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An Antimicrobial Cathelicidin Peptide, Human CAP18/LL-37, Suppresses Neutrophil Apoptosis via the Activation of Formyl-Peptide Receptor-Like 1 and $P2X_7$[1]

Isao Nagaoka,[2]* Hiroshi Tamura,[†] and Michimasa Hirata[‡]

Peptide antibiotics possess the potent antimicrobial activities against invading microorganisms and contribute to the innate host defense. An antibacterial cathelicidin, human cationic antibacterial protein of 18 kDa/LL-37, not only exhibits potent bactericidal activities against Gram-negative and Gram-positive bacteria, but also functions as a chemoattractant for immune cells, including neutrophils. During bacterial infections, the life span of neutrophils is regulated by various pathogen- and host-derived substances. In this study, to further evaluate the role of LL-37 in innate immunity, we investigated the action of LL-37 on neutrophil apoptosis. Neutrophil apoptosis was assessed using human blood neutrophils based on the morphological changes. Of note, LL-37 dose dependently (0.01–5 μg/ml) suppressed neutrophil apoptosis, accompanied with the phosphorylation of ERK-1/2, expression of Bcl-x_L (an antiapoptotic protein), and inhibition of caspase 3 activity. Interestingly, LL-37-induced suppression of neutrophil apoptosis was attenuated by the antagonists for formyl-peptide receptor-like 1 (FPRL1) and $P2X_7$ nucleotide receptor. Of importance, the agonists for FPRL1 and $P2X_7$ apparently suppressed neutrophil apoptosis. Collectively, these observations indicate that LL-37 cannot only kill bacteria, but also modulate (suppress) neutrophil apoptosis via the activation of FPRL1 and $P2X_7$ in bacterial infections. Suppression of neutrophil apoptosis results in the prolongation of their life span, and may be advantageous for host defense against bacterial invasion. *The Journal of Immunology,* **2006, 176: 3044–3052.**

Neutrophils play an important role as an effector of inflammation, tissue injury, and host defense against microbial infection (1). The lifetime of neutrophils, terminally differentiated blood cells, is relatively short, and they constitutively undergo apoptosis (2). Apoptotic neutrophils are phagocytosed by macrophages without release of proinflammatory mediators, leading to the limitation of tissue injury and resolution of inflammatory process (2). In this context, it is interesting to note that spontaneous apoptosis of neutrophils is inhibited in patients with sepsis, systemic inflammatory syndrome, and acute respiratory distress syndrome by the action of various pathogen- and host-derived substances, such as bacterial products (i.e., Gram-negative LPS; LPS), cytokines, and chemokines (i.e., IL-1β and IL-8) (3–7). The suppressed neutrophil apoptosis results in the prolongation of their life span and causes the uncontrolled release of cytotoxic metabolites and proinflammatory substances (i.e., reactive oxygen species and proteases), which leads to the amplification of systemic inflammation, tissue injury, and organ failure observed in those disorders (8, 9). In contrast, neutrophil apoptosis can be accelerated by Fas ligand, reactive oxygen species, immune complexes, and bacterial toxins (such as *Pseudomonas aeruginosa* exotoxin, pyocyanin) produced at the sites of inflammation and infection (10–13). Inappropriate induction of neutrophil apoptosis is likely to deplete neutrophil numbers and functions, thereby impairing host defense and favoring bacterial invasion and persistence.

Mammalian cells express a number of peptide antibiotics (such as defensins) that function as effector components in the innate host defense system. They are found in blood, secretions, epithelial tissues, and neutrophil granules, and exhibit potent antimicrobial activities against both Gram-positive and Gram-negative bacteria, fungi, and certain viruses (14–17). Among these peptides, cathelicidin is a novel family of antimicrobial peptides, characterized by the highly conserved cathelin-like prosequences and variable C-terminal sequences that correspond to the mature antibacterial peptides (18). Approximately 30 cathelicidin members have been identified from various mammalian species; however, only one cathelicidin, human cationic antibacterial protein of 18 kDa (hCAP18),[3] has been found in humans, and its C-terminal mature antibacterial peptide, called LL-37, which comprises 37 aa residues (L^1LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES^{37}), has been identified (19, 20). In addition to exhibiting potent antibacterial activities against both Gram-positive and Gram-negative bacteria (21), LL-37 can bind to LPS and blunt its biological activities (19, 22). Furthermore, LL-37 possesses the ability to chemoattract immune and inflammatory cells, including neutrophils, monocytes, and T cells via the action on a low affinity formyl-peptide receptor, formyl-peptide receptor-like 1 (FPRL1) (23). In addition, LL-37 is demonstrated to promote the processing and release of IL-1β from monocytes via the activation of $P2X_7$, a nucleotide receptor (24).

*Department of Host Defense and Biochemical Research, Juntendo University, School of Medicine, Tokyo, Japan; [†]Seikagaku, Tokyo, Japan; and [‡]Insititute of Ohtaka Enzyme, Hokkaido, Japan

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[2] Address correspondence and reprint requests to Dr. Isao Nagaoka, Department of Host Defense and Biochemical Research, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail address: nagaokai@med.juntendo.ac.jp

[3] Abbreviations used in this paper: hCAP18, human cationic antibacterial protein of 18 kDa; Bz-ATP, benzoylbenzoyl-ATP; EGFR, epidermal growth factor receptor; FPRL1, formyl-peptide receptor-like 1; Ox-ATP, oxidized ATP.

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Given that LL-37 is a multifunctional molecule (25, 26) and acts on neutrophils as a chemoattractant (23), we hypothesized that LL-37 may have a potential to modulate the lifetime (apoptosis) of neutrophils. Thus, we investigated the effect of LL-37 on neutrophil apoptosis in vitro, and further evaluated the actions of antagonistic and agonistic agents for FPRL1 or $P2X_7$ receptor. In this study, we provide the evidence that LL-37 can suppress neutrophil apoptosis possibly via the activation of FPRL1 and $P2X_7$.

Materials and Methods

Reagents

LPS (from *Escherichia coli* 0111:B4), an Annexin V^{FITC} apoptosis detection kit, a caspase 3 assay kit, 2′-3′-*O*-(4-benzoyl-benzoyl)-ATP (benzoylbenzoyl-ATP (Bz-ATP)), ATP-2′,3′-dialdehyde (oxidized ATP (Ox-ATP)), ATP, apyrase, and 3,3′,5,5′-tetramethylbenzidine liquid substrate system were purchased from Sigma-Aldrich; KN-93 (2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesufonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine)) and AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) were purchased from Calbiochem. A 37-mer peptide of hCAP18 (LL-37; L^1LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVP RTES^{37}), an FPRL-1 antagonist WRW^4 (Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$) (27), and FPRL1 agonists WKYMVm (Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$) (28) and MMK-1 (LESIFRSLLFRVM) (28) were synthesized by the solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu Scientific Instruments) by fluorenylmethoxycarbonyl chemistry, and purified to homogeneity by reversed phase HPLC on a Cosmosil 5C18 column (Nacalai Tesque), using a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid, as described before (22). The molecular masses of synthesized peptides were confirmed on a mass spectrometer (model TSQ 700; Thermo Quest Finnigan). Tissue culture supplies were obtained from BD Discovery Labware.

