Antimicrobial cathelicidin polypeptide CAP11 suppresses the production and release of septic mediators in D-galactosamine-sensitized endotoxin shock mice

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Abstract

Endotoxin shock is a severe systemic inflammatory response that is caused by the augmented production and release of septic mediators. Among them, inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 play a pivotal role. In addition, anandamide, an endogenous cannabinoid and high-mobility group box-1 (HMGB1), a non-histone chromosomal protein has recently been recognized as members of septic mediators. We previously reported that cationic antibacterial polypeptide of 11-kDa (CAP11), an antimicrobial cathelicidin peptide (originally isolated from guinea pig neutrophils), potently neutralizes the biological activity of LPS and protects mice from lethal endotoxin shock. In this study, to clarify the protective mechanism of CAP11 against endotoxin shock, we evaluated the effects of CAP11 on the production and release of septic mediators in vitro and in vivo using a murine macrophage cell line RAW264.7 and a D-galactosamine-sensitized murine endotoxin shock model. LPS stimulation induced the production of inflammatory cytokines and anandamide and release of HMGB1 from RAW264.7 cells. Importantly, CAP11 suppressed the LPS-induced production and release of these mediators by RAW264.7 cells. Moreover, LPS administration enhanced the serum levels of HMGB1, anandamide and inflammatory cytokines in the endotoxin shock model. Of note, CAP11 suppressed the LPS-induced increase of these mediators in sera, and LPS binding to CD14-positive cells (peritoneal macrophages), accompanied with the increase of survival rates. Together these observations suggest that the protective action of CAP11 on endotoxin shock may be explained by its suppressive effect on the production and release of septic mediators by CD14-positive cells possibly via the inhibition of LPS binding to the targets.

Introduction

Sepsis is caused by polymicrobial infections and associated with severe systemic inflammatory response syndrome that leads to multiple organ failure (such as acute lung injury and renal failure) and septic shock (1–3). LPS, also called endotoxin, is a major component of the outer membrane of Gram-negative bacteria and is the most potent activator of mononuclear phagocytes (macrophage and monocyte) (1). LPS triggers excessive response of innate immunity that stimulates mononuclear phagocytes to secrete inflammatory cytokines [such as tumor necrosis factor (TNF-α), IL-1β and IL-6], which play a crucial role in the pathogenesis of septic shock. Additionally, high-mobility group box-1 (HMGB1) and anandamide are reported as mediators of endotoxin/septic shock (4–8).

HMGB1 is a highly conserved, non-histone nuclear protein with multiple functions. HMGB1 binds with DNA and participates in the gene transcription in the cells. Moreover, HMGB1 functions as a cytokine in the extracellular milieu (4, 6, 9); HMGB1 up-regulates pro-inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) in human mononuclear cells and neutrophils (10, 11) and adhesion molecules (such as intercellular cell...
adhesion molecule-1 and vascular cell adhesion molecule-1) in endothelial cells (12). Accordingly, HMGB1 is proposed to play a crucial role in endotoxin/septic shock (13).

Anandamide, an endogenous cannabinoid, induces the characteristic feature of septic shock such as hypotension (7, 14). Furthermore, serum anandamide level is increased in patients with endotoxin/septic shock (8, 15). Moreover, anandamide modulates the serum cytokine levels (such as TNF-α) in LPS-stimulated RAW264.7 cells (16). Thus, anandamide is also regarded as a mediator of endotoxin/septic shock. Since pro-inflammatory substances are overproduced and involved in the pathogenesis of endotoxin/septic shock, therapeutic strategies have targeted the blockade of pro-inflammatory molecules; however, most of the strategies have been unsuccessful (17, 18). Thus, the development of novel agents with therapeutic potential for endotoxin/septic shock is explored.

