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Glycogen Synthase Kinase-3Beta Suppresses Tumor Necrosis Factor-Alpha Expression in Cardiomyocytes During Lipopolysaccharide Stimulation

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Abstract This study was to investigate the role of glycogen synthase kinase-3beta (GSK-3β) in cardiomyocyte tumor necrosis factor-alpha (TNF-α) expression induced by lipopolysaccharide (LPS). In cultured neonatal mouse cardiomyocytes, LPS induced TNF-α expression and increased GSK-3β activation. Inhibition of GSK-3β by SB216763 or by over-expression of a dominant negative mutant of GSK-3β significantly enhanced TNF-α expression in LPS-stimulated cardiomyocytes, in association with an increase in p65 phosphorylation. In contrast, over-expression of GSK-3β by adenoviral vectors containing wild-type GSK-3β or a constitutively active GSK-3β attenuated TNF-α expression induced by LPS. Further evidence to support the inhibitory role of GSK-3β in TNF-α expression is that protein kinase B (Akt) signaling, an upstream inhibitor of GSK-3β, promotes TNF-α expression in LPS-stimulated cardiomyocytes and this action of Akt signaling can be mimicked by GSK-3β inactivation. Our study demonstrates that GSK-3β plays an inhibitory role in cardiomyocyte TNF-α expression during LPS stimulation, and it may be a potential therapeutic target for sepsis. J. Cell. Biochem. 104: 329–338, 2008.

Key words: cytokine; glycogen synthase kinase-3β; lipopolysaccharide; signal transduction; cardiomyocytes

Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine with multiple biological actions [Tracey and Cerami, 1993; Meldrum, 1998], impairs cardiac contractile function in intact animals, isolated hearts and cardiomyocytes [Oral et al., 1997; Bozkurt et al., 1998; Grandel et al., 2000]. TNF-α-induced myocardial dysfunction has two distinct phases. The early phase, which occurs within minutes after TNF-α exposure, is related to sphingosine production leading to disturbances of intercellular Ca^{2+} homeostasis [Yokoyama et al., 1993]. The late phase, which occurs hours after TNF-α exposure, is mediated by iNOS expression, apoptosis [Stein et al., 1996; Song et al., 2000], and inhibition of both pyruvate dehydrogenase activity and mitochondrial function [Zell et al., 1997; Li et al., 2001]. Therefore, understanding regulation of TNF-α expression may point to novel therapeutic strategies to treat or prevent myocardial dysfunction.

Lipopolysaccharide (LPS) of Gram-negative bacteria has been recognized as a causative agent in myocardial depression during sepsis [Natanson et al., 1989; Suffredini et al., 1989]. Cardiomyocytes produce TNF-α in response to LPS exposure [Kapadia et al., 1995; Grandel et al., 2000; Peng et al., 2003a]. It has been known that the signaling pathways of LPS induced TNF-α expression in cardiomyocytes involve Toll-like receptor-4 (TLR-4) and nuclear factor-kappa B (NF-κB) [Monick and Hunninghake, 2003; Peng et al., 2005a; Hoebe et al.,

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2006]. We have recently demonstrated that NAD(P)H oxidase-mediated mitogen activated protein kinase (MAPK) signaling plays an important role in cardiomyocyte TNF-α expression during LPS stimulation [Peng et al., 2003a,b, 2005b]. However, the regulation of TNF-α expression remains to be fully elucidated in cardiomyocytes.

Glycogen synthase kinase-3beta (GSK-3β) is a serine/threonine kinase that was originally identified as a kinase that is involved in glucose metabolism, but subsequent research has determined that it acts on a wide variety of substrates, including transcription factors and is a key regulator in many signaling pathways [Cohen and Frame, 2001]. It has been demonstrated that GSK-3β is involved in regulation of NF-κB signaling [Hoeflich et al., 2000; Demarchi et al., 2003; Haefner, 2003; Takada et al., 2004], and thus it may be important in regulation of pro-inflammatory cytokines expression in sepsis. Studies have shown that several inhibitors of GSK-3β attenuate proinflammatory cytokine expression including TNF-α in the plural exudates [Cuzzocrea et al., 2006] and colon [Whittle et al., 2006] during acute systemic inflammation and in monocytes during LPS stimulation [Martin et al., 2005], suggesting GSK-3β may potentiate TNF-α expression. However, a recent study challenged those previous observations by showing that over-expression of GSK-3β reduces the nuclear half-life of NF-κB and inhibits TNF-α expression in lung tissues from LPS-stimulated mice [Vines et al., 2006]. In the heart, previous study has indicated that GSK-3β may play a role in TNF-alpha expression during LPS stimulation [Vines et al., 2006]. Nevertheless, it remains unclear whether GSK-3β contributes to TNF-alpha expression in cardiomyocytes.

