Cyclic *Limulus* Anti-lipopolysaccharide (LPS) Factor-Derived Peptide CLP-19 Antagonizes LPS Function by Blocking Binding to LPS Binding Protein

Yao Liu, Bing Ni, Jian-dong Ren, Jian-hong Chen, Zhi-qiang Tian, Min Tang, Di Li and Peiyuan Xia

Department of Pharmacy, Southwest Hospital, Third Military Medical University; a Department of Immunology, Third Military Medical University; Chongqing 400038, China; and a Department of Pharmacy, General Hospital of Chengdu Military Command; Chengdu 610083, China.

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Inflammation and septic shock due to endotoxins from Gram-negative bacteria infection continue to pose significant challenges to human healthcare. It is, therefore, necessary to develop therapeutic strategies targeting endotoxins, such as lipopolysaccharide (LPS), to prevent their potentially systemic effects. Pathogenesis due to Gram-negative bacteria involves LPS binding to the host LPS-binding protein (LBP), causing detrimental downstream signaling cascades. Our previous study showed that CLP-19, a synthetic peptide derived from the *Limulus* anti-LPS factor (LALF), could effectively neutralize LPS toxicity; however, the detailed mechanisms underlying this anti-LPS effect remained unexplained. Thus, we carried out investigations to determine how the CLP-19 neutralizes LPS toxicity. CLP-19 was found to block LPS binding to LBP in a dose-dependent manner, as evidenced by competitive enzyme-linked immunosorbent assay (ELISA). In peripheral blood mononuclear cells, CLP-19 blocked LPS-induced phosphorylation of mitogen activated protein kinase (MAPK) signaling proteins p38, extracellular signal-regulating kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK)1/2. Furthermore, CLP-19 potency in LPS antagonism *in vitro* and *in vivo* was directly associated with its ability to block the LPS-LBP interaction. Taken together, the results suggested that CLP-19’s inhibitory effect on LPS-LBP binding and on the subsequent MAPK pathway signaling may be responsible for its anti-LPS mechanism. This peptide appears to represent a potential therapeutic agent for clinical treatment of sepsis.

Key words cyclic *Limulus* peptide; lipopolysaccharide; lipopolysaccharide binding protein; *Limulus* anti-lipopolysaccharide factor; tumor necrosis factor alpha

Sepsis manifests from an exaggerated cascade of otherwise normal molecular and cellular events following an inflammatory response to pathogenic infection. In particular, Gram-negative bacteria infections, such as *Escherichia coli* related urinary tract infection or *Staphylococcus aureus*-related skin infection, are commonly associated with sepsis onset. Gram-negative bacteria outer membrane is primarily composed of lipopolysaccharide (LPS), which binds to and activates a variety of host cell types, including macrophages, neutrophils and endothelial cells. LPS-mediated immune cell activation culminates in production and secretion of pro-inflammatory cytokines, such as interleukins and tumor necrosis factor-alpha (TNF-α); moreover, this event is strongly associated with systemic progression of sepsis to involvement of a cardiovascular component, known as septic shock, which is considered to be a key step towards sepsis death. Interestingly, LPS binding and its immune effects are dependent upon interaction with a host soluble serum protein, the LPS binding protein (LBP). It is hypothesized that a therapeutic agent that possesses the ability to block this interaction will be efficacious towards treating septic events, including the life-threatening septic shock.

The *Limulus* anti-LPS factor (LALF), a small basic peptide found in hemocytes of the marine chelicerates *Tachypleus tridentatus* and *Limulus polyphemus*, has been demonstrated to inhibit endotoxin-mediated activation of the coagulation cascade, another key event in sepsis pathophysiology. The LALF sequence that lies between amino acids 31 and 52 represents the region responsible for LALF-LPS binding. Peptides derived from LALF 31–52 bind to LPS with high affinity in a dose-dependent manner. Importantly, these peptides elicit a significant protective effect in the sepsis mouse model, prolonging life span and decreasing mortality. Based upon a detailed analysis of LALF structural features, we designed and synthesized a 19 amino acid cyclic peptide, CLP-19, that showed remarkable properties of LPS neutralization and inhibition of TNF-α production in response to LPS stimulation. Herein, we describe our subsequent studies to characterize the CLP-19 peptide’s ability to interfere with LPS and its potential to neutralize LPS toxicity *in vivo*. The functional interactions between CLP-19, LBP and LPS were evaluated during the process of LPS antagonism by competitive enzyme-linked immunosorbent assay (ELISA). The capacity of CLP-19 to protect LPS-challenged animals and to suppress TNF-α release stimulated by LBP-mediated signaling cascades was determined in a mouse endotoxemic model. CLP-19 was determined to block LBP binding to LPS, thereby inhibiting subsequent signaling processes that are associated with sepsis pathophysiology and mortality.

