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ORIGINAL ARTICLE

GSK3β Inhibitor Peptide Protects Mice from LPS-induced Endotoxin Shock

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Background: Glycogen synthase kinase 3β (GSK3β) is a ubiquitous serine/threonine kinase that is regulated by serine phosphorylation at 9. Recent studies have reported the beneficial effects of a number of the pharmacological GSK3β inhibitors in rodent models of septic shock. Since most of the GSK3β inhibitors are targeted at the ATP-binding site, which is highly conserved among diverse protein kinases, the development of novel non-ATP competitive GSK3β inhibitors is needed. **Methods:** Based on the unique phosphorylation motif of GSK3β, we designed and generated a novel class of GSK3β inhibitor (GSK3i) peptides. In addition, we investigated the effects of a GSK3i peptide on lipopolysaccharide (LPS)-stimulated cytokine production and septic shock. Mice were intraperitoneally injected with GSK3i peptide and monitored over a 7-day period for survival. **Results:** We first demonstrate its effects on LPS-stimulated pro-inflammatory cytokine production including interleukin (IL)-6 and IL-12p40. LPS-induced IL-6 and IL-12p40 production in macrophages was suppressed when macrophages were treated with the GSKi peptide. Administration of the GSK3i peptide potently suppressed LPS-mediated endotoxin shock. **Conclusion:** Collectively, we present a rational strategy for the development of a therapeutic GSK3i peptide. This peptide may serve as a novel template for the design of non-ATP competitive GSK3 inhibitors.
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INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a ubiquitous serine/threonine kinase that is regulated by serine phosphorylation at 21 in GSK3α and 9 in GSK3β (1-3). GSK3 phosphorylates a broad range of substrates such as glycogen synthase (4), nuclear factor of activated T-cells (NFATc) (5), cAMP response element binding (CREB) (6), c-Jun (7) and c-Myc (8), and also inactivates many of these substrates. Through this activity, GSK3 regulates many cellular functions, including glycogen metabolism, cell-cycle control and cell proliferation (3,9). Among the GSK3 isoforms, α and β, GSK3β gained prominence as a potential drug target in various disease areas including type 2 diabetes (10,11), Alzheimer's disease (12), mood disorders (13) and cancer (14). Recently, GSK3β was also identified as a regulator of the immune system, suggesting it might be an attractive therapeutic target in inflammatory and autoimmune diseases (15-18).

Due to the growing evidence of GSK3β as a potential therapeutic target in multiple diseases (19,20), several approaches such as high-throughput screening, virtual computer simulations, and structure-based drug design have been used to develop GSK3β kinase inhibitors (21). Most of these inhibitors are a class of ATP-competitive inhibitors. However, a major drawback to the use of the inhibitors is their limited specificity, and therefore there is a concern that such inhibitors exert undesired side effects (22-25).

To overcome these issues, we developed a cell-permeable peptide, the GSK3β inhibitor (GSKi) peptide. We hypothesized that small unique peptides derived from the N-terminal phosphorylation motif of GSK3β containing serine 9 may

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serve as a pseudo-substrate of GSK3 β in cells (26-28).

In this study, we have investigated the effects of a novel GSK3i peptide in a LPS-induced septic shock model. We found that inhibition of GSK3 β by the inhibitor peptide decreased LPS-mediated pro-inflammatory cytokine production. In addition, administration of the GSK3i peptide protected mice from LPS-induced endotoxin shock. Therefore, the GSK3i peptide may be useful in the development of selective therapeutic agents for the treatment of septic shock and other related inflammatory diseases.

MATERIALS AND METHODS

Cell culture

Murine bone marrow-derived macrophages (BMDMs) were obtained from the femur of 6~8 week-old C57BL/6 male mice. Bone marrow cells were flushed out from the bone marrow cavity, suspended in DMEM (Hyclone) that was supplemented with 20% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin. After 1 day, non-adherent cells were cultured in the presence of 10 ng/ml recombinant human M-CSF (R&D Systems). After 7 days, a homogeneous population of adherent macrophages was obtained.

