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Glycogen Synthase Kinase 3 Regulates IL-1β Mediated iNOS Expression in Hepatocytes by Down-Regulating c-Jun

Jaganathan Lakshmanan,* Baochun Zhang, Ikenna C. Nweze, Yibo Du, and Brian G. Harbrecht

Hiram C. Polk Jr., MD, Department of Surgery and Price Institute of Surgical Research, School of Medicine, University of Louisville, Louisville 40202, Kentucky

ABSTRACT

Excessive nitric oxide from the inducible nitric oxide synthase (iNOS) increases shock-induced hepatic injury, hepatic dysfunction, inflammation, and mortality in animal models. Cytokines increase the expression of iNOS in hepatocytes, but the signaling mechanisms involved are not completely understood. We have previously demonstrated that Akt mediates the inhibitory effect of cAMP and insulin on cytokine-induced hepatocyte iNOS expression. We hypothesized that glycogen synthase kinase 3 (GSK3), a target of Akt phosphorylation, would regulate hepatocyte iNOS expression. In cultured rat hepatocytes, GSK3 inhibitors decreased IL-1β mediated nitric oxide (NO) production and iNOS protein expression, while the phosphatidylinositol 3-kinase (PI3K)/Akt pathway inhibitor LY294002 increased the cytokine-mediated NO production and iNOS expression. Over-expression of the constitutively active form of GSK3β enhanced IL-1β-mediated iNOS expression. GSK3 catalyzes the phosphorylation of c-Jun at the c-terminal Thr239 that facilitates c-Jun degradation. Inhibition of GSK3 with SB216763 and lithium chloride significantly reduced, whereas blocking PI3K/Akt increased phosphorylation of c-Jun at Thr239. The levels of total-c-Jun and c-Jun phosphorylated at Ser63 inversely correlated with c-Jun phosphorylated at Thr239, GSK3 activation and iNOS expression. Over-expression of a dominant negative c-Jun not only caused an increase in IL-1β-mediated *iNOS* promoter activity and iNOS protein expression but was also able to reverse the SB216763-mediated suppression of iNOS. These results demonstrate that GSK3, a downstream target of Akt, regulates IL-1β-stimulated iNOS expression in hepatocytes by directly phosphorylating c-Jun in an inhibitory manner. J. Cell. Biochem. 116: 133–141, 2015.

KEY WORDS: GSK3; NITRIC OXIDE SYNTHASE; C-JUN; HEPATOCYTES

Hepatic inducible nitric oxide synthase (iNOS) expression is up-regulated in response to proinflammatory stimuli such as shock, sepsis, and ischemia/reperfusion (IR) injury [Hierholzer et al., 1988; Menezes et al., 1999]. Endothelial nitric oxide (NO) prevents hepatic tissue injury during shock and sepsis by maintaining hepatic perfusion [Harbrecht et al., 1995; Kim et al., 1997], but excessive NO from the inducible isoform is deleterious and causes hepatic dysfunction, hepatic injury, and upregulates hepatic inflammation [Hierholzer et al., 1988; Menezes et al., 1999; Tu et al., 2003]. Therefore, understanding the regulation of iNOS has potential therapeutic implications. We have shown that cytokines stimulate hepatic iNOS expression, but glucagon, cAMP, and insulin down-regulate cytokine-induced iNOS production in hepatocytes [Harbrecht et al., 1996, 2001, 2004, 2012]. Akt plays a pivotal role in the effects of cAMP and insulin on hepatocyte iNOS expression [Harbrecht et al., 1996, 2001; Zhang et al., 2011; Harbrecht et al., 2012] but the pathyway(s), signaling intermediates, and molecular mechanisms that link Akt to iNOS have not been clearly defined.

Glycogen Synthase Kinase 3 (GSK3) is a well-established downstream target of Akt in hepatocytes that plays an important role in maintaining normal cellular metabolism and cellular energetics through its effects on numerous intracellular signaling pathways and transcription factors [Ali et al., 2001]. GSK3 has also been implicated in regulating pathological conditions such as inflammation and cancer [Dugo et al., 2005, 2006, 2007; Forde and Dale, 2007; Mai et al., 2009; Rayasam et al., 2009; Beurel et al., 2010; Grassilli et al., 2013]. GSK3 is a serine-threonine kinase that has two isoforms (α and β) and is constitutively active in hepatocytes due to

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*Correspondence to: Jaganathan Lakshmanan, PhD, Hiram C. Polk Jr., MD, Department of Surgery, 511 S Floyd Street, MDR, RM#317, Louisville 40202, KY. E-mail: j0laks01@louisville.edu
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phosphorylation at Tyrosine residues (279 for α and 216 for β) [Hughes et al., 1993; Murai et al., 1996]. GSK3 activity is decreased by phosphorylation at inhibitory serine sites (21 for α and 9 for β) or dephosphorylation at activating sites [Wang et al., 1994; Cross et al., 1995; Pap and Cooper, 1999; Ali et al., 2001]. Akt-mediated inhibitory phosphorylation of GSK3 is one mechanism for the regulation of GSK3 activity by hormones such as insulin and alters the activation of downstream GSK3 substrates including c-Jun, NF-κB, CREB, C/EBP, and β-catenin [Frame and Cohen, 2001; Jope and Bijur, 2002; Götschel et al., 2008]. We hypothesized that in hepatocytes, GSK3, a well-established downstream target of Akt, would regulate the expression of iNOS. Our data demonstrate that GSK3 upregulates hepatocyte iNOS expression by phosphorylation dependent inhibition of c-Jun.