MATERIALS AND METHODS

Mice Inbred Kunming mice were obtained from the Experimental Animal Center of Third Military Medical University (Chongqing, China). The mean weight of mice on the day of the experiments was 20 ± 2 g.

Reagents *Escherichia coli* O111:B4-isolated LPS, polymyxin B (PMB) and the 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system were purchased from Sigma-
Aldrich (St. Louis, MO, U.S.A.). ELISA kits for recombinant human LBP and human TNF-α were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Ficoll-Hyphaque density media was from Hoo Yang Biological Manufacture Co., Ltd. (Tianjin, China). Kinetic turbidimetric assay kit for *Limulus* amebocyte lysate (LAL) was from Zhanjiang A&C Biological (Zhanjiang, China).

**Antibodies** Mouse anti-human LBP monoclonal antibody (mAb) 6G3 was from HyCult Biotech (Uden, Netherlands). Anti-phospho-p38 (ab4822), anti-phospho-extracellular signal-regulating kinase (ERK)1/2 (ab50011), anti-phospho-c-Jun N-terminal kinase (JNK)1/2 (ab4821) and anti-phospho-nuclear factor (NF)-κB p65 (ab28810) were from Abcam (Cambridge, MA, U.S.A.). Horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin G (IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

**Preparation of Peptides** Head-to-tail cyclized peptide CLP-19 (CRKPTFRRLWKIKFKFKC; molecular weight: 2511.1 Da) was synthesized by using the Symphony peptide synthesis system (Protein Technologies, Inc., Tucson, AZ, U.S.A.), which proceeds by a stepwise solid phase peptide assembly starting from an Fmoc-Lys (Boc)-Wang resin. After drying, the resins were cleaved by application of a trifluoroacetic acid cocktail solution, and the peptide was purified by high performance liquid chromatography (purity: 98.4%; by Shen Zhen Hybio Engineering, Shenzhen, China) and dissolved in phosphate-buffered saline (PBS) as a working solution.

**Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)** PBMCs were obtained from healthy volunteers, who provided informed consent, as previously described. Briefly, blood was collected by venipuncture and anticoagulated with heparin. Diluted samples (1:1 with Hank’s balanced salt solution (HBSS)) were layered onto Ficoll-Hyphaque (1.077 g/ml) and centrifuged at 400 **g** for 20 min. PBMCs were then harvested from the plasma/Ficoll-Hyphaque interface, washed three times in HBSS, counted using a hemocytometer, and resuspended in RPMI 1640 to the desired density.

**Competitive ELISA Assay** All the related reagents were prepared in PBS. LPS pre-coated 96-well ELISA plates were prepared as described elsewhere. Bovine serum albumin (BSA; 1%) was added to reduce non-specific binding. After the plates were washed, either: (1) various concentrations of CLP-19 or PMB were added along with LBP (100 ng/ml), respectively, and plates were incubated for 30 min at 37°C; (2) various concentrations of CLP-19 were pre-incubated with immobilized LPS for 30 min at 37°C, after which LBP was added and plates were incubated for an additional 30 min at 37°C; or (3) LBP (100 ng/ml) was incubated at 37°C for various time periods, and various concentrations of CLP-19 were added followed by additional incubation for 1 h at 37°C. Then, the plates were washed extensively with PBS containing 0.1% Tween-20 (PBST) and anti-LBP mAb (1:5000 in PBS containing 0.1% BSA) was added and incubated for 1 h at 37°C. After another round of PBST washing, the HRP-conjugated goat anti-mouse IgG (1:5000 in PBS containing 0.1% BSA) was added and incubated for 1 h. Finally, TMB was added and incubation carried out for 5 min. The reaction was terminated by addition of 0.1 M sulfuric acid, and absorbance at 450 nm was quantitated in a microtiter plate reader.

