Consequences of Alteration in Leucine Zipper Sequence of Melittin in Its Neutralization of Lipopolysaccharide-induced Proinflammatory Response in Macrophage Cells and Interaction with Lipopolysaccharide

Raghvendra M. Srivastava, Saurabh Srivastava, Manish Singh, Virendra Kumar Bajpai, and Jimut Kanti Ghosh

From the Molecular and Structural Biology Division, Electron Microscopy Unit, Sophisticated Analytical Instrument Facility, Central Drug Research Institute, Council of Scientific and Industrial Research, Chattar Manzil Palace, P. O. Box 173, Lucknow 226001, India

Background: Bee venom antimicrobial peptide, melittin, neutralizes LPS-induced proinflammatory response in macrophage cells.

Results: Alteration in the leucine zipper sequence of melittin impaired its anti-LPS property and interaction with LPS.

Conclusion: The leucine zipper sequence of melittin plays a crucial role in maintaining its antiendotoxin properties.

Significances: The results suggest an overlap of structural requirements for the cytotoxic and antiendotoxin properties of melittin.

The bee venom antimicrobial peptide, melittin, besides showing versatile activity against microorganisms also neutralizes lipopolysaccharide (LPS)-induced proinflammatory responses in macrophage cells. However, how the amino acid sequence of melittin contributes in its anti-inflammatory properties is mostly unknown. To determine the importance of the leucine zipper sequence of melittin in its neutralization of LPS-induced inflammatory responses in macrophages and interaction with LPS, anti-inflammatory properties of melittin and its three analogues and their interactions with LPS were studied in detail. Two of these analogues, namely melittin Mut-1 (MM-1) and melittin Mut-2 (MM-2), possess leucine to alanine substitutions in the single and double heptadic leucine residue(s) of melittin, respectively, whereas the third analogue is a scrambled peptide (Mel-SCR) that contains the amino acid composition of melittin with minor rearrangement in its leucine zipper sequence. Although MM-1 partly inhibited the production of proinflammatory cytokines in RAW 264.7 and rat primary macrophage cells in the presence of LPS, MM-2 and Mel-SCR were negligibly active. A progressive decrease in interaction of melittin with LPS, aggregation in LPS, and dissociation of LPS aggregates with alteration in the leucine zipper sequence of melittin was observed. Furthermore, with alteration in the leucine zipper sequence of melittin, these analogues failed to exhibit cellular responses associated with neutralization of LPS-induced inflammatory responses in macrophage cells by melittin. The data indicated a probable important role of the leucine zipper sequence of melittin in neutralizing LPS-induced proinflammatory responses in macrophage cells as well as in its interaction with LPS.

Sepsis or septicemia is a systemic inflammatory response syndrome induced by bacterial remains like lipoteichoic acids, CpG nucleotides, and predominantly by lipopolysaccharide in the course of bacterial infections. Elderly, immunocompromised, and critically ill patients are more vulnerable to microbial infection and thus sepsis. It is a major reason of mortality in ICU hospitalized patients affecting ≈750,000 persons yearly throughout the world (1, 2). The lipid part of LPS, known as lipid A, is an acylated molecule and plays a central role in LPS-induced inflammatory responses (3, 4) in host cells. The over-stimulation of defense cells (monocytes, macrophages, and polymorphonuclear leukocytes) by LPS invokes unregulated cytokine secretions such as TNF-α, interleukins, and prostaglandins, which establish the confined inflammation (5). This local inflammation if unrestricted can lead to sepsis or septic shock (6, 7).

The aggregated state of LPS helps serum proteins like LBP, sCD14, and sMD2 to recognize and transfer it to cell membrane receptors, like mCD14 and TLR4-MD2 complexes, from where it stimulates signaling cascade leading to proinflammatory responses by immune cells, primarily by macrophages (3, 4, 5, 8, 9). Various therapeutic approaches are being discovered and investigated for restricting the detrimental effects of microbial invasions and its consequences. The antibacterial molecules like naturally occurring antimicrobial peptides, their analogues, and designed novel antimicrobial peptides (6–9) that can neu-
neutralize LPS-induced inflammatory response in immune cells are considered prospective alternatives to conventional antibiotics.

Melittin, a 26-residue amphipathic, α-helical and cationic peptide from venom of the honey bee (Apis mellifera) is one of the well studied molecules of its class. The broad spectrum antibacterial activity along with potent immunomodulatory functions make it a suitable candidate for development of novel antimicrobial or immunomodulatory agents as well as to study its mechanism of action. Melittin is reported to play a pivotal role in anti-inflammatory activities of bee venom (10); for example, neutralization of LPS-induced inflammatory responses in macrophages as well as in synovocytes by melittin is well known (10). Although there are reports on interactions of melittin with lipid A of LPS (11), as well as with potential intracellular targets like p50 and IκB kinase-α as a plausible basis of the immunomodulatory property of melittin (10, 12), very little is known about the amino acid sequences of the peptide that contribute to detoxification of LPS by melittin. Our earlier report (13) demonstrated the role of the leucine zipper motif in maintaining the hemolytic activity of melittin, which was later found to be applicable for the toxicity of several other naturally occurring peptides also (14–16). Encouraged by the previous observations we considered that it would be interesting to explore the importance of this motif in melittin in neutralizing the LPS-induced inflammatory response in macrophage cells along with understanding the underlying mechanism of action. For this purpose, the previously designed analogues (13), viz. melittin Mut-1 (MM-1) and melittin Mut-2 (MM-2), were employed. A recently designed (17) scrambled analogue (Mel-SCR) of melittin was also employed to dissect the role of the leucine zipper sequence from the role of the mean hydrophobicity of melittin in its immunomodulatory functions. We observed a progressive decrease in the activity of melittin analogues with leucine to alanine residue(s) substitutions in the leucine zipper sequence with respect to their interaction with LPS, peptide-induced disaggregation of LPS aggregates, decline in LPS-induced cytokine production, and other cellular responses in macrophage cells. Murine macrophage cells, RAW 264.7, as well as rat primary macrophage cells were utilized to examine the LPS-induced cytokine and cellular responses in the presence of melittin and its analogues.

EXPERIMENTAL PROCEDURES

Materials

Rink amide MBHA resin (loading capacity, 0.63 mmol/g) and all N-α-Fmoc- and side chain-protected amino acids were purchased from Novabiochem. Coupling reagents for peptide synthesis, 1-hydroxybenzotriazole, N,N′-diisopropylcarbodiimide, 1,1,3,3-tetramethyluronium tetrafluoroborate, and N,N′-diisopropylethylamine, were purchased from Sigma, whereas N,N′-dimethylformamide, and piperidine were of standard grades and procured from reputed local commercial sources. Acetonitrile (HPLC grade) was from Merck, whereas trifluoroacetic acid (TFA) was purchased from Sigma. TAMRA was purchased from Invitrogen.

Escherichia coli 0111:B4 lipopolysaccharide (L3012), FITC-LPS E. coli 0111:B4 (F3665), and sodium nitroprusside (228710) were from Sigma, whereas p-phenylenediamine (151830) and paraformaldehyde (150146) were from MP Biomedicals. Components of Griess reagents, viz. sulfanilamide (S92510) and p-naphthylethenediamine dihydrochloride (N9125), were purchased from Sigma. 2′,7′-Dichlorodihydrofluorescein diacetate (5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate diethyl ester) (D-399) and DAF-FM diacetate (4-amino-5-methylamino-2,7-difluorofluorescein diacetate) (D23844) were purchased from Molecular Probes/Invitrogen. DAPI (157574), a-phenylenediamine dihydrochloride (P0029–50G), Ponceau S (81460), and BCP/NBT(203790) were from Fluka and Calbiochem, respectively. The remaining reagents were of analytical grade and procured locally; buffers were prepared in Milli-Q (USF-ELGA) water.