Antibodies

Biotin-conjugated mouse anti-human IL-1β mAb (CRM56), mouse anti-human IL-1β mAb (CRM57), biotin-conjugated mouse anti-human TNF-α mAb (MAB11), mouse anti-human TNF-α mAb (MAB1), and HRP-conjugated avidin were purchased from eBioscience; mouse anti-phosphorylated ERK mAb (E-4 that recognizes both ERK-1 and ERK-2 phosphorylated at Tyr^{204}), rabbit anti-ERK polyclonal Ab (K-23 that recognizes both ERK-1 p44 and ERK-2 p42), and mouse anti-Bcl-x_L mAb (H-5) from Santa Cruz Biotechnology; mouse anti-human IL-1β mAb (clone 8516.31), mouse anti-human TNF-α mAb (clone 1825.12), mouse anti-human IL-8 mAb (clone 6217), and biotinylated goat anti-human IL-8 polyclonal Ab from Genzyme Techne; mouse anti-human epidermal growth factor receptor (EGFR) mAb (clone LA1) from Upstate Biotechnology; HRP-conjugated goat anti-mouse IgG/IgM from Chemicon International; HRP-conjugated goat anti-rabbit IgG from Organon Teknika; HRP-conjugated streptavidin from Zymed Laboratories; FITC-conjugated mouse anti-human CD3 mAb (S4.1) and allophycocyanin-conjugated mouse anti-human CD20 mAb (HI47) from Caltag Laboratories; PE-conjugated mouse anti-human CD14 mAb (MY4) from Beckman Coulter; and mouse purified control IgG from Jackson ImmunoResearch Laboratories.

Cell preparation

This study was approved by the Institutional Human Subject's Review Board (Juntendo University, School of Medicine). Informed consent was obtained from healthy volunteers, and blood was drawn from cubital vein. Neutrophils and mononuclear cells were isolated from heparinized blood by dextran sedimentation of erythrocytes, followed by centrifugation over Ficoll-Paque Plus (Amersham Biosciences) density gradient (29). Purities were determined by differential cytospin counts with May-Grünwald-Giemsa stain, and forward light scatter/side light scatter gating of cells stained with FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD14 mAb, and allophycocyanin-conjugated anti-CD20 mAb using a flow cytometer (FACSVantage; BD Biosciences). Neutrophil fraction contained 94.0 ± 2.0% of neutrophils, 5.7 ± 2.1% of eosinophils, 0.15 ± 0.36% of lymphocytes, and 0.15 ± 0.29% of monocytes ($n = 24$); mononuclear cell fraction contained 85.8 ± 2.3% lymphocytes, 11.4 ± 1.1% monocytes, and 2.8 ± 0.6% neutrophils ($n = 4$). After washing with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.4)), cells were suspended at 10^6 cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sanko Junyaku); FBS contained <5 pg/100 ml LPS, as certified by the manufacturer.

Assessment of neutrophil apoptosis

Neutrophils (10^6 cells/ml) were incubated in the presence or absence of LL-37 (0.01∼5 μg/ml) or LPS (*E. coli* O111:B4, 10 ng/ml) at 37°C for 18 h in RPMI 1640–10% FBS in 5% CO_2 in Falcon 2063 tubes. To further evaluate the involvement of FPRL1 and $P2X_7$ in the LL-37-induced suppression of neutrophil apoptosis, neutrophils were incubated with 1 μg/ml LL-37 at 37°C for 18 h in the presence or absence of an FPRL1 antagonist WRW^4 (10 μM) (27) or $P2X_7$ inhibitors (100 μM Ox-ATP and 5 μM KN-93) (30–32). Moreover, neutrophils were directly incubated with FPRL1 agonists (0.1∼10 μM WKYMVm and MMK-1) (28) or a $P2X_7$ agonist (50∼500 μM Bz-ATP) (30, 31) at 37°C for 18 h. After incubation, cells were cytocentrifuged (Cytospin 4; ThermoShandon) and stained with May-Grünwald-Giemsa. A minimum of 300 neutrophils/slide was examined by light microscopy on duplicate cytospins, and apoptotic neutrophils were identified based on morphological changes characteristic of apoptosis, such as chromatin condensation, formation of rounded nuclear profiles, cell shrinking, membrane blebbing, and presence of cytoplasmic vacuolization (29, 33). Alternatively, cells were stained with Annexin V^{FITC} and propidium iodide, according to the manufacturer's instruction (Sigma-Aldrich). After a 10-min incubation in the dark, cells were analyzed by flow cytometry (FACSVantage). Apoptotic neutrophils were defined as annexin V-positive but propidium iodide-negative cells, and viable neutrophils as annexin V- and propidium iodide-negative cells (34). Results were expressed as a percentage of apoptotic cells. Because the two methods for the assessment of neutrophil apoptosis (morphological changes and annexin V binding) closely correlated with each other (29, 33), neutrophil apoptosis was evaluated essentially based on the morphological changes.

Measurement of caspase 3 activity

Caspase 3 activity was measured with a colorimetric assay kit (Sigma-Aldrich), as previously reported (29). In brief, after incubation with various agents (18 h, 37°C), neutrophils (3×10^6 cells) were lysed in 60 μl of lysis buffer (50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (pH 7.4), 5 mM 3-[(3-cholamidopropyl)dimethlyammonio]-1-propanesulfonate, and 5 mM DTT) and disrupted on ice by sonication. The sonicates were centrifuged (17,400 × *g* for 10 min), and the supernatants (10 μl; 5×10^5 cell equivalents, containing ∼20 μg of protein) were incubated with 2 mM acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide substrate in the absence or presence of 200 μM acetyl-Asp-Glu-Val-Asp-al, a specific inhibitor for caspase 3 at 37°C for 2 h in a total volume of 100 μl of assay buffer. Caspase 3 activity was measured at 405 nm in a model 3550-UV microplate reader (Bio-Rad), and expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h.

The protein contents were determined with a bicinchoninic acid protein assay kit (Pierce).

Western blot analysis of the phosphorylation of ERK-1/2 and expression of Bcl-x_L

Neutrophils (10^6 cells/ml) were incubated in the absence or presence of LL-37 (1 μg/ml) or LPS (10 ng/ml) for 4 h at 37°C in RPMI 1640–10% FBS. After washing with PBS containing 5 mM EDTA and 2 mM Na_3VO_4, cells were lysed in 30 μl of lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate, and 1 mM diisopropyl fluorophosphate) containing 1/25 v/v Complete (Roche Diagnostic Systems). The lysates were then mixed with 30 μl of SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.005% bromphenol blue, and 5% 2-ME), disrupted on ice by sonication, and centrifuged (17,400 × *g* for 10 min). The supernatants were denatured for 3 min at 100°C, and aliquots (20 μl; containing ∼3×10^5 cell equivalents) were subjected to SDS-PAGE on a 7.5–20% linear gradient of polyacrylamide under reducing condition. The resolved proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membrane (Millipore) using a Trans-Blot SD apparatus (Bio-Rad). The blots were blocked in Block Ace (Dainippon Pharmaceutical), and probed with mouse anti-phosphorylated ERK mAb (E-4; 1/500 dilution) or anti-Bcl-x_L mAb (H-5; 1/500). The blots were further probed with HRP-conjugated goat anti-mouse IgG/IgM (1/5000), and phosphorylated ERK-1/2 and Bcl-x_L were finally detected with SuperSignal West Pico chemiluminescent substrate (Pierce) (29).