Mammalian cells express a number of peptide antibiotics that function as effector components in the innate host defense system. Defensins and cathelicidins are the two major classes of antimicrobial peptides that can kill a broad spectrum of invading microorganisms, including both Gram-positive and Gram-negative bacteria, fungi and viruses (19-21). Previously, we isolated cationic antibacterial polypeptide of 11-kDa (CAP11), a member of cathelicidins, from guinea pig neutrophils (22). CAP11 adopts an amphipathic α-helical structure with the hydrophilic (positively charged) and hydrophobic facets, which facilitates the interaction with microbial cell-surface anionic lipids and negatively charged amphipathic LPS; thus, CAP11 can not only kill bacteria but also neutralize the biological activity of LPS (23). Of note, CAP11 potently binds with LPS and interferes with its association with LPS-binding peptide to suppress the activation of CD14/toll-like receptor-4 expressing cells, thereby protecting mice from endotoxin shock (23). Furthermore, we have revealed that CAP11 can suppress the anamidine production from LPS-stimulated RAW264.7 mouse macrophage-like cells by inhibiting the induction of anamidine-synthesizing enzyme activity (24). In this study, to clarify the protective mechanism of CAP11 against endotoxin shock, we have evaluated the effect of CAP11 on the LPS-induced production of cytokines and anamidine and the release of HMGB1 in vitro and in vivo using a murine macrophage cell line RAW264.7 and a Δ-galactosamine-sensitized mouse endotoxin shock model.

Methods

Reagents

LPS (from Escherichia coli serotype O111:B4) and D-(-)-galactosamine hydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, USA); Alexa Fluor® 488-labeled LPS (from E. coli serotype O55:B5) was from Molecular Probe (Eugene, OR, USA). CAP11 (a 43-mer peptide of G1-LRRKKFRKRKQKLGRKIGKTGRKVWKAWREYG-QIPYPCRI43) and a control peptide of CAP11 with the same amino acid composition but scrambled amino acid sequence (scrubbed CAP11 (SCAP11); G1IKRLKKYWWGTRQEA-KGKTRRRFRRKVQIRPLRCPRG43) were synthesized by a solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by fluorenylmethoxycarbonyl chemistry and purified, as described previously (23). A FlexSpectra mouse inflammation cytokine cytometric bead array kit was purchased from BD Bioscience (San Jose, CA, USA).

Quantification of HMGB1 released from LPS-stimulated RAW264.7 cells

A murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, endotoxin level <10 EU ml⁻¹). Cell Culture Technologies, Herndon, VA, USA). Quantitative analysis of HMGB1 in the culture supernatants of RAW264.7 cells was performed by western blotting. Cells (10⁵ cells per well) were seeded into a 48-well cell culture plate and incubated overnight at 37°C, 5% CO₂. Then, cells were incubated without or with LPS (100 ng ml⁻¹) in the absence or presence of CAP11 (0.1 or 1 µg ml⁻¹) in RPMI 1640-2% FBS. Culture supernatants were recovered 24 h after the incubation, and 60-µl aliquots were subjected to 12% SDS-PAGE and then migrated proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membrane was blocked in 10% skimmed milk and probed with rabbit anti-HMGB1 polyclonal antibody (20 ng ml⁻¹, ab11972, abcam, Cambridge, MA, USA). The membrane was washed and further probed with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA, USA), and HMGB1 was finally detected using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Quantification of inflammatory cytokines secreted from LPS-stimulated RAW264.7 cells

RAW264.7 cells seeded into a 96-well cell culture plate were incubated without or with LPS (10 ng ml⁻¹) in the absence or presence of CAP11 (0.1 or 1 µg ml⁻¹) in RPMI 1640-10% FBS. Culture supernatants were recovered 5 h after the incubation, and inflammatory cytokines (TNF-α and IL-6) in culture media were quantitated by an ELISA using a Ready-SET-Go! ELISA set for TNF-α and IL-6 (eBioscience, San Diego, CA, USA). IL-1β was also measured by ELISA using a Duo Set ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). The detection limits of these cytokines were 16 pg ml⁻¹.

