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IFN-γ Synergizes With LPS to Induce Nitric Oxide Biosynthesis Through Glycogen Synthase Kinase-3-Inhibited IL-10

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ABSTRACT

Interferon-γ (IFN-γ) plays a crucial role in innate immunity and inflammation. It causes the synergistic effect on endotoxin lipopolysaccharide (LPS)-stimulated inducible nitric oxide synthase (iNOS)/NO biosynthesis; however, the mechanism remains unclear. In the present study, we investigated the effects of glycogen synthase kinase-3 (GSK-3)-mediated inhibition of anti-inflammatory interleukin-10 (IL-10). We found, in LPS-stimulated macrophages, that IFN-γ increased iNOS expression and NO production in a time-dependent manner. In addition, ELISA analysis showed the upregulation of tumor necrosis factor-α and regulated on activation, normal T expressed and secreted, and the downregulation of IL-10. RT-PCR further showed changes in the IL-10 mRNA level as well. Treating cells with recombinant IL-10 showed a decrease in IFN-γ/LPS-induced iNOS/NO biosynthesis, whereas anti-IL-10 neutralizing antibodies enhanced this effect, suggesting that IL-10 acts in an anti-inflammatory role. GSK-3-inhibitor treatment blocked IFN-γ/LPS-induced iNOS/NO biosynthesis but upregulated IL-10 production. Inhibiting GSK-3 using short-interference RNA showed similar results. Additionally, treating cells with anti-IL-10 neutralizing antibodies blocked these effects. We further showed that inhibiting GSK-3 increased phosphorylation of transcription factor cyclic AMP response element binding protein. Inhibiting protein tyrosine kinase Pyk2, an upstream regulator of GSK-3β, caused inhibition on IFN-γ/LPS-induced GSK-3β phosphorylation at tyrosine 216 and iNOS/NO biosynthesis. Taken together, these findings reveal the involvement of GSK-3-inhibited IL-10 on the induction of iNOS/NO biosynthesis by IFN-γ synergized with LPS. J. Cell. Biochem. 105: 746–755, 2008.

KEY WORDS: IFN-γ; LPS; iNOS; NO; GSK-3; IL-10; MACROPHAGE

Endotoxin lipopolysaccharide (LPS), the microbial component of Gram-negative bacteria, causes severe inflammatory responses through a mechanism involving a Toll-like receptor (TLR)-mediated pathway [Cohen, 2002; Triantafilou and Triantafilou, 2002]. Patients with endotoxemia-induced sepsis present life-threatening multiple organ failure and multiple organ dysfunction syndrome (MOF/MODS) [Balk, 2000; Sessler et al., 2004]. Overproduction of inflammatory cytokines, chemokines, free radicals, and cytotoxic factors contribute to the development of MOF/MODS [Marshall, 2001; Cohen, 2002; Netea et al., 2003]. Furthermore, the involvement of excessive nitric oxide (NO) has been also reported [Kirkeboen and Strand, 1999; Titheradge, 1999; ter Steege et al., 2000].

NO, a short-lived free radical, is synthesized from L-arginine by NO synthase (NOS) and is known as the intercellular messenger in a variety of biological functions such as anti-microbial infection, vascular homeostasis, neurotransmission, and anti-tumor activity [Mori and Gotoh, 2000; Muriel, 2000]. The roles of NO in septic

Abbreviations used: IFN-γ, interferon-γ; LPS, lipopolysaccharide; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; GSK-3, glycogen synthase kinase-3; IL-10, interleukin-10; TLR, Toll-like receptor; MOF/MODS, multiple organ failure and multiple organ dysfunction syndrome; TNF-α, tumor necrosis factor-α; RANTES, regulated on activation, normal T expressed and secreted; CREB, cyclic AMP response element binding protein; LiCl, lithium chloride; BIO, 6-bromo-indirubin-3'-oxime; siRNA, short interference RNA.

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pathogenesis are controversial [Kirkeboen and Strand, 1999; Titheradge, 1999]. Inducible NOS (iNOS)-derived NO is generally exhibited in LPS-activated monocytes/macrophages, vascular endothelial cells, and vascular smooth muscle cells [Salkowski et al., 1997; Kirkeboen and Strand, 1999; ter Steege et al., 2000]. NO and its derivates play important roles in inflammatory cytokine production [Zidek, 2001] and may induce cytotoxicity [Kirkeboen and Strand, 1999]. Since N(omega)-nitro-L-arginine methyl ester, an inhibitor of NO synthesis, has been used in clinical trials for the treatment of sepsis, the harmful effects of excessive NO are the promising targets of anti-sepsis [Avontuur et al., 1998; Hollenberg, 1998].

Interferon-γ (IFN-γ), a pro-inflammatory cytokine mainly produced by T helper type 1 cells and activated natural killer cells, is crucial for innate and adaptive immunity from its role of regulating macrophage activation and MHC expression [Bach et al., 1997]. IFN-γ synergizes with iNOS/NO biosynthesis in LPS-stimulated macrophages [Lorsbach et al., 1993; Gao et al., 1997; Held et al., 1999; Chan and Riches, 2001; Huang et al., 2004]. The synergistic mechanisms are currently under intensive investigation. It has been reported that IFN-γ signaling positively regulates iNOS mRNA expression [Lorsbach et al., 1993; Gao et al., 1997]. The actions of IFN-γ have been speculated to cause the completed activation of transcription factors of iNOS, including NF-κB [Held et al., 1999], mitogen-activated protein kinases [Chan and Riches, 2001], signal transducer and activator of transcription 1 [Huang et al., 2004], and interferon regulatory factor-1 [Koide et al., 2007], under different experimental conditions.

