Structure and Function of Papiliocin with Antimicrobial and Anti-inflammatory Activities Isolated from the Swallowtail Butterfly, *Papilio xuthus*\(^*\)

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Papiliocin is a novel 37-residue cecropin-like peptide isolated recently from the swallowtail butterfly, *Papilio xuthus*. With the aim of identifying a potent antimicrobial peptide, we tested papiliocin in a variety of biological and biophysical assays, demonstrating that the peptide possesses very low cytotoxicity against mammalian cells and high bacterial cell selectivity, particularly against Gram-negative bacteria as well as high anti-inflammatory activity. Using LPS-stimulated macrophage RAW264.7 cells, we found that papiliocin exerted its anti-inflammatory activities by inhibiting nitric oxide (NO) production and secretion of tumor necrosis factor (TNF)-\(\alpha\) and macrophage inflammatory protein (MIP)-2, producing effects comparable with those of the antimicrobial peptide LL-37. We also showed that the innate defense response mechanisms engaged by papiliocin involve Toll-like receptor pathways that culminate in the nuclear translocation of NF-\(\kappa\)B. Fluorescent dye leakage experiments showed that papiliocin targets the bacterial cell membrane. To understand structure–activity relationships, we determined the three-dimensional structure of papiliocin in 300 mm dodecylphosphocholine micelles by NMR spectroscopy, showing that papiliocin has an \(\alpha\)-helical structure from Lys\(^3\) to Lys\(^{21}\) and from Ala\(^{25}\) to Val\(^{36}\), linked by a hinge region. Interactions between the papiliocin and LPS studied using tryptophan blue-shift data, and saturation transfer difference-NMR experiments revealed that Trp\(^2\) and Phe\(^5\) at the N-terminal helix play an important role in attracting papiliocin to the cell membrane of Gram-negative bacteria. In conclusion, we have demonstrated that papiliocin is a potent peptide antibiotic with both anti-inflammatory and antibacterial activities, and we have laid the groundwork for future studies of its mechanism of action.

Antimicrobial peptides (AMPs\(^2\)) are important components of the innate immune systems in all living organisms (1–3). Many of these peptides rapidly kill invading pathogens by causing membrane permeabilization, although this may not be their sole mode of action (1–5). Although the detailed mechanisms have not been fully elucidated, the antibiotic action of most AMPs appears to involve depolarization or permeabilization of the bacterial cell membrane (1–6). Recent reports on intracellular targeting mechanisms of some AMPs have suggested that these peptides may target nonmembrane intracellular components by inhibiting the activity of specific molecular targets essential to bacterial growth (6, 7). Studies of innate immune systems of invertebrates, insects in particular, have revealed the importance of AMPs for the defense of these organisms against bacteria. Insects are extremely resistant to bacterial infections (8–10). A majority of such insect defensive peptides are produced in either fat bodies or hemocytes and then released into the hemolymph (9, 10). To date, more than 200 antibacterial peptides have been identified in insects. Most AMPs from insects can be divided into three categories (11–14). The largest category consists of peptides with intramolecular cysteine disulfide bonds forming hairpin-like \(\beta\)-sheets or \(\alpha\)-helical/\(\beta\)-sheet mixed structures. The second group has amphipathic \(\alpha\)-helical structures. The third group includes proline-rich or glycine-rich peptides (9, 14–16).

In insects, the cecropins constitute a large family of cationic \(\alpha\)-helical AMPs that are active against Gram-positive and Gram-negative bacteria and fungi (17–19). Among the insect-derived AMPs, the first \(\alpha\)-helical form discovered was cecropin, which was found to contribute an indispensable proportion of the innate immunity of insects (14). Cecropin was first isolated in 1981 from the hemolymph of the bacterially challenged, diapausing pupae of the giant silk moth *Hyalophora cecropia* (20). Since then, a number of cecropin-like peptides, composed of

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\(‡\) The abbreviations used are: AMP, antimicrobial peptide; DQF-COSY, double-quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; CH, cholesterol; DPC, dodecylphosphocholine; PC, \(\alpha\)-phosphatidylcholine; PG, \(\alpha\)-phosphatidylglycerol; SUV, small unilamellar vesicle; LLV, large unilamellar vesicle; hRBCs, human red blood cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-\(\kappa\)B, nuclear factor kappa B; NO, nitric oxide; MIC, minimum inhibitory concentration; iNOS, inducible nitric-oxide synthase; EG, egg yolk; STD, saturation transfer difference; TLR, toll-like receptor.
35–39 amino acids, have been identified in other insects, including *Bombyx mori* (21), *Drosophila melanogaster* (22), *Musca domestica* (23), *Acalolepta luxuriosa* (24), and *Helicoverpa armigera* (25). The tertiary structure of cecropin A revealed by nuclear magnetic resonance (NMR) spectroscopy has been shown to consist of an N-terminal amphipathic α-helix linked to a more hydrophobic C-terminal α-helix by a hinge region (26, 27).

In recent years, there has been a rapid increase in the emergence of microbes that are resistant to conventionally used antibiotics. The need to treat infections caused by multidrug-resistant bacteria has propelled an urgent search for new antibiotics. Among the possible candidates, AMPs have attracted increased clinical attention and expanded research interest (4, 28). Because the mechanism of action of AMPs differs from that of other therapeutic antibiotics, as a class, they may be effective against microorganisms that are resistant to currently available antibiotics (1–7).

There has been extensive progress in the identification of a broad array of diverse polypeptides implicated in many aspects of the host response to infection and other inflammatory stimuli (29). Some AMPs are known to interact with lipopolysaccharide (LPS) with high affinity (30). The biophysical properties of AMPs and their mode of interaction with LPS determine their biological function, the susceptibility of bacteria to them, as well as the ability of LPS to activate the immune system. A few cathelicidin AMPs, such as LL-37, indolicidin, and bactenecin, have the potential to inhibit LPS-induced cellular cytokine and nitric oxide (NO) release by binding directly to LPS or blocking the binding of LPS to LPS-binding protein (30, 31). Upon infection, human cathelicidin LL-37 is released from its precursor hCAP-18 (human cationic antimicrobial protein, 18 kDa) by the action of proteases (32, 33). Patients lacking LL-37 are reported to be more susceptible to infections (34), and LL-37 has also been shown to associate with LPS and protect rats from sepsis caused by bacteria (35). Therefore, AMPs are attractive therapeutic candidates for treating endotoxin shock and sepsis caused by Gram-negative bacterial infections.

We recently isolated papiliocin, a novel 37-residue peptide (RWKIFKKIEKVKGRNVRDGIIKAGPAVAVGQAATVVK-NH₂) from the larval of the swallowtail butterfly *Papilio xuthus* (36). Papiliocin exhibits 78.4% sequence homology with cecropin A from the giant silk moth *H. cecropia* (KWKIFKKIEKVKQNRDGIIKAGPAVAVGQAATVIK-NH₂) (37). In this study, we provide the first verification that papiliocin is a potent peptide antibiotic with both anti-inflammatory and antibacterial activities. We measured the toxicity of papiliocin toward bacteria and mammalian cells, and we also tested its ability to permeate model phospholipid membranes and investigated its modes of action. The anti-inflammatory activity of papiliocin was established by examining inhibition of nitrite production and inducible nitric-oxide synthase (iNOS) mRNA expression in LPS-stimulated RAW264.7 cells, a mouse macrophage cell line. We also investigated the innate defense response mechanisms engaged by papiliocin. Finally, we studied the tertiary structure of papiliocin using NMR spectroscopy and investigated the interaction between papiliocin and LPS to understand structure-function relationships underlying antimicrobial and anti-inflammatory activities.

