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Glycogen Synthase Kinase-3β Indirectly Facilitates Interferon-γ-Induced Nuclear Factor-κB Activation and Nitric Oxide Biosynthesis

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ABSTRACT

Either glycogen synthase kinase (GSK)-3β or nuclear factor (NF)-κB regulates interferon (IFN)-γ-induced nitric oxide (NO) biosynthesis; however, the inter-regulation between GSK-3β and NF-κB is unknown. We have previously shown that IFN-γ-activated GSK-3β negatively regulates Src homology-2 domain-containing phosphatase (SHP) 2 to facilitate Janus kinase (Jak) 2-signal transducer and activator of transcription (STAT) 1 activation. Because Jaks-IFN-inducible dsRNA-activated serine–threonine protein kinase (PKR) axis signaling is essential for IFN-γ-activation of NF-κB, in this study we investigate the potential mechanism for GSK-3β-facilitated NF-κB activation in IFN-γ-stimulated RAW264.7 murine macrophages. Pharmacological inhibitors of GSK-3β or NF-κB signaling, such as the inhibitor of κB (IκB) kinase β (IKKβ) and IκBα, inhibited IFN-γ-induced inducible NO synthase (iNOS) and thus NO biosynthesis. Inhibiting GSK-3β decreased IFN-γ-induced NF-κB phosphorylation (Ser536) and activation. The upstream regulators for GSK-3β activation, including okadaic acid-sensitive protein phosphatase and proline-rich tyrosine kinase 2, were also important for IFN-γ-induced IκBα phosphorylation (Ser32) and degradation. Under IFN-γ stimulation, Jak2–PKR axis signaling induced IκBα inactivation as well as iNOS/NO biosynthesis. It is notable that inhibiting GSK-3β caused SHP2-mediated dephosphorylation of PKR (Thr446), IKKβ (Ser180), and NF-κB (Ser536). Taken together, we provide the first evidence to demonstrate that GSK-3β indirectly facilitates IFN-γ-induced NF-κB activation by inhibiting SHP2, in turn activating the PKR–IKKβ–IκBα axis signaling pathway. J. Cell. Biochem. 111: 1522–1530, 2010.

KEY WORDS: IFN-γ; GSK-3β; SHP2; NF-κB; iNOS; NO; MACROPHAGE

Glycogen synthase kinase-3β (GSK-3β), originally identified as a potent regulator of glycogenesis, also regulates cellular inflammation induced by Toll-like receptor (TLR) ligands, cytokines, and ischemia/reperfusion, and is involved in the pathogenesis of diabetes, cancer, and neurological disorders [Beurel et al., 2010]. In searching for a novel target for anti-inflammation therapy, inhibition of GSK-3β has been identified as a strategy to protect hosts from endotoxemia-induced sepsis [Dugo et al., 2005; Wang et al., 2009], experimental colitis [Whittle et al., 2006], type II collagen-induced arthritis [Cuzzocrea et al., 2006], ovalbumin-induced asthma [Bao et al., 2007], experimental autoimmune encephalomyelitis [De Sarno et al., 2008], and Gram-negative or Gram-positive bacterial infection [Cheng et al., 2009; Zhang et al., 2009]. As demonstrated in GSK-3β knockout mice, the regulation of transcription factor nuclear factor (NF)-κB activation by GSK-3β is shown [Hoeflich et al., 2000]. GSK-3β regulates the signaling pathways that pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β [Takada et al., 2004] and endotoxin

Abbreviations used: IFN-γ, interferon-γ; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; GSK-3, glycogen synthase kinase-3; BIO, 6-bromo-indirubin-3′-oxime.

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lipopolysaccharide (LPS) [Takada et al., 2004; Martin et al., 2005; Huang et al., 2009; Wang et al., 2009] use to activate NF-κB, which then promotes inflammatory activation. Mitogen-activated protein kinase (MAPK) kinase kinase (MEKK) 1, an upstream kinase of MAPK which causes inhibitor of κB (IκB) kinase (IKK) complex and NF-κB activation, is directly activated by GSK-3β [Kim et al., 2003; Takada et al., 2004]; however, the molecular mechanisms for GSK-3β-regulated NF-κB signaling are undocumented because there is no further evidence to explain the direct or indirect role of GSK-3β.

Inducible nitric oxide synthase (iNOS)-derived NO, a short-lived free radical, is generally produced in inflammatory stimulus-activated monocytes/macrophages [Weinberg et al., 1995]. The pro-inflammatory cytokine interferon (IFN)-γ causes excessive inflammatory responses, including production of cytokines and chemokines, as well as iNOS/NO biosynthesis [Schroder et al., 2004], and decreases the activity and expression of anti-inflammatory IL-10 [Herrero et al., 2003; Schroder et al., 2004; Lin et al., 2008]. It has been reported that IFN-γ signaling positively regulates iNOS mRNA expression [Gao et al., 1997]. The actions of IFN-γ have been speculated to cause a completed activation of transcription factors of iNOS, including NF-κB [Kleinert et al., 1998], signal transducer and activator of transcription 1 (STAT1) [Kleinert et al., 1998; Blanchette et al., 2003], and interferon regulatory factor (IRF)-1 [Kamijo et al., 1994]. For IRF-1 expression, IFN-γ activates Janus kinases (Jaks)–STAT1, which then regulates IFN-γ-inducible gene expression [Schroder et al., 2004]. It is now known that the suppressor of cytokine signaling proteins [Krebs and Hilton, 2000] and dual-phosphatase Src homology-2 domain-containing phosphatase 2 (SHP2) [You et al., 1999] deactivates IFN-γ-activated Jaks–STAT1 signaling. However, the mechanisms for feedback regulation are not well understood.