IFN-γ causes excessive production of tumor necrosis factor-α (TNF-α), IFN-inducible protein 10, monocyte chemoattractant protein-1, and regulated on activation, normal T expressed and secreted (RANTES) [Schroder et al., 2004], but decreases the activity and expression of anti-inflammatory interleukin (IL)-10 [Donnelly et al., 1995; Herrero et al., 2003; Schroder et al., 2004]. IL-10, a potent anti-inflammatory cytokine, enables inhibition of the synthesis of the pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, IL-18, GM-CSF, and IL-10 itself), chemokines (IL-8, IP-10, MIP-2, MCP-1, and RANTES), and soluble mediators of inflammation such as platelet-activating factor and prostaglandins [Hu et al., 1999; Moore et al., 2001]. In addition, IL-10 shows the inhibitory effects on LPS- [Ledeboer et al., 2000; Molina-Holgado et al., 2001b], IFN-γ- [Cunha et al., 1992], and IFN-γ/LPS [Molina-Holgado et al., 2001a] induced iNOS/NO biosynthesis. Generally, IL-10-activated suppressor of cytokine signaling protein 3, a negative regulator of IFN-γ and TLR4 signaling, provides negative feedback regulation against inflammatory activation [Moore et al., 2001].

Potentiation of TNF-α production by IFN-γ is coupled to IL-10 downregulation [Donnelly et al., 1995]. A recent study has reported that the mechanism by which IFN-γ synergizes with TLR2-mediated NF-κB activation and TNF-α production is regulated by inhibiting IL-10 in a glycogen synthase kinase-3 (GSK-3)-regulated manner [Hu et al., 2006, 2007]. The pro-inflammatory effects of GSK-3 are widely speculated and may be useful for treating septic MOF/MODS [Muhl and Pfeilschifter, 2006; Dugo et al., 2007a,b]. Actually, inhibiting GSK-3 protects cells against TNF-α, endotoxemia, experimental colitis, type II collagen-induced arthritis, zymosan, and ovalbumin-induced asthma [Takada et al., 2004; Dugo et al., 2005, 2006; Cuzzocrea et al., 2006; Whittle et al., 2006; Bao et al., 2007; Cuzzocrea et al., 2007]. In TLR-mediated inflammatory activation, inhibiting GSK-3 causes IL-10 upregulation [Martin et al., 2005; Woodgett and Ohashi, 2005]. It has previously been demonstrated that GSK-3 negatively regulates cyclic AMP responsive element binding protein (CREB), a transcription factor of IL-10, and that this is followed by IL-10 downregulation [Martin et al., 2005; Woodgett and Ohashi, 2005; Hu et al., 2006, 2007]. In the present study, we investigated the synergistic mechanisms of IFN-γ that upregulates LPS-induced iNOS/NO biosynthesis in macrophages. The involvement of IL-10 and its regulatory mechanisms which are controlled by GSK-3 and CREB are also further investigated.

MATERIALS AND METHODS

CELL CULTURE

RAW264.7 murine macrophages and BV2 immortalized murine microglial cells 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.

ANTIBODIES AND REAGENTS

Rabbit polyclonal antibodies specific for iNOS, and mouse monoclonal antibody to β-actin, were purchased from Chemicon International, Inc. (Temecula, CA). Rabbit monoclonal antibodies against GSK-3α/β and rabbit anti-mouse Pyk2 and phospho-Pyk2 at tyrosine 402 (Tyr402) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Goat anti-mouse phospho-CREB at serine 133 (Ser133) and phospho-GSK-3β at tyrosine 216 (Tyr216) and rabbit anti-mouse CREB antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing anti-IL-10 was from R&D Systems (Minneapolis, MN). HRP-conjugated goat anti-mouse or anti-rabbit IgG were from Invitrogen Corp. (Carlsbad, CA). Recombinant mouse cytokines IFN-γ and IL-10 were obtained from PeproTech (Rocky Hill, NJ). LPS from *Escherichia coli* strain O111:B4 were purchased from Calbiochem (San Diego, CA). The GSK-3 inhibitors lithium chloride (LiCl) and 6-bromo-indirubin-3'-oxime (BIO), Pyk2 inhibitor tyrphostin A9, and other chemical reagents were obtained from Sigma–Aldrich Co. (St Louis, MO).

NITRITE ASSAY

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After treatment, we assessed NO production by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4) as previously described [Liu et al., 2002]. The concentration of nitrite was measured using spectrophotometry (Spectra MAX 340PC; Molecular Devices Corporation, Sunnyvale, CA) at 540 nm, and the nitrite concentration was calculated using a standard curve of sodium nitrite with ELISA software (Softmax Pro; Molecular Devices).

