Cathelicidin LL-37 peptide regulates endothelial cell stiffness and endothelial barrier permeability

Fitzroy J. Byfield,1 Qi Wen,1 Katarzyna Leszczyńska,2 Alina Kulakowska,1,3 Zbigniew Namiot,4 Paul A. Janmey,1 and Robert Bucki1

1Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania; and 3Department of Diagnostic Microbiology, 2Department of Neurology, and 4Department of Physiology, Medical University of Białystok, Białystok, Poland

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Byfield FJ, Wen Q, Leszczyńska K, Kulakowska A, Namiot Z, Janmey PA, Bucki R. Cathelicidin LL-37 peptide regulates endothelial cell stiffness and endothelial barrier permeability. Am J Physiol Cell Physiol 300: C105–C112, 2011. First published October 13, 2010; doi:10.1152/ajpcell.00158.2010.—LL-37 peptide is a multifunctional host defense molecule essential for normal immune responses to infection or tissue injury. In this study we assess the impact of LL-37 on endothelial stiffness and barrier permeability. Fluorescence microscopy reveals membrane localization of LL-37 after its incubation with human umbilical vein endothelial cells (HUVECs). A concentration-dependent increase in stiffness was observed in HUVECs, bovine aortic endothelial cells (BAECs), human pulmonary microvascular endothelial cells, and mouse aorta upon LL-37 (0.5–5 μM) addition. Stiffening of BAECs by LL-37 was blocked by P2X7 receptor antagonists and by the intracellular Ca2+ chelator BAPTA-AM. Increased cellular stiffness correlated with a decrease in permeability of HUVEC cell monolayers after LL-37 addition compared with nontreated cells, which was similar to the effect observed upon treatment with sphingosine 1-phosphate, and both treatments increased F-actin content in the cortical region of the cells. These results suggest that the antiinflammatory effect of LL-37 at the site of infection or injury involves an LL-37-mediated increase in cell stiffening that prevents increased pericellular permeability. Such a mechanism may help to maintain tissue fluid homeostasis.

endothelium; cytoskeleton

THE LL-37 PEPTIDE is the only known cathelicidin-derived peptide expressed in humans. It is present in airways, digestive tract, genitourinary system, circulating blood, and skin (17). The final step of LL-37 production involves proteolysis of human cathelicidin (hCAP-18) by protease 3, which generates the active peptide from its COOH-terminal part (31). hCAP-18 is an 18-kDa protein predominantly found in neutrophils, in the cells of bone marrow, and in epithelial cells (1). Within neutrophils, hCAP-18 is stored as an inactive proform in peroxidase-negative granules. Production of cathelicidin occurs constitutively with a significant increase during infection (9). Recent studies have demonstrated that, in addition to its antimicrobial activity (34), LL-37 performs many activities related to innate immunity, including the induction or modulation of cytokine production, alteration of gene expression in host cells, and inhibition of proinflammatory responses of host cells to bacterial components such as lipopolysaccharide and lipoteichoic acid (5). It has been reported that LL-37 induces IL-8 production in a mitogen-activated protein kinase-dependent process (32), stimulates mast cells to mobilize intracellular calcium and releases histamine in a G protein/phospholipase C-dependent manner (28), and stimulates regeneration of human corneal epithelium in a process that involves activation of G protein-coupled receptors (15). However, it is not clearly defined whether LL-37’s effects require binding to specific transmembrane receptors or occur by other mechanisms (4, 6, 32, 33).

In the past decade, increased attention has been directed to understanding the pathophysiology of molecular determinants that govern cellular and extracellular matrix stiffness (20). Multiple extracellular stimuli mediated by receptors at the plasma membrane converge on the cytoskeleton, and its reorganization results in changes of stiffness. Attenuation of thrombin-induced permeability in endothelial cell (EC) monolayers correlates well with stiffness changes from the cell center to its periphery (3). Phorbol ester-mediated remodeling of actin cytoskeletal mechanics was found to result in pulmonary artery endothelial barrier dysfunction (24). Endothelial barrier-protecting mediators such as PGE2 and sphingosine 1-phosphate (S1P) can produce cytoskeletal rearrangements that promote interendothelial junction reformation (35). These findings indicate that regulation of cell stiffness through actin remodeling has consequences for cell function and is tightly regulated by cell signaling. Motivated by a previous study suggesting the receptor-mediated immunomodulatory activity of LL-37 at epithelial surfaces, we characterize here LL-37’s effects on the stiffness of various endothelial cell types. A concentration-dependent increase in cell stiffness was observed upon LL-37 (0.5–5 μM) addition to bovine aortic endothelial cells (BAECs), human umbilical vein endothelial cells (HUVECs), pulmonary microvascular endothelial cells, and mouse aorta. These observations indicate that the antiinflammatory effect of LL-37 peptide at the site of infection or injury may be enforced through an LL-37-mediated increase in endothelial cell stiffening that prevents increases in pericellular permeability.