GSK-3β is catalytically active in cells under un-stimulated conditions. The activity of GSK-3β is regulated by multiple mechanisms [Cohen and Frame, 2001]. Protein kinase B, also known as Akt, is one of the most important kinase that has been identified as upstream inhibitory regulator of GSK-3β [Jiang et al., 2005]. Akt has been shown to modulate TNF-α expression during LPS stimulation in non-cardiomyocytes [Guha and Mackman, 2002; Strassheim et al., 2004; Zhang and Daynes, 2007; Zhang et al., 2007]. However, the role of Akt and its signaling-mediated GSK-3β inactivation in LPS-induced TNF-α expression has not been demonstrated in cardiomyocytes.

The present study was to investigate the role of GSK-3β in LPS-induced TNF-a expression in cardiomyocytes and to determine the contribution of Akt-dependent GSK-3β inactivation pathway. Our results suggest that GSK-3β limits LPS-induced TNF-a expression in cardiomyocytes and Akt signaling acts upstream of GSK-3β in regulation of TNF-a expression during LPS stimulation.

MATERIALS AND METHODS

Neonatal Mouse Cardiomyocyte Culture

Wild-type C57BL/6 mice were purchased from the Jackson Laboratory. All animal breeding, husbandry and experimental procedures were reviewed and approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the Canadian Council on Animal Care guidelines.

Cardiomyocytes cultures were prepared as described previously with modifications [Peng et al., 2003a]. Briefly, neonatal mouse hearts were removed and then minced in sodium bicarbonate, Ca^{2+}, and Mg^{2+}-free Hanks balanced salt solution (D-Hanks, Sigma, St. Louis, MO). Cardiomyocytes were dispersed with 22.5 μg/ml liberase 4 (Roche, Laval, Quebec) in D-Hanks at 37°C. Cells were collected by centrifugation at 200*g* for 5 min and resuspended in 10% FCS-containing M199 medium (Sigma). Removal of non-cardiomyocytes was achieved through 90 min of preplating. The cardiomyocytes were seeded in 1% gelatin pre-coated 24-well plates. Cells were incubated in a humidified atmosphere at 37°C with 5% CO_2. After 48 h of culture, treatments with various drugs were performed.

Reagents

LPS (Salmonella typhosa), SB216763, and LY294002 were purchased from Sigma (Sigma–Aldrich Canada Ltd., Ontario). Adenoviral vectors containing either the gene for wild-type GSK-3β (Adv-wtGSK), constitutively active GSK-3β (Adv-caGSK) or dominant negative GSK-3β (Adv-dnGSK) were kindly provided by Dr. Morris Birnbaum (University of Pennsylvania Medical School, Philadelphia, PA). Adenoviral vectors containing either the gene for dominant negative Akt (Ad-dnAKT) or

a green fluorescence protein (Ad-GFP) were purchased from Applied Biological Materials, Inc. (Canada).

Adenoviral Infection of Neonatal Mouse Cardiomyocytes

Cardiomyocytes were infected with adenoviral vectors Ad-dnGSK, Ad-caGSK, Ad-wtGSK or Ad-dnAKT at a multiplicity of infection (MOI) of 20–100 pfu/cell. Ad-GFP was used as a control. Adenovirus-mediated gene transfer was implemented as previously described [Peng et al., 2003a, 2005a]. All experiments were performed after 24 h of adenoviral infection.

Analysis of TNF-α mRNA by Real-Time Reverse-Transcription PCR (RT-PCR)

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Invitrogen Canada, Inc., Ontario) following the manufacturer's instructions. Real-time RT-PCR for TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the same primers as previously described [Peng et al., 2003a].

Measurement of TNF-α Protein Levels

TNF-α release into the media was measured using an ELISA kit (ALPCO Diagnostics) for mouse TNF-α as described in our previous reports [Peng et al., 2003a].

Measurement of GSK-3β, Akt and p65 Phosphorylation

Assessment of the phosphorylation of GSK-3β (Ser9, Akt (Thr308)) and p65 (Ser536) was accomplished by Western blot analysis using antibodies against phospho-GSK-3β,phospho-Akt and phospho-p65 (Cell Signaling, Danvers, MA), as described previously [Peng et al., 2003a].

Statistical Analysis

All data were given as mean ± SD from at least three independent experiments. Measurements for all in vitro experiments were made in duplicate or triplicate. Differences between two groups were compared by unpaired Student's *t*-test. For multi-group comparisons, ANOVA followed by Student–Newman–Keuls test was performed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

GSK-3β Phosphorylation is Decreased in Cardiomyocytes During LPS Stimulation

Phosphorylation of GSK-3β at Ser9 leads to its inactivation whereas dephosphorylation leads to its activation [Cohen and Frame, 2001]. To examine the effect of LPS on GSK-3β activation, cardiomyocytes were incubated with LPS (1 μg/ml) for 20, 60, 120 min, and GSK-3β phosphorylation was determined by Western blot analysis. LPS treatment did not affect total GSK-3β protein expression but significantly decreased GSK-3β phosphorylation in cardiomyocytes in a time-dependent manner (Fig. 1A). To further confirm this result, we measured GSK-3β phosphorylation in in vivo heart. Adult mice were given an intraperitoneal (i.p.) injection of LPS (4 mg/kg) or same volume of phosphate-buffered saline (PBS). Four hours after LPS challenge, heart tissues were collected for measurement of GSK-3β phosphorylation by Western blot analysis. GSK-3β phosphorylation was also reduced in in vivo heart during endotoxemia (Fig. 1B). This data suggests that LPS increased GSK-3β activation in cardiomyocytes.