### MATERIALS AND METHODS

**Peptide Synthesis**—Papiliocin was prepared by solid-phase synthesis using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The peptide was purified by reversed-phase preparative HPLC on a C₁₈ column (20 × 250 mm; Shim-pack) using an appropriate 0–90% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. Analytical HPLC with an ODS column (4.6 × 250 mm; Shim-pack) revealed that purified peptides were more than 98% homogeneous (data not shown). The molecular mass of purified peptides was determined by matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Kyoto, Japan). The concentration of papiliocin was quantified using a UV spectrometer.

**Antibacterial Activity**—*Escherichia coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637), *Salmonella typhimurium* (KCTC 1926), *Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917), and *Staphylococcus aureus* (KCTC 1621) were purchased from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). Minimum inhibitory concentrations (MICs) of papiliocin against bacteria were determined using a broth microdilution assay and compared with those of melittin and cecropin A. Briefly, single colonies of bacteria were inoculated into Luria-Bertani (LB) and cultured overnight at 37 °C. An aliquot of the culture was transferred to 10 ml of fresh LB and incubated for an additional 3–5 h at 37 °C until mid-logarithmic phase. 2-Fold serial dilutions of peptides in 1% peptone were prepared. Diluted peptides (100 μl) were added to 100 μl of cells (2 × 10⁶ cfu/ml) in 96-well microtiter plates and incubated at 37 °C for 16 h. The lowest concentration of peptide that completely inhibited growth was defined as the MIC. MIC values were calculated as the average of triplicate measurements in three independent assays.

Salt resistance tests for papiliocin were also performed at fixed concentrations of NaCl, CaCl₂, or MgCl₂. MIC against six standard bacterial strains (*E. coli* KCTC 1682, *P. aeruginosa* KCTC 1637, *S. typhimurium* KCTC 1926, *B. subtilis* KCTC 3068, *S. epidermidis* KCTC 1917, and *S. aureus* KCTC 1621) were determined in the presence of 100–200 mM NaCl, 1–5 mM CaCl₂, and 1–5 mM MgCl₂.

**Hemolytic Activity**—The hemolytic activity of papiliocin was tested against human red blood cells (hRBCs). Fresh hRBCs were washed three times with phosphate-buffered saline (PBS) (35 mM phosphate buffer containing 150 mM NaCl, pH 7.4) by centrifugation for 10 min at 1000 × g and then resuspended in PBS. Peptide solutions were then added to 50 μl of hRBCs in PBS to give a final volume of 100 μl and a final erythrocyte concentration of 4% (v/v). The resulting suspension was incubated with agitation for 1 h at 37 °C. The samples were then centrifuged at 1000 × g for 5 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm. No hemolysis (blank) and 100% hemolysis controls consisted of hRBCs suspended in PBS.
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and 0.1% Triton X-100, respectively. Percent hemolysis was calculated using Equation 1,

\[
\text{Hemolysis (\%) = \frac{(OD_{405\text{nm, sample}} - OD_{405\text{nm, zero lysis}})}{(OD_{405\text{nm, 100\% lysis}} - OD_{405\text{nm, zero lysis}})} \times 100 \quad (\text{Eq. 1})
\]

Cytotoxicity against RAW264.7 Cells—The mouse macrophage-derived RAW264.7 cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (100 units/ml penicillin and 100 \( \mu \)g/ml streptomycin) at 37 °C in a humidified 5% CO₂ atmosphere. Cultures were passaged every 2–3 days by brief trypsin treatment and visualized with an inverted microscope. The cytotoxicity of peptides against mammalian cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported previously (38), with minor modifications. Briefly, cells were seeded on 96-well microplates at a density of 1 \( \times 10^5 \) cells/well in 100 \( \mu \)l of RPMI 1640 medium containing 10% FBS. Plates were incubated for 24 h at 37 °C in 5% CO₂. Serial 2-fold dilutions of peptide solutions (100 \( \mu \)l) in RPMI 1640 medium were added; wells containing cells without peptides served as controls. After incubating plates for 1 day, 20 \( \mu \)l of an MTT solution (5 mg/ml) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. Precipitated MTT formazan was dissolved by adding 20 \( \mu \)l of a 20% (w/v) SDS solution containing 0.01 \( \text{m} \) HCl and incubating plates for 4 h. Absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA). Cell survival, expressed as a percentage, was calculated as the ratio of A₅₇₀ for cells treated with peptide to that of cells only.

Quantification of Nitrite Production in LPS-stimulated RAW264.7 Cells—Nitrite accumulation in culture media was used as an indicator of NO production (39). RAW264.7 cells were plated at a density of 1 \( \times 10^6 \) cells/ml in 96-well culture plates and stimulated with LPS (20 ng/ml) from E. coli O111:B4 (Sigma) in the presence or absence of peptides for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2% phosphoric acid) and incubated at RT for 10 min. Nitrite production was determined by measuring absorbance at 540 nm and converted to nitrite concentrations by reference to a standard curve generated with NaNO₂.

Quantification of Inflammatory Cytokine (TNF-α and MIP-2) Production by ELISA in LPS-stimulated RAW264.7 Cells—Antibodies against mouse tumor necrosis factor-α (mTNF-α) and mouse macrophage inflammatory protein-2 (mMIP-2) were immobilized on immunoplates by incubating with 0.2–0.8 \( \mu \)g/ml solutions of antibody in PBS overnight at room temperature (RT). Plates were washed once with PBS, 0.1% Tween 20 (PBST) and blocked by incubating with 200 \( \mu \)l of blocking solution (3% bovine serum albumen (BSA), 0.02% NaN₃ in PBS) overnight at RT. The supernatants from LPS-stimulated RAW264.7 cells co-incubated with serial-diluted peptide for 18 h were added to the wells of pre-coated plates and incubated for 2 h at RT. After washing plates three times with PBST, biotinylated anti-mTNF-α antibody (0.4 \( \mu \)g/ml), diluted in 0.1% BSA, was added, and plates were incubated for 2 h. Plates were then washed three times with PBST and further incubated with streptavidin peroxidase (0.3 \( \mu \)g/ml) diluted in PBS. After washing, SureBlue 3,3’,5,5’-tetramethylbenzidine peroxidase substrate (Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD) was added. The enzyme reaction was allowed to proceed at RT for color development and was stopped by adding 100 \( \mu \)l of 1 \( \text{m} \) \( \text{H}_2\text{SO}_4 \). The absorbance at 450 nm was detected using a microplate reader. All values represent the means ± S.D. of at least three independent experiments (40).

Reverse Transcription (RT)-PCR—Mouse RAW264.7 cells were plated in 6-well plates (5 \( \times 10^5 \) cells/well) and cultured overnight. Cells were stimulated without (negative control) or with 20 ng/ml LPS in the presence or absence of peptide in RPMI 1640 medium supplemented with 1% penicillin/streptomycin for 3 h. After stimulation, cells were detached from the wells by cold PBS and washed once with PBS. Competitive RT-PCR was performed as described previously (41). Briefly, total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, and equal amounts of total RNA were reverse-transcribed into cDNA using oligo(dT)₁₅ primers. The indicated targets were amplified from the resulting cDNA by PCR using the following specific primers: IL-1β, 5’-CTG TCC TGA GAG CAT CC-3’ (sense) and 5’-TGT CCA TTG AGG TGG AGA GC-3’ (antisense); IL-6, 5’-ACA AGT CCG GAG AGA AGA CT-3’ (sense) and 5’-GGG TGA TCT TGG TCC TTA GC-3’ (antisense); macrophage inflammatory protein (MIP)-1, 5’-ATG AAG CTC TGC GTG TCT GC-3’ (sense) and 5’-TGA GGA GCA AGG ACG TCT CT-3’ (antisense); MIP-2, 5’-ACA CTT CAG CCT AGC GCC AT-3’ (sense) and 5’-CAG GTG AGT TAG CCT TGC CT-3’ (antisense); TNF-α, 5’-GGT CTT CGT CCT TCC TCA TCT ACG G-3’ (sense) and 5’-GGT AGA GAA TGG ATG AAC ACC-3’ (antisense); and iNOS, 5’-CTG TCC TGA GAG CAT CC-3’ (sense) and 5’-GCC CAC TCT TGA GCA GTC-3’ (antisense). The primers for glyceraldehyde-3-phosphate (GAPDH), used as an internal control, were 5’-ACC ACA GTC CAT GCC ATC AC-3’ (sense) and 5’-TCC ACC ACC CTG TTT GTC TA-3’ (antisense). PCR was performed using the following cycling conditions: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1.5 min, and 94 °C for 1 min, and a final extension step of 72 °C for 5 min. Amplified products were electrophoresed on 1% agarose gels, and bands were visualized by UV illumination of ethidium bromide-stained gels.