MATERIALS AND METHODS

Materials. Rhodamine B-LL-37, LL-37 (LLGDFRKSSKEKIGKEFKRIVQRKDFLRNLVPRTES), and WLB2 (RWWRVRVRWWRVVRVVRVRVRWVRVRVVRWVRVR) peptides were purchased from Bachem (King of Prussia, PA). RK-31 (RKSKEKGFVRKIVQKRDFLRNLVPRTES) and KR-20 (KRIVQRKDFLRNLVPRTES) peptides were from Peptide2.0 (Chantilly, VA). Human thrombin (T-6848), BAPTA-AM (A1076), adenosine 5-triphosphate, periodate-oxidized sodium salt (ox-ATP) (A6779), and S1P (S9666) were obtained from Sigma (St. Louis, MO). Permeability
stress and is taken to be 0.5 for all samples. To determine endothelial cell applied stress and the extensional strain in the direction of the applied force to the material and the resulting stress. The Poisson ratio is defined as the ratio of transverse strain to the normal strain. Young's modulus is the inverse ratio between the strain (ε) and the stress (σ) in linear elastic deformation. The Poisson ratio is typically between 0 and 0.5, and Young's modulus is defined as the ratio of stress to strain in the material.

Cell culture and aorta preparation. BAECs were cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% calf serum (Hyclone, Logan, UT), and HUVECs and human pulmonary microvascular endothelial cells (HPMECs) were cultured in EGM-2 media at 37°C with 5% CO₂. Cells were seeded and allowed to spread for 24 h before experiments were performed. When required, BAECs were incubated with BAPTA-AM, a selective chelator of intracellular Ca²⁺, (10 µM for 30 min), or with the P2X7 receptor antagonists KN-62 (15 and 30 nM for 15 min) or ox-ATP (100 µM for 30 min) at 37°C before LL-37 stimulation. Male C57BL/6 mice (5–6 mo old) were fully sedated and then killed by cervical dislocation. The thoracic aorta was isolated, adventitia were carefully removed, and the tissue was cut into 3 mm long, 23 µm wide, 0.6 µm thick) with a pyramidal area (1.6-µm² area) for indentation. The spring constant of the cantilever, calculated by resonance measurements, was typically 0.06 N/m (DNP, Veeco). To quantify cellular stiffness, the first 600 nm of tip deflection from the horizontal (∆d) were fitted with the Hertz model modified for a cone

\[
\Delta d = \frac{k}{4A} + \Delta z + \frac{1}{2} \sqrt{\left(\frac{k}{A}\right)^2 + 4\frac{k\Delta z}{A}} \quad \text{and}
\]

\[
A = \frac{2}{\pi} \tan(\alpha) \frac{E}{1 - \nu^2}
\]

where k and Δz are the bending rigidity and the vertical indentation of the cantilever, E is Young's modulus, α is cone tip angle, and ν is the Poisson ratio. Young's modulus is the inverse ratio between the strain (ε/2) applied to the material and the resulting stress. The Poisson ratio is defined as the ratio of compression strain in the direction normal to the applied stress and the extensional strain in the direction of the applied stress and is taken to be 0.5 for all samples. To determine endothelial cell stiffness, AFM measurements were made on single cells by indenting on three positions of the peripheral cell body within a period of 30 min after agonist addition. The average elastic modulus for each condition was calculated by averaging the three measurements for each cell followed by averaging all obtained values. The AFM stiffness measurements made in isolated mouse aorta were done by taking 100 measurements over a 25 μm × 25 μm area and averaging them together.

Cell permeability assay. One of the most specific functions of the endothelial layer is to act as a selective barrier between blood/lymph and tissues. A standardized assay to evaluate permeability of cultured EC is to measure the passage of an easily detectable tracer between two compartments separated by a cell layer. To evaluate the permeability of HUVECs, we used FITC-dextran as a tracer. HUVECs were grown in collagen-coated permeability assay inserts according to the manufacturer’s recommendations. A confluent HUVEC monolayer after 72 h of growth was subjected to agonist treatment (LL-37, human thrombin, or S1P) in the presence of FITC-dextran. Monolayer permeability was assessed on the basis of fluorescence intensities measured using a multiple plate reader (Fluoroskan Ascent, Lab-systems) in the cell culture medium of the lower chamber.