**Western Blot** PBMCs were treated with LPS (100 ng/ml) and with or without CLP-19 (25, 50, 100 μg/ml) at 37°C for 30 min. PBMC (25, 50, 100 μg/ml) treatment was used as a control. Total cellular proteins were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred to polyvinylidene fluoride membranes which were then blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% skim milk for 1 h at room temperature. Appropriate primary antibody (anti-phospho-ERK1/2, anti-phospho-JNK1/2, or anti-phospho-p38, respectively) was added and the reaction incubated overnight at 4°C. Immunoreactive proteins were detected by HRP-conjugated secondary antibody and an enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, U.S.A.).

**Evaluation of LPS-Induced TNF-α Release from PBMCs** PBMCs were cultured for 2 h in 96-well plates (1.5×10⁵ cells/well) containing RPMI 1640 supplemented with 10% normal human serum, which served as the source of endogenous LBP. Then, one of the following reaction mixtures was added to the wells: (1) pre-incubated (30 min, 37°C) CLP-19 at various concentrations with LPS (100 ng/ml); (2) CLP-19 (100 μg/ml) or LPS (100 ng/ml) added at different intervals, (I) CLP-19 added 30 min before LPS, (II) CLP-19 mixed with LPS for 30 min and the two added simultaneously, or (III) CLP-19 added 30 min after LPS; (3) cells stimulated with LPS (100 ng/ml) alone and (4) untreated (used as positive and negative controls, respectively). All reactions were incubated for 4 h at 37°C, after which the supernatant from each well was collected and TNF-α level was determined by ELISA.

**Evaluation of LALF Peptides in LPS-Challenged Mice** A Kunming mouse model of experimental endotoxemia was established to characterize the efficacy of our synthetic peptide in protecting an immunologically-intact host against serious LPS attack. Experimental endotoxemia was induced by intraperitoneal (i.p.) injection of LPS (1 μg/kg) 1 h after d-galactosamine (600 mg/kg) sensitization. One-hundred percent mortality of these mice was experienced within 72 h, in the absence of therapeutic intervention (peptide treatment). Dose-dependence was evaluated according to the methods previously described. To evaluate time-dependence of the peptide treatment, mice (n=10 per group) were administered an i.p. injection of peptides (2 mg/kg) at either: 1 h before LPS; simultaneously with LPS; or 1 h after LPS. Survival was recorded every 4 h over a period of 72 h. The research was conducted in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals.

**Statistics and Presentation of Data** Each experiment was repeated at least twice and each data point represents the mean of at least three independent samples tested in parallel. The values are expressed as mean±standard deviation (S.D.). Intergroup differences were determined by one-way analyses of variance. The student’s t-test was used to determine significance of differences in the cytokine concentrations. Survival data were analyzed by Chi-squared exact test. A p-value <0.05 was considered significant and <0.01 was considered very significant.
RESULTS

CLP-19 Blocks the Binding of LBP to LPS in Vitro

Both PMB and CLP-19 were able to significantly block the binding of LBP to LPS, in a dose-dependent manner (Fig. 1A). Even at the lowest concentration (10 μg/ml), CLP-19 inhibited the LPS-LBP binding rate by more than 50%. Moreover, the inhibiting effects of both peptides reached a steady state (10—15% of the LPS-LBP binding rate) when their concentrations were greater than 40 μg/ml. No additive effects were observed when CLP-19 was added simultaneously with LBP or when it was pre-incubated with LPS for 30 min prior to LBP addition (p>0.05).

We next investigated whether CLP-19 could disrupt the bound state of LPS-LBP. To this end, one of three doses (20, 40, 80 μg/ml) of CLP-19 was added to pre-bound LPS-LBP; only a slight disruption of the complexed LPS-LBP was detected (Fig. 1B). However, the disruptive (inhibitory) effects of CLP-19 were time-dependent, as evidenced by the three concentrations elicits gradually weaker effects as the interval between CLP-19 additions after LBP exposure to LPS was prolonged. Interestingly, no dose-dependence was observed as there was no difference among the effects of the three doses given at the same time points (p>0.05). This result indicates that CLP-19 was unable to sufficiently disrupt the pre-formed LPS-LBP complexes even when the concentration of CLP-19 was 200—800 times higher than that of LBP.