RT-PCR

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After treatment, we assessed mRNA expression using RT-PCR. Total cellular RNA from cells was extracted using a reagent (Trizol; Invitrogen) according to the manufacturer's instructions. We quantified RNA concentrations using spectrophotometry at 260 nm (U-2000; Hitachi, Tokyo, Japan). cDNA was prepared using reverse transcription, and PCR was done using a thermal cycler (GeneAmp PCR system 2400; PerkinElmer, Fremont, CA). According to previous published sequences [Hollenbach et al., 2004] and self-designed sequences using the Primer3 online software [Rozen and Skaletsky, 2000], we used the following oligonucleotide primers for:

mouse iNOS—
sense: 5′-CCCTTCCGAAGTTTCTGGCAGCAGCG-3′ and
antisense: 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′,
IL-10—
sense: 5′-ACCTGGTAGAAGTGATGCCCCAGGCA-3′ and
antisense: 5′-CTATGCAGTTGATGAAGATGTCAAA-3′, and
β-ACTIN—
sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and
antisense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

The PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and viewed with UV light. The expression of mRNA was quantified using densitometry with LabWorks Image Acquisition and Analysis Software (UVP, Upland, CA)

WESTERN BLOT ANALYSIS

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After treatment, cell lysates were prepared from a buffer containing 1% Triton X-100, 50 mM of Tris (pH 7.5), 10 mM of EDTA, 0.02% NaN_3, and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany). After they had been freeze–thawed once, the cell lysates were centrifuged at 12,000 rpm at 4°C for 20 min. The supernatants were then collected and boiled in sample buffer for 5 min. After they had undergone SDS–PAGE, proteins were transferred to PVDF membrane (Millipore, Billerica, MA), blocked at 4°C overnight in PBS–T (PBS plus 0.05% Tween-20) containing 5% skim milk, and probed with primary antibodies at 4°C for 1 h. After they had been washed with PBS–T, blots were incubated with a 1:5,000 dilution of HRP-conjugated secondary antibodies at 4°C for 1 h. The protein bands were visualized using ECL (Pierce Biotechnology Inc., Rockford, IL) and the relative signal intensity was quantified using densitometry with LabWorks analysis software (UVP).

ELISA

Cells were seeded in 96-well plates and 6-well plates at a density of 3×10^4cells/well and 5×10^5 cells/well, respectively. After treatment, we used commercial ELISA kits (R&D Systems) to detect the concentrations of mouse TNF-α (catalog number: DY410), RANTES (catalog number: DY478), and IL-10 (catalog number: DY417) in cell-conditioned culture medium according to the manufacturer's instructions. Regarding the different detection limits on standard preparation, cell-conditioned culture medium was regularly diluted and the concentrations were calculated.

IMMUNOSTAINING OF CREB PHOSPHORYLATION

Cells were seeded in 96-well plates at a density of 3×10^4 cells/well. After treatment, cells were fixed with 1% formaldehyde in PBS at room temperature for 10 min. After they had been washed twice with PBS, the cells were stained with goat anti-mouse phospho-CREB (Ser133) at a final concentration of 1 μg/ml at room temperature for 1 h and then incubated with a mixture of Alexa Fluor 594-conjugated donkey anti-goat IgG, respectively, plus 4,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) at a concentration of 5 μg/ml each at room temperature for 1 h. After they had been washed with PBS, the cells were visualized under a fluorescent microscope (IX71, Olympus, Japan).

GSK-3 siRNA

GSK-3β expression was silenced using GSK-3 siRNA kits according to the manufacturers' instructions (Cell Signaling Technology, Inc., and Upstate Biotechnology, Charlottesville, VA). Briefly, before short-hairpin RNA transfection, 10^6 cells (cultured in 24-well plates at a density of 1×10^5 cells/well) were washed with serum-free DMEM and then cultured with 2 μl of Lipofectamine 2000 and various doses of siRNA in 24-well plates. After 6 h of incubation, the cells were washed with DMEM containing 10% FBS and maintained for an additional 24 h before the experiments.

STATISTICAL ANALYSIS

Data were analyzed using the Student's *t*-test with commercially available statistical software (SigmaPlot 8.0 for Windows; Systat Software, Inc., San Jose, CA). Statistical significance was set at $P < 0.05$.

RESULTS

IFN-γ UPREGULATED iNOS/NO BIOSYNTHESIS AND PRODUCTION OF TNF-α AND RANTES, BUT DOWNREGULATED IL-10 EXPRESSION IN LPS-STIMULATED MACROPHAGES

We examined the effects of IFN-γ on LPS-induced iNOS/NO biosynthesis. Using a Griess reaction assay, we measured NO production by determining the production of nitrite. Treating RAW264.7 mouse macrophages and BV2 mouse microglial cells with IFN-γ in addition to LPS (IFN-γ/LPS) showed significantly ($P < 0.05$) higher NO production in a time-dependent manner (Fig. 1A), when compared to treatment with LPS- or IFN-γ alone. We found, using RT-PCR, that IFN-γ synergized with iNOS mRNA expression (0.11 with LPS only, 0.63 with IFN-γ only, and 0.82 with IFN-γ/LPS) in RAW264.7 cells 3 h after LPS stimulation (Fig. 1B) and, using Western blotting, that IFN-γ synergized with iNOS protein expression (0.19 with LPS only, 0.07 with IFN-γ only, and 3.36 with IFN-γ/LPS) in LPS-treated RAW264.7 cells at 6 h (Fig. 1C). Taken together, these results provide strong evidence that IFN-γ