Thereafter, the blots were stripped by incubating in the stripping buffer (2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 100 mM 2-ME) for 30 min at 60°C, and ERK-1/2 proteins contained in each sample were detected by reprobing with rabbit anti-ERK polyclonal Ab (K-23; 1/1000) and HRP-conjugated goat anti-rabbit IgG (1/5000).

Assay for the effect of apyrase and quantification of ATP in the culture supernatants

To test the participation of extracellularly released ATP in the LL-37-induced suppression of neutrophil apoptosis, neutrophils (10^6 cells/ml) were incubated with 1 μg/ml LL-37 or 5 mM ATP (as a positive control) at 37°C for 18 h in the presence or absence of 10 U/ml apyrase, an enzyme that hydrolyzes ATP to AMP. Alternatively, neutrophils were incubated with exogenously added ATP (0.001~5 mM) at 37°C for 18 h, and then neutrophil apoptosis was quantitated.

In addition, extracellularly released ATP was quantified by the sensitive firefly luciferase assay, as described previously (35). In brief, 10 μl aliquots of culture supernatants from LL-37-treated neutrophils (0.01~5 μg/ml; 37°C, 10 min and 18 h) or standard ATP were added to 0.1 ml of reaction buffer (50 mM Tris-acetate, 2 mM EDTA, 60 mM DTT, 0.072% BSA, and 10 mM magnesium acetate (pH 7.7)) containing 1 μg/ml luciferase (Roche Molecular Biochemicals) and 0.1 mM D-luciferin (Wako Pure Chemical). Luminescence was measured with a Lumat LB9501 luminometer (Berthold Technologies), and light output was given as the integral relative light units over the 10-s measuring period. The concentration of ATP was calculated from a standard curve.

Measurement of cytokine production by neutrophils and mononuclear cells

Neutrophils or mononuclear cells (10^6 cells/ml) were incubated in the absence or presence of LL-37 (0.01~5 μg/ml) for 18 h at 37°C in RPMI 1640–10% FBS. Culture supernatants were collected and used for quantification of IL-1β, TNF-α, and IL-8 by ELISA (29).

Microtiter plates (96-well half area flat bottom; Corning Glass) were coated with 25 μl/well anti-human IL-1β (CRM56) or anti-TNF-α (MAB1) mAb (1/250 dilution in 1× coating buffer; eBioscience) overnight at 4°C. After washing with PBS-0.05% Tween 20, plates were blocked with 1× Assay Diluent (eBioscience) for 1 h at room temperature. The plates were then washed, added with culture supernatants (25 μl/well), and incubated for 2 h at room temperature. After washing, the plates were incubated with a combination of biotin-conjugated anti-human IL-1β (CRM57) or anti-TNF-α (MAB11) mAb (1/250 dilution in 1× Assay Diluent; 1 h) and HRP-conjugated avidin (1/250 dilution in 1× Assay Diluent; 30 min). For detection of IL-8, microtiter plates were coated with anti-IL-8 mAb (clone 6217; 2 μg/ml diluted in PBS) and blocked with Block Ace. After incubation with culture supernatants, the plates were further incubated with a combination of biotin-conjugated anti-IL-8 polyclonal Ab (20 ng/ml in Block Ace) and HRP-conjugated streptavidin (500 ng/ml in Block Ace). IL-1β, TNF-α, or IL-8 was finally detected by incubation with 3,3′,5,5′-tetramethylbenzidine liquid substrate (25 μl/well) for ~15 min. The reaction was terminated by the addition of 25 μl/well 2 M H_2SO_4, and absorbance at 450 and 570 nm was quantitated in a microplate reader. The detection limits of the ELISAs were <15 pg/ml for IL-1β, TNF-α, and IL-8.

To further evaluate a role of inflammatory cytokines in the LL-37-induced suppression of neutrophil apoptosis, neutrophils (10^6 cells/ml) were incubated with LL-37 (0.01~5 μg/ml) at 37°C for 18 h in the presence or absence of 10 μg/ml neutralizing anti-IL-1β mAb (clone 8516.31), anti-TNF-α mAb (clone 1825.12), anti-human IL-8 mAb (clone 6217), or mouse control IgG, and neutrophil apoptosis was quantitated.

Statistical analysis

Data are expressed as mean ± SD, and analyzed for significant difference by a one-way ANOVA with multiple comparison test (Prism 4; GraphPad). Differences were considered statistically significant at $p < 0.05$.

Results

Effects of LL-37 on neutrophil apoptosis and caspase 3 activity

Before looking at the actions of LL-37, we determined the spontaneous apoptosis of neutrophils. When neutrophils were incubated alone for 18 h, they exhibited characteristic features of apoptosis, such as chromatin condensation, formation of rounded nuclear profiles, cell shrinking, and presence of cytoplasmic vacuolization (Fig. 1*B*). Alternatively, neutrophil apoptosis was evaluated by flow cytometry using FITC-annexin V and propidium iodide staining. Incubation of neutrophils alone for 18 h substantially induced apoptosis defined as annexin V positive, but propidium iodide negative (Fig. 1*E*). Evaluation of neutrophil apoptosis based on the morphological changes revealed that >50% of neutrophils underwent apoptosis after incubation alone for 18 h (resting vs control; $p < 0.001$) (Fig. 2*A*). LPS (10 ng/ml) used as a control stimulus

