Effect of Human Cationic Antimicrobial Protein 18 Peptide on Endotoxin-Induced Uveitis in Rats

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Purpose. Human cationic antimicrobial protein 18 (hCAP18, 18 kDa) was originally identified in leukocytes on the basis of its antimicrobial activity. The peptide composed of the 27 C-terminal amino acids of hCAP18 ($hCAP18_{109-135}$) binds lipopolysaccharide (LPS). The purpose of the present study was to investigate the effects of hCAP18 peptide on endotoxin-induced uveitis (EIU) in rats.

Methods. EIU was induced by footpad injection of LPS. Each rat was injected intravenously with 1, 10, or 100 μg hCAP18 peptide in 0.1 mL of PBS immediately after LPS injection in male Lewis rats. At 24 hours after LPS injection, enzyme-linked immunosorbent assay was performed to evaluate concentrations of protein, nitric oxide (NO), tumor necrosis factor (TNF)-α, prostaglandin (PG)-E2, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 in aqueous humor. Also, EIU was evaluated by counting inflammatory cells in aqueous humor.

Results. hCAP18 peptide at 10 and 100 μg significantly suppressed an LPS-induced increase in the number of inflammatory cells and the levels of protein, NO, TNF-α, PGE2, MCP-1, and MIP-2. The anti-inflammatory effect of 10 μg hCAP18 peptide was as strong as that of 100 μg hCAP18 peptide. Treatment with 1 μg hCAP18 peptide did not suppress EIU, compared with the LPS group.

Conclusions. The present results indicate that hCAP18 peptide suppresses development of EIU. A possible mechanism for the ocular anti-inflammatory effect of hCAP18 peptide is that it suppresses onset of LPS-triggered inflammatory reactions by binding directly to LPS. (*Invest Ophthalmol Vis Sci.* 2003;44: 4412–4418) DOI:10.1167/iovs.03-0246

Lipopolysaccharide (LPS), a major glycolipid component of the outer membrane of Gram-negative bacteria, induces a generalized proinflammatory response during infection. Systemic injection of a sublethal dose of LPS induces bilateral acute ocular inflammation in susceptible strains of rats and mice.[1,2] This endotoxin-induced uveitis (EIU) is an animal model for acute anterior uveitis in the human. In general, EIU peaks 24 hours after LPS injection and subsides within the next 48 hours. EIU is characterized by percolation of proteins from the serum and by infiltration of macrophages and neutrophils into the eye.[2] In Lewis rats with EIU, acute inflammation develops mainly in the anterior chamber (iridocyclitis), and inflammatory cells may infiltrate the vitreous humor and retina.[3] The exact mechanism responsible for EIU has yet to be determined, although cytokines seem to play important roles.[4–7]

Cytokines are signaling proteins released by cells; they serve as important mediators. The cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 have been detected in rabbits, rats, and mice with EIU.[8–10] TNF-α is involved in the lethality induced by LPS.[11–13] A considerable body of evidence supports a model in which LPS or LPS-containing particles (including intact bacteria) form complexes with a serum protein known as LPS-binding protein. The LPS in this complex is subsequently transferred to CD14, which is another protein that binds LPS. The latter is found on the plasma membrane of most myeloid cell types and in the serum in its soluble form. LPS binding to these two forms of CD14 results in the activation of myeloid and nonmyeloid cell types, respectively.[14–17]

Host defense against infection involves a multitude of factors and cells that together form the elements of the immune system. The most intensively investigated components of innate immunity are antibacterial peptides. Antibacterial peptides are found in insects, plants, and mammals, where they exhibit activity against both Gram-positive and -negative bacteria and against fungi and viruses.[18–20] Among these peptides, granulysin, histatins, lactoferricin, and α- and β-defensins have been found in humans.[18] Cathelicidins, a novel family of antibacterial proteins, have recently been identified in epithelial tissues and myeloid cells of humans and animals.[20] Cathelicidins consist of a putative N-terminal signaling peptide, a highly conserved cathelin-like domain in the middle, and a less conserved C-terminal antimicrobial domain corresponding to the mature antibacterial peptide.[20] Approximately 30 cathelicidins have been identified in mammals. However, only cathelicidin, human cationic antibacterial protein 18 (hCAP18, 18 kDa), has been found in humans thus far; its C-terminal mature antibacterial peptide, comprising 27-amino-acid residues, has been identified in human neutrophil granules. hCAP18 is mostly amphipathic, retaining both cationic and hydrophobic faces, and these features facilitate interactions with negatively charged surface membranes.[21,22]

As far as we know, there have been no reports of the effects of hCAP18 (which has LPS-binding effects) on ocular inflammation. In the present study, we investigated the effects of hCAP18 peptide on LPS-induced uveitis in rats.

