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PEGylated lysozymes with anti-septic effects in human endothelial cells and in mice

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ABSTRACT

High mobility group box 1 (HMGB1) was recently shown to be an important extracellular mediator of severe vascular inflammatory disease, sepsis. Lysozyme (LYZ) has been shown to bind to bacterial lipopolysaccharide (LPS) and have a potential for playing a role in the therapy of inflammatory diseases. However, the effect of LYZ on HMGB1-induced septic response has not been investigated. Moreover, PEGylation effects on the antiseptic activity of LYZ are not known. Here, we show, for the first time, the anti-septic effects of PEGylated LYZ (PEG-LYZ) in HMGB1-mediated inflammatory responses *in vitro* and *in vivo*. Among four mono-PEGylated LYZs with different PEGylation sites (N-terminus, Lys^{13}, Lys^{33}, and Lys^{97}), N-terminally PEGylated LYZ showed the highest activity. Subsequently, among three N-terminally PEGylated LYZs prepared with aldehyde-activated PEGs of 5, 10, and 20 kDa, 5 kDa-PEG-conjugated LYZ (P5-K^1-LYZ) showed the highest antiseptic activity. The data showed that P5-K^1-LYZ post-treatment effectively suppressed LPS-mediated release of HMGB1. P5-K^1-LYZ also inhibited HMGB1-mediated hyperpermeability in human endothelial cells. Furthermore, P5-K^1-LYZ reduced the cecal ligation and puncture (CLP)-induced release of HMGB1 and septic mortality. Collectively, these results suggest P5-K^1-LYZ as a candidate therapeutic agent for the treatment of vascular inflammatory diseases via inhibition of the HMGB1 signaling pathway.

1. Introduction

Sepsis is a systemic inflammatory response to presumed or known infection [1]. It is a leading cause of in-hospital death in adults, and its incidence is increasing worldwide [1]. High mobility group box1 (HMGB1) is a non-histone chromosomal protein with high electrophoretic mobility [2]. Its pro-inflammatory properties, acquired upon its cellular release and consequent receptor stimulation, contribute to the pathogenesis of various human diseases [3,4]. The recent discovery of HMGB1, as a critical mediator of sepsis, stimulated an increasing interest in inflammation research field [3,4]. HMGB1 binds to several transmembrane receptors, such as receptor for advanced glycation end products (RAGE) and toll like receptor (TLR)-2 and TLR-4, and activates NF-κB and extracellular regulated kinase (ERK) 1 and 2 [5,6]. HMGB1 accumulates during sepsis, leading to multiple organ collapse and death [7]. Therefore, HMGB1 is a therapeutic target for the clinical management of sepsis.

Lysozyme (LYZ) is a bacteriolytic enzyme found in animal tissues and serum as well as in tears, saliva, breast milk and mucus. LYZ from hen egg white, which consists of 129 amino acids cross-linked with four disulfide bonds, has been widely used as an antibacterial agent in many foods and pharmaceuticals [8]. LYZ has been shown to bind to bacterial lipopolysaccharide (LPS) with a high affinity and inhibits various biological activities of LPS [9]. The binding of LYZ to LPS inhibited TNF-α production and lowered mortality of septic shock model mice caused by LPS [10]. These findings suggest the potential of LYZ for the treatment of endotoxin shock.

Protein modification using poly (ethylene glycol, PEG), which is known as PEGylation, is an approach of increasing importance for

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enhancing the therapeutic efficacy of protein biopharmaceuticals by increasing their circulation half-life, enhancing stability against proteolytic enzymes, reducing immunogenicity, and lowering toxicity [11]. In numerous studies, PEGylated proteins were demonstrated to possess better pharmacokinetic properties and efficacy than native protein products [12,13]. LYZ has been also PEGylated in several studies, but most studies have been focused on the use of LYZ as a model protein for purification process optimization and physicochemical properties evaluation of PEGylated proteins [14,15]. Recently, Wu et al. investigated the efficacy of PEG for preventing lethal gut-derived sepsis in mice due to intestinal *Pseudomonas aeruginosa* [16]. Ackland et al. reported that low-molecular-weight PEG (200–400 Da) reduced inflammatory cytokine expression, pyrexia, and mortality in both LPS and zymosan models of sepsis [17]. Zaborin et al. demonstrated that phosphorylated PEG prevents lethal sepsis in mice inoculated with multiple highly virulent pathogenic organisms from hospitalized patients [18]. Based on the biological effect of each LYZ and PEG, we hypothesized that PEGylated LYZ (PEG-LYZ), the combined form of LYZ and PEG, could give synergistic effect on the treatment of sepsis. To the best of our knowledge, we describe here, for the first time, the effects of PEG-LYZ on HMGB1 release, HMGB1-mediated proinflammatory responses, and the molecular mechanisms underlying the barrier protective effects of PEG-LYZ both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents

LYZ from chicken egg white (L7651), bacterial lipopolysaccharide (LPS; serotype: 0111:B4, L5293), Evans blue dye, crystal violet, antibiotics (penicillin G and streptomycin), sodium cyanoborohydride, sinapinic acid, α-cyano-4-hydroxycinnamic acid, dithiothreitol, and endoproteinase Lys-C were purchased from Sigma (St. Louis, MO). Monomethoxy-PEG-aldehydes with molecular weight (MW) of 5, 10, or 20 kDa ($mPEG_{5K}$-ALD, $mPEG_{10K}$-ALD, or $mPEG_{20K}$-ALD, respectively) and *N*-hydroxysuccinimidyl mPEG with MW of 10 kDa ($mPEG_{10K}$-NHS) were obtained were provided by NOF Corporation (Tokyo, Japan). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade and were used as obtained commercially.

2.2. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bioscience (Charles City, IA) and used in cell culture at passages 3–5 as described previously [19–21]. Human neutrophils were freshly isolated from whole blood (15 mL) obtained by venipuncture from five healthy volunteers, and maintained as previously described [22,23].

2.3. Permeability assay in vitro

Endothelial cell permeability after exposure to indicated LYZ or PEG-LYZs from 0 to 200 nM was quantified by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers using a modified two-compartment chamber model, as previouslhy described [24]. HUVECs were plated (5×10^4/well) in 3-μm pore size, 12-mm diameter transwells and incubated for three days. Confluent monolayers of HUVECs were treated with HMGB1 (1 μg/mL) for 16 h, and then treated with indicated LYZ or PEG-LYZs for 6 h.

2.4. In vivo permeability assays

For the *in vivo* study, male mice were anesthetized with zoletil (tiletamine and zolazepam 1:1 mixture, 30 mg/kg) and rompun (xylazine, 10 mg/kg). CLP-operated mice or mice pretreated with HMGB1 (2 μg/mouse, i.v.) for 16 h were injected with indicated LYZ or PEG-LYZs (0.57, 1.43, 2.86 or 5.72 μg per mouse i.v.), as previously described [24].

*The online supplement provides more information regarding methods.

3. Results and discussion

3.1. Preparation and characterization of PEG-LYZ

LYZ from hen egg white has seven primary amino groups, i.e. N-terminal amino group and six lysine residues, which offer potential sites for PEGylation using mPEG-ALD and mPEG-NHS. The PEGylation reaction with mPEG-NHS forms an amide bond between protein's amino group and PEG, whereas mPEG-ALD preferentially reacts with N-terminal amine and thus forms a secondary amine linkage (Fig. 1A). In the cation-exchange chromatography of reaction mixture of LYZ and $mPEG_{10K}$-NHS, two distinct peaks corresponding to PEG-LYZs except unmodified LYZ were observed (Fig. 1B). The two peaks were identified as mono-PEG_{10K}-LYZs modified at Lys^{33} (P10-K^{33}-LYZ) and Lys^{13} (P10-K^{13}-LYZ) by measuring the molecular masses of intact PEG-LYZs and their Lys-C digests with MALDI-TOF MS (Supplementary Table 1). In the cation-exchange chromatography of reaction mixture of LYZ and $mPEG_{10K}$-ALD, two peaks corresponding to PEG-LYZs except unmodified LYZ were also observed (Fig. 1C). As expected, main peak (retention time: 17.3 min) was identified as N-terminally PEGylated LYZ (P10-K^1-LYZ) and other small peak (retention time: 20.2 min) was identified as mono-PEG conjugate modified at Lys^{97} (P10-K^{97}-LYZ) by MALDI-TOF MS of intact forms and Lys-C-digested PEGylated fragments (Supplementary Table 1). The production ratio of P10-K^1-LYZ and P10-K^{97}-LYZ based on their peak areas on cation-exchange chromatogram was 81:19. Although the PEGylation reaction with mPEG-ALD at acidic pH is generally known to be site-specific for N-terminal amine of protein, slight PEGylation at Lys residues is often occurred, as also found in PEGylation study of other investigator [25].

