100 μl of PBS containing 1% bovine serum albumin (BSA), 100 μl of PBS containing 1, 3, 10, or 30% FBS was added, and the plates were then incubated for 1 hour at 37°C. They were next washed, and 25 nM anti-LBP mAb 6G3 in 50 μl of PBS containing 0.1% BSA was added; and incubation was then continued for 1 hour at 37°C. The mAb solution was rinsed out, and 50 μl of HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences) diluted 1,000-fold in PBS containing 0.1% BSA. Finally, binding of LBP to the immobilized-lipid A was detected by incubation with 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma). The reaction was stopped by addition of 100 μl of 0.18 M sulfuric acid, and absorbances at 450 and 570 nm were quantified in a micro plate reader. When surfactin was added, the lipid A-coated microtiter plates were preincubated for 1 hour at 37°C with 0.3, 1, 3 or 10 $\mu\text{g}/\text{well}$ surfactin in 100 μl of PBS containing 0.1% BSA. After the solution has been removed, 100 μl of PBS containing 10% FBS was added; and the plates were incubated for 1 hour at 37°C. Binding of LBP was determined as described above.

SPR Analysis

Realtime bio-interaction analysis of surfactin or polymyxin B with lipid A was measured by surface plasmon resonance (SPR) using a Biacore X (Biacore AB, Uppsala, Sweden). The immobilization of lipid A onto the HPA biosensor chip (Biacore AB) was carried out as described previously with slight modifications [21]. Briefly lipid A at 0.5 mg/ml in water was sonicated at 37°C for 15 minutes before being immobilized. After the HPA chip has been washed with

40 mM of *n*-octyl β -D-glucoside for 5 minutes at a flow rate of 5 $\mu\text{l}/\text{minute}$, the lipid A was injected into the flow cell at 1 $\mu\text{l}/\text{minute}$ until the saturation level was achieved. After immobilization, 2 mM NaOH was injected at 20 $\mu\text{l}/\text{minute}$ in 1-minute pulses into the flow cell to remove excess lipid A so that only a monolayer of lipid A remained. Washing was continued until the basal SPR response unit (RU) in the sensorgram stably returned to the baseline. Typically, around 1000 RU per flow-cell surface coating of lipid A was obtained. For the reference cell, dimyristoylphosphatidylcholine (DMPC; Sigma) at 0.5 mg/ml in water was used for coating in the same way as the lipid A. For the binding analysis, samples in the running buffer (10 mM Tris-HCl, pH 7.0, 100 mM NaCl) were injected for 2 minutes at a flow rate of 20 $\mu\text{l}/\text{minute}$. Association and dissociation curves were obtained from the Biacore software. The surface of the sensor chip was regenerated by the injection of 20 μl of 2 mM NaOH. All Biacore experiments were carried out at 25°C, with the samples kept on ice before the injection. The value of the apparent binding affinity of surfactin or polymyxin B for lipid A was calculated by fitting the sensorgrams of kinetic injections to the bivalent binding model with Biacore evaluation software version 3.0.

Results

Inhibition of Leukemic Cell Adhesion to LPS-stimulated HUVEC by Surfactin

In the course of our screening of cell adhesion inhibitors, we found that the filtrate from *Bacillus* sp. BML752-121F2 showed marked inhibitory activity toward HL-60 cell adhesion to LPS-stimulated HUVEC. From the culture broth of BML752-121F2 the active principle was isolated through chromatographic separations. Spectroscopic analysis of the purified compound revealed that the compound was identical to a known cyclic lipopeptide, surfactin C₁ (Fig. 1). In the present study, we employed commercially available surfactin (Sigma), which is mainly composed of surfactin C₁.

Treatment of HUVEC with LPS increased the adhesion of HL-60 cells to them. The addition of surfactin at 3 $\mu\text{g}/\text{ml}$ prior to LPS stimulation markedly decreased the HL-60 cell-HUVEC adhesion (Fig. 2A). The IC₅₀ value was determined to be 1.10 $\mu\text{g}/\text{ml}$, as shown in Fig. 2B. Furthermore surfactin also inhibited the LPS-induced cell adhesion of human monocyte cell line THP-1 cells or human T cell leukemia Jurkat cells to HUVEC (THP-1: IC₅₀ value 1.45 $\mu\text{g}/\text{ml}$; Jurkat: IC₅₀ value 1.43 $\mu\text{g}/\text{ml}$; data not shown). Lipid A, the active component of LPS, also