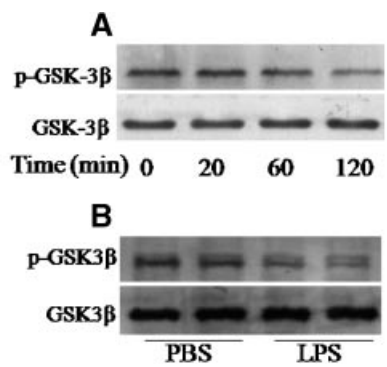

Fig. 1. Effect of LPS on GSK-3β phosphorylation. **A**: Cultured cardiomyocytes were incubated with LPS (1 μg/ml) or vehicle for 20, 60, 120 min. Phosphorylation of GSK-3β was measured by Western blot analysis using anti-body against phosphor-GSK-3β (Ser9). A representative blot from 2 independent experiments shows a reduction of GSK-3β phosphorylation after LPS treatment. **B**: Adult mice were given an intraperitoneal (i.p.) injection of LPS (4 mg/kg) or same volume of phosphate-buffered saline (PBS). Four hours after LPS challenge, heart tissues were collected for measurement of GSK-3β phosphorylation by Western blot analysis. A representative blot from three different hearts in each group shows a decrease in GSK-3β phosphorylation after LPS treatment.

GSK-3β Inhibition Enhances TNF-α Expression in LPS-Stimulated Cardiomyocytes

To investigate the role of GSK-3β in TNF-α expression, cardiomyocytes were pre-incubated with a selective inhibitor of GSK-3β, SB216763 (10 μM) for 30 min, followed by LPS (1 μg/ml) for 4 h. TNF-α mRNA was analyzed by real-time RT-PCR. As shown in Figure 2A, SB216763 did not affect the basal levels of TNF-α mRNA. However, it significantly enhanced LPS-induced TNF-α mRNA in cardiomyocytes by 38% ($P < 0.05$). This data suggests that inhibition of GSK-3β promotes LPS-stimulated TNF-α expression. To substantiate this result, we employed adenoviral vector containing a dominant negative mutant of GSK-3β, Ad-dnGSK, to specifically block GSK-3β activity. Infection of Ad-dnGSK did not alter basal TNF-α mRNA but significantly increased TNF-α mRNA expression by 70% ($P < 0.05$) in LPS-stimulated cardiomyocytes, compared to Ad-GFP (Fig. 2B). This was associated with a significant enhancement by 50% ($P < 0.05$) in TNF-α protein production in Ad-dnGSK-infected cardiomyocytes (Fig. 2C). The effects of SB216763 and Ad-dnGSK on GSK-3β activity were verified by measuring β-catenin protein using Western blot analysis since GSK-3β is an important upstream regulator of β-catenin [Chen et al., 2000; Hagen et al., 2002]. Our results showed that both SB216763 treatment and infection with Ad-dnGSK significantly increased β-catenin protein (Fig. 2D), suggesting an inhibition of GSK-3β. Thus, these results suggest that GSK-3β suppresses TNF-α expression induced by LPS in cardiomyocytes.

Over-Expression of GSK-3β Decreases TNF-α Expression in LPS-Stimulated Cardiomyocytes

We then investigated whether over-expression of GSK-3β limits TNF-α expression in LPS-stimulated cardiomyocytes. We first used