When four mono-PEG-LYZs (P10-K^{33}-LYZ, P10-K^{13}-LYZ, P10-K^1-LYZ, and P10-K^{97}-LYZ) were initially tested in cell-based assays, P10-K^1-LYZ showed the highest biological activity and thus the additional N-terminally mono-PEGylated LYZs with different size were subsequently prepared with $mPEG_{5K}$-ALD and $mPEG_{20K}$-ALD. As like P10-K^1-LYZ, the PEGylation site of mono-PEG-LYZs prepared with $mPEG_{5K}$-ALD and $mPEG_{20K}$-ALD (P5-K^1-LYZ and P20-K^1-LYZ, respectively) was identified by MALDI-TOF MS analysis of Lys-C-digested PEGylated fragment (Supplementary Table 1). Supplementary figure 1 shows the mass spectrometric, chromatographic and electrophoretic characterization of three N-terminally PEGylated LYZs (P5-K^1-LYZ, P10-K^1-LYZ, and P20-K^1-LYZ) by MALDI-TOF MS, reversed-phase HPLC, and MCGE. These analytical techniques are useful tools for checking identity and purity of PEGylated proteins. MCGE is an electrophoresis technique based on lab-on-a-chip technology with several advantages over conventional techniques because it is highly automated, offers rapid sample analysis, and requires minimal sample consumption, in addition to being highly precise and sensitive with laser-induced fluorescence [26]. The separation principle of MCGE is similar to that of SDS-PAGE where proteins are separated based on relative molecular size in the presence of SDS. Recently, MCGE was successfully used for characterizing PEGylated forms of proteins, such