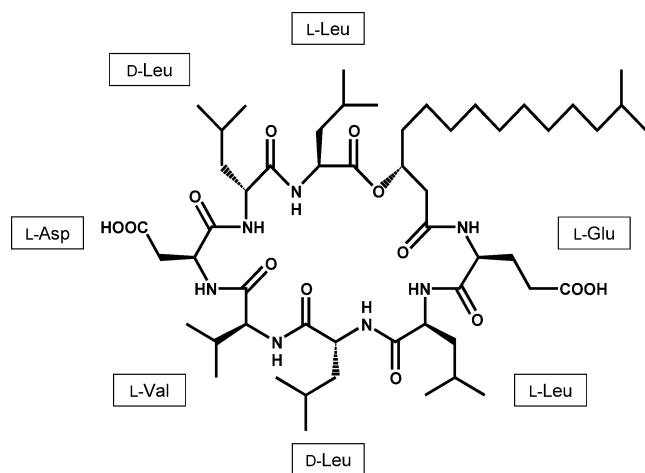


Fig. 1 Structure of surfactin C₁.

induced the adhesion of HL-60 cells to HUVEC, which was again inhibited by surfactin (Fig. 2B). However, surfactin did not inhibit TNF- α - or IL-1 β -induced cell adhesions. Therefore, surfactin was shown to inhibit LPS-induced cell adhesion specifically.

Effects of Surfactants on LPS-Activity and Cell Viability

Surfactin is an amphiphilic compound and has surface-activating potential [22]. So, we studied whether the cell adhesion could be inhibited by general surface-activating compounds. We tested the effects on cell adhesion of sodium *n*-dodecyl sulfate (SDS), which is an anionic surfactant like surfactin, cetyltrimethylammonium chloride that is a cationic surfactant; and Triton X-100, Tween-20, and *n*-octyl β -D-glucoside, which are nonionic surfactants. As shown in Fig. 3 and Table 1, all the tested detergents inhibit cell adhesion while exhibiting cytotoxicity at similar concentrations. Only the exception is surfactin. But this can not exclude the possibility that surfactin inhibits the cell adhesion *via* its general detergent activity. Surfactin did not influence the viability of HUVEC up to 30 μ g/ml after 24 hours, with or without LPS. These results showed that inhibition of surfactin on the cell adhesion may not be attributable to its general detergent activity.

Inhibition of LPS-Induced Expression of Adhesion Molecules by Surfactin

LPS induced the expression of adhesion molecules such as ICAM-1 and VCAM-1. Western blotting analysis revealed that surfactin inhibited 1 μ g/ml LPS-induced expression of ICAM-1 and VCAM-1 at 3 μ g/ml almost completely (Fig. 4). LPS did not induce E-selectin in our preparation of HUVEC.

Inhibition of the Interaction of Lipid A/LBP by Surfactin

Using lipid A-coated plates, we next examined the effects of surfactin on the binding of lipid A to LBP. When the lipid A-coated plates were incubated with FBS, serum LBP bound to the lipid A-coated plates in a serum concentration dose-dependent manner (Fig. 5A). When the lipid A-coated plates were pretreated with surfactin the binding of LBP to the lipid A-coated plates was inhibited in a dose-dependent manner by surfactin (Fig. 5B). Thus, surfactin is likely to interact with either lipid A or LBP.

Direct Interaction between Surfactin and Lipid A

Since surfactin did not inhibit TNF- α or IL-1 β -induced signaling, it is likely that surfactin directly acts on LPS or LBP to inhibit the signal transduction of LPS. So, we looked into the real-time interaction between surfactin and lipid A by using surface plasmon resonance (SPR) analysis. The increase in resonance units (RU) by binding of surfactin to immobilized-lipid A demonstrated the direct interaction between surfactin and lipid A (Fig. 6A). We also employed polymyxin B, which is known to bind to lipid A [23]. Prominent binding of polymyxin B to immobilized-lipid A also occurred in a dose-dependent manner (Fig. 6B). However, a rapid and time-dependent decrease in the binding signal for surfactin and lipid A interaction was detected. Thus, compared with that with polymyxin B, the interaction with surfactin was shown to be reversible. The association rate (k_a), dissociation rate (k_d) and the apparent dissociation constant (K_D) of surfactin binding to lipid A were calculated to be $2.27 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $2.46 \times 10^{-3} \text{ s}^{-1}$ and $1.09 \times 10^{-7} \text{ M}$, respectively. Those parameters of polymyxin B binding to lipid A were calculated to be $7.86 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (k_a), $1.30 \times 10^{-4} \text{ s}^{-1}$ (k_d) and $1.65 \times 10^{-11} \text{ M}$ (K_D). These kinetic parameters also indicate that the binding of surfactin showed slower association and faster dissociation compared with polymyxin B.