Antibodies

For protein expression we used immunoblotting for mouse anti-iNOS2 (610328, BD Transduction), rabbit anti-TNFα polyclonal (654300, Calbiochem), IL-1β rabbit polyclonal (sc-7884), COX-2 mouse monoclonal (sc-19999), anti-phospho-IκBα mouse monoclonal (OP-142, Calbiochem), and NFκB p65 rabbit polyclonal (sc-372, Santa Cruz Biotechnology). For MAPK phosphorylation experiments mouse anti-JNK-SAPK-612540 (phospho-Thr183/phospho-Tyr185), mouse anti-p38 MAPK-612280 (phospho-Thr180/phospho-Tyr182), and phospho-ERK1/2 (phospho-Thr202/phospho-Tyr204) were purchased from BD Transduction Laboratories. Mouse anti-β-actin monoclonal (CP01) was procured from Calbiochem. For immunoblotting and ELISA experiments, alkaline phosphatase-conjugated anti-mouse (401212), anti-rabbit (401352), and peroxidase-conjugated anti-mouse (401215) and anti-rabbit (401315) were from Calbiochem. Fluorescent-labeled antibodies anti-mouse Alexa Fluor 488 (A11001) and anti-rabbit Alex Fluor 488 (A11008) were purchased from Molecular Probes and Invitrogen. For MAPK phosphorylation experiments mouse anti-JNK-SAPK-612540 (phospho-Thr183/phospho-Tyr185), mouse anti-p38 MAPK-612280 (phospho-Thr180/phospho-Tyr182) and phospho-ERK1/2 (phospho-Thr202/phospho-Tyr204) were purchased from BD Transduction; alkaline phosphatase-conjugated anti-mouse (401215) and anti-rabbit (401315) were from Calbiochem. Fluorescent-labeled antibodies anti-mouse Alexa Fluor 488 (A11001) and anti-rabbit Alexa Fluor 488 (A11008) were purchased from Molecular Probes and Invitrogen. For MAPK phosphorylation experiments mouse anti-JNK-SAPK-612540 (phospho-Thr183/phospho-Tyr185), mouse anti-p38 MAPK-612280 (phospho-Thr180/phospho-Tyr182) and phospho-ERK1/2 (phospho-Thr202/phospho-Tyr204) were purchased from BD Transduction; alkaline phosphatase-conjugated anti-mouse (401212) and anti-rabbit (401352) were from Calbiochem.

Cell Culture

Raw 264.7, a murine macrophage-like cell line, was obtained from the National Center for Cell Sciences, Pune, India. Iscove’s modified Dulbecco’s medium (MED 216) and fetal bovine serum (EU-000-F) were purchased from Sera Laboratories, West Sussex, United Kingdom. The Invitrogen × 100 antibiotic-antimycotic (15240) was used. Rat primary bone marrow macrophage cells were obtained by inducing differentiation in bone marrow cells isolated from femurs of healthy rats aged ~4 weeks. The differentiation was induced by previously known
methods using the cell supernatant of L929 murine fibroblast cells (18, 19). The culture and processing of these differentiated cells were performed as RAW 264.7 macrophages. Sterile polystyrene tissue culture flasks (690175), 96-well plates (655180), and 24-well plates (662–160) were procured from Greiner Bio-one, whereas 6-well plates (3506) were from Corning. Trypsin EDTA (25200) was purchased from Invitrogen. The cell line was maintained in an Innova CO₂ incubator.

Peptide Synthesis, Fluorescent Labeling, and Purification

Stepwise solid phase syntheses of all the peptides were conducted manually on rink amide MBHA resin (0.15 mmol) utilizing standard Fmoc chemistry (20, 21) and reported previously (17, 23). Labeling at the N terminus of peptides with a fluorescent probe, cleavage of the labeled and unlabeled peptides from the resin, and their precipitation and purification by reverse phase HPLC were achieved by standard procedures (17, 23). The purified peptides were ~95% homogeneous. Experimental molecular masses of the peptides, detected by ESI/MS analysis, corresponded closely with the desired values.

Assay for NO Neutralizing Activity

RAW 264.7 cells were plated at 5 × 10⁵ cells/well in 24-well plates and then incubated with LPS (1 μg/ml) in the presence of 10 μg/ml of melittin wild type (Mel), melittin Mut-1 (MM-1), melittin Mut-2 (MM-2), and melittin scrambled (Mel-SCR). The cells with and without LPS addition were taken as positive and negative controls for LPS-induced and basal levels of nitric oxide production, respectively. Nitric oxide production was measured using Griess reagent. 100 μl of Griess reagent was added with 100 μl of culture supernatant to observe nitrate accumulation after the respective treatments (22). Absorbance was then measured at 548 nm using a 96-well microtiter plate reader from Quant Bio-Tek Instruments, VT. Fresh culture media was used as media-control for all experiments. For the determination of NO production with peptide and LPS treatments, the assay was done in triplicate and the average values were considered for each set.

1 × 10⁵ RAW264.7 cells were seeded in flat bottom 96-well plates and treated with 10 μg/ml of melittin, MM-1, MM-2, and Mel-SCR for 6, 12, 18, 24, 30, and 36 h for time-dependent NO production upon LPS stimulation (1 μg/ml). Nitric oxide inhibitions by melittin and its mutants were also investigated at different concentrations by treating cells with 1 μg/ml of LPS along with 1.5, 3, 5, 7.5, and 10 μg/ml of peptides for 24 h incubations. The inhibitory effect of melittin and its analogues on LPS-induced intracellular nitric oxide accumulation was studied with confocal laser scanning microscopy after a 12-h treatment of 1 μg/ml of LPS with cells (5 × 10⁵) treated with 10 μM DAF-FM-acetate (23, 24) 1 h prior to addition of LPS and peptides (10 μg/ml). After treatment, the cells were fixed with 4% para-formaldehyde and mounted on glass slides using 90% glycerol containing 1 mg/ml of p-phenylenediamine.

In another experiment, to check the effect of melittin and its analogues on LPS-pretreated RAW 264.7 macrophage cells, we treated cells with LPS (1 μg/ml) for 1 h and 30 min prior to peptide treatments for 24 h. Nitric acid production of the cells was measured by recording the absorbance at 548 nm of the culture supernatant after employing the Griess reagent as described above (22).

Measurement of Inhibition of LPS-induced Oxidation Stress

Approximately 2 × 10⁵ RAW 264.7 cells in 6-well culture plates were stimulated with 1 μg/ml of LPS with and without melittin wild type and its analogues for 1 h and during the last 15 min of incubation 10 μM 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein-diaceitate acetyl ester (Molecular Probes) was added (25, 26). Cells were extensively washed and re-suspended in 4 ml of PBS, pH 7.4. A 400-μl aliquot from each sample was used for analysis of signals produced by accumulated 2′,7′-dichlorodihydrofluorescein with excitation and emission wavelengths of 488 and 525 nm, respectively, in a Fluorescence Spectrometer (PerkinElmer Life Science) (27, 55).

Immunoblotting Experiments for iNOS2 and TNF-α

RAW 264.7 macrophage cells (approximately 2 × 10⁵) were stimulated with 1 μg/ml of LPS in the presence of melittin and its analogues (10 μg/ml) in 24-well plates for 24 h. LPS-treated and untreated cells were taken as positive and negative controls, respectively, representing the stimulated and unstimulated levels of protein expressions. For immunoblotting, cells were harvested, washed with ice-cold PBS, pH 7.4, and lysed in Laemmli sample buffer (100 mmTris, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromphenol blue, 20% glycerol, and 200 mm Na-mercaptoethanol). Lysates were resolved on a SDS-PAGE 8% gel for iNOS2 and 12% for TNFα and transferred to the nitrocellulose membrane (Immobilins, Millipore). After blocking with 3% BSA for 3 h at room temperature, the membrane was incubated with primary Abs for 2 h, washed 3 times with TBST (10 mm Tris, pH 8.0, and 150 mm NaCl solution containing 0.05% Tween 20), and incubated with the appropriate alkaline phosphatase-conjugated secondary Ab. Signals were developed afterward with the substrate NBT/5-bromo-4-chloro-3-indolyl phosphate (Calbiochem). β-Actin served as loading control for Western blot experiments.

Measurement of Cytokine Expression Levels in Supernatant

Enzyme-linked immunosorbent assays were performed to estimate the secreted TNF-α and IL-1β in LPS-treated cells in the presence of melittin and its analogues after a 12-h incubation. Levels of these cytokines in culture supernatant of untreated and LPS-treated cells were taken as minimum and maximum to calculate the percentage of inhibition by melittin and its analogues (28). The dilution of primary Abs was 1:300 for both TNF-α and IL-1β, whereas compatible horseradish peroxidase-conjugated secondary Abs were diluted 1:1000 with 1% BSA. 3% BSA was used for blocking. A 1 mg/ml of o-phenylenediamine dihydrochloride solution with 0.5 μl/ml of 30% hydrogen peroxide in citrate buffer, pH 5.0, was used as substrate for calorimetric analysis of horseradish peroxidase activity. The reaction was stopped by 0.2 N H₂SO₄. Final readings were taken at 492 nm in a BIOTEK microtiter plate reader.

Cytometric Bead Array Experiment

The generalized effect of melittin wild type and its analogues on proinflammatory secretions induced by LPS was studied...
with the Mouse Inflammation Kit (Cytometric Bead Array kit, BD Biosciences number 552364) and CBA analysis was performed on a BD FACSCaliber flow cytometer (BD Biosciences). The cells were treated as stated earlier, the supernatant was collected, diluted, and the samples were prepared per the manufacturer’s protocol. The results presented resemble a pattern given elsewhere (29, 30).

