Inflamm. res. 54 (2005) 451–457
1023–3830/05/110451–7
DOI 10.1007/s00011-005-1378-1

Inflammation Research

Original Research Papers

LBP inhibitory peptide reduces endotoxin-induced macrophage activation and mortality

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Received 9 March 2005; returned for revision 8 April 2005; returned for final revision 12 July 2005; accepted by A. Falus 14 July 2005

Abstract. *Objective and Design:* The aim of this study was to investigate whether P12, a lipopolysaccharide (LPS)-binding protein (LBP) inhibitory peptide could reduce LPS induced inflammation *in vitro* and *in vivo.*
Material and Methods: Human monocyte-like cell line (U937 cells) was grown in RPMI 1640 and stimulated with PMA in order to induce differentiation to the macrophage stage. A total of 70 *Kunming* mice (8–12 wk old) were used in our experiments. The effects of P12 on the binding of LPS to U937 cells and alveolar macrophages (AMs) were determined by flow cytometric analysis. Nuclear factor kappa B (NF-kappa B) translocation was evaluated with subunit P65 by Western blotting. The production of tumor necrosis factor-alpha (TNF-α), alanine transaminase (ALT), and nitric oxide (NO) as measured by ELISA, enzymatic activity assay, and enzymatic assay with nitrate reductase. Differences among groups were determined using one-way ANOVA test and Fisher exact test.
Treatment: U937 cells were treated with LPS, LBP, and indicated concentrations of P12. Mice were administered LPS intraperitoneally and P12 via the tail vein.
Results: P12 inhibited the binding of FITC-conjugated LPS (FITC-LPS) to U937 cells and AMs. NF-kappa B translocation and the production of TNF-α, ALT, and NO induced by LPS was also significantly suppressed by P12.Furthermore P12 protected mice from LPS-induced death.
Conclusions: The results suggest that blockade of LBP at inflammation sites might attenuate LPS-induced circulatory shock. This results in a beneficial effect in a mouse model of endotoxemia.

Key words: Lipopolysaccharide – Lipopolysaccharide-binding protein – Nuclear factor kappa B

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Introduction

Activation of macrophages can occur by recognition of the structural features of microbes, including the bacterial cell wall component lipopolysaccharide [LPS (endotoxin)] [1]. After its release from bacteria, LPS can bind to a specific binding protein, known as LPS-binding protein (LBP) [2, 3]. LBP can transfer monomeric LPS to CD14 [4]. The binding of the LPS/LBP complex to CD14 and toll like receptors (TLRs) may result in the transfer of a signal across the cell membrane and activation of nuclear factor kappa B (NF-kappa B), as well as extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase, leading to a high level of cytokine production and nitric oxide (NO) release. These mediators, which orchestrate inflammatory reactions in the lung, are also considered to be essential for the host defense system [5–9]. LPS-mediated stimulation of CD14-positive cells can be enhanced 100–1000 fold when LBP is added to serum-free systems and therefore LBP has been thought to participate in the pathogenesis of septic shock [10, 11]. However, LBP can also detoxify LPS by transferring LPS into high-density lipoprotein (HDL) particles *in vitro* [12], and HDL-bound LPS lacks cell stimulatory activity. Therefore, LBP is a two-edge sword in inflammatory reactions.

In our previous studies, we obtained phage clones with the sequence of WKXRKXFXKXXG (homologus with 91–102 amino acids WKVRKSFFKLQG of LBP) and with the sequence of FHTRWNYWPYLH (able to mimic the anti-inflammation sites of LBP) which could block the inflammatory and anti-inflammatory activities of LBP, and synthesized peptide with the sequence of WKVRKSFFK-LQG (named as LBP inhibitory peptide, namely P12) [13, 14]. In this study, we tested the hypothesis that blocking of LBP at the inflammation sites may protect against inflammatory responses associated with low-dose LPS, and thus reduce mortality. We examined the effects of LBP blockade

with P12 on the human monocyte-like cell line U937 cells and a mouse model of LPS induced endotoxemia.

Materials and methods

Reagents

LPS (from *E. coli* 0111:B4), FITC-conjugated LPS (from *E. coli* 0111:B4), and D-galactosamine (D-gal) were purchased from Sigma. NF-kappa B P65 primary antibody was purchased from Santa Cruze. ELISA kit for tumor necrosis factor-alpha (TNF-α) was purchased from Jingmei Chemical. NO detection kit and ALT detection kit were purchased from Nanjing Bioengineering Institute. All materials used in cell culture were certified LPS free and tested by the Limulus amoebocyte lysate assay method to confirm the absence of detectable LPS.