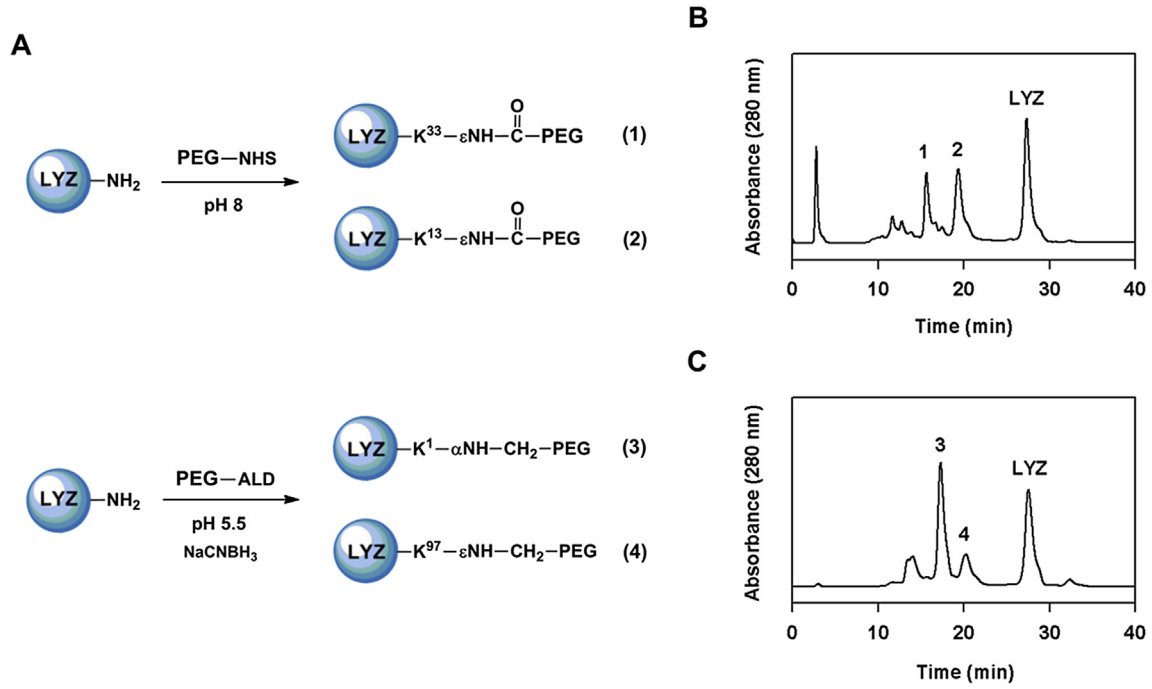

Fig. 1. PEGylation of LYZ and isolation of mono-PEGylated LYZs. PEGylation reaction of LYZ with mPEG-NHS at pH 8 and mPEG-ALD in the presence of sodium cyanoborohydride ($NaCNBH_3$) at pH 5.5 (A). The PEGylation reaction with mPEG-NHS forms an amide bond between protein's amino group and PEG, whereas mPEG-ALD preferentially reacts with N-terminal α-amine and forms a secondary amine linkage. Cation-exchange chromatograms of PEGylation reaction mixtures of LYZ with $mPEG_{10K}$-NHS (B) and $mPEG_{10K}$-ALD (C). Peaks 1: P10-K^{33}-LYZ, 2: P10-K^{13}-LYZ, 3: P10-K^{1}-LYZ, 4: P10-K^{97}-LYZ.

as α-lactalbumin, bovine serum albumin, human growth hormone, and granulocyte-colony stimulating factor [27,28]. Each spectrum, chromatogram and electropherogram of P5-K^1-LYZ, P10-K^1-LYZ and P20-K^1-LYZ demonstrates that they were successfully prepared with high purity (Supplementary Fig. 1).

3.2. Effect of PEG-LYZs on LPS- and CLP-mediated release of HMGB1

Previous studies have shown that LPS stimulates the release of HMGB1 from human endothelial cells [29,30]. In agreement with these previous results, LPS (100 ng/mL) stimulated the release of HMGB1 from HUVECs (Fig. 2A and C). To investigate the effects of PEGylation of LYZ on LPS-mediated release of HMGB1, endothelial cells were stimulated with 100 ng/mL LPS for 16 h before treatment with increasing concentrations of LYZ and PEG-LYZs for 6 h. The results shown in Fig. 2A indicate that P10-K^1-LYZ among four PEG_{10K}-LYZs (P10-K^1-LYZ, P10-K^{13}-LYZ, P10-K^{33}-LYZ, and P10-K^{97}-LYZ) is the most effective for inhibiting the LPS-induced release of HMGB1 from endothelial cells, and the optimal effects of LYZ and P10-K^1-LYZ was observed at concentrations greater than 20 nM. LYZ or PEG_{10K}-LYZs treatment alone did not affect HMGB1 release (Fig. 2A). To confirm this effect *in vivo*, we used a standardized mouse model of sepsis, in which severe sepsis was induced by CLP because this model closely resembles human sepsis [31]. As shown in Fig. 3B, P10-K^1-LYZ among PEG_{10K}-LYZs also the most highly inhibited CLP-induced release of HMGB1 in mice. In both assays, the rank order of activity was P10-K^1-LYZ > LYZ > P10-K^{13}-LYZ > P10-K^{97}-LYZ > P10-K^{33}-LYZ (Fig. 2A and B). Therefore, N-terminally mono-PEGylated LYZs with different size were subsequently prepared with $mPEG_{5K}$-ALD and $mPEG_{20K}$-ALD, and their effects on HMGB1 release in HUVECs and mice were investigated (Fig. 2C and D). Among N-terminally mono-PEGylated LYZs with different size (P5-K^1-LYZ, P10-K^1-LYZ, and P20-K^1-LYZ), P5-K^1-LYZ showed the highest activity for inhibiting the LPS-induced release of HMGB1 from endothelial cells and CLP-induced release of HMGB1 in mice.