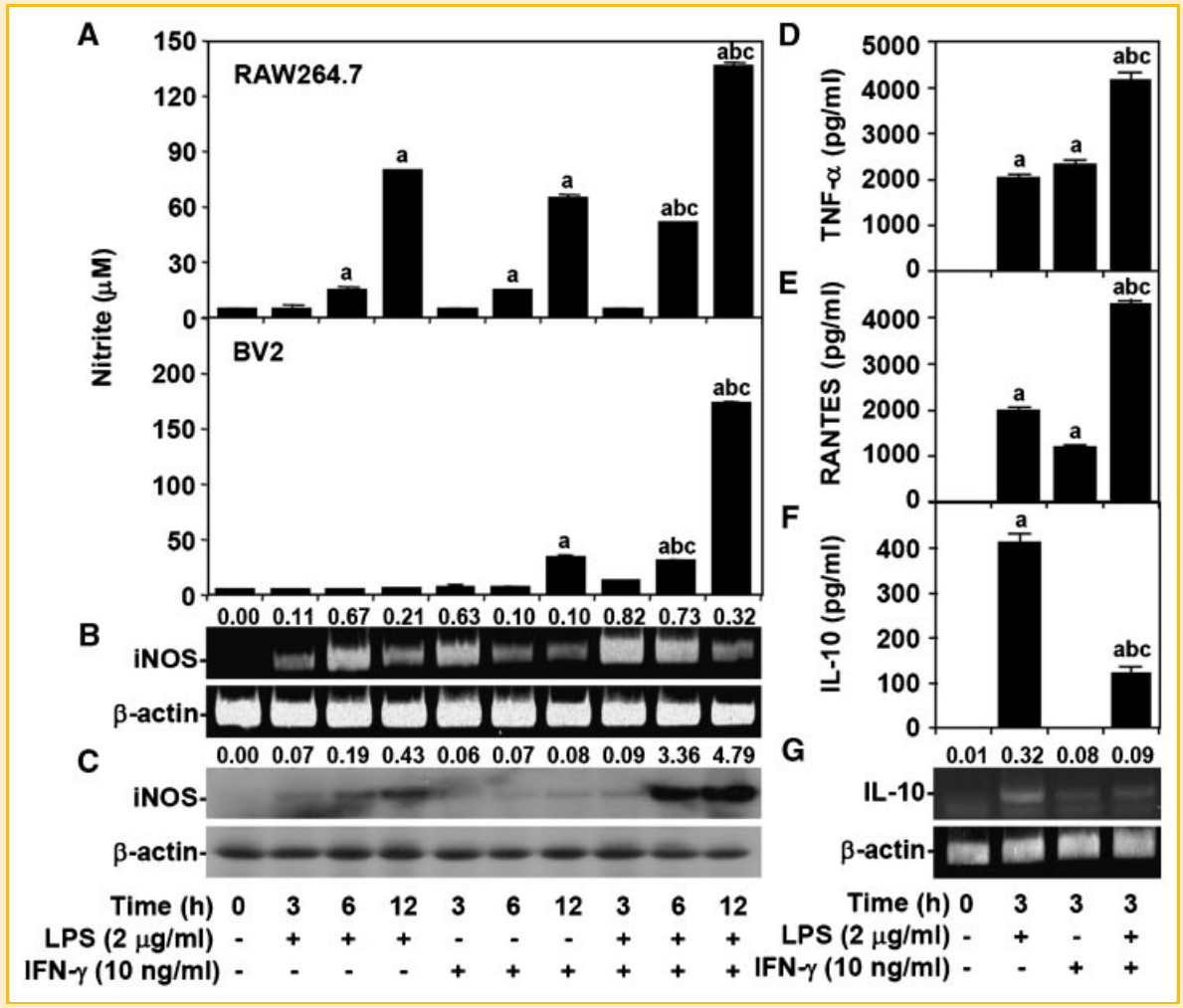

Fig. 1. The effects of IFN-γ on LPS-induced iNOS/NO biosynthesis and TNF-α, RANTES, and IL-10 production in macrophages. A: Mouse RAW264.7 macrophages (5×10^5) and BV2 mouse microglial cells (5×10^5) were treated with the indicated doses of LPS, IFN-γ, or IFN-γ/LPS for 3, 6, and 12 h. After the supernatant had been collected, we determined NO production [referred to as "the detection of nitrite (NO_2^-)"] using Griess reagent as described in Materials and Methods Section. Sodium nitrite ($NaNO_2$) was used for the standard calculation of nitrite concentration. The data are means ± SD obtained from three individual cultures. [a] $P < 0.05$ compared to untreated group; [b] $P < 0.05$ compared to the LPS group; [c] $P < 0.05$ compared to the IFN-γ group. B,C: For the indicated time periods, we used RT-PCR to determine iNOS mRNA expression and used Western blotting to determine iNOS protein expression in LPS-, IFN-γ-, or IFN-γ/LPS-stimulated RAW264.7 cells. β-Actin was the internal control. The ratio of iNOS to β-actin is shown. Data shown are representative of three individual experiments. D–G: RAW264.7 cells (3×10^4) were treated with the indicated doses of LPS, IFN-γ, or IFN-γ/LPS for 3 h. After the supernatant had been collected, we used ELISA to determine TNF-α, RANTES, and IL-10 production. The data are means ± SD obtained from three individual cultures. [a] $P < 0.05$ compared to untreated group; [b] $P < 0.05$ compared to the LPS group; [c] $P < 0.05$ compared to the IFN-γ group. In addition, we used RT-PCR to determine IL-10 mRNA expression. β-Actin was the internal control. The ratio of IL-10 to β-actin is shown. Data shown are representative of three individual experiments.

synergized with LPS-induced iNOS expression, thereby increasing NO production in macrophages.