Quantification of anandamide released from LPS-stimulated RAW264.7 cells

Anandamide, an endogenous cannabinoid, was quantitated with a liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) system, as previously described (25). RAW264.7 cells (6 × 10⁵ cells per well) were seeded in a six-well culture plate and incubated without or with LPS (10 ng ml⁻¹) in the absence or presence of CAP11 (0.1 or 1 µg ml⁻¹) in RPMI 1640 containing 10% FBS. After 1 h, both culture supernatants and cells were recovered and immediately injected into 5 ml of acetonitrile. Anandamide was extracted in acetonitrile, evaporated to dryness and dissolved in acetonitrile. Anandamide parent ion (with atomic mass unit of 348.3/621.1) in the samples was detected and analyzed by MS/MS using deuterium-labeled anandamide (d₈-anandamide, Cayman Chemical, Ann Arbor, MI, USA) as an internal standard.
standard and a MS/MS system software (Analyst version 1.3.2; Applied Biosystems, Foster City, CA, USA). The detection limit of anandamide was 1 pg per sample.

Assay for the binding of LPS to RAW264.7 cells

RAW264.7 cells (10⁶ cells ml⁻¹) were incubated in RPMI 1640–10% FBS with Alexa488-labeled LPS (100 ng ml⁻¹) at 37°C for 15 min in the absence or presence of CAP11 or SCAP11 (0.1 or 1 µg ml⁻¹). Cells were then washed twice with ice-cold PBS, and the LPS binding was analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). The mean fluorescent intensity was measured in each group, and LPS binding was expressed as a percentage of that of Alexa488-labeled LPS alone.

ω-Galactosamine-sensitized endotoxin shock model

A ω-galactosamine-sensitized mouse model (26), which is highly susceptible to LPS, was utilized to assess the potential of CAP11 to suppress inflammatory reaction in vivo. Male C57BL/6 mice aged 7 weeks (Sankyo Laboratories, Tokyo, Japan) were intra-peritoneally injected with 400 µl of ω-galactosamine (18 mg, dissolved in saline) or ω-galactosamine + LPS (100 ng) without or with CAP11 (5 or 50 µg) and survival rates were monitored at 6, 24, 48 and 72 h after the injection. Alternatively, 1 h (for cytokines and anandamide quantification) and 5 h (for HMGB1 quantification) after LPS challenge, mice were anesthetized by intra-peritoneal injection of pentobarbital, and blood was collected by cardiac puncture for the assays of cytokines, HMGB1, and anandamide. All procedures with mice were approved by the ethic committee in Juntendo University School of Medicine and performed according to the institutional guidelines.

Quantification of serum HMGB1

Serum HMGB1 levels were quantitated with 10-µl aliquots of sera (20-fold diluted with sample dilution buffer) using an HMGB1 ELISA Kit II (Shino-Test Corp., Kanagawa, Japan), according to the manufacturer’s instruction. The detection limit of HMGB1 was 1 ng ml⁻¹. Furthermore, changes in the serum HMGB1 levels were confirmed by SDS–PAGE/western blotting using 3 µl sera, as described above.

Quantification of serum cytokine levels

Sera were prepared from blood by centrifugation at 1000 × g for 10 min and diluted 10-fold with assay diluent. Then, 50-µl aliquots were assayed for cytokines using a cytometric bead array system (CBA, BD Bioscience), according to the manufacturer’s instruction. The system includes six fluorescently distinguishable capture beads coated with antibody against six analytes (TNF-α, IL-6, IL-10, IL-12p70, MCP-1 and IFN-γ) and detects these cytokines by ELISA on microbeads. IL-1β was also measured by ELISA using a Duo Set ELISA kit (R&D Systems). The detection limits were 7.3 pg ml⁻¹ for TNF-α, 5 pg ml⁻¹ for IL-6, 17.5 pg ml⁻¹ for IL-10, 10.7 pg ml⁻¹ for IL-12p70, 52.7 pg ml⁻¹ for MCP-1, 2.5 pg ml⁻¹ for IFN-γ and 16 pg ml⁻¹ for IL-1β.

