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Isolation of Heptadepsin, a Novel Bacterial Cyclic Depsipeptide that Inhibits Lipopolysaccharide Activity

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Summary

Lipopolysaccharide (LPS) is considered to cause various inflammatory reactions. We searched among microbial secondary metabolites for compounds that could inhibit LPS-stimulated adhesion between human umbilical vein endothelial cells (HUVEC) and human myelocytic cell line HL-60 cells. In the course of our screening, we isolated a novel cyclic depsipeptide, which we named heptadepsin, from the whole culture broth of *Paenibacillus* sp. The addition of heptadepsin prior to LPS stimulation decreased HL-60 cell-HUVEC adhesion without showing any cytotoxicity. It also inhibited the cellular adhesion induced by lipid A, the active component of LPS, but it did not inhibit TNF-α or IL-1β-induced cell adhesion. The result of surface plasmon resonance (SPR) analysis revealed that heptadepsin interacted with lipid A directly. Thus, heptadepsin, a novel naturally occurring cyclic heptadepsipeptide, was shown to inactivate LPS by direct interaction with LPS.

Introduction

The vascular endothelium is intimately involved in a wide variety of physiological processes, including coagulation/anticoagulation, maintenance of the vascular tone, and immune reactions. However, it is also involved in various pathological processes, including atherosclerosis, inflammation of other tissues, and blood-borne tumor metastasis [1]. In the case of atherosclerosis, the pathogenesis involves a series of critical events including endothelial dysfunction, infiltration of monocytes into the vessel wall, differentiation of monocytes, and vascular remodeling [2]. The first critical step is the recruitment of circulating monocytes into the inflamed endothelium of the blood vessel. Recruitment of blood circulating cells into the endothelium plays an important role more generally at sites of inflammation [3]. Under normal circumstances, blood leukocytes do not adhere to the endothelium. However, under inflammatory conditions, exogenous stimuli such as bacterial lipopolysaccharide (LPS) or endogenous stimuli such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) often activate endothelial cells, thereby enhancing the adhesion of leukocytes [4].

LPS is one of the major constituents of the outer membranes of gram-negative bacteria and is recognized as a key molecule in the pathogenesis of endotoxin shock seen in inflammatory syndromes associated with gram-negative bacterial infections. LPS and its active component, lipid A, are commonly known to bind to the Toll-like receptor (TLR) 4 on the cell surface of monocytes, macrophages, neutrophils, and endothelial cells in mammals [5]. Especially in endothelial cells, LPS induces (1) production of proinflammatory cytokines such as IL-6 [6], IL-8 [7], and IL-1β [8], (2) upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [9], and (3) expression of tissue factor [10]. TLR4 was identified as the putative transmembrane receptor for LPS that activates nuclear factor-κB (NF-κB) signaling [11–13]. NF-κB is known to play a critical role in the development of the inflammatory response by upregulating the expression of many inflammatory cytokines and cell adhesion molecules.

The recruitment of leukocytes into the inflamed endothelium involves inflammatory stimulus-induced expression of multiple cell adhesion molecules on the surface of endothelial cells. The most important of these molecules involved in this process are ICAM-1, VCAM-1, and E-selectin [4, 9]. Only a small amount of ICAM-1 is present on the endothelium without stimulation [14], and VCAM-1 and E-selectin are absent entirely [15, 16]. However, they are immediately expressed in response to extracellular inflammatory stimulation [17]. Each stimulus induces its selective expression of these adhesion molecules, whose expression may contribute to the selective recruitment of leukocyte subtypes specific to the endothelial cells at sites of inflammation. ICAM-1 interacts with the ligand CD11a/CD18 (LFA-1), which is expressed on neutrophils, lymphocytes, and monocytes, and VCAM-1 interacts 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 is implicated in the pathogenesis of gram-negative bacteria infections and its attendant vascular complications. Because it triggers an inflammation in bowels, including Crohn disease [18, 19] and periodontitis [20, 21], LPS is considered to be one of the most common and potent pathogenic factor of blood vessels. Therefore, searching for molecules that can inhibit LPS-induced endothelial cell dysfunction should contribute to the discovery of new antiinflammatory agents.

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As microorganisms produce a variety of compounds of low molecular weight having unique structures, they are ideal sources of novel bioactive secondary metabolites. Thus, in the present study, we searched among 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 novel cyclic depsipeptide named heptadepsin from the whole culture broth of *Paenibacillus* sp. BML771-113F9. Our data suggested that heptadepsin interacts with LPS directly.

Results

Isolation of Heptadepsin

We constructed the screening system for low molecular weight compounds that inhibit the adhesion of HL-60 cells to LPS-stimulated HUVEC. We searched such compounds among several thousand samples from natural sources, such as culture broths of *Streptomyces*, bacteria, and plant extracts. We took into account only the samples without cytotoxicity. The hitting rate was less than 1/1000. In the course of our screening, the filtrate from *Paenibacillus* sp. BML771-113F9 revealed marked inhibitory activity toward HL-60 cell adhesion to LPS-stimulated HUVEC. From the whole culture broth of *Paenibacillus* sp. BML771-113F9, the active principle was isolated through the several chromatographic separations described in the Experimental Procedures. Spectroscopic analysis of the active compound revealed that the compound was a novel cyclic heptadepsipeptide, and so we named it heptadepsin (Figure 1A). Related known naturally occurring cyclic heptadepsipeptides include HUN-7293, which inhibits inducible cell-adhesion molecule expression as does heptadepsin [22]. However, the amino acid composition of HUN-7293 is much different from that of heptadepsin, containing Leu and unusual tryptophan and leucines [23].

Physico-Chemical Properties

Heptadepsin was obtained as a white amorphous powder, and its physico-chemical properties are summarized in Supplemental Table S1. It was easily soluble in MeOH and DMSO, slightly soluble in water, and insoluble in $CHCl_3$, EtOAc, and *n*-hexane. It gave a positive reaction with the Sakaguchi reagent [24, 25], but was negative in the ninhydrin test. The color reactions and ^1H and ^{13}C NMR data suggested that heptadepsin was a cyclic depsipeptide having a guanidino group. The UV spectrum of heptadepsin showed absorption at 210 nm. The IR spectrum of heptadepsin exhibited typical absorption bands of amides at 1658, 1635, and 1539 cm^{-1} and an ester at 1753 cm^{-1}. The molecular formula of heptadepsin was determined to be $C_{45}H_{81}N_{11}O_{12}$ by HRFAB-MS (pos.): *m/z* 968.6133 $(M + H)^+$ ($C_{45}H_{82}N_{11}O_{12}$ calculated mass 968.6144).