Western Blotting—RAW264.7 cells were seeded in 6-well plates (3 \( \times 10^5 \) cells/well) and incubated in RPMI 1640 medium (WelGENE, Daegu, Korea) supplemented with 10% FBS (Invitrogen) and antibiotics (100 units/ml penicillin, 100 \( \mu \)l/ml streptomycin; Invitrogen) at 37 °C under 5% CO₂. Cultured cells were stimulated with 20 ng/ml LPS for 3 h, after which cells were incubated overnight with papiliocin (25 \( \mu \)M). After incubation, cells were washed twice with PBS and detached with ice-cold PBS. The collected cells were centrifuged at 1000 rpm for 5 min at 4 °C. Cell pellets were resuspended in 100 \( \mu \)l of
lysates specific for toll-like receptor 4 (TLR4, 1:2000; Cell Signaling Technology), and the remaining pellets using the NE-PERTM nuclear extraction reagent (Pierce). Equal amounts of protein (20 μg) were separated by SDS-PAGE on a 10% gel and transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Billerica, MA). The membrane was blocked by incubating with 5% skim milk in TBST (25 mM Tris, 3 mM KCl, 140 mM NaCl, 0.1% Tween 20) for 1 h at RT and then incubated with antibodies specific for toll-like receptor 4 (TLR4, 1:2000; Cell Signaling Technology, Beverly, MA), nuclear factor κB (NF-κB; 1:2000, Cell Signaling Technology), and β-actin (1:5000, Sigma). After washing with TBST buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:30,000; Sigma) secondary antibodies, as appropriate. Signals were detected using an enhanced chemiluminescence (ECL) detection system (GE Healthcare).

Calcein Leakage Assay—Calcein-entrapped large unilamellar vesicles (LUVs) composed of egg yolk 1-α-phosphatidylcholine (PC)/egg yolk 1-α-phosphatidylglycerol (PG) (7:3, w/w), or egg yolk PC/cholesterol (CH) (10:1, w/w) were prepared by vortexing dried lipids in dye buffer solution (70 mM calcein, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA). The suspension was subjected to 10 cycles of freeze-thaw in liquid nitrogen and extruded through polycarbonate filters (two stacked 100-nm pore size filters) using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. Passing through a Sephadex G-50 column usually resulted in approximately a 10-fold dilution of lipid vesicles. The eluted calcein-entrapped vesicles were diluted further to achieve the desired final lipid concentration of 64 μM for experiments. The leakage of calcein from LUVs was monitored by measuring fluorescence intensity using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. All fluorescence experiments were performed in quartz cuvettes with a 1-cm path length using a model RF-5301PC spectrophotometer (Shimadzu, Kyoto, Japan). Vesicles dissolved in Tris buffer containing 10% Triton X-100 (20 μl) were used to establish 100% dye release. The percentage of dye leakage caused by peptides was calculated in Equation 2,

\[
dye\% = \frac{F - F_o}{F_t - F_o} \times 100
\]

where \(F\) is the fluorescence intensity of peptide-treated vesicles, and \(F_o\) and \(F_t\) are fluorescence intensities without the peptides and with Triton X-100, respectively.

Tryptophan Fluorescence Blue Shift—Small unilamellar vesicles (SUVs) were prepared according to a standard procedure using the required amounts of egg yolk PC/PG (7:3, w/w) or egg yolk PC/CH (10:1, w/w). Dry lipids were dissolved in chloroform, deposited as a film on the wall of a glass vessel, and then lyophilized overnight. Dried thin films were resuspended in Tris-HCl buffer by vortexing. The lipid dispersions were then sonicated in an ice water bath for 10–20 min using a titrated ultrasonicator until the suspension became transparent. SUVs were added to a peptide solution (5 μM final concentration) in 10 mM Tris buffer, pH 7.4, 0.1 mM EDTA, and 150 mM NaCl and maintained at 25 °C with continuous stirring in a total volume of 2 ml. The lipid concentration of SUV was 1.0 μM. Tryptophan residues of each peptide were excited at 280 nm, and emission spectra were recorded in the range 300–400 nm using a 5-nm bandpass filter. Blue shifts for papiliocin were also measured in 8.0 μM LPS and 1.5 mM dodecylphosphocholine (DPC) in 10 mM sodium phosphate buffer.

Fluorescence Quenching Experiments—Intrinsic tryptophan fluorescence studies of papiliocin were performed in 10 mM sodium phosphate buffer at a peptide concentration of 5 μM and at pH 6.0. The interaction of papiliocin with LPS, the major component of the outer surface membrane of almost all Gram-negative bacteria, was investigated by recording the fluorescence emission spectra of the peptide as a function of the concentration of LPS compared with that in DPC. Tryptophan fluorescence was quenched by adding acrylamide (5 μM stock) to a final concentration of 0.2 μM. Fluorescence quenching for papiliocin was also measured in EYPC/EYPG SUVs and EYPC/CH SUVs in 10 mM Tris buffer, pH 7.4. Decreases in fluorescence intensities were measured at the corresponding emission maxima of the peptide or peptide-lipid complex. The results of the quenching reactions were analyzed by fitting to the Stern-Volmer equation, \(F_o/F = 1 + K_{SV}[Q]\), where \(F_o\) and \(F\) are the fluorescence intensities at the appropriate emission wavelengths in the absence and presence of quencher, respectively; \(K_{SV}\) is the Stern-Volmer quenching constant, and \([Q]\) is the molar quencher concentration.

Interaction of Papiliocin with FITC-labeled LPS Aggregates—The interactions of papiliocin with fluorescein isothiocyanate (FITC)-conjugated LPS were studied by exciting 0.5 μM FITC-LPS at 480 nm and monitoring the change in the emission of FITC at 515 nm in the presence of different concentrations of papiliocin (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 8.0, and 9.6 μM). Samples were prepared in 10 mM phosphate buffer at pH 6.0.

Circular Dichroism Analysis—CD experiments were performed using a J810 spectropolarimeter (Jasco, Tokyo, Japan) with a 1-mm path length cell. The CD spectra of the peptides were recorded at 25 °C in 0.1-nm intervals from 190 to 250 nm. CD spectroscopy was used to investigate the secondary structure adopted by peptides in the following membrane mimetic environments: 15% hexafluorosopropyl/water solution, 15 mM DPC micelles, 100 mM SDS micelles, and 100 μM LPS. The peptide concentration was 50 μM for all CD experiments. For each spectrum, the data from 10 scans were averaged and smoothed using J810 software. CD data were expressed as the mean residue ellipticity (θ) in degrees cm² dmol⁻¹.

NMR Analyses and Structure Calculations—The spectra of papiliocin showed severe spectral overlapping in SDS micelles and LPS micelles. Therefore, the peptide was dissolved at 1.0 mM in 0.45 ml of 9:1 (v/v) H₂O/D₂O, 20 mM phosphate buffer, pH 5.9, containing 300 mM DPC micelles prepared from perdeuterated DPC (Cambridge Isotope Laboratories, Andover, MA).