Peptide synthesis

Cell-permeable peptides were synthesized by Peptron (Daejeon, Korea). Peptides were purified by preparative reverse-phase HPLC and were more than 95% pure with the expected amino acid composition and mass spectra. Immediately before use, the peptides were dissolved in phosphate buffered saline (PBS) to prepare stock solutions that were between 5 and 10 mM.

Measurement of cytokines

The level of mouse interleukin (IL)-6 and IL-12p40 in culture supernatants and sera were measured using enzyme-linked immunosorbent assay (ELISA) kits from BD biosciences (San Jose, CA, USA) according to the manufacturer's instructions.

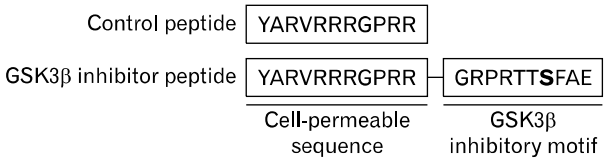

Figure 1. Structure of the GSKi peptide conjugated with the Hph-1 protein transduction domain. Amino acid sequences corresponding to residues 3~12 of GSK3 β were chosen for the design of a GSKi peptide. The serine 9 residue, which can be phosphorylated by PKB/Akt, is highlighted in bold. The control peptide contains 11-mer of the protein transduction domain.

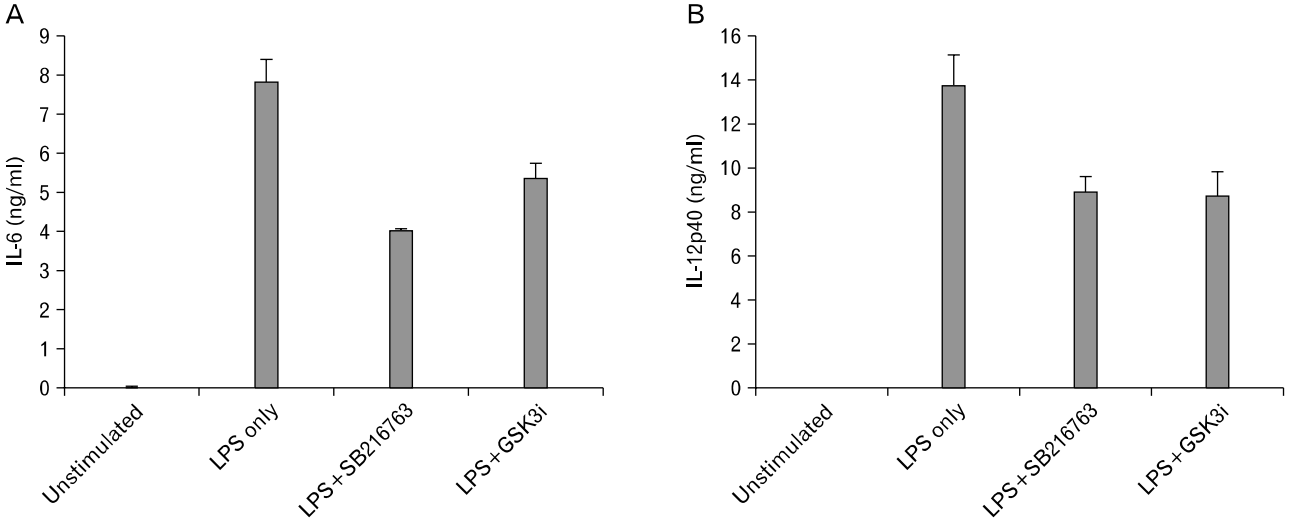

Figure 2. The GSKi peptide decreased pro-inflammatory cytokines production after LPS stimulation. BMDMs were pre-incubated for 2 hours with medium only and either 10 μM SB216763 or 5 μM GSK3i peptide, and then stimulated with 1 μg/ml LPS for 20 hours. Cell-free supernatants were analyzed by ELISA for production of pro-inflammatory cytokines; IL-6 (A) or IL-12p40 (B). Data represent mean $\pm$ s.d. and are representative of at least three experiments.

