
Ciornei, Cristina; Egesten, Arne; Bodelsson, Mikael

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Effects of human cathelicidin antimicrobial peptide LL-37 on lipopolysaccharide-induced nitric oxide release from rat aorta in vitro

C. D. CIORNEI1, A. EGESEN2 and M. BODELSSON1

1Department of Anaestheiology and Intensive Care, Lund University, Lund, 2Department of Medical Microbiology, Malmö University Hospital, Malmö, Sweden

Background: Lipopolysaccharides (LPS), released by Gram-negative bacteria, cause vascular expression of inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) production and septic shock. Human cathelicidin antimicrobial peptide (LL-37) can bind and neutralize LPS. We wanted to study whether LL-37 affects LPS or interleukin-1β (IL-1β)-induced production, release and function of NO in intact rat aorta rings and cultured rat aorta smooth muscle cells.

Methods: Isolated segments of thoracic aorta and cultured cells were incubated in the presence of LPS, LL-37, LPS + IL-37, IL-1β, IL-1β + IL-37 or in medium alone. Smooth muscle contraction in response to phenylephrine and accumulation of the degradation products of NO, nitrate and nitrite, were measured in aorta segments. Levels of iNOS were assessed by Western blot and cytotoxic effects were detected by measurement of DNA fragmentation in cultured cells. Number of viable cells were determined after Trypan blue treatment.

Results: Both LPS and IL-1β reduced contractility in response to phenylephrine and increased NO production as well as iNOS expression. LL-37 inhibited the LPS depression of vascular contractility induced only by LPS. LL-37 reduced both the LPS- and IL-1β-induced NO production and iNOS expression. LL-37 at high concentrations induced DNA fragmentation and decreased the number of living cells.

Conclusion: IL-37 reduces NO production induced by LPS and IL-1β. The reduction does not seem to result only from neutralization of LPS but also from a cytotoxic effect, possibly via induction of apoptosis.

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Key words: Apoptosis; endotoxin; hCAP-18; iNOS; interleukin; lipopolysaccharide; LL-37; nitric oxide; rat aorta; vascular smooth muscle.


Septic shock is a syndrome caused by bacteria, virus or fungi entering the blood stream. The clinical picture includes hypotension and multiple organ dysfunction (1).

Lipopolysaccharide (LPS, endotoxin), a major component of the cell wall of Gram-negative bacteria, is released into the circulation following bacterial lysis. Lipopolysaccharide induce dilation of the blood vessels, which contributes to the hypotension during septic shock (2). This dilation is in part the result of an LPS-induced increase of the production of nitric oxide (NO) in the blood vessel wall, which relaxes the vascular smooth muscle cells (3). The formation of NO is catalyzed by the enzyme inducible nitric oxide synthase (iNOS), which is up-regulated by LPS and certain proinflammatory cytokines such as interleukin-1β (IL-1β) (4, 5).

Agents that are able to neutralize the effects of LPS may be of clinical importance in the treatment of septic shock. One of them could be human cathelicidin antimicrobial peptide (hCAP-18) (6–8), which is produced by human neutrophils but can also be found in a variety of human tissues (9, 10). hCAP-18 belongs to the protein family of cathelicidins, which have bactericidal activity by means of their high-affinity binding to the outer bacterial cell wall (8).

The COOH-terminal of this 140 amino acid residue protein contains the antimicrobial and LPS-binding domains (7). Naturally occurring cleavage gives rise to a 37-residue peptide (LL-37), which accounts for the biological actions in vivo (11, 12). It has been shown that LL-37 and LL-37 analogs neutralize the effect of LPS in vitro and in vivo (7, 13–16). Along with conventional antibiotics, LL-37 may be a potential candidate...
in the treatment of septic shock as a result of its dual action: bactericidal activity and neutralization of LPS.

As a result of the importance of the circulatory changes present during Gram-negative septic shock we undertook this study in order to test the hypothesis that LL-37 can inhibit the effects of LPS on the smooth muscle contractility, NO production and iNOS expression in the blood vessel wall. The study verifies this hypothesis but also suggests that cytotoxic effects, possibly mediated via the apoptosis pathway, might limit the clinical use of the naturally occurring peptide.