MATERIALS AND METHODS

MATERIALS

Williams Medium E, penicillin, streptomycin, L-glutamine, and HEPES were all from Life Technologies (Carlsbad, CA). Insulin was from Lilly (Indianapolis, IN). Polyclonal antibody to iNOS was purchased from BD Bioscience (Billerica, MA). Antibodies to total-Gsk3α/β, Gsk3α/β phosphorylated at Ser21/Ser9 (S21/S9), total Akt, Akt phosphorylated at Ser473 (S473), total c-Jun and c-Jun phosphorylated at Ser63 (S63) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to GSK3α/β phosphorylated at Tyr279/Tyr216 (Y279/Y216) and c-Jun phosphorylated at Thr239 (T239) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SB216763, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), and LY294002 were from Calbiochem (San Diego, CA). Human recombinant interleukin 1β (IL-1β) was from Dupont (Boston, MA) and lithium chloride (LiCl) from Sigma (St. Louis, MO). The pGL3-Basic firefly, pGL4.74 *Renilla* vectors and reagents for luciferase assays were purchased from Promega Corporation (Madison, WI). pcDNA3.1-TAM67 dominant negative (DN) c-Jun was a gift from Dr. Michael J. Birrer MD, PhD at the National Cancer Institute. The adenoviral vectors expressing constitutively active human Gsk3β (Ser9 to Ala; S9A) (pAdEasy-mRFP-GSK3β-S9A; Addgene plasmid 24521) and pAdEasy-GFP were from Addgene (Cambridge, MA). Chemiluminescence detection reagents were from Pierce (Rockford, IL) and all other reagents were from Sigma (St. Louis, MO).

PRIMARY HEPATOCYTE ISOLATION AND CULTURE

Primary hepatocytes were isolated from male Sprague-Dawley rats (200–250 g) using the modified collagenase perfusion technique as previously described [Zhang et al., 2004b]. All animal care was in accordance with the University of Louisville's Animal Care and Use Committee and followed guidelines prescribed by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Purified hepatocytes (98% pure with >95% viability by trypan blue exclusion) were plated on collagen-coated 6-well plates in Williams Medium E with L-arginine (0.5 mM), L-glutamine (2 mM), HEPES (15 mM) penicillin, streptomycin, and 10% low endotoxin calf serum (HyClone Laboratories, Logan, UT) for 4 h. The cells were then washed to remove non-attached cells and incubated with insulin-free media with 5% calf serum. After 16 h of additional incubation, the experimental conditions were established. Conditions were performed in duplicate or triplicate and experiments were repeated at least three times to ensure reproducibility. In experiments involving SB216763, TDZD-8, and LY294002, control hepatocytes and those treated with IL-1β alone received 0.001% DMSO in the culture media as vehicle control, equivalent to the amount present in hepatocytes treated with the inhibitors.

WESTERN BLOT

Hepatocytes were washed with ice-cold PBS and then scraped from the plate in 500 μl of lysis buffer (20 mM Tris–HCl, pH7.5, 150 mM NaCl, 1mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After 30 min at 4 °C, the lysates were centrifuged (15,000 × *g* for 15 min) and stored at −80 °C until use. Proteins (50 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h. Primary antibodies were diluted and incubated with membranes for 1–2 h at room temperature or overnight at 4 °C with agitation. After washing three times with TBS-T, secondary antibodies were incubated at 1:10,000 dilutions for 1 h. After five additional washes with TBS-T, the bands were visualized using chemiluminescence according to the manufacturer's instructions. Membranes were first probed for the phosphorylated form (p-Gsk3, p-Akt, and p-c-Jun) of the protein, and then stripped and re-probed for total levels of the respective protein using the appropriate antibody. The membranes were stripped and re-probed for β-actin, which served as internal control. Quantification of band intensities was done using ImageJ software.

RNA EXTRACTION AND Q-PCR

Total RNA was isolated from hepatocytes using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. TaqMan Reverse Transcription reagent (Applied Biosystems) was used to generate cDNA from total RNA. The rat *iNOS* probe (Rn00561646_m1) for Q-PCR was purchased from Life Technologies (Grand Island, NY). Samples were analyzed using a StepOne Plus PCR machine (Applied Biosystems) in triplicate for each set of experiments, and the average values were used for quantification. *GAPDH* (Rn01775763_g1, Life Technologies) was used as an endogenous control. The comparative CT method (cycle threshold) was used for quantification of gene expression. Analysis was performed using the StepOne software (Applied Biosystems) according to the manufacturer's instructions.

LUCIFERASE REPORTER PLASMID AND LUCIFERASE ASSAY

The 1.7 kb 5′-flanking region, including the TATA box of the rat *iNOS* promoter, was amplified and inserted into pGL3-Basic vector (Promega) to create the pGL3-RiNOS-Luc vector. Hepatocytes were plated in 6-well plates (1×10^6 cells/well) and transfected with plasmids using LipofectAMINE. Briefly, for the luciferase reporter assay, the hepatocytes were co-transfected with the pGL3-RiNOS-

Luc reporter plasmid (2 μg) and 0.1 μg pGL4.74 *Renilla* luciferase-expressing plasmid using 10 μL of LipofectAMINE for 5 h. The cells were allowed to recover overnight. Twenty-four hours after transfection, the cells were treated with IL-1β in the absence or presence of the GSK3 inhibitor SB216763. Untreated control hepatocytes and those receiving IL-1β alone received 0.001% DMSO, equivalent to the amount present in SB216763-treated hepatocytes. After 24 h, the cells were lysed with 125 μL of Promega Cell Lysis buffer and the supernatant assayed for firefly and *Renilla* luciferase activity using kits from Promega. The luciferase activity was measured using a Berthold LB 960 Centro XS^3 luminometer. For experiments with the dominant negative c-Jun plasmid, the hepatocytes were transfected with 1 μg each of the pGL3-RiNOS-Luc reporter plasmid and the pcDNA3.1-TAM67 DN c-Jun plasmid using the LipofectAMINE transfection reagent and luciferase activity was assessed as described above. The firefly luciferase values were normalized to the corresponding *Renilla* luciferase activity to control for transfection efficiency. The data represent the mean ± SEM of three independent experiments. For studies involving TAM67DN c-Jun and the GSK3 inhibitor SB216763, primary hepatocytes were transfected with 2 μg of the pcDNA3.1-TAM67 DN c-Jun plasmid or the pcDNA3.1 control plasmid using the LipofectAMINE transfection reagent. Twenty-four hours after transfection, the cells were treated with IL-1β in the absence or presence of the GSK3 inhibitor SB216763. Untreated hepatocytes and those receiving IL-1β alone received 0.001% DMSO as a vehicle control, equivalent to the amount present in SB216763-treated hepatocytes. The cells were harvested after 18 h of treatment and protein was extracted for iNOS Western blot analysis.