Structure Determination of Heptadepsin

The molecular formula of heptadepsin was determined to be $C_{45}H_{81}N_{11}O_{12}$ from HRFAB-MS and NMR spectra. The ^1H and ^{13}C NMR spectra of heptadepsin were measured in DMSO-d_6, and those data are summarized in Supplemental Table S2. They appeared similar to those of fusaricidins produced by *Bacillus polymyxa* [26] or circulocins produced by *Bacillus circulans* [27], but were not identical. The multiplicities of carbon signals were determined by DEPT experiments, and all bond correspondence between ^1H and ^{13}C signals was determined by DEPT and HMQC experiments. Detailed analysis of 2D ^1H-^1H COSY, HMQC, HMBC, and ROESY data for heptadepsin revealed the presence of 7 amino acids: alanine 1 (Ala1), asparagine (Asn), serine 1 (Ser1), alanine 2 (Ala2), isoleucine (Ile), valine (Val), and serine 2 (Ser2). As described in the Experimental Procedures, the chirality of these amino acids was determined by acid hydrolysis (6 N, 105°C, 17 hr) of heptadepsin. This result indicated the presence of D-Ala, D-Asn, L-Ser, L-Ile, and D-Val. Ser1 and Ser2 were both L-Ser, and Ala1 and Ala2 were both D-Ala, so the absolute stereochemistry of them was the same. In addition, amino acid analysis of L-Ile by HPLC (column; Hitachi custom ion exchanging resin #2622) showed that it was not L-*allo*-Ile.

The sequence of the 7 amino acids and GHHD residue in heptadepsin was determined on the basis of ^1H-^{13}C HMBC data shown in Figure 1B. The respective two-bond correlations from amide NHs of Val, Ile, Ala1, Ser2, Asn and Ala2 to C-1 carbonyls of Ser1, Val, Ile, Ala1, Ser2, and Asn allowed the sequence to be established as Ser1-Val-Ile-Ala1-Ser2-Asn-Ala2. The three-bond correlation between H-3 (Ser1) and C-1 (Ala2) across the ester linkage was also observed by changing the measurement condition of the PI1 value from 60 to 120 ms, which established the cyclic heptadepsipeptide system.

A long chain acid terminating in a guanidino group was suggested to have a linear chain of 14 methylenes between the β-hydroxy and the guanidino groups on the basis of NMR spectra as shown in Supplemental Table S2 and Figure 1. Additional support for this fragment was obtained by FAB-MS analysis and by the color reaction of an EtOAc extract of an acid hydrolysate (6 N, 105°C, 17 hr) of heptadepsin with the Sakaguchi reagent. The FAB-MS spectrum of the acid hydrolysate contained a prominent ion at *m/z* 344, corresponding to the $(M+H)^+$ for 17-guanidino-3-hydroxyheptadecanoic acid (GHHD). In addition, the acid hydrolysate gave a positive reaction with the Sakaguchi reagent. Thus, this long chain acid was concluded to have a guanidino group. The absolute configuration of C-3 of GHHD remains to be determined.

The attachment of GHHD to the amino group on Ser1 was confirmed by the two-bond correlation between the amide NH of Ser1 and C-1 of GHHD. Additional two- or three-bond correlations in the HMBC spectrum and ROE cross peaks in the ROESY spectrum supported the structure of heptadepsin indicated in Figure 1.

Inhibition of Leukemic Cell Adesion to LPS-Stimulated HUVEC

When HUVEC were unstimulated or pretreated with 3 μg/ml heptadepsin alone, no prominent HL-60 cell adhesion was observed. Treatment of HUVEC with 1 μg/ml LPS for 4 hr significantly increased the adhesion of HL-60 cells. However, the addition of heptadepsin at 3 μg/ml prior to LPS stimulation markedly decreased the HL-60

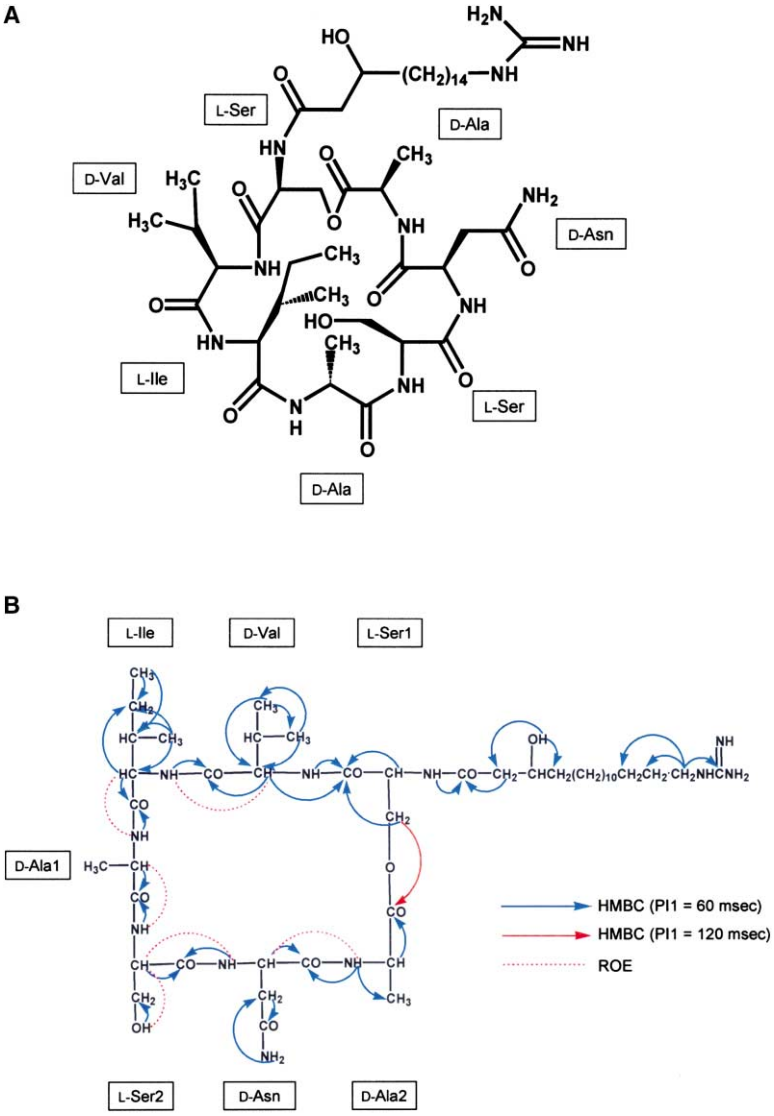

Figure 1. Structure Determination of Heptadepsin

(A) Structure of heptadepsin. (B) HMBC and ROE correlations of heptadepsin.

cell-HUVEC adhesion (Figure 2A). The IC_{50} value was determined to be 0.92 μg/ml, as shown in Figure 2B. Furthermore, incubation with 1 μg/ml LPS for 4 hr increased the adhesion of human monocyte cell line THP-1 cells or human T cell leukemia Jurkat cells, and heptadepsin also inhibited these leukemic cell adhesions to HUVEC (THP-1: IC_{50} = 1.29 μg/ml; Jurkat: IC_{50} = 1.57 μg/ml; data not shown). Lipid A, the active component of LPS, also induced the adhesion of HL-60 cells to HUVEC, which was again inhibited by heptadepsin (Figure 2B). Next, we employed stimulants other than LPS, such as TNF-α and IL-1β. As shown in Figure 2B, 10 ng/ml TNF-α or 10 ng/ml IL-1β induced the adhesion of HL-60 cells to HUVEC; however, heptadepsin did not inhibit these adhesions. Therefore, heptadepsin appeared to act specifically on LPS or lipid A.