F-actin and immunofluorescent staining. For visualization of F-actin, β-catenin, and VE-cadherin distribution, cells after various treatments were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature and stained with 1:100 rhodamine-labeled phalloidin (Invitrogen) in PBS for 30 min, mouse polyclonal antibody to VE cadherin (1:500), and rabbit polyclonal antibody to β-catenin (1:500) for 1 h. Samples were then rinsed with PBS and incubated with fluorescently labeled secondary antibody (1:100). Fluorescence images were taken using a LEICA DM IRBE.

Evaluation of LL-37 concentration in different body fluids. Samples of different body fluids were obtained from individuals admitted to the Department of Neurology at the Medical University of Bialystok, and the Department of Internal Medicine and Gastroenterology at the District Hospital in Bialystok. All specimens were centrifuged (2,000 g, 20 min), and the supernatants were frozen. The Medical University of Bialystok Ethics Committee for Research on Humans and Animals approved the study and written consent was obtained from all subjects. LL-37 concentration was measured using a sandwich enzyme-linked immunosorbent assay according to the manufacturer’s instructions. The detection limit was 0.1 ng/ml. According to previous reports, digestion of secreted LL-37 by pepsin or by a serine protease–dependent mechanism will generate multiple novel antimicrobial peptides distinct from the cathelicidin LL-37 (19, 25). Therefore, the presence of these LL-37 digestion products in body fluids might interfere with ELISA assessment of LL-37 concentration due to potential antibody cross-reaction. To address this issue, we performed an ELISA analysis of synthetic LL-37 peptide after its 3-h digestion with pepsin (0.5 mg/ml) at pH ~1.5, and two LL-37 fragments (KR-20 and KR-31) recently identified as a physiological component of sweat (25).

Statistical analysis. Data are reported as means ± SD from three to six experiments. Data analysis was performed using one-way analysis of variance (ANOVA) tests with a post hoc Bonferroni analysis test.

RESULTS

LL-37 localizes to the plasma membrane. A punctuate staining pattern was observed in HUVECs that were treated with LL-37 then stained with anti-LL-37 antibodies (Fig. 1B) compared with nontreated control (Fig. 1A). This distribution of LL-37 fluorescence may indicate a direct binding of LL-37 to specific receptors as well as nonuniform interaction with the endothelial plasma membrane. Preincubation of HUVECs with unlabeled LL-37 did not affect the staining pattern or intensity of rhodamine-B LL-37-labeled peptide (results not shown), indicating that ability of membrane insertion also governs LL-37 interaction with membrane. Additionally, the staining was resistant to subsequent rinsing. The ability of LL-37 to activate plasma membrane receptors was recently reported (12); however, insertion of LL-37 peptide into the lipid bilayer cannot be disregarded because of LL-37’s high membrane activity that is related to its general electrostatically driven anti-bacterial effects (7).

LL-37 increases stiffness of endothelial cells. In agreement with previous studies showing enhanced peripheral stiffness in agonist-stimulated human pulmonary cells associated with increased EC barrier properties (3), we observed a dose-depen-
dent increase in the elastic moduli of HPMECs, HUVECs, and BAECs after treatment with LL-37 (concentration ranging from 0.5 to 5 μM) (Fig. 1, C–E). These data were generated on the basis of analysis of the cell deformation as a result of the stress applied by AFM tip up to 600 nm. Because this depth corresponds primarily to the submembrane cytoskeletal region, and the stiffness of the plasma membrane can be neglected because of its low value compared to the cytoskeleton, our measurement reflects primarily the changes in cytoskeleton stiffness induced by LL-37 (3).

Antagonism of P2X7 receptor or calcium chelation inhibits LL-37-induced stiffening. Previous studies have demonstrated the importance of the P2X7 receptor, a member of the P2X family of nucleotide-dependent ion channels, in the effect of
LL-37 on mammalian cell physiology (12, 33). To determine whether P2X7 activation plays a role in LL-37’s stiffening effect, BAECs were pretreated with KN-62, a potent noncompetitive antagonist of the P2X7 receptor. This treatment resulted in a dose-dependent inhibition of LL-37’s effect. Furthermore, this observation was confirmed with the use of ox-ATP, an irreversible antagonist of the P2X7 receptor (Fig. 2A). Additionally, the LL-37-induced increase in cell stiffness requires an increase in intracellular Ca\(^{2+}\) as indicated by the inhibitory effect of the calcium-specific chelator BAPTA-AM on LL-37-induced cell stiffening (Fig. 2B).