**MAPK Phosphorylation Experiments**

We investigated the phosphorylation states of component proteins of MAPK pathways in LPS-stimulated cells treated simultaneously with peptides for 1 h. Cells treated with LPS and with no treatments served as positive and negative controls, respectively, for this experiment. The cell lysate was prepared as described and proteins were immunoblotted with their respective antibodies for the detection of phosphorylated proteins. The normal, non-phosphorylated proteins were also probed in the same samples by the respective antibodies and served as controls for the experiment. We used mouse anti-JNK-SAPK-612540 (phospho-Thr183/phospho-Tyr185), mouse anti-p38 MAPK-612280 (phospho-Thr180/phospho-Tyr182), and phospho-ERK1/2 (phospho-Thr202/phospho-Tyr204) purchased from BD Transduction Laboratories. Alkaline phosphatase-conjugated anti-mouse (401212) and anti-rabbit (401352) were from Calbiochem (31).

**Detection of NFkB Activation**

**Immunoblotting Experiment**—The effect of melittin as well as its mutants on LPS-induced activation of NFkB was investigated through immunoblotting and immunofluorescence experiments. For immunoblotting, 2 × 10⁶ cells were seeded in a 6-well plate for adherence. In freshly supplemented medium, peptides (10 μg/ml) and LPS (1 μg/ml) were added, the LPS-treated and untreated cells were assumed as positive and negative controls for translocation. Following the treatments, cells were washed twice in ice-chilled PBS, pH 7.4. The cells were incubated in 0.4 ml of ice-cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF) with freshly added protease inhibitor mixture (Sigma, S8820) for 15 min, then 12.5 ml of 10% Nonidet P-40 was added and the contents were vortexed and then centrifuged for 1 min (14,000 × g) at 4 °C. The supernatant was saved as cytosolic lysate and stored at −80 °C. The nuclear pellet was resuspended in 50 ml of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) with freshly added protease inhibitor mixture for 30 min with intermittent mixing. The tubes were centrifuged for 5 min (14,000 × g) at 4 °C, and the supernatant (nuclear extract) was stored at −80 °C (32). The nuclear lysate Ponceau S (33) and the cytosolic lysate β-actin was used as loading controls.

**Immunofluorescence Microscopy**—RAW 264.7 macrophage cells were seeded on sterile coverslips for proper adherence prior to LPS and peptide treatments. Afterward the cells were washed twice with sterile PBS, pH 7.4, and supplemented with fresh media. Cells were then treated with LPS and the peptides and incubated for 4–5 h for NFkB activation. The untreated and LPS-treated cells were considered as negative and positive controls for the experiment. Cells were washed with chilled PBS and later fixed with 4% paraformaldehyde for 15 min at 4 °C, the formaldehyde was quenched in 0.1 M glycine in PBS for 10 min. Subsequently, cells were incubated for 5 min in 0.1% Triton X-100 for permeabilization. Following permeabilization cells were washed in PBS containing 1% bovine serum albumin (BSA) and labeled with antibody against p65 subunit of NFkB (1:200) for 1 h, then extensively washed with PBS containing 1% BSA, and the primary antibodies were detected by anti-rabbit Alexa Fluor 488. The nucleus was stained by DAPI. 90% Glyceraldehyde 3-phosphate dehydrogenase was used as mounting media. Detection of fluorochromes was carried out using a Zeiss LSM-510 META confocal laser scanning microscope with a ×63 1.4 NA (oil) Plan apochromate lens.

**Circular Dichroism (CD) Spectroscopy**

The CD spectra of the wild type melittin and its analogues in the presence of LPS were measured with a Jasco J-500A spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. The spectra were scanned at 30 °C in a capped, quartz optical cell with a 0.2-cm path length. Spectra were obtained at wavelengths of 250–190 nm. Mean residue ellipticities were expressed as [θ] (degrees of square centimeters per decimole). The peptides (20 μM) were scanned in the presence or absence of LPS (25 μM) dissolved in PBS (150 mM NaCl) (34). The fractional helicities were calculated with the help of mean residue ellipticity values at 222 nm by Equation 1 (35, 36).

\[
F_h = -\frac{[\theta]_{222} - [\theta]_0}{[\theta]_{100} - [\theta]_0} \quad (\text{Eq. 1})
\]

Where [θ]₂₂₂ was the experimentally observed mean residue ellipticity at 222 nm. The values for [θ]₁₀₀ and [θ]₀ that correspond to 100 and 0% helix contents were considered to have mean residue ellipticity values of −32,000 and −2,000, respectively, at 222 nm.

**Endotoxin Neutralization Assay (LAL Assay)**

The ability of melittin and its designed analogues to bind the LPS was assessed using a quantitative chromogenic limulus amoebocyte lysate (LAL) using the QCL-1000 (LONZA) kit. Endotoxin neutralization experiments were carried out following the protocols recommended by the manufacturer. Stock solutions of peptides were prepared in the pyrogen-free water provided with the kit. Peptides concentrations of 7.5, 15, and 25 μM were incubated with 1 endotoxin unit of LPS in a flat bottom nonpyrogenic 96-well tissue culture plate at 37 °C for 30 min to allow peptide binding to LPS. A total of 50 μl of this mixture was then added to an equal volume of LAL reagent (50 μl), and the mixture was incubated for 10 min followed by the addition of 100 μl of LAL chromogenic substrate (Ac-Ile-Ala-Arg-p-nitroaniline). The reaction was terminated by the addition of 25% acetic acid, and the yellow color that developed due to cleavage of the substrate was measured spectrophotometrically at 410 nm. The reduction of absorbance at 410 nm as a function of peptide concentrations is directly proportional to the inhibition of LPS by the peptide. All assays were repeated twice, and average values are reported (37, 38).
Antidendotoxin Property of Melittin

Effect of Peptides on LPS Binding to Macrophages—FITC-labeled LPS was incubated with melittin and its analogues in an equimolar ratio for 1 h at 4 °C, afterward \( \sim 10^5 \) macrophage cells were treated with the LPS/peptide mixture for 30 min at room temperature with gentle shaking. The cells were washed with ice-chilled PBS, pH 7.4, extensively to remove the unbound LPS from the samples. Binding, localization, and penetration of FITC-LPS in the presence of melittin or its analogues was monitored by measuring the mean fluorescence of 10,000 cells for each sample; the untreated cells served as autofluorescence in log FL1 height in a BD Biosciences FACS Caliber flow cytometer.

For another study regarding the LPS-peptide interaction, FITC-LPS and rhodamine-labeled peptides were incubated in an equimolar ratio for 30 min at room temperature with gentle shaking. The LPS/peptide mixture was added on \( \sim 10^5 \) cells suspended in Iscove’s modified Dulbecco’s complete media containing 5% FBS and incubated for 15 min with gentle shaking in 1.5-ml centrifuge tubes. Later the samples were washed extensively with chilled PBS to remove excess fluorophores and without fixation, cells were mounted on slides of 90% glycerol with p-phenylenediamine as the autofading agent and images of the cells were recorded by confocal laser scanning microscopy as described.

Effect of Melittin and Its Analogues on Aggregated Form of FITC-LPS

The assay was carried out as described previously (39). LPS FITC (0.5 μg/ml) was treated with increasing concentrations of peptides from 0 to 48 μM. The changes in the emission of FITC (515 nm) in 10 mM sodium phosphate buffer, pH 6.9, were monitored using a fluorescence spectrometer (PerkinElmer LS55) with excitation wavelength set at 488 nm and excitation and emission slits of 8 and 6 nm, respectively. The emissions of both sodium phosphate buffer and peptides alone were taken for background subtractions. Dissociation of the aggregates results in an increase in the fluorescence of FITC due to dequenching (40).

RESULTS

Melittin Analogues—We previously identified and characterized a leucine zipper motif in melittin (13). The role of this sequence element in melittin was addressed by designing two of its novel analogues; in one a leucine residue at position 13 (MM-1) was replaced with an alanine residue and in the other (MM-2) two leucine residues at positions 6 and 13 were replaced with two alanine residues (13). These two analogues have been tested in the present investigation for their ability to neutralize the LPS-induced immune response in murine macrophage cells, RAW 264.7, and rat primary macrophage cells. Furthermore, another novel analogue of melittin has been designed (17) recently that possesses the same amino acid composition of melittin; only the positions of the hydrophobic amino acids are interchanged with each other to disturb its leucine zipper sequence. This scrambled melittin analogue (Mel-SCR) has the same mean hydrophobicity as its parent molecule; also the positions of the cationic and other polar residues in this as well as in MM-1 and MM-2 are identical to melittin. Fig. 1, A–C, describes the sequences of the peptides employed in this study, their helical wheels, and physicochemical properties, respectively.