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Materials and Methods

hCAP18 Peptide

hCAP18 peptide was synthesized, purified, and characterized by Peptide Institute, Inc. (Osaka, Japan), using methods described elsewhere.[23] Briefly, the active domain of hCAP18 was synthesized as a C-terminal amide of 27 amino acids ($hCAP18_{109-135}$: FRK SKEKIGKEFK RIVQRIKDFL RNLV). This peptide was purified by reversed-phase high-performance liquid chromatography on a YMC-Pak ODS column (YMC Co. Ltd., Kyoto, Japan). The peptides were also confirmed by reversed-phase high-performance liquid chromatography (100 μg/mL, eluant: 20%–70% acetonitrile gradient [CH_3CN] in 0.1% trifluoroacetic acid, flow rate: 1.0 mL/min, detector 220 nm) on a Zorbax 330SB-C18 column (4.6 mm inner diameter $\times$ 150 mm). The verification of the level of purity, as assessed by peak integration at 14.34 seconds, was 96.3% (ESI-MS: MW, 3376.9, theoretical MW, 3377.1) for C-terminal domain of hCAP18. Stock peptide solutions were prepared by dissolving vacuum-dried material in Hanks' balanced salt solution (HBSS, pH 7.4; Sigma-Aldrich, St. Louis, MO) at 400 μg/mL.

Animals and EIU Induction

Eight-week-old male Lewis rats were used. The rats weighed 180 to 220 g. EIU was induced by injecting one footpad with 200 μg of LPS (from *Salmonella typhimurium*; Sigma-Aldrich) diluted in 0.1 mL of sterile saline.

In the hCAP18 group, each rat was injected intravenously with 1, 10, or 100 μg hCAP18 peptide in 0.1 mL of PBS (pH7.4) immediately after LPS injection. In the LPS group, each rat was injected intravenously with 0.1 mL of PBS immediately after LPS injection. In the control group, each rat was injected intravenously with 0.1 mL of PBS without LPS injection.

Animals were handled and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Number of Infiltrating Cells and Protein Concentration in Aqueous Humor

The number of cells infiltrating into the aqueous humor and the protein concentration in the aqueous humor were used as indicators of the degree of anterior inflammation. At 24 hours after LPS injection, rats were killed, and the aqueous humor was collected immediately, as described elsewhere.[24] Briefly, the aqueous humor was collected from both eyes through an anterior chamber puncture (15–20 μL/rat) using a 30-gauge needle under a surgical microscope. In this study, each aqueous humor sample was diluted 30-fold with PBS.

For cell counting, an aqueous humor sample was suspended in an equal amount of Türk stain solution, and the cells were counted by hemocytometer under a light microscope. The number of cells per field (equivalent to 0.1 mL) was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. The total protein concentration in an aqueous humor sample was measured using a BCA protein assay reagent kit (Pierce, Rockford, IL).

The aqueous humor samples were stored in ice water until used. Cell count and total protein concentration were measured on the day of sample collection.

Determination of NO Levels in Aqueous Humor

The total level of nitrate plus nitrite in the aqueous humor was measured with a total nitrite colorimetric assay kit (OXIS International, Portland OR) according to the manufacturer's instructions. An aqueous humor sample (from both eyes, 15–20 μL/rat) was diluted up to 50 μL and used for an assay. The NO assay was performed once or twice for each concentration of hCAP18 peptide.

Levels of TNF-α, PGE2, IL-6, MCP-1, and MIP-2 in Aqueous Humor

Levels of TNF-α, PGE2, IL-6, MCP-1, and MIP-2 in the aqueous humor were assessed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (PGE2; R&D Systems, Minneapolis, MN, or BioSource International, Camarillo, CA) according to the manufacturer's instructions. ELISA was performed in duplicate. The data represent the mean of eight assay results $\pm$ SD.