Next, we investigated the effects of P5-K^1-LYZ on the expression of the HMGB1 receptors TLR2, TLR4, and RAGE in HUVECs. As shown in Supplementary Figure 2A, HMGB1 treatment induced a 4-fold increase in the expression of each receptor in HUVECs. P5-K^1-LYZ treatment markedly inhibited TLR2, TLR4 and RAGE expression. To test the effects of P5-K^1-LYZ on cellular viability, MTT assays were performed in HUVECs treated with P5-K^1-LYZ for 24 h. P5-K^1-LYZ did not affect cell viability at the concentrations used (up to 200 nM) (Supplementary Figure 2B). High plasma concentrations of HMGB1 in patients with inflammatory diseases are known to be related to poor prognosis and high mortality. In addition, pharmacological inhibition of HMGB1 is known to improve survival in animal models of acute inflammation in response to endotoxin challenge [32]. Therefore, the prevention of LPS- or CLP-induced release of HMGB1 by P5-K^1-LYZ suggests that P5-K^1-LYZ can be used for the treatment of vascular inflammatory diseases.

3.3. Effect of PEG-K^1-LYZs on LPS-, HMGB1-, or CLP-mediated barrier disruption

A permeability assay was performed to determine the effects of PEG-K^1-LYZs on the barrier integrity of HUVECs. Treatment with LYZ or PEG-K^1-LYZs (200 nM) alone did not alter barrier integrity (Fig. 3A). In contrast, LPS is known to induce the cleavage and disruption of endothelial membrane barriers [33,34]. HUVECs were treated with various concentrations of LYZ or PEG-K^1-LYZs for 6 h after the addition of LPS (100 ng/mL). As shown in Fig. 3A, PEG-K^1-LYZs downregulated LPS-mediated membrane disruption in a dose-dependent manner. HMGB1 is also known to induce cleavage and disruption of barrier integrity [35,36]. Treatment with PEG-K^1-LYZs resulted in a dose-dependent decrease in HMGB1-mediated membrane disruption (Fig. 3B). To confirm this effect *in vivo*,