ADENOVIRUS-MEDIATED EXPRESSION OF CONSTITUTIVELY ACTIVE GSK3β

The pAdEasy-mRFP-GSK3β (S9A) adenoviral vector expressing the constitutively active human Gsk3β and the pADdEasy-GFP control plasmid vectors were from Addgene Inc. (Cambridge, MA). Adenovirus particles were amplified and purified using the ViraBind Adenovirus purification kit (Cell Biolabs, Inc). Viral titer was determined using the QuickTiter Adenovirus Titer ELISA Kit (Cell BioLabs, Inc) according to the manufacturer's instructions. The hepatocytes were infected with pAdEasy-GFP control plasmid or the pAdEasy-mRFP-GSK3β (S9A) plasmid (MOI of 1:20). After 6 h of culture, the media containing adenovirus was replaced with fresh media and the cells were allowed to recover for 48 h. The hepatocytes were then stimulated with IL-1β for 60 and 180 min, the proteins extracted and Western blot analyses were performed. Unstimulated hepatocytes served as controls. For iNOS expression studies involving the constitutively active GSK3β, the hepatocytes were allowed to recover for 24 h after infection with the adenovirus. The hepatocytes were then stimulated with 200 U of IL-1β for 24 h after which proteins were extracted and analyzed by Western blot for iNOS and β-actin levels.

NO_2^- MEASUREMENT

Hepatocyte culture supernatant NO_2^- was measured as an index of NO production by the Griess reaction as described [Harbrecht et al., 2001].

STATISTICAL ANALYSIS

Data are presented as the mean ± SEM. Statistical significance was determined using the two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

EFFECT OF GSK3 INHIBITORS ON IL-1β-MEDIATED INOS INDUCTION

To test the hypothesis that GSK3 regulates iNOS expression, rat primary hepatocytes were incubated with GSK3 inhibitors (SB216763, TDZD-8, LiCl) for 30 min and then cultured with 200 U/mL of IL-1β for 24 h to induce iNOS. Each GSK3 inhibitor tested caused a significant decrease in IL-1β-induced supernatant NO_2^- and iNOS protein levels (Fig. 1A). We then tested the effect of GSK3 inhibition on *iNOS* mRNA expression and *iNOS* promoter activation using SB216763. SB216763 significantly reduced IL-1β-induced *iNOS* mRNA levels (Fig. 1B) and *iNOS* promoter activation (Fig. 1C).

GSK3 activity is increased by phosphorylation at Y279 for GSK3α and Y216 for GSK3β and GSK3 is constitutively active in hepatocytes [Hughes et al., 1993; Murai et al., 1996]. We therefore measured the effect of IL1β on GSK3 phosphorylation. Western blot analysis demonstrated prominent phosphorylation at Y279/Y216 in protein extracts from untreated control hepatocytes while extracts from IL-1β treated hepatocytes showed no change in GSK3α/β phosphorylation at these sites (Fig. 2A). IL-1β did, however, increase phosphorylation of GSK3 at the inhibitory S21 (α) and S9 (β) sites within 15 min (Fig. 2A) and this persisted for up to 3 h of culture. The level of the inhibitory GSK3 phosphorylation peaked at 15 min after the addition of IL-1β and decreased significantly at 180 min, but was still above control levels (Fig. 2A). As SB216763 decreases GSK3 activity, we also measured the effect of SB216763 on GSK3 phosphorylation. Interestingly, we found that SB216763 decreased the inhibitory GSK3 phosphorylation in primary hepatocytes (Fig. 2B) suggesting an autoregulatory effect of GSK3 activity. This finding is consistent with the work of others [Ren et al., 2011].

ROLE OF AKT IN GSK3 REGULATION OF INOS

Insulin increases inhibitory GSK3 phosphorylation at S21/S9 through an Akt-mediated mechanism [Cross et al., 1995; Pap and Cooper, 1999]. We have shown that cAMP and insulin decrease iNOS expression through increased Akt activation [Zhang et al., 2011; Harbrecht et al., 2012]. We therefore tested the hypothesis that Akt-mediated GSK3 phosphorylation regulates subsequent iNOS expression. Hepatocytes were treated with IL-1β in the presence or absence of LY294002 (10 μM), an inhibitor of phosphatidylinositol 3 kinase (PI3K)/Akt, and then assayed for NO_2^- and iNOS protein levels. LY294002 had no effect on iNOS in unstimulated hepatocytes (Fig. 3A). In the presence of LY294002, IL-1β caused a small but consistent increase in supernatant NO_2^- levels and iNOS protein expression (Fig. 3A) similar to our previous findings [Zhang et al., 2011]. Treating hepatocytes with IL-1β alone caused an increase in Akt phosphorylation that decreased with time. Inhibiting PI3K with LY294002 decreased Akt phosphorylation in control cells and significantly decreased the IL-1β-induced increase in Akt