Erythrocyte Agglutination Assay

LPS-binding activity was examined using a method described elsewhere.[25] A 1-mL volume of 1% erythrocytes (human O type) was

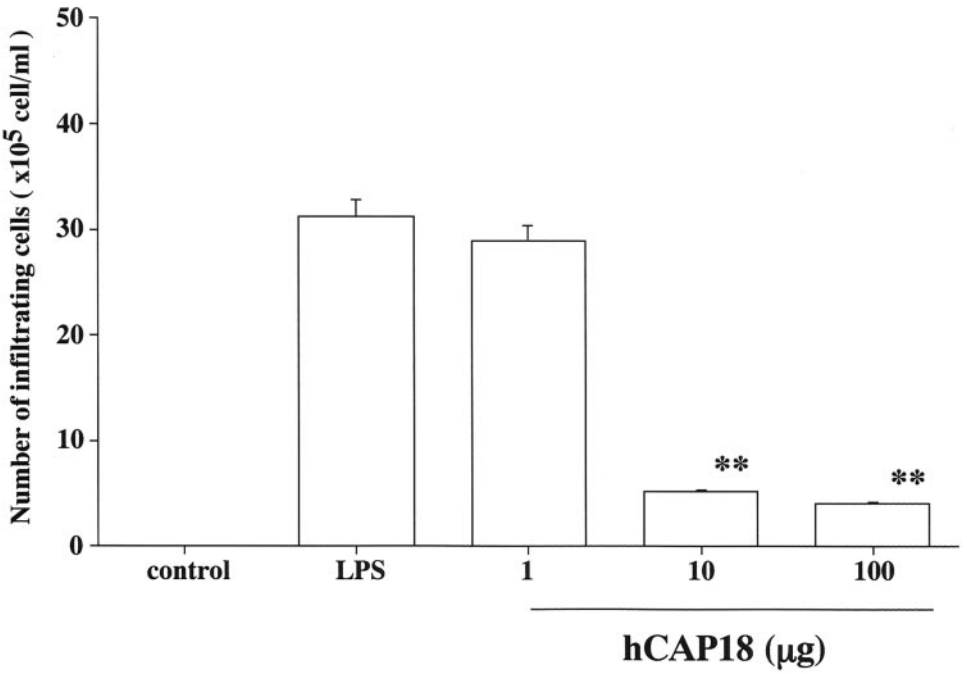

Figure 1. Effect of hCAP18 peptide on cellular infiltration in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Cell number is expressed as the mean $\pm$ SD of results in eight rats. **Significantly different from LPS group ($P < 0.01$). No infiltrating cells were detected in the control group.

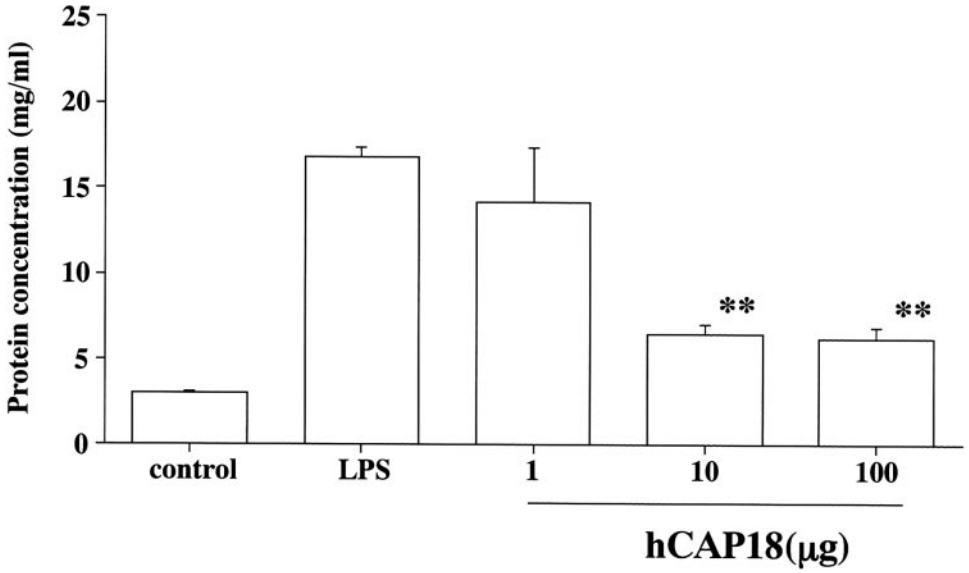

FIGURE 2. Effect of hCAP18 peptide on protein concentration in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of results in eight rats. **Significantly different from LPS group ($P < 0.01$).