**Methods**

**Material**
The Institutional Review Board for the care of animal subjects approved the study and the care and handling of the animals were in accord with National Institutes of Health guidelines. Male Sprague-Dawley rats (250 g body weight) were anesthetized with halothane (Astra, Södertälje, Sweden) and bled to death. The thoracic aorta was removed, cleaned of adherent fat and cut into 3-mm long cylindrical segments.

**Cell culture**
Rat vascular smooth muscle cells were isolated from thoracic aorta by the explant method (17). In short, aorta segments were aseptically removed, trimmed and longitudinally cut open. The endothelium was removed by gently rubbing the intimal surface with a sterile cotton pad. Approximately 3×3-mm sections were placed with the intimal side down into 28-cm² Petri dishes. Dulbecco’s Modified Eagle’s Medium (DMEM) containing fetal bovine serum (10%), penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and amphotericin-B (250 ng ml⁻¹, all from Life Technologies, Täby, Sweden) was added to cover the tissue without disturbing the orientation of the explants. The dishes were incubated in a humidified incubator at 37°C with 5% CO₂ in air.

Vascular cells were allowed to grow out from the aorta sections for 8 days after which the sections were removed. When confluence was reached, the cells were harvested using 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA, both from Sigma-Aldrich, St. Louis, MO), rinsed in Hanks’ Balanced Salt Solution (Life Technologies) and transferred to 25-cm² flasks (first passage). The cells were cultured to confluence in DMEM with 10% fetal bovine serum and antibiotics, as above. At subsequent passages the cells were seeded at a ratio of 1:4. Subcultured cells were found to have a mean doubling time of approximately 30h. The cells were identified as smooth muscle cells by their characteristic hill and valley appearance in culture and by their expression of an approximately 40-kDa protein with immunoreactivity corresponding to smooth muscle α-actin as determined by Western blot (see below) using a monoclonal anti-smooth muscle α-actin antibody (Cat No. A-2547, Sigma-Aldrich). The cells used for the experiments in the present study were from passages 5 or 6.

**Incubations**
Intact aorta segments intended for contraction experiments were incubated at 37°C for 5h in 2 ml of DMEM without phenol red (ICN, Costa Mesa, CA) with penicillin (2000 U ml⁻¹) and streptomycin (0.2 mg ml⁻¹, both from Sigma-Aldrich) continuously aerated with 5% CO₂ in O₂. Intact aorta segments intended for measurement of nitrate/nitrite accumulation were incubated for 24 h in aerated DMEM without phenol red containing L-arginine (1 mM, substrate for NO synthesis) and penicillin (2000 U ml⁻¹) and streptomycin (0.2 mg ml⁻¹, all from Sigma-Aldrich). Cells cultured to confluence on 28-cm² Petri dishes (for Western blot) or 2-cm² wells in a 24-well plate (for DNA fragmentation and cytotoxicity experiments) were incubated for 16 or 48 h in DMEM with phenol red, penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and amphotericin-B (250 ng ml⁻¹, all from Life Technologies) but without fetal bovine serum in an atmosphere of 5% CO₂ in air.

The segments and cells were incubated in the presence of the following combinations of compounds: (a) LPS; (b) IL-1β; (c) LL-37; (d) LL-37 + IPS; (e) LL-37 + IL-1β; or (f) neither LL-37, LPS nor IL-1β (control). The concentrations of LPS and IL-1β used were determined from initial pilot experiments and were chosen because they were found to be the lowest concentrations giving a clear response compared to the control.

**Measurement of smooth muscle contraction**
The first set of experiments aimed to investigate the effects of incubation with LPS (200 ng ml⁻¹), IL-1β (0.6 ng ml⁻¹) and/or LL-37 (1, 10, 30 or 100 μg ml⁻¹) on circular smooth muscle contraction. After incubation, the segments were placed on two L-shaped hooks in 2-ml tissue baths. The baths contained Krebs-Ringer solution aerated with 11.5% CO₂ in O₂ at a rate giving PCO₂ 5.0 kPa, PO₂ approximately
40 kPa and pH 7.4. The Krebs-Ringer solution contained (mM): Na⁺ 143, K⁺ 5.9, Cl⁻ 128, Ca²⁺ 2.5, HCO₃⁻ 25.0, Mg²⁺ 1.2, SO₄²⁻ 1.2, H₂PO₄⁻ 1.2, glucose 5.5 and EDTA 0.024. The temperature was thermostatically maintained at 37°C. One of the hooks was connected to a Grass FT03C force-displacement transducer (Grass Medical Instruments, Quincy, MA) for isometric measurement of tension. The vessel tension was recorded on Grass polygraph model 7d. The segments were stretched to a pretension of 20 mN and allowed to equilibrate for 1 h. Thereafter, potassium chloride (90 mM) was added and the resulting smooth muscle contraction was used to check the viability of the segments. After wash out, the α₁-adrenoceptor agonist, phenylephrine (10⁻⁹–10⁻⁴ M), was added cumulatively in 10 log units. The resulting contraction was registered and concentration-response curves drawn. The experiments were performed first in the absence and then after wash out, during which the vessel tension returned to baseline, in the presence of the iNOS inhibitor L-N⁶ nitroarginine methyl ester (L-NAME 0.3 mM, added 10 min prior to phenylephrine).

