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Phospholipase C Activator m-3M3FBS Protects against Morbidity and Mortality Associated with Sepsis

Sang Doo Kim,*† Hak Jung Kim,*† Jae Woong Shim,* Ha Young Lee,*†
Sung Kyun Lee,*† Soonil Kwon,*† Young Su Jung,*† Suk-Hwan Baek,‡ Joon Seong Park,§
Brian A. Zabel,‖ and Yoe-Sik Bae*‡,‖

Although phospholipase C (PLC) is a crucial enzyme required for effective signal transduction and leukocyte activation, the role of PLC in polymicrobial sepsis remains unclear. In this study, we show that the direct PLC activator m-3M3FBS treatment significantly attenuates vital organ inflammation, widespread immune cell apoptosis, and mortality in a mouse sepsis model induced by lethal cecal ligation and puncture challenge. Mechanistically, m-3M3FBS–dependent protection was largely abolished by pretreatment of mice with the PLC-selective inhibitor U-73122, thus confirming PLC agonism by m-3M3FBS in vivo. PLC activation enhanced the bactericidal activity and hydrogen peroxide production of mouse neutrophils, and it also enhanced the production of IFN-γ and IL-12 while inhibiting proinflammatory TNF-α and IL-1β production in cecal ligation and puncture mice. In a second model of sepsis, PLC activation also inhibited the production of TNF-α and IL-1β following systemic LPS challenge. In conclusion, we show that agonizing the central signal transducing enzyme PLC by m-3M3FBS can reverse the progression of toxic shock by triggering multiple protective downstream signaling pathways to maintain organ function, leukocyte survival, and to enhance microbial killing. The Journal of Immunology, 2012, 189: 2000–2005.

Sepsis is a systemic inflammatory disease induced by live bacteria or bacterial products such as LPS (1). In the United States, >500,000 patients per year develop sepsis, and the incidence rate is increasing ∼1.5% per year (2). Moreover, the overall mortality associated with sepsis ranges from 30 to 70% (2). Thus, despite >20 y of extensive research, sepsis remains the major cause of death in intensive care units (3). Evidence now indicates that sepsis-induced lethality is accompanied by an inability to regulate the inflammatory response due to substantial impairment of the innate immune system during early sepsis (i.e., the first 6 h) (4–6). Additionally, excessive lymphocyte apoptosis also occurs during sepsis, resulting in clinical signs of multiorgan failure (7, 8), and the levels of the proinflammatory cytokines TNF and IL-1β are substantially increased (9–11). We and others therefore hypothesize that effective treatment of sepsis requires an approach that enhances bactericidal activity, prevents systemic cytokine imbalance, and blocks widespread lymphocyte apoptosis.

Phospholipase C (PLC) is a crucial enzyme required for effective conversion of extracellular stimuli into intracellular signals that result in leukocyte activation. PLC hydrolyzes phosphatidylinositol biphosphate into inositol-1,4,5-trisphosphate and diacylglycerol, which then mediate intracellular calcium release and activation of protein kinase C, respectively (12). Increases in intracellular calcium and protein kinase C activation subsequently mediate various intracellular signaling events, including the activation of phospholipase A2, phospholipase D, and MAPKs. Diverse cellular responses including superoxide generation, cytokine secretion, and cell survival are modulated by PLC-mediated signaling events (13–15).

Given its central role in leukocyte activation, PLC is therefore an attractive therapeutic target in sepsis. We previously identified m-3M3FBS, a small molecule that significantly enhanced superoxide production by neutrophils in vitro, likely resulting from direct PLC agonism (16). Reactive oxygen species such as superoxide anion are important weapons for bacteria killing activity of neutrophils (17). Given the essential role of superoxide anion in host defense against bacterial infection, as well as superoxide anion producing activity of a PLC activator (m-3M3FBS), we investigated the in vivo efficacy of m-3M3FBS in preclinical mouse models of sepsis and define the mechanisms of septic protection by this novel PLC pharmacoenhancer.