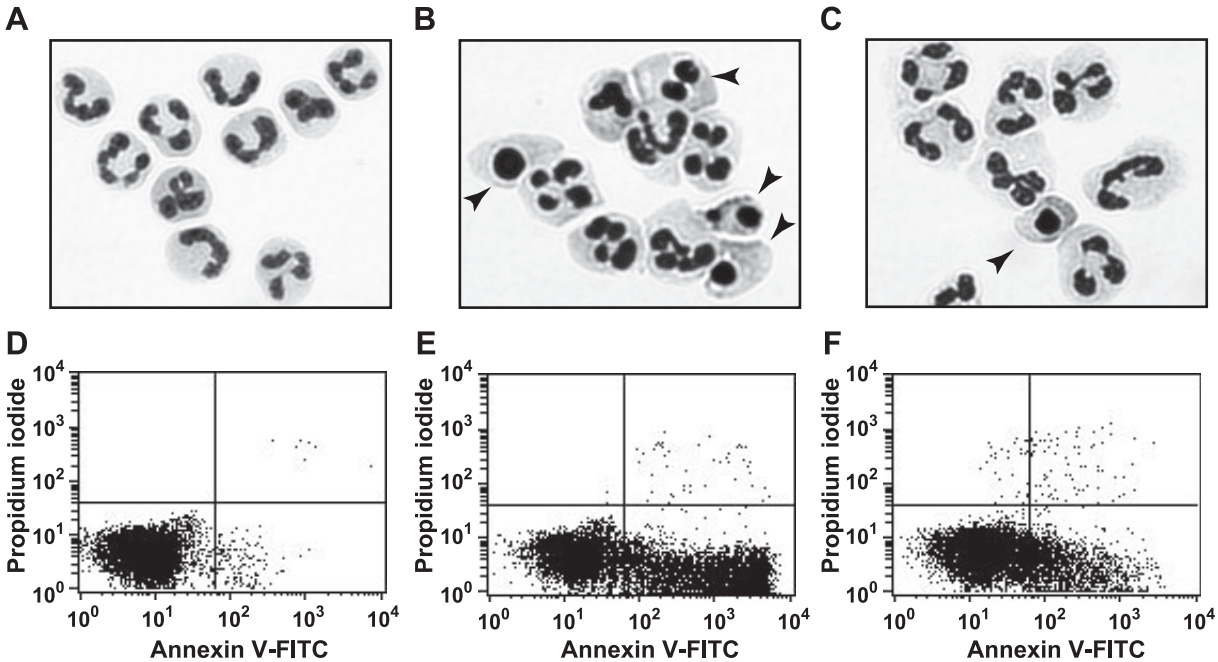

FIGURE 1. Assessment of neutrophil apoptosis by the morphological changes and FITC-annexin V/propidium iodide staining. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (*B* and *E*) or presence of LL-37 (1 μg/ml; *C* and *F*). Neutrophils were also incubated for 18 h at 4°C in the absence of LL-37 (*A* and *D*). After incubation, neutrophil apoptosis was assessed by the morphological changes (*A–C*) and FITC-annexin V/propidium iodide staining (*D–F*). Apoptotic neutrophils exhibit characteristic features of chromatin condensation, formation of rounded nuclear profiles, cell shrinking, and presence of cytoplasmic vacuolization (as indicated by arrowheads), and were defined as annexin V-positive but propidium iodide-negative cells. Shown are representative data.

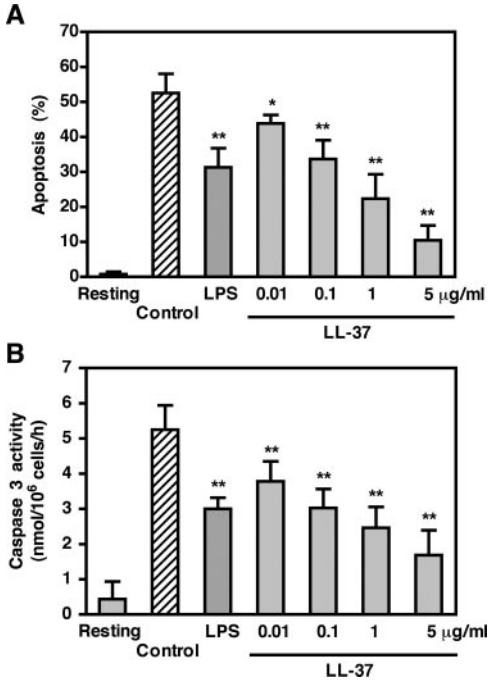

FIGURE 2. Effects of LL-37 on neutrophil apoptosis and caspase 3 activity. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of LL-37 (0.01~5 μg/ml) or LPS (10 ng/ml). Neutrophils were also incubated for 18 h at 4°C in the absence of LL-37 or LPS (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells (*A*). Alternatively, caspase 3 activity was assayed by incubating neutrophil lysates with acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide substrate in the absence or presence of acetyl-Asp-Glu-Val-Asp-al, a specific caspase 3 inhibitor at 37°C for 2 h. Caspase 3 activity is expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h (*B*). Data are the mean ± SD of 4–18 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of LL-37 or LPS. *, $p < 0.05$; **, $p < 0.001$.

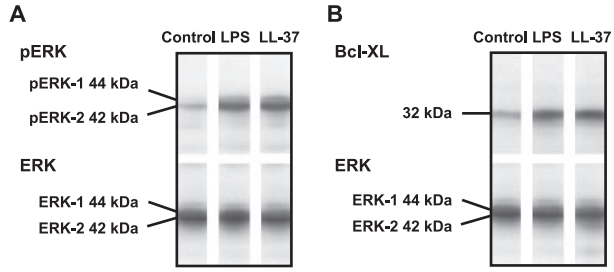

FIGURE 3. Effects of LL-37 on the phosphorylation of ERK and expression of Bcl-x_L. Neutrophils (10^6 cells/ml) were incubated for 4 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of LL-37 (1 μg/ml) or LPS (10 ng/ml). Phosphorylation of ERK and expression of Bcl-x_L were detected by probing with mouse anti-phosphorylated ERK mAb (*A*) or anti-Bcl-x_L mAb (*B*), and HRP-conjugated goat anti-mouse IgG/IgM. To confirm that equal amounts of proteins were analyzed in each sample, the blots were stripped, and ERK-1/2 proteins were detected by reprobing with rabbit anti-ERK polyclonal Ab and HRP-conjugated goat anti-rabbit IgG. Anti-phosphorylated ERK mAb can recognize both ERK-1 and ERK-2 phosphorylated at Tyr^{204} (pERK-1 and -2; *upper half* of *A*), and rabbit anti-ERK polyclonal Ab detects both ERK-1 p44 and ERK-2 p42 (ERK-1 and -2; *lower halves* of *A* and *B*). Data are from one of four separate experiments.

reduced neutrophil apoptosis from 52.6 ± 5.5% ($n = 10$) to 31.2 ± 5.5% (control vs LPS; $p < 0.001$). Interestingly, spontaneous apoptosis of neutrophils was inhibited by incubation with LL-37 (Fig. 1, *C* and *F*); LL-37 dose dependently suppressed neutrophil apoptosis, and neutrophil apoptosis was reduced to 10.4 ± 4.2% by 5 μg/ml LL-37 (control vs LL-37; $p < 0.001$) (Fig. 2*A*).

Next, we evaluated the activation of caspase 3, a key executor for apoptosis (36). Consistent with the changes in the number of apoptotic cells, caspase 3 activity was increased from 0.44 ± 0.50 to 5.25 ± 0.69 nmol/10^6 cells/h after 18 h of incubation (resting vs control, $p < 0.001$) (Fig. 2*B*), and the activity was reduced to 3.00 ± 0.31 nmol/10^6 cells/h by LPS stimulation (10 ng/ml) (control vs LPS; $p < 0.001$). Of importance, LL-37 dose dependently suppressed the activation of caspase 3; caspase 3 activity was reduced to 1.69 ± 0.71 nmol/10^6 cells/h by 5 μg/ml LL-37 (control vs LL-37; $p < 0.001$).