Discussion

In the present study, we looked among microbial secondary metabolites for compounds that could inhibit HL-60 cell adhesion to LPS-stimulated HUVEC. Among several thousands of microbial culture filtrates tested, we isolated a known inhibitory lipopeptide, surfactin, from the culture broth of *Bacillus* sp. BML752-121F2. The inhibition of LPS signal transduction by surfactin was shown to be due to the direct interaction between surfactin and lipid A.

Surfactin is a lipopeptide antibiotic produced by *Bacillus subtilis*, and it was first identified as a potent inhibitor of

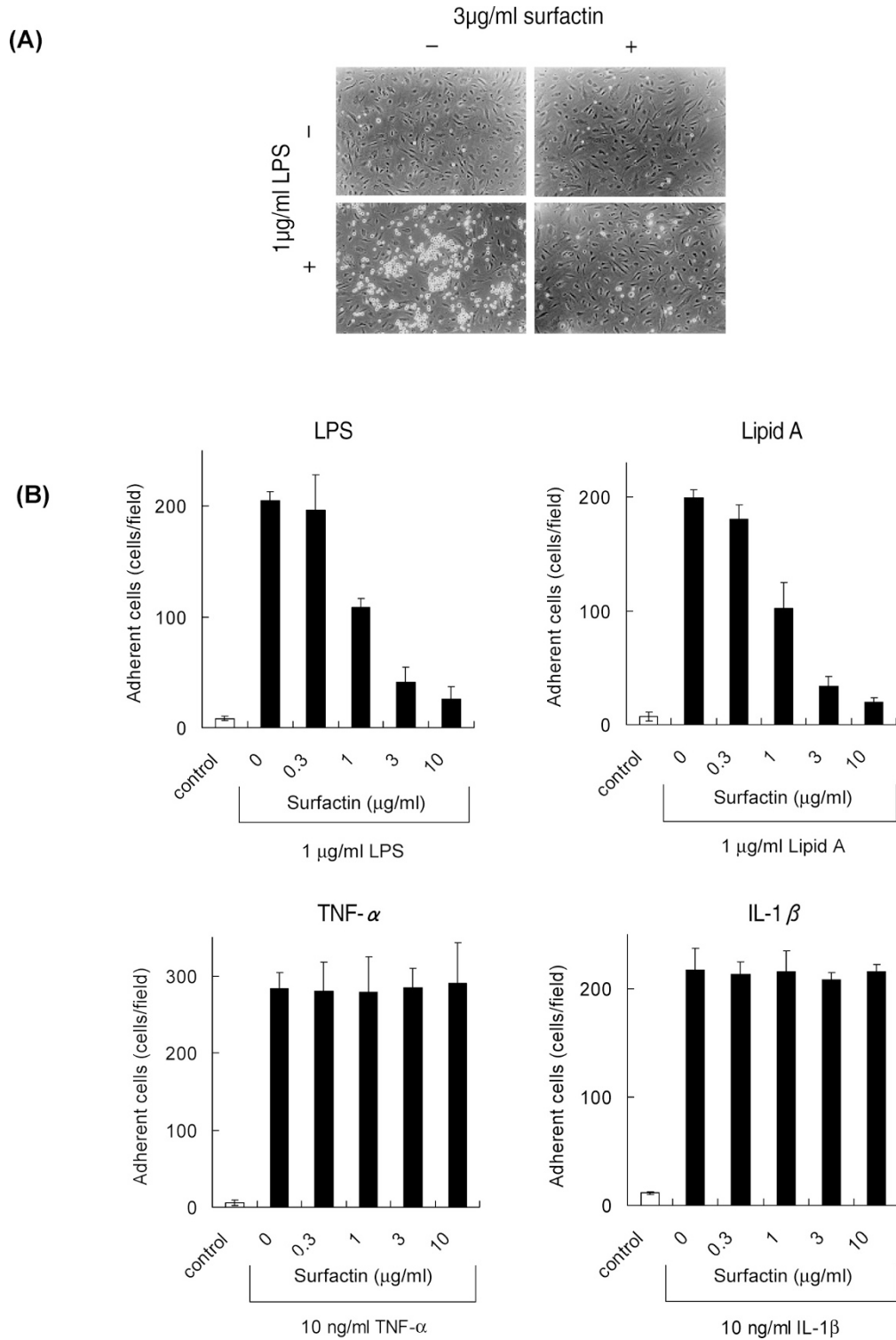


Fig. 2 Inhibition of HL-60 cell adhesion to LPS-stimulated HUVEC by surfactin.