sensitized by incubation with 0.2 mL of LPS solution (100 μg /mL in HBSS) at 37°C for 30 minutes. After sensitization, erythrocytes were washed twice in HBSS for 5 minutes at 100 rpm. A 50-μL volume of a 1.0% suspension of sensitized erythrocytes was mixed with an equal volume of a twofold serial dilution of hCAP18 peptide in a U-bottomed microtiter plate and incubated at 37°C for 1 hour. Plates were stored at 4°C overnight. Activity of hCAP18 peptide was expressed as minimum agglutinating concentration (MAC). In the result, the MAC of hCAP18 peptide for LPS from *S. typhimurium* was 5.0 μg/mL.

Statistical Analysis

Data are expressed as the mean ± SD. Data were analyzed by analysis of variance (ANOVA). The Tukey-Kramer test was used as a post hoc comparison to compare two treatment groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Number of Inflammatory Cells in Aqueous Humor

In the control group, no infiltrating cells were detected in the aqueous humor. In the LPS group, the number of inflammatory cells that had infiltrated into the aqueous humor 24 hours after LPS injection was $31.3 \pm 6.2 \times 10^5$ cells/mL (mean ± SD, $n = 8$). Rats treated with 10 or 100 μg hCAP18 peptide showed a significantly reduced number of inflammatory cells, compared with the LPS group: 10 μg, $5.4 \pm 4.3 \times 10^5$ cells/mL; 100 μg, $4.1 \pm 0.7 \times 10^5$ cells/mL, both $P < 0.01$ (Fig. 1).

Aqueous Humor Protein Concentration

In the control group, protein concentration in the aqueous humor was 3.0 ± 0.1 mg/mL and in the LPS group, 16.8 ± 0.6 mg/mL. In rats treated with 10 or 100 μg hCAP18 peptide, protein concentration was significantly lower than that of the LPS group: 10 μg, 6.5 ± 0.6 mg/mL; 100 μg, 6.2 ± 0.7 mg/mL, both $P < 0.01$ (Fig. 2). Treatment with 1 μg hCAP18 peptide only slightly reduced protein concentration (14.2 ± 3.2 mg/mL), compared with the LPS group; the difference was not significant.

Levels of NO

In the control group, NO concentration was 2.0 ± 1.6 μM and in the LPS group, 185.6 ± 63.5 μM ($n = 8$). In the hCAP18

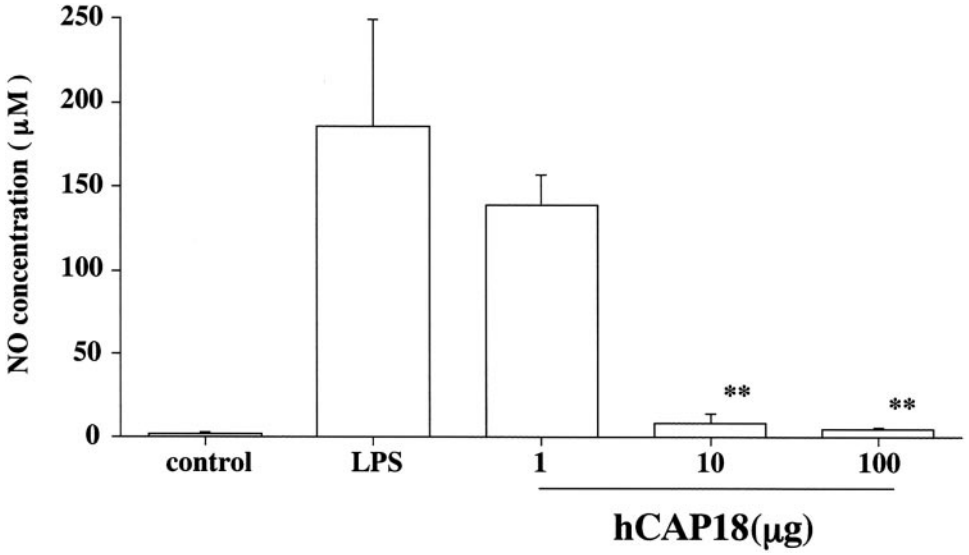

FIGURE 3. Effect of hCAP18 peptide on NO levels in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of results in eight rats. **$P < 0.01$ significantly different from LPS group.