Effects of LL-37 on the phosphorylation of ERK and expression of Bcl-x_L

To clarify the mechanism for the action of LL-37, we investigated the signaling molecules that mediate suppression of apoptosis.

First, we looked at the effect of LL-37 on the phosphorylation of ERK, a member of mitogen-activated kinase family. As shown in Fig. 3*A*, LL-37 (1 μg/ml) stimulation strikingly enhanced the phosphorylation of ERK-1/2. Furthermore, we evaluated the effect of LL-37 on the expression of Bcl-x_L, an antiapoptotic protein. LL-37 (1 μg/ml) markedly induced the expression of Bcl-x_L (Fig. 3*B*).

As expected (29), LPS (10 ng/ml) used as a control stimulus substantially augmented the phosphorylation of ERK and expression of Bcl-x_L (Fig. 3).

Involvement of FPRL1 and $P2X_7$ in the LL-37-induced suppression of neutrophil apoptosis

It has been demonstrated that LL-37 uses FPRL1 as a receptor to chemoattract neutrophils, monocytes, and T cells (23). In addition, LL-37 is reported to promote the processing and release of IL-1β from monocytes via the activation of $P2X_7$ receptor (24). Thus, we determined the involvement of FPRL1 and $P2X_7$ in the LL-37-induced suppression of neutrophil apoptosis.

First, we evaluated the effects of FPRL1 antagonist and $P2X_7$ inhibitors. As shown in Fig. 4*A*, inhibitory agents for FPRL1 (WRW^4) (10 μM) (27) and $P2X_7$ (Ox-ATP, 100 μM; KN-93, 5 μM) (30–32) significantly reversed the LL-37-induced suppression of neutrophil apoptosis (LL-37 vs WRW^4 + LL-37, Ox-ATP + LL-37, or KN-93 + LL-37; $p < 0.001$), although these agents did not induce neutrophil apoptosis by themselves (control vs WRW^4, Ox-ATP, or KN-93; $p > 0.05$). Similarly, FPRL1 antagonist (WRW^4, 10 μM) and $P2X_7$ inhibitors (Ox-ATP, 100 μM; KN-93, 5 μM) obviously attenuated the LL-37-induced inhibition of caspase 3 activity (LL-37 vs WRW^4 + LL-37, Ox-ATP + LL-37, or KN-93 + LL-37; $p < 0.001$), although these agents gave no effect on the enzyme activity by themselves (control vs WRW^4, Ox-ATP, or KN-93; $p > 0.05$) (Fig. 4*B*). These observations apparently suggest that FPRL1 and $P2X_7$ are involved in the LL-37-induced suppression of neutrophil apoptosis.

Next, to further determine the involvement of FPRL1 and $P2X_7$ in the suppression of neutrophil apoptosis, neutrophils were directly incubated with the FPRL1 and $P2X_7$ agonists, and apoptosis was evaluated. As shown in Fig. 5*A*, agonistic agents for FPRL1 (WKYMVm, 0.1~10 μM; MMK-1, 0.1~10 μM) (28) and $P2X_7$ (Bz-ATP, 50~500 μM) (30, 31) dose dependently suppressed neutrophil apoptosis (control vs WKYMVm, MMK-1, or Bz-ATP; $p < 0.001$). Notably, the combinations of FPRL1 and $P2X_7$ agonists cooperatively suppressed neutrophil apoptosis as well as the activation of caspase 3 (WKYMVm, MMK-1, or Bz-ATP vs

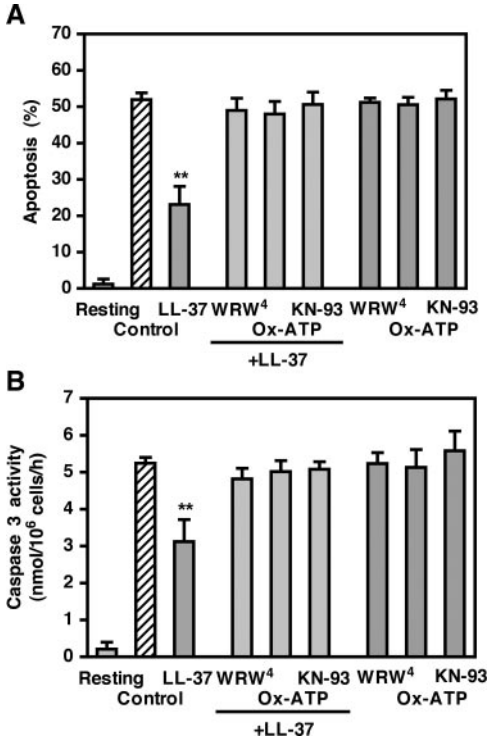

FIGURE 4. Effects of FPRL1 and $P2X_7$ antagonists on the LL-37-induced suppression of neutrophil apoptosis and caspase 3 activity. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of LL-37 (1 μg/ml), WRW^4 (10 μM), Ox-ATP (100 μM), KN-93 (5 μM), or their combination (+LL-37; 10 μM WRW^4 and 1 μg/ml LL-37, 100 μM Ox-ATP and 1 μg/ml LL-37, or 5 μM KN-93 and 1 μg/ml LL-37). Neutrophils were also incubated for 18 h at 4°C in the absence of LL-37, FPRL1, or $P2X_7$ antagonists (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells (*A*). Alternatively, caspase 3 activity was assayed, and expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h (*B*). Data are the mean ± SD of 4–12 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of LL-37, WRW^4, Ox-ATP, KN-93, or their combination. **, $p < 0.001$.