LBP inhibitory peptides (P12) with the sequence of WKVRKSFFKLQG were synthesized by solid phase method on a peptide synthesizer by F-moc chemistry in our laboratory. An amino group was added to P12 terminal for the purpose of the enhancement of P12 stability. Then P12 was purified by high performance liquid chromatography (HPLC), dialyzed into PBS, and stored at –70℃. The molecular weight of P12 was 1523.6 and the relative binding activity to LPS was one thirty-three of LBP, but the molecular weight of LBP was about 51 kDa, so the relative binding activity to LPS of P12 was higher than that of LBP in the same mass concentration.

Cell preparation and stimulation

Human monocyte-like cell line U937 cells (kindly provided by Doctor Xuzhi, Institute of Respiratory Diseases, Xinqiao Hospital, Third Military Medical University, China) were grown in RPMI 1640 modified to containing 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate and containing 10 % fetal calf serum (FCS) in cell culture plates. Cells were stimulated for 48 h with 10 ng/ml phorbol myristate acetate (PMA) in order to induce their differentiation to macrophage like phenotype stage [15, 16]. The PMA-treated U937 cells became adherent, ceased to proliferate, and exhibited increased ability to mediate chemotaxis, phagocytosis, superoxide anion production.

Murine alveolar macrophages (AMs) were collected as previously described [17]. Briefly, AMs were harvested from mice by bronchoalveolar lavage (BAL). The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge T catheter. BAL was performed by instilling two 0.5-ml aliquots of sterile saline. The cells were spun at 200 × *g* to pellet cells, and then washed twice with complete RPMI 1640. Total cells were counted from each sample using a hemocytometer. Cells were pooled, washed, and then resuspended in RPMI 1640 in a final concentration of 1×10^5 cells/ml. Cells were then cultured in cell culture plates for 2 h and washed with RPMI 1640 for removing the nonadherent cells. Adherent monolayer cells were stained with Giemsa stain and trypan blue to clarify the purity and viability of AMs and in all cases in which the recovered cells were > 95 % viable.

Cells were treated with phosphate buffer (PBS, control group), 100 ng/ml LPS in the presence of 100 ng/ml LBP (LPS group), and 10, 100 and 1000 μg/ml P12 (P12-treated group). In some experiments, cell supernatants were retained for conditioned media experiments or enzyme-linked immunosorbent assay (ELISA).

Establishment of endotoxemic mice model

The Animal Care and Use Committee of the Third Military Medical University approved all experiments. *Kunming* mice (8–12 wk of age, 26–32 g) were purchased from the animal center of Third Military Medical University and were acclimatized for 1 wk in the animal housing facility before experiments. For all experiments, mice were administered with 25 mg of D-gal intraperitoneally (i.p.) [18]. At 15 min after the administration, mice were injected i.p. with LPS (50 ng/mice) dissolved in 100 μl saline and immediately mice were then injected with P12 *via* the tail vein. Mice in the 5 groups (n = 8 in each group) were treated with LPS plus D-gal (LPS group), LPS plus D-gal plus indicated concentrations of P12 (10, 100, and 1000 μg/kg), and saline (control group), respectively. At 2 h and 7 h after LPS challenge, mice were bled retroorbitally and 2 ml blood drawn was allowed to clot for 1 h on ice, and then centrifuged. The sera were stored in small aliquots at –70 °C. For subsequent determination of TNF-α, NO, and liver enzymes, fresh aliquots were thawed. A part of lung tissues was collected and frozen in liquid nitrogen for protein isolation, and another part of lung tissues was put into 10 % buffered formalin for immediate fixation. Pulmonary neutrophils were quantified by morphometric analysis in histologic sections. Paraffin-embedded 5-μm sections of lungs were cut and stained with hematoxylin and eosin. Neutrophil emigration was quantitated by counting the number of neutrophils in 200 randomly selected alveoli and expressed as the number of neutrophils per 100 alveoli. Another 30 mice were divided into three groups (n = 10 in each group) for investigation of the mortality.

Toxicity tests

Trypan blue exclusion and release of lactate dehydrogenase (LDH) indicating cytolysis revealed the toxicity of P12 to U937 cells.