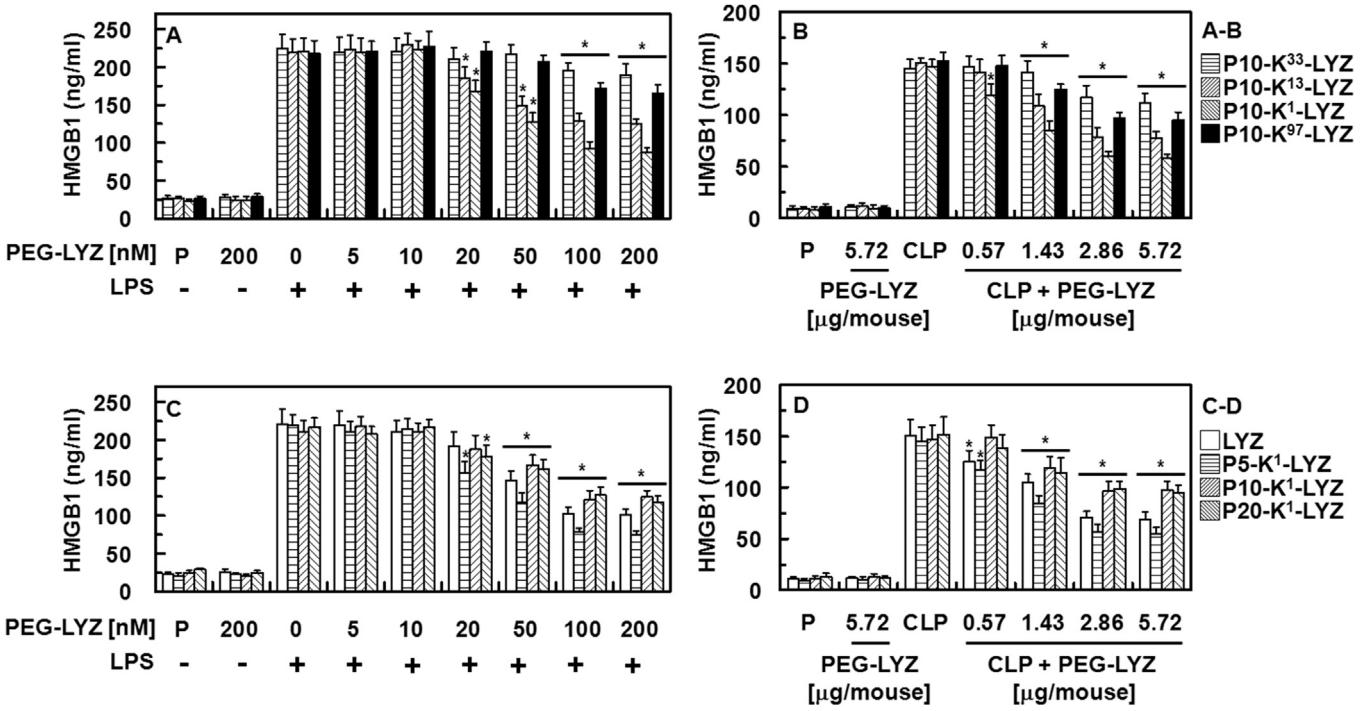

Fig. 2. The effects of PEG-LYZs on HMGB1 release and the expression of HMGB1 receptors. (A, C) HUVECs were treated with the indicated concentrations of PEG-LYZs for 6 h after stimulation with LPS (100 ng/mL) for 16 h. HMGB1 release was then measured by ELISA. (B, D) Male C57BL/6 mice underwent CLP, and were then administered PEG-LYZs (0.57–5.72 μg per mouse) intravenously (i.v.) 12 h after CLP (n = 5). Mice were euthanized 24 h after CLP. Serum HMGB1 levels were measured by ELISA. $^{*}p < 0.05$ versus LPS alone (A, C) or CLP (B, D). P, PBS vehicle control.

HMGB1- or CLP-induced vascular permeability in mice was evaluated. As shown in Fig. 3C and D, PEG-K^1-LYZs markedly inhibited HMGB1- or CLP-induced peritoneal leakage of dye. Among three PEG-K^1-LYZs (P5-K^1-LYZ, P10-K^1-LYZ, and P20-K^1-LYZ), P5-K^1-LYZ also showed the highest activity for inhibiting LPS-, HMGB1-, and CLP-mediated barrier disruption and its activity was slightly higher than native LYZ. HMGB1 is known to induce proinflammatory responses by promoting phosphorylation of p38 MAPK [37,38]. To