Phase-sensitive two-dimensional experiments, including double quantum-filtered correlation spectroscopy (DQF-
COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY), were performed using time-proportional phase incrementation (42–45). For TOCSY and NOESY experiments, 450–512 transients with 4 K complex data points were collected for each increment with a relaxation delay of 1.2 s between successive transients. The data along the \( t_1 \) dimension were zero-filled to 1 K before two-dimensional Fourier transformation. TOCSY experiments were performed using 50- and 80-ms MLEV-17 spin-lock mixing pulses. Mixing times of 80, 150, and 250 ms were used for NOESY experiments. For DQF-COSY experiments, 512 transients with 4 K complex data points were collected for each increment, and the data along the \( t_1 \) dimension were zero-filled to 4 K before two-dimensional Fourier transformation. The \( ^{3}J_{HN} \) coupling constants were measured from the DQF-COSY spectra with a spectral width of 6502.630 Hz and a digital resolution of 1.02 Hz/point. Chemical shifts were expressed relative to the 4,4-dimethyl-4-silapentane-1-sulfonate signal at 0 ppm. Intramolecular hydrogen bonding in the peptides was investigated by calculating temperature coefficients from the TOCSY experiments at 298, 303, 308, and 313 K. All NMR spectra were recorded on a Bruker 500 or 800 MHz spectrometer at KBSI (Bruker, Rheinstetten, Germany). NMR spectra were processed with NMRPipe (46) and visualized with Sparky (47).

Structure calculations of the peptides were carried out using the standard protocol of the Cyana2.1 program in a LINUX environment (48). A total of 500 structures was calculated using the torsion angle dynamics protocol. The structures were sorted according to the final value of the target function, and the best 20 structures were analyzed in terms of distance and angle violations. The chemical shifts, NMR-derived constraints, and coordinates are deposited in the Biological Magnetic Resonance Bank (BMRB; accession number, 17500). The atomic coordinates for 20 final structures have been deposited with the Protein Data Bank under the file name 2LA2.

**Saturation Transfer Difference (STD)-NMR Experiments—** Peptide was added at a concentration of 0.5 mM to 15 \( \mu M \) LPS in D\(_2\)O at pH 5.9. LPS was prepared by lyophilization from 99.9% D\(_2\)O. STD-NMR experiments were recorded on a Bruker 500 MHz spectrometer at a temperature of 298 K. The STD-NMR spectra were obtained with 512 scans and selective saturation of LPS resonances at \( 2.0 \) ppm (40 ppm for reference spectra). A cascade of 40 selective Gaussian-shaped pulses of 45-ms duration and a 100-\( \mu \)s delay between each pulse were used in all STD-NMR experiments with a total saturation time of 2 s. Subtraction of the two spectra (on resonance-off resonance) leads to the difference spectrum, which contains signals arising from the saturation transfer. Therefore, spectral differences primarily constituted resonances belonging to peptide protons bound to LPS micelles.

**RESULTS**

Papiliocin and cecropin A were synthesized as described in Table 1. Papiliocin has a net charge of +8 and hydrophobicity of −1.48, whereas cecropin A has a net charge of +7 and hydrophobicity of −1.37, calculated according to the combined consensus scale (49). Even though the sequence homology between the two peptides is very high, the N-terminal helix of papiliocin has higher cationicity than that of cecropin A, whereas the

**TABLE 1**

| Peptides                  | Net charge | Hydrophobicity | Hydrophobicity (sum of all residue hydrophobicity indices divided by the number of residues, according to combined consensus scale) |
|---------------------------|------------|----------------|-----------------------------------------------------------------------------------------------------------------------|
| Papiliocin, RWKIFKKIEKVGRNVRDGIIKAGPAVAVVGQAATVVK-NH\(_2\)  | +8         | 1.48           | 0.73                                                                                                                  |
| Cecropin A, KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH\(_2\)  | +7         | 1.37           | 0.71                                                                                                                  |

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C-terminal helix of papiliocin is somewhat more hydrophobic than that of cecropin A. Overall, the relative hydrophobicities of papiliocin and cecropin A were very similar, as determined by measuring retention times in reversed-phase-HPLC; retention time was 19.1 min for papiliocin and 20.0 min for cecropin A. A helical wheel diagram of papiliocin (Fig. 1) shows the amphipathicity of the N-terminal helix (1–21) in papiliocin, with the hydrophobic residues in the upper region and the hydrophilic residues in the lower region. As shown in Fig. 1, the C-terminal helix (25–37) is hydrophobic and is not amphipathic.

**Antimicrobial Activity**—The antimicrobial activities of papiliocin were examined against a representative set of bacterial strains, including three Gram-negative species (E. coli, S. typhimurium, and P. aeruginosa) and three Gram-positive species (B. subtilis, S. epidermidis, and S. aureus), and compared with the activities of melittin, which is known to have profound antibacterial activity against all bacterial strains, as indicated in Table 2. Papiliocin demonstrated broad spectrum antibacterial activity. However, papiliocin showed much higher antibacterial activities against Gram-negative bacteria than against Gram-positive bacteria. Surprisingly, papiliocin and cecropin A were more potent than melittin against all Gram-negative bacteria tested but were much less potent than melittin against Gram-positive bacteria. The antimicrobial activity of papiliocin was somewhat higher than that of cecropin A against Gram-negative bacteria and Gram-positive bacteria. These differences may result from differences in the net charge and hydrophobicity of the two peptides.

**Salt Resistance Tests**—MICs of papiliocin for bacteria were determined in the presence of NaCl, CaCl2, and MgCl2 and compared with those of melittin. As summarized in Table 3, papiliocin exhibited strong antibacterial activity against Gram-negative bacteria that was largely unchanged over a range of NaCl concentrations from 100 to 200 mM. Against all Gram-positive bacteria, there was significant increase in the MICs of papiliocin in the presence of NaCl. The MICs of papiliocin against all Gram-negative bacteria increased 2-fold in the presence of 1–5 mM CaCl2 and 1–5 mM MgCl2, whereas those for melittin increased substantially. The reported concentrations of calcium and magnesium in human body fluids are on the order of 1 mM (50). At this concentration of CaCl2 and MgCl2, papiliocin showed good antimicrobial activity, exhibiting an MIC of 1–2 μM against all Gram-negative bacteria. Therefore, papiliocin is salt-resistant and can be described as calcium- and magnesium-resistant against Gram-negative bacteria in a physiological environment.

**Hemolytic Activity and Cytotoxicity against Mammalian Cells**—We then tested the hemolytic activity of papiliocin. Dose-response curves for the hemolytic activity of the peptides, shown in Fig. 2A, reveal that papiliocin lacked hemolytic activity, even at 100 μM. Next, we examined the cytotoxicity of papiliocin and antimicrobial activities against standard bacterial strains

### Table 2

| Bacterial strains | Papiliocin (μM) | Melittin (μM) | Cecropin A (μM) |
|------------------|-----------------|---------------|-----------------|
| E. coli          | 0.25            | 4.0           | 0.50            |
| S. typhimurium   | 0.50            | 8.0           | 1.0             |
| P. aeruginosa    | 1.0             | 8.0           | 1.0             |
| GM              | 0.58            | 6.7           | 0.83            |
| MHC             | 200             | 0.39          | 200             |
| Therapeutic index (MHC/GM) | 340 | 0.059 | 240 |

### Table 3

| Salt | Concentration (M) | Papiliocin | Melittin | Papiliocin | Melittin | Papiliocin | Melittin |
|------|-------------------|------------|----------|------------|----------|------------|----------|
| none |                   | 0.50       | 4.0      | 0.50       | 4.0      | 1.0        | 8.0      |
| NaCl | 1.0               | 2.0        | 4.0      | 0.50       | 8.0      | 1.0        | 8.0      |
| 5.0  | 2.0               | 16         | 32       | 1.0        | 32       | 1.0        | 32       |
| CaCl2| 3.0               | 2.0        | >32      | 1.0        | >32      | 2.0        | >32      |
| MgCl2| 3.0               | 2.0        | >32      | 1.0        | >32      | 2.0        | >32      |

### Table 3 (continued)

| Bacterial strains | Papiliocin | Melittin | Papiliocin | Melittin | Papiliocin | Melittin |
|------------------|------------|----------|------------|----------|------------|----------|
| None             | 32         | 4.0      | 2.0        | 1.0      | 16         | 4.0      |
| NaCl             | 100        | >32      | 4.0        | 16       | 2.0        | >32      | 4.0      |
| 150              | >32        | 4.0      | 16         | 2.0      | >32        | 4.0      |
| 200              | >32        | 4.0      | 16         | 2.0      | >32        | 4.0      |

* MICs were determined in three independent experiments performed in triplicate with a standard deviation of 14.0%.