Flow cytometric analysis (FACS)

P12 in indicated concentrations were incubated for 15 min at 37 °C with FITC-conjugated LPS (FITC-LPS, 100 ng/ml), then the complex were added into the culture of U937 cells (5×10^5 cells/ml) in presence of LBP (100 ng/ml). After 15 min at 37 °C, cells were washed with phosphate buffer (PBS) and the binding of FITC-LPS was analyzed by FACS, and the median fluorescence intensity (MFI) was determined, representing the LPS transfer to cells. In some experiments, U937 cells and AMs were treated with P12 (100 μg/ml P12) 15 min before LPS treatment or at 15 min and 60 min after LPS treatment in presence of LBP.

NF-kappa B translocation

Isolation of Cytoplasmic Protein and Nuclear Protein [19–21]

U937 cells and the isolated lung tissues frozen in liquid nitrogen were homogenized in 400 μl of ice-cold Buffer A (pH at 7.9, 10 mmol/L Hepes-NaOH, 0.05 mmol/L EGTA, 10 mmol/L KCl, 0.1 mmol/L EDTA, and 1.5 mmol/L $MgCl_2$) with a 0.1 % volume of Nonidet P-40 and protease inhibitor cocktail (1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mg/ml pepstatin). At 10 min after incubation on ice, the homogenates were centrifuged at 12000 rpm for 10 seconds at 4 °C. The supernatant was cytoplasmic protein. The pellets were washed with PBS and then the crude nuclear pellets were resuspended in 50 μl of Buffer B (20 mmol/L Hepes-NaOH, 0.05 mol/L EGTA, 0.42 mmol/L NaCl, 0.2 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 25 % vol/vol glycerol, and 0.5 mmol/L PMSF) with protease inhibitor cocktail (as described above) and incubated on ice for 30 min. Nuclear extracts were recovered following centrifugation at 12 000 rpm for 2 min at 4 °C, and then stored at –70 °C and subjected to immunodetection.

Western blotting

The protein lysate-containing samples were subjected to electrophoresis on 10 % polyacrylamide gel. Start wells were loaded with an equivalent amount of protein (20 μg/lane) or with the molecular weight markers. The separated proteins were electrotransferred from the gel to polyvinylidene difluoride (PVDF) filter using the equipment for Western blot semidry analysis. The filter was incubated with the primary polyclonal

antibody to NF-kappa B P65 diluted at 1:300 and then, with the secondary anti-goat-HRP antibody at 1:300. The immunoreactive bands were visualized by addition of 3, 3-diamibezidine (DAB). Integrated Density Value (IDV) was read using a densitometer.

Determination of TNF-α Concentration and Hepatic Injury Indicated by ALT Activity

Determination of TNF-α in the supernatant of U937 cells and sera from mice were carried out using a commercial cytokine ELISA kit according to the manufacture's instructions. Since hepatic failure is the reason for LPS toxicity in LPS/D-gal treated mice, the influence of P12 treatment on serum concentration of ALT of mice was determined by enzymatic activity assay.

NO Assay

To determine whether P12 could affect the production of NO by U937 cells and mice, NO in the cell culture supernatant or sera from mice was determined. Because nitric oxide could be spontaneously oxidized to both nitrite and nitrate, serum NO was measured by enzymatic assay with nitrate reductase as previously described [22]. Briefly, 100 μL samples were equilibrated with 250 μL of 100 mmol/L potassium phosphate buffer (pH 7.5), 50 μL of distilled water, 50 μL of 0.2 mmol/L flavin adenine dinucleotide, and 10 μL of 12 mmol/L β-nicotinamide adenine dinucleotide phosphate, in the presence or absence of 40 μL of 500 U/L nitrate reductase. After a 45-min incubation at room temperature, the absorbance was recorded.

Mortality of D-gal-sensitized mice treated with LPS with or without P12

A total of 30 animals were divided into three groups (n = 10 in each group): i.p. injection of LPS plus D-gal (LPS group), LPS plus D-gal Plus P12 at the dose of 100 μg/kg (P12 group), and control saline (control group), respectively. Mortality was monitored at 12 h, 24 h, and 36 h after injection.

Statistical analysis

Differences among groups were determined using one-way ANOVA test and Fisher exact test. A p value of 0.05 was considered to be of significance. Values are presented as mean ± SEM.

Results

Toxicity tests

Toxicity tests revealed that P12 at the maximum concentration (1000 μg/ml) failed to display any toxicity towards U937 cells.