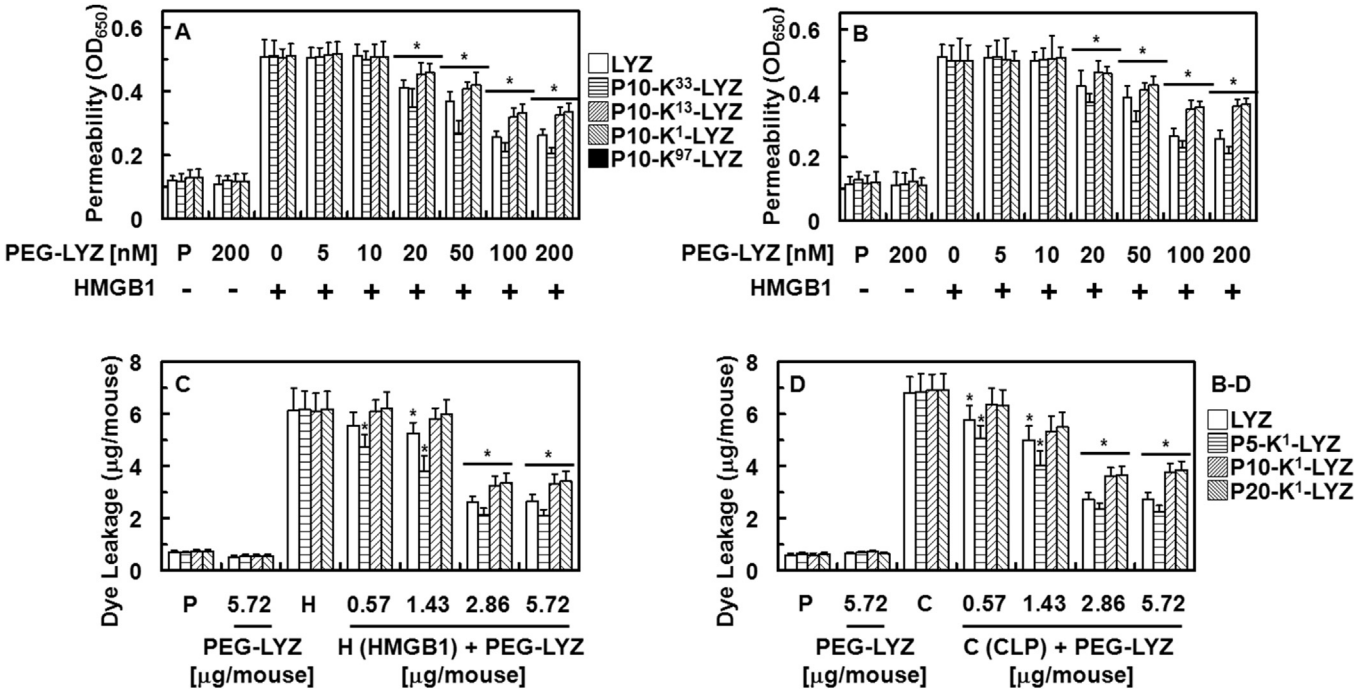

Fig. 3. Effects of PEG-K^1-LYZs on HMGB1-mediated permeability *in vitro* and *in vivo*. (A, B) HUVECs were stimulated with LPS (A, 100 ng/mL for 4 h) or HMGB1 (B, 1 μg/mL for 16 h), and then treated with different concentrations of LYZ or PEG-K^1-LYZs for 6 h. Permeability was then monitored by measuring the flux of Evans blue dye-bound albumin across HUVECs. (C, D) The effects of LYZ or PEG-K^1-LYZs (0.57–5.72 μg per mouse) injected in HMGB1- (C, 2 μg/mouse, i.v.) or CLP- (D, at 24 h after CLP) induced vascular permeability in mice were examined by measuring the amount of Evans blue dye in peritoneal washes (expressed in μg per mouse, n = 5). Results are expressed as the mean ± SEM of five independent experiments. $^{*}p < 0.05$ versus LPS (A), HMGB1 (B, C), or CLP (D). P, PBS vehicle control.

determine whether PEG-K^1-LYZ inhibits HMGB1-induced activation of p38 MAPK in HUVECs, cells were activated with HMGB1 and incubated with P5-K^1-LYZ, and then phosphorylated p38 MAPK levels were determined. As shown in Supplementary Figure 3, HMGB1 upregulated the expression of phosphorylated p38, which was significantly inhibited by P5-K^1-LYZ treatment. These findings demonstrate that PEG-K^1-LYZ treatment inhibited HMGB1-mediated endothelial disruption and maintained human endothelial cell barrier integrity in mice.

3.4. The protective effect of PEG-K^1-LYZs in CLP-induced septic mortality

Sepsis, a systemic response to a serious infection, has a poor prognosis when it is associated with organ dysfunction, hypoperfusion, or hypotension [4,39]. Based on the above-mentioned findings, we hypothesized that treatment with PEG-K^1-LYZs would reduce mortality in CLP mouse sepsis model. To determine whether PEG-K^1-LYZs protect mice from CLP-induced lethality, mice were administered LYZ or PEG-K^1-LYZs after CLP surgery. Twenty-four hours after the CLP operation, the animals manifested signs of sepsis, including shivering, bristled hair, and weakness. Administration of LYZ or PEG-K^1-LYZs once at two different doses (2.86 or 5.72 μg per mouse, 12 h after CLP) did not prevent CLP-induced death (data not shown). Thus, in a subsequent experiment, LYZ or PEG-K^1-LYZs was administered twice, once 12 h after CLP and a second time 50 h after CLP, and Kaplan–Meier survival analysis revealed a significant increase in this treatment group ($p < 0.0001$, Fig. 4). Among animal groups treated with LYZ and three PEG-K^1-LYZs, P5-K^1-LYZ-treated groups showed the higher survival rate than other groups in both doses 2.86 and 5.72 μg per mouse. The final survival rates of P5-K^1-LYZ-treated groups at 132 h after CLP were 20% for the 2.86 μg per mouse group and 40% for the 5.72 μg per mouse group. These marked benefits of P5-K^1-LYZ administration, including suppression of HMGB1 release and HMGB1-mediated inflammatory responses, offer a therapeutic strategy for the management of sepsis and septic shock.