Endotoxin shock model

All animal study protocols were approved by the Animal Care Committee of Ewha Laboratory Animal Genomics Center. The endotoxin shock model used in this study has been described previously (15). In brief, male C57BL/6 mice (6~8 weeks of age; 18~20 g body weight) were injected intraperitoneally with an LD_{100} (10 μg/ml) of *E. coli* K235 LPS (Sigma) in 200 μl of PBS containing 0.1% DMSO. Mice survival was monitored over a 7-day period.

RESULTS

Inhibitory effect of the GSKi peptide on LPS-induced pro-inflammatory cytokine production

We designed a cell-permeable GSKi peptide spanning the serine 9 phosphorylation motif of GSK3 β that was fused with recently characterized cell-permeable sequences derived from the human transcription factor Hph-1 (Fig. 1) (29,30). Since GSK3 β is known key regulator of pro-inflammatory cytokine production (16), we examined the ability of the GSKi peptide to regulate cytokine production in response to LPS stimulation. BMDMs from male 6~8 week-old mice were pre-incubated for 2 hours with either 5 μM GSKi peptide or 10 μM SB216763 as a positive control, and then the cells were stimulated with 1 μg/ml LPS for 20 hours. The control peptide containing the cell-permeable sequences only did not affect cytokine production stimulated by LPS (data not shown). As shown in Fig. 2, the presence of the GSK3i peptide was shown to attenuate pro-inflammatory cytokine production; IL-6 and IL-12p40. These inhibitory effects were comparable to that of SB216763 which is a well-characterized pharmacological inhibitor of GSK3. These results demonstrate that the GSKi peptide can regulate LPS-mediated pro-inflammatory cytokine production.

The GSKi peptide protects mice from endotoxin shock

To test the therapeutic potential of the GSKi peptide on septic shock, the effects of the peptide on an experimental LPS-induced endotoxin shock model were investigated. Mice that were given 30 mg/kg of the GSKi peptide before receiving a 100% lethal dose (LD_{100}) of LPS showed significantly improved survival, compared with the control group given LPS (Fig. 3). This protective effect was comparable to that of SB216763. The control peptide did not affect LPS-induced septic shock (data not shown).

Next, we examined whether GSK3 β inhibition regulated the pro-inflammatory cytokine production in mice given an LD_{100} of LPS. As shown in Fig. 4, we found that the serum level of the pro-inflammatory cytokine IL-6 was significantly reduced in LPS-challenged mice given the GSK3i peptide rela-

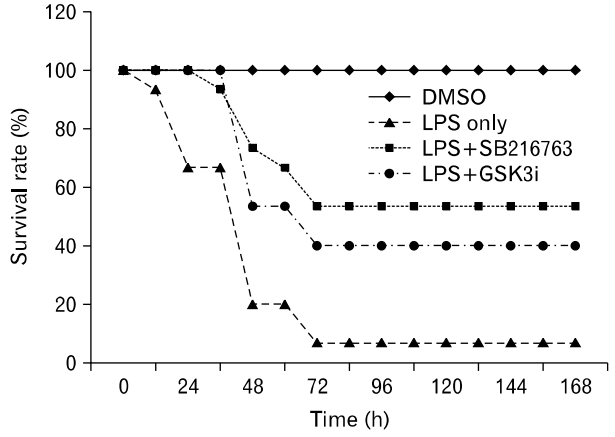

Figure 3. The GSK3i peptide protected mice from LPS-induced endotoxin shock. 25 mg/kg of the GSK3 inhibitor SB216763 (n=15) or 30 mg/kg of the GSK3i peptide (n=15) were administered intraperitoneally to mice before injection of 15 mg/kg of the *E.coli* K235 LPS insult, which is the lethal dose (LD_{100}). Sham-immunized mice were given only PBS containing 0.1% DMSO (n=4). LPS control (n=15). Administration of the GSK3 inhibitor SB216763 or GSK3i peptide protected mice from an LD_{100} of LPS given therapeutically. Survival of mice following LPS challenge was monitored for 7 days.