Histological changes

In the lung tissues of endotoxemic mice, significantly increased neutrophil emigration was seen, as well as swelling of endothelial cells, thrombosis, angiorrhexia (rupture of blood vessel), and bleeding and edema with proteinaceous liquid in the alveolar space. However, mice administered with P12 revealed decreased neutrophil emigration as compared with those given LPS ($P < 0.05$), slight swelling of endothelial cells, thrombosis, angiorrhexia, and diminished bleeding and edema with proteinaceous liquid in the alveolar space (Fig. 1).

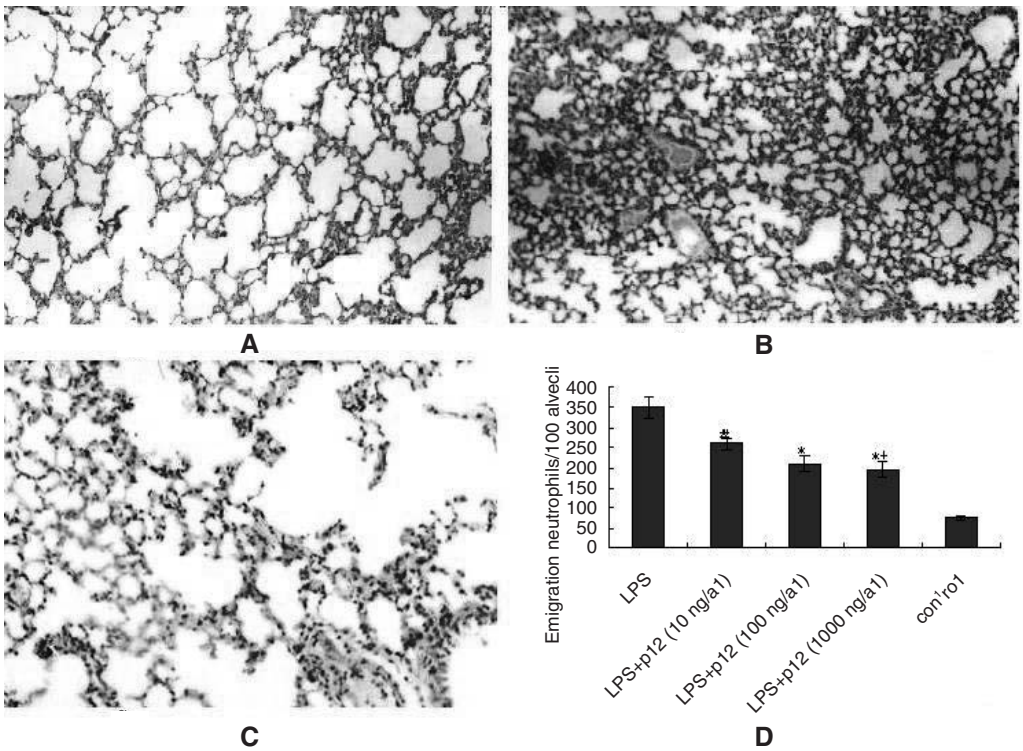

Fig. 1. Hematoxylin and eosin stained histological lung sections of mice. (A) Biopsy of the lung tissues taken from mice treated with D-gal. (B) Biopsy of the lung tissues taken from mice treated with D-gal and LPS. (C) Biopsy of the lung tissues taken from mice treated with D-gal plus LPS plus P12 (100 μg/kg). (D) Mice administered LPS revealed increased neutrophil emigration. Blockade of LBP decreased the neutrophil emigration after LPS challenge. Data are presented as mean SEM. (#: $p > 0.05$ and *: $p < 0.05$ versus LPS group, +p < 0.05 versus control group).

Effects of P12 on the binding of FITC-LPS to U937 cells and AMs

We first examined the effects of P12 in indicated concentrations on the binding of FITC-LPS to U937 cells and AMs by FACS analysis. P12 (100 ng/ml, 1000 ng/ml) inhibited the binding of FITC-LPS to cells when FITC-LPS was incubated with P12 in the presence of LBP. Interestingly, P12 couldn t inhibit the binding of FITC-LPS to cells when added at 60 min after FITC-LPS treatment in presence of LBP. These observations indicated that P12 could bind to FITC-LPS and thereby inhibit the interaction of FITC-LPS with LBP in a dose and time dependent manner (Fig. 2).