It is notable that the synergy of IFN-γ with TLR-mediated NF-κB activation and inflammatory responses, including TNF-α and IL-6 production and iNOS/NO biosynthesis, is regulated by the inhibition of IL-10 in a GSK-3β-regulated manner [Hu et al., 2006; Lin et al., 2008; Beurel and Jope, 2009]. GSK-3β is in turn negatively regulated by phosphoinositide 3-kinase/Akt-mediated serine phosphorylation (Ser9), which is inhibited by okadaic acid-sensitive serine/threonine protein phosphatases, such as PP1 and PP2A [Lin et al., 2007]. In addition, proline-rich tyrosine kinase 2 (Pyk2) positively regulates GSK-3β through phosphorylation of tyrosine residue 216 [Hartigan et al., 2001; Lin et al., 2008]. We previously showed that IFN-γ activates GSK-3β via the PP2A- and Pyk2-mediated pathways [Tsai et al., 2009]. Furthermore, IFN-γ activates GSK-3β, which then directly regulates STAT3 [Beurel and Jope, 2008] and indirectly regulates Jak2–STAT1 by inhibiting SHP2 [Tsai et al., 2009]. The molecular mechanisms for IFN-γ-activated NF-κB are still unclear [Gough et al., 2008] because IFN-γ is usually not an efficient activator of NF-κB in many types of cell. Notably, IFN-inducible dsRNA-activated serine–threonine protein kinase (PKR), which associates with the IKK complex, is able to induce NF-κB activation [Zamanian-Daryoush et al., 2000; Deb et al., 2001; Gough et al., 2008]. Concerning the mechanism for Jaks-regulated PKR in NF-κB activation [Deb et al., 2001; Gough et al., 2008; Pyo et al., 2008] and for GSK-3β-inhibited SHP2 in facilitating IFN-γ activation of Jaks [Tsai et al., 2009], we hypothesize that there is an inter-regulated mechanism for GSK-3β-regulated NF-κB signaling through SHP2 inactivation and PKR–IKKβ activation. In the present study, we provide the first evidence to show that activated GSK-3β indirectly facilitates IFN-γ-induced NF-κB activation, which then regulates the biosynthesis of iNOS/NO. The involvement of SHP2 and its regulatory mechanisms on IFN-γ-activated PKR–IKKβ–IκBα–NF-κB, which are controlled by GSK-3β, are also further investigated.

MATERIALS AND METHODS

CELL CULTURE

RAW264.7 murine macrophages were obtained from C. C. Huang, MD (Department of Pediatrics, National Cheng Kung University, Tainan, Taiwan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), 50 U/ml of penicillin, and 50 μg/ml of streptomycin in a humidified atmosphere with 5% CO_2 and 95% air. Human peripheral blood mononuclear cells (PBMCs) were centrifuged in tubes containing Ficoll-Pague (Amersham Biosciences, Piscataway, NJ). Isolated PBMCs were grown in RPMI (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin before the experiments.

ANTIBODIES AND REAGENTS

Rabbit polyclonal antibodies specific for iNOS and mouse monoclonal antibodies against β-actin were purchased from Chemicon International, Inc. (Temecula, CA). Rabbit anti-mouse IKKβ, IKKβ (Ser180), IκBα, IκBα (Ser32), NF-κB, and NF-κB (Ser536), PKR, PKR (Thr446), and SHP2 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were obtained from Chemicon International, Inc. Recombinant murine cytokine IFN-γ was obtained from PeproTech (Rocky Hill, NJ). The GSK-3 inhibitors 6-bromoindirubin-3′-oxime (BIO) and AR-A014418 (AR), the NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC) and caffeic acid phenethyl ester (CAPE), the IKKβ inhibitor BMS-345541 (BMS), the IκBα phosphorylation inhibitor Bay 11-7082 (BAY), the Jak2 inhibitor AG490, the PKR inhibitor 2-aminopurine (2-AP), the PP2A inhibitor okadaic acid (OA), the Pyk2 inhibitor Tyrphostin A9 (A9), the SHP2 inhibitor NSC-87877 (NSC), and other chemical reagents were obtained from Sigma–Aldrich Co. (St Louis, MO) and dissolved in DMSO prior to dilution in PBS and used in experiments. All drug treatments in cells were assessed for their cytotoxic effects using cytotoxicity assays before the experiments were conducted. Non-cytotoxic dosages were used in this study.

CYTOTOXICITY ASSAY

To evaluate cell damage, lactate dehydrogenase (LDH) activity was assayed using a colorimetric assay (Cytotoxicity Detection Kit; Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. Aliquots of culture media were transferred to 96-well microplates. A microplate reader (Spectra MAX 340PC; Molecular Devices Corporation, Sunnyvale, CA) was used to measure the absorbance at 620 nm with a reference wavelength of 450 nm and

data were analyzed with Softmax Pro software (Molecular Devices Corporation).

WESTERN BLOT ANALYSIS

Harvested cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% NaN_3, and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany). Following one freeze–thaw cycle, cell lysates were centrifuged at 10,000*g* at 4°C for 20 min. Lysates were boiled in a sample buffer for 5 min. The proteins were then subjected to SDS–PAGE and transferred to PVDF membranes (Millipore, Billerica, MA) using a semi-dry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with a 1:1,000 dilution of primary antibodies, including those against phospho-IKKβ (Ser180), phospho-IκBα (Ser32), phospho-NF-κB (Ser536), IKKβ, IκBα, NF-κB, iNOS, and β-actin, at 4°C overnight. The membranes were then washed with 0.05% PBS–Tween 20 and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. After washing, the membranes were soaked in ECL solution (PerkinElmer Life Sciences, Inc., Boston, MA) for 1 min and then exposed to film (BioMax; Eastman Kodak, Rochester, NY), and the relative signal intensity was quantified using ImageJ software (version 1.41o) from W. Rasband (National Institutes of Health, Bethesda, MD) (http://rsb.info.nih.gov/ij/).

DETECTION OF NO PRODUCTION

Production of NO was assessed as the accumulation of nitrite (NO_2^-) in the medium using a colorimetric reaction with the Griess reagent [Lin et al., 2008]. Briefly, culture supernatants were mixed with an equal (1:1) volume of Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4). The absorbance was measured at 540 nm using a 96-well microplate reader (Spectra MAX 340PC), and the data were analyzed (Softmax Pro software). $NaNO_2$ was dissolved in double-distilled H_2O and used for the standard control (from 1 to 50 μM).