Substitution of Leucine Residues at Heptadic Positions Progressive Reduced the Inhibitory Activity of Melittin for LPS-induced Nitric Oxide Production in RAW 264.7 Macrophage Cells—To examine the effects of substitutions of leucine by alanine residue(s) at the heptadic positions of melittin, we stimulated the macrophage cells (RAW 264.7) with LPS with simultaneous addition of either melittin or its analogues. LPS-stimulated RAW 264.7 cells showed significant production of nitric oxide as compared with the control, nonstimulated cells (Fig. 2A). However, in the presence of melittin the level of LPS-induced nitric oxide in these cells was almost comparable with that of the non-LPS-stimulated cells (Fig. 2, A and B). The result indicated the neutralization (~95%) of the LPS-induced NO response in macrophage cells by melittin as reported by others also (31). Although, the single and double alanine-substituted analogues (MM-1 and MM-2) showed ~35 and ~5% neutralization of LPS-induced nitric oxide production in RAW 264.7 cells, respectively (Fig. 2B). Interestingly, Mel-SCR showed an insignificant effect on LPS-induced NO production and proved to be negligibly active like MM-2 in neutralizing the NO response in LPS-stimulated macrophage cells (Fig. 2, A and B). The dose-response activity of the peptides at their various concentrations toward neutralization of nitric oxide in LPS-stimulated cells is shown in Fig. 2C.

DAF-FM diacetate is a cell-permeant probe that passively diffuses across cellular membranes. Once inside the cells, it is deacetylated by intracellular esterases to become DAF-FM. The fluorescence quantum yield of DAF-FM increases after reacting with nitric oxide and this helps to observe the accumulation of intracellular NO. Melittin showed nearly absolute abrogation in the level of intracellular nitric oxide accumulation. However, MM-1, MM-2, and Mel-SCR exhibited the pattern of weak inhibition of intracellular NO accumulation that was similar to that observed for LPS-induced NO production in culture supernatants in the presence of these melittin analogues (supplemental Fig. S1).

The results suggested the partial efficacy of MM-1 and the almost negligible effect of MM-2 and Mel-SCR toward the LPS-induced NO production in RAW264.7 macrophage cells. Thus, NO estimation assays (Fig. 2) evidently showed that the substitution of leucine residues by alanine residues in the leucine zipper sequence of melittin abrogated the ability of mellitin to neutralize LPS-induced nitric oxide production in RAW 264.7 cells. Furthermore, the scrambled melittin analogue with the impaired leucine zipper sequence, but having the same amino acid composition as melittin, negligibly neutralized the nitric oxide response in the same cells.

Melittin Analogues Exhibited Declined Attenuation for Expressions of iNOS and TNF-α in LPS-stimulated Cells—Melittin significantly reduces LPS-induced overexpression of stress-associated proteins and cytokines (31). The effect of leucine-substituted melittin analogues in suppressing LPS-induced cytokine expressions was studied by immunoblotting experiments. It was observed that MM-1 partly inhibited the
expression levels of these proteins, whereas MM-2 and Mel-SCR failed to suppress their expression significantly.

iNOS (inducible nitric-oxide synthase or NOS2) mediates the production of nitric oxide from L-arginine. Expression of iNOS or NOS2 is enhanced after activation by endotoxins or cytokines and generates copious amounts of NO presumably to help kill or inhibit the growth of invading microorganisms or neoplastic tissue (41, 42). Melittin was reported (43) to inhibit LPS-induced iNOS up-regulation, which we observed in the present experiment also (Fig. 3A). In contrast to melittin, a sharp decline for MM-1 in inhibiting the expression level of iNOS in LPS-stimulated RAW 264.7 cells was observed (Fig. 3A). Both MM-2 and Mel-SCR were negligibly active in inhibiting the iNOS expression level in LPS-stimulated RAW 264.7 cells (Fig. 3A). The effects of melittin and its analogues on the intracellular state of the LPS-induced proinflammatory mediator TNFα, which is markedly enhanced by LPS treatment, was examined (Fig. 3B). Again a remarkable decrease in the activity of these melittin analogues in suppressing the expression of this proinflammatory protein was observed as compared with the parent molecule (Fig. 3B).Taken together the substitution of leucine residues in the heptadic positions of the leucine zipper sequence impaired the ability of melittin to suppress the expression of these proinflammatory proteins in LPS-stimulated RAW 264.7 cells.

Differences in the Activities of Melittin and Its Analogues toward Secretion of Proinflammatory Mediators—The extracellular accumulations of proinflammatory cytokines were measured in LPS-stimulated RAW 264.7 cells following treatment with melittin and its analogues. Results obtained using ELISA for TNF-α (Fig. 4A) and interleukin-1β (Fig. 4B) suggested accumulations of these cytokines as a result of replacement of melittin by its analogues. Furthermore, the data were in agreement to the intracellular levels of cytokines as studied by immunoblotting (Fig. 3B) experiments. Along with ELISA, anti-inflammatory properties of melittin and its analogues against LPS in RAW 264.7 cells were determined by measuring proinflammatory cytokines with the help of...
Antiendotoxin Property of Melittin

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIGURE 2. Effect of melittin and its analogues on the production of NO in LPS-stimulated RAW 264.7 cells.** Panel A, the amount of nitric oxide produced in LPS-stimulated RAW 264.7 cells, ctrl and LPS represent untreated control and LPS (1 μg/ml)-treated cells, respectively, whereas Mel, MM-1, MM-2, and Mel-SCR represent the amount of NO produced by LPS-treated cells in the presence of these peptides (10 μg/ml). The micromolar (μM) values of nitric oxide were determined using a standard sodium nitrite curve. Panel B, percentage inhibition of NO production in LPS-stimulated cells in the presence of different peptides. Panel C, nitric oxide produced in LPS-stimulated cells in the presence of increasing concentrations of melittin and its analogues.

of a CBA kit. The results clearly indicate the difference between melittin and its analogues with respect to their anti-inflammatory properties (Fig. 4C and supplemental Fig. S2). As observed in the case of NO neutralization, MM-1 was ~30% active to melittin but MM-2 and Mel-SCR peptides were significantly inactive in neutralizing TNF-α, IL-6, and MCP-1 in RAW 264.7 cells.

**State of LPS-stimulated Oxidative Stress with Treatment of Melittin and Its Analogues in RAW 264.7 Cells**—LPS causes a significant increase in cellular oxidative stress (44) and these reactive oxygen species play an important role in activation of NFκB. We observed that melittin neutralized LPS-induced reactive oxygen species generation, whereas its analogue MM-1 was found to partially neutralize oxidative stress but MM-2 and Mel-SCR almost completely failed to inhibit ROS augmentation in these LPS-stimulated cells (supplemental Fig. S3).

**The Analogues of Melittin Are Less Efficient in Inhibiting LPS-stimulated NFκB Activation in RAW 264.7 Cells**—LPS induces activation of the NFκB (nuclear factor κ light chain enhancer of activated B cells) protein complex that controls transcription of many inflammatory genes (45–47). NFκB in its inactive state remains associated with IκBα by interacting with the C terminus of ankyrin repeats. Translocation of NFκB to the nucleus requires phosphorylation of the IκBα protein followed by its ubiquitination and degradation (48). The signal-induced phosphorylation and hence degradation of IκBα is considered a key event that ultimately leads to the activation of NFκB (49). We observed that melittin significantly inhibited LPS-stimulated activation and translocation of the p65 subunit of NFκB into the nucleus (Fig. 5, A and B). This is evident by the Western blotting experiment, which showed that the majority of p65 subunit of NFκB in LPS-stimulated RAW 264.7 cells remained in the cytoplasm in the presence of melittin (Fig. 5A). However, the substitution of heptadic leucine by alanine residue(s) progressively attenuated the ability of mellitin to inhibit translocation of the NFκB p65 subunit to the nucleus, as indicated by the simultaneous decrease in the p65 level in the cytoplasm and its increase in the nucleus in the same LPS-stimulated cells in the presence of melittin analogues (Fig. 5, A and B). The scrambled peptide, Mol-SCR, was also unable to stop the translocation of NFκB p65 (Fig. 5, A and B). To understand the basis of differences in properties of melittin and its analogues in activating NFκB, degradation of IκBα was investigated in LPS-stimulated cells in the presence of melittin and its analogues. A remarkable variation in the degradation of IκBα in the presence of melittin and its analogues in LPS-stimulated cells was observed (Fig. 5C), which probably explains the differences among these peptides with respect to activation of NFκB in LPS-stimulated cells in the presence of these peptides. Melittin inhibited the degradation of IκBα significantly in LPS-stimulated RAW 264.7 cells (Fig. 5C). However, with substitution of leucine residues the extent of inhibition of degradation of IκBα observed in the presence of melittin decreased significantly; even Mel-SCR inhibited IκBα degradation to a significantly lower extent than melittin (Fig. 5C). Further support on the differences among melittin and its analogues in inhibiting LPS-induced translocation of NFκB into the nucleus of macrophage cells was obtained by immunofluorescence microscopic studies by employing an
antibody of the p65 subunit of NFκB, also described under “Experimental Procedures.” Translocation of NFκB into the nucleus was prominently observed in LPS-stimulated cells (Fig. 5D). However, melittin significantly inhibited LPS-induced translocation of NFκB into the nucleus, which remained in the cytoplasm. On the other hand, when melittin was replaced by its alanine-substituted analogues, translocation of NFκB into the nucleus of RAW 264.7 cells was again observed (Fig. 5D). The nucleus of the cells were stained by DAPI.