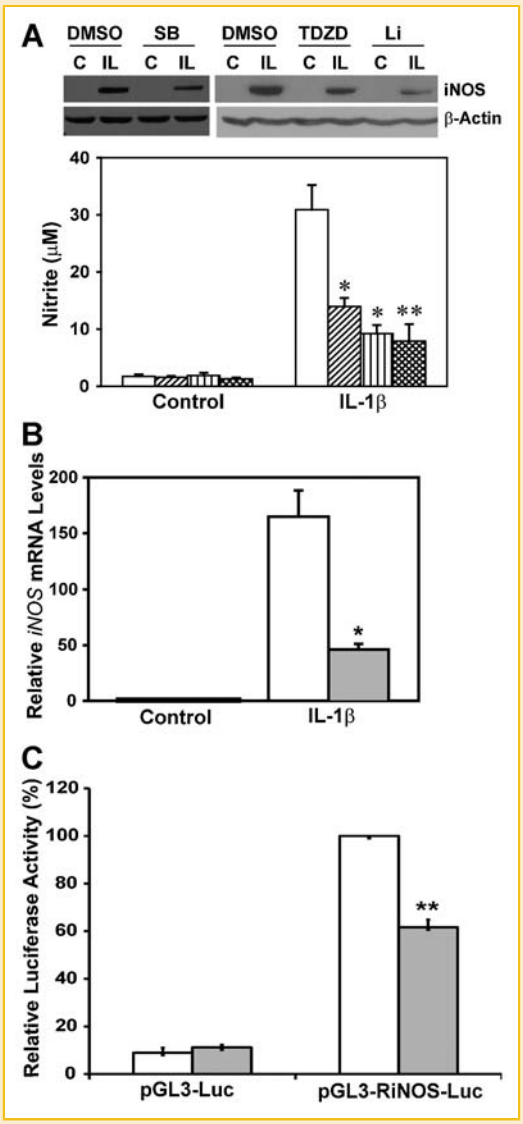

Fig. 1. Effect of GSK3 inhibitors on iNOS expression. A: Rat primary hepatocytes were pre-incubated for 30 min with GSK3 inhibitors (SB216763, 25 μM, ▨; TDZD-8, 100 μM, ▥; LiCl, 20 mM, ▩) and then stimulated with 200 U of IL-1β. After 24 h, the supernatants were analyzed for nitrite by the Griess method. Supernatants from cells treated with 0.001% DMSO served as control (□). The cell-lysates were collected and analyzed for iNOS protein by Western blots. The blots shown are representative of three independent experiments. B: *iNOS* mRNA was extracted after 180 min of culture with 200 U of IL-1β in the presence of 0.001% DMSO (control; white bars) or GSK3 inhibitor 25 μM SB216763 (grey bars). Quantitation was done by Q-PCR method using TaqMan probes. *GAPDH* was used as the endogenous control. C: Rat primary hepatocytes were transfected with pGL3-Basic or pGL3-RiNOS-Luc vector along with control pGL4.74 *Renilla* luciferase vector using LipofectAMINE reagent. The cells were allowed to recover over-night and then pre-treated with media containing 0.001% DMSO (white bars) or 25 μM SB216763 (grey bars) for 30 min and then stimulated with 200 U of IL-1β. After 24 h, the cells were lysed and the lysates were assayed for firefly and *Renilla* luciferase activities. The firefly luciferase values were normalized to the corresponding *Renilla* luciferase activity to account for transfection efficiency differences. In A, B, and C the data represents the mean ± standard error of three independent measurements. *$P<0.05$ and **$P<0.005$.

phosphorylation and decreased downstream phosphorylation of GSK3α/β at S21/S9 (Fig. 3B). LY294002 had no effect on the phosphorylation of GSK3α/β at the activating Y279/Y216 site (data not shown). Furthermore, treating hepatocytes with LiCl, which is known to directly inhibit GSK3 as well as increase inhibitory GSK3 phosphorylation by activating PI3K and Akt, had the opposite effect. Lithium chloride slightly increase Akt and inhibitory GSK3 phosphorylation in unstimulated hepatocytes, but markedly increased IL-1β induced Akt phosphorylation and downstream inhibitory phosphorylation of GSK3 (Fig. 3C). The ERK1/2 pathway inhibitor PD95059 and a PKCζ inhibitor did not have any significant effect on GKS3 α/β phosphorylation at S21/S9 either alone or with IL-1β (data not shown) suggesting that these pathways play no significant role in GSK3 regulation in hepatocytes. To confirm the effects of GSK3 activation, we used a constitutively active GSK3β construct (S9A) in which the inhibitory serine 9 site was mutated to alanine and could not be phosphorylated by Akt. Expression of the constitutively active GSK3β (S9A) (pAdEasy-mRFP-GSK3β-S9A) in hepatocytes had no effect on iNOS expression in unstimulated hepatocytes, but augmented the IL-1β mediated induction of iNOS compared to hepatocytes infected with the control pAD-GFP plasmid (Fig 3D). These results demonstrate that IL-1β regulates GSK3 in hepatocytes through Akt and that inhibition of GSK3 activity is associated with decreased iNOS expression.

The effect of Akt on GSK3 has been well described [Cross et al., 1995; Pap and Cooper, 1999], but whether GSK3 regulates Akt in a feedback manner has not been established. To evaluate this relationship, we measured Akt phosphorylation in hepatocytes cultured with SB216763. Inhibition of GSK3 with SB216763 increased Akt phosphorylation in hepatocytes cultured in media alone and also in hepatocytes stimulated with IL-1β (Fig. 3E). To study this relationship further, we transfected hepatocytes with an adenoviral plasmid containing the constitutively active GSK3β (S9A) construct. Over-expression of the constitutively active GSK3β was associated with significantly decreased Akt phosphorylation in hepatocytes cultured in media alone and also in hepatocytes stimulated with IL-1β (Fig. 3F). These data demonstrate that GSK3 activity in hepatocytes decreases Akt phosphorylation.

GSK3 REGULATES INOS EXPRESSION THROUGH C-JUN

c-Jun is one of several established substrates of GSK3 [Morton et al., 2003] and we have previously shown that c-Jun acts as a transcriptional repressor of *iNOS* in hepatocytes [Zhang et al., 2004a]. Deletion of GSK3 decreases cytokine-stimulated JNK activation in fibroblasts while agonists that decrease GSK3 activity in hepatocytes increase c-Jun DNA binding, suggesting that GSK3 can increase c-Jun activation [Auer et al., 1998; Takada et al., 2004] In macrophages, GSK3 phosphorylates c-Jun at the C-terminal T239 that directs c-Jun to undergo polyubquination and proteasomal degradation to decrease total c-Jun levels [Morton et al., 2003]. We hypothesized that GSK3 would regulate iNOS expression in hepatocytes through effects on c-Jun. To test this hypothesis, we inhibited GSK3 activity with SB216763 and measured phosphorylation of c-Jun at the inhibitory T239 site. SB216763 significantly decreased phosphorylation of c-Jun at T239 at both 60 and 180 min in both unstimulated and IL-1β stimulated hepatocytes (Fig. 4A)