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Cells were incubated in 300 μl of buffer A (10 mM HEPES [pH 7.9], 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM sodium fluoride, and 1 mM sodium orthovanadate) on ice for 10 min and then gently shaken for 10 s. The pellet containing the crude nuclei was collected using centrifugation at 12,000*g* for 10 s, resuspended in 30 μl of buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM sodium chloride, 1.5 mM magnesium chloride, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM sodium fluoride, and 1 mM sodium orthovanadate) by vortexing for 15 s, and then incubated on ice for 20 min. After the cells had been centrifuged at 12,000*g* for 2 min, the supernatant containing the nuclear proteins was collected, quantified (BCA Protein Assay Reagent; Pierce), and stored in aliquots at −70°C. The EMSA used the following oligonucleotides as probes:

NF-κB (f): 5′-CAA ATG TGG GAT TTT CCC ATG AGT;
NF-κB (r): 5′-GAC TCA TGG GAA AAT CCC ACA TTT G.

The forward and reverse oligonucleotides (30 pmol) were placed in 23 μl of DNA polymerase buffer (Klenow 1X; Promega), heated at 94°C for 2 min, and then annealed at room temperature for 30 min. The annealed double-stranded oligonucleotides were end-labeled using a fill-in reaction with Klenow polymerase. One unit of Klenow and 40 μCi of [α-^{32}P] dCTP (PerkinElmer) were added to the annealed oligonucleotides, and then the mixture was incubated at 30°C for 15 min. The labeled oligonucleotides were purified using G-50 columns (Sephadex; PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). The DNA binding reaction was done at 4°C for 30 min in a mixture containing 3 μg of nuclear extract, 10 mM Tris-Cl [pH 7.5], 50 mM sodium chloride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM magnesium chloride, 4% glycerol, 0.05 μg of poly(dI-dC)-poly (dI-dC) (PerkinElmer), and 2×10^4 cpm of ^{32}P-labeled double-stranded oligonucleotides. Samples were analyzed on a 4% polyacrylamide gel (acrylamide/bis-acrylamide 29:1 in 0.5× Tris borate–EDTA buffer) at 10 V/cm for 2 h. The gel was dried and analyzed using quantitative autoradiography computer densitometry.

LUCIFERASE REPORTER ASSAY

For the analysis of the NF-κB promoter using a luciferase reporter assay, transient transfection was performed using GeneJammer transfection reagent (Stratagene). Briefly, cells were co-transfected with an NF-κB-promoter-driven luciferase reporter (0.2 μg) and 0.01 μg of *Renilla* luciferase-expressing plasmid (pRL-TK; Promega). Twenty hours after transfection, cells were treated with IFN-γ for 0.5 h with or without a 0.5-h BIO pre-treatment and were lysed and harvested for luciferase and *Renilla* measurement using the Dual-Glo luciferase assay system (Promega). For each lysate, the firefly luciferase activity was normalized to the *Renilla* luciferase activity to assess transfection efficiencies.

LENTIVIRAL-BASED RNAI TRANSFECTION

PKR and SHP2 knockdown in RAW264.7 was performed using lentiviral transduction to stably express short hairpin RNAs (shRNA). shRNA clones were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. The mouse library should be referred to as TRC-Mm 1.0. The construct that was most effective in RAW264.7 cells (TRCN0000274652, containing the shRNA target sequence 5′-GTGACCCACAGACATTGTATT-3′ for mouse PKR and TRCN0000029875, containing the shRNA target sequence 5′-CCTGATGAGTATGCGCTCAAA-3′ for mouse SHP2) and the control shLuc (shRNA target sequence TRCN0000072247 5′-GAATCGTCGTATGCAGTGAAA-3′ for luciferase) were used to generate recombinant lentiviral particles. The preparation protocol has been detailed in our previous study [Tsai et al., 2009].

STATISTICAL ANALYSIS

Values are expressed as means ± SD. Groups were compared using Student's two-tailed unpaired *t*-test or one-way ANOVA analysis

followed by the Dunnet post hoc test, as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

INHIBITING GSK-3β OR NF-κB DOWNREGULATED IFN-γ-INDUCED INOS/NO BIOSYNTHESIS

IFN-γ induces iNOS/NO biosynthesis through NF-κB [Kleinert et al., 1998; Schroder et al., 2004]. Our current results show that GSK-3β is essential for iNOS/NO biosynthesis in IFN-γ-stimulated macrophages [Tsai et al., 2009]. In this study, the inter-regulation of GSK-3β-activated NF-κB in IFN-γ-induced iNOS/NO biosynthesis was investigated. To investigate the effect of GSK-3β using the GSK-3 inhibitors 6-bromo-indirubin-3′-oxime (BIO) and AR-A014418 (AR), we used the Griess reaction, which confirmed that both BIO and AR significantly ($P < 0.05$) reduced IFN-γ-induced NO production (Fig. 1A) in RAW264.7 murine macrophages. Additionally, Western blot analysis showed that BIO (1.07 with IFN-γ only vs. 0.21 with IFN-γ + BIO) and AR (1.03 with IFN-γ only vs. 0.23 with IFN-γ + AR) inhibited IFN-γ-induced iNOS expression (Fig. 1A). These results indicate that GSK-3β, and possibly GSK-3α, are essential for enabling IFN-γ-induced iNOS/NO biosynthesis. To examine whether NF-κB activation is involved in the signal transduction pathway leading to iNOS/NO biosynthesis caused by IFN-γ, the NF-κB inhibitors PDTC and CAPE were used. Figure 1B shows that treatment of cells with PDTC (0.15 with IFN-γ only vs. 0.02 with IFN-γ + PDTC) or CAPE (0.37 with IFN-γ only vs. 0.00 with IFN-γ + CAPE) effectively inhibited iNOS/NO biosynthesis. We also evaluated the effects of the IKKβ and IκBα phosphorylation inhibitors BMS-345541 (BMS) and Bay 117082 (BAY), respectively. Comparable with PDTC and CAPE, BMS (0.62 with IFN-γ only vs. 0.13 with IFN-γ + BMS; Fig. 1C) and BAY (0.12 with IFN-γ only vs. 0.01 with IFN-γ + AY; Fig. 1D) had significant effects on IFN-γ-induced iNOS/NO biosynthesis, suggesting that NF-κB signaling was responsible for iNOS/NO biosynthesis. Treatment of cells with BIO (10 μM), AR (25 μM), PDTC (30 μM), CAPE (10 μM), BMS (3 μM), or BAY (10 μM) did not affect cell viability, which was assessed using the LDH assay (data not shown). These results confirm that the biosynthesis of iNOS/NO by IFN-γ