Inhibition of leukemic cell adhesion to LPS-stimulated HUVEC by heptadepsin might be expected to be due to a decrease in LPS-induced adhesion molecule expression. So, we examined the effect of heptadepsin on LPS-induced expression of adhesion molecules such as ICAM-1 and VCAM-1. Western blotting analysis revealed that heptadepsin inhibited 1 μg/ml LPS-induced expression of ICAM-1 and VCAM-1 at 3 μg/ml almost completely (Figure 2C). LPS did not induce E-selectin in our preparation of HUVEC.

Heptadepsin Was Not Cytotoxic or Growth Inhibitory toward HUVEC

To exclude the possibility that heptadepsin inhibited adhesion by its cytotoxicity to HUVEC, we examined the cytotoxicity and growth inhibition by using a trypan blue dye exclusion assay and MTT assay, respectively. Heptadepsin did not show any cytotoxicity after 24 hr or growth inhibition after 72 hr toward HUVEC up to 30 μg/ml, with or without LPS (Figure 3).

Inhibition of Fluorescent-Conjugated LPS Binding to TLR4-Overexpressing Ba/F3 Cells

Then, we examined the effect of heptadepsin on the binding of fluorescent-conjugated LPS to the cells overexpressing the LPS receptor, TLR4. We employed mouse pro B Ba/F3 cells stably expressing mouse TLR4, mouse MD-2, and mouse CD14. Fluorescence micro-

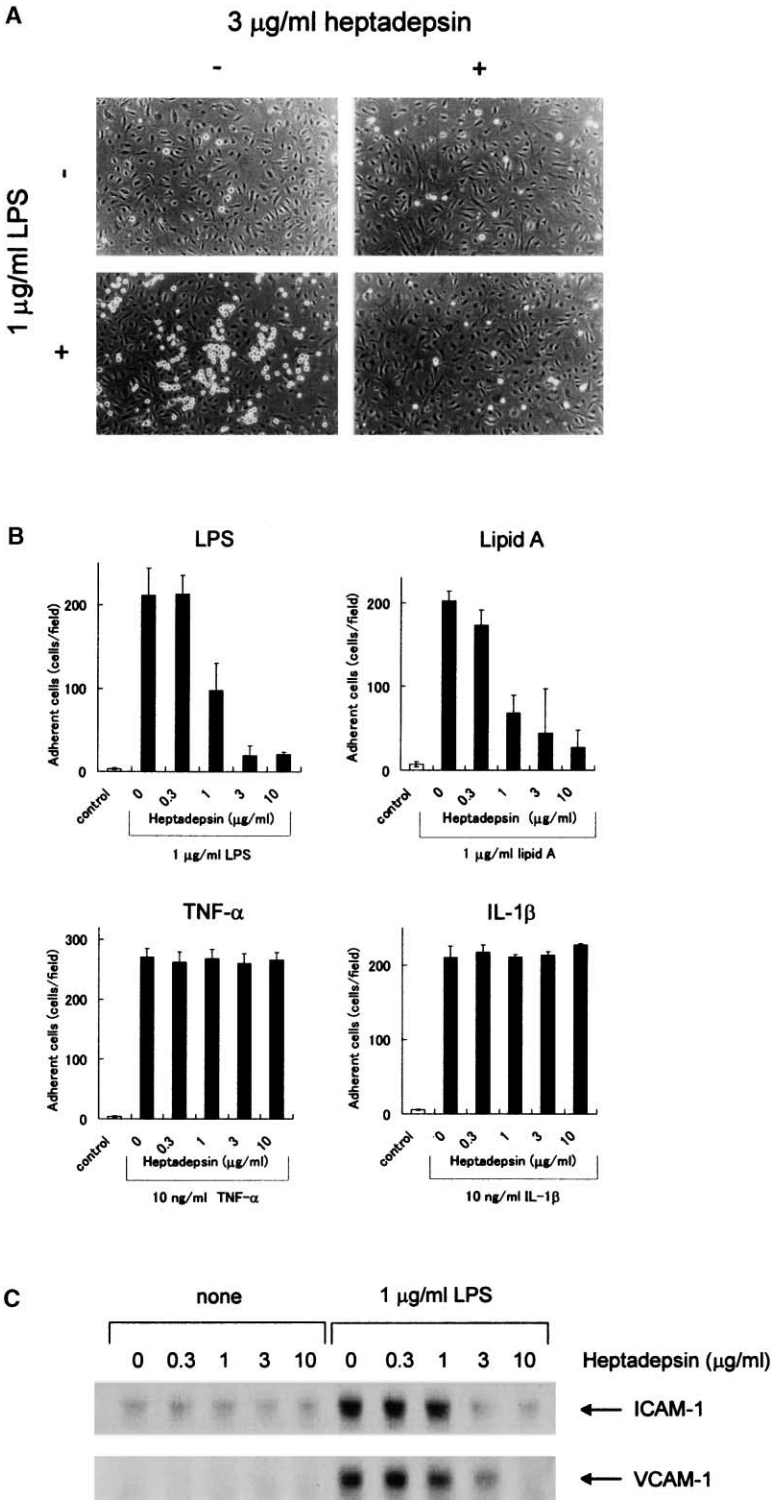

Figure 2. Inhibition of HL-60 Cell Adhesion to LPS-Stimulated HUVEC by Heptadepsin

(A) HUVEC were preincubated or not with 3 μg/ml heptadepsin for 2 hr, treated or not with LPS (1 μg/ml) for 4 hr, and, after washing, incubated with HL-60 cells for 1 hr.

(B) HUVEC were preincubated with the indicated concentrations of heptadepsin for 2 hr and then treated (solid columns) or not (open columns) with 1 μg/ml LPS, 1 μg/ml lipid A, 10 ng/ml TNF-α, or 10 ng/ml IL-1β for 4 hr. After 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 ± SD of triplicate determinations.

(C) Adhesion molecule expression in LPS-activated HUVEC was inhibited by heptadepsin. HUVEC were treated with the indicated concentrations of heptadepsin for 2 hr and then stimulated or not with 1 μg/ml LPS for 4 hr. The cell lysates were analyzed by Western blotting with anti-ICAM-1 and anti-VCAM-1 antibodies.

scopic analysis revealed the binding of Alexa-LPS to the Ba/F3 transformants, and addition of heptadepsin prior to Alexa-LPS markedly decreased the binding of Alexa-LPS to the cells in a dose-dependent manner (Figure 4A). An excess amount of nonlabeled LPS (cold LPS) also decreased the fluorescence. FACS analysis of the binding of Alexa-LPS to the cell surface receptor gave the similar results, as shown in Figure 4B. It was confirmed that heptadepsin did not interact with the fluorescence of Alexa Fluor 488 by in vitro analysis using a fluorescence spectrophotometer (data not shown). Furthermore, heptadepsin did not decrease the viability of Ba/F3 transformants up to 3 μg/ml, with or without LPS at 24 hr treatment (data not shown). Thus, heptadepsin was demonstrated to block the binding of LPS to the cell surface overexpressing TLR4.