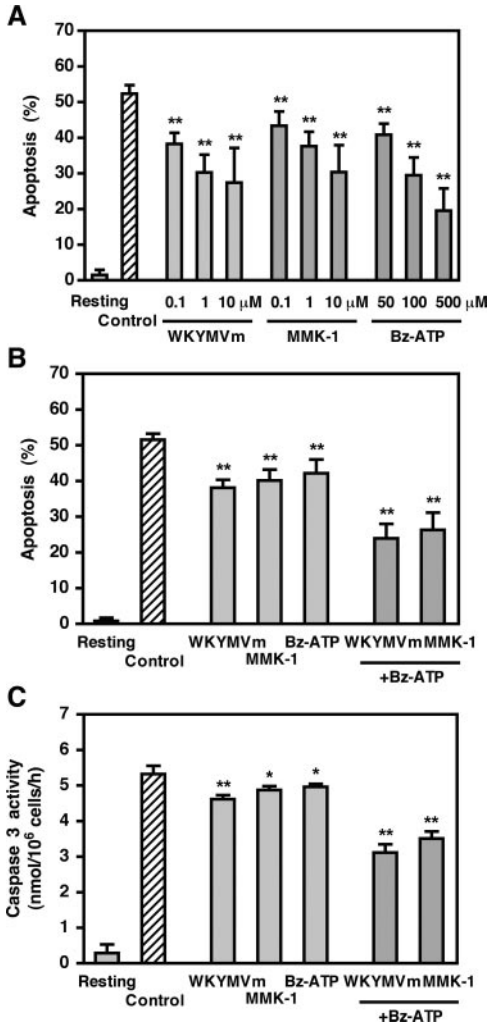

FIGURE 5. Effects of FPRL1 and $P2X_7$ agonists on neutrophil apoptosis and caspase 3 activity. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of WKYMVm (0.1~10 μM), MMK-1 (0.1~10 μM), or Bz-ATP (50~500 μM) (*A*). Alternatively, neutrophils were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of WKYMVm (0.1 μM), MMK-1 (1 μM), Bz-ATP (50 μM), or their combination (+Bz-ATP; 0.1 μM WKYMVm and 50 μM Bz-ATP, or 1 μM MMK-1 and 50 μM Bz-ATP) (*B* and *C*). Neutrophils were also incubated for 18 h at 4°C in the absence of FPRL1 or $P2X_7$ agonists (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells (*A* and *B*). Caspase 3 activity was also assayed, and expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h (*C*). Data are the mean ± SD of 4–15 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of WKYMVm, MMK-1, Bz-ATP, or their combination. *, $p < 0.05$; **, $p < 0.001$.

WKYMVm + Bz-ATP, or MMK-1 + Bz-ATP; $p < 0.001$) (Fig. 5, *B* and *C*). These observations most likely indicate that the activation of FPRL1 and $P2X_7$ in concert acts to induce the suppression of neutrophil apoptosis.

However, it is unclear whether both FPRL1 and $P2X_7$ are activated in parallel to promote the survival of neutrophils. It is postulated that $P2X_7$ activation is downstream of FPRL1, if a $P2X_7$ antagonist inhibits the FPRL1 activation. In contrast, FPRL1 activation is assumed to be downstream of $P2X_7$, if an FPRL1 antagonist inhibits the $P2X_7$ activation. Thus, we evaluated the effects of FPRL1 antagonist and $P2X_7$ inhibitors on the suppression of neutrophil apoptosis induced by FPRL1 and $P2X_7$ agonists. As expected, FPRL1 antagonist (WRW^4, 10 μM) and $P2X_7$ inhibitors (Ox-ATP, 100 μM; KN-93, 1 μM) attenuated the FPRL1 (WKYMVm, 1 μM; MMK-1, 5 μM) and $P2X_7$ (Bz-ATP, 100 μM) agonist-induced suppression of neutrophil apoptosis, respectively (WKYMVm vs WRW^4 + WKYMVm, MMK-1 vs WRW^4 + MMK-1, Bz-ATP vs Ox-ATP + Bz-ATP or KN-93 + Bz-ATP; $p < 0.001$) (Fig. 6). Importantly, $P2X_7$ inhibitors (Ox-ATP and KN-93) also abrogated the FPRL1 agonist (WKYMVm and MMK-1)-induced suppression of neutrophil apoptosis (WKYMVm vs Ox-ATP + WKYMVm or KN-93 + WKYMVm, MMK-1 vs Ox-ATP + MMK-1 or KN-93 + MMK-1; $p < 0.001$), although an FPRL1 antagonist (WRW^4) did not affect the action of $P2X_7$ agonist (Bz-ATP) (Bz-ATP vs WRW^4 + Bz-ATP; $p > 0.05$). These observations suggest a possibility that $P2X_7$ is likely to be activated downstream of FPRL1.

Roles of ATP and inflammatory cytokines in the LL-37-induced suppression of neutrophil apoptosis

Because LL-37 stimulation is shown to release ATP from monocytes (24), it is possible that ATP is released from neutrophils during incubation with LL-37 and acts on $P2X_7$, thereby inducing

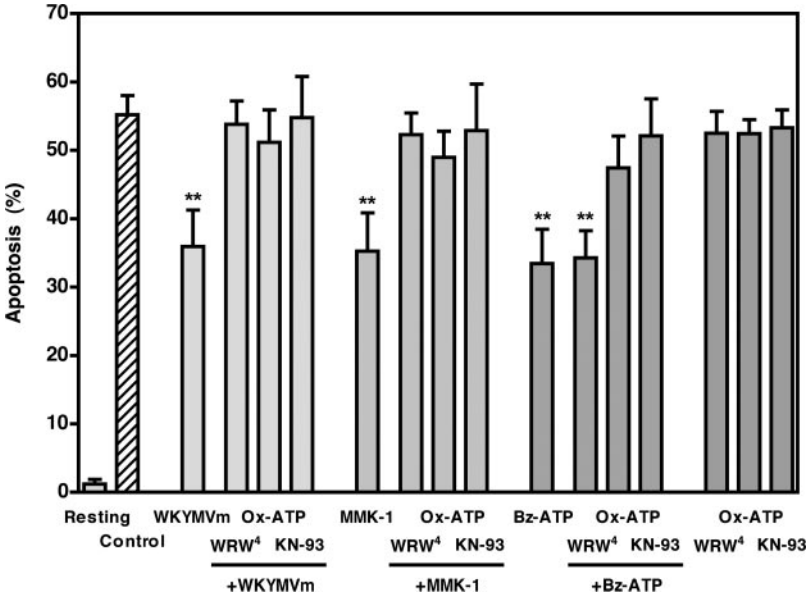

FIGURE 6. Effects of FPRL1 antagonist and $P2X_7$ inhibitors on the suppression of neutrophil apoptosis induced by FPRL1 and $P2X_7$ agonists. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of WKYMVm (1 μM), MMK-1 (5 μM), Bz-ATP (100 μM), WRW^4 (10 μM), Ox-ATP (100 μM), KN-93 (1 μM), or their combination (WRW^4 + WKYMVm, Ox-ATP + WKYMVm, KN-93 + WKYMVm; WRW^4 + MMK-1, Ox-ATP + MMK-1, KN-93 + MMK-1; WRW^4 + Bz-ATP, Ox-ATP + Bz-ATP, KN-93 + Bz-ATP). Neutrophils were also incubated for 18 h at 4°C in the absence of FPRL1 and $P2X_7$ agonists, or FPRL1 and $P2X_7$ antagonists (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells. Data are the mean ± SD of 5–11 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of WKYMVm, MMK-1, Bz-ATP, WRW^4, Ox-ATP, KN-93, or their combination. **, $p < 0.001$.

suppression of apoptosis. Thus, we asked whether ATP participates in the observed effect of LL-37 on neutrophil apoptosis. Of note, exogenously added ATP exhibited an antiapoptotic activity at >0.1 mM (control vs 0.1 mM ATP; $p < 0.05$) (Fig. 7*A*). As expected, the addition of an ATP-degrading enzyme apyrase completely attenuated the action of exogenous ATP on neutrophil apoptosis (ATP vs ATP + apyrase; $p < 0.001$); however, apyrase never affected the LL-37-induced suppression of neutrophil apoptosis (LL-37 vs LL-37 + apyrase; $p > 0.05$) (Fig. 7*B*). Consistent with this, the levels of ATP detected in the culture supernatants of neutrophils (10^6 cells/ml) were only 10~50 nM after incubation with LL-37 (0.01~5 μg/ml), which were too much lower than those required to prevent neutrophil apoptosis. These observations apparently indicate that ATP, if any, released from neutrophils is unlikely to participate in the suppression of neutrophil apoptosis during incubation with LL-37.