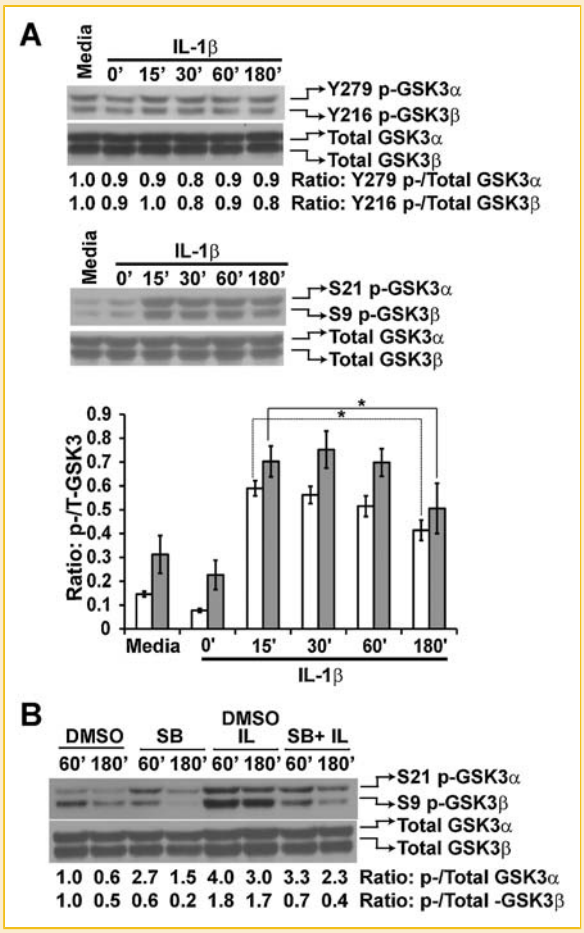

Fig. 2. Effect of IL-1β on GSK3 phosphorylation. A: Rat primary hepatocytes were treated with 200 U of IL-1β for the indicated times and cell lysates were collected and analyzed for p-GSK3α/β (activating Y279/216; inhibitory S21/9) and total GSK3α/β protein levels. Lysates from hepatocytes treated with 0.001% DMSO alone served as controls. The open bars in the graph represent the ratio of inhibitory S21 phosphorylation to total GSK3α levels and the grey bars represent the ratio of inhibitory S9 phosphorylation to total GSK3β levels. The density of each band was determined using the ImageJ software. The numbers represent the ratio of p-Y279/216 GSK3α/β band intensity to total GSk3α/β band intensity. The error bars represent the standard deviation of the quantifications from three independent experiments and the asterisk represent $P < 0.05$. B: Rat hepatocytes were treated with SB216763 or media containing DMSO (0.001%) for 30 min and then stimulated with 200 U of IL-1β for the indicated time. Cell lysates were extracted and analyzed for p-GSK3α/β (S21/9) and total GSK3 α/β levels. The density of the band was determined using ImageJ software as described above. The blots and numbers below them are representative of three independent experiments.

suggesting that inhibition of GSK3 activity decreases c-Jun proteosomal degradation. Stimulation with IL-1β caused a decrease in c-Jun phosphorylation at T239, but the magnitude was small when compared with the decrease caused by the GSK3 inhibitor SB216763 (Fig. 4A). This decrease in c-Jun phosphorylation at T239 by IL-1β is also consistent with the observed increase in GSK3α/β S9/21 phosphorylation and Akt phosphorylation upon stimulation with IL-1β (Fig. 3B). When we blocked the Akt-mediated inhibitory phosphorylation of GSK3 with LY294002, we detected greatly increased phosphorylation of c-Jun at the T239 site in hepatocytes both with and without IL-1β stimulation (Fig. 4B).

We also measured total c-Jun by Western blot to assess whether T239 phosphorylation was associated with changes in total cellular c-Jun levels. Inhibition of GSK3 by SB216763 significantly increased total c-Jun in hepatocytes stimulated with IL-1β at 180 min (Fig. 4A). Further, SB216763 treatment increased the phosphorylation of c-Jun at the activating S63 site (Fig. 4A). This increase in phosphorylation at S63 paralleled that of total c-Jun with no change in the ratio of S63p-c-Jun/total c-Jun. These data demonstrate that inhibition of GSK3 activity decreases c-Jun phosphorylation at the inhibitory T239 site, increases c-Jun phosphorylation at the activating S63 site, and increases total hepatocyte c-Jun levels. Lithium chloride, an activator of the Akt pathway and inhibitor of GSK3, was able to mimic the effect the GSK3 inhibitor SB216763 on T239 c-Jun phosphorylation (Fig. 4C).

To confirm the role of c-Jun in regulating iNOS expression in response to IL-1β, we transfected a dominant negative c-Jun plasmid (pcDNA3.1-TAM67 DN c-Jun) into hepatocytes together with pGL3.1-RiNOS-Luc reporter plasmid and stimulated the cells with IL-1β. We found that the TAM67 DN c-Jun significantly increased the *iNOS* promoter activity in response to IL-1β (Fig.4D) and reversed the inhibitory effect of the GSK3 inhibitor SB216763 on iNOS expression (Fig. 4E). Taken together, these results demonstrate that GSK3 regulates iNOS expression in hepatocytes and does so, at least in part, by regulating c-Jun degradation and total c-Jun levels.

DISCUSSION

GSK3 regulates iNOS induction in TNF-stimulated hepatocytes and in macrophages [Götschel et al., 2008; Tsai et al., 2009]. In these systems, GSK3 was shown to mediate its effect through Jak2/STAT-1 and NF-κB to regulate iNOS/NO [Tsai et al., 2009; Kai et al., 2010; Tsai et al., 2011]. GSK3 regulates a number of other pathways that could also be important in iNOS expression including CREB, MEKK1 and β-catenin [Frame and Cohen, 2001; Jope and Bijur, 2002; Kim et al., 2003; Götschel et al., 2008]. We have previously demonstrated that some of the signaling pathways regulating iNOS activation in hepatocytes are different compared to other cells that express iNOS and so we tested the role of GSK3 in IL-1β-mediated hepatocyte iNOS expression.