IFN-γ modulates the production of a variety of cytokines and chemokines during inflammation [Donnelly et al., 1995; Herrero et al., 2003; Schroder et al., 2004]. We then investigated the effects of IFN-γ on LPS-induced pro-inflammatory TNF-α and RANTES and anti-inflammatory IL-10 expression. ELISA analysis demonstrated that RAW264.7 cells with IFN-γ/LPS treatment showed a significant ($P < 0.05$) increase in TNF-α (Fig. 1D) and RANTES (Fig. 1E) and a significant ($P < 0.05$) decrease in IL-10 (Fig. 1F). RT-PCR yielded similar results: cells treated with IFN-γ/LPS showed lower IL-10 mRNA expression (0.32 with LPS only, 0.08 with IFN-γ only, and 0.09 with IFN-γ/LPS; Fig. 1G). These results strongly indicate that IFN-γ upregulated TNF-α and RANTES but downregulated IL-10 production in LPS-stimulated macrophages.

IL-10 INHIBITED IFN-γ-SYNERGIZED WITH LPS-INDUCED iNOS/NO BIOSYNTHESIS

Inhibition of IFN-γ signaling by anti-inflammatory cytokine IL-10 has been reported [Ledeboer et al., 2000; Molina-Holgado et al., 2001b]. To investigate the mechanism through which IFN-γ augments LPS-induced iNOS/NO biosynthesis, we further evaluated the effects of downregulated IL-10. We found that exogenous IL-10 significantly ($P < 0.05$) inhibited LPS-, IFN-γ-, or IFN-γ/LPS-induced NO production (Fig. 2A) as well as iNOS expression (Fig. 2B) in RAW264.7 cells. Furthermore, anti-IL-10 neutralizing antibodies,

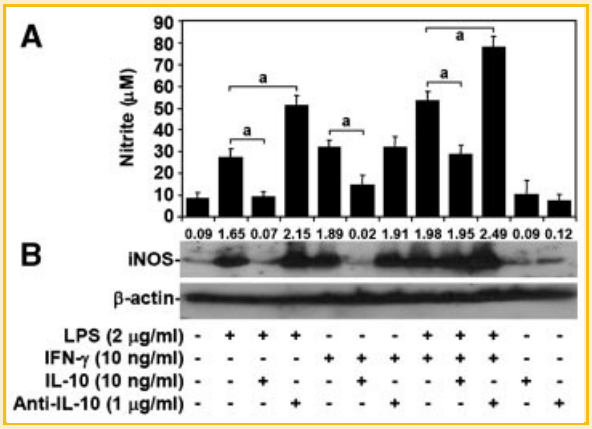

Fig. 2. The effects of IL-10 on iNOS/NO biosynthesis in LPS-, IFN-γ-, or IFN-γ/LPS-stimulated macrophages. Mouse RAW264.7 cells (5×10^5) were pre-treated with the indicated doses of recombinant IL-10 or anti-IL-10 neutralizing antibodies for 30 min and then treated with the indicated doses of LPS, IFN-γ, or IFN-γ/LPS for 12 h. A: After the supernatant had been collected, we determined NO production using Griess reagent. The data are means ± SD obtained from three individual cultures. $^aP < 0.05$. B: We used Western blotting to determine iNOS protein expression. β-Actin was the internal control. The ratio of iNOS to β-actin is shown. Data shown are representative of three individual experiments.

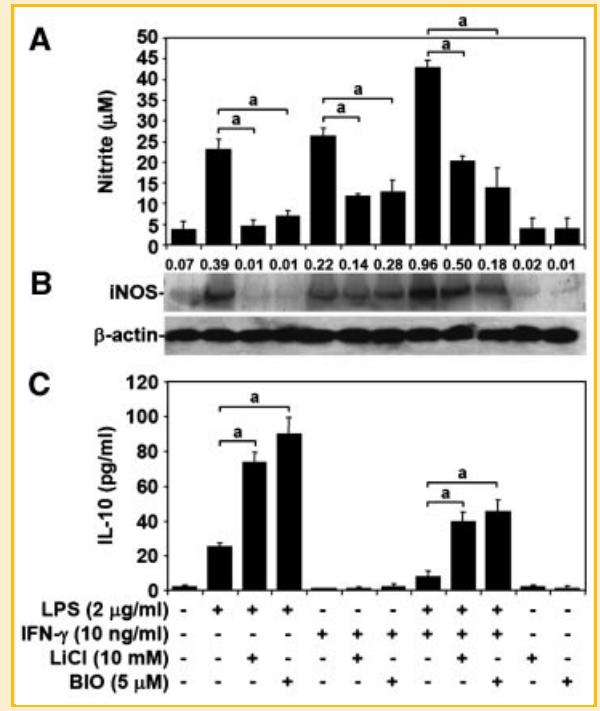

Fig. 3. The effects of inhibiting GSK-3 on iNOS/NO biosynthesis and IL-10 production in LPS-, IFN-γ-, or IFN-γ/LPS-stimulated macrophages. Mouse RAW264.7 cells (5×10^5) were pre-treated with the indicated doses of GSK-3 inhibitors lithium chloride (LiCl) and 6-bromo-indirubin-3′-oxime (BIO) for 30 min and then treated with the indicated doses of LPS, IFN-γ, or IFN-γ/LPS for 12 h. A: After the supernatant had been collected, we determined NO production using Griess reagent. The data are means ± SD obtained from three individual experiments. $^aP < 0.05$. B: We used Western blotting to determine iNOS protein expression. β-Actin was the internal control. The ratio of iNOS to β-actin is shown. Data shown are representative of three individual experiments. C: We used ELISA to determine IL-10 production. The data are means ± SD obtained from three individual cultures. $^aP < 0.05$.

but not control IgG (data not shown), significantly ($P < 0.05$) enhanced NO production (Fig. 2A) and iNOS expression (1.98 with IFN-γ/LPS only, 2.49 with IFN-γ/LPS + anti-IL-10; Fig. 2B) in IFN-γ/LPS-treated RAW264.7 cells. These results indicate that IL-10 is critical for the negative regulation of iNOS/NO biosynthesis in IFN-γ/LPS-stimulated macrophages.