Materials and Methods

Animals and sepsis model

Male wild-type ICR mice were used as an experimental sepsis model as previously described (18). All experiments involving animals adhered to guidelines and received the approval of the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University. For cecal ligation and puncture (CLP), mice were anesthetized with Zoletil (50 mg/kg, i.p.) and Rompun (10 mg/kg, i.p.). After which a small abdominal midline incision was made to expose the cecum. The cecum was then ligated below the ileocecal valve, punctured twice through both surfaces (once for measurement of cytokine production) using a 22-gauge needle, and the abdomen was closed. Sham CLP mice were subjected to the same procedure but without ligation and puncture of the cecum. Survival was monitored once daily for 10 d.
Measurement of bactericidal activity in vivo

Twenty-four hours after CLP, peritoneal lavage fluid was collected and cultured overnight on blood agar base plates (Trypticase Soy Agar Deep; Becton Dickinson) at 37°C. The numbers of CFUs were counted as described previously (18).

Quantification of pulmonary edema

The extent of pulmonary edema was quantified by evaluating the wet/dry (W/D) weight ratio of the lung as described previously (19). Whole harvested wet lungs were weighed and then placed in an oven for 48 h at 60°C. The dry weight was then measured and the W/D weight ratio calculated.

Tissue histology

Mice were subjected to CLP surgery and given PBS or m-3M3FBS at a dose of 1.5 mg/kg 2 h later. The mice were then euthanized 24 h after surgery, after which their lungs were fixed, sectioned, and stained with H&E for morphological analysis.

Isolation of neutrophils and measurement of H2O2

Mouse neutrophils were isolated from peripheral blood using Histopaque-1077 solution (Sigma-Aldrich) as described previously (20). Freshly isolated neutrophils from normal mice were stimulated with various concentrations of m-3M3FBS for 10 min in the presence of cytochalasin B (5 μM). To investigate the role of PLC, neutrophils were preincubated with U-73122 (10 μM) or its inactive analog (U-73343) for 30 min prior to adding m-3M3FBS (10 μM) for 10 min. H2O2 in the supernatant was measured using an H2O2 assay kit (Molecular Probes).

Neutrophil bactericidal activity

Neutrophil bactericidal activity was measured according to the method previously described (18). Neutrophils were incubated at 37°C on 13-mm plastic coverslips in 60-mm plastic culture dishes (1×106 neutrophils/coverslip) for 1 h. Nonadherent cells were removed with PBS. Adherent neutrophils were incubated with 106 opsonized Escherichia coli for 1 h. After washing away the unengulfed E. coli, the number of viable bacteria in the neutrophils was determined before and after incubation with several concentrations of m-3M3FBS or vehicle for 1 h. The percentage of bacteria killed was calculated as 100 × (1 – number of CFUs after m-3M3FBS stimulation/number of CFUs before m-3M3FBS stimulation). To investigate the role of PLC, neutrophils were preincubated with U-73122 (10 μM) or its inactive analog (U-73343) (10 μM) for 30 min prior to adding m-3M3FBS (10 μM) for 1 h.

TUNEL assay

TUNEL assay was performed in paraffin–embedded tissue sections, which were first deparaffinized using a standard histological protocol. The sections were then permeabilized with Triton X-100 at 4°C for 2 min and flooded with TdT enzyme and digoxigenin-dUTP reaction buffer (TUNEL) reagent for 60 min at 37°C. The percentage of apoptotic cells (TUNEL-positive cells) was determined by counting 500 splenocytes under a light microscope (21).

Immunohistochemistry

We performed immunohistochemistry in paraffin–embedded tissue sections that were first deparaffinized using a standard histological protocol. After incubation with primary Abs against cleaved caspase-3 (Cell Signaling Technology), all sections were stained with fluorochrome-conjugated secondary Ab.

Cytokine measurement

To measure CLP-induced cytokine production in peritoneal lavage fluid, mice were given m-3M3FBS at 2, 14, 26, and 38 h after CLP. Peritoneal lavage fluid was collected at various times between 4 and 72 h after CLP, and the cytokines present in the peritoneal fluid were measured by ELISA (BD Biosciences Pharmingen).

