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 GSK3 β inhibitors in rodent models of septic shock. Since most of the GSK3 β inhibitors are targeted at the ATP-binding site, which is highly conserved among diverse protein kinases, the development of novel non-ATP competitive GSK3 β inhibitors is needed. Methods: Based on the unique phosphorylation motif of GSK3 β, we designed and generated a novel class of GSK3 β 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.

[Immune Network 2010;10(3):99-103]

INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a ubiquitous serine/threonine kinase that is regulated by serine phosphorylation at 21 in GSK3 α and 9 in GSK3 β (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, GSK3 β 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, GSK3 β 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 GSK3 β 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 GSK3 β 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 GSK3 β inhibitor (GSKi) peptide. We hypothesized that small unique peptides derived from the N-terminal phosphorylation motif of GSK3 β containing serine 9 may...
serve as a pseudo-substrate of GSK3β 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 GSK3β 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 GSK peptide conjugated with the Hph-1 protein transduction domain. Amino acid sequences corresponding to residues 3~12 of GSK3β were chosen for the design of a GSK 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±s.d. and are representative of at least three experiments.
Effects of GSK3β Inhibitor Peptide on Septic Shock
Ryeojin Ko, et al.

IMMUNE NETWORK http://www.ksimm.or.kr Volume 10 Number 3 June 2010

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 (LD100). Sham-immunized mice were given only PBS containing 0.1% DMSO (n=4). The control group given LPS (n=15). Administration of the GSK3 inhibitor SB216763 or GSK3i peptide protected mice from an LD100 of LPS given therapeutically. Survival of mice following LPS challenge was monitored for 7 days.

RESULTS

Inhibitory effect of the GSK3i peptide on LPS-induced pro-inflammatory cytokine production

We designed a cell-permeable GSK3i peptide spanning the serine 9 phosphorylation motif of GSK3β that was fused with recently characterized cell-permeable sequences derived from the human transcription factor Hph-1 (Fig. 1) (29,30). Since GSK3β is known key regulator of pro-inflammatory cytokine production (16), we examined the ability of the GSK3i 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 GSK3i 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 GSK3i peptide can regulate LPS-mediated pro-inflammatory cytokine production.

The GSK3i peptide protects mice from endotoxin shock

To test the therapeutic potential of the GSK3i 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 GSK3i peptide before receiving a 100% lethal dose (LD100) 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 GSK3β inhibition regulated the pro-inflammatory cytokine production in mice given an LD100 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-
tive to that of the control mice given LPS. Thus, these results demonstrate that a cell-permeable inhibitor peptide that targets GSK3β 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 GSK3β. Our study has shown that the peptide motif spanning serine 9 of GSK3β natively offers novel exciting approaches for target-selective inhibition. GSK3β phosphopeptide corresponding to residue 7-14 of GSK3β blocks LPS-induced cytokine production and septic shock. The central role of the GSK3β pathway in innate immune responses has been well documented (31). Specifically, it has been shown that GSK3β is involved in the PI3K/Akt pathway and mediates cytokine production in TLR signaling (15). Moreover, many reports have elucidated important roles for the GSK3β in immune diseases such as arthritis, colitis, multiple sclerosis and sepsis (32,33) suggesting that GSK3β 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 GSK3β on the N-terminal serine 9 residue (37,38). After serine 9 phosphorylation, the phosphorylated N-terminal residues of GSK3β 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 GSK3β and inhibits its enzyme activity. Consistent with our data, a phosphopeptide corresponding to residue 7-14 of GSK3β inhibited GSK3 activity in vitro, whereas the nonphosphorylated 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 determined 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.

**ACKNOWLEDGEMENTS**

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-C00538).

**CONFLICTS OF INTEREST**

The authors have no financial conflict of interest.

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