A second set of experiments was performed in order to assess any direct effect of LL-37 on smooth muscle. Segments of rat aorta were mounted in organ baths, allowed to equilibrate and tested with potassium chloride as above. To some segments, LL-37 was added cumulatively in 10 log units (10⁻¹²–10⁻⁶ g ml⁻¹) and smooth muscle contraction was measured. Other segments were first precontracted with endothelin-1 to a stable submaximal contraction. LL-37 was then added cumulatively in 10 log units (10⁻¹⁰–10⁻⁴ g ml⁻¹) and smooth muscle relaxation was measured.

**Measurement of nitrate/nitrite accumulation**

Nitric oxide is rapidly oxidized to nitrite and nitrate (18). The NO release from the segments is therefore reflected in the accumulation of nitrate and nitrite in the incubation medium. The segments were incubated with LPS (1 ng ml⁻¹) or IL-1β (0.2 ng ml⁻¹) for 24 h. LL-37 (1, 10 or 100 μg ml⁻¹) was added at the beginning of the incubation (‘preincubation’) or immediately before analysis of the nitrate/nitrite content (‘postincubation’). This protocol was used in order to exclude any interaction of LL-37 with the nitrate/nitrite assay.

After incubation, the aorta segments were removed, briefly blotted on a paper cloth and weighted. The incubation medium was centrifuged at 11 000 × g for 5 min at room temperature. All nitrate in 100 μl of the supernatant was reduced to nitrite with nitrate reductase (2 μM) and reduced β-nicotinamide adenine dinucleotide phosphate (NADPH, 20 nmol, both from Sigma-Aldrich) in 70 μl of 20 mM potassium phosphate buffer (pH 7.40) during 2 h at room temperature. Higher levels of NADPH and/or its oxidized form was found to quench the color reaction of the Griess reagent (see below). However, the presently used concentration of NADPH was insufficient to reduce the highest concentrations of nitrate. Therefore, glucose-6-phosphate dehydrogenase (8 μM) and glucose-6-phosphate (40 nmol, both from Sigma-Aldrich) was present to reduce and thereby recycle the oxidized NADPH.

After reduction, 100 μl of Griess reagent (40 mg ml⁻¹; Sigma-Aldrich) was added and optical density at 550 nm was measured using a spectrophotometer. Standard curves were determined by analyzing DMEM with different concentrations of sodium nitrate (Assay Designs, Michigan, USA).

**Western blot**

After incubation of confluent cells cultured on 28 cm² Petri dishes with LPS (0, 0.1, 1 or 10 ng ml⁻¹), IL-1β (0, 0.01, 0.1 or 1 ng ml⁻¹) and/or LL-37 (1, 10 or 100 μg ml⁻¹), the incubation medium was replaced by a lysis buffer containing phosphate buffered saline (PBS) with Triton X-100 (1%), a protease inhibitor cocktail (20 μl ml⁻¹) and benzamidine hydrochloride (10 mM, all from Sigma-Aldrich). The cells were removed from the Petri dishes with a cell scraper. The cell suspension was frozen and thawed two times to enhance cytology and was then centrifuged for 5 min at 11 000 × g at room temperature to remove particular debris.

Total protein concentration was determined on the basis of the Biuret reaction combined with colorimetric detection of the cuprous cation using a reagent containing bichinonic acid (19) (Pierce, Rockford, IL). Twenty μg protein was electrophoretically separated in a 7.5% SDS polyacylamide gel, stacking 4% (20), together with protein size standards (Kaleidoscope, Bio-Rad, Hercules, CA).