Effects of P12 on NF-kappa B translocation

NF-kappa B translocation was estimated using the levels of P65 in the nucleus and cytoplasm from U937 cells and the whole lung of mice at 2 h after LPS treatment. In LPS group, NF-kappa B translocation increased as compared with that in the control group. However, NF-kappa B translocation was inhibited in U937 cells and mice treated with P12 as compared with that in cells and mice challenged with LPS (Fig. 3).

Influence of P12 on TNF-α and ALT Release

We found elevated concentrations of TNF-α in U937 cells and mice challenged with LPS at 2 h and the difference between P12 group and LPS group was also significant at the time point of 2 h. However, we didn't find elevated concentrations of ALT at 2 h after LPS injection (Fig. 4). Unfortunately, no difference in ALT concentrations was observed between LPS group and P12 group.

Effects of P12 on NO production

To further investigate the effects of P12 on LPS-induced inflammation, we quantitated NO. In unstimulated cells and mice the concentrations of NO were low. In response to LPS, there was a marked increase in levels of NO. However P12 treatment significantly reduced the production of NO in U937 cells and endotoxemic mice in dose dependent manner although the concentration did not return to the baseline (Fig. 5).

Mortality of LPS-treated mice with or without P12

At 12 h after LPS treatment, 6 out of 10 mice died, whereas only 1 out of 10 mice treated with LPS and LBP inhibitory peptide died. A further 12 h observation revealed that only one mouse in each group died, but there was no death at 36 h after injection of LPS with or without P12, and that 30 % of endotoxemic mice, but 80 % of the mice treated with both LPS and P12 survived. Therefore P12 reduced LPS-induced mortality ($P < 0.05$ by Fisher exact test comparing mice receiving P12 with endotoxemic mice). Protection should be associated with low circulating levels of TNF-α and NO in all survivors.

Discussion

The control of acute and chronic inflammatory disorders is currently a major problem in clinical practice. Since much effort has been invested in the development of therapeutic strategies that can block appropriate parts of the inflammatory network [23, 24], the search for sepsis therapies has proved to be disappointing.

The pathway of macrophage activation is thought to be a major contributor to the development of LPS-induced sepsis