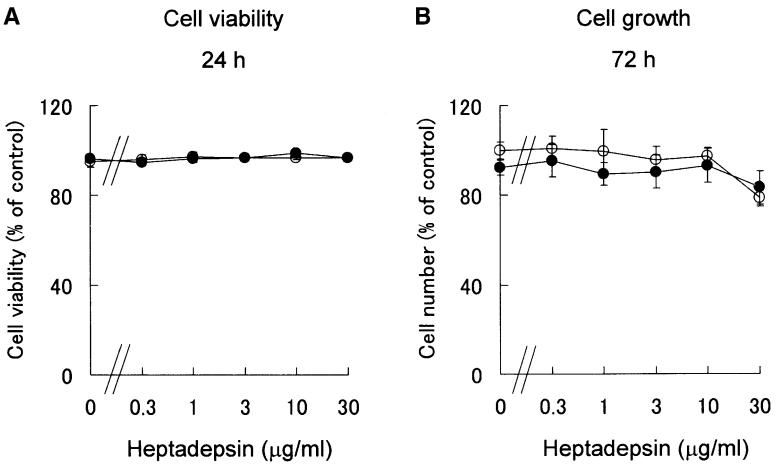

Figure 3. Effects of Heptadepsin on Viability and Growth of HUVEC

(A) HUVEC were treated with the indicated concentrations of heptadepsin for 24 hr in the absence (open circles) or presence (solid circles) of 1 μg/ml LPS, and cell viability was determined by trypan blue dye exclusion. Values are the means ± SD of quadruplicate determinations.

(B) HUVEC were treated with the indicated concentrations of heptadepsin for 72 hr in the absence (open circles) or presence (solid circles) of 1 μg/ml LPS, and the cell number was determined by using the MTT assay. Values are the means ± SD of triplicate determinations.

Effect of Heptadepsin on Taxol-Induced NF-κB Activation in Ba/F3 Cells

Taxol, an antitumor agent derived from a plant, was reported to mimic the action of LPS in mice [28, 29]. TLR4 and MD-2 were demonstrated to be necessary for the response to taxol [30–32]. Thus, both taxol and LPS require the TLR4-MD-2 complex for their signal transduction. To study whether heptadepsin acts on the LPS receptor, we examined the effect of heptadepsin on taxol-induced activation of NF-κB, the downstream signaling of TLR4. As a result, although heptadepsin inhibited LPS-induced NF-κB activation in Ba/F3 cells (Figure 5A), it did not inhibit taxol-induced NF-κB activation (Figure 5B). These results suggested that heptadepsin did not interact with LPS receptors.

Analysis of the Direct Interaction between Heptadepsin and LPS

Since heptadepsin did not inhibit TNF-α, IL-1β, or taxol-induced signaling, it is likely that heptadepsin directly acts on LPS, especially on lipid A, to inactivate the activity of LPS. To examine this possibility, we looked into the real-time interaction between heptadepsin and lipid A by using surface plasmon resonance (SPR) analysis. A strong binding signal was observed when heptaepsin was applied to immobilized lipid A on the HPA sensor chip (Figure 6A). The rapid increase of resonance unit (RU) by binding of heptadepsin to lipid A indicated the associated changes in mass during the reaction, demonstrating the direct interaction between heptadepsin and lipid A. Because time-dependent dissociation was observed, the type of interaction between heptadepsin and lipid A is likely to be reversible. Polymyxin B, known to bind to lipid A, induced the resonance in our SPR analysis, but ring-opened heptadepsin that was inactive to inhibit HL-60/HUVEC adhesion did not (data not shown). We also calculated the kinetic parameters such as k_a, k_d, and K_D using BIAcore evaluation software version 3.0. The dissociation rate (k_d), the association rate (k_a), and the apparent dissociation constant (K_D) of heptadepsin binding to lipid A were calculated to be 2.04×10^{-3} s^{-1}, 10.4 M^{-1} s^{-1}, and 1.97×10^{-4} M, respectively.

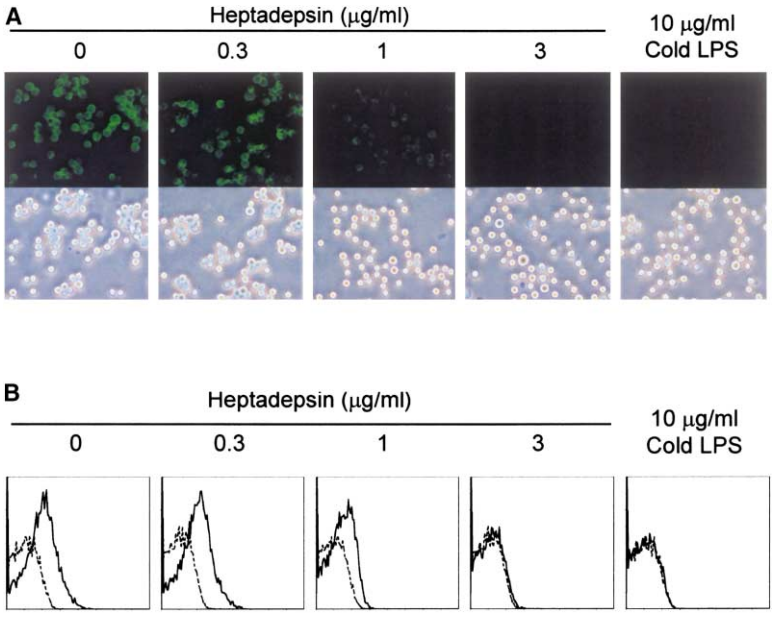

Figure 4. Inhibition of Fluorescent LPS Binding to Ba/F3 Cells Expressing TLR4-MD-2 and CD14 by Heptadepsin

(A) Ba/F3 transformants were incubated with the indicated concentrations of heptadepsin or 10 μg/ml cold LPS for 30 min. Then, the cells were treated with 1 μg/ml Alexa Fluor 488-conjugated LPS for 30 min. After washing, binding of Alexa-LPS to the cells was analyzed by fluorescence (upper panel) and phase contrast (lower panel) microscopy.

(B) FACS analysis for the binding of Alexa-LPS to Ba/F3 transformants was carried out after the cells were cultured as in (A). The histograms represent the fluorescence of Ba/F3 transformants treated with (bold line) or without (dotted line) 1 μg/ml Alexa-LPS in the presence of the indicated concentrations of heptadepsin or 10 μg/ml cold LPS.