The separated proteins were transferred to a PVDF membrane (Hybond ECL, Amersham Pharmacia, Freiburg, Germany) using a Bio-Rad Mini protein tank blot equipment (21). The membranes were blocked with 5%-non-fat milk (ICN) in 2 M Tris buffered saline (TBS) with 0.1% Tween-20 (ICN) for one hour at room temperature and subsequently exposed to a polyclonal rabbit anti-iNOS antibody 1/1000 (Santa Cruz Biotechnology, Santa Cruz, CA) at room
temperature for 1 h in blocking solution. After repetitive washings, membranes were incubated with a biotinylated goat antirabbit antibody 1/5000 (Santa Cruz) for another hour in TBS. Detection was performed using ECL Plus reagents and Hyper Film™ (both from Amersham Pharmacia).

DNA fragmentation and cytotoxicity experiments
Confluent cells cultured in 2-cm² wells were incubated in serum free DMEM for 16 h with LL-37 (1, 10, 30 or 100 μg ml⁻¹) or H₂O₂ (10 μM). H₂O₂ has previously been found to induce apoptosis in cultured smooth muscle cells and was used as a positive control in these experiments (22). After incubation, the cells were lysed in the culture wells and the DNA fragments of the lysate were bound to a microtiter plate coated with monoclonal anti-histone antibodies. The bound DNA fragments were then detected by peroxidase-conjugated monoclonal anti-DNA antibodies and 2,2’-azino-di-[3-ethylbenzthiazoline sulfonate] (Cell Death Detection ELISA, Roche Molecular Biochemicals, Mannheim, Germany). Optical density was determined at 415 nm and is expressed as percentage of untreated controls. Cell viability of separate cells after 48 h incubation as above was determined using Trypan blue (Sigma-Aldrich) on trypsinized cells by counting in a hemocytometer.

Drugs
LPS (from E. coli, strain 0111:B4, Difco, Detroit, MI); interleukin-1β (IL-1β, Alexis, San Diego, CA); LL-37 (synthetic, generous gift from Johan Malm, M.D., Ph.D., Department of Clinical Chemistry, Malmö University Hospital); L-N⁵-nitroarginine methyl ester (L-NAME), L-phenylephrine hydrochloride and endothelin-1 (all from Sigma-Aldrich, St. Louis, MO).

Results

Smooth muscle contraction
Phenylephrine induced a concentration-dependent smooth muscle contraction that was significantly weaker after incubation with LPS compared to control. Co-incubation with LL-37 at 100 μg ml⁻¹ significantly attenuated the effect of LPS (Fig.1A). In the presence of the nitric oxide inhibitor, L-NAME, the hyporeactivity to phenylephrine induced by LPS was diminished. The effect of LL-37 on LPS-induced hyporeactivity to phenylephrine was not seen in the presence of L-NAME (not shown, n = 6).

Similar to after incubation with LPS, the phenylephrine-induced contraction was significantly weaker in the presence of IL-1β compared to control (Fig.1B). LL-37 did not affect the IL-1β-induced suppression of the phenylephrine-induced contraction. L-NAME diminished the effect of IL-1β incubation on contraction induced by phenylephrine (not shown, n = 4).

Incubation with LL-37 alone did not affect the contraction induced by phenylephrine (not shown, n = 5). LL-37 added directly to the organ bath neither induced contraction of resting aorta segments nor relaxed aorta segments precontracted by endothelin-1 (not shown, n = 2).

Nitrate/nitrite production
Incubation with LPS or IL-1β significantly increased the production of nitrate/nitrite from the aorta segments compared to control (Fig.2, ‘postincubation’ data). Co-incubation with LL-37 at 10 and 100 μg ml⁻¹ significantly reduced the LPS-induced nitrate/nitrite production (Fig.2A). Co-incubation with LL-37 100 μg ml⁻¹ also significantly reduced the IL-1β-induced nitrate/nitrite production (Fig.2B).

Western blot
The anti-iNOS antibodies detected a band corresponding to the molecular weight of iNOS (approximately 135 kDa) in lysates of vascular smooth muscle cells. The band was clearly visible after incubation with LPS at 1 and 10 ng ml⁻¹ and IL-1β at 1 ng ml⁻¹ but not at lower concentrations, indicating concentration-dependent up-regulation of iNOS (Fig.3). LL-37 inhibited both the LPS and IL-1β up-regulation of iNOS as detected by Western blot.