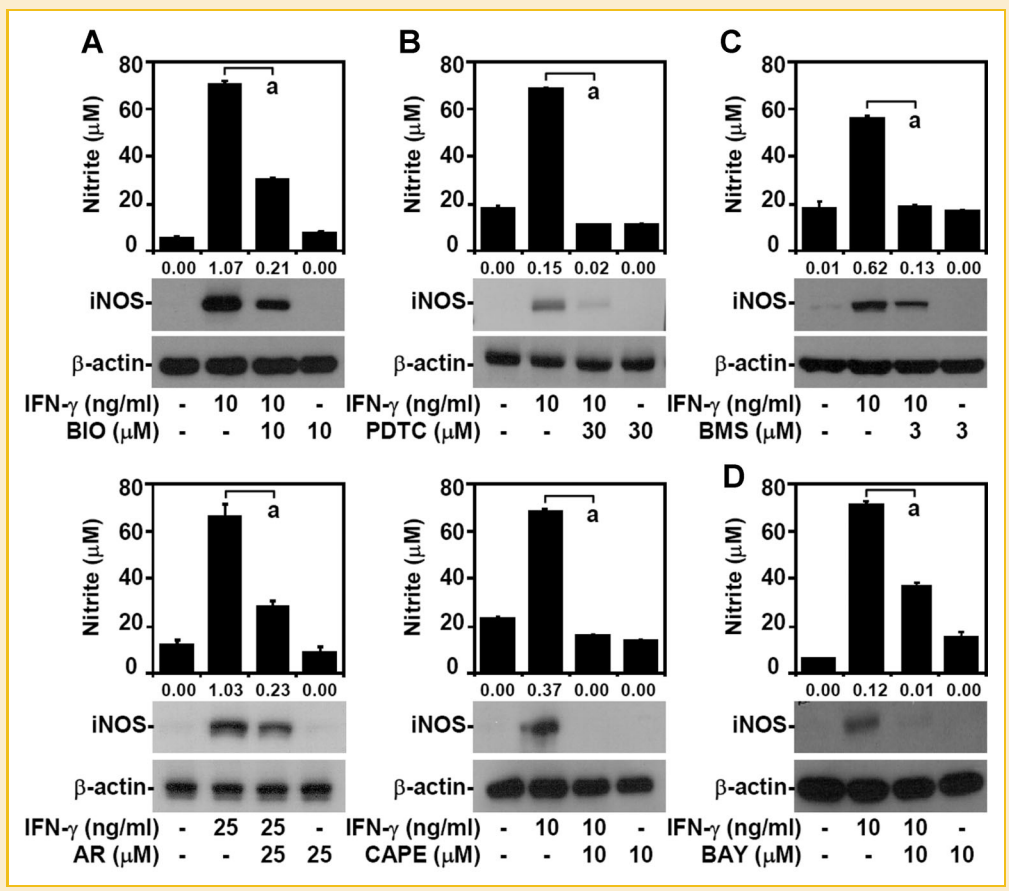

Fig. 1. Inhibiting GSK-3β and NF-κB signaling reduces IFN-γ-induced iNOS/NO biosynthesis. RAW264.7 cells (5×10^4 cells/well in 96-well culture plates or 1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for 24 h with or without (A) GSK-3β inhibitors 6-bromoindirubin-3′-oxime (BIO, 10 μM) and AR-A014418 (AR, 25 μM), (B) NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC, 30 μM) and caffeic acid phenethyl ester (CAPE, 10 μM), (C) IKKβ inhibitor BMS-345541 (BMS, 3 μM), and (D) IκBα inhibitor Bay 11-7082 (BAY, 10 μM) pre-treatment for 0.5 h. Griess reagent was used to detect generation of NO. Data, obtained from triplicate cultures, are means ± SD. $^{a}P < 0.05$. Western blot analysis was used to determine the expression of iNOS. β-Actin was used as the internal control. The ratio of iNOS to β-actin is shown. Data are representative of three individual experiments.

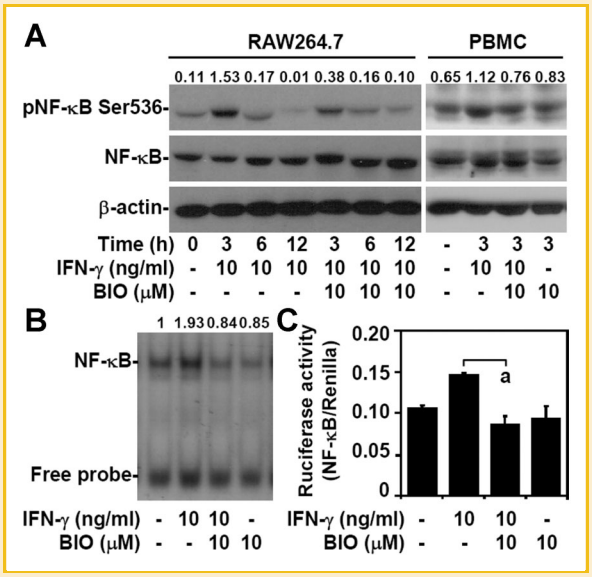

Fig. 2. Inhibiting GSK-3β decreases IFN-γ-induced NF-κB phosphorylation (Ser536) and activation. RAW264.7 cells (1×10^6 cells/well in 6-well culture plates or 5×10^4 cells/well in 96-well culture plates) or primary human PBMCs (1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for the indicated time periods with or without BIO (10 μM) pre-treatment for 0.5 h. A: Western blot analysis was used to determine the phosphorylation of NF-κB (pNF-κB Ser536). β-Actin was used as the internal control. The ratio of pNF-κB to NF-κB is shown. Data are representative of three individual experiments. B: Nuclear proteins were extracted from cells after they had been treated IFN-γ (10 ng/ml) for 0.25 h and co-treated with or without BIO (10 μM). An EMSA assay was used to analyze the activation of NF-κB. The fold increase is shown as compared to the untreated group. C: Luciferase reporter assay of NF-κB promoter transactivation for 0.25-h post-treatment was used to detect activation of NF-κB. Data, obtained from triplicate cultures, are means ± SD. $^aP < 0.05$.