Cytokine release from neutrophils in vitro

Mouse splenocytes (3×106 cells/0.3 ml) were placed in RPMI 1640 medium containing 5% FBS in 24-well plates and kept in a 5% CO2 incubator at 37°C. The splenocytes were then incubated with LPS (100 ng/ml) for 3 h in the presence or absence of m-3M3FBS (10 μM). LPS (100 ng/ml) was added to the cells 30 min later, and cell-free supernatants were collected, centrifuged, and measured for IL-1β or TNF-α production by ELISA (BD Biosciences Pharmingen) according to the manufacturer’s instructions.
**Statistical analysis**

Survival data were analyzed using the log-rank test. All other data were evaluated using ANOVA or t test. The Bonferroni test was used for post hoc comparisons, and statistical significance was set a priori at \( p < 0.05 \).

**Results**

**Administration of m-3M3FBS protects against sepsis-induced mortality**

Therapeutic treatment of mice with m-3M3FBS significantly protected against mortality induced by CLP in a dose-dependent manner (Fig. 1A). Survival was greatly improved when 1.5 mg/kg m-3M3FBS was injected 2 h after CLP and at 12-h intervals three or four additional times (Fig. 1B). To check whether the treatment can be delayed, we tested the therapeutic effect of m-3M3FBS by administrating it 2, 6, 12, or 24 h after CLP. Beneficial activity of m-3M3FBS was observed in 2–10 h (Fig. 1C). Given these results, our subsequent experiments were performed in CLP mice using 1.5 mg/kg m-3M3FBS beginning 2 h after CLP and at 12-h intervals three additional times.

Although we previously showed that m-3M3FBS likely acts directly on PLC to enhance superoxide production, others have reported that it may have off-target effects as well (22). Therefore, to confirm that the mechanism of action of m-3M3FBS (50 \( \mu \)M) or U-73343 (10 \( \mu \)M) was added 30 min prior to the addition of m-3M3FBS stimulation. The number of viable bacteria in the neutrophils was then counted. (E) Neutrophils were stimulated with vehicle (PBS) or m-3M3FBS (1–50 \( \mu \)M) for 1 h. (F) U-73122 (10 \( \mu \)M) or U-73343 (10 \( \mu \)M) was added 30 min prior to the addition of m-3M3FBS (50 \( \mu \)M). The amount of \( \text{H}_2\text{O}_2 \) produced from neutrophils was measured using a \( \text{H}_2\text{O}_2 \) assay kit. Data are expressed as the means ± SEM \( [n = 8 \text{ for (A)–(C); } n = 16 \text{ for (D)–(F)}] \). *\( p < 0.05 \), ***\( p < 0.001 \) by t test.

Because in the clinical setting septic patients always receive antibiotics, we tested the effect of m-3M3FBS treatment on survival in mice subjected to CLP in the presence of concomitant treatment with appropriate antibiotic regimen (gentamicin plus cephalosporin). Combination of m-3M3FBS with the antibiotics additionally increased survival rate (Fig. 1E).

To confirm the in vivo efficacy of m-3M3FBS, we tested the PLC activator in a second model of sepsis. Therapeutic administration of m-3M3FBS significantly enhanced the survival in mice injected with LPS (60 mg/kg i.p.) compared with vehicle-treated controls (Supplemental Fig. 1A). Once again, treatment with the PLC inhibitor U-73122 abolished the anti-septic activity of m-3M3FBS (Supplemental Fig. 1B).

Mortality after sepsis is associated with inflammation of vital organs, resulting in severe alveolar congestion and extensive thrombotic lesions in the lungs, for example. m-3M3FBS treatment dramatically improved the pulmonary histopathology (Fig. 1F), and the W/D ratio, an indicator of acute lung inflammation, was also significantly reduced (Fig. 1G).