INHIBITING GSK-3 DOWNREGULATED IFN-γ-SYNERGIZED WITH LPS-INDUCED iNOS/NO BIOSYNTHESIS BUT REVERSED IL-10 PRODUCTION

IFN-γ facilitates LPS-induced iNOS/NO biosynthesis through multifactorial pathways [Lorsbach et al., 1993; Gao et al., 1997; Held et al., 1999; Chan and Riches, 2001; Huang et al., 2004; Schroder et al., 2004; Koide et al., 2007]. Our results show that downregulated IL-10 in IFN-γ/LPS-stimulated macrophages may be essential for enhancing iNOS/NO biosynthesis. The mechanisms by which IFN-γ inhibits IL-10 expression remained unclear until GSK-3 was recently demonstrated to be involved [Hu et al., 2006, 2007]. To investigate the effect of GSK-3, using the GSK-3 inhibitors, LiCl and 6-bromo-indirubin-3′-oxime (BIO), we showed that LiCl and BIO significantly ($P < 0.05$) reduced LPS-, IFN-γ-, or IFN-γ/LPS-induced NO production (Fig. 3A) in RAW264.7 cells. Additionally, LiCl and BIO also inhibited IFN-γ-synergized with LPS-induced iNOS expression (0.96 with IFN-γ/LPS only, 0.50 with IFN-γ/LPS + LiCl, and 0.18 with IFN-γ/LPS + BIO; Fig. 3B). These results strongly indicate that GSK-3 is essential for enabling LPS-, IFN-γ-, or IFN-γ/LPS-induced NO production as well as iNOS expression.

To further confirm the effects of GSK-3 on IL-10 inhibition, in LPS- and IFN-γ/LPS-stimulated RAW264.7 cells, we found that, using ELISA, treating cells with LiCl and BIO showed significantly ($P < 0.05$) higher IL-10 production (Fig. 3C). In addition, we observed that LiCl and BIO significantly ($P < 0.05$) reversed IFN-γ-mediated inhibition of LPS-induced IL-10 production.

GSK-3 WAS ESSENTIAL FOR IFN-γ-SYNERGIZED WITH LPS TO INDUCE iNOS/NO BIOSYNTHESIS THROUGH A MECHANISM INVOLVING IL-10 DOWNREGULATION

To exclude the indeterminate effects of GSK-3 inhibitors, we silenced GSK-3 expression using short interference RNA (siRNA) and then investigated the effects on IFN-γ-synergized with LPS to induce iNOS/NO biosynthesis and IFN-γ-downregulated IL-10 production. The rates of transfection efficiency were 96.6% and 97.9%, respectively, in the control and GSK-3 siRNA groups (data not shown). Western blot analysis showed downregulated GSK-3α/β expression in the GSK-3 siRNA group (Fig. 4A). We found that silencing GSK-3 reduced the expression of iNOS (1.61 with IFN-γ/LPS + control siRNA vs. 0.00 with IFN-γ/LPS + GSK-3 siRNA; Fig. 4A) as well as the production of NO (Fig. 4B) in IFN-γ/LPS-treated RAW264.7 cells. We also found, using ELISA, that silencing GSK-3 augmented IL-10 production (Fig. 4C). These results strongly indicate the indispensable role of GSK-3 in INF-γ-synergized with

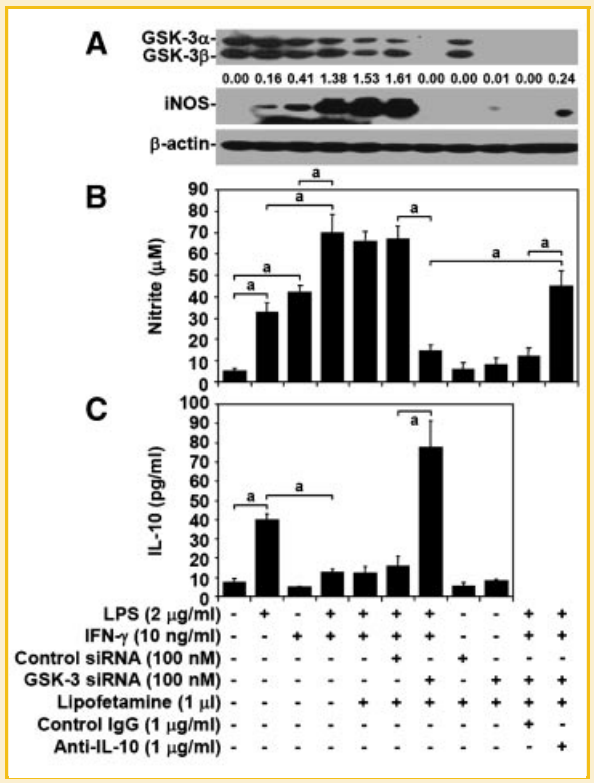

Fig. 4. The effects of GSK-3 on iNOS/NO biosynthesis and IL-10 production in IFN-γ/LPS-stimulated macrophages. Mouse RAW264.7 cells (5×10^5) were pre-treated with control siRNA (100 nM) or GSK-3 siRNA (100 nM) for 24 h and then treated with the indicated doses of IFN-γ/LPS plus 1 μg/ml of control IgG or anti-IL-10 neutralizing antibodies for 12 h. A: We used Western blotting to determine GSK-3α/β and iNOS protein expression. β-Actin was the internal control. The ratio of iNOS to β-actin is shown. Data shown are representative of three individual experiments. B: After the supernatant had been collected, we determined NO production using Griess reagent. The data are means ± SD obtained from three individual cultures. $^aP < 0.05$. C: We used ELISA to determine IL-10 production. Cells treated with Lipofetamine 2000 were used for the negative controls. The data are means ± SD obtained from three individual cultures. $^aP < 0.05$.