* The geometric means (GM) of the MIC values from all six bacterial strains are shown.

* The minimal peptide concentration produces hemolysis. When no detectable hemolysis was observed at 100 μM, a value of 200 μM was used to calculate the therapeutic index.

* The ratio of the MHC (μM) over the geometric mean of the MIC (μM) is given. Larger values indicate greater cell selectivity.
Papiliocin against RAW264.7 macrophage cells, assessing the effects on cell growth by measuring the mitochondrial reduction of MTT to a colored product by live cells. As shown in Fig. 2B, melittin exhibited remarkable cytotoxicity at its MIC, whereas papiliocin was not toxic at its MIC. At 12.5 μM papiliocin, the survival rate of RAW264.7 cells was greater than 90%, and an analysis of dose-response curves yielded an IC₅₀ of 58 μM. However, melittin, which is known to have strong antimicrobial activities against all bacteria, and a second control peptide, LL-37, which is known to possess strong anti-inflammatory activity, were very cytotoxic against mammalian cells. Therefore, papiliocin was highly selective for bacterial cells, whereas melittin and LL-37 were not.

Inhibition of NO Production in LPS-stimulated RAW264.7 Cells—LPS, also termed endotoxin, is the major component of the outer membrane of Gram-negative bacteria. Release of LPS from these bacteria during sepsis causes septic shock by inducing the production of higher concentration of systemic pro-inflammatory cytokines and NO (51). To assess the potential anti-inflammatory activity of papiliocin, we indirectly measured peptide inhibition of NO production in RAW264.7 macrophages stimulated with 20 ng/ml LPS by quantifying nitrite concentration. As shown in Fig. 3A, papiliocin significantly inhibited NO production by LPS-stimulated RAW264.7 macrophages at 1.0–10.0 μM; the responses to cecropin A and the cathelicidin-derived AMP, LL-37, are presented as controls. Notably, the inhibitory activity of papiliocin and cecropin A against NO production was comparable with that of LL-37.

FIGURE 2. A, dose-response curve for the hemolytic activity of papiliocin toward human erythrocytes. B, dose-response curves for the cytotoxicity of papiliocin (●), melittin (○), and LL-37 (▲) toward macrophage-derived RAW264.7 cells.

FIGURE 3. A, inhibition of nitrite production by papiliocin, cecropin A, and LL-37 in LPS-stimulated RAW264.7 cells. B, inhibition of TNF-α production by papiliocin and LL-37 in LPS-stimulated RAW264.7 cells. C, inhibition of MIP-2 production by papiliocin and LL-37 in LPS-stimulated RAW264.7 cells.
Inhibition of TNF-α and MIP-2 Production in LPS-stimulated RAW264.7 Cells—Because papiliocin showed inhibitory activity against NO production, we further tested its anti-inflammatory activities. To accomplish this, we measured the inhibitory effect of papiliocin on the production of the inflammatory cytokines, TNF-α and MIP-2 in LPS (20 ng/ml)-stimulated RAW264.7 cells. A quantitative analysis of TNF-α (Fig. 3B) and MIP-2 (Fig. 3C) concentrations revealed that both papiliocin and cecropin A clearly inhibited both TNF-α and MIP-2 production in LPS-stimulated RAW264.7 cells and were more effective in this respect than the positive control, LL-37.

Inhibition of iNOS mRNA Expression in LPS-stimulated RAW264.7 Cells—LPS induces the expression of iNOS and the inflammatory cytokines, IL-1β, IL-6, MIP-1, MIP-2, and TNF-α, in macrophages. The effects of papiliocin on the expression of iNOS and inflammatory cytokines induced in RAW264.7 macrophages by stimulation with 20 ng/ml LPS were examined over 6 h by RT-PCR. As shown in Fig. 4, papiliocin effectively suppressed the expression of iNOS as well as that of all inflammatory cytokine genes. These data are in agreement with the observed inhibition of NO production and cytokine production by papiliocin, confirming that the cecropin-like peptide papiliocin possesses potent anti-inflammatory activity.

Inhibition of TLR4 Expression in LPS-stimulated RAW264.7 Cells—We next investigated the inflammatory response pathways of papiliocin. Members of the Toll-like receptor (TLR) family are transmembrane proteins that play a critical role in signal transduction (52). TLRs recognize specific substances derived from pathogenic molecules, after which activated TLRs trigger the recruitment of downstream signaling proteins, such as MyD88, TIRAP/Mal (toll-interleukin 1 receptor domain-containing adaptor protein/MyD88 adapter-like), and TRIF (TIR domain-containing adapter protein inducing IFN-β), leading to the secretion of various inflammatory proteins, including NO, IL-1β, and TNF-α. Therefore, TLR-mediated signal transduction is closely associated with the induction of immune and inflammatory responses (52–54). TLR4, in particular, is known to induce the expression of genes during an inflammatory response (53). To address the potential role of TLR proteins in LPS-stimulated RAW264.7 macrophages, we analyzed cytoplasmic extracts for TLR4 expression by Western blotting. As shown in Fig. 5, LPS induced the expression of TLR4, an effect that was suppressed by papiliocin. LPS of pathogenic bacteria is known to binds to TLR4 on cytoplasmic membranes and trigger expression of a variety of inflammatory products, such as TNF-α, IL-1β, and NO. These data thus suggest that papiliocin may modulate the binding of LPS to TLR4 in LPS-stimulated macrophage-derived RAW264.7 cells, thereby inhibiting the inflammatory response.

Inhibition of the NF-κB Pathway in LPS-stimulated RAW264.7 Cells—Activation of TLR4 leads to phosphorylation (activation) of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinase (JNK). The phosphorylated signal transduction molecules ultimately induce translocation of the transcription factor NF-κB from the cytoplasm to the nucleus, stimulating its transcriptional activity (55, 56). To investigate the association of this transcription factor with the inflammatory cellular response, we analyzed nuclear extracts for the presence of NF-κB by Western blotting. As shown in Fig. 5B, NF-κB was highly expressed in LPS-stimulated cells. Notably, LPS-induced NF-κB expression was dramatically decreased in the presence of papiliocin, suggesting that papiliocin prevents translocation of NF-κB from the cytoplasm to the nucleus and implying that NF-κB-mediated signal transduction is a downstream of papiliocin in LPS-stimulated RAW264.7 cells. Taken together, the results presented in Fig. 5, A and B, indicate that papiliocin modulates the activation of TLR4 and NF-κB in LPS-stimulated RAW cells. Given the involvement of TLR4 and NF-κB in the inflammatory pathway, these data suggest that papiliocin is a candidate anti-inflammatory agent.