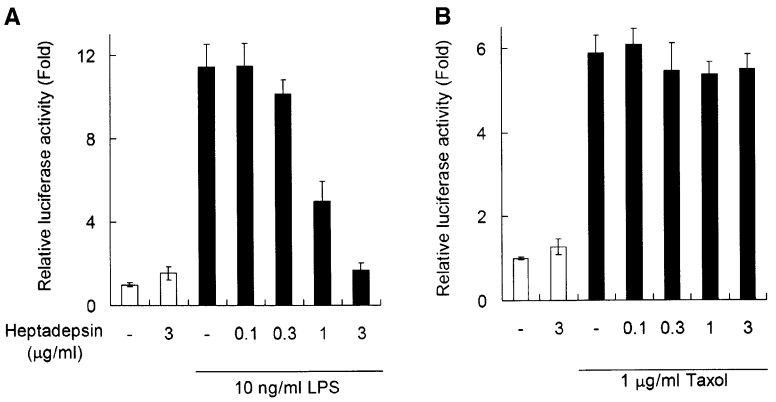

Figure 5. Effect of Heptadepsin on Taxol-Induced NF-κB Activation in Ba/F3 Cells Expressing TLR4-MD-2 and CD14

Ba/F3 transformants were incubated with the indicated concentrations of heptadepsin for 30 min. Then, the cells were treated with or without 10 ng/ml LPS (A), or 1 μg/ml taxol (B) for 4 hr. The luciferase activity was measured with a luminometer. Values are the means ± SD of quadruplicate determinations.

These results showed that heptadepsin interacted with lipid A directly to inhibit the LPS activity.

We also looked into the interaction between heptadepsin and LPS using heptadepsin absorpsion by LPS. To study whether LPS could inactivate heptadepsin, we employed a large molecule-remaining filter and centrifugation to remove LPS-bound heptadepsin (Figure 6B). Heptadepsin and LPS were mixed and incubated in a cell-free condition for 1 hr at 37°C, and then the mixed solution was ultrafiltered in a centrifugal filter device. In this procedure, molecules bound to LPS stayed in the retentate with LPS. If heptadepsin bound to LPS, it would not pass through the filter membrane of the centrifugal filter device, and thus the filtrate should not show any inhibitory activity toward cell adhesion. For the control experiments, we employed bovine serum albumin (BSA) and lipoteichoic acid (LTA). As a result, the heptadepsin fraction that had been pretreated with LPS showed no inhibitory activity toward HL-60 cell adhesion to LPS-stimulated HUVEC. On the other hand, heptadepsin pretreated with BSA or LTA maintained its inhibitory activity (Figure 6C). Previously, we confirmed that LPS itself did not pass through the filter membrane of the centrifugal filter device after ultrafiltration (data not shown). Moreover, the molecular weight of LPS was presumed to be over 3000 [33], which is the nominal molecular weight limit in daltons of the centrifugal filter device we used. In addition, the molecular weight of BSA is 68,000, and that of LTA used in this study is also over 3,000 [34], and so they could not pass through the filter membrane.

From these results, heptadepsin was concluded to interact with LPS directly, as shown in Figure 7. Thus, heptadepsin appears to be a unique inhibitor of LPS signal transduction, interacting with LPS itself.

Discussion

We looked among microbial secondary metabolites for compounds that could inhibit HL-60 cell adhesion to LPS-stimulated HUVEC. Among several thousand microbial culture filtrates tested, we isolated a novel compound, heptadepsin, from the culture broth of *Paenibacillus* sp. BML771-113F9. *Paenibacillus* arose from strains of *Bacillus* reported to produce other bioactive cyclic peptides [35]. Bioactive cyclic peptides produced by *Bacilli* can be classified into two groups. The first group has a common feature in that the N terminus of the peptide chain is bound to the long chain acid via the amide bond, and the carboxyl group on the C terminus forms a lactone with the β-hydroxy group, as seen in surfactin [36] and lichenysins [37], or forms a lactam with the β-amino group, as seen in iturins [38] and bacillomycins [39]. In these compounds, the long chain acid is incorporated into the ring system. The second group includes fusaricidins [26], circulocins [27], pipastatins [40], colistins [41], and octapeptins [42], in which the N terminus of the cyclic peptide is bound to the long chain acid by the amide bond lying outside the ring system. Heptadepsin belongs to the second group and contains a heptadepsipeptide core and a long chain acid with a terminal guanidino group. Although *Bacilli* produce many cyclic peptides, there has been no compound among them reported to inhibit LPS-induced cell adhesion. Actually, we tested the effect of fusaricidins A and B, which also belong to the second group of the cyclic peptides, on the cell adhesion between HL-60 cells and LPS-stimulated HUVEC. However, they showed no inhibitory activity (data not shown).

Our present study demonstrated that heptadepsin showed significant inhibitory activity against HL-60 cell adhesion to LPS-stimulated HUVEC (Figure 2) and that the inhibition occurred without toxicity or growth inhibition (Figure 3). This biological activity was proved to be caused by the inhibition of LPS-induced expression of adhesion molecules ICAM-1 and VCAM-1 in HUVEC (Figure 2C). Until now, only the naturally occurring cyclic heptadepsipeptide HUN-7293, isolated from a fungal broth, had been reported to inhibit inducible cell-adhesion molecule expression [23]. HUN-7293 is structurally different from heptadepsin in the following two points: its amino acid content and the absence of a long chain acid lying outside the cyclic system. HUN-7293 inhibited TNF-α-induced expression of ICAM-1 and VCAM-1 in human microvascular cell line HMEC-1 [22]. Because heptadepsin did not inhibit the signal transduction of TNF-α or IL-1β (Figure 2B), the mechanisms of the actions of heptadepsin and HUN-7293 must be different.

We found that heptadepsin blocked the binding of fluorescent-conjugated LPS to its receptors by using Ba/F3/TLR4/MD-2/CD14 cells (Figure 4). In addition, we revealed that heptadepsin directly interacted with lipid A by the SPR analysis and in vitro adsorption assay