Suppressive actions of CAP11 on septic mediators

After collection of blood by cardiac puncture, blood samples (0.5 ml) were immediately injected into 5 ml of acetonicitrite. Then, blood anandamide was extracted in acetonicitrite and quantified, as described above.

Assay for the binding of LPS to mouse peritoneal macrophages

Male C57BL/6 mice aged 7 weeks were intra-peritoneally injected with 400 µl of Alexa488-labeled LPS (100 ng) and ω-galactosamine (18 mg) without or with CAP11 (5 or 50 µg). After 4 h, mice were euthanized by diethylether, and peritoneal macrophages were collected by rinsing peritoneal cavity with ice-cold PBS. Collected macrophages were centrifuged (190 × g, 5 min) and washed twice in ice-cold PBS. Macrophages were identified with FITC-conjugated anti-mouse CD14 mAb (4C1, BD Pharmingen, San Diego, CA, USA) and R-phycocerythrin-conjugated anti-mouse F4/80 mAb (CI:A3-1, AbD Serotec, Oxford, UK), and the binding of Alexa488-labeled LPS to macrophage was analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA). Number of macrophages with >10¹ of fluorescent intensity was measured in each group (Alexa488-labeled LPS/ω-galactosamine alone or with CAP11), and LPS binding was expressed as a percentage of that from Alexa488-labeled LPS/ω-galactosamine-injected mice.

Statistical analysis

Data are shown as mean ± SE. Statistical significance was determined by one-way analysis of variance analysis (Graphpad Prism, La Jolla, CA, USA), unless otherwise noted. Survival of mice after LPS administration was assessed by a χ² test (Graphpad Prism). A P value of <0.05 was considered to be significant.

Results

Effects of antimicrobial CAP11 on the production and release of septic mediators from LPS-stimulated RAW264.7 cells

To evaluate whether CAP11 modulates the levels of septic mediators, we first examined the effect of CAP11 on the release of HMGB1 from LPS-stimulated RAW264.7, a murine macrophage-like cell. As shown in Fig. 1, LPS stimulation (100 ng ml⁻¹) enhanced the HMGB1 release from RAW264.7 cells. Of note, CAP11 (0.1 and 1 µg ml⁻¹) dose dependently suppressed the HMGB1 release (P < 0.05). As shown in Fig. 2, LPS (10 ng ml⁻¹) also stimulated RAW264.7 cells to produce anandamide detected by LC/MS/MS, as previously reported (24). Similar to the effect on HMGB1 release, 1 µg ml⁻¹ CAP11 suppressed the anandamide production almost to the resting level (P < 0.01). Furthermore, the effect of CAP11 on cytokine production was determined. As shown in Fig. 3, CAP11 dose dependently suppressed the production of TNF-α and IL-6 by LPS-stimulated RAW264.7 cells (P < 0.001 at 1 µg ml⁻¹ CAP11). We also tried to measure IL-1β production by LPS-stimulated RAW264.7 cells; however, IL-1β could not be detected in the culture supernatants even after the LPS stimulation (data not shown).
Further, to clarify the suppressive effect of CAP11, we examined the effect of CAP11 on the LPS binding to RAW264.7 cells by using Alexa488-labeled LPS. In accordance with its effects on the production and release of septic mediators (HMGB1, anandamide and cytokines), CAP11 (1 μg ml\(^{-1}\)) significantly inhibited the LPS binding (\(P < 0.001\)) (Fig. 4).