Peptide-induced Permeabilization of Lipid Vesicles—To investigate the mechanism of action of papiliocin, we measured the membrane-permeabilizing ability of papiliocin by monitoring the release of the fluorescent marker, calcein, from LUVs with different composition. We employed zwitterionic LUVs composed of 7:3 (w/w) EYPC/EYPG and negatively charged LUVs composed of 10:1 (w/w) EYPC/CH and negatively charged LUVs composed of 7:3 (w/w) EYPC/EYPG. The percentage of...
calcein leakage 2 min after exposure to the peptide was used as a measure of membrane permeability. The dose-response curves of peptide-induced calcein release presented in Fig. 6 show that papiliocin permeated negatively charged vesicles, which mimic bacterial cell component membranes, much more effectively than zwitterionic vesicles, which mimic the major components of the outer leaflet of human erythrocytes. In contrast, melittin showed no bacterial cell selectivity in these assays.

**Tryptophan Fluorescence Blue Shift**—The tryptophan residues in several types of AMPs are reported to be critical for the antibacterial and/or hemolytic activities of these agents (57–59). To investigate the interactions between tryptophan and the phospholipid membrane, we monitored fluorescence emissions of Trp2 in papiliocin in Tris-HCl buffer, pH 7.4, or in the presence of SUVs composed of the mammalian membrane-mimetic zwitterionic phospholipid, EYPC/CH (10:1, w/w) or the negatively charged phospholipid, EYPC/EYPG (7:3, w/w), which mimics the bacterial membrane. As listed in Table 4, melittin, which has both high antibacterial activity and high cytotoxicity, showed large blue shifts in both types of vesicles as well as both micelles, implying that it is not cell-selective. Papiliocin exhibited a much larger blue shift in the negatively charged vesicles and LPS micelles than in the zwitterionic phospholipid vesicles and DPC micelles, consistent with enhanced selectivity for bacterial cells. Papiliocin in LPS showed a much lower Stern-Volmer quenching constant compared with that for papiliocin in neutral micelles.

**Quenching Experiments**—To assess the solvent exposure of Trp2, we performed fluorescence quenching studies using the neutral quencher acrylamide. Papiliocin showed much lower Stern-Volmer quenching constants (KSV) in micelles or SUVs compared with those in aqueous buffer solution (60). As shown in Fig. 7, A and C, papiliocin in negatively charged lipids, such as LPS micelles or EYPC/EYPG SUVs, showed much lower KSV than in neutral lipids, such as DPC micelles or EYPC/CH SUVs, indicating that the Trp2 residue is deeply buried in negatively charged membranes but is largely exposed in neutral membranes. In good agreement with the cytotoxicity data, melittin showed similar Stern-Volmer quenching constants (KSV) in negatively charged and neutral lipids (Fig. 7, B and D). Blue shift data as well as quenching data imply that papiliocin is inserted more deeply into the negatively charged bacterial cell membrane than into the neutral membrane, resulting in high bacterial cell selectivity. These data also imply that Trp2 in papiliocin may play an important role in the antibacterial activity and bacterial cell selectivity of this peptide.

**FITC-labeled LPS Aggregates**—Interactions of AMPs with LPS results in the dissociation of large LPS aggregates into smaller sizes, an effect that can be monitored as an increase in fluorescence using FITC-conjugated LPS (30, 61). Using this approach, we tested the interaction of papiliocin with LPS. As shown in Fig. 8, which presents changes in the intensity of FITC-LPS fluorescence as a function of the concentration of papiliocin, the addition of papiliocin caused a dose-dependent increase in FITC-LPS fluorescence, suggesting that the interaction of papiliocin with LPS resulted in the dissociation of LPS aggregates.

**CD Measurements**—To investigate the secondary structures of papiliocin in membrane-like environments, we analyzed the CD spectra of the peptide dissolved under a variety of membrane-mimicking conditions. As shown in Fig. 9, the peptide showed unordered structures in aqueous solution, but exhibited conformational changes in SDS micelles, DPC micelles, a
15% HFIP/water solution, and LPS micelles. In all membrane
mimetic environments, papiliocin exhibited characteristic dou-
ble negative maxima at 205 and 220 nm, suggesting that it can
adopt a significant degree of \( \alpha \)-helical structure under these con-
ditions. Papiliocin had more \( \alpha \)-helical structure in SDS and
LPS micelles than in neutral DPC micelles. These results
suggest that papiliocin has a more folded structure in nega-
tively charged environments due to electrostatic interaction
between the positively charged residues in papiliocin and the
negatively charged lipid.

**FIGURE 7.** Stern-Volmer plots of acrylamide quenching of tryptophan fluorescence of papiliocin (A and C) and melittin (B and D) in the presence of sodium phosphate buffer, pH 6.0 (●), 8.0 \( \mu \)M LPS (○), 1.5 mM DPC (▼), Tris buffer, pH 7.4 (▲), EYPC/EYPG (7:3, w/w) SUV (■), and EYPC/CH SUVs (10:1, w/w) (◇).

**FIGURE 8.** Enhancement of the intensity of FITC-labeled LPS as a function of papiliocin concentration. AU, absorbance unit.

**FIGURE 9.** CD spectra of peptides (50 \( \mu \)M, pH 4.1) in \( H_2 O \), a 15% HFIP/water solution, 100 mM SDS micelles, 50 mM DPC micelles, and 100 \( \mu \)M LPS.

**Resonance Assignments**—We performed CD experiments at different concentrations of DPC micelles (5–150 mM) for 50 \( \mu \)M papiliocin. At peptide/lipid ratios from 1:100 to 1:3000, CD spectra of peptides were similar, showing a similar degree of \( \alpha \)-helical structure. NMR experiments performed using 1 mM peptide in 100–300 mM DPC micelle indicated that the best...
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NMR spectra were obtained in 300 mM DPC micelles at 303 K. Therefore, 1 mM peptide in 300 mM DPC micelles was used for structural determination of papiliocin by NMR.

We performed sequence-specific resonance assignments using mainly DQF-COSY, TOCSY, and NOESY data (63), determining chemical shifts of papiliocin in 300 mM DPC micelles at 303 K, using 4,4-dimethyl-4-silapentane-1-sulfonate as a reference. Fig. 10 shows NOESY spectra with sequential assignments for papiliocin in the NH-NH region. Sequential NOE connectivities and other NMR data are illustrated in Fig. 11. A number of nonsequential NOE connectivities characteristic of an \( \alpha \)-helix were observed for papiliocin. Papiliocin had \( d_{\alpha N}(i, i + 3) \) and \( d_{\alpha N}(i, i + 4) \) connectivities and other NMR data are illustrated in Fig. 11. A number of nonsequential NOE connectivities characteristic of an \( \alpha \)-helix were observed for papiliocin. Papiliocin had \( d_{\alpha N}(i, i + 3) \) and \( d_{\alpha N}(i, i + 4) \) connectivities from Lys\(^3\) to Ala\(^{22}\) and from Ala\(^{25}\) to Lys\(^{37}\). These results suggest the presence of two helices extending in these regions. The \( ^1H \) chemical shift deviation, determined based on the method of Wishart et al. (64), showed a dense grouping of four or more \( \pm 1 \) chemical shift index values not interrupted by a \( +1 \) value, indicating the presence of an \( \alpha \)-helix in these regions. In the case of papiliocin, most of the residues have a negative \( ^1H_\alpha \) chemical shift index, implying that papiliocin has a stable \( \alpha \)-helix structure. Pro\(^{24}\) adopts only a trans configuration, as judged by the NOE between Gly\(^{23}\) C\(_\beta\)Hs-Pro\(^{22}\) C\(_\beta\)Hs, whereas a \( d_{\alpha N}(i, i + 1) \) NOE, which is characteristic of the cis isoform, was not observed.