Inhibition of LPS-Mediated Phosphorylation of p38, ERK1/2 and JNK1/2 by CLP-19

To understand the biological significance of the CLP-19 mediated suppression of LPS activity, we used human macrophage cultures that are known to constitutively express the membrane-bound LPS receptors, CD14 and TLR4. Western blot analysis showed that LPS stimulation over a 30 min time course led to progressive phosphorylation of p38, ERK1/2 and JNK1/2, the major members of the mitogen-activated protein kinase pathway.
We next examined the effect of CLP-19 on LBP–LPS complex-driven MAPK signaling. As shown in Fig. 2, stimulation of PBMCs with 100 ng/ml LPS alone caused increased phosphorylation of p38, ERK1/2 or JNK1/2. When CLP-19 or PMB was added to the LPS treatment, however, phosphorylated p38, ERK1/2 and JNK1/2 decreased in a dose-dependent manner. Most importantly, treatment with the highest dose (100 μg/ml) of CLP-19 caused an almost complete reduction in phosphorylated p38, ERK1/2 and JNK1/2, as compared with the untreated control.

**CLP-19 Inhibits TNF-α Production in PBMCs** Since CLP-19 exhibited a robust ability to block the binding of LPS-LBP, we examined its effects on TNF-α release from LPS-stimulated PBMCs. As shown in Fig. 3, when cells were treated with the mixture of CLP-19 and LPS TNF-α secretion was reduced; this finding was in agreement with previously published results from the RAW264.7 cell line, which also demonstrated blocking LPS binding to LBP. The effect showed a dose–response relationship from 10 to 40 μg/ml, and no increases were observed when the peptide concentration exceeded 40 μg/ml (Fig. 3A). On the other hand, when the highest dose of CLP-19 (100 μg/ml) was added 30 min before LPS, TNF-α secretion was inhibited by 52.5%, and when added together with LPS the inhibition rate was 68.3%; when CLP-19 (100 μg/ml) was added 30 min after LPS the inhibition rate of TNF-α secretion was only 8.8% (Fig. 3B).

**CLP-19 Protects Mice from LPS Challenge** In our previous study, we found that CLP-19 treatment was most effective in protecting mice from LPS-induced death when administered at a dose of 2 mg/kg. In order to further explore the time-dependent effectiveness of the peptide treatment, in this study we treated LPS-challenged mice with CLP-19 or PMB either prior to, together with, or following LPS injection. A significant protective difference was observed between the groups of peptide intervention administered at various times (p<0.05; Fig. 4). Mice receiving the simultaneous CLP-19 or PMB treatment with LPS-challenge exhibited 72 h survival rates of 90% and 80%, respectively. No significant difference was observed between the survival rates of CLP-19 or PMB. In contrast, there was no significant protective effect associated with CLP-19 or PMB when administered as 1h pre-treatment (40% vs. 30% survival rate at 72 h, respectively) or 1 h after LPS-challenge (50% survival rate at 72 h for both).

**DISCUSSION**

Previously, our lab developed CLP-19, a synthetic cyclic peptide from the *Limulus* anti-LPS factor capable of neutralizing LPS *in vivo*. Here, we sought to characterize the molecular mechanism by which CLP-19 antagonized LPS and to determine its potential for therapeutic protection from LPS induced illness in an animal model. Our results indicated that CLP-19 blocks LPS-induced responses by interfering with LPS binding to LBP, which otherwise mediates LPS transport to CD14 and activation of key signaling processes. By using a competitive ELISA we demonstrated that CLP-19 inhibited LPS-LBP binding, regardless of whether the peptide was introduced simultaneously with or prior to LBP. On the other hand, the peptide was largely incapable of disrupting the pre-complexed LPS-LBP. Importantly, the ability of CLP-19 to block the binding of LPS to LBP correlated with our previous results that demonstrated this peptide can effectively antagonize LPS *in vitro* and *in vivo*.

Previous studies by others have examined similarly derived synthetic peptides from LBP and cationic antimicrobial peptides. Two potential mechanisms to explain peptide-mediated LPS detoxification have emerged from those studies, namely inhibition of LPS-LBP binding and blockade of the LPS-LBP complex binding to its receptor. It is important to note that the LALF and its derived peptides, which we study, have a significantly high affinity for LPS and appear to have superior neutralizing effects on LPS. Therefore, we investigated whether our synthetic LALF-derived CLP-19 peptide functioned by interfering with the LPS-LBP interaction.