(A) HUVEC were preincubated or not with 3 $\mu\text{g/ml}$ surfactin for 2 hours, treated or not with LPS (1 $\mu\text{g/ml}$) for 4 hours, and, after having been washed, incubated with HL-60 cells for 1 hour. (B) HUVEC were preincubated with the indicated concentrations of surfactin for 2 hours, then treated (solid columns) or not (open columns) with 1 $\mu\text{g/ml}$ LPS, 1 $\mu\text{g/ml}$ lipid A, 10 ng/ml TNF- α , or 10 ng/ml IL-1 β for 4 hours. After a washing, HL-60 cells were added to the HUVEC. One hour later, the adhesion of HL-60 cells to HUVEC was determined by measuring the number of adherent cells in 1 microscopic field. Values are the means \pm S.D. of triplicate determinations.

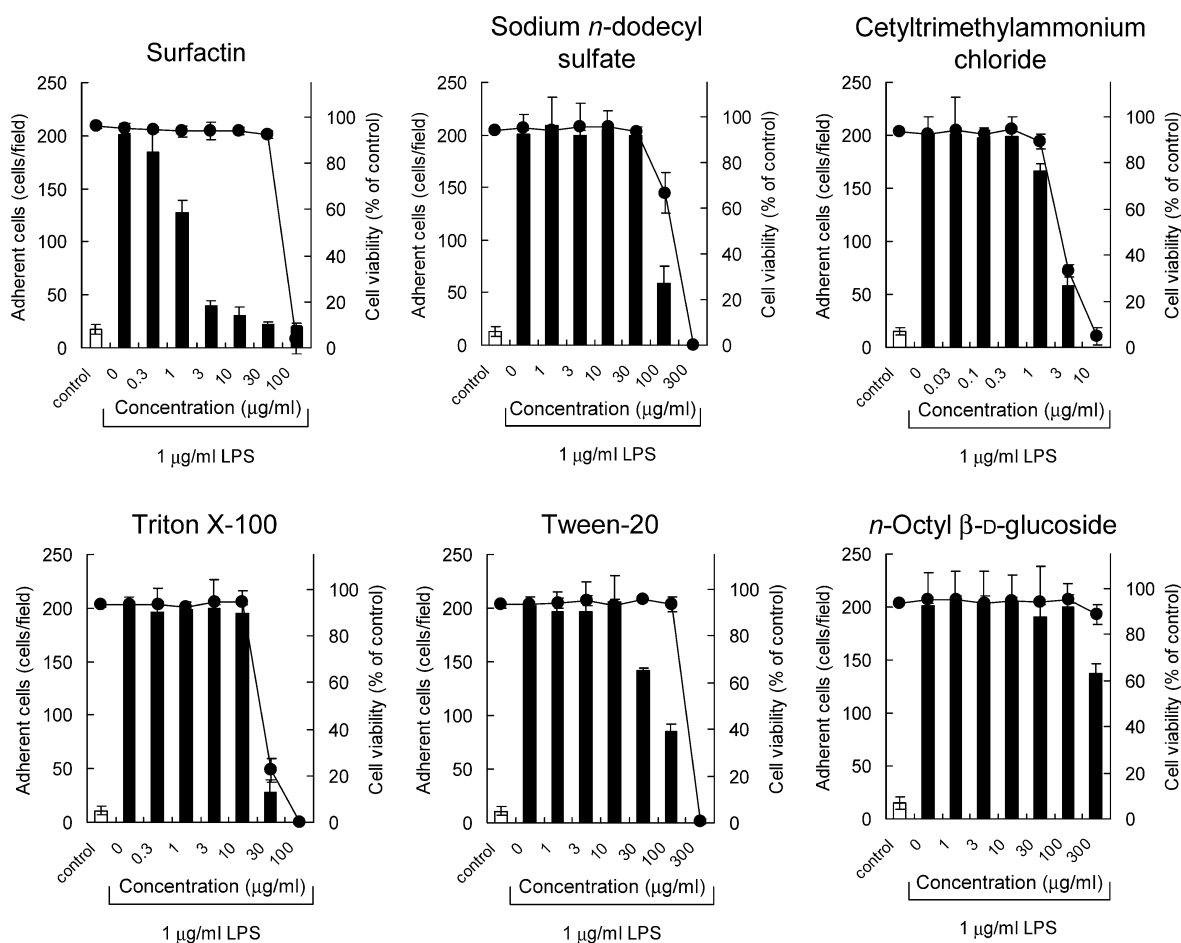


Fig. 3 Effects of various surfactants on cell adhesion and viability of HUVEC.