Furthermore, it could be possible that proinflammatory cytokines (such as IL-1β, TNF-α, and IL-8) are produced and induce the suppression of neutrophil apoptosis during incubation with LL-37, because these cytokines are shown to repress neutrophil apoptosis (6, 7). To test this possibility, we measured the cytokine production by neutrophils and mononuclear cells (10^6 cells/ml) after incubation with LL-37 (0.01~5 μg/ml). The production levels of IL-1β, TNF-α, and IL-8 were <16, <16, and ~60 pg/ml for neutrophils, and <16 pg/ml, ~30 pg/ml, and 2 ng/ml for mononuclear cells, respectively. Furthermore, the effects of neutralizing mAbs (10 μg/ml) against IL-1β, TNF-α, and IL-8 were determined on the apoptosis of neutrophils during incubation with LL-37 (0.01~5 μg/ml); however, these mAbs gave no substantial effect on the LL-37-induced suppression of neutrophil apoptosis (data not shown). Given that IL-1β, TNF-α, and IL-8 exhibit antiapoptotic actions on neutrophils at >500 pg/ml (29), these findings suggest that the cytokine production by neutrophils and contaminated mononuclear cells (<0.3% in the neutrophil preparation) is too low to affect the apoptosis of neutrophils during incubation with LL-37.

Discussion

Peptide antibiotics, as the effectors in the innate host defense system, exhibit antimicrobial activities against a broad spectrum of microbes, including both Gram-positive and Gram-negative bacteria, fungi, and viruses (14–17). LL-37/hCAP18 is the only one human peptide that belongs to a cathelicidin family of antimicrobial peptides (18–20). LL-37 not only displays the potent antibacterial activities against Gram-positive and Gram-negative bacterial, but also can bind to LPS and neutralize its biological activities (19, 21, 22). Furthermore, LL-37 possesses the ability to chemoattract neutrophils, monocytes, and T cells (23).

In this study, we determined the effect of LL-37, a multifunctional antimicrobial peptide (25, 26), on the apoptosis of neutrophils. The results indicated that LL-37 can suppress neutrophil apoptosis, accompanied with the phosphorylation of ERK-1/2, expression of Bcl-x_L, and inhibition of caspase 3 activity. In addition, the present data suggest that the activation of FPRL1 and $P2X_7$ is involved in the LL-37-induced suppression of neutrophil apoptosis. It has been shown that the activation of ERK, a member of mitogen-activated kinase family, generates the survival signals via the up-regulation of antiapoptotic proteins of Bcl-2 family (such as Bcl-x_L) to prolong the life span of cells (7, 37). Moreover, apoptosis is modulated through the expression of Bcl-x_L that regulates the activation of caspase 3, one of death proteases functioning as the central executioners of apoptosis (36, 38, 39). Of importance, the activation of FPRL1 or $P2X_7$ is demonstrated to induce the phosphorylation of ERK-1/2 in human monocytic cells and neutrophils (40, 41). Thus, it is feasible to assume that the stimulation of neutrophils with LL-37 induces the phosphorylation of ERK-1/2 and the subsequent expression of antiapoptotic protein Bcl-x_L, which inhibits caspase 3 activity and suppresses neutrophil apoptosis via the activation of FPRL1 or $P2X_7$.

Cationic antimicrobial peptides (such as defensins and cathelicidins) kill the invaded microorganisms by perturbing their membranes; the action of those peptides is not receptor mediated, but involves a less specific interaction with microbial membrane components, because the peptides target cell surface anionic lipids such as phosphatidyl glycerol and cardiolipin that are abundant in microorganisms (42, 43). In contrast, the mammalian cell membrane is mainly composed of electrically neutral phospholipids such as phosphatidylcholine and sphingomyelin, for which the affinity of antimicrobial peptides is generally low (42). Interestingly, human β-defensin-2 is shown to chemoattract T cells, immature dendritic cells, and TNF-α-primed neutrophils through the action on the cell surface CCR6 (44, 45). In addition, human neutrophil peptides

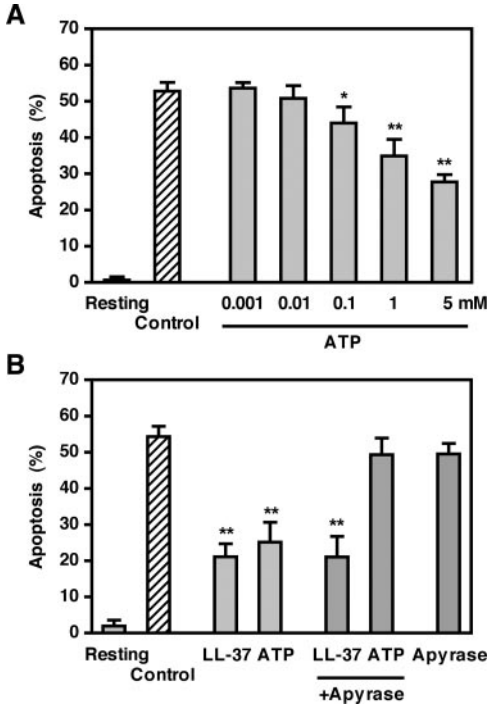

FIGURE 7. Effect of apyrase on the LL-37-induced suppression of neutrophil apoptosis. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of ATP (0.001~5 mM) (*A*). Alternatively, neutrophils were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of LL-37 (1 μg/ml), ATP (5 mM), apyrase (10 U/ml), or their combination (+Apyrase; 1 μg/ml LL-37 and 10 U/ml apyrase, or 5 mM ATP and 10 U/ml apyrase) (*B*). Neutrophils were also incubated alone for 18 h at 4°C in the absence of LL-37, ATP, or apyrase (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells. Data are the mean ± SD of 4–10 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of LL-37, ATP, apyrase, or their combination. *, $p < 0.05$; **, $p < 0.0001$.