**LL-37 increases stiffness of aortic tissue.** The physiological relevance of LL-37’s ability to regulate endothelial cell permeability in vitro is supported by data from experiments in which the mechanical properties of endothelial layers in freshly isolated mouse aorta were probed using AFM (Fig. 3A). In this study, similar to observations in endothelial cell culture, an increase in the stiffness of aorta endothelium was observed upon treatment with 2 \(\mu\)M LL-37 for 30 min (Fig. 3B). However, after addition of WLBU2, which, compared with LL-37, has higher positive charge and higher membrane surface activity, we did not observe an increase in aorta endothelial cell stiffness.

**LL-37 reduces basal HUVEC monolayer permeability coincident with remodeling of actin cytoskeleton and adherens junction proteins.** In agreement with the previous findings, we observed an increase of basal HUVEC monolayer permeability after addition of human thrombin (0.5 U/ml) (22) and a decrease after addition of S1P (1 \(\mu\)M) (3). S1P has emerged as an effective barrier-protective agonist for cultured endothelial cells and intact microvessels. LL-37-induced permeability changes in HUVECs were similar to those observed after S1P addition, suggesting the ability of LL-37 to enhance endothelial cell barrier strength (Fig. 4). To link S1P and LL-37-induced modulation of EC permeability with cellular morphological changes, we determined the organization of cytoskeletal F-actin and the distribution of \(\beta\)-catenin and VE-cadherin at cell-cell junctions (27). S1P and LL-37 treatment results in increased cortical actin bundle concentration evident as an increase in phalloidin staining (Fig. 5). A previous study by Mehta et al. (22) showed an increase in \(\beta\)-catenin and VE-
**DISCUSSION**

Increasing evidence suggests that cathelicidin LL-37 has pleiotropic immunomodulatory functions under physiologically relevant conditions such as the presence of serum, negatively charged biopolymers, or millimolar concentrations of divalent cations that strongly compromise its antibacterial activity (14, 38). The importance of immunomodulatory activity of LL-37 is also supported by murine models of infection in which mice subjected to intraperitoneal injection of bacteria were subsequently treated with LL-37 in comparison to treatment with synthetic LL-37 variants that were designed to ablate its bacteria-killing activity but retain selected immunostimulatory effects. In this model, LL-37 as well as its synthetic immunomodulatory analog decreased blood bacteria load (4) to similar extents. The ability of LL-37 peptide to increase cell stiffness and enforce endothelial barrier permeability shown here might also help explain the pleiotropic actions of LL-37 in physiological and disease stages and is in agreement with a recent study describing LL-37 involvement in reestablishment of intestinal barrier integrity (29) and ability of neutrophil-delivered substances to modulate vasomotion (18).

Because LL-37 is continuously present in the circulation it is likely that, in healthy subjects, it may contribute to the maintenance of tissue fluid homeostasis. S1P was recently identified as a dominant factor that effectively decreases pericellular fluid traffic, and silencing the S1P1 receptor, Tiam1, or both α-ac- tinin isoforms 1 and 4 was found to inhibit S1P-induced cortical F-actin rearrangement and S1P-mediated barrier enhancement (21, 30). An opposing function on endothelial permeability has been described for several agonists such as thrombin, histamine, and some cytokines. S1P and LL-37 can both effectively prevent EC permeability, indicating the existence of redundant pathways to assure efficient regulation of this important process. Such redundancy would explain why dysfunction of tissue fluid homeostasis was not reported in cathelicidin-deficient (Cnlp−/−) mice lacking the cathelicidin cathelin-related antimicrobial peptide (CRAMP), a structural and functional homolog of LL-37. A previous study reported that, in plasma, LL-37 is bound to apolipoprotein A-1 (ApoA-1) with a $K_d$ in the range of 0.6–2.4 μM, and that ApoA-1 might function as a buffer preventing cytotoxic effects of LL-37 (36, 37).