**m-3M3FBS enhances bacterial clearance and \( \text{H}_2\text{O}_2 \) generation in phagocytes**

Because CLP-induced lethality is positively correlated with bacterial colony counts in peripheral blood and peritoneal fluid (1), and because m-3M3FBS enhances bacterial superoxide production by neutrophils, we asked whether m-3M3FBS enhances bacterial clearance. m-3M3FBS treatment almost completely eliminated intravascular bacteria (Fig. 2A). m-3M3FBS also significantly reduced the number of i.p. bacteria by 80% as well (Fig. 2B). PLC activator in a second model of sepsis. Therapeutic administration of m-3M3FBS significantly enhanced the survival in mice injected with LPS (60 mg/kg i.p.) compared with vehicle-treated controls (Supplemental Fig. 1A). Once again, treatment with the PLC inhibitor U-73122 abolished the anti-septic activity of m-3M3FBS (Supplemental Fig. 1B).
inhibitor U-73122 abolished the bactericidal activity of m-3M3FBS, confirming that pharmacoenhancement of PLC is responsible for the protective effects of m-3M3FBS. To confirm that m-3M3FBS treatment works directly on neutrophils to enhance increased bacteria killing, freshly isolated mouse neutrophils were allowed to ingest *E. coli* for 1 h, followed by stimulation with 1–50 μM m-3M3FBS for 20 min. The bactericidal activity of the neutrophils was markedly enhanced in a dose-dependent manner (Fig. 2C). Preincubation of mouse neutrophils with PLC-selective inhibitor (U-73122) prior to the addition of m-3M3FBS completely blocked m-3M3FBS–stimulated bactericidal activity (Fig. 2D).

Because the primary mechanism of bacteria killing by phagocytes requires H₂O₂ production (23), we evaluated the effect of m-3M3FBS on H₂O₂ generation in mouse neutrophils. m-3M3FBS increased the production of H₂O₂ in neutrophils in a concentration-dependent manner (Fig. 2E), which was almost completely blocked by pretreatment with U-73122 (Fig. 2F).

**FIGURE 3.** m-3M3FBS protects against widespread CLP-induced leukocyte apoptosis via inhibition of caspase-3 activity. (A) m-3M3FBS (1.5 mg/kg) was injected two times into CLP mice 2 and 14 h after CLP. The spleen, which was collected 24 h after sham, CLP plus PBS, or CLP plus m-3M3FBS administration was used for H&E staining (original magnification ×400). (B) The spleens from the mice described in (A) were used for TUNEL assay. (C) TUNEL-positive cells from spleen of the mice described in (A) were quantified. (D) The spleens from the mice described in (A) were used for immunohistochemistry with cleaved caspase-3 Ab (original magnification ×100). The data are representative of eight mice per group (A, B, D). Data are expressed as the means ± SEM (n = 8) (C). *p < 0.05, ***p < 0.001 by t test compared with CLP plus vehicle.

**FIGURE 4.** Effect of m-3M3FBS on CLP-induced cytokine production. m-3M3FBS (1.5 mg/kg) was injected four times into CLP mice at 2, 14, 26, and 38 h after CLP. Separate groups of animals were given sham, CLP plus PBS, or CLP plus m-3M3FBS treatment. Peritoneal fluids were collected at several different times after CLP. The amounts of cytokines in the peritoneal fluid were determined by ELISA analysis. (A) IL-1β, (B) TNF-α, (C) IL-12, (D) IFN-γ. Data are expressed as the means ± SEM [n = 8 for (A)–(D)]. *p < 0.05, **p < 0.01, ***p < 0.001 by ANOVA compared with CLP alone.
m-3M3FBS inhibits CLP-induced apoptosis of splenocytes and thymocytes

CLP-induced sepsis caused cell death of splenocytes and thymocytes, indicated by small compacted nuclei (pyknosis) with multiple nuclear fragments (apoptotic bodies) in apoptotic lymphocytes (Fig. 3A); however, these effects were significantly inhibited by m-3M3FBS (Fig. 3A). These results are also reflected in DNA fragmentation analysis (TUNEL) (Fig. 3B, 3C). To quantify the apoptotic cell numbers, we counted TUNEL-positive cell number and found that the administration of m-3M3FBS strongly inhibited CLP-induced apoptosis from thymus and spleen (Fig. 3C). Similar results were observed when caspase-3 activation was used as a marker of splenocyte apoptosis. CLP caused substantial caspase-3 activation in splenocytes, which was inhibited by in vivo treatment with m-3M3FBS (Fig. 3D).

Effect of m-3M3FBS on CLP-induced cytokine production

The effect of m-3M3FBS on CLP-induced cytokine production in peritoneal fluid was measured from 4 to 72 h after CLP (Fig. 4). CLP induced a dramatic increase in proinflammatory cytokine production (IL-1β and TNF-α) within 24 h (Fig. 4A, 4B). Treatment with m-3M3FBS significantly decreased IL-1β and TNF-α levels (Fig. 4A, 4B). However, the level of IL-12p70 was significantly increased by injection of m-3M3FBS (Fig. 4C). The level of IFN-γ was significantly increased by injection of m-3M3FBS at the early time (4 h after CLP) but the IFN-γ level was much lower in m-3M3FBS at the late time (24 h after CLP) (Fig. 4D).