Columns: HUVEC were preincubated with the indicated concentrations of surfactants for 2 hours, then treated (solid columns) or not (open columns) with 1 $\mu\text{g/ml}$ LPS for 4 hours. Then, the adhesion of HL-60 cells to HUVEC was determined by measuring the number of adherent cells as described above. Values are the means \pm S.D. of triplicate determinations. Circles; HUVEC were treated with the indicated concentrations of surfactants for 24 hours in the presence of 1 $\mu\text{g/ml}$ LPS, and the cell viability was determined by trypan blue dye exclusion. Values are the means \pm S.D. of quadruplicate determinations.

Table 1 Effects of surfactants on LPS-induced HL-60-HUVEC adhesion and viability of HUVEC

Compounds	IC ₅₀ value of cell adhesion ($\mu\text{g/ml}$)	IC ₅₀ value of cell viability ($\mu\text{g/ml}$)
Surfactin	1.38	55.00
Sodium <i>n</i> -dodecyl sulfate	69.20	138.00
Cetyltrimethylammonium chloride	0.29	0.25
Triton X-100	18.60	20.90
Tween-20	70.80	178.00
<i>n</i> -Octyl β -D-glucoside	>300.00	>300.00

fibrin clotting [24]. Surfactin contains the structure of a cyclic heptapeptide and a lipid portion represented by a mixture of several β -hydroxy fatty acids with chain lengths of 13~15 carbon atoms [25]. The main component that we isolated was surfactin C₁ (Fig. 1). It is an amphiphilic compound and known to be one of the most powerful biosurfactants [22, 26]. Surfactin was also reported to inhibit phospholipase A₂ (PLA₂) [27], and to enhance plasminogen activation [28]. In addition, it is less toxic than other surfactants as judged from the results of an acute toxicity study in mice (LD₅₀ value at >100 mg/kg, i.v.) [29]. However, the interaction with LPS has been entirely not known before. The activity of surfactin observed in the present research was not due to its surface-activating potential, since other surfactants were not active in inhibition of LPS-activity (Fig. 3, Table 1). As mentioned

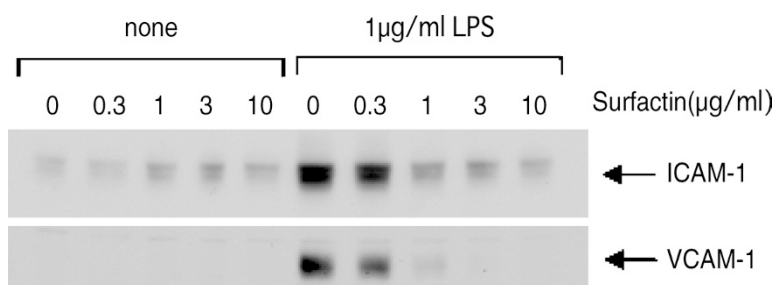


Fig. 4 Inhibition of adhesion molecule expression in LPS-activated HUVEC by surfactin.

HUVEC were treated with the indicated concentrations of surfactin for 2 hours and then stimulated or not with 1 $\mu\text{g/ml}$ LPS for 4 hours. The cell lysates were analyzed by Western blotting with anti-ICAM-1 and anti-VCAM-1 antibodies.

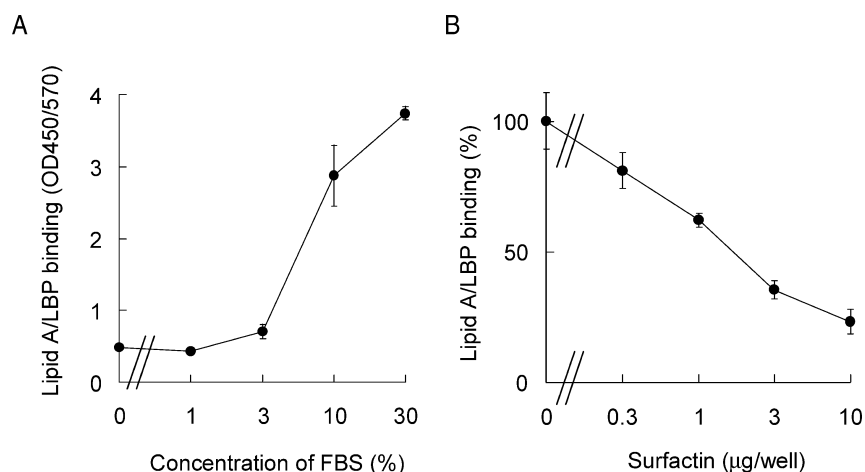


Fig. 5 Inhibition of interaction of lipid A with LBP by surfactin.