LPS to induce iNOS/NO biosynthesis. In addition, inhibiting GSK-3 also reversed IL-10 downregulation under IFN-γ/LPS stimulation.

To investigate the mechanisms inhibiting the GSK-3 reduction iNOS/NO biosynthesis, we further examined the effects of upregulated IL-10. We found that anti-IL-10 neutralizing antibodies, but not control IgG, significantly ($P < 0.05$) reversed the downregulation of iNOS expression (0.00 with IFN-γ/LPS + GSK-3 siRNA + control IgG compared to 0.24 with IFN-γ/LPS + GSK-3 siRNA + anti-IL-10; Fig. 4A) and NO production (Fig. 4B) in IFN-γ/LPS-treated cells while GSK-3 was silenced. These results strongly demonstrate that IL-10 is critical for inhibiting the GSK-3-mediated downregulation on iNOS/NO biosynthesis.

INHIBITING GSK-3 CAUSED TRANSCRIPTION FACTOR CREB PHOSPHORYLATION AT SERINE 133

To further investigate the roles of GSK-3 signaling on IL-10 production, we next examined the phosphorylation of CREB at serine 133, a downstream substrate of GSK-3 and a critical transcription factor of IL-10 [Martin et al., 2005; Woodgett and Ohashi, 2005; Hu et al., 2006, 2007]. Western blot analysis showed lower phosphorylation of CREB at serine 133 (Ser133; 0.31 with LPS only compared to 0.14 with LPS + IFN-γ) in LPS-treated RAW264.7 cells following 0.5 h with IFN-γ co-treatment (Fig. 5A). Immunocytochemical staining yielded similar results: IFN-γ downregulated LPS-induced CREB phosphorylation (Fig. 5B). We also found that LiCl and BIO enhanced CREB phosphorylation in IFN-γ/LPS-treated cells. These results indicate that IFN-γ causes CREB deactivation through a GSK-3-mediated pathway.

IFN-γ/LPS CAUSED Pyk2-MEDIATED GSK-3 PHOSPHORYLATION AT TYROSINE 216 AND THE INDUCTION OF iNOS/NO BIOSYNTHESIS

Protein tyrosine kinase Pyk2 causes phosphorylation of GSK-3β at tyrosine 216, an active site of GSK-3β [Frame and Cohen, 2001; Hartigan et al., 2001; Jope and Johnson, 2004; Sayas et al., 2006]. To investigate whether GSK-3 activation is required for the mechanism of IFN-γ-synergized with LPS to induce iNOS/NO biosynthesis following IL-10 downregulation, we then evaluated the roles of Pyk2 signaling. Western blot analysis showed that Pyk2 inhibitor tyrphostin A9 blocked IFN-γ/LPS-induced Pyk2 phosphorylation at tyrosine 402 (Tyr402; 0.36 with IFN-γ/LPS only, compared to 0.14 with IFN-γ/LPS + tyrphostin A9; Fig. 6A) and GSK-3β phosphorylation at tyrosine 216 (Tyr216; 0.39 with IFN-γ/LPS only, compared to 0.11 with IFN-γ/LPS + tyrphostin A9; Fig. 6B). We further found that tyrphostin A9 significantly ($P < 0.05$) reduced IFN-γ/LPS-induced NO production (Fig. 6C) as well as iNOS expression (0.89 with IFN-γ/LPS only, compared to 0.36 with IFN-γ/LPS + tyrphostin A9; Fig. 6D). IFN causes calcium/calmodulin-dependent protein kinase II (CaMKII)-mediated Pyk2 activation [Wang et al., 2008]. Treating cells with CaMKII inhibitor KN93 showed a decrease in IFN-γ/LPS-induced iNOS expression (data not shown). Taken together, these results strongly indicate that IFN-γ causes synergistic actions on LPS-induced iNOS/NO biosynthesis through a mechanism involving Pyk2-mediated GSK-3 activation.