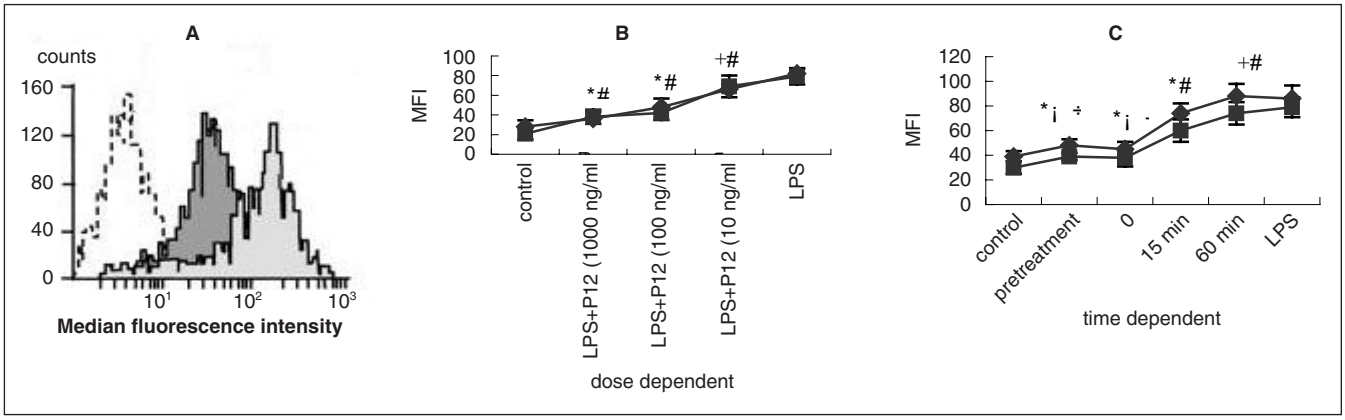

Fig. 2. Influence of P12 on the binding of FITC-LPS to U937 cells and AMs. (A) U937 cells were incubated with PBS (white graph, control group), FITC-LPS in the presence of LBP together with 100 μg/ml P12 (deep gray graph, P12-treated group) and FITC-LPS in the presence of LBP (light gray graph, LPS group). (B) Dose-dependent inhibition of binding of FITC- LPS to U937 cells and AMs by increasing concentrations of P12 as indicated. U937 cells (5×10^5 cells/ml) were incubated with 100 ng/ml FITC-conjugated LPS in the absence or presence of 10–1000 ng/ml P12 in RPMI 1640 for 15 min at 37 °C. After washing, the binding of FITC-LPS was analyzed by flow cytometry, and median fluorescence intensity was determined. Binding of LPS was expressed as a percentage of that obtained using U937cells incubated with FITC-LPS in the absence of P12. Data are presented as mean ± SEM. (C) Time-dependent inhibition of binding of FITC-LPS to U937 and AMs by P12. U937 cells and AMs were treated with P12 (100 μg/ml) at 15 min before LPS (pretreatment), together with LPS (0), and at 15 or 60 min after LPS pretreatment (15 min and 60 min). Data are presented as mean ± SEM. ◆: U937 cells; ■: AMs; *: $p < 0.05$ and +: $p > 0.05$, *versus* LPS group; #: $p < 0.05$ and △: $p > 0.05$, *versus* control group. Data are presented as mean ± SEM.

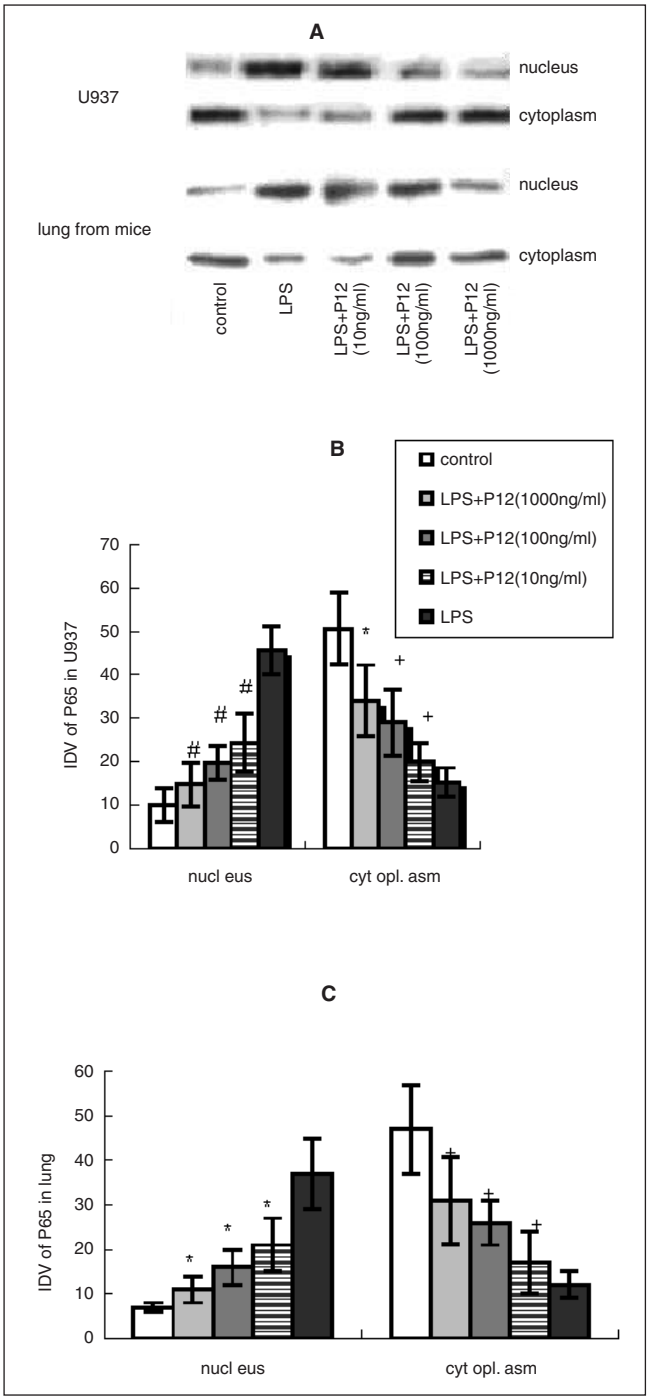

Fig. 3. Effects of P12 on NF-kappa B translocation
Western blotting showed P65 in the cytoplasm and nucleus (A). Increased P65 in the nucleus but decreased P65 in the cytoplasm from U937 cells (B) and lung tissues of mice (C) after LPS challenge revealed increased NF-kappa B translocation. Blockade of LBP with P12 significantly decreased the NF-kappa B translocation after LPS challenge. Data are presented as mean ± SEM. #: $p < 0.05$, *versus* in the nucleus of U937 cells in LPS group; *: $p > 0.05$ and +: $p < 0.05$, *versus* in the cytoplasm of U937 cells in LPS group; **: $p < 0.05$, *versus* in the nucleus of lung tissues in LPS group; ++: $p < 0.05$, *versus* in the cytoplasm of lung tissues in LPS group.