(A) Lipid A/LBP binding was examined by incubating 100 μl of PBS containing 1, 3, 10, or 30% FBS in lipid A-coated 96 well microtiter plates for 1 hour. After incubation, bound LBP was detected as TMB reaction products obtained with anti-LBP MAb 6G3 and HRP-conjugated sheep anti-mouse IgG. (B) Lipid A-coated microtiter plates were preincubated with surfactin in 100 μl of PBS for 1 hour. After the removal of the medium, the plates were incubated with 100 μl of PBS containing 10% FBS for 1 hour. Lipid A/LBP binding was expressed as a percentage of that obtained by incubation with PBS containing 10% FBS in the absence of surfactin. Values are the means \pm S.D. of quadruplicate determinations.

above, surfactin is a known inhibitor of PLA_2 . Surfactin inhibited cytosolic PLA_2 purified from bovine platelets with an IC_{50} of 8.5 μM (8.8 $\mu\text{g/ml}$) [27]. PLA_2 was reported to be activated by inflammatory stimuli such as LPS in endothelial cells, and activated PLA_2 was shown to lead to increased leukemic cell adhesion *via* the expression of adhesion molecules [30]. Therefore, the inhibition of PLA_2 may also contribute to the inhibitory activity of surfactin on cell adhesion.

Recently, we reported that a novel naturally-occurring cyclic heptadepsipeptide, heptadepsin, inhibited LPS activity [18]. Similar to polymyxin B, heptadepsin was demonstrated to inactivate LPS by its direct interaction with LPS. Compared with polymyxin B and heptadepsin, surfactin showed more reversible interaction with lipid A.

Since surfactin could inhibit the LBP binding and LPS-induced cellular effects at the concentration necessary to interact with lipid A, the reversible interaction may be sufficient to inactivate the lipid A activity.

Thus, surfactin, a naturally-occurring cyclic lipopeptide, was found to be a selective inhibitor of LPS signal transduction. Having minimal cytotoxicity, surfactin may have a therapeutic potential for the treatment of LPS-triggered syndromes such as Gram-negative bacterial sepsis and periodontitis.

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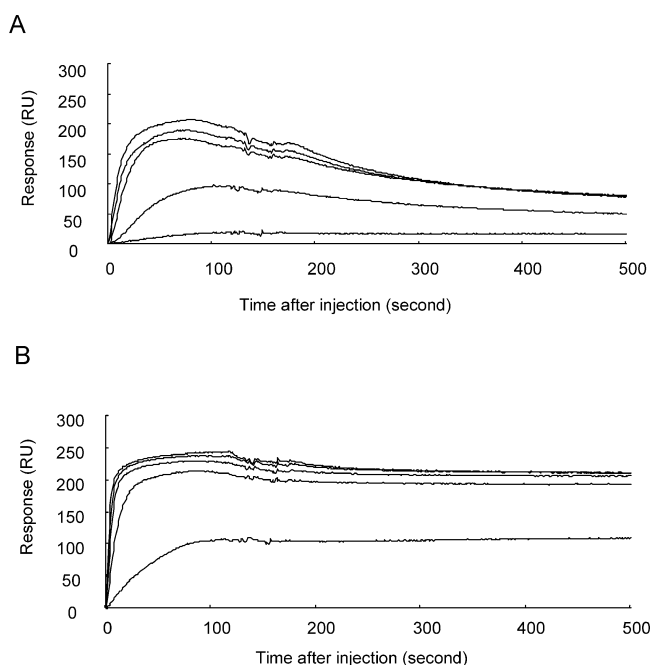


Fig. 6 SPR analysis for the interaction of surfactin or polymyxin B with immobilized-lipid A.

Sensorgrams indicate the association and dissociation phases of the reactions with surfactin (A) or polymyxin B (B). Each chemical was added at 1, 2, 3, 4, 5 µg/ml (from bottom to top) for 2 minutes at 20 µl/minute over the monolayer of lipid A immobilized on the HPA sensor chip.

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