In the present study we demonstrate that the effect of GSK3 on iNOS in hepatocytes involves, at least in part, GSK3-mediated effects on c-Jun. We show that GSK3 phosphorylates c-Jun at T239, which facilitates its degradation and that inhibiting GSK3 increases total c-Jun associated with decreased iNOS expression. In our experiments, we stimulated hepatocytes with IL-1β which is the best single cytokine stimulus for inducing iNOS [Geller et al., 1995]. We demonstrate that GSK3 over-expression increased iNOS whereas GSK3 inhibitors attenuated the IL-1β mediated induction of iNOS expression. In this study, we found that the phosphorylation of GSK3 at the inhibitory S21/S9 site increased upon IL-1β treatment, but there was no discernible decrease in phosphorylation at the activating Y279/Y219 sites (Figs. 2A and B) similar to that observed

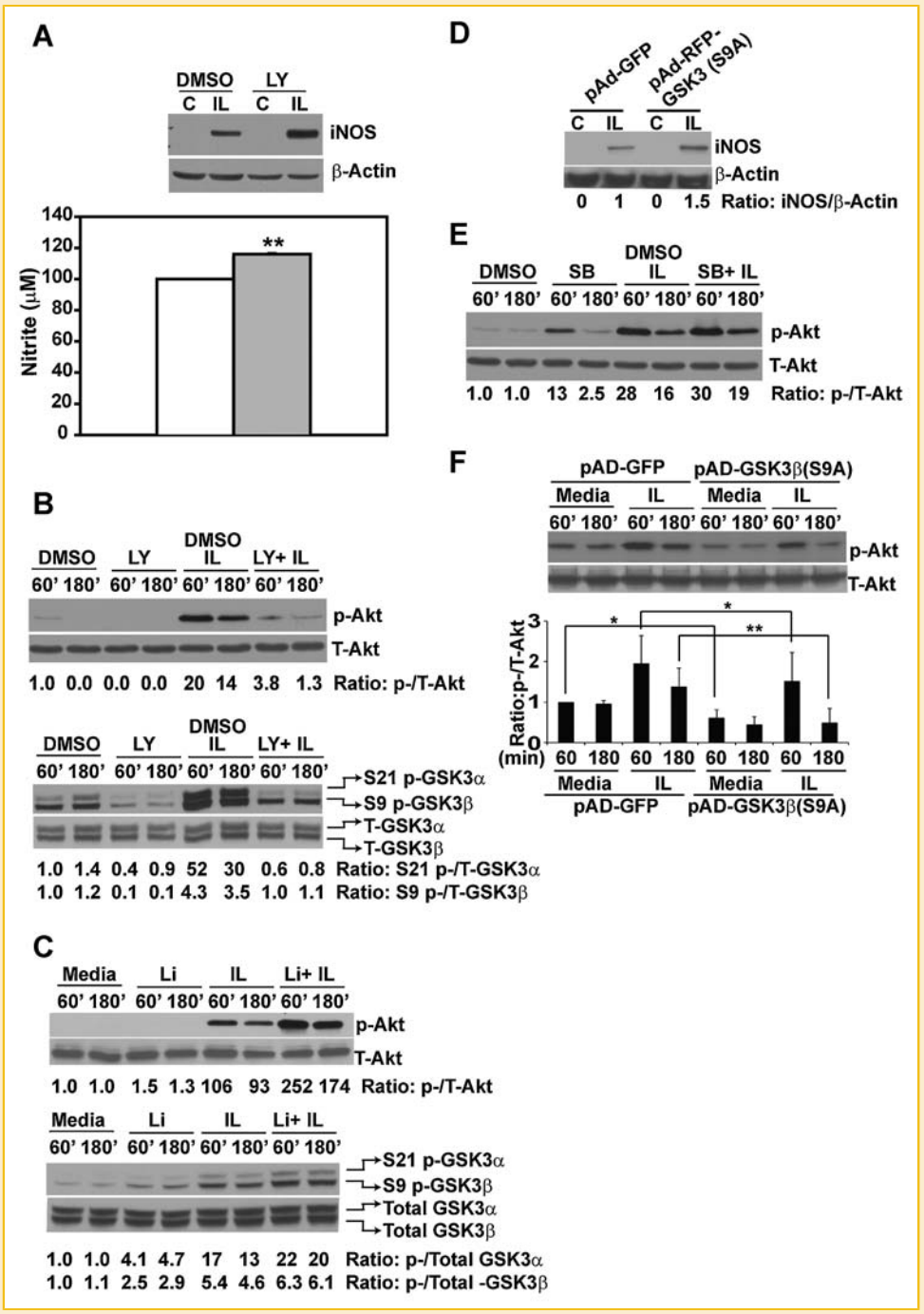

Fig. 3. PI3K inhibitor increases nitrite and iNOS expression. A: Rat primary hepatocytes were pre-incubated for 30 min with PI3K inhibitor LY294002 (10 μM; grey bar) or media containing 0.001% DMSO (open bar) and then stimulated with 200 U of IL-1β. After 24 h, the supernatants were analyzed for nitrite by the Griess method and the cell lysates were analyzed for iNOS protein levels by Western blot. The bars in the graph represent the mean ± standard error of three independent measurements. B: Hepatocytes were pre-incubated with LY294002 (10 μM) or media containing DMSO (0.001%) for 30 min, and then stimulated with 200 U of IL-1β for 60 and 180 min. The cells were lysed at indicated time-points and analyzed by Western blot for phosphorylated and total Akt levels (upper panel) and GSK3α/β (S 21/9) and total GSK3α/β levels (lower panel). C: Hepatocytes were pretreated with 20 mM LiCl for 30 min and then stimulated with 200 U of IL-1β. Proteins were then collected at the indicated time points and analyzed by Western blot for p-Akt (S473), total Akt, p-GSK3, and total GSK3 levels. The band intensities quantified using ImageJ software. D: Hepatocytes were infected with pAd-GFP (control) or pAd-RFP-GSK3β (S9A) with MOI of 1:20. After 6 h of culture, the media containing adenovirus was replaced with fresh media and the cells were allowed to recover for 24 h. The cells were then stimulated with 200 U of IL-1β and after 24 h cell extracts were prepared for Western blot analysis of iNOS and β-actin levels. E: Hepatocytes were pretreated with 0.001% DMSO or 25 μM SB216763 for 30 min and then stimulated with 200 U of IL-1β. Proteins were then collected at the indicated time points and analyzed by western blot for p-Akt (S473) and total Akt levels. F: Hepatocytes were infected with pAd-GFP (control) or pAd-RFP-GSK3β (S9A) as above and allowed to recover for 48 h. The cells were then treated with 200 U of IL-1β for 60 and 180 min, after which cell extracts were prepared for Western blot analysis of p-Akt and total Akt levels. The graph shows the ratio of p-Akt/total Akt. The blots shown in are representatives of three independents experiments. In A and F, the graph represents the mean ± standard error of three independent measurements. The asterisk in A represents $P < 0.0001$ versus hepatocytes stimulated with IL-1β alone. In E, the asterisk represents $P < 0.05$.