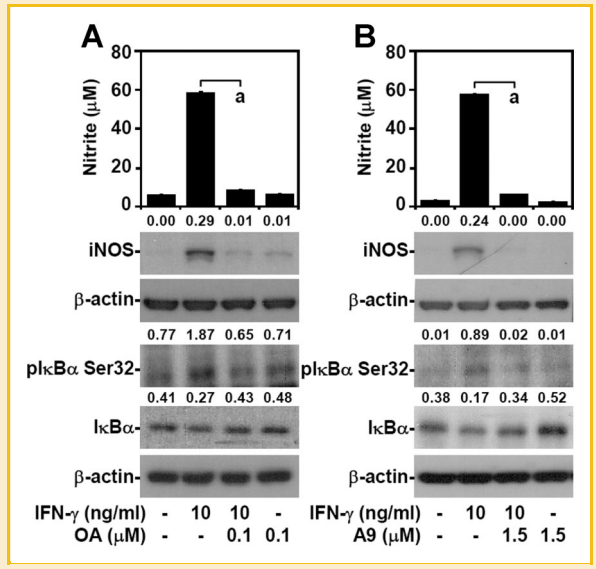

Fig. 3. Inhibiting okadaic acid-sensitive protein phosphatase and Pyk2 reduces IFN-γ-induced IκBα inactivation and iNOS/NO biosynthesis. RAW264.7 cells (5×10^4 cells/well in 96-well culture plates or 1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for 0.25 or 24 h with or without (A) okadaic acid (OA, 0.1 μM) and (B) Pyk2 inhibitor Tyrphostin A9 (A9, 1.5 μM) pre-treatment for 0.5 h. Griess reagent was used to detect generation of NO. Data, obtained from triplicate cultures, are means ± SD. $^aP < 0.05$. Western blot analysis was used to determine the expression of iNOS for 24 h post-treatment and phosphorylation of IκBα (pIκBα Ser32) and degradation of IκBα for 0.25 h post-treatment. β-Actin was used as the internal control. The ratios of iNOS to β-actin, pIκBα to IκBα, and IκBα to β-actin are shown, respectively. Data are representative of three individual experiments.

stimulation is highly dependent on GSK-3β, as well as on NF-κB signaling.

INHIBITING GSK-3β REDUCED IFN-γ ACTIVATION OF NF-κB

Because iNOS/NO biosynthesis is regulated by IFN-γ-activated GSK-3β and NF-κB, we hypothesized that there could be inter-regulation of these two signal molecules. Previous studies showed that GSK-3β regulates NF-κB activation caused by TNF-α [Takada et al., 2004] and LPS [Takada et al., 2004; Martin et al., 2005; Huang et al., 2009; Wang et al., 2009]. We further examined whether GSK-3β acts upstream of IFN-γ-induced NF-κB activation. We used Western blotting to demonstrate that inhibiting GSK-3β using BIO reduced IFN-γ-induced phosphorylation of NF-κB (Ser536) 3 h after treatment (1.53 with IFN-γ only vs. 0.38 with IFN-γ + BIO; Fig. 2A, left). Similar results can be demonstrated using primary human PBMCs (1.12 with IFN-γ only vs. 0.76 with IFN-γ + BIO; Fig. 2A, right). Furthermore, using EMSA (1.93 with IFN-γ only vs. 0.84 with IFN-γ + BIO; Fig. 2B) and luciferase-based NF-κB promoter assays (Fig. 2C), results showed that BIO significantly ($P < 0.05$) inhibited IFN-γ-activated NF-κB. These results, for the first time, showed that GSK-3β facilitates IFN-γ-induced NF-κB activation.

BLOCKAGE OF GSK-3β SIGNALING REDUCED IFN-γ ACTIVATION OF NF-κB

To further confirm the role of GSK-3β signaling, the upstream regulators of IFN-γ-activated GSK-3β, including okadaic acid (OA)-sensitive protein phosphatase and tyrosine kinase Pyk2 [Tsai et al., 2009], were investigated for their effects on IFN-γ-induced NF-κB activation. Western blotting showed that the protein phosphatase inhibitor OA (Fig. 3A) and the Pyk2 inhibitor Tyrphostin A9 (A9) (Fig. 3B) suppressed IFN-γ-induced IκBα phosphorylation (Ser32) (1.87 or 0.89 with IFN-γ only, 0.65 with IFN-γ + OA, and 0.02 with IFN-γ + A9) and degradation (0.27 or 0.17 with IFN-γ only, 0.43 with IFN-γ + OA, and 0.34 with IFN-γ + A9). In addition, IFN-γ-induced iNOS/NO biosynthesis, as demonstrated by Western blotting (0.29 or 0.24 with IFN-γ only, 0.01 with IFN-γ + OA, and 0.00 with IFN-γ + A9) and the Griess reaction, respectively, was also inhibited by treatment of OA and A9. These results indicate that GSK-3β signaling is critical for facilitating IFN-γ-induced NF-κB activation.