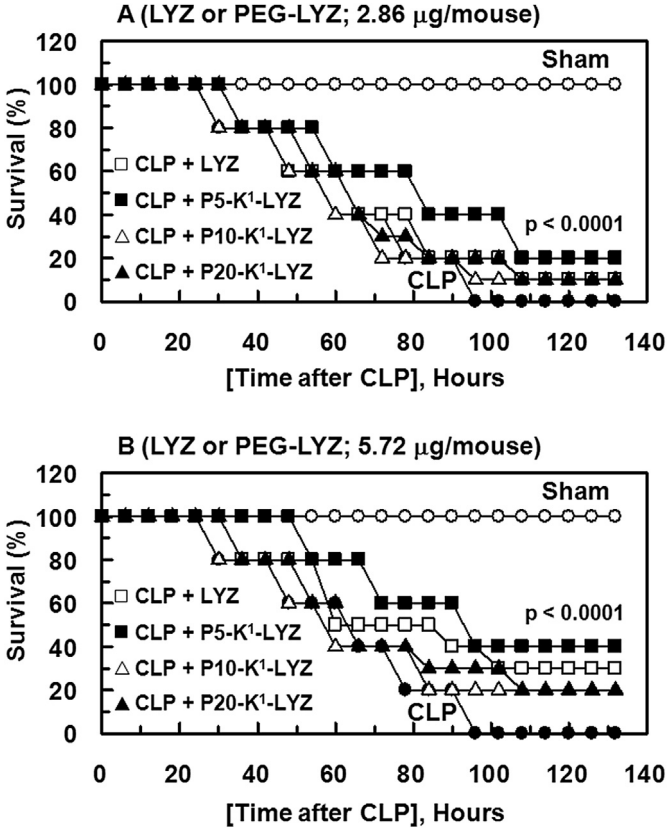

Fig. 4. Effects of PEG-K1-LYZs on lethality after CLP. Male C57BL/6 mice (n = 20) were administered LYZ or PEG-K^1-LYZs (A, 2.86 or B, 5.72 μg per mouse) intravenously at 12 h and 50 h after CLP. Animal survival was monitored every 6 h after CLP for 132 h. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline. Kaplan–Meier survival analysis was used for determination of overall survival rates versus CLP-treated mice.

Sepsis is a systemic inflammatory response syndrome, resulting from microbial infection. A wide array of proinflammatory cytokines, including TNF-α, IL-1β, interferon-γ, and macrophage migration inhibitory factor, either individually or in combination, contribute to the pathogenesis of lethal systemic inflammation [40]. For instance, neutralizing antibodies against TNF-α [41] reduce lethality in an animal model of endotoxemic/bacteremic shock. However, the early kinetics of systemic TNF-α accumulation makes its targeting difficult in the clinical setting [41], which prompted the investigation of other late proinflammatory mediators such as HMGB1 as potential therapeutic targets for sepsis. The prevailing theories of sepsis as a deregulated systemic inflammatory response are supported by extensive studies employing various animal models of sepsis, including endotoxemia and peritonitis induced by CLP [42]. In murine models of sepsis, HMGB1 is first detectable in the circulating blood 8 h after the onset of the disease, and subsequently reaches a plateau at 16–32 h after onset [43]. This late appearance of circulating HMGB1 precedes and parallels with the onset of animal lethality from endotoxemia or sepsis, and distinguishes it from TNF-α and other early proinflammatory cytokines [43]. Therefore, in this study, LYZ and PEG-LYZs were administered 16 h after the inflammatory challenge.

In summary, our results demonstrate that N-terminally PEG-conjugated LYZ, PEG-K^1-LYZ, inhibits both LPS- and CLP-mediated release of HMGB1, the expression of the HMGB1 receptors, and HMGB1-mediated barrier disruption by increasing barrier integrity and inhibiting CAMs expression. Among PEG-K^1-LYZ with different size (P5-K^1-LYZ, P10-K^1-LYZ, and P20-K^1-LYZ), P5-K^1-LYZ showed the highest antiseptic activity. The protective effects of P5-K^1-LYZ were confirmed in a mouse model of sepsis, in which P5-K^1-LYZ treatment reduced HMGB1-induced mortality. Our findings indicate that P5-K^1-LYZ is a candidate for use in the treatment of severe vascular inflammatory diseases such as septic shock.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.167.

Transparency document

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