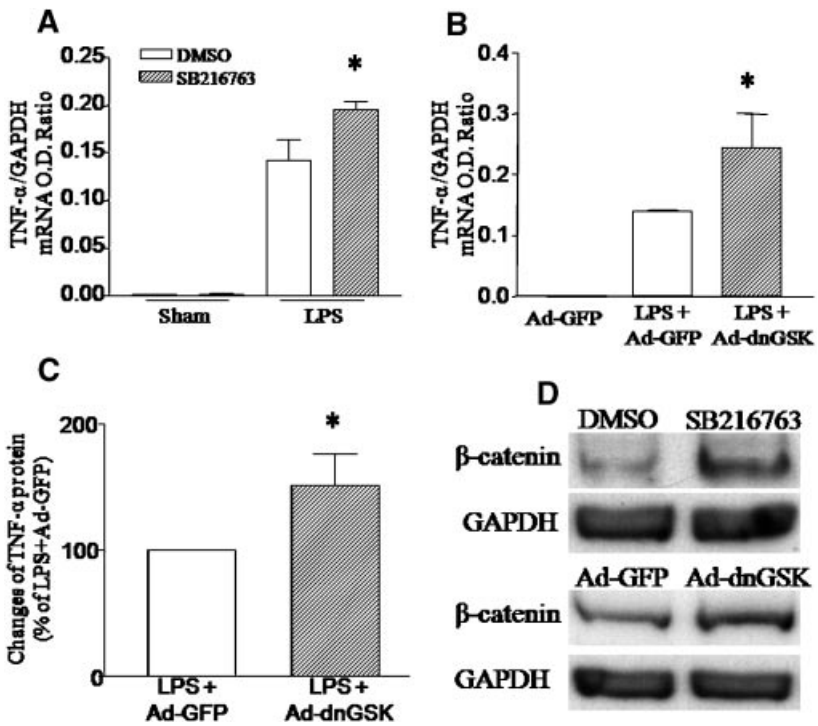

Fig. 2. Effects of GSK-3β inhibitors on LPS-induced TNF-α expression. **A**: Cardiomyocytes were pre-incubated with SB216763 (10 μM) or DMSO for 30 min, and then treated with LPS (1 μg/ml) or vehicle. Four hours after LPS treatment. TNF-α mRNA was analyzed by real-time PCR and expressed as TNF-α to GAPDH ratio. **B**,**C**: Cardiomyocytes were infected with Ad-GFP or Ad-dnGSK for 24 h and then incubated with LPS (1 μg/ml) or vehicle for 4 h. TNF-α mRNA and protein levels in culture media were measured by real-time PCR and ELISA, respectively. Ad-dnGSK infection significantly enhanced LPS-induced TNF-α mRNA (B) and protein release (C). **D**: Cardiomyocytes were infected with Ad-dnGSK or Ad-GFP for 24 h, or incubated with SB216763 or DMSO for 2 h. β-catenin protein was measured by Western blot analysis. A representative blot from three different experiments shows an increase in the levels of β-catenin protein after GSK-3β inhibition. Data are mean ± SD of four to six independent experiments. *$P < 0.05$ versus LPS + Ad-GFP.

Ad-wtGSK adenovirus to determine the effect of GSK-3β over-expression on TNF-α expression. Cardiomyocytes were infected with Ad-wtGSK or Ad-GFP and then stimulated with LPS (1 μg/ml). Ad-wtGSK infection significantly decreased TNF-α mRNA expression by 31% ($P < 0.05$), compared to Ad-GFP (Fig. 3A). Similarly, over-expression of a constitutively active GSK-3β in cardiomyocytes by Ad-caGSK significantly attenuated LPS-induced TNF-α mRNA and protein by 35% and 30% ($P < 0.05$), respectively (Fig. 3A,B). Both Ad-wtGSK and Ad-caGSK infection had no evident effect on basal levels of TNF-α expression in cardiomyocytes. These data suggest that over-expression of GSK-3β down-regulates TNF-α expression in LPS-stimulated cardiomyocytes.

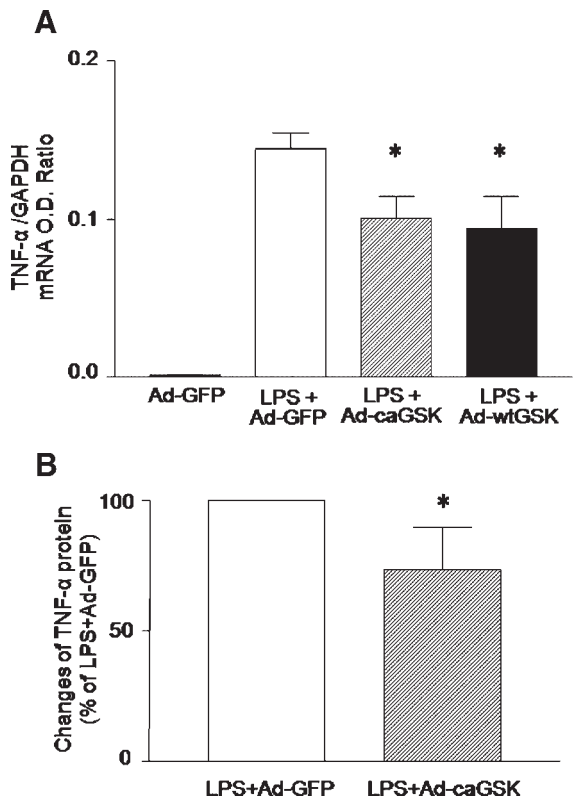

Fig. 3. Effects of GSK-3β over-expression on LPS-induced TNF-α expression. After 24 h culture, cardiomyocytes were infected with Ad-wtGSK, Ad-caGSK or Ad-GFP for 24 h. The cells were then incubated with LPS (1 μg/ml) or vehicle for 4 h. TNF-α mRNA and protein levels in culture medium were measured by real-time PCR and ELISA, respectively. LPS-induced TNF-α mRNA (**A**) and protein release (**B**) were significantly decreased in Ad-wtGSK and Ad-caGSK compared to Ad-GFP infected cardiomyocytes. Data are mean ± SD of four to six independent experiments. *$P < 0.05$ versus LPS + Ad-GFP.