INHIBITING GSK-3β REDUCED IFN-γ ACTIVATION OF NF-κB THROUGH SHP2-MEDIATED INACTIVATION OF PKR–IKKβ SIGNALING

The mechanism for IFN-γ activation of NF-κB is still unclear. Signaling of Jaks regulates PKR, and PKR causes IKKβ and NF-κB activation [Zamanian-Daryoush et al., 2000; Deb et al., 2001; Gough

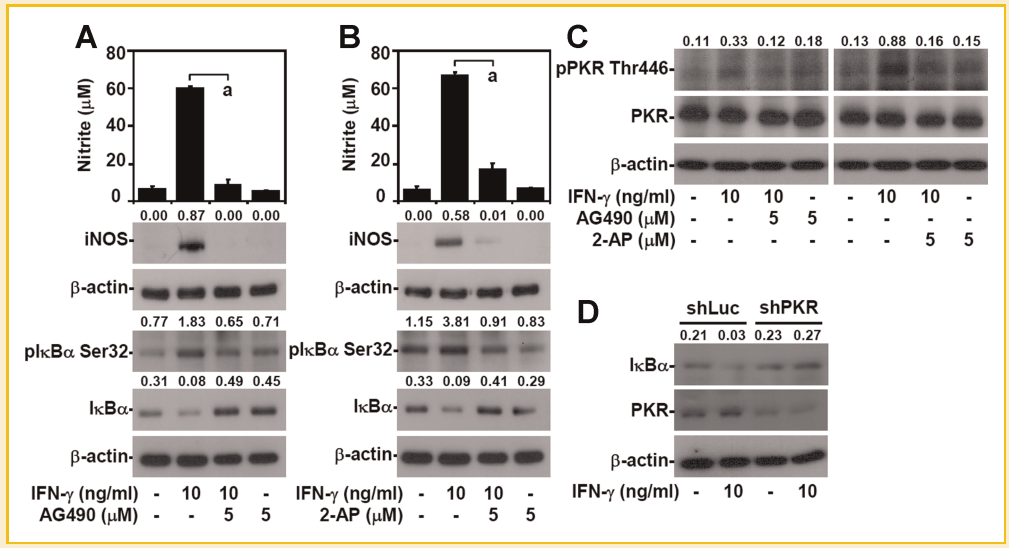

Fig. 4. Inhibiting Jak2 and PKR suppresses IFN-γ-induced IκBα inactivation and iNOS/NO biosynthesis. RAW264.7 cells (5×10^4 cells/well in 96-well culture plates or 1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for 0.25 or 24 h with or without (A,C) Jak2 inhibitor AG490 (5 μM) and (B,C) PKR inhibitor 2-aminopurine (2-AP, 5 μM) pre-treatment for 0.5 h. Griess reagent was used to detect generation of NO. Data, obtained from triplicate cultures, are means ± SD. $^aP < 0.05$. Western blot analysis was used to determine the expression of iNOS for 24-h post-treatment and phosphorylation of IκBα (pIκBα Ser32) and PKR (pPKR Thr446) and degradation of IκBα for 0.25 h post-treatment. β-Actin was used as the internal control. The ratios of iNOS to β-actin, pIκBα to IκBα, IκBα to β-actin, and pPKR to PKR are shown, respectively. Data are representative of three individual experiments. D: PKR knockdown (shPKR). RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for 0.25 h. Western blot analysis was used to determine the expression of IκBα and PKR. β-Actin was used as the internal control. The ratio of IκBα to β-actin is shown. shRNA for luciferase (shLuc) was used as a control.

et al., 2008; Pyo et al., 2008]. To further confirm the role of IFN-γ activation of Jak2 and PKR on the activation of IKKβ-NF-κB signaling, Western blotting showed that the Jak2 inhibitor AG490 (Fig. 4A) and the PKR inhibitor 2-aminopurine (2-AP) (Fig. 4B) suppressed IFN-γ-induced IκBα phosphorylation (Ser32) (1.83 or 3.81 with IFN-γ only, 0.65 with IFN-γ + AG490, and 0.91 with IFN-γ + 2-AP) and degradation (0.08 or 0.09 with IFN-γ only, 0.49 with IFN-γ + AG490, and 0.41 with IFN-γ + 2-AP). In addition, IFN-γ-induced iNOS/NO biosynthesis, as demonstrated by Western blotting (0.87 or 0.58 with IFN-γ only, 0.00 with IFN-γ + AG490, and 0.01 with IFN-γ + 2-AP) and by the Griess reaction, respectively, was also inhibited by treatment of AG490 and 2-AP. For confirming the regulation of PKR by IFN-γ-activated Jak2, Western blotting showed that AG490 blocked IFN-γ-induced phosphorylation of PKR (Thr446; 0.33 or 0.88 with IFN-γ only, 0.12 with IFN-γ + AG490, and 0.16 with IFN-γ + 2-AP; Fig. 4C). Silencing PKR in RAW264.7 cells using lentiviral-based short hairpin RNA (shRNA) re-confirmed the role of PKR by showing that a lack of PKR caused defects in IFN-γ-induced IκBα degradation (0.03 with IFN-γ + shLuc vs. 0.28 with IFN-γ + shPKR; Fig. 4D). These results confirm that Jak2 regulation of PKR is critical for facilitating IFN-γ-induced NF-κB activation.

Our previous studies demonstrated that GSK-3β inactivation of SHP2 is also critical for IFN-γ activation of Jak2 as well as for iNOS/NO biosynthesis [Tsai et al., 2009]. Using the SHP2 inhibitor NSC-87877 (NSC), we found that NSC reversed BIO-inhibited IFN-γ-induced phosphorylation of PKR (Thr446) (0.25 with IFN-γ only, 0.08 with IFN-γ + BIO, and 0.28 with IFN-γ + BIO + NSC), IKKβ (Ser180) (0.53 with IFN-γ only, 0.19 with IFN-γ + BIO, and 0.51 with IFN-γ + BIO + NSC), and NF-κB (Ser536) (1.34 with IFN-γ only, 0.69 with IFN-γ + BIO, and 1.31 with IFN-γ + BIO + NSC) 3 h after treatment (Fig. 5A). Silencing SHP2 in RAW264.7 cells re-confirmed the role of SHP2 by showing that a lack of SHP2 caused defects in BIO-inhibited IFN-γ-induced IκBα degradation (0.72 with IFN-γ + BIO + shLuc vs. 0.53 with IFN-γ + BIO + shSHP2; Fig. 5B). These results indicate that GSK-3β facilitates IFN-γ-activated PKR–IKKβ–NF-κB signaling through SHP2 inactivation (Fig. 5C).