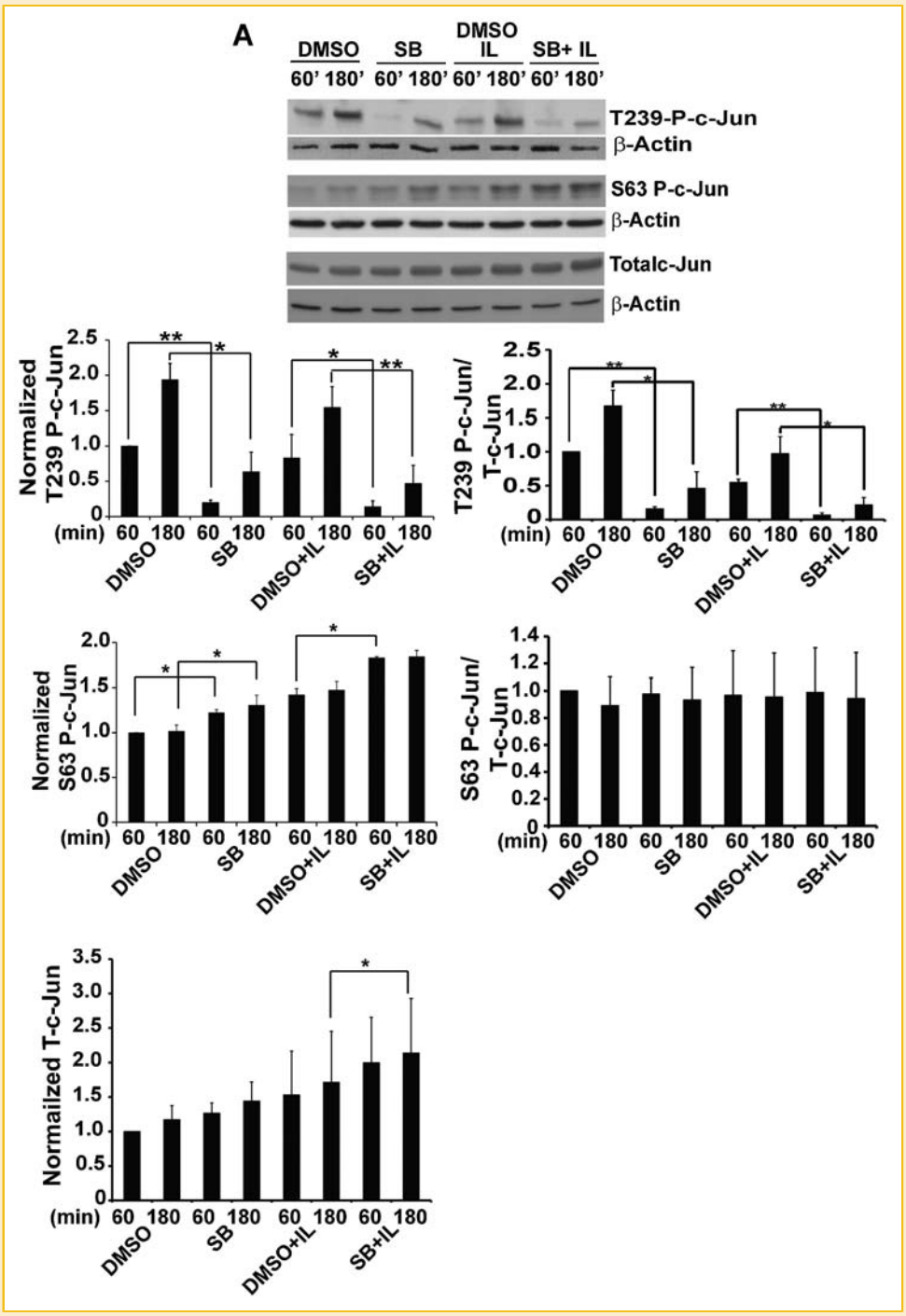

Fig. 4. c-Jun is regulated by GSK3 in hepatocytes stimulated with IL-1β to produce iNOS. A: Hepatocytes were pre-incubated with GSK3 inhibitor SB216763 (25 μM) or in DMSO (0.001%) for 30 min and then stimulated with 200 U of IL-1β. Cell lysates were prepared after 60 and 180 min of cytokine stimulation and Western blot analysis on the protein extracts were performed with antibodies for phosphorylated c-Jun at T239, c-Jun phosphorylated at S63, total c-Jun, and β-actin levels. The band intensities were quantified using ImageJ software as described above. The levels of phosphorylated c-Jun and total c-Jun were normalized against β-actin levels. Error bars represent standard deviation. B: Hepatocytes were pre-incubated in PI3K inhibitor LY294002 (10 μM) or DMSO (0.001%) for 30 min and then stimulated with IL-β for 60 and 180 min. The cells were lysed and the lysates were then subjected to Western blot analysis for phosphorylated c-Jun (T239), total c-Jun, and β-actin levels and quantitated as above. C: Hepatocytes were preincubated with 20 mM LiCl or media alone for 30 min and then stimulated with IL-1β. The cells were harvested at 60 and 180 min post-stimulation and analyzed for T239 p-c-Jun, total-c-Jun, and β-actin levels by Western blot as described above. D: Rat primary hepatocytes were co-transfected with pGL3-Basic or pGL3-RiNOS-Luc vector along with control pGL4.74 *Renilla* luciferase vector in the presence of pcDNA4.0 (control) or pcDNA3.1-TAM67 DN c-Jun plasmid using LipofectAMINE reagent. The cells were allowed to recover and one set was treated with 200 U of IL-1β. After a further 24 h, the cells were lysed and supernatants assayed for firefly and *Renilla* luciferase activities. The bars in the graph represent the mean ± standard error of three independent measurements. E: Rat primary hepatocytes were transfected with either 2 mg of pcDNA4.0 (control) or pcDNA3.1-TAM67 DN c-Jun plasmid using LipofectAMINE reagent. The cells were allowed to recover and pretreated with SB216763 or media containing 0.001% DMSO for 30 min. The cells were then treated with 200 U of IL-1β. After a further 24 h, the cells were lysed and iNOS and β-actin protein levels estimated by Western blotting. The blots shown are a representative of three independent experiments. In A and D, single and double asterisk represents $P < 0.05$ and $P < 0.005$, respectively.

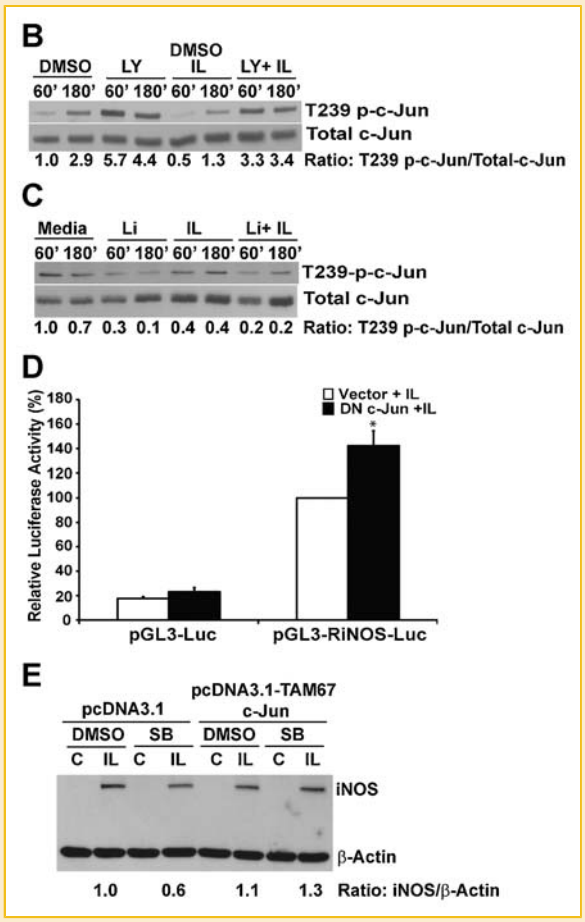

Fig. 4. (*Continued*)