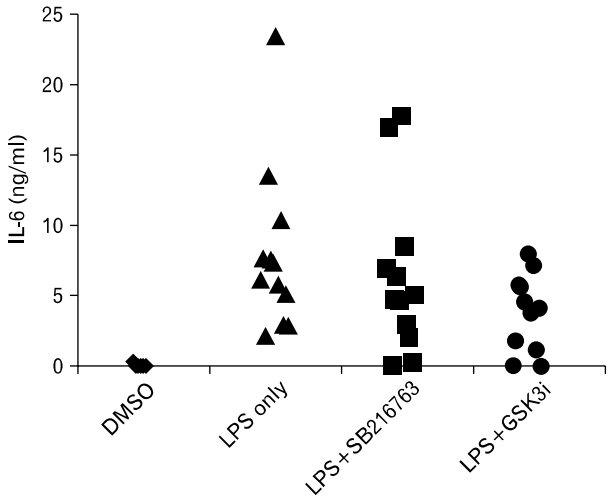

Figure 4. *In vivo* IL-6 in plasma, measured by ELISA 12 hours after mice were given an LD_{100} of LPS. Sham-immunized mice were given only PBS containing 0.1% DMSO (n=3). n=12 for LPS only, SB216763, and GSKi peptide, respectively.

tive to that of the control mice given LPS. Thus, these results demonstrate that a cell-permeable inhibitor peptide that targets GSK3 β may be useful in the treatment of septic shock.

DISCUSSION

In this study, we present a new strategy for developing a novel inhibitor peptide that targets GSK3 β. Our study has shown that the peptide motif spanning serine 9 of GSK3 β blocks LPS-induced cytokine production and septic shock. The central role of the GSK3 β pathway in innate immune responses has been well documented (31). Specifically, it has been shown that GSK3 β is involved in the PI3K/Akt pathway and mediates cytokine production in TLR signaling (15). Moreover, many reports have elucidated important roles for the GSK3 β in immune diseases such as arthritis, colitis, multiple sclerosis and sepsis (32,33) suggesting that GSK3 might be an attractive therapeutic target in inflammatory diseases. In this regard, our studies suggest that the GSKi peptide may be used as a novel tool for studying GSK3 inhibition.

The exact mechanism of how the GSK3i peptide regulates inflammatory response such as cytokine production is not fully understood. In resting cells, GSK3 is highly active, but its enzyme activity can be inhibited by the PI3-kinase-dependent pathway in response to various ligands stimulation (34-36). It has been well established that PKB/Akt is responsible for the direct phosphorylation of GSK3 β on the N-terminal serine 9 residue (37,38). After serine 9 phosphorylation, the phosphorylated N-terminal residues of GSK3 β inhibit the enzyme by binding to the active site as a pseudo-substrate (26-28). Since the GSKi peptide contains a serine 9 residue that can be phosphorylated by PKB/Akt in cells, it may act as a pseudo-substrate that binds in the active site of GSK3 β and inhibits its enzyme activity. Consistent with our data, a phosphopeptide corresponding to residue 7-14 of GSK3 β inhibited GSK3 activity in vitro, whereas the nonphsphorylated peptide did not (27,34).

The development of bioactive peptides as therapeutic alternatives offers novel exciting approaches for target-selective pharmacotherapy (39,40). The *in vivo* pharmacodynamics of the GSKi peptide was not detemined in this study. However, our data demonstrated that the GSKi peptide acted *in vivo* to protect mice against LPS-induced shock. Such phenomenon indicates that a more detailed assessment of the *in vivo* delivery and pharmacokinetic profiles of the GSKi peptide may lead to the design of effective therapeutic reagents directed against septic shock.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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