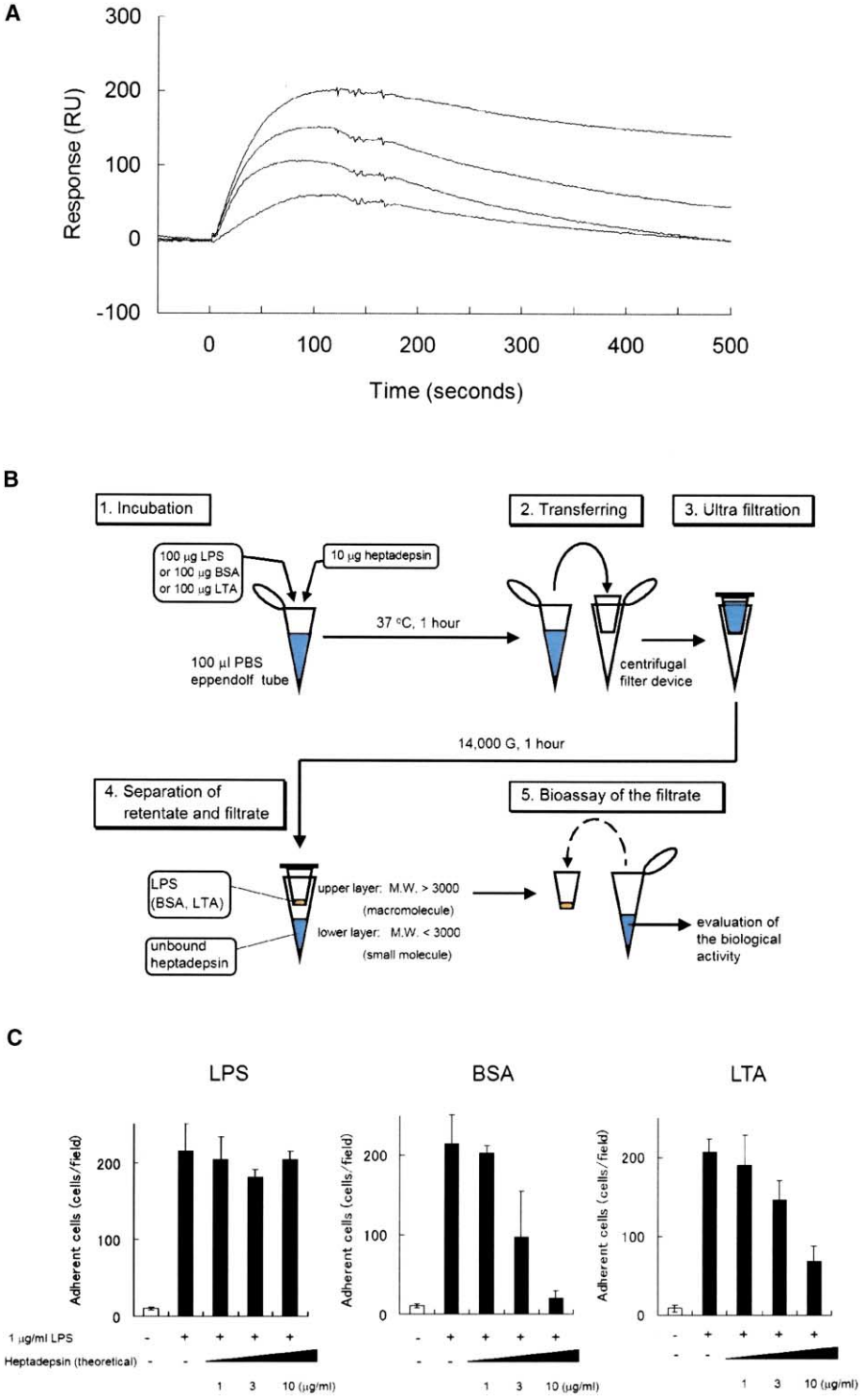

Figure 6. Interaction of Heptadepsin and LPS

(A) SPR analysis of the direct binding of heptadepsin and immobilized lipid A. Sensorgrams indicate the association and dissociation phases of the reactions when heptadepsin (1, 3, 4, and 5 μg/ml, from bottom to top) was injected for 2 min at 20 μl/min over the monolayer of lipid A immobilized on the HPA sensor chip.

(B) Procedure of the heptadepsin adsorption assay.

(C) Heptadepsin was mixed with LPS, BSA, or LTA for 1 hr, and then the mixture was ultrafiltered. The filtrate was then assayed for inhibitory activity toward HL-60 cell adhesion to LPS-stimulated HUVEC. HUVEC were preincubated with the indicated theoretical concentrations of heptadepsin pretreated with LPS, BSA, or LTA for 2 hr and then treated (solid columns) or not (open columns) with 1 μg/ml LPS for 4 hr. After the HUVEC had been washed, HL-60 cells were added. 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 ± SD of triplicate determinations.

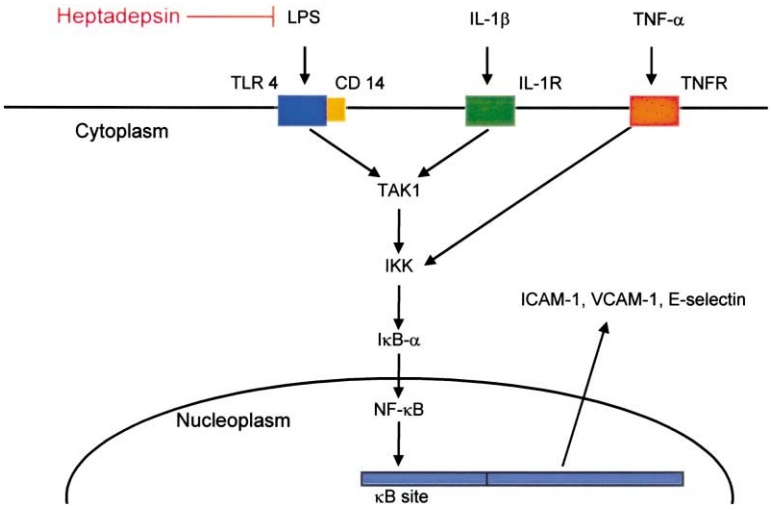

Figure 7. Scheme of Signal Transduction on Ligand-Induced Expression of Adhesion Molecules

using centrifugal filter devices (Figure 6). These results strongly indicated that heptadepsin directly interacted with LPS to block the downstream of LPS-TLR4 signal transduction. Until now, several naturally occurring compounds, mostly peptides, were reported to inhibit LPS signal transduction by neutralizing LPS itself. One such compound that possesses LPS-neutralizing activity is polymyxin B [43]. Polymyxin B is a naturally occurring cationic cyclic decapeptide isolated from *Bacillus polymyxa* [44]. It consists of a positively charged cyclic peptide ring and a long chain acid tail. The LPS-neutralizing activity of polymyxin B is due to its high-affinity binding to the highly conserved hydrophobic domain of LPS, which is lipid A [45]. By the SPR analysis, polymyxin B was indicated to possess the ability to bind to lipid A and LPS directly [46, 47]. Because polymyxins were comparatively toxic antibiotics, the cyclic peptides (nonapeptide) derived from polymyxin B were studied for reducing the toxicity [48]. Although polymyxin B and its derivatives inhibit the biological effects of LPS, their therapeutic applications are still very limited because of the toxicity.

Another known compound having LPS-neutralizing activity is cathelicidin. Cathelicidin is a novel family of antibacterial endogenous peptides isolated from the epithelial tissues and myeloid cells of human and animal species [49]. About 30 cathelicidin members from various mammalian species have been identified, and one of them, hCAP18 (human cationic antibacterial protein of 18 kDa), was characterized from a human source. Its carboxyl terminus, called LL-37, which comprises 37 amino acid residues, was recently identified in humans, and LL-37 and its derivatives were reported to possess LPS-neutralizing activity [50]. Originally, cathelicidin was thought to function in the innate host defense against microbial infections by impairing the membranes of targeted organisms [51]. It had been found that cathelicidin and its derivatives could block LPS signal transduction and reduce the mortality associated with endotoxemia in the murine model [52]. Recently, the mechanism by which the cathelicidin family blocks the biological activities of LPS was elucidated: they possess both LPS- and CD14 binding activities and block the binding of LPS to CD14-expressing cells [50, 53]. Because heptadepsin was shown to possess LPS-interacting activity, the mechanism by which heptadepsin blocks the biological activities of LPS may be similar to that of the cathelicidin family.

In addition, neutralizing antibodies against LPS and LPS binding protein (LBP) have been shown to suppress the cascade of LPS-induced inflammatory reactions [54–56].