with other cell types [Martin et al., 2005; Rodionova et al., 2007; Rådinger et al., 2009]. While the IL-1β-mediated phosphorylation of Akt and GSK3 occur during iNOS induction (Fig.2), our previous works demonstrate that Akt negatively regulates iNOS expression [Zhang et al., 2011; Harbrecht et al., 2012]. These divergent observations coupled with the results using LY294002 (Fig.3) suggest that IL-1β not only induces iNOS expression but also upregulates autoregulatory pathways such as Akt signaling, that could limit NO production in hepatocytes.

We demonstrate that in hepatocytes, GSK3 down-regulates c-Jun by phosphorylation at T239 that leads to polyubiquitination and proteosomal degradation. We found that IL-1β by itself caused a small decrease in c-Jun T239 phosphorylation in hepatocytes and this could be due to the observed increase in GSK3 phosphorylation at S9/21 upon IL-1β treatment. Additionally, the decrease in T239 phosphorylation could also be due to activation of phosphatases that are independent of GSK3 activity similar to that observed in RAW macrophages treated with LPS or anisomycin [Morton et al., 2003]. In primary hepatocytes too, we found that GSK3 inhibition caused a sustained and pronounced decrease in c-Jun T239 phosphorylation with a concomitant increase in total c-Jun and activating S63 c-Jun phosphorylation similar to the effect seen in a pancreatic cell line [Marchand et al., 2012]. The observed increase in total-c-Jun level upon treatment with GSK3 inhibitors SB216763 and LiCl (Figs. 4B and C) is consistent with the dephosphorylation of c-Jun at T239 and stabilization of the protein as reported in previous studies [Wei et al., 2005; Tullai et al., 2011]. We further showed that a dominant-negative c-Jun was able to increase *iNOS* promoter activity, iNOS expression and block the effects of GSK3 inhibition, confirming the role of c-Jun in mediating the effects of GSK3 on iNOS expression. Whether the effects on iNOS expression by c-Jun are due to its participation as a suppressive factor at AP-1 sites in the *iNOS* promoter or as an inducer of other genes/transcription factors that act as inhibitory intermediaries in iNOS expression will require further study. The current results with GSK3 are consistent with our previous findings on the importance of c-Jun in iNOS expression in hepatocytes [Zhang et al., 2004a] and validate our hypothesis that the Akt-mediated inhibition of GSK3 is an important mechanism in hepatocyte iNOS regulation.

In conclusion, we demonstrate that GSK3, a downstream target of Akt, mediates the effect of IL-1β in inducing hepatocyte iNOS expression by regulating c-Jun. These signaling pathways may play an integral role in vivo in modulating hepatocyte's response to mediators of inflammation during sepsis and shock.

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