DISCUSSION

We previously demonstrated that GSK-3β facilitates IFN-γ activation of Jak2–STAT1 and inflammation through SHP2 inactivation [Tsai et al., 2009]. We therefore hypothesize that GSK-3β plays a critical role in facilitating IFN-γ signaling and bioactivities. Activation of NF-κB is believed to play a necessary role in developing inflammation induced by IFN-γ [Schroder et al., 2004; Gough et al., 2008]. However, the mechanism of IFN-γ-activation of NF-κB until PKR regulation of IKKβ is involved is still unknown [Zamanian-Daryoush et al., 2000; Deb et al., 2001; Gough et al., 2008]. In the present work, we provide the first evidence to demonstrate that GSK-3β and possibly GSK-3α indirectly facilitate IFN-γ-activation of NF-κB in RAW264.7 murine macrophages (Fig. 2), which is critical for iNOS/NO biosynthesis (Fig. 1), by regulating PKR-mediated IKKβ–IκBα signaling (Fig. 4). Furthermore, we showed that the activation of SHP2 is inhibited by GSK-3β to facilitate Jak2-regulated PKR activation (Figs. 4 and 5). Based on these findings, we have created a schematic summary for GSK-3β-regulated NF-κB signaling and its effects on IFN-γ-induced iNOS/NO biosynthesis (Fig. 5C). However, the mechanisms of GSK-3β

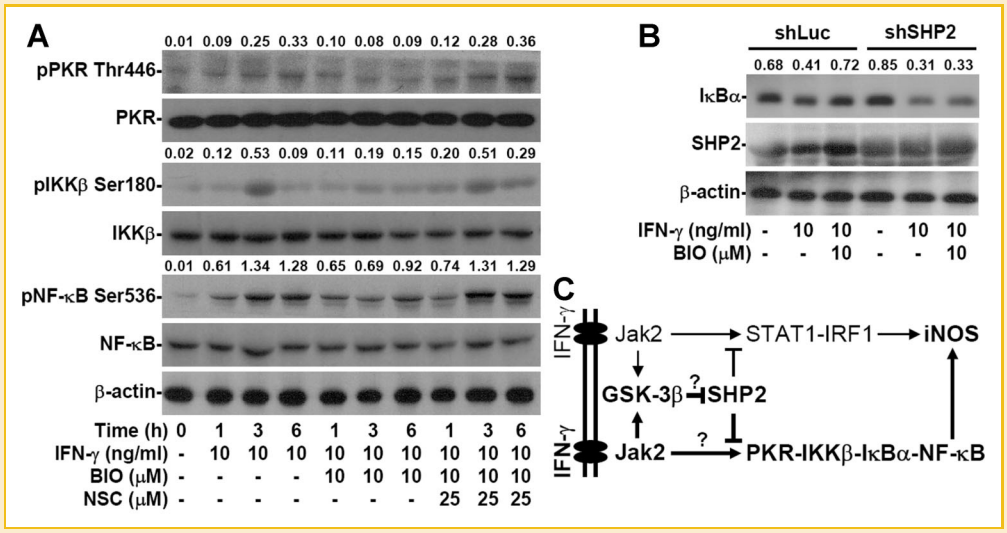

Fig. 5. Inhibiting SHP2 reverses GSK-3β inhibition and induces the blockade of IFN-γ-induced phosphorylation of PKR (Ser446), IKKβ (Ser180), and NF-κB (Ser536). A: RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for the indicated time periods with or without BIO (10 μM) and SHP2 inhibitor NSC-87877 (NSC, 25 μM) pre-treatment for 0.5 h. Western blot analysis was used to determine the phosphorylation of PKR (pPKR Thr446), IKKβ (pIKKβ Ser180), and NF-κB (pNF-κB Ser536). β-Actin was used as the internal control. The ratios of pPKR to PKR, pIKKβ to IKKβ, and pNF-κB to NF-κB are shown, respectively. Data are representative of three individual experiments. B: SHP2 knockdown (shSHP2). RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were treated with IFN-γ (10 ng/ml) for 0.25 h with or without BIO (10 μM) pre-treatment for 0.5 h. Western blot analysis was used to determine the expression of IκBα and SHP2. β-Actin was used as the internal control. The ratio of IκBα to β-actin is shown. shRNA for luciferase (shLuc) was used as a control. C: Schematic model for GSK-3β-facilitated IFN-γ-induced NF-κB activation and iNOS/NO biosynthesis. IFN-γ activates Jak2–STAT1 to facilitate iNOS/NO biosynthesis. In addition, transactivation of NF-κB is critical for iNOS expression while IFN-γ induces PKR-regulated IKKβ–IκBα–NF-κB signaling. Our results show that IFN-γ activates GSK-3β to facilitate SHP2 inactivation followed by induction of Jak2-regulated PKR activation. However, the mechanisms of GSK-3β inhibition of SHP2 and Jak2 activation of PKR are still unclear.

regulation of SHP2 and Jak2 regulation of PKR need further investigation.

Our present work demonstrates a novel role for GSK-3β in IFN-γ activation of NF-κB in an indirect manner. This is consistent with TNF-α activation of NF-κB indirectly by GSK-3β-regulated MEKK1–MAPK–IKKβ signaling [Takada et al., 2004]. However, it is still unclear whether GSK-3β has a role in post-modification of MEKK1, a GSK-3β-activated kinase [Kim et al., 2003], during IFN-γ stimulation. Interestingly, IFN-γ-activated Pyk2, an upstream kinase involved in GSK-3β activation, is required for activation of MEKK and MAPKs [Takaoka et al., 1999; Halfter et al., 2005]. It is speculated that IFN-γ may activate Pyk2–GSK-3β for MEKK1 activation; this needs further investigation. Because NF-κB signaling is important for IFN-γ-induced production of monokines [Horton et al., 2002], IFN-inducible protein 10 [Shultz et al., 2009], CXC ligand 10 (CXCL10), CXCL9 [Hiroi and Ohmori, 2003], and Fcγ-receptor-I expression [Karehed et al., 2007] and iNOS/NO biosynthesis [Saura et al., 1999], we therefore hypothesize that GSK-3β has a potential regulatory effect on these molecules based on our findings from this work; this needs further investigation.