Newly discovered heptadepsin was found to be a selective inhibitor of LPS signal transduction, interacting with LPS itself. Recently, much attention has focused on the low molecular weight compounds that possess LPS-neutralizing activities. Because the molecular weight of heptadepsin is lower than that of other known LPS-neutralizing agents, and because of its minimal cytotoxicity, heptadepsin may have therapeutic potential for the treatment of LPS-triggered syndromes. Thus, heptadepsin is an attractive candidate for adjunctive therapy of diseases associated with LPS, such as periodontitis, gram-negative bacterial sepsis, and atherosclerosis.

Significance

Molecules that can inhibit adhesion molecule expression in LPS-treated endothelial cells should be useful as antiinflammatory agents. So, we searched among microbial secondary metabolites for compounds that could inhibit LPS-stimulated adhesion between human umbilical vein endothelial cells (HUVEC) and HL-60 cells. As a result, we isolated a novel cyclic depsipeptide, which we named heptadepsin, from the whole culture broth of *Paenibacillus* sp. The addition of heptadepsin prior to LPS stimulation decreased HL-60 cell-HUVEC adhesion with an IC_{50} value of 0.92 μg/ml. One of the characteristics of heptadepsin is its low toxicity, and heptadepsin did not show any cytotoxicity or growth inhibition toward HUVEC, even at 30 μg/ml. It also inhibited the cellular adhesion induced by lipid A, the active component of LPS. However, heptadepsin did not inhibit HL-60 cell-HUVEC adhesion when HUVEC were activated by TNF-α or IL-1β. Interestingly, heptadepsin was found to interact with lipid A

or LPS directly. Binding of heptadepsin to lipid A was clearly demonstrated by the surface plasmon resonance (SPR) analyses. We set up the assay system for binding of LPS and the ligand, in which the ligand bound to high molecular weight LPS can be removed by filtration. Heptadepsin was shown to bind to LPS in this assay system. These results strongly suggested that heptadepsin interacted with LPS directly to inhibit the LPS activity. Thus, heptadepsin, a novel naturally occurring cyclic heptadepsipeptide, was shown to be a new selective inhibitor of LPS-induced signal transduction, acting by direct interaction with LPS. Because the molecular weight of heptadepsin is lower than that of other known LPS-neutralizing agents, and because of its minimal cytotoxicity, heptadepsin may have therapeutic potential for the treatment of LPS-triggered syndromes, such as periodontitis and gram-negative bacterial sepsis.

Experimental Procedures

Materials

HUVEC were purchased from Cell Systems (Lake Kirkland, WA). HL-60 cells were obtained from the Japanese Collection of Research Bioresources. The mouse pro B cell line Ba/F3 stably expressing mouse TLR4, mouse MD-2, mouse CD14, and p55IgκLuc, an NF-κB-dependent luciferase reporter construct, were prepared as described previously [57]. LPS (*Escherichia coli* Serotype 055:B5), lipid A (1, 4′-diphosphoryl form, *Escherichia coli* F-583), taxol (Paclitaxel; from *Taxus brevifolia*), BSA, and LTA (*Staphylococcus aureus*) were purchased from Sigma Chemical Co. (St. Louis, MO). TNF-α was purchased from Genzyme-Techne (Cambridge, MA), and IL-1β from Pepro Tech EC Ltd. (London, UK). Polyclonal antibodies against ICAM-1 and VCAM-1 came from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated LPS (*Escherichia coli* Serotype 055:B5) was purchased from Molecular Plobes (Eugene OR). HPA biosensor chip was obtained from BIAcore AB (Uppsala, Sweden).

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 μg/ml kanamycin, 100 units/ml penicillin G, 30 μg/ml L-glutamine, and 2.25 g/l $NaHCO_3$. Ba/F3 cells expressing TLR4-MD-2 and CD14 were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS, 100 μg/ml kanamycin, 100 units/ml penicillin G, 30 μg/ml L-glutamine, 2.25 g/liter $NaHCO_3$, 100 μM 2-mercaptoethanol, and IL-3, as described previously [57].

Fermentation

A slant culture of *Paenibacillus* sp. BML771-113F9 was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a seed medium composed of 2.5% lactose (Kanto Chemical Co., Tokyo, Japan), 7.5% polypeptone (Wako Pure Chemical, Tokyo, Japan), 0.5% meat extract (Kyokuto, Tokyo, Japan), 0.5% yeast extract (Wako Pure Chemical), 0.2% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, and 0.2% $CaCO_3$, adjusted to pH 7.4 before sterilization. The inoculated medium was incubated at 27°C for 1 day on a rotary shaker. The seed culture (2.2 ml) was then inoculated into a 500 ml Erlenmeyer flask containing 110 ml of the same medium, and incubated at 27°C for 4 days on a rotary shaker at 180 rpm.

Isolation and Purification

After the culture period, the whole broth of *Paenibacillus* sp. BML771-113F9 (1 liter, pH 6.4) was washed with an equal volume of $CHCl_3$ and then extracted three times with an equal volume of 1-BuOH. The combined 1-BuOH layers were concentrated in vacuo to give a brown residue (4.67 g). The residue was purified by column chromatography on silicagel 60 (Merck, Darmstadt, Germany) prepacked with 1-BuOH. The active fraction was eluted with a mixture of 1-BuOH, MeOH, H_2O (7:1:1, 1 liter), and concentrated in vacuo to give a yellow powder (212 mg). This yellow powder was dissolved in MeOH and chromatographed on Sephadex LH-20 (100 ml) with MeOH (200 ml). After concentration of the active fractions in vacuo to dryness, the residue (18.4 mg) was dissolved in MeOH and rechromatographed on Sephadex LH-20 (100 ml) with MeOH (200 ml). The active fraction was concentrated in vacuo to yield 13.1 mg of heptadepsin as a pure white powder.

Acid Hydrolysis and Determination of the Chirality of Amino Acids

Pure heptadepsin (2.6 mg) was hydrolyzed with 6 N HCl (1 ml) in a sealed tube at 105°C for 17 hr. The hydrolyzate was diluted with H_2O (2 ml) and washed with diethyl ether (3 ml) and EtOAc (3 ml). Evaporation of the aqueous layer gave a residue (1.1 mg) containing several ninhydrin-positive compounds detected following thin-layer chromatography (TLC) or high-voltage paper electrophoresis (HVPE): TLC Rf 0.45, 0.40, 0.28, and 0.22; HVPE Rm 1.0, 0.90–0.85, 0.80. Four known amino acids, isoleucine (TLC Rf 0.45, HVPE Rm 1.0), valine (Rf 0.40, Rm 0.89), alanine (Rf 0.28, Rm 0.10), and serine (Rf 0.22, Rm 0.88) were isolated by preparative TLC. Furthermore, aspartic acid (Rf 0.34, Rm 0.78), which had come from an asparagine residue in heptadepsin, was obtained by preparative HVPE. TLC was performed on a silica gel plate (Merk Art. 5626) developed with PrOH/H_2O (7:3) and stained with ninhydrin spray (Wako Pure Chemical). HVPE was carried out with a CAMAG HVE system at 3300 V for 15 min, with HCOOH/CH_3COOH/H_2O (25:75:900, pH 1.8) used as the electrolyte solution. The paper was stained with ninhydrin reagent, and the relative mobility (Rm) with respect to alanine was calculated. Chirality of amino acids was determined with a chiral HPTLC plate (Merk, HPTLC plate CHIR Art. 14101) developed with MeCN/MeOH/H_2O (4:1:1), and the Rf value was compared with that of the authentic D- or L-amino acid. L-Isoleucine (authentic D, Rf 0.48; L, 0.61), D-valine (authentic D, Rf 0.48; L, 0.58), D-alanine (authentic D, Rf 0.42; L, 0.49), L-serine (authentic D, Rf 0.47; L, 0.51), and D-aspartic acid (authentic D, Rf 0.49; L, 0.54) were identified.