Effect of m-3M3FBS on LPS-induced proinflammatory cytokine production

To determine whether m-3M3FBS acts directly on leukocytes to inhibit proinflammatory cytokine production, we treated freshly isolated splenocytes with LPS (prototypical of microbial signals) for 100 ng/ml. The levels of TNF-α, IL-1β, and IL-6 following LPS stimulation were significantly decreased in m-3M3FBS–treated cells compared with LPS only treated cells in a concentration-dependent manner (Fig. 5A–C).

Because administration of m-3M3FBS enhanced IFN-γ and IL-12 production in the CLP model in vivo (Fig. 4), we next asked whether there was a direct effect of m-3M3FBS IFN-γ and IL-12 production in mouse splenocytes. Stimulation of mouse splenocytes with several different concentrations of m-3M3FBS caused a dose-dependent increase in IFN-γ and IL-12 production (Fig. 5D, 5E). The results indicate that m-3M3FBS directly stimulates the production of IFN-γ and IL-12.

Discussion

In this study, we demonstrated that therapeutic administration of m-3M3FBS after induction of sepsis by CLP effectively inhibited CLP-induced lethality in mice. The therapeutic effect of m-3M3FBS in our experimental animal models of sepsis was mediated by at least three different mechanisms: 1) increase in bactericidal activity, 2) inhibition of leukocyte apoptosis, and 3) inhibition of proinflammatory cytokine secretion.

To defend against invading microorganism, the innate immune cells produce intracellular toxic mediators including reactive oxygen species. In this study, we demonstrated that m-3M3FBS enhanced H2O2 production, which was associated with enhanced bacterial clearance; U-73122 (a PLC inhibitor) but not U-73343 (an inactive analog) significantly inhibited m-3M3FBS–induced H2O2 production in neutrophils. From the data, we suggest that the bactericidal effect enhanced by m-3M3FBS is related to augmentation of the production of bactericidal mediators within phagocytes. Previously, PLC was reported to play an important role against infectious agents. Graham et al. (15) demonstrated that the activation of integrin-induced PLC-γ2 is important to the activation of NADPH oxidase, resulting in the production of reactive oxygen species and stimulation of host defense in mouse neutrophils. This result supports our assertion that the activation of PLC enhances...
the generation of reactive oxygen species, resulting in a therapeutic effect against experimental sepsis.

In terms of cytokine profile change after administration of m-3M3FBS, we observed that m-3M3FBS augmented the production of Th1 type cytokines (IL-12 and IFN-γ) in CLP mice (Fig. 4). Previous reports demonstrated that increases in IFN-γ, IL-12, and IL-2 levels have beneficial effects against sepsis (24, 25). IL-12 also has been known to act as an important mediator in the defense against infectious agents (25–27). In our previous report, we also demonstrated that IL-12 is essentially required for the therapeutic action of the immune-stimulating peptide WKYMVm against sepsis (28). Given our findings and previous reports, we think that increased IFN-γ and IL-12 might contribute on anti-septic activity of m-3M3FBS.

m-3M3FBS induced a dramatic change in the levels of several cytokines in the early stages of the CLP model. Proinflammatory cytokine (IL-1β and TNF) levels were decreased by m-3M3FBS treatment (Fig. 4). Others have shown that decreases in certain proinflammatory cytokines, such as IL-1β and TNF, contribute to an increase in survival in sepsis models (29–31). The inhibitory effect of m-3M3FBS on the production of proinflammatory cytokines against LPS was also observed from mouse spleenocytes (Fig. 5A–C). The results suggest that the activation of PLC by m-3M3FBS may negatively regulate TLR4-induced signaling in splenocytes.

In conclusion, we report that therapeutic administration of m-3M3FBS against CLP sepsis model effectively inhibited CLP-induced lethality in mice. Our data suggest that the target molecule for m-3M3FBS, PLC, can be a useful target for the development of therapeutic drugs against sepsis and microbial infectious diseases.

Disclosures
S.D.K. and Y.-S.B. have pending patent applications. The other authors have no financial conflicts of interest.

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