IFN-γ enables the induction of iNOS expression as well as NO production for antiviral activities [Guidotti et al., 2000; Jin et al., 2004; Jarasch et al., 2005]. The potential action of IFN-γ has been suggested to be activation of several transcriptional factors that regulate iNOS gene expression, including STAT1 [Kleinert et al., 1998; Blanchette et al., 2003], IRF-1 [Kamijo et al., 1994], and NF-κB [Kleinert et al., 1998]. A complex regulatory cascade involving these transcription factors has been demonstrated [Ganster et al., 2001]. Based on results from our previous work [Tsai et al., 2009] and from this study (Figs 1 and 2), GSK-3β facilitates IFN-γ-induced iNOS/NO biosynthesis not only directly by enhancing transcriptional activity of STAT1 and IRF-1 but also indirectly by causing NF-κB activation. We hypothesize that there is a common role for GSK-3β in regulating IFN-γ-activated Jak2–STAT1–IRF-1 as well as Jak2–PKR–IKKβ–IκBα–NF-κB.

In J774 murine macrophages, IFN-γ effectively activates NF-κB and causes NF-κB nuclear translocation [Blanchette et al., 2003]. Consistent with this finding, we showed that IFN-γ induces phosphorylation of IKKβ, IκBα, and NF-κB, degradation of IκBα, and transactivation of NF-κB in RAW264.7 (Figs. 2–5). However, IFN-γ is generally not an activator of NF-κB at most dosages, in most cell types, and in most time periods. Nakashima et al. [1999] showed that the contribution of Jak2 signaling to IFN-γ-induced iNOS/NO biosynthesis is greater than that of NF-κB. Although IFN-γ activates NF-κB [Blanchette et al., 2003], activation of NF-κB is not required for IFN-γ-induced iNOS/NO biosynthesis. In addition, Hurgin et al. [2007] reported that the basal level of NF-κB, which is activated by constitutively expressed IL-1α but not by IFN-γ, is essential for IFN-γ-mediated antiviral and immunoregulatory activities. However, our preliminary data showed that IFN-γ was able to activate NF-κB independently of IL-1 and TNF-α (data not shown). Notably, IKKβ, but not NF-κB p65, is essential for IFN-γ-induced target gene expression [Sizemore et al., 2004]. Inconsistent with the previous findings, we and others [Karehed et al., 2007; Shultz et al., 2009] have shown that IKKβ, IκBα, and NF-κB are important for IFN-induced gene expression. The cell type and

treatment conditions may explain the inconsistent results and need further investigation.

While PKR is essential for IKKβ activation [Zamanian-Daryoush et al., 2000; Deb et al., 2001; Gough et al., 2008], we demonstrated that PKR, IKKβ, and IκBα are critical for IFN-γ-induced iNOS/NO biosynthesis (Figs. 1 and 4). Our results further showed that inhibiting PKR suppressed IFN-γ-induced IκBα degradation, suggesting a role for PKR in facilitating NF-κB signaling (Fig. 4). We also provided evidence to show that GSK-3β is able to facilitate Jak2-regulated PKR activation through SHP2 inactivation (Figs. 4 and Fig 55). Although the mechanism for GSK-3β inhibition of SHP2 remains unclear, we hypothesize that GSK-3β is a key factor in IFN-γ activation of NF-κB and iNOS/NO biosynthesis in an indirect manner by regulating SHP2–Jak2–PKR–IKKβ–IκBα signaling. The mechanisms of Jak2-regulated PKR activation need further investigation, as the involvement of Jaks signaling has been demonstrated in PKR activation of NF-κB [Deb et al., 2001; Gough et al., 2008; Pyo et al., 2008].

We previously [Lin et al., 2008; Tsai et al., 2009] demonstrated that OA-sensitive protein phosphatases (PPases), which cause dephosphorylation of GSK-3β (Ser9) and Pyk2, further leading to phosphorylation of GSK-3β (Tyr216), are critical for IFN-γ activation of GSK-3β. In the present work, both the OA-sensitive PPase inhibitor OA and the Pyk2 inhibitor A9 decreased IFN-γ-induced iNOS/NO biosynthesis, as well as IκBα phosphorylation and degradation (Fig. 3). These results are consistent with previous studies, in that IFN-γ-induced iNOS mRNA expression requires the activity of PP1 and PP2A [Dong et al., 1995], and that IFN-γ-activated Pyk2 mediates activation of Jak2–STAT1 and MAPK [Takaoka et al., 1999; Halfter et al., 2005]. We hypothesize that GSK-3β activation, which is regulated by IFN-γ-activated PPases and Pyk2, is also required for IFN-γ-activated NF-κB signaling.

An emerging role of GSK-3β has currently been widely demonstrated in pathogens [Cheng et al., 2009; Zhang et al., 2009], TLR ligands [Martin et al., 2005; Huang et al., 2009; Wang et al., 2009], physiological stress, and cytokine-induced inflammatory responses [Takada et al., 2004; Tsai et al., 2009]. Because NF-κB is critical for inflammatory activation and is potentially regulated by GSK-3β, inhibiting GSK-3β has become a novel strategy for anti-inflammation. In conclusion, our results demonstrate the mechanisms by which IFN-γ induces iNOS/NO biosynthesis; this involves GSK-3β inhibition of SHP2 followed by the activation of Jak2–PKR–IKKβ–IκBα–NF-κB signaling. Generation of NO is both beneficial and harmful to the host, depending on the pathogenesis and actual state of a disease [Kirkeboen and Strand, 1999]. For treatment of inflammation caused by IFN-γ, GSK-3β may be a potential therapeutic target. By contrast, viral infection-elicited IFN-γ-resistance may result from the blockade of iNOS/NO biosynthesis, which is critical for IFN-γ-induced anti-viral activities mediated by GSK-3β inactivation. This hypothesis needs further investigation.

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