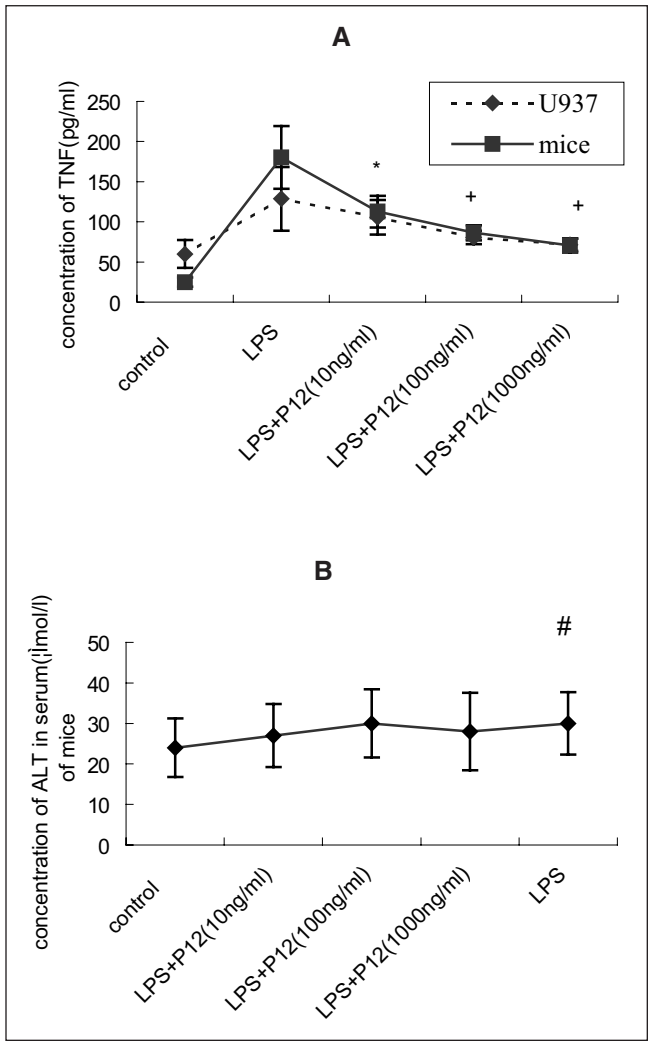

Fig. 4. Effects of P12 on LPS-mediated cytokine release and liver function. TNF-α and ALT concentrations in sera were determined by ELISA (A) and enzymatic assay (B). *: $p > 0.05$ and +: $p < 0.05$ *versus* TNF-α production at 2 h in LPS group; #: $p > 0.05$ *versus* ALT production at 2 h in the control group.

[25]. Innate defense is both multifaceted and redundant. The LBP-initiated pathway is essential for defense, because it is required for the induction of an inflammatory reaction. However, the amplification of LPS effects mediated by LBP may be detrimental to the host [26]. Blockade of LBP activity with polyclonal or monoclonal antibodies blocked the activation of macrophages by LPS [27, 28], suggesting that LBP may contribute to the toxicity of low-dose LPS, and that the transfer of LPS to HDL may not be sufficient to prevent the activation of cells *via* the LPS/LBP/CD14 pathway [29]. However, high concentrations of LBP in sera of patients with severe sepsis or septic shock can inhibit the LPS response in human monocytes, demonstrating an inhibitory role of high concentrations of LBP in the LPS-induced inflammatory response [30]. To further investigate the role of LBP in innate immunity, we generated LBP inhibitory peptide which include inflammatory sites of LBP.