Structure of Papiliocin—To calculate the tertiary structures of papiliocin, we used the following experimental restraints: sequential \((|i - j| = 1)\), medium range \((1(|i - j| \leq 5)\), long range \((|i - j| > 5)\), and intraresidual distance, hydrogen bonding restraints, and torsion angle restraints. Superimposition of the 20 lowest energy structures of papiliocin in 300 mM DPC micelles over the backbone atoms showed that the root mean square deviations from the mean structures for residues Lys\(^3\) to Lys\(^{21}\) were 0.24 \( \pm 0.13 \) Å for the backbone atoms (N, Ca, C\(^\prime\), and O) and 1.05 \( \pm 0.13 \) Å for all heavy atoms, respectively (Fig. 12A); for residues Ala\(^{23}\) to Val\(^{36}\), the corresponding values were 0.11 \( \pm 0.03 \) and 0.44 \( \pm 0.11 \) Å. Fig. 12B shows the average structure, and Fig. 12C shows a head-on view of the amphipathic helix of papiliocin from residue Lys\(^3\) to Lys\(^{21}\). According to a Procheck analysis, papiliocin has a flexible bent structure at Ala\(^{22}\)-Gly\(^{23}\)-Pro\(^{24}\) and amphipathic \( \alpha \)-helices from Lys\(^3\) to Lys\(^{21}\) and Ala\(^{25}\) to Val\(^{36}\). Greater than 99% of residues are in the allowed region in Ramachandran plots, as summarized in Table 5.

NMR Studies of Papiliocin Bound to LPS—To investigate the mode of papiliocin interaction with LPS, we performed STD-NMR experiments to identify residues close to the LPS aggregates. Fig. 13 shows the one-dimensional spectrum of free papiliocin and the STD spectrum of papiliocin bound to LPS in D\(_2\)O. Intermolecular saturation transfer between the peptide and the LPS micelles resulted in a difference in the intensity of resonances between LPS-bound papiliocin and free papiliocin. The largest STD effect was observed for the aromatic ring protons of Trp\(^2\) and Phe\(^5\), indicating their close contacts with LPS. Other STD effects that were not assigned were also evident in the aliphatic region. These data confirm that Trp\(^2\) and Phe\(^5\) in the N-terminal helix play important roles in interacting with the bacterial cell membrane as well as in interactions with LPS in infected cell membranes.

DISCUSSION

In this study, we verified that papiliocin is a potent peptide antibiotic with antibacterial activities as well as anti-inflammatory activities. We also determined the structure of papiliocin and investigated its mode of action. Papiliocin showed good antibacterial activity against standard bacterial strains. The therapeutic potential of peptide antibiotic drugs lies in the ability of the peptide to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. This
property is conveyed by the concept of the therapeutic index, which is the ratio of the minimally effective concentration against human cells (in this case hRBCs) to the minimally effective concentration against bacterial cells. These values are summarized in Table 2, which presents the minimal concentrations of peptide producing hemolysis of hRBCs (MHCs), and the minimal concentrations that are toxic to bacteria (MICs), averaged across all bacterial strains, to obtain the geometric means values that form the basis of the therapeutic index (MHC/geom- etric means). A high therapeutic index is thus an indication of two preferred characteristics of the peptide as follows: a high MHC (low hemolysis) and a low MIC (high antimicrobial activity). If we include only the MIC against Gram-negative bacteria, the therapeutic index of papiliocin is 340, which is much higher than the value 12 against Gram-positive bacteria. These data also showed that higher cationicity of N-terminal helix of pap-

TABLE 5

| Distance restraints     | 163 | 197 |
|-------------------------|-----|-----|
| Intraresidue (i-j=0)    |     |     |
| Sequential ([i]=[j]-1)  |     |     |
| Medium-range (2<|j|<4) | 177 |     |
| Long-range (5<|j|<10)  | 6   |     |
| Total                   | 543 |     |
| H-bond                  | 40  |     |
| Angular restraints (Φ)  | 30  |     |
| Mean Cyma target function (Å²) | 0.012 ± 0.0005 |

deviation from mean structure

| Residues 3–21 | 0.24 ± 0.13 | 0.11 ± 0.03 |
| Residues 25–36| 1.05 ± 0.13 | 0.44 ± 0.11 |

Ramachandran plot for the mean structure

| Residues in the most favorable and additionally allowed region (%) | 99.7 |
| Residues in the generously allowed region (%) | 0.3 |
| Residues in the disallowed region (%) | 0 |

FIGURE 12. A, superpositions of the 20 lowest energy structures calculated from the NMR data for papiliocin in 300 mM DPC micelles. The backbone atoms of residues Lys⁴ to Lys²¹ are superimposed. B, ribbon diagram of the average structure of papiliocin in 300 mM DPC micelles. The hydrophobic residues are indicated in red, and the hydrophilic residues are shown in blue. C, head-on view of the amphiphilic N-terminal helix from Lys³ to Lys²¹ of papiliocin.

It has been reported that the NaCl concentration in the environment of the epithelial cells of cystic fibrosis patients is 120 mM and that human β-defensin is inactivated in the high salt environment of the cystic fibrosis airway surface fluid (65). Moreover, interactions between cationic peptides and the LPS surface components of the outer membrane of Gram-negative bacteria are inhibited by high concentrations of divalent cations, such as calcium and magnesium (66). Salt resistance experiments showed that papiliocin retained its antibacterial activity against all Gram-negative bacteria at high salt concentrations and under serum conditions. Therefore, papiliocin is highly salt-resistant in a physiological environment. Because high ionic concentrations generally prevail in body fluids of patients suffering from inflammatory disease, papiliocin may be a potent antibiotic candidate for use in patients with cystic fibrosis or inflammatory disease.

Helical wheel diagrams of papiliocin, with the hydrophobic residues in the upper region and the hydrophilic residues in the lower region, are shown in Fig. 1. Papiliocin has a highly positively charged sequence. At physiological pH values, 12 of its 37 residues are charged, including six Lys residues, three Arg residues, and an amidated C-terminal, which collectively carry +10 charges, and one Glu and one Asp residue, which carry −2 charges, resulting in a net charge at physiological pH of +8. As shown in Fig. 1 and Table 1, the C-terminal helix from residues 25 to 37 is very hydrophobic, and the hydrophobic sector is much larger compared with the hydrophilic sector. In contrast, the N-terminal helix has a larger hydrophilic sector than hydrophobic sector. This is a common feature found in all cecropin family members. The tertiary structure of papiliocin determined by NMR spectroscopy revealed that papiliocin has an amphipathic α-helix from Lys³ to Lys²¹ linked by a hinge region to a hydrophobic α-helix from Ala²⁵ to Val³⁶. Fig. 12C shows a head-on view of the amphiphilic α-helix of papiliocin from Lys³ to Lys²¹. This amphipathicity of papiliocin is important because the hydrophilic sector faces toward the negatively charged head
groups of the lipid membrane, and the hydrophobic side chains, including the aromatic rings of Trp and Phe, have close contact with the acyl chains of the hydrophobic lipid. Papiliocin has a helix-hinge-helix structure in DPC micelles, which is very similar to those of other cecropins (26, 67, 68). This typical structural characteristic is likely important for the high antimicrobial and anti-inflammatory activity of papiliocin.

The structures of cecropins have been determined by NMR spectroscopy. Cecropin A and analogues of cecropin B (cecropin B1 and B1a) from H. cecropia have a helix-hinge-helix motif structure in a HFIP/water solution (24, 68, 69). Even though cecropin P1 does not have a bent structure and is composed of one straight helix in a HFIP/water solution, similar to other cecropins, cecropin P1 also has an amphipathic α-helix at the N terminus and hydrophobic α-helix at the C terminus (69). In this study, the tertiary structure of papiliocin, which is in the cecropin A family, determined in 300 mM DPC micelles was confirmed to adopt a helix-hinge-helix motif. The bent angle between the two helices of papiliocin is about 45–80°, indicating a flexibility that might be important for the antimicrobial and anti-inflammatory activities of papiliocin as well as its interaction with the membrane surface (26, 67, 68).