Cell Adhesion Assay

HUVEC were seeded at 4×10^4 cells/well in 48-well collagen-coated plates (Costar) and cultured overnight at 37°C in 5% CO_2. The cells were preincubated with several concentrations of heptadepsin for 2 hr and then treated with the stimulant (1 μg/ml LPS, 1 μg/ml lipid A, 10 ng/ml TNF-α, or 10 ng/ml IL-1β) for 4 hr. Then, the medium was removed, and the wells were washed with phosphate buffered saline (PBS) twice. Next, leukemic cells (HL-60, THP-1, Jurkat) were added at 6×10^4 cells/well to the treated HUVEC monolayers. After 1 hr, nonadhering leukemic cells were removed by three washes with PBS. Then, the number of adherent cells in 1 microscopic field was counted.

Cell Viability and Growth

HUVEC were seeded at 1.6×10^5 cells/well in 24-well collagen-coated plates (Costar) and cultured overnight. Then, heptadepsin was added to the cells for 24 hr, and the cell viability was determined after staining the cells with trypan blue. For the cell growth assay, HUVEC were seeded at 2×10^3 cells/well in 96-well collagen-coated plates (Costar) and cultured overnight. Then, heptadepsin was added, and incubation was continued for 72 hr. Cell proliferation was measured by using the MTT assay.

Western Blotting Analysis

HUVEC (8×10^5 cells) were treated with LPS for the designed periods. Then, the cells were scraped off and suspended in lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μM Na_3VO_4, 1% NP-40, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 mM PMSF). The supernatants were combined with loading buffer (150 mM Tris, 30% glycerol, 3% bromophenol blue, 3% SDS, 15% 2-mercaptoethanol) and electrophoresed in 9% polyacrylamide gels. The gels were electrophoretically transferred to PVDF mem-

branes (Amersham Biosciences, Piscataway, NJ) at 4°C for 3 hr. 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 hr. The blotted membranes were next washed six times with 0.2% Tween 20 in TN buffer (50 mM Tris-HCl [pH 7.5] and 137 mM NaCl) and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) for 1 hr. Immunoreactive proteins were visualized with the ECL chemiluminescence system (Perkin Elmer Life Sciences, Boston, MA), and exposed to Fuji Medical X-ray film HR-H (Fuji Photo Film, Tokyo, Japan).

Fluorescence Microscopic Analysis

Ba/F3 cells expressing TLR4-MD-2 and CD14 were inoculated at 1×10^5 cells/well in 12-well plates (Costar) and incubated with heptadepsin or 10 μg/ml LPS for 30 min at 37°C in 5% CO_2. Then, the cells were treated with 1 μg/ml Alexa Fluor 488-conjugated LPS for 30 min and then washed twice with PBS. Binding of fluorescent LPS to the cells was analyzed by fluorescence microscopy (Eclipse E600; Nikon, Tokyo, Japan). The cells were photographed through a B2 (emission at 450–490 nm, absorption at 520 nm, Nikon) filter at a magnification of 200×.

FACS Analysis

Ba/F3 cells expressing TLR4-MD-2 and CD14 were inoculated at 1×10^6 cells/well in 12-well plates and incubated with heptadepsin or 10 μg/ml LPS for 30 min at 37°C in 5% CO_2. Then, the cells were treated with 1 μg/ml Alexa Fluor 488-conjugated LPS for 30 min and then washed twice with PBS. Cellular binding of fluorescent LPS was assayed by flow cytometry (EPICS Altra, Beckman Coulter, Fullerton, CA) using EXPO2 software (Beckman Coulter).

κB/Luciferase Reporter Gene Assay

Ba/F3 cells expressing TLR4-MD-2 and CD14 were inoculated at 1×10^5 cells/well in 48-well plates. After the preincubation with heptadepsin for 30 min, the cells were treated with 10 ng/ml LPS or 1 μg/ml taxol for 4 hr at 37°C in 5% CO_2. Next, the cells were harvested and lysed in 50 μl of lysis buffer (25 mM Tris-HCl [pH 7.8], 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100), and then luciferase activity was measured using 5 μl of lysate and 50 μl of luciferase assay substrate (Promega, Madison, WI). The luminescence was quantified with a luminometer (Lumat 9501; Berthold Japan, Tokyo, Japan).

SPR Analysis

Real-time biointeraction analysis of heptadepsin with lipid A was measured by surface plasmon resonance (SPR) using BIAcore X (BIAcore AB). The immobilization of lipid A onto the HPA biosensor chip was carried out as described previously, with slight modifications [58]. Lipid A at 0.5 mg/ml in water was sonicated at 37°C for 15 min before immobilization. Briefly, after the HPA chip was washed with 40 mM of n-octyl-β-D-glucoside for 5 min at a flow rate of 5 μl/min, lipid A was injected into a flow cell at 1 μl/min until saturation level was achieved. After immobilization, 2 mM NaOH was injected at 20 μl/min in 1 min 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) at 0.5 mg/ml in water was coated in the same way with lipid A. For the binding analysis, samples in the running buffer (TBS; 10 mM Tris-HCl [pH 7.0], 100 mM NaCl) were injected for 2 min at a flow rate of 20 μl/min. Association and dissociation curves were obtained on a BIAcore X. The surface of the sensor chip was regenerated by injection of 20 μl of 2 mM NaOH. All BIAcore experiments were carried out at 25°C, while the samples were kept in ice before injection. The value of apparent binding affinity of heptadepsin for lipid A was calculated by fitting the sensorgrams of kinetic injections using the bivalent binding model with BIAcore evaluation software version 3.0.

Evaluation of the Interaction of Heptadepsin with LPS

Heptadepsin (10 μg) was dissolved in 100 μl PBS along with 100 μg of LPS, BSA, or LTA and then incubated for 1 hr at 37°C. Thereafter, the mixed solution was put into a centrifugal filter device (Microcon YM-3; Millopore Co., Bedford, MA) having a nominal molecular weight limit (NMWL) of 3,000 daltons, and centrifuged at 14,000 × g. Finally, we collected the filtrate and examined its inhibitory activity toward the HL-60 cell adhesion to LPS-stimulated HUVEC.

Supplemental Data

Supplemental Data, consisting of two tables for the physico-chemical properties and the NMR data, are available at http://www.chembiol.com/cgi/content/full/11/8/1059/DC1.

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