Inhibition of Akt Signaling Attenuates TNF-α Expression Induced by LPS

GSK-3β activity is inversely regulated by phosphorylation at a specific serine residue [Cohen and Frame, 2001], and Akt, as a major upstream kinase, therefore serves as an inhibitor. Phosphatidylinositol-3 kinase (PI3K) is an upstream regulator of Akt. Thus, the PI3K/Akt pathway is an important endogenous inhibitor of GSK-3β. Since our data showed that LPS decreased GSK-3β phosphorylation in cardiomyocytes (Fig. 1), we examined if Akt phosphorylation is also down-regulated in response to LPS. In line with GSK-3β, Akt phosphorylation was reduced in both LPS-treated cardiomyocytes and in vivo heart during endotoxemia (Fig. 4).

To further demonstrate the role of GSK-3β in TNF-α expression, we first investigated the contribution of PI3K/Akt pathway to LPS-induced TNF-α expression. To explore the role of PI3K, we used a selective inhibitor of PI3K, LY294002. Cardiomyocytes were pre-incubated with LY294002 (10 μM), and then treated with LPS (1 μg/ml). Pre-treatment of LY294002 did not alter the basal levels of TNF-α but significantly decreased LPS-induced TNF-α mRNA and protein by 37% and 46% ($P < 0.05$), respectively (Fig. 5A,B). We also examined the role of Akt in TNF-α expression. Cardiomyocytes

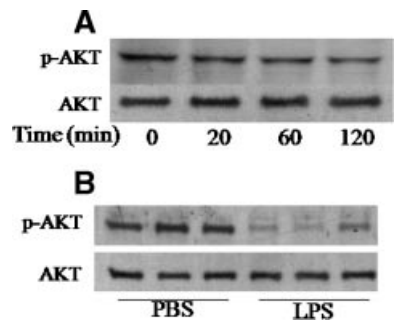

Fig. 4. Effect of LPS on Akt phosphorylation. **A**: Cultured cardiomyocytes were incubated with LPS (1 μg/ml) or vehicle for 20, 60, 120 min. Phosphorylation of Akt was measured by Western blot analysis using anti-bodies against phosphor-Akt (Thr308). A representative blot from three independent experiments shows a slight reduction of Akt phosphorylation after LPS treatment. **B**: Adult mice were given an intraperitoneal (i.p.) injection of LPS (4 mg/kg) or same volume of phosphate-buffered saline (PBS). Four hours after LPS challenge, heart tissues were collected for measurement of Akt phosphorylation by Western blot analysis. A representative blot from three different hearts in each group shows a decrease in GSK-3β phosphorylation after LPS treatment.