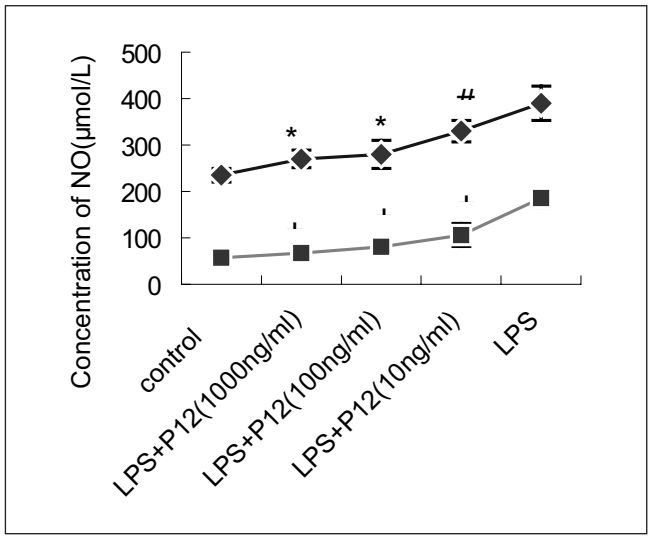

Fig. 5. Effects of P12 on LPS-mediated nitric oxide production in U937 cells and mice. At 2 h after LPS challenge, sera were collected and nitric oxide concentration was determined. Data are presented as mean ± SEM. ◆: U937 cells; ■: AMs; *: $p < 0.05$ and #: $p > 0.05$, *versus* LPS-treated U937 cells; +: $p > 0.05$, *versus* LPS-treated mice.

When bacterial activity is controlled by antibiotic therapy, blocking the function of LBP might decrease neutrophil-mediated lung injury rather than increase tissue damage due to bacterial infection [28]. It has been well documented that activation of myelomonocytic cells by LPS *via* LBP occurs only at low-dose LPS (no more than 100 ng/ml).To gain insight into the mechanisms responsible for protection from lethal endotoxemia induced by low-dose LPS of LBP inhibitory peptide, we examined the effect of P12 on the binding of LPS to LBP and NF-kappa B translocation and TNF-α production. On the basis of our *in vitro* study, we suggest that P12 significantly inhibited LBP activity by blocking the binding of LPS and prevented the binding of FITC-LPS to U937 cells when P12 was preincubated with FITC-LPS but not when FITC-LPS/LBP complexes were formed according to FACS.

NF-kappa B can be activated by various stimuli including microbial products, and in turn regulates the inducible expression of many cytokines, chemokines and adhesion molecules. We observed decreased NF-kappa B translocation after P12 treatment using lung homogenates, but it remains unclear which types of pulmonary cells are responsible for this change. We think that LBP inhibitory peptide may work via suppressing NF-kappa B translocation.

Furthermore, P12 suppressed LPS-induced TNF-α production and nitric oxide release although blockade of LBP did not return these values to the baseline. However, cytokines and chemokines are known to potentiate the functions of macrophages, resulting in activation of anti-microbial mechanisms, including generation of superoxide and reactive oxygen, or enhancement of phagocytosis, with an increased ability to kill microorgamisms. TNF-α production is dependent on LBP in the initial steps, when LPS-triggered macrophage activation occurs [31]. TNF-α stands as a key cytokine and netrphil activation and recruitment is of paramount important. It has been revealed that nitric oxide reduces neutrophil recruitment during endotoxemia and that nitric oxide reacts with oxygen and superoxide to form nitrogen dioxide, a potent pulmonary irritant, and peroxynitrite, a cytotoxic oxidant, respectively. We think it possible that reduced production of TNF-α and nitric oxide might have contributed to the attenuated lung inflammation after adminstration of LPS.

The *in vivo* activity of P12 was very similar to that measured *in vitro.* Administration of P12 inhibited NF-kappa B translocation in lung and suppressed TNF-α production and protected D-gal-sensitized mice from death. It is known that the lethal effects of LPS in mice sensitized with D-gal are due to hepatic failure, so we detected the ALT production in mice. Unfortunately, we found that P12 couldn t reduce the ALT production.

In summary, we have produced LBP inhibitory peptide that could block the inflammatory function of LBP. The present study revealed that LBP blockade with LBP inhibitory peptide attenuate LPS-induced circulatory shock and protect mice from lethal endotoexmia induced by low-dose LPS. These investigations suggest that the 91–102 amino acids of LBP might be the inflammatory site and LBP inhibitory peptide with the sequence of WKVRKSFFKLQG could inhibit the inflammatory function of LBP. Our experimental data should broaden understanding of the pathophysiology of LBP in Gram- negative infections and suggest a novel approach to the endotoexmia.

Acknowledgements. We would like to thank our colleagues Mr. Zhang Fang and Mr. Jiang Peng, Institute of Respiratory Diseases, Xinqiao Hospital, for their excellent technical assistance. This work was supported by a grant from the National Natural Science Foundation of China (No. 30170366).

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