Effect of Alteration in Leucine Zipper Motif of Melittin on Its Inhibitory Effects on MAPK Pathways—MAP kinases play a critical role in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stress. In addition, they play a critical role in NF-κB activity modulation. To investigate the molecular basis of the differences in NF-κB activation in the presence of melittin and its analogues in LPS-stimulated RAW 264.7 cells, we studied the phosphorylation of p38, ERK1/2, and JNK in these cells. RAW 264.7 cells were treated with peptides for 45 min in the presence of 1 μg/ml of LPS. Total cell lysates were then probed with phosphor-specific antibodies for p38, ERK1/2, and JNK. Phosphorylation of p38, ERK1/2, and JNK increased in RAW 264.7 cells treated with LPS alone (Fig. 6). However, melittin treatment reduced phosphorylated p38, ERK1/2, and JNK levels in LPS-stimulated RAW 264.7 cells, whereas its analogues MM1, MM2, and Mel-SCR failed to do so. No changes in the expression of nonphosphorylated ERK, JNK, and p38 kinase were observed in cells treated with LPS or LPS and peptide together (Fig. 6). Thus the data suggest that melittin prevented LPS to induce MAPK pathways and subsequently NFκB translocation; however, the analogues with the impaired leucine zipper sequence showed a reduced level of inhibition on LPS-induced activation of MAPK pathways in RAW 264.7 cells.

Significant Difference in the Secondary Structures of Melittin and Its Analogues in the Presence of LPS—Many LPS neutralizing antimicrobial peptides adopt predominant secondary structures especially α-helices in the presence of LPS (34). The degree of helicity of the peptides could depend on their inclination to interact, bind, and oligomerize in the presence of LPS. To determine whether there is a difference between melittin and its analogues in their secondary structures in the presence of LPS, CD experiments were performed. We observed a significant induction of helical structure in melittin in the presence of LPS (Fig. 7) as compared with that in PBS. Interestingly, the helix contents for the analogues of melittin in the presence of LPS were much lesser as compared with that observed for melittin (Fig. 7). In the presence of a fixed concentration of LPS (~25 μM), melittin (20 μM) adopted ~63% helicity, whereas MM-1, MM-2, and Mel-SCR exhibited ~43, 24, and 35% helicities, respectively, at the same concentrations.

Melittin but Not Its Analogues Dissociate FITC-LPS Aggregation—Sepsis is a complex disorder and its augmentation depends on the physical state of LPS. A direct correlation has been found between the ability of amphipathic α-helical AMPs to disaggregate LPS oligomers and their ability to neutralize LPS-induced proinflammatory response (50). It was investigated how melittin and its analogues dissociate LPS aggregates in an aqueous environment. We found that melittin was aptly able to dissociate the FITC-LPS aggregates in all used concentrations in a fluorometric study where dequenching of the aggregated fluorescence of the LPSs was recorded with increasing peptide concentrations (Fig. 8), as reported by others (40). However, the melittin analogues examined here were less active than melittin in inducing disaggregation in LPS aggregates, suggesting that these alterations in the leucine zipper sequence significantly impaired melittin-induced disaggregation of LPS oligomers (Fig. 8). However, MM-1 with the single leucine substitution was relatively active among the analogues in inducing disaggregation in LPS oligomers.

Binding of Melittin and Its Analogues to LPS in Aqueous Environment—The effect of alteration in the leucine zipper sequence of melittin on its binding to LPS was examined by performing the chromogenic LAL assay with melittin and its analogues by employing an endotoxin detection kit. Binding of the peptides to LPS was determined by measuring their efficacy.
Antiendotoxin Property of Melittin

FIGURE 4. Effect of melittin and its analogues onto the secretion of cytokines in LPS-stimulated RAW 264.7 cells. Panels A and B show the percentage inhibition of levels of LPS-induced secretions of TNF-α and IL-1β, respectively, in the presence of melittin and its analogues by ELISA experiments. Panels C–E, estimation of the effect of melittin and its analogues on LPS-stimulated proinflammatory secretions of TNF-α, IL-6, and MCP-1 by a cytometric bead array.

to inhibit LPS-induced activation of the LAL enzyme. Melittin showed significant binding to LPS as evidenced by the substantial inhibition of activation of the LAL enzyme (Fig. 9A). MM-1 showed lesser activity as compared with melittin in inhibiting LPS-mediated activation of the LAL enzyme, suggesting that melittin binding to LPS decreases as a result of substitution of leucine by alanine in the leucine zipper sequence. This is further evident by the insignificant efficacy of MM-2 to inhibit activation of the LPS-induced LAL enzyme. Interestingly, Mel-SCR also failed to inhibit the LPS-induced activation of the LAL enzyme (Fig. 9A) appreciably, suggesting a crucial role of the amino acid sequence of melittin in binding to LPS.

Effect of Amino Acid Substitutions in the Leucine Zipper Sequence of Melittin on Its Interaction with LPS onto the Macrophage Cell Surface—It was observed that substitution of heptadric leucine residues in melittin significantly disturbed its helical structure in the presence of LPS, as well as these melittin analogues did not dissociate the LPS aggregates like the parent molecule. Detailed flow cytometric studies were performed to find out the influence of melittin and its analogues on the binding of LPS to macrophages cells. Incubation of LPS with melittin significantly reduced the binding of LPS to RAW 264.7 cells (Fig. 9B). This is evident by the observation that fluorescence of Raw 264.7 cells bound to FITC-LPS that were incubated with melittin was much lower than fluorescence of the cells incubated with the same amount of FITC-LPS without any melittin pre-treatment. However, in the same experiment replacement of melittin with its analogues yielded different results. As shown in Fig. 9B, melittin analogues, particularly MM-2 and Mel-SCR, did not significantly inhibit binding of FITC-LPS to RAW264.7 cells.

By confocal microscopy we explored the binding of the Rho-labeled peptides to FITC-LPS on the surface of RAW 264.7 cells. We observed that melittin significantly colocalized with FITC-LPS on RAW 264.7 cell surfaces as indicated by the appearance of an appreciable yellow color (supplemental Fig. S4). Rho-MM-1 also co-localized to some extents with FITC-LPS, but the degree of co-localization of Rho-MM-2 and Rho-SCR-Mel with FITC-LPS was not significant.

DISCUSSION

The results presented show a significant impact of substitutions of leucine by alanine residue(s) in the leucine zipper sequence of melittin on its neutralization of the LPS-induced proinflammatory response in the macrophage cell line, RAW 264.7 (Figs. 2–4 and supplemental Figs. S1 and S2). The single alanine-substituted melittin analogue (MM-1) although partly neutralized the cytokines produced in the presence of LPS, MM-2, and Mel-SCR were negligibly active. The failure of Mel-SCR, which possesses the amino acid composition of the native peptide, in attenuating the LPS-induced cytokine response probably indicated a crucial role of the leucine zipper sequence in maintaining the ability of melittin to neutralize the LPS-induced proinflammatory response in RAW 264.7 cells. Experiments with rat primary macrophage cells also support the
FIGURE 5. Western blot analysis of the effect of melittin and its analogues on translocation of NFκB into the nucleus and degradation of IκBα in LPS-stimulated RAW 264.7 cells. Panel A, Western blot of the cytosolic fraction of lysates of LPS-stimulated RAW 264.7 cells for p65 of NFκB in the absence and presence of melittin and its different analogues as indicated; β-actin used as internal control. Panel B, Western blot of the nuclear fraction of the above cell lysates against the same p65 antibody; treatment of the respective peptides are as indicated; Ponceau S was used as loading control. Panel C, Western blot analysis of the whole cell extract of LPS-stimulated cells in the absence and presence of different peptides against IκBα antibody; β-actin was used as internal control. Panel D, confocal laser scanning microscopy study of nuclear translocation of the p65 subunit of NFκB in LPS-stimulated RAW 264.7 cells in the absence and presence of melittin and its analogues. DIC, FITC, DAPI, and MERGED represent the bright field image of cells, p65 subunit of NFκB recognized by AF-488-conjugated antibody, nucleus, and fluorescence of all the fluorochromes in single window, respectively. Ctrl represents the RAW 264.7 cells not treated with either LPS or peptides; LPS, on the left-hand side of the image, represents the cells treated with only LPS but no peptide; peptides used to treat the LPS-stimulated RAW 264.7 cells are shown in the left-hand side of the images.
observations on RAW 264.7 cells related to neutralization of the proinflammatory response by melittin and its analogues. Both NO and TNF-α responses of LPS stimulated rat bone marrow-derived macrophage cells in the presence of melittin and its analogues showed a similar pattern (supplemental Fig. S5) as observed with RAW 264.7 cells. Thus the data showed a possible general effect of substitution in the leucine zipper sequence of melittin on its neutralization of the proinflammatory response of LPS in macrophage cells. Furthermore, melittin or its analogues by themselves did not induce any proinflammatory response in macrophage cells as evidenced by no appreciable production of NO in rat primary macrophage cells in the presence of any of these peptides (data not shown).