Most bacterial cell-selective AMPs bind strongly and permeate more efficiently into negatively charged phospholipid membranes, which mimic bacterial membranes, than into zwitterionic phospholipid membranes, which are the major components of the outer leaflet of human erythrocytes (57, 70). Non-cell-selective peptides bind and efficiently permeate both negatively charged and zwitterionic phospholipid membranes (57, 70). Recent experiments have suggested that the antimicrobial activity of cecropin A is related to changes in membrane permeability induced by the peptide (71–73). To address the antimicrobial mechanism of action of papiliocin, we compared its ability to damage mammalian and microbial mimetic membranes. Experiments employing a fluorescent dye entrapped within LUVs of varying phospholipid compositions showed that papiliocin, at its MIC, induced very strong leakage of dye from negatively charged bacterial cell membrane-mimetic phospholipid vesicles, but it promoted much lower leakage of the dye from neutral vesicles, which mimic mammalian cell membranes (Fig. 6). These results suggest that the bactericidal action of papiliocin is attributable to perturbation of the bacterial cell membrane.

Papiliocin showed high antibacterial activity against Gram-negative bacteria, as summarized in Table 2. The cell wall of Gram-negative bacteria is a relatively thin structure with distinct layers. The main component of the Gram-negative cell wall is LPS (endotoxin), which consists of a core region to which are attached repeating units of polysaccharide moieties. LPS acts as an extremely strong stimulator of macrophages, which are part of the innate immunity of diverse organisms (74, 75), and is linked to the pyrogenic effects of Gram-negative infections. LPS covers more than 90% of the outer leaflet of the bacterial cell surface and binds to surface TLR4 molecules, triggering the secretion of a variety of inflammatory products, including TNF-α, IL-1β, and NO, which contribute to the pathophysiology of septic shock and other immune diseases (30, 76).

A variety of biophysical techniques has recently been used to understand the interactions between AMPs and LPS and to explore the mechanisms of membrane perturbation and endotoxin neutralization (77, 78). Cathelicidin-derived AMPs with outstanding anti-inflammatory activity, such as LL-37, CAP18, SMAP-29, and fowlicidin-1, are also known to have high LPS-binding affinity and neutralize LPS, measured in quantitative chromogenic Limulus amoebocyte lysate assays (79). Recent transferred NOE, STD-NMR, and Carr-Purcell-Meiboom-Gill relaxation-dispersion experiments have determined the three-dimensional structure of pardaxin4 (Pa4), identified the amino acid residues responsible for interacting with LPS, and established the binding kinetics of the Pa4-LPS complex (78). The interaction of AMPs with LPS has also been examined using
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CD, isothermal titration calorimetry, infrared, dynamic light scattering, and $^{31}$P NMR techniques (77–79). $^{15}$N and $^{31}$P solid-state NMR spectroscopy have been used to investigate the orientation and structure-function relationships of LL-37, pardaxin, MSI-78, and MSI-594 in phospholipid bilayer membranes too (80–82).

To investigate the interactions between papiliocin and the phospholipid membrane, we monitored fluorescence emissions of tryptophan residues in papiliocin. Papiliocin exhibited considerably smaller blue shifts in zwitterionic vesicles than in negatively charged vesicles. This finding suggests that Trp$^2$ in papiliocin does not penetrate deeply into the zwitterionic phospholipid vesicles, which mimic mammalian membranes, resulting in high bacterial cell selectivity. Fluorescence quenching data indicated that Trp$^2$ of papiliocin is deeply buried in negatively charged membranes but is largely exposed in neutral membranes. In this study, we also investigated interactions between papiliocin and LPS using fluorescence and NMR spectroscopy, demonstrating that papiliocin dequenched the fluorescence intensity of FITC-labeled LPS and implying that papiliocin dissociated the aggregated form of LPS. Tryptophan fluorescence blue shift data also showed that papiliocin has a much larger blue shift in LPS than in DPC micelles. Furthermore, papiliocin in LPS showed a much lower Stern-Volmer quenching constant compared with that for papiliocin in neutral micelles, implying that papiliocin interacts tightly with negatively charged LPS. STD-NMR experiments showed that Trp$^2$ and Phe$^5$ are in close contact with LPS. Collectively, these data imply that Trp$^2$ and Phe$^5$ in the N-terminal helix play important roles in interacting with the bacterial cell membrane as well as with the LPS, which activates the immune system. All these results implied that strong interaction between papiliocin and LPS may lead to a plausible disruption of LPS structures, and this may be the reason for the susceptibility of Gram-negative bacteria to papiliocin as well as its high anti-inflammatory activity.

The tryptophan residues in melittin, IsCT, and mastoparan B have been reported to be critical for their antibacterial and/or hemolytic activities (57–59). In the AMPs, the tryptophan residues are located in the hydrophobic core of the peptides, exhibit restricted motion in the membrane, and are involved in hydrophobic interactions with the acyl chains of phospholipid (57–59). In the case of papiliocin, which does not show cytotoxicity, the tryptophan residue is located at position 2 in the N terminus, a rather flexible region. Therefore, the anchoring of Trp$^2$ and Phe$^5$ into the membrane, as well as the electrostatic interactions between the positively charged Lys or Arg residues in the amphipathic helix at the N terminus and anionic phospholipid head groups, primarily mediate binding to the bacterial cell membrane. Thereafter, the flexibility or bending potential induced by the hinge region in the central part of papiliocin may allow the hydrophobic α-helix of the C terminus to insert deeply into the lipid bilayer.

LL-37 is the only member of the cathelicidin family of host defense peptides known to be expressed in humans (51, 83, 84). The mechanism of action of LL-37 has been studied extensively by Hancock and co-workers (85, 86), who showed that LL-37 modulates a number of pathways of LPS-stimulated gene expression and cytokine production. LL-37 is known to suppress the nuclear translocation of NF-κB, ultimately modulating the expression of genes regulated by this transcription factor. Alternatively, it has been suggested that LL-37 regulates gene expression entirely at the transcriptional level by suppressing several inflammatory genes, including p50 and TNFAIP2 (tumor necrosis factor α-induced protein), thereby reducing the expression of the proinflammatory cytokine, TNF-α. LL-37 alone also modulates MAPK pathways, which are also associated with inflammatory responses, and can associate with LPS, inhibiting binding to LPS-binding protein, MD2(Lymphocyte antigen 96), or subunits of TLR4 associations that ultimately suppress downstream pathways (83). It has been reported that cecropin B enhances the activity of β-lactams in Gram-negative septic shock rat models (62). However, neither the anti-inflammatory activities of cecropin-like peptides nor their underlying mechanism of action have been studied. Here, we provide the first investigation of the anti-inflammatory activity and associated mechanism of a cecropin-like peptide, papiliocin. Papiliocin significantly inhibited NO production and dramatically decreased TNF-α and MIP-2 secretion by LPS-stimulated mouse macrophage-derived RAW264.7 cells. Notably, the activity of papiliocin was comparable with that of the positive control, LL-37. Papiliocin also inhibited LPS-induced production of NO and inflammatory cytokine (IL1-β, TNF-α, MIP1 and -2, and IL-6) mRNA in LPS-stimulated RAW264.7 cells. Similar to the inflammatory cascade of the cathelicidin LL-37 peptide, we showed that papiliocin triggers the suppression of LPS-induced TLR4 and NF-κB expression, implying that papiliocin is related to inflammatory responses through effects on the TLR4/NF-κB pathway. Specifically, these data suggest an inflammatory cascade of papiliocin in which LPS stimulates TLR4 receptors, which, once activated, then recruit several downstream signaling molecules.

In conclusion, papiliocin has high antibacterial and anti-inflammatory activities without exhibiting cytotoxicity against mammalian cells. These properties make papiliocin a potent peptide antibiotic suitable for treating the endotoxin shock and sepsis caused by Gram-negative bacterial infections. Structure-function relationships responsible for antibacterial and anti-inflammatory activities will be investigated further based on the solution structure of papiliocin, and the mechanism underlying the anti-inflammatory activity of papiliocin will be investigated in detail.

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