Cytotoxicity and DNA fragmentation
H₂O₂ and LL-37 at 100 μg ml⁻¹ caused a significant DNA fragmentation in cultured smooth muscle cells.
Fig. 1. Phenylephrine-induced smooth muscle contraction in rat aorta. Phenylephrine induced a concentration-dependent smooth muscle contraction. (A) The contraction was significantly weaker after 5-hour incubation with lipopolysaccharide (LPS) (200 ng ml⁻¹) compared with control (P = 0.001). Co-incubation with LL-37 at 100 μg ml⁻¹, but not lower concentrations, significantly attenuated the effects of LPS (n = 6, P = 0.004). (B) The contraction was significantly weaker after 5-hour incubation with IL-1β (0.6 ng ml⁻¹, P = 0.030, n = 4). Co-incubation with LL-37 did not affect the IL-1β-induced suppression of the phenylephrine contraction. Values are means ± SE mean. Two-way repeated measures ANOVA.

Fig. 2. Production of the nitric oxide end products nitrate and nitrite from segments of rat aorta following 24-hour incubation as measured with the Griess reagent. The production of nitrate/nitrite was significantly higher after incubation with LPS (1 ng ml⁻¹) or IL-1β (0.2 ng ml⁻¹) compared with control (P = 0.031 and P = 0.010 for LPS and IL-1β, respectively). (A) LL-37 at 10 and 100 μg ml⁻¹ added before the incubation (preincubation) significantly reduced the nitrate/nitrite production induced by LPS (1 ng ml⁻¹) compared with LL-37 added after the incubation (postincubation, n = 6, P = 0.031 for both 10 and 100 μg ml⁻¹). (B) LL-37 at 100 μg ml⁻¹ added before the incubation (preincubation) significantly reduced the nitrate/nitrite production induced by IL-1β (0.2 ng ml⁻¹) compared with LL-37 added after the incubation (postincubation, n = 7, P = 0.016) Wilcoxon’s signed-rank test. There was no difference in the nitrate/nitrite values between the different concentrations of LL-37 used in the postincubation experiments, indicating that LL-37 itself does not, to any major extent, affect the determination of nitrate/nitrite.

Discussion

In the present study, we have shown that incubation of the aorta rings with LPS or IL-1β reduces the contractile response to phenylephrine via an
NO-dependent mechanism, as previously demonstrated (4, 23–25). Co-incubation with LL-37 attenuated the effect of LPS. This suggests that LL-37 neutralized the NO-mediated LPS-induced hyporeactivity to phenylephrine in the rat aorta segments. Co-incubation with LL-37 did not affect the IL-1β-induced reduction of the contraction in response to phenylephrine. Furthermore, we did not find any direct effects of LL-37 on vascular smooth muscle contraction. This indicates that the action of LL-37 is LPS-specific, probably because of binding of LPS to LL-37 in solution, thereby inhibiting its effects on NO production. LL-37 has previously been found to bind and neutralize the effects of LPS on the procoagulant activation of limulus amoebocytes (13) and the tumor necrosis factor-α (TNF-α) expression in the murine macrophage cell line RAW 264.7 in vitro (16). Larrick and colleagues (7) demonstrated that a COOH truncated LL-37 analog (CAP 18[40-135]) inhibits LPS-induced NO release from RAW 264.7 cells. The present results suggest that the naturally occurring peptide binds LPS. An alternative explanation for our findings could be the binding of LL-37 to the cell-surface-bound LPS receptor, CD14, thereby inhibiting the LPS-induced activation of NO synthesis as demonstrated in RAW 264.7 cells (16). However, the expression of CD14 in vascular tissue seems to be absent or very low (26, 27).