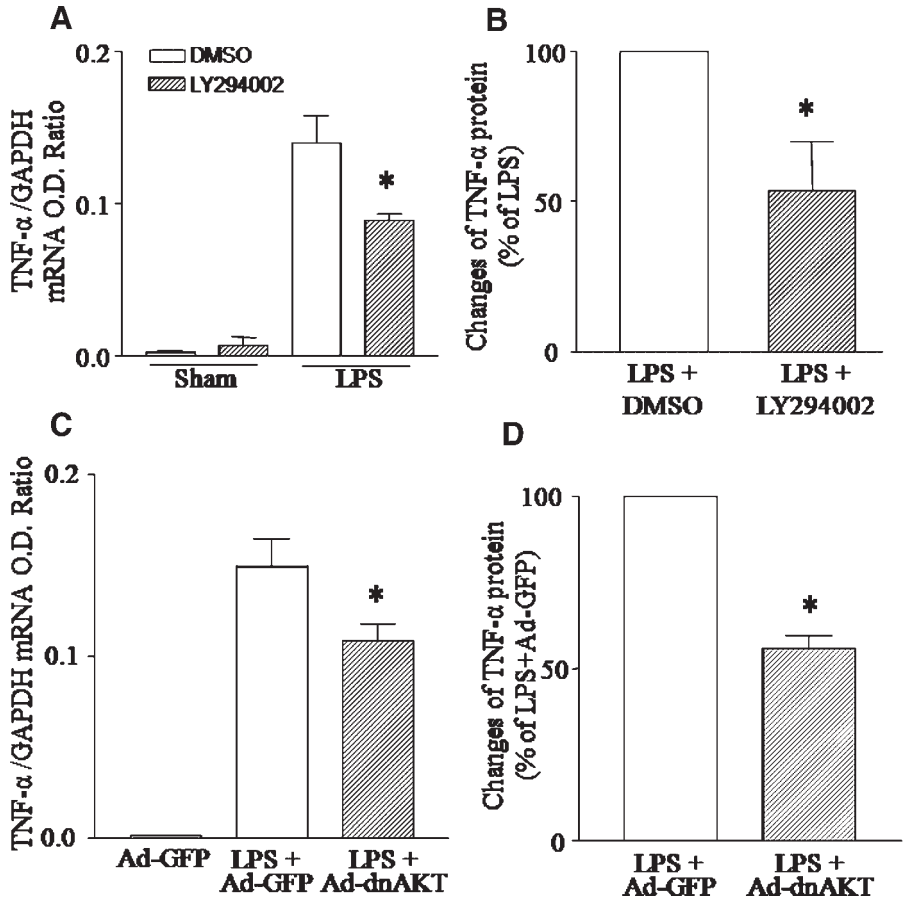

Fig. 5. Role of PI3K/Akt pathway in TNF-α expression during LPS stimulation. **A**,**B**: Cardiomyocytes were pre-incubated with LY294002 (10 μM) or DMSO for 30 min, followed by LPS (1 μg/ml) or vehicle for 4 h. TNF-α mRNA was measured by real-time PCR and expressed as TNF-α to GAPDH ratio (A). The TNF-α protein levels in culture medium were determined by ELISA (B). **C**,**D**: Cardiomyocytes were infected with Ad-dnGSK or Ad-GFP for 24 h and then incubated with LPS (1 μg/ml) or vehicle for 4 h. TNF-α mRNA (C) and protein (D) were measured by real-time PCR and ELISA, respectively. Data are mean ± SD of four to six independent experiments. $^*P < 0.05$ versus LPS + DMSO or LPS + Ad-GFP.

were infected with Ad-dnAKT or Ad-GFP and then incubated with LPS (1 μg/ml). In line with LY294002, Ad-dnAKT infection attenuated LPS-induced TNF-α mRNA and protein by 30% and 44% ($P < 0.05$), respectively (Fig. 5C,D). Ad-dnAKT alone did not affect basal TNF-α expression. These data suggest that PI3K/Akt signaling contributes to cardiomyocyte TNF-α expression during LPS stimulation.

We then investigated if effects of PI3K/Akt inhibitors on TNF-α expression can be reversed by GSK-3β inactivation. Cardiomyocytes were pre-incubated with SB216763 (10 μM) or LY294002 (10 μM) either alone or in combination, and then treated with LPS (1 μg/ml) for 4 h. TNF-α mRNA and protein were determined. Consistently, LY294002 attenuated LPS-induced TNF-α expression. This inhibitory effect of LY294002 on TNF-α expression was absent when cardiomyocytes were concomitantly incubated with SB216763 during LPS stimulation (Fig. 6). This result suggests an important role of PI3K/Akt-dependent GSK-3β inactivation in the enhancement of TNF-α expression induced by LPS.

GSK-3β Inactivation Enhances p65 Phosphorylation During LPS Stimulation

GSK-3β has been shown to regulate NF-κB activation [Hoeflich et al., 2000; Demarchi et al., 2003; Haefner, 2003; Takada et al., 2004]. NF-κB is an important transcription factor for TNF-α expression induced by LPS. Studies have shown that p65 phosphorylation promotes NF-κB activation upon stimuli [Yang

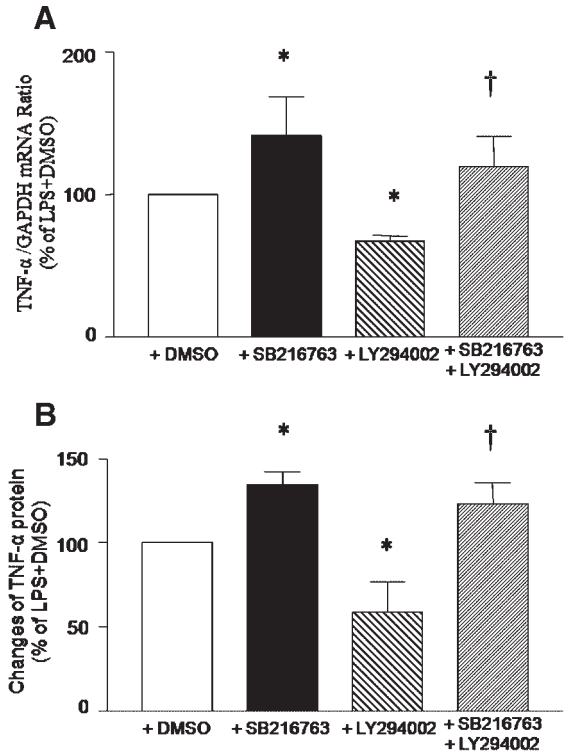

Fig. 6. Role of PI3K/Akt-dependent GSK-3β inactivation in TNF-α expression during LPS stimulation. Cardiomyocytes were pre-incubated with SB216763 and LY294002 either alone or in combination for 30 min, and then treated by LPS (1 μg/ml). Four hours after LPS treatment, TNF-α mRNA (**A**) and protein (**B**) were measured by real-time PCR and ELISA, respectively. Data are mean ± SD of four to five independent experiments. $^{*}P < 0.05$ versus +DMSO, $^{\dagger}P < 0.05$ versus +LY294002.