The ability of LL-37 to induce endothelial cell stiffness in the presence of serum at a concentration that effectively prevents its bacteria-killing activity (8) suggests that LL-37 effects may be mediated via activation of plasma membrane receptors. It is also likely that LL-37-binding affinity to membrane receptors is higher than its binding to ApoA-1, and that circulating complexes of LL-37/ApoA-1 serve as the source of LL-37 necessary to maintain tonic enforcement of endothelial

**Fig. 4.** Effect of LL-37, sphingosine 1-phosphate (SIP; 1 μM), and human thrombin (THR; 0.5 U/ml) on monolayer permeability of macromolecules. HUVEC monolayers grown in Transwell plates on semipermeable membranes were treated with different agonists in the presence of FITC-dextran for 6 h. AU, arbitrary units. Data shown are means ± SD of 3–4 independent experiments. *Statistically significant ($P < 0.05$) compared with control group.

**LL-37 concentration in different body fluids.** LL-37 concentrations were measured in body fluids that were stored frozen at −80°C. Analyzed samples included 3 saliva samples, 8 bronchoalveolar lavage (BAL) samples, 12 plasma samples, 12 cerebrospinal fluid (CSF) samples, and 16 ascites samples. Saliva and plasma samples were obtained from healthy volunteers and the CSF samples were from patients undergoing diagnostic lumbar puncture; however, the LL-37 analysis was performed using selected specimens from patients with conditions that do not alter standard CSF clinical tests [idiopathic cephalgia, ischialgia due to discopathy, and idiopathic (Bell’s) facial nerve palsy or entrapment radial neuropathy]. Ascites samples were collected from eight patients diagnosed with liver cirrhosis and eight patients with abdominal cancer. The range of LL-37 concentrations was 0.15–6.1 μM in saliva, 0.16–1.9 μM in BAL, 0.2–0.5 μM in plasma, 0.01–0.7 μM in CSF, and 0–4.4 μM in ascites. These data indicate that LL-37 blood concentration is in the range at which we observed LL-37 effects on endothelial cell stiffness and HUVEC permeability. ELISA analysis of LL-37 peptide subjected to pepsin digestion suggests that its shorter fragments such as RK-SKEKIGKE, FKRIQVRKID, and LVPRTES do not account for the measured LL-37 concentration (data not shown). However, the RK-31 peptide, a product of postsecretory processing of LL-37 peptide by a serine protease (25), was recognized by the antibody used in this LL-37 ELISA kit. This result suggests that RK-31 fragments might contribute to the detected LL-37 concentration.
membrane strength. Currently, three membrane receptors—
formyl peptide receptor-like 1 (FPRL1) (10), P2X7 (26), and
epidermal growth factor receptor (EGFR) (32)—have been
reported to be engaged upon LL-37 cell activation. Two of
these receptors are included in a list of receptors suggested to
be involved in regulation of endothelial permeability that
includes S1P1 and -2 receptors, PAR1, D1, -2, and -5 dopamine
receptors, bradykinin type 2 receptor, VEGF receptor, and
ATP-activated receptors (23). However, among the ligands for
these receptors, only S1P through S1P1 produces a clear
increase in endothelial barrier strength compared with data
generated using purines or growth factors, which show both
barrier-protective and enhanced permeation activity. Endothe-
lial cells express FPRL1, but direct FPRL1 involvement in
endothelial barrier regulation has not been fully studied. Activ-
ation of P2X7 receptors, however, induces signals to the
cytoskeleton that result in similar changes to those induced by
S1P, and those are involved in the LL-37-mediated decrease of
EC permeability. However, the involvement of LL-37 peptide
in regulation of vascular homeostasis may also occur through
an indirect mechanism and may be endothelium dependent.
Accordingly, vasodilation of precontracted endothelin-1 hu-
man omental veins upon LL-37 treatment was observed upon
LL-37-mediated release of nitric oxide and endothelium-de-
duced hyperpolarizing factor (2).

It is notable that the concentration of LL-37 in plasma
samples was within the concentration range that induced
tissue fluid. Such an accumulation is usually mediated by
inflammatory cytokines that can also upregulate LL-37 pep-
tide production. This sequence of events would create a
negative feedback loop in which cytokine-mediated increase
in LL-37 production counters the cytokine-mediated increase in EC permeability. At inflammatory sites, LL-37-mediated modulation of EC permeability could modulate chemotaxis, adjuvant, angiogenic, and wound-healing effects. Defining the biological properties and functions of LL-37 and other host defense peptides would advance our knowledge of innate immunity and potentially establish new strategies for therapeutic intervention.

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DISCLOSURES

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REFERENCES