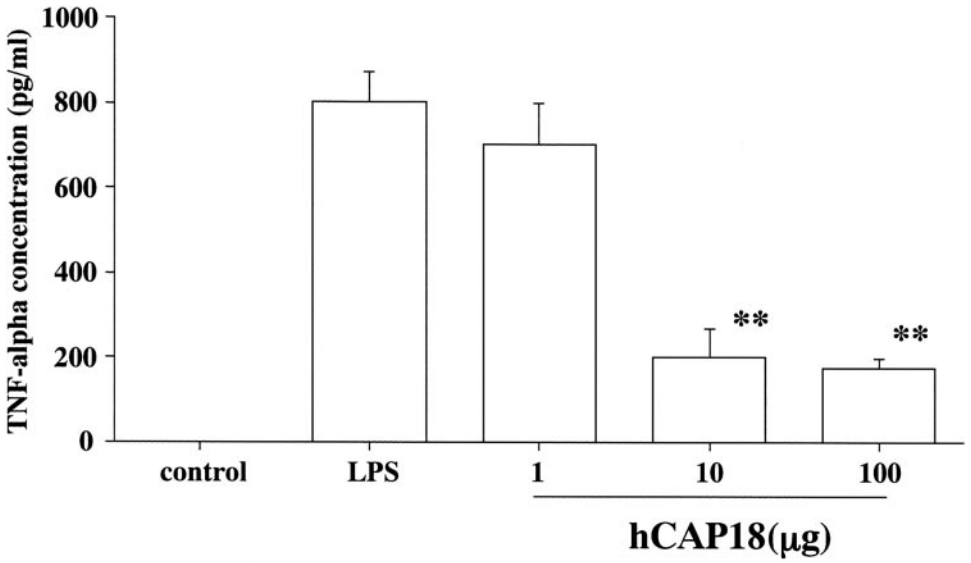

FIGURE 4. Effect of hCAP18 peptide on TNF-α concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of results in eight rats. **Significantly different from LPS group ($P < 0.01$).

peptide group, NO concentration decreased in a dose-dependent fashion. In rats treated with 10 or 100 μg hCAP18 peptide, NO concentration was significantly lower than that in the LPS group: 10 μg, 8.5 ± 6.1 μM; 100 μg, 4.7 ± 1.6 μM, both $P < 0.01$ (Fig. 3). Treatment with 1 μg hCAP18 peptide only slightly reduced NO concentration (139.3 ± 17.8 μM), compared with the LPS group; the difference was not significant.

Levels of TNF-α in Aqueous Humor

TNF-α was not detected in the control group. In the LPS group, TNF-α concentration in the aqueous humor was 807.3 ± 71.1 pg/mL. In the hCAP18 peptide group, TNF-α concentration decreased in a dose-dependent fashion. Treatment with hCAP18 peptide significantly reduced TNF-α concentration, compared with the LPS group: 1 μg, 706.0 ± 97.9 pg/mL, 10 μg, 205.9 ± 65.0 pg/mL, $P < 0.01$; 100 μg, 176.1 ± 24.3 pg/mL, $P < 0.01$ (Fig. 4).

PGE2 Concentration

In the control group, PGE2 concentration in the aqueous humor was 0.1 ± 0.3 ng/mL and in the LPS group, 60.4 ± 27.6 ng/mL. Treatment with hCAP18 peptide significantly reduced PGE2 concentration, compared with the LPS group: 1 μg, 18.2 ± 6.4 ng/mL; 10 μg, 7.5 ± 0.2 ng/mL; 100 μg, 3.7 ± 0.3 ng/mL, all $P < 0.01$ (Fig. 5).

IL-6 Concentration

In the LPS group, IL-6 concentration was approximately 20 times higher than that of the control group. IL-6 concentration was significantly reduced by hCAP18 peptide, compared with the LPS group: 10 μg, 98.4 ± 15.5 pg/mL; 100 μg, 84.2 ± 8.1 pg/mL, both $P < 0.01$ (Fig. 6). Treatment with 1 μg hCAP18 peptide did not reduce IL-6 concentration, compared with the LPS group.