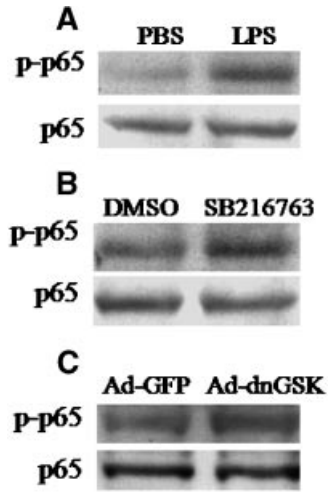

Fig. 7. Effects of GSK-3β inhibition on p65 phosphorylation during LPS stimulation in cardiomyocytes. Cardiomyocytes were infected with Ad-dnGSK or Ad-GFP for 24 h, or pre-incubated cardiomyocytes with SB216763 or vehicle (DMSO) for 30 min, and then treated with LPS or PBS. Two hours late, p65 phosphorylation at the residue Ser536 was determined by Western blot analysis. A representative blot from three different experiments shows that LPS treatment increased p65 phosphorylation (**A**), the levels of phosphorylated p65 were enhanced by either SB216763 (**B**) or Ad-GSK infection (**C**).

et al., 2003; Hall et al., 2005; Sasaki et al., 2005]. To study if GSK-3β modulates p65 phosphorylation, we infected cardiomyocytes with Ad-dnGSK or Ad-GFP for 24 h, or pre-incubated cardiomyocytes with SB216763 or vehicle for 30 min. We then treated these cardiomyocytes with LPS or PBS. Two hours later, p65 phosphorylation at the residue Ser536 was determined by Western blot analysis. LPS treatment increased p65 phosphorylation (Fig. 7A). The levels of phosphorylated p65 were further enhanced by either SB216763 (Fig. 7B) or Ad-GSK infection (Fig. 7C). These data suggest that GSK-3β inactivation promotes p65 phosphorylation in LPS-stimulated cardiomyocytes.

DISCUSSION

The present study demonstrates a critical role of GSK-3β in the regulation of TNF-α expression in cardiomyocytes during LPS stimulation. Several lines of evidence support this. First, we found that LPS increased GSK-3β activation and inhibition of GSK-3β enhanced LPS-induced TNF-α expression in cardiomyocytes. When GSK-3β was over-expressed in LPS-stimulated cardiomyocytes, TNF-α expression was significantly decreased. These data suggest that GSK-3β plays an inhibitory role in LPS-induced TNF-α expression in cardiomyocytes and thus, up-regulation of GSK-3β activation by LPS may represent a novel negative feedback mechanism in regulation of TNF-α expression. This conclusion is further supported by the following evidence: blocking PI3K/Akt pathway significantly down-regulated TNF-α expression and this effect of blocking PI3K/Akt pathway on TNF-α expression was absent when GSK-3β was inhibited during LPS stimulation. Thus, our data also suggest that PI3K/Akt-dependent inactivation of GSK-3β promotes LPS-induced TNF-α expression in cardiomyocytes.

GSK-3β has been shown to regulate NF-κB activation [Hoeflich et al., 2000; Demarchi et al., 2003; Haefner, 2003; Takada et al., 2004], a known key transcription factor for the genes involved in the production of proinflammatory mediators. GSK-3β-null cells have diminished NF-κB activity [Hoeflich et al., 2000; Martin et al., 2005] and pharmaceutical inhibitors of

GSK-3β down-regulate NF-κB DNA binding activity [Dugo et al., 2005; Cuzzocrea et al., 2006; Whittle et al., 2006]. These studies suggest that GSK-3β has the potential to modulate proinflammatory genes expression [Dugo et al., 2007]. Several reports have shown that chemical inhibitors of GSK-3β can reduce the levels of the pro-inflammatory cytokines in acute systemic inflammation [Dugo et al., 2005, 2006; Cuzzocrea et al., 2006; Whittle et al., 2006], suggesting a positive regulation by GSK-3β. In our study, we over-expressed dominant negative GSK-3β to down-regulate and constitutively active or wild-type GSK-3β to up-regulate GSK-3β activity. Our data show that down-regulation of GSK-3β enhances and, in contrast, up-regulation of GSK-3β suppresses LPS-induced TNF-α expression in cardiomyocytes. These results have provided definitive evidences that suggest an inhibitory role of GSK-3β in TNF-α expression during LPS stimulation. Our studies further demonstrate that either SB216763 or over-expression of a dominant negative mutant GSK-3β enhances p65 phosphorylation in LPS-stimulated cardiomyocytes. Since p65 phosphorylation is associated with NF-κB activation[Yang et al., 2003; Hall et al., 2005; Sasaki et al., 2005], our data suggest that the role of GSK-3β in TNF-α expression is likely mediated through modulation of p65 phosphorylation (Ser536). GSK-3β has also been shown to down-regulate TNF-α expression during LPS-stimulation by a recent study [Vines et al., 2006]. However, in disagreement with our results, their study demonstrated that over-expression of GSK-3β reduced the residence time of p56 in the nucleus in association with an increase in p65 phosphorylation (Ser536) in human endothelial cells during TNF-α stimulation. Our results as to the inhibitory role of GSK-3β in TNF-α expression are different from those observations by using chemical inhibitors. It is currently unclear whether this discrepancy results from different models or cell types used in different studies or the inhibition by chemical inhibitors is not entirely because of their effects on GSK-3β as chemical inhibitors often show nonselective activity.