In addition, we confirmed that a control scrambled peptide (SCAP11) did not affect the LPS-induced production and release of septic mediators from RAW264.7 cells at 0.1 and 1 μg ml\(^{-1}\) (Figs 1, 3 and 4).
Effects of CAP11 on the inflammatory responses in a D-galactosamine-sensitized endotoxin shock model

We previously revealed that CAP11 protects mice from endotoxin shock (23). Furthermore, the present study has shown that CAP11 can inhibit the production and release of inflammatory mediators from LPS-stimulated macrophage-like cells \textit{in vitro} (Figs 1–3). Thus, we tried to elucidate the mechanism for the protective action of CAP11 on endotoxin shock by evaluating the effect of CAP11 on septic mediator production and release \textit{in vivo} using a D-galactosamine-sensitized murine endotoxin shock model. As shown in Fig. 5, 55 and 100% of the mice died at 6 and 24 h, respectively, after the administration of D-galactosamine and LPS. Notably, administration of CAP11 (5 and 50 μg) increased the survival rate to 100% at 6 h \((P < 0.05)\), and 50 μg CAP11 increased the rate to 91% at 24–72 h \((P < 0.01)\). Furthermore, we quantified the serum levels of septic mediators. Serum HMGB1 level was strikingly elevated from the undetectable levels to \(\sim 200 \text{ ng ml}^{-1}\) by LPS administration \((P < 0.001)\) (Fig. 6A). Consistent with the protective actions of CAP11 on endotoxin shock, CAP11 administration (50 μg) markedly suppressed the serum HMGB1 level \textit{in vivo} \((P < 0.05)\). Similarly, the serum levels of TNF-α, IL-6, IL-10 and MCP-1 apparently increased by LPS injection, and CAP11 administration repressed the serum levels of these cytokines \((P < 0.001)\) for the effect of 50 μg CAP11 on TNF-α, IL-6, IL-10 and MCP-1 (Fig. 7A–D). In contrast, serum levels of anandamide as well as IL-12p70 and IFN-γ were only marginally increased in LPS-injected mice, and the suppressive effect of CAP11 on these mediators was not obvious (Figs 6B, 7E and F). Further, we measured the \textit{in vivo} production of IL-1β in a D-galactosamine-sensitized murine endotoxin shock model; however, IL-1β could not be detected in sera even after the LPS administration (100 ng per mouse) (data not shown).

In addition, we confirmed that SCAP11 neither affected the survival rate of D-galactosamine-sensitized endotoxin shock mice nor the increase in the serum levels of septic mediators \textit{in vivo} (Figs 5–7).

Effect of CAP11 on the LPS binding to peritoneal macrophages

To estimate the effect of CAP11 on the binding of LPS to CD14-positive cells \textit{in vivo}, we assessed the LPS binding to mouse peritoneal macrophage after injection of Alexa488-labeled LPS. Consistent with the effects on the serum cytokine and HMGB1 levels, administration of 50 μg CAP11 significantly suppressed the LPS binding to peritoneal macrophages \((P < 0.001)\) (Fig. 8). In separate experiments, we confirmed that the LPS binding to peritoneal macrophages is primarily caused by CD14 (an LPS receptor), because a neutralizing anti-CD14 antibody (4C1; a gift from Dr Y. Adachi, Tokyo University of Pharmacy and Life Sciences) can almost completely suppress the LPS binding (data not shown). These observations indicate that CAP11 likely prevents the binding of LPS to CD14-positive cells and suppresses the production and release of septic mediators.
in vivo, thereby exhibiting protective action on endotoxin shock.

Discussion

Endotoxin/septic shock is a severe and abnormal condition that is induced during serious infections with Gram-negative bacteria (1–3). We previously revealed that a cathelicidin peptide CAP11 (originally isolated from guinea pig neutrophils) potently neutralizes the biological activity of LPS and protects mice from lethal endotoxin shock (23). In this study, to elucidate the mechanism for the action of CAP11 on endotoxin shock, we determined the effects of CAP11 on the production and release of septic mediators (HMGB1, anandamide and cytokines) in vitro and in vivo using a murine macrophage-like cell RAW264.7 and a d-galactosamine-sensitized mouse endotoxin shock model.

Inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-10, IL-12 and MCP-1 are released by mononuclear phagocytes (macrophages) shortly after the exposure to LPS, and they play a pivotal role in the pathogenesis of lethal systemic inflammation in endotoxin/septic shock (1). TNF-α, IL-1β, IL-6 and IL-12 activate neutrophils, lymphocytes and vascular endothelial cells up-regulate cellular adhesion molecules and induce the production of lipid mediators, nitric oxide and reactive oxygen species, whereas IL-10 negatively regulates these responses. In addition, MCP-1, as a chemokine, activates inflammatory cell (especially macrophage) to migrate into the tissues. The present study revealed that LPS stimulation induced the production of anandamide and cytokines (TNF-α and IL-6) and release of HMGB1 from RAW264.7 cells, and CAP11 strikingly suppressed the LPS-induced production and release of these mediators.
Mide is more rapidly metabolized in vivo than in vitro (31, 32). Thus, it could be speculated that the anandamide-producing action of LPS is more obvious in vitro than in vivo, and similarly the suppressive effect of CAP11 on the anandamide production can be more clearly appreciated in vitro than in vivo.

We have already confirmed that CAP11 suppresses the LPS binding to RAW264.7 cells in vitro (23). Thus, we evaluated the LPS binding to CD14-positive cells using peritoneal macrophages as target cells. The results indicated that CAP11 administration inhibits the binding of LPS to peritoneal macrophages, accompanied with the increase of survival rates in a D-galactosamine-sensitized endotoxin shock model.

Together these observations suggest that the protective action of CAP11 on endotoxin shock may be explained by its suppressive effect on the production and release of septic mediators by CD14-positive cells possibly via the inhibition of LPS binding to the targets.

CAP11 is known to adopt an amphipathic α-helical structure with the hydrophilic (positively charged) and hydrophobic facets, which facilitates the interaction with negatively charged amphipathic LPS to neutralize its biological activity (23). A control scrambled peptide of CAP11 (SCAP11) neither adopted a positively charged amphipathic structure nor exhibited LPS-neutralizing activity in vitro; SCAP11 did not affect the LPS-induced release and production of septic mediators (HMGB1 and cytokines) from RAW264.7 cells and the LPS binding to the cells. Moreover, SCAP11 neither affected the survival rate of D-galactosamine-sensitized endotoxin shock mice nor the increase in the serum levels of septic mediators in vivo. These observations indicate that the LPS-neutralizing activity of CAP11 is based on its unique amphipathic structure.

Endotoxin/septic shock is an uncontrolled fatal condition that arises from harmful or damaging host response to infections. Components of innate immune response that are normally concerned with host defense against infection can, under circumstances, cause cell and tissue damage (1–3). Excess production of inflammatory mediators leads to the amplified systemic inflammation, tissue injury, organ failure and septic shock (1–3). Based on these findings, therapeutic strategies have mainly targeted the blockade of pro-inflammatory mediators; however, most of the strategies have been unsuccessful (17, 18). The present study indicates that CAP11 can suppress the cytokine and anandamide production and HMGB1 release from LPS-activated RAW264.7 cells via the inhibition of LPS binding to target cells. Furthermore, CAP11 obviously repressed the elevation of serum HMGB1 and cytokine levels and protects mice from endotoxin lethality possibly via the inhibition of LPS binding to CD14-positive target cells. Consequently, CAP11 could be expected as a therapeutic agent for endotoxin/septic shock with a potential to regulate the production and release of septic mediators from LPS-stimulated cells via the suppression of LPS binding to CD14-positive target cells. However, endotoxinemia is not necessarily detected in the blood of patients with sepsis or septic shock (33). Moreover, sepsis is caused by not only Gram-negative bacteria but also Gram-positive bacteria and fungi (34). Thus, it is expected that CAP11 unlikely exerts a therapeutic action on sepsis or septic shock, the pathogenesis of which is irrelevantly associated with LPS.
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References