Levels of MCP-1 and MIP-2

In the LPS group, the level of MCP-1 was approximately 10 times higher than that of the control group. Treatment with 10 or 100 μg hCAP18 peptide significantly reduced MCP-1 levels, compared with the LPS group: 10 μg, 572.7 ± 218.8 pg/mL; 100 μg, 438.6 ± 157.0 pg/mL, both $P < 0.01$ (Fig. 7). Treatment with 10 μg or 100 μg hCAP18 peptide significantly reduced MIP-2 concentration, compared with the LPS group:

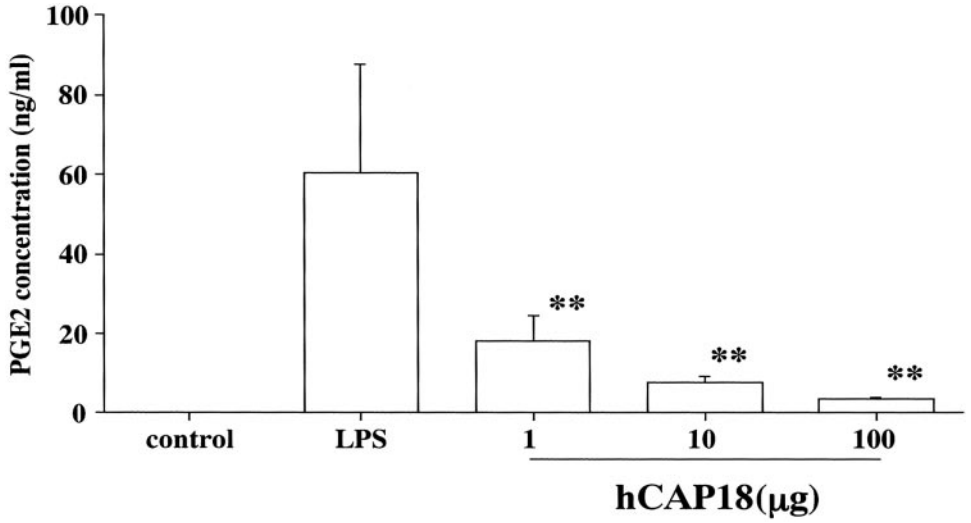

FIGURE 5. Effect of hCAP18 peptide on prostaglandin E2 concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of results in eight rats. **Significantly different from LPS group ($P < 0.01$).

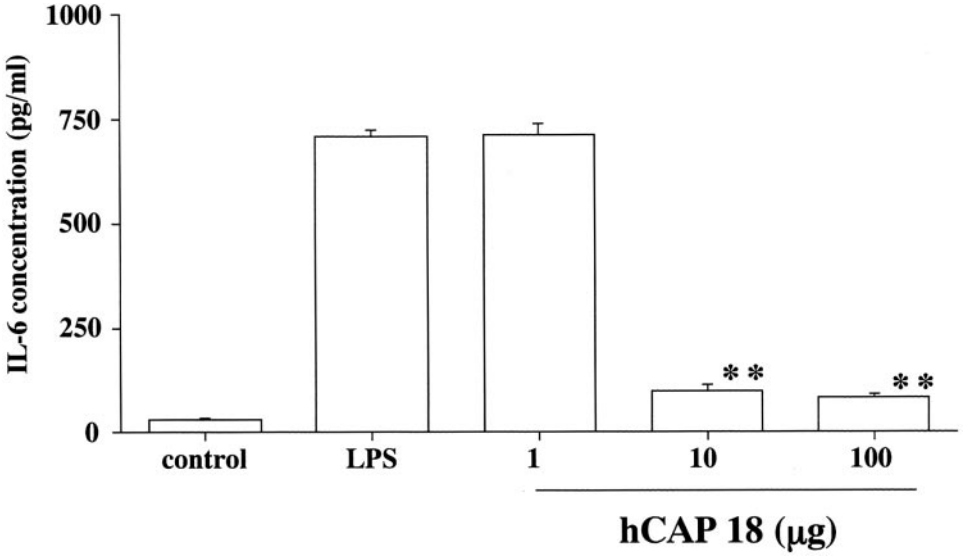

FIGURE 6. Effect of hCAP18 peptide on IL-6 concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of eight rats. **Significantly different from LPS group ($P <$ 0.01).

10 μg, 310.2 ± 84.9 pg/mL; 100 μg, 378.9 ± 115.6 pg/mL, both $P < 0.01$ (Fig. 8).

DISCUSSION

The present results show that hCAP18 peptide significantly suppressed LPS-induced increases in the number of inflammatory cells in the aqueous humor and the levels of NO, TNF-α, PGE2, and IL-6.