(defensins) are reported to induce IL-8 production from A549 lung epithelial cells via the activation of $P2Y_6$ nucleotide receptor (46). In contrast, LL-37 functions as a chemoattractant for neutrophils, monocytes, and T cells via the interactions with FPRL1, a low affinity formyl-peptide receptor (23). Moreover, LL-37 is shown to promote the processing and release of IL-1β from monocytes via the activation of $P2X_7$ nucleotide receptor (24). In addition, it is known that neutrophils express both FPRL1 and $P2X_7$ on their surfaces (28, 47). Thus, we addressed the issue as to whether the LL-37-induced suppression of neutrophil apoptosis is mediated by FPRL1 and/or $P2X_7$. The present data demonstrated that the suppressive effect of LL-37 on neutrophil apoptosis was inhibited by both an FPRL1 antagonist (WRW^4) (27) and $P2X_7$ inhibitors (Ox-ATP and KN-93) (30–32). Furthermore, FPRL1 agonists (WKYMVm and MMK-1) (28) and a $P2X_7$ agonist (Bz-ATP) (30, 31) collaboratively suppressed the neutrophil apoptosis. Collectively, these observations suggest that the LL-37 induces the suppression of neutrophil apoptosis via the activation of both FPRL1 and $P2X_7$. In addition, the suppression of FPRL1 activation by $P2X_7$ antagonists suggests that $P2X_7$ is likely to be activated downstream of FPRL1. Alternatively, LL-37 is reported to *trans* activate EGFR and release IL-8 from airway epithelial cells (48). In this study, however, we revealed that a neutralizing anti-EGFR mAb (clone LA1; 10 μg/ml) and a EGFR tyrosine kinase inhibitor AG1478 (1 μM) (48) never affected the action of LL-37 on neutrophil apoptosis (data not shown), suggesting that the EGFR activation is unlikely to be involved in the suppression of neutrophil apoptosis by LL-37.

To date, FPRL1 is proposed as the only receptor to which LL-37 can directly bind (28), whereas the direct interaction of LL-37 with a nucleotide receptor $P2X_7$ is not determined (24). Thus, LL-37 could be speculated to facilitate the extracellular release of ATP from the cells (24), which indirectly activates $P2X_7$ and subsequently suppresses neutrophil apoptosis. However, the present findings indicated that an ATP-hydrolyzing enzyme apyrase did not affect the LL-37-induced suppression of neutrophil apoptosis, and the released levels of ATP in the extracellular medium were too low to induce the suppression of neutrophil apoptosis. In another aspect, $P2X_7$ is shown to participate in the modulation of cellular reactions, such as macrophage responses to LPS, and the C-terminal portion of $P2X_7$ is thought to be essential for the functions of this receptor (49, 50). In fact, the $P2X_7$ C-terminal domain contains multiple protein-protein and protein-lipid interaction motifs with potential importance to the intracellular signaling and LPS action, and is able to directly bind with LPS, an amphipathic lipid-sugar molecule (51). However, LPS would have to be internalized in the cell before binding to $P2X_7$, because the C-terminal domain of $P2X_7$ is predicted to be located inside the cell (50, 51). Based on these observations, it is tempting to speculate that LL-37, an amphipathic peptide, may also penetrate into the cell membrane and bind with the $P2X_7$ C-terminal domain via the protein-protein interaction, thereby activating intracellular signaling to induce the suppression of neutrophil apoptosis. However, it cannot be ruled out that LL-37 interacts with the ligand-binding site of $P2X_7$, because the action of LL-37 was inhibited by Ox-ATP, an irreversible $P2X_7$ blocker that reacts with unprotonated lysine residues located in the vicinity of the ATP binding site of the receptor (52). It remains to be clarified whether LL-37 can activate $P2X_7$ through a direct interaction or an as of yet-undefined indirect mechanism.

hCAP18/LL-37 was first identified in neutrophils and later shown to be expressed in various squamous epithelia, lung epithelial cells, keratinocytes in inflamed skin, specific lymphocytes (NK cells, $\gamma\delta$T cells, and B cells) and monocytes, and skin mast cells (53–55). Interestingly, hCAP18/LL-37 can be locally induced at sites of inflammation and infection within epithelial cells, and invading neutrophils are likely to represent an additional source for the peptide (53, 56). In this context, it has been reported that the concentration of hCAP18/LL-37 increases up to 15 μg/ml in bronchoalveolar fluids of patients with pulmonary or systemic infections (56, 57). Moreover, the plasma level of hCAP18/LL-37 is shown to be 1.18 μg/ml in healthy individuals (58). Importantly, the present study indicated that LL-37 can suppress neutrophil apoptosis at >0.01 μg/ml in vitro. Thus, it is likely to be expected that the apoptosis of neutrophils could be modulated in vivo at the sites of inflammation or infection, or in the normal blood by LL-37 present in the local extracellular milieu. Consistent with this, it has been demonstrated that antiapoptotic genes are up-regulated, but proapoptotic genes are down-regulated in neutrophils that transmigrated to the inflammatory skin lesions in vivo in response to various chemotactic factors such as LL-37 (59).

Clearance of neutrophils from inflamed tissues is critical for the resolution of inflammation. Clinical studies have indicated that spontaneous apoptosis of neutrophils is inhibited in patients with sepsis, systemic inflammatory syndrome, and acute respiratory distress syndrome by the actions of various bacterial products, cytokines, and chemokines detected in these disorders (3–7). Activated neutrophils with prolonged survival are assumed to cause the amplified systemic inflammation, tissue injury, and organ failure via

the uncontrolled release of cytotoxic metabolites and proinflammatory substances (8, 9). From this point of view, LL-37 is supposed to exert a harmful effect during inflammation by suppressing apoptosis and prolonging life span (survival) of neutrophils, which may lead to the amplification of inflammatory reactions. In contrast, physiological process of neutrophil apoptosis can be subverted by bacterial pathogens during infections (60). Inappropriate or premature apoptosis of neutrophils could deplete cell numbers and functions, impairing host defense and favoring bacterial persistence in infections. In this context, it has been reported that neutrophil apoptosis is accelerated and neutrophil-mediated host defense is impaired in vivo during infection with *P. aeruginosa* by the action of pyocyanin, a predominant phenazine exotoxin (61). Considering its antiapoptotic actions, LL-37 is expected to exert an advantageous effect on host defense against bacterial infections by prolonging the life span of neutrophil, a major phagocyte that kills the invaded bacteria. It remains to be determined what effect(s) LL-37 can exhibit in the processes of inflammation and infection via its regulatory action on neutrophil apoptosis.

As for the effects of cathelicidin peptides on mammalian cell apoptosis, a porcine peptide PR-39 is shown to inhibit the apoptosis of murine RAW264.7 cells (62). In contrast, bovine peptides BMP-27/28 and a 27-mer peptide of hCAP18/LL-7 (corresponding to F^{6}-V^{32}) induce the apoptosis of tumor cells such as leukemia and squamous carcinoma cells (63, 64). However, the mechanisms and receptors involved in the antiapoptotic and proapoptotic actions of these peptides are not clarified.

LL-37 is originally identified as an antimicrobial peptide, which participates in the innate immune system, capable of protecting host from invasive microbial infections and neutralizing Gram-negative LPS (19–22, 65), and now regarded as a multifunctional molecule that links the innate immune response to the adaptive immune system by exerting various immunomodulatory actions, such as production of cytokines and chemokines, induction of immune cell chemotaxis, and alteration of dendritic cell differentiation (25, 26). The present study has demonstrated for the first time an additional function of LL-37 to prolong the life span of neutrophils possibly via the activation of FPRL1 and $P2X_7$ receptor. This finding provides a novel insight into the role of LL-37 in the regulation of neutrophil life span (apoptosis) as well as the innate and adaptive host defense systems.

Disclosures

The authors have no financial conflict of interest.

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