CLP-19 exhibited a LPS-neutralizing potency equal to that of the well-characterized cationic anti-LPS peptide PMB.
The results from our binding assay indicated that both CLP-19 and PMB had a significant dose-dependent inhibitory effect on LBP binding to immobilized LPS. Furthermore, the inhibitory effect of CLP-19 was powerful when added before or together with LBP; however, CLP-19 was unable to sufficiently disrupt pre-formed LPS-LBP complexes, even when the concentration of CLP-19 was 200—800 times higher than that of LBP.

PBMCs, which isolated from human blood, are more suitable to simulate clinical conditions after stimulation of LPS than mouse macrophage cell line RAW264.7. We also observed that CLP-19 was able to reduce TNF-α secretion from LPS-stimulated human PBMCs in the presence of healthy human serum (normally containing ca. 7 μg/ml LBP). The dose–response results of TNF-α suppression were in excellent accordance with our LPS-LBP binding measurements described above. Moreover, CLP-19 exerted a superior inhibitory effect on TNF-α release when pre-incubated with LPS before the interaction with LBP. In our studies, human serum served as the source of LBP and provided sufficient amounts of LBP in the experimental wells. When CLP-19 was added first into the wells in the presence of serum, followed by LPS addition, the peptide and LBP bound to LPS simultaneously, leading to more or less LPS-LBP binding and affecting TNF-α release. Otherwise, on account of LPS neutralization that occurred during pre-incubation with CLP-19, minimal amounts of unbound LPS were available to bind with LBP; this hypothesis might explain why the inhibition rate of CLP-19 tended to be significantly higher under this particular experimental condition. In addition, when CLP-19 was added after cells had been pre-incubated with LPS, only slight inhibition of TNF-α release was observed. These results further verified that CLP-19 was mostly ineffective for interfering with complexed LPS-LBP, and suggested that CLP-19 acted as a competitive binding inhibitor not an active disruptor of pre-existing binding.

The ability of CLP-19 to interfere with the LPS-LBP complex was also verified by evaluating the downstream signal related MAPK family members. Western blot analysis revealed that the phosphorylation status of p38, JNK1/2 and ERK1/2 was down-regulated by CLP-19. Since p38, JNK1/2 or ERK1/2 signaling occurs upstream of the inflammatory response and septic shock, inhibition of the phosphorylation of major members of the MAPK family reflects the likelihood of CLP-19 to block overexpression of cytokines, hence preventing septic shock.

We speculate that CLP-19 and LBP may share the same binding sites on LPS, or sites that are proximal and capable of influencing each other. It is worthy to note that the results from our two experimental approaches were not fully concordant. In detail, the binding experiment indicated that no difference in blocking effect existed between the conditions when CLP-19 was added before or together with LBP, while the inhibition of TNF-α release was unequal when CLP-19 was added at various times. In fact, the concentration of LBP in the medium was relatively higher than that used in the binding assay (100 ng/ml), which may account for the disparity between the results of these two experiments.

Our in vitro findings (reduced TNF-α, and dose- and time-dependent properties) were recapitulated in LPS-challenged mice upon CLP-19 treatment. It was also notable that the prophylactic and therapeutic effects of CLP-19 were contrary to what was exhibited in vitro. When added before LPS, CLP-19 showed an obvious effect of antagonizing LPS, whereas it was nearly ineffective in helping animals to combat LPS exposure. The possible explanation for this event is that the small size and cationic nature of CLP-19 and its half-life time is 21 min in bloodstream. Furthermore, while CLP-19 had little impact on the LPS-induced response in vitro when added after the interaction of LPS and LBP, it exerted a moderate preventative activity in mice under the corresponding condition; we presumed this could be due to the clearance of LPS by some molecules in the blood. Furthermore, the other researchers found LALF also have immunoregulatory effects such as down regulation of inflammatory protein HMGB1 in sepsis model. CLP-19 which derived from LALF, may have similar effect such as immunoregulation effect on LPS induced sepsis, and this effect can partially protected mice whenever it be administrated.

Taken together, our findings revealed that CLP-19 can inhibit LPS-induced TNF-α production and can protect mice from lethal LPS attack; furthermore, these properties are associated with CLP-19’s ability to block LPS from binding to LBP. This idea is also supported by a previous study in which the abilities of LBP monoclonal antibodies to interfere with the LPS-LBP interaction were found to be responsible for LPS antagonism. Therefore, we hypothesize that LPS-LBP binding blockade mediated by CLP-19 may be useful as a strategy for the treatment of sepsis.

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