One of the important events in the LPS-induced proinflammatory response in macrophage cells is the translocation of NF-κB into the nucleus, which has been implicated in the regulation of expression of many proinflammatory agents. In contrast to melittin, MM-1 partly inhibited translocation of NF-κB into the nucleus, whereas both MM-2 and Mel-SCR were negligibly active in preventing its translocation into the nucleus (Fig. 5, A, B, and D), which matched well with the cytokine response in LPS-stimulated cells in the presence of these peptides. Furthermore, evidence of NF-κB translocation into the nucleus of LPS-stimulated RAW 264.7 cells in the presence of these melittin analogues was complemented with degradation of IκBα (Fig. 5C). Additionally we observed a significant inhibition of the phosphorylation of all three components of MAPK pathways in the presence of melittin (Fig. 6) as reported by others (31), which could play a crucial role in inhibiting the degradation of IκB. However, MM-1, MM-2, and Mel-SCR failed to inhibit phosphorylations of the components of MAPK pathways appreciably (Fig. 6) and probably therefore could not stop the degradation of IκB.

How melittin neutralizes the LPS-induced proinflammatory response is apparently a matter of debate. However, the majority of antimicrobial peptides, except cathelicidin-derived LL-37 (50–52), directly interact with LPS and thus do not allow LPS to interact with LBP or other LPS receptors to start the LPS-induced signaling events so that LPS-induced production of cytokine and other proinflammatory agents do not take place (6, 7, 50, 53–56). When melittin was added to rat primary macrophage cells after an hour of pretreatment with LPS, antiendoxin property of the peptide was lost (~75 ± 5%) significantly (data not shown). Melittin added to the cells after a relatively shorter preincubation period (~15 and 30 min) with LPS showed a revival of its antiendoxin activity (~81 ± 5 and 72 ± 5% inhibition) (data not shown). The results suggest a probable crucial role of LPS-melittin interaction in neutralizing the proinflammatory response in macrophage cells. Interaction of melittin as well as its analogues with LPS was studied in an aqueous environment and also onto the macrophage cell surface. Melittin adopted a significant helical structure in the presence of LPS. The CD studies (Fig. 7) suggest that replacement of heptadic leucine residue(s) by alanine residue(s) or impairment in the leucine zipper sequence of melittin disturbs its interaction and/or secondary structure in the presence of LPS (Fig. 7). Studies in the literature indicate that many of the LPS-neutralizing peptides adopt well defined structures in the presence of LPS (8, 34, 37, 57) and a LPS-binding motif was recognized from the x-ray structural studies of E. coli outer membrane protein, FhuA, complexed with LPS (58, 59). A significant difference between melittin and its analogues was also observed when these peptides interacted with LPS on the surface of RAW 264.7 cells. Flow cytometric studies (Fig. 9B) revealed that melittin disturbed the binding of FITC-LPS to RAW 264.7 cells, whereas melittin analogues including its scrambled analogue showed lesser influence on the binding of LPS to macrophage cells. Probably, preincubation resulted in binding of endotoxin to the peptide and thus its binding to macrophage cells was prevented. The same experiment showed a significant difference between melittin and its analogues. Furthermore, confocal microscopic studies (supplemental Fig. S4) with Rho-labeled peptides and FITC-labeled LPS indicated that with substitution of leucine by...
Recent studies have demonstrated that antimicrobial peptides capable of neutralizing the LPS-induced cytokine response also dissociate the aggregates of LPS (37, 50). MM-1, MM-2, and Mel-SCR were much less efficient than melittin in inducing disaggregation of FITC-LPS, which consequently matched with their neutralization of LPS-induced proinflammatory responses (Fig. 8). The results of the LAL assay (Fig. 9A) suggested that substitution in the leucine zipper sequence of melittin significantly impaired its binding to LPS in an aqueous environment. The results probably suggest that proper arrangements of hydrophobic amino acids in melittin are crucial for its interaction with LPS. This is also consistent with a previous observation (11) that the interaction of melittin with LPS is endothermic indicating an important contribution of hydrophobic forces in this event (60).

Recent NMR studies show the involvement of its C-terminal of melittin in its interaction with lipid A, the lipid component of LPS (11). Unlike in the presence of lipid vesicles, only the C-terminal of melittin showed a helical structure in the presence of lipid A (11). Yet the present study showed that substitution of leucine by alanine residue(s) at the leucine zipper sequence of melittin or impairment of this sequence element, which is located at the N-terminal, significantly abrogates the interaction of melittin with LPS as well as neutralization of the LPS-induced proinflammatory response by melittin. Therefore, it is most likely that substitution in the leucine zipper sequence of melittin changes the overall conformation or assembly of melittin in the aqueous environment, which could further affect its interaction with lipid A or LPS. Indeed it was already reported that self-assembly of these two melittin analogues were significantly disturbed in an aqueous environment (13) and the Mel-SCR also showed a significantly lower self-association property than its parent molecule (supplemental Fig. S6). The present study is also supported by a report that showed that the aggregated peptides are more efficient in inducing disaggregation of LPS aggregates and detoxification of this endotoxin response (28, 61). Interestingly, melittin and its analogues showed differences in their self-association properties in the presence of LPS.

FIGURE 8. Dissociation of FITC-LPS aggregates in the presence of increasing concentrations of melittin and its analogues. Increase in fluorescence (in arbitrary units) of FITC-LPS has been plotted with respect to the peptide concentration in micromolar. The change in FITC emission after each treatment was monitored until emission reached equilibrium. The FITC-LPS concentration (LPS-FITC 0.5 μg/ml) was the same for each experiment and the collected data with a particular peptide (name marked in the x-axis) have been shown in the y-axis of each plot.
also. CD studies clearly indicated that melittin self-assembled in the LPS micelle, whereas its self-association property progressively decreased with substitution of leucine residues in its leucine zipper sequence (supplemental Fig. S7). Although the mechanism is not clearly understood, the aggregated state of melittin could be involved in disaggregating or detoxifying LPS. Nevertheless, the results for the first time indicate that the leucine zipper sequence of melittin could assist in its self-association in LPS micelles. Besides, it was investigated whether the analogues of melittin have any influence on neutralization of the proinflammatory response in LPS-stimulated macrophage cells. For this purpose, neutralization of the NO response in LPS-stimulated rat primary macrophage cells by melittin was measured in the presence of its analogues also. We observed that neutralization of the NO response in these cells by a mixture of melittin and MM-2 or melittin and Mel-SCR was very close to only melittin at the same concentration, indicating that these two analogues did not show any appreciable influence on the anti-inflammatory properties of melittin (supplemental Fig. S8). Also, the observation is consistent with our data on negligible anti-inflammatory properties of these melittin analogues. However, when melittin was mixed with MM-1, neutralization of the NO response in the same cells was to some extent more than the algebraic sum of the neutralization caused by the individual peptides (supplemental Fig. S8, columns with checkered pattern and gray columns with square pattern). The result possibly indicates co-assembly between melittin and the single alanine-substituted MM-1, which further shows to some extent a synergistic effect toward NO neutralization by a mixture of these peptides. This is probably possible because MM-1 partly retains the leucine zipper sequence of melittin. However, noteworthy is that neutralization of the NO response by a combination of melittin and MM-1 was significantly less as compared with that when the mixture of these two peptides was replaced by melittin alone (supplemental Fig. S8, black column). The data further indicate that the molecular assembly of melittin that neutralizes the LPS-induced pro-inflammatory response in these macrophage cells cannot be restored by a combination of melittin and any of its analogues. However, the fact that in comparison to MM-2 and Mel-SCR, MM-1, shows at least some synergistic effect to the activity of melittin, it probably signifies the role of the leucine zipper sequence of melittin in neutralizing the NO response produced in LPS-stimulated macrophage cells. Interestingly, we observed an appreciable enhancement of hemolytic activity of melittin and the MM-1 mixture against human red blood cells as compared with the algebraic sum of their individual hemolytic activities; whereas MM-2 and Mel-SCR did not have any influence on the hemolytic activity of melittin (data not shown).
A novel analogue of melittin (Mel-SCR-1) in which the positions of proline and an adjacent heptadic leucine residue were interchanged with each other also did not show appreciable efficacy to neutralize LPS-induced NO response in RAW 264.7 cells (supplemental Fig. S9). This analogue did not show an appreciable self-assembly property (supplemental Fig. 6C) in the aqueous environment, a significant secondary structure in the presence of LPS, and also did not induce substantial disaggregation of LPS aggregates (data not shown). The data once again suggest that the specific amino acid sequence rather than mean hydrophobicity of melittin is important for its anti-LPS activity.