LPS, also known as an endotoxin, is an outer membrane component of Gram-negative bacteria. LPS has strong biological activity and plays a key role in the pathogenesis of endotoxin shock associated with various syndromes.[26,27] LPS induces monocytes, macrophages, and other types of cells to produce and release potent inflammatory mediators. One of these, TNF-α, appears to be essential for the development of endotoxin-induced inflammation.[3,28] Endotoxin-induced inflammation (e.g., endotoxin shock) can occur during antibiotic therapy for bacterial infections underlying septic syndrome. Some antibiotics not only kill bacteria but also stimulate the release of LPS from the outer membrane of the dying bacteria, thereby causing endotoxin shock.[29,30] Thus, a drug that suppresses LPS activity may be useful as a treatment for endotoxin shock or sepsis caused by Gram-negative bacterial infections. Several strategies for preventing the cascade of LPS-induced inflammatory reactions have been tested, including neutralizing antibodies against LPS or cytokines.[31,32] Much attention has been focused on the low-molecular-weight cationic antibacterial peptides that possess both antibacterial and LPS-neutralizing activity. These peptides function as an innate host defense against microbial infections by attacking the membranes of organisms.[33–35]

Structure-activity relationship studies using various natural and synthetic model peptides have revealed that the potencies and spectra of the peptides can be influenced by interrelated structural and physicochemical parameters, such as charge (cationicity), hydrophobicity, and amphipathicity.[36] Replacing amino acids to increase the hydrophobicity caused the hydrophobic sector in the helix to be extended and the resultant analogues showed potent LPS-binding activity.[37] The active domain of hCAP18 is amphipathic, retaining both cationic (positively charged) and hydrophobic faces. The structures are

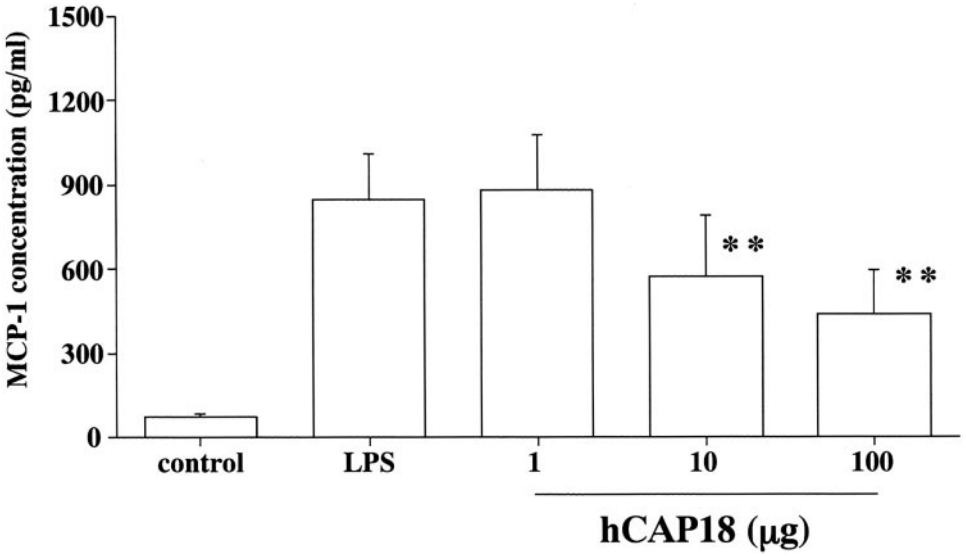

FIGURE 7. Effect of hCAP18 peptide on MCP-1 concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of eight rats. **Significantly different from LPS group ($P <$ 0.01).