The activity of GSK-3β is regulated by PI3K/Akt pathway. Studies have shown that PI3K/Akt pathway may play a role in modulation of TNF-α expression during LPS stimulation. For example, inhibition of PI3K/Akt decreases LPS-induced TNF-α expression in macrophages [Kim et al., 2006], suggesting that PI3K/Akt promotes TNF-α expression. In contrast, activation of PI3K/Akt limits the LPS induction of TNF-α expression in human monocytes [Zhang et al., 2007]. These studies suggest that the contribution of PI3K/Akt pathway to TNF-α expression may be cell or tissue specific. The role of PI3K/Akt in TNF-α expression has not been shown in cardiomyocytes. The present study aimed to investigate the role of PI3K/Akt pathway as an upstream inhibitory regulator of GSK-3β in LPS-induced TNF-α expression. Using cultured cardiomyocytes, we demonstrated: (1) LPS consistently decreased phosphorylation of Akt and GSK-3β; (2) Inhibition of PI3K activity attenuated TNF-α expression; (3) Blocking Akt activation decreased TNF-α expression. These results suggest that PI3K/Akt signaling induces TNF-α expression in LPS-stimulated cardiomyocytes. More importantly, we showed that the inhibitory effect of blocking PI3K/Akt on TNF-α expression was reversed by GSK-3β inactivation in LPS-stimulated cardiomyocytes. This suggests that PI3K/Akt-dependent inactivation of GSK-3β plays a part in LPS-induced TNF-α expression. It is important to mention that previous studies have shown a conflict result as to Akt phosphorylation during LPS stimulation. Hickson-Bick et al. [2006] demonstrated that LPS dramatically induced Akt phosphorylation in cultured neonatal rat cardiomyocytes whereas Chagnon et al. [2005] reported that LPS did not alter total phosphorylation of Akt but significantly decreased nuclear Akt phosphorylation in heart. Our data clearly showed that LPS decreased Akt phosphorylation (Thr308) and subsequently attenuated GSK-3β phosphorylation in both cultured neonatal mouse cardiomyocytes and in vivo heart. It is currently unknown whether this discrepancy is due to the detection of different phosphorylation sites of Akt since individual phosphorylation site of Akt may be differentially regulated [Bayascas and Alessi, 2005; Gao et al., 2005].

The significance of the finding that GSK-3β inactivation enhances LPS-induced TNF-α expression in cardiomyocytes in sepsis is presently unclear. High levels of TNF-α have been demonstrated to impair myocardial function [Oral et al., 1997; Bozkurt et al., 1998; Grandel et al., 2000]. However, increased TNF-α expression has also been shown to induce manganese

superoxide dismutase expression which may reduce oxidative stress and tissue injury during sepsis [Jones et al., 1997]. Therefore, GSK-3β inactivation-mediated enhancement of TNF-α production may also confer some beneficial effects. In addition, Akt-mediated GSK-3β inactivation is a well-known survival pathway. A recent study has shown that Akt activation and subsequent GSK-3β inactivation improve cardiomyocyte survival and function through increasing anti-apoptotic factors in endotoxemia [Chagnon et al., 2005]. This result suggests a protective action of Akt-mediated GSK-3β inactivation on septic heart. In this regard, a reduction of Akt activation and subsequent increase in GSK-3β activation induced by LPS (Figs. 1 and 4) may be one of the mechanisms for myocardial dysfunction in sepsis. However, the pathophysiological significance of the Akt/GSK-3β pathway in sepsis requires further investigation.

In conclusion, our results demonstrate that GSK-3β limits LPS-induced TNF-a expression and Akt signaling acts upstream of GSK-3β in regulation of TNF-a expression in cardiomyocytes. The role of GSK-3β in TNF-a expression is associated with p65 phosphorylation in LPS-stimulated cardiomyocytes. Although the patho-physiological significance of GSK-3β is currently incompletely understood in myocardial dysfunction during sepsis, the finding that GSK-3β suppresses LPS-induced TNF-α expression in cardiomyocytes in addition to a recent report [Chagnon et al., 2005] suggests that GSK-3β may be a potential therapeutic target for sepsis.

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