DISCUSSION

In the present work, we demonstrate that the synergistic mechanisms of IFN-γ, by enhancing LPS-induced iNOS/NO biosynthesis, are, at least in part, dependent on IL-10 inhibition (Fig. 2). Generally, IFN-γ enables the induction of iNOS expression as well as NO production. Also, IFN-γ synergistically induces iNOS/NO biosynthesis in LPS-stimulated macrophages [Lorsbach et al., 1993; Gao et al., 1997; Held et al., 1999; Chan and Riches, 2001; Huang et al., 2004; Schroder et al., 2004; Koide et al., 2007]. The potential synergistic actions of IFN-γ have been suggested to be responsible for the activation of several transcriptional factors that regulate iNOS gene expression, including NF-κB, mitogen-activated protein kinase-regulated signal transducer, activated protein 1 (AP-1), and interferon regulatory factor-1 [Held et al., 1999; Chan and Riches, 2001; Huang et al., 2004; Koide et al., 2007]. Based on our studies, it is likely that IFN-γ upregulates LPS-induced iNOS/NO biosynthesis, not only directly, by enhancing transcriptional activity, but also

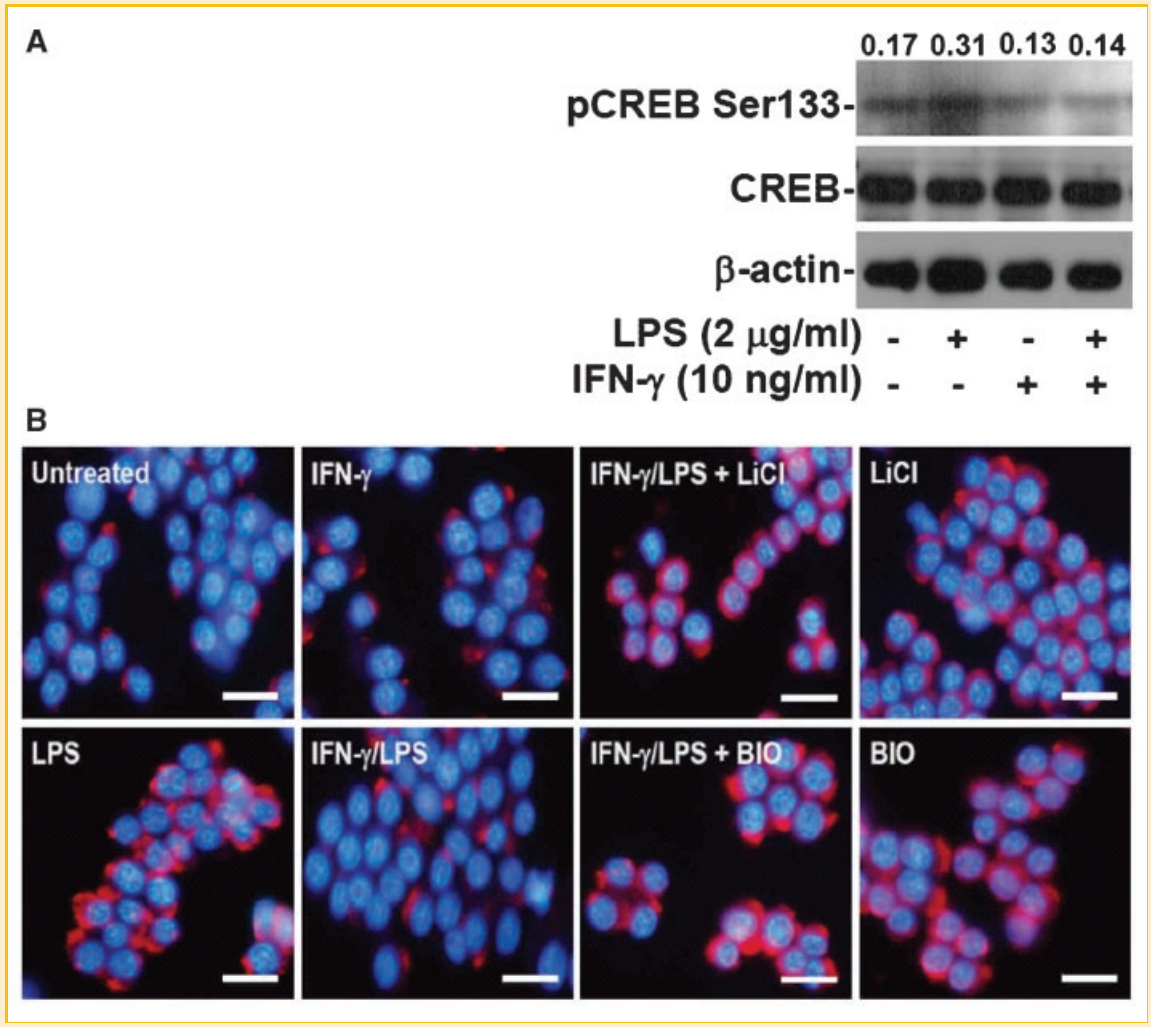

Fig. 5. The effects of inhibiting GSK-3 on CREB phosphorylation at serine 133 in IFN-γ/LPS-stimulated macrophages. A: Mouse RAW264.7 cells (5×10^5) were treated with the indicated doses of LPS, IFN-γ, or IFN-γ/LPS for 0.5 h. We used Western blotting to determine CREB (Ser133) phosphorylation. β-Actin was the internal control. The ratio of phosphorylated CREB to CREB is shown. Data shown are representative of three individual experiments. B: We used immunocytochemical staining and then fluorescent microscopic analysis to detect the phosphorylation of CREB (Ser133; *red*) in cells with 0.5-h post-treatment using primary antibodies followed by Alexa Fluor 594-conjugated secondary antibodies. With 4,6-diamidino-2-phenylindole (DAPI) counterstaining, data shown are representative of three individual experiments. DAPI (*blue*) was used for nuclei staining. The scale bar is 50 μm.

indirectly, by causing IL-10 downregulation. Therefore, we hypothesize that IL-10 has an anti-inflammatory role in IFN-γ-synergized with LPS signaling and that IFN-γ causes inhibition of IL-10 production.

It has been shown that IFN-γ causes IL-10 downregulation