To verify the involvement of NO in the vascular actions of LPS, IL-1β and LL-37 we studied the effects of these compounds on the accumulation of the NO degradation products nitrate and nitrite. As expected, LPS and IL-1β increased the production of nitrate/nitrite in the vessel segments (25). LL-37 reduced the LPS-induced nitrate/nitrite production, which confirms that LL-37 can inhibit the LPS-induced NO production in rat aorta. It seemed like LL-37 at 10 μg ml⁻¹ was more effective in inhibiting the NO response to LPS than LL-37 at 100 μg ml⁻¹ (Fig. 2A). However, this tendency was not statistically significant. The concentrations of LL-37 required to inhibit the effects of LPS in our study were similar to those found by Turner et al. and Nagaoka et al. in non-vascular models (13, 16). In contrast to the results from the contraction experiments with phenylephrine in which LL-37 did not affect the vascular response to IL-1β, LL-37 at the highest concentration used reduced the IL-1β-induced NO production. In the pilot experiment preceding the contraction experiments, a 5-h incubation time with LPS or IL-1β was found to be sufficient for a clear effect of these compounds to be registered. However, 24 h incubation was required for the accumulation of detectable amounts of nitrate and nitrite. One possible explanation for the inhibitory action of LL-37 at high concentrations on IL-1β-induced NO production could be that LL-37 during prolonged exposure acts not only as an LPS scavenger but could also have other effects such as the inhibition of vascular protein synthesis.

This was confirmed in the Western blot experiments aiming to assess the amount of immunoreactive iNOS in cultured vascular smooth muscle cells. These experiments were performed after 48 h exposure to LPS or IL-1β in order to obtain a clear increase in the iNOS expression in the controls. In these experiments, LL-37 inhibited the LPS- and IL-1β-induced iNOS up-regulation to the same extent. This suggests that during prolonged exposure, the general inhibition of protein synthesis is so profound that it obscures any effects of LPS-binding by LL-37. On the other hand, LL-37 affected the LPS-induced but not IL-1β-induced
Fig. 4. (A) Effect of LL-37 on DNA fragmentation in cultured rat aorta smooth muscle cells after 16 h incubation. H$_2$O$_2$ and LL-37 at 100 µg ml$^{-1}$ induced a significant DNA fragmentation compared with controls ($n=5$, $P<0.05$). (B) Effect of LL-37 on the viability of cultured rat aorta smooth muscle cells determined by Trypan blue after 48 h incubation. LL-37 at 100 µg ml$^{-1}$ significantly decreased the density of attached living cells ($n=5$, $P<0.05$). Friedman’s repeated measures ANOVA on ranks followed by Dunnett’s post hoc test.

reduction in smooth muscle contractility after 5 h exposure, suggesting that the protein synthesis inhibition is slowly developing. It was early recognized that LL-37 and LL-37 analogs have direct effects on eukaryotic cells. Johansson and colleagues (28) observed an increased fluorescein staining by fluorescein diacetate of human peripheral leukocytes and the T-cell line MOLT after incubation with LL-37 at concentrations of approximately 100 µg ml$^{-1}$. They concluded that LL-37 is toxic not only to bacteria but also to eukaryotic cells. Later it was demonstrated that LL-37 has affinity to the eukaryotic cell membrane and causes hemolysis (29) although conflicting data has been published (30).

Indeed, in our study after a 48 h incubation period, the number of attached viable cells was decreased by LL-37 in a concentration-dependent manner, indicating that LL-37 was cytotoxic in our model. Cell death was preceded by DNA fragmentation, which together with an impeded protein synthesis, is a typical feature of programmed cell death: apoptosis (31). Although this hypothesis remains to be confirmed, a pro-apoptotic effect of LL-37 would be a property that LL-37 shares with two bovine cathelicidins: BMAP-27 and BMAP-28 (32).

The use of LL-37 in its naturally occurring form as an adjunctive treatment of sepsis caused by Gram negative bacteria may thus be limited by its cytotoxicity. However, removal of the four N-terminal hydrophobic amino acid residues seems to reduce the hemolytic activity of LL-37 but does not affect its antimicrobial properties (29). The effect on LPS binding capacity and any beneficial effects on sepsis by the truncated peptide are not known. A systematic investigation of LL-37 analogs in this respect might prove fruitful.

In conclusion, this is, to our knowledge, the first study showing that LL-37 reduces vascular NO production induced by LPS and IL-1β. The reduction seems to be the result of, on one hand, neutralization of LPS, and on the other hand a cytotoxic effect evident after prolonged exposure at higher concentrations.

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Address:
Mikael Bodelsson
Department of Anesthesiology and Intensive Care
University Hospital
SE-221 85 Lund
Sweden
e-mail: mikael.bodelsson@anest.lu.se