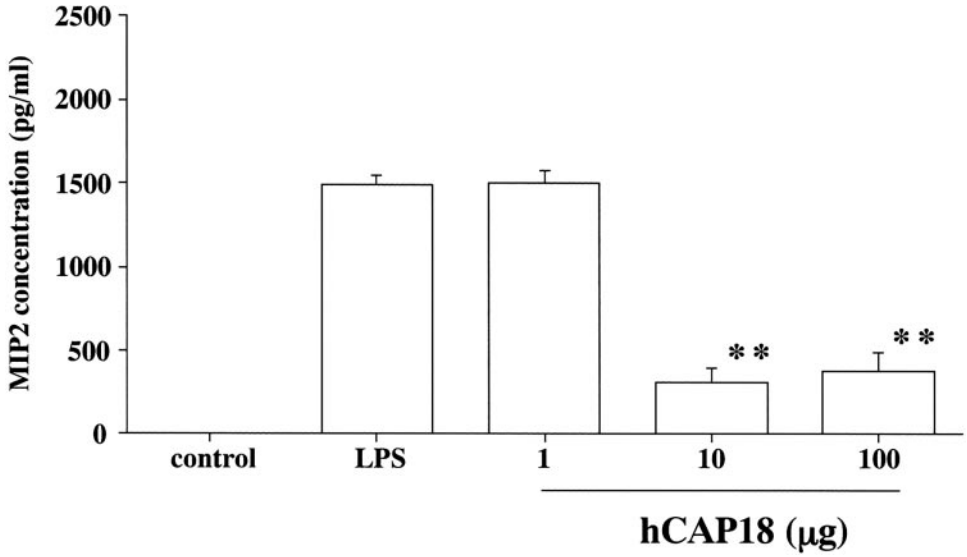

FIGURE 8. Effect of hCAP18 peptide on MIP-2 concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each data point represents the mean ± SD of eight rats. **Significantly different from LPS group ($P < 0.01$).

thought to be important for interaction with a negatively charged amphipathic LPS, lipid-A moiety.

EIU is an animal model of acute anterior inflammation that is caused by an injection of LPS.[1] In this model, vascular endothelium, macrophages, and other cells may be directly activated by LPS. Although this model is not an actual simulation of clinical uveitis (like Behçet's disease or Reiter's syndrome), LPS-induced cytokine activation with inflammatory reactions appears to mimic the acute phase of anterior uveitis.

We determined the dose needed to cause a reduction in LPS efficacy by erythrocyte agglutination assay. The LPS-binding activity of the peptide was varied with LPS characteristics (Isogai E, unpublished data, 2002). In this study, the MAC of hCAP18 peptide for LPS from *S. typhimurium* was 5.0 μg/mL. Kirikae et al.[30] reported that preincubation of LPS with hCAP18 peptide (LPS-to–peptide ratio, 1:1, intraperitoneally), attenuates the lethal toxicity of LPS in D-Gal N-sensitized mice. In our EIU model, 200 μg LPS was needed. As a general rule, the level of a drug in the blood after intravenous administration is higher than that after intraperitoneal administration. Therefore, we believed that anti-inflammatory effects could be observed with 100 μg hCAP18 peptide. Fortunately, the anti-inflammatory effect of this peptide on EIU occurred at a dosage of 100 μg. For this reason, hCAP18 peptide doses with a geometric ratio of 10 were established. This rationale for dose setting mostly matched the results of the MAC of hCAP18 peptide against LPS in vitro.

These findings suggest the following: First, a possible mechanism for the anti-inflammatory effect of hCAP18 peptide is that it suppresses onset of LPS-triggered inflammatory reactions by binding directly to LPS. Second, under this experimental condition (including the strain and quantity of LPS), 10 μg hCAP18 peptide is sufficient to counteract the effects of LPS in rats significantly.

A possible mechanism for the ocular anti-inflammatory effect of hCAP18 peptide is that it suppresses the onset of LPS-triggered inflammatory reactions by binding directly to LPS. hCAP18 may bind to the lipid-A moiety of LPS and inhibit interaction between LPS and LBP, which transports LPS into $CD14^+$ cells, thereby suppressing the binding of LPS to $CD14^+$ cells[27] and possibly attenuating toll-like receptor–mediated $CD14^+$ cell activation.[27] Nagaoka et al.[27,37] recently reported that a CAP18/LL-37-derived antimicrobial peptide (an 18mer that is part of our 27mer peptide and the 37mer active domain) suppresses the binding of LPS to $CD14^+$ cells and attenuates production of TNF-α by these cells. Furthermore, they reported that these peptides effectively suppress LPS-induced TNF-α production and protect mice from lethal endotoxin shock in the murine endotoxin shock model. The CAP18 active domain thus exerts strong anti-LPS activities against both endotoxin shock and EIU.

In summary, in the present study that hCAP18 peptide suppressed development of EIU. hCAP18 peptide suppressed NO, PGE2, TNF-α, MCP-1, and MIP-2 concentration in aqueous humor. These findings suggest that hCAP18 peptide may be a promising agent for the treatment of inflammation.

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