The present study unambiguously indicates that antimicrobial and antiendotoxin properties of antimicrobial peptides do not depend on the same parameters that also support other studies in the literature (8, 62). Although our previous study (13) showed that substitutions of one or two leucine by alanine residues in the leucine zipper sequence of melittin did not significantly influence its bactericidal activity against the selected bacteria, the same melittin analogues exhibited significantly lesser efficacy to neutralize the LPS-induced proinflammatory response in macrophage cells as compared with that of melittin. Even Mel-SCR and Mel-SCR-1 with the same amino acid composition of melittin failed to neutralize the LPS-induced proinflammatory response in RAW 364.7 and rat primary macrophage cells. Binding of the cationic antimicrobial peptides to LPS, which constitutes the major portion of the outer layer of E. coli, is the first step for exhibiting their bactericidal activities, which is followed by their interaction with the cytoplasmic membrane of the bacteria resulting in damage of the membrane structure and finally lysis of bacteria. However, the extent of binding of the antimicrobial peptides to LPS does not necessarily determine the minimum inhibitory concentration values of the peptides against Gram-negative bacteria. For example, several Fowlicidin 1 analogues (63) with significantly lower affinity for LPS exhibited comparable bactericidal activity against Gram-negative bacteria as the native peptide. Also some analogues with similar affinity for LPS showed lower bactericidal activity than the native peptide (63). This is consistent with observations of other researchers (8, 62) as well as the present investigation that revealed a decrease in interaction of melittin analogues with LPS although they exhibited comparable bactericidal activity against Gram-negative bacteria as the native peptide (13).

One interesting observation of the present study is that amino acid substitutions that affect the cytotoxicity of melittin also influenced its anti-LPS activity. The result is in agreement with the reports that indicate an overlap between the cytotoxicity determining and LPS-interacting segments of antimicrobial peptides like SMAP-29 or Fowlicidin-1 (63, 64). Cytotoxicity of these membrane-active antimicrobial peptides is correlated with their ability to permeabilize phospholipid membrane composed of zwitterionic lipids that are the main components of the mammalian cell membrane. The alanine-substituted melittin analogues as well as the scrambled melittin analogue showed significantly decreased potency to permeabilize the zwitterionic lipid vesicles (13, 17). Binding of amphipathic antimicrobial peptides to LPS, which contains both polysaccharide and lipid A, is associated with both hydrophilic and hydrophobic interactions. In the analogues of melittin the positions of its polar and cationic charged residues remained intact, therefore, substitution in the leucine zipper sequence of melittin probably impaired its hydrophobic interactions with LPS. Our results support a recent study on the design and characterization of antiendotoxins and antimicrobial peptides that revealed the importance of hydrophobic amino acids at specific positions of these peptides rather than their global hydrophobicity (39). The results also showed that some of the endotoxin neutralizing peptides did not possess the usual amphipathic amino acid sequences but contains the cationic and hydrophobic amino acid residues at proper positions to interact with LPS (39).

It is to be mentioned that quite some time ago a cecropin/melittin hybrid peptide was reported to bind LPSs, such as polymixin B, and exhibit anti-LPS activity in RAW 264.7 cells (9, 51, 65). Incidentally, that melittin analogue also possessed the leucine zipper sequence, although the results did not reveal the importance of the amino acid sequence in any of these peptides.

In summary the results presented here showed a significant impact of substitution of leucine by alanine residues in the leucine zipper sequence of melittin in its neutralization of the LPS-induced proinflammatory response in the macrophage cell line, RAW 264.7, and rat primary macrophage cells as well as in its interaction with LPS in different environments. To our knowledge, this is the first time that this structural element has been implicated in antiendotoxin properties of an antimicrobial peptide. The results also showed the consequences of these amino acid substitutions in cellular events associated with neutralization of the LPS response in these macrophage cells by melittin. The work opens up further avenues to look into whether this motif has similar implications in other antiendotoxin peptides or not. However, the results described here suggest an apparent overlap of structural requirements for the cytotoxic and antiendotoxin properties of melittin. Consequently it points toward the requirement of further studies for designing noncytotoxic analogues of melittin with antiendotoxin properties. The results depicted here suggest an important role of hydrophobic leucine residues in the leucine zipper sequence of melittin in neutralizing the LPS response in macrophage cells and interaction with LPS. The data could aid significantly in the design of peptides with anti-LPS or LPS-binding properties. In this context, it is to be mentioned that although not pointed out in the related text, a careful look reveals that several leucine-rich designer peptides with anti-LPS property possess this motif (34). Although further studies are required in understanding the role of this motif in these molecules, the data probably indicates that one can design anti-LPS or LPS-binding molecules based on this structural element.

Acknowledgments—We acknowledge the assistance of A. L. Vishwakarma in recording the flow cytometry profiles. J. K. G. dedicates this article to his mentor Prof. Cornelis Murre, Division of Biological Sciences, University of California, San Diego, CA, with deep respect.
REFERENCES

1. Hancock, R. E., and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24, 1551–1557
2. Martin, G. S., Mannino, D. M., Eaton, S., and Moss, M. (2003) The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348, 1546–1554
3. Kitchens, R. L., and Thompson, P. A. (2005) Modulatory effects of scCD14 and LBP on LPS-host cell interactions. J. Endotoxin Res. 11, 225–229
4. Vasselon, T., Hailman, E., Thieringer, R., and Detmers, P. A. (1999) Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J. Exp. Med. 190, 509–521
5. Kennedy, M. N., Mullen, G. E., Leifer, C. A., Lee, C., Mazzoni, A., Dileepan, K. N., and Segal, D. M. (2004) A complex of soluble MD-2 and lipopolysaccharide serves as an activating ligand for Toll-like receptor 4. J. Biol. Chem. 279, 34698–34704
6. Rifkind, D., and Palmer, J. D. (1966) Neutralization of endotoxin toxicity in chick embryos by antibiotics. J. Bacteriol. 92, 815–819
7. Hirata, M., Shimomura, Y., Yoshida, M., Morgan, J. G., Palings, I., Wilson, D., Yen, M. H., Wright, S. C., and Larrick, J. W. (1994) Characterization of a rabbit cationic protein (CAP18) with lipopolysaccharide inhibitory activity. Infect. Immun. 62, 1421–1426
8. Papo, N., and Shai, Y. (2005) A molecular mechanism for lipopolysaccharide protection of Gram-negative bacteria from antimicrobial peptides. J. Biol. Chem. 280, 10378–10387
9. Scott, M. G., Gold, M. R., and Hancock, R. E. (1999) Interaction of cationic peptides with lipoteichoic acid and Gram-positive bacteria. Infect. Immun. 67, 645–6453
10. Park, H. J., Lee, S. H., Son, D. J., Oh, K. W., Kim, K. H., Song, H. S., Kim, G. J., Oh, G. T., Yoon, D. Y., and Hong, J. T. (2004) Antiatherogenic effect of bee venom. Inhibition of inflammation mediator generation by suppression of NF-κB through interaction with the p50 subunit. Arthritis Rheum. 50, 3504–3515
11. Bhunia, A., Domadia, P. N., and Bhattacharjya, S. (2007) Structural and thermodynamic analyses of the interaction between melittin and lipopolysaccharide. Biochim. Biophys. Acta 1768, 3282–3291
12. Park, H. J., Son, D. I., Lee, C. W., Choi, M. S., Lee, U. S., Song, H. S., Lee, J. M., and Hong, J. T. (2007) Melittin inhibits inflammatory target gene expression and mediator generation via interaction with IkB kinase. Biochem. Pharmacol. 73, 237–247
13. Asthana, N., Yadav, S. P., and Ghosh, J. K. (2004) Dissection of antibacterial and toxic activity of melittin. A leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. J. Biol. Chem. 279, 55042–55050
14. Zhu, W. L., Song, Y. M., Park, Y., Eaton, S., and Moss, M. (2003) The epidermology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348, 1546–1554
15. Ahmad, A., Azmi, S., Srivastava, R. M., Pandey, B. K., Yadav, V., and Ghosh, J. K. (2009) Structure-function study of cationic-derived bovine antimicrobial peptide BMAP-28. Design of its cell-selective analogues by amino acid substitutions in the heptad repeat sequences. Biochimie Acta 1788, 2411–2420
16. Vandenberg, M. J., van Dam, B., Groot, B. S., Grimmeren, J. M., Valerio, D., Bout, A., and Quax, P. H. (2001) Simultaneous detection of NOS-3 protein expression and nitric oxide production using a flow cytometer. Anal. Biochem. 290, 283–291
17. Pandey, B. K., Ahmad, A., Asthana, N., Azmi, S., Srivastava, R. M., Yadav, V., and Ghosh, J. K. (2009) Design of nontoxic analogues of cathelicidin-derived bovine antimicrobial peptide BMAP-27. The role of cathelicidin-derived bovine antimicrobial peptide BMAP-27. J. Biol. Chem. 283, 22907–22917
18. Yu, Z., and Shah, D. M. (2007) Curcumin down-regulates Ets-1 and Bcl-2 expression in human endometrial carcinoma HECl-1-A cells. Gynecol. Oncol. 106, 541–548
19. Rosenfeld, Y., Sahl, H. G., and Shai, Y. (2008) Parameters involved in antimicrobial and endotoxin detoxification activities of antimicrobial peptides. Biochemistry 47, 6468–6478
20. Todd, A. B., Kurten, R. C., McCullough, S. S., Brock, R. W., and Hinson, J. A. (2004) Quercetin induces apoptosis of Trypanosoma brucei gambiense and decreases the proinflammatory response of human macrophages. Antimicrob. Agents Chemother. 48, 924–929
21. Reid, A. B., Turner, R. C., McCullough, S. S., Brock, R. W., and Hinson, J. A. (2005) Mechanisms of acetaminophen-induced hepatotoxicity. Role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J. Pharmacol. Exp. Ther. 312, 509–516
22. Devadas, S., Zariatskaya, L., Rhee, S. G., Oberley, L., and Williams, M. S. (2002) Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation. Selective regulation of mitogen-activated protein kinase activation and fas ligand expression. J. Exp. Med. 195, 59–70
23. Afaq, F., Adhami, V. M., Ahmad, N., and Mukhtar, H. (2003) Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-α. Toxicol. Sci. 100, 259–266
24. Turnbull, I. R., McDunn, J. E., Takai, T., Townsend, R. C., Cobb, J. P., and Colonna, M. (2005) DAP12 (KARAP) amplifies inflammation and increases mortality from endotoxia and septic peritonitis. J. Exp. Med. 202, 363–369
25. Mamani-Matsuda, M., Rambert, J., Malvy, D., Lejoly-Boisseau, H., Davidouède, S., Thiolat, D., Coves, S., Courtois, P., Vincendeau, P., and Mossa-layi, M. D. (2004) Quercetin induces apoptosis of Trypanosoma brucei gambiense and decreases the proinflammatory response of human macrophages. Antimicrob. Agents Chemother. 48, 924–929
26. Colonna, M. (2005) DAP12 (KARAP) amplifies inflammation and increases mortality from endotoxia and septic peritonitis. J. Exp. Med. 202, 566–570
27. Privat, C., Lantoinie, F., Bedioui, F., Millanvoye van Brussel, E., Devynck, J., and Devermey, M. A. (1997) Nitric oxide production by endothelial cells. Comparison of three methods of quantification. Life Sci. 61, 1193–1202
28. Mamani-Matsuda, M., Rambert, J., Malvy, D., Lejoly-Boisseau, H., Davouldé, S., Thiolat, D., Coves, S., Courtois, P., Vincendeau, P., and Mossalayi, M. D. (2004) Quercetin induces apoptosis of Trypanosoma brucei gambiense and decreases the proinflammatory response of human macrophages. Antimicrob. Agents Chemother. 48, 924–929
29. Schillke, S., Waibler, Z., Mende, M. S., Zoccatelli, G., Vieths, S., Toda, M.

Antendotoxin Property of Melittin
and Scheurer, S. (2010) Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC. *Mol. Immunol.* **48**, 341–350.

39. Bhunia, A., Mohanram, H., and Bhattacharjya, S. (2009) Lipopolysaccharide bound structures of the active fragments of fowlcidein-1. A cathelicidin family of antimicrobial and antiendotoxin peptide from chicken, determined by transferred nuclear Overhauser effect spectroscopy. *Biopolymers* **92**, 9–22.

40. de Haas, C. J., van Leeuwen, H. J., Verhoeof, J., van Kessel, K. P., and van Strijp, J. A. (2000) Analysis of lipopolysaccharide (LPS) binding characteristics of serum components using gel filtration of FITC-labeled LPS. *J. Immunol. Methods* **242**, 79–89.

41. Petruson, K., Stalfors, J., Jacobsson, K. E., Ny, L., and Petruson, B. (2005) Structural origin of nuclear factor κB in peripheral blood mononuclear cells, even during activation. *J. Surg. Res.* **100**, 127–134.

42. Kadowaki, S., Chikumi, H., Yamamoto, H., Yoneda, K., Yamasaki, A., Sato, K., and Shimizu, E. (2004) Down-regulation of inducible nitric-oxide synthase by lysophosphatidic acid in human respiratory epithelial cells. *Biochem. J.* **382**, 51–59.

43. Hayter, J. B., Rivera, M., and McGroarty, E. J. (1987) Neutron scattering analysis of bacterial lipopolysaccharide phase structure. Changes at high pH. *J. Biol. Chem.* **262**, 5100–5105.

44. Pattanaik, U., and Prasad, K. (1996) Endotoxin and oxidative stress. *Ann. N.Y. Acad. Sci.* **793**, 506–510.

45. Downey, J. S., and Han, J. (1998) Cellular activation mechanisms in septic shock. *Front. Biosci.* **3**, d468–476.

46. Gilmore, T. D. (2006) Introduction to NF-κB. Players, pathways, and perspectives. *Ontogene* **25**, 6680–6684.

47. Perkins, N. D. (2007) Integrating cell signalling pathways with NF-κB and IKK function. *Nat. Rev. Mol. Cell Biol.* **8**, 49–62.

48. Moynagh, P. N. (2005) The NF-κB pathway. *J. Cell Sci.* **118**, 4589–4592.

49. Turpin, P., Hay, R. T., and Dargemont, C. (1999) Characterization of IkBα nuclear import pathway. *J. Biol. Chem.* **274**, 6804–6812.

50. Rosenfeld, Y., Papo, N., and Shai, Y. (2006) Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. *J. Biol. Chem.* **281**, 1636–1643.

51. Scott, M. G., Dressel, A. C., Buurman, W. A., Hancock, R. E., and Gold, M. R. (2000) Cutting edge, cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS-binding protein. *J. Immunol.* **164**, 549–553.

52. Nagaoka, I., Hirota, S., Niyonsaba, F., Hirata, M., Adachi, Y., Tamura, H., and Heumann, D. (2001) Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-α by blocking the binding of LPS to CD14+ cells. *J. Immunol.* **167**, 3329–3338.

53. Gough, M., Hancock, R. E., and Kelly, N. M. (1996) Antiendotoxin activity of cationic peptide antimicrobial agents. *Infect. Immun.* **64**, 4922–4927.

54. Giacometti, A., Circioni, O., Ghiselli, R., Mocchegiani, F., Del Prete, M. S., Viticchi, C., Kamysz, W., LEmpicka, E., Saba, V., and Scalise, G. (2002) Potential therapeutic role of cationic peptides in three experimental models of septic shock. *Antimicrob. Agents Chemother.* **46**, 2132–2136.

55. Tsuzuki, H., Tani, T., Ueyama, H., and Kodama, M. (2001) Lipopolysaccharide. Neutralization by polymyxin B shuts down the signaling pathway of nuclear factor κB in peripheral blood mononuclear cells, even during activation. *Science* **282**, 2125–2220.

56. Japelj, B., Pristovsek, P., Majerle, A., and Jerala, R. (2005) Structural origin of endotoxin neutralization and antimicrobial activity of a lactoferrin-based peptide. *J. Biol. Chem.* **280**, 16955–16961.

57. Japelj, B., Pristovsek, P., Majerle, A., and Jerala, R. (2005) Structural origin of endotoxin neutralization and antimicrobial activity of a lactoferrin-based peptide. *J. Biol. Chem.* **280**, 16955–16961.

58. Di, P., Wohland, T., Ho, B., and Ding, J. L. (2004) Perturbation of lipopolysaccharide (LPS) micelles by Sushi 3 (S3) antimicrobial peptide. The importance of an intermolecular disulfide bond in S3 dimer for binding, disruption, and neutralization of LPS. *J. Biol. Chem.* **279**, 50150–50156.

59. Giacometti, A., Circioni, O., Ghiselli, R., Mocchegiani, F., Orlando, F., Silvestri, C., Bozzi, A., Di Giulio, A., Luzi, C., Mangoni, M. L., Barra, D., Saba, V., Scalise, G., and Rinaldi, A. C. (2006) Interaction of antimicrobial peptide temporin I with lipopolysaccharide in vitro and in experimental rat models of septic shock caused by Gram-negative bacteria. *Antimicrob. Agents Chemother.* **50**, 2478–2486.

60. Tack, B. F., Sawai, M. V., Kearney, W. R., Robertson, A. D., Sherman, M. A., Wang, W., Hong, T., Boo, L. M., Wu, H., Waring, A. J., and Lehrer, R. I. (2002) SMAP-29 has two LPS-binding sites and a central hinge. *Eur. J. Biochem.* **269**, 1181–1189.

61. Xiao, Y., Dai, H., Bommineni, Y. R., Soulsages, J. L., Gong, Y. X., Prakash, O., and Zhang, G. (2006) Structure-activity relationships of fowlcidein-1, a cathelicidin antimicrobial peptide in chicken. *FEBS J.* **273**, 2581–2593.

62. Piers, K. L., and Hancock, R. E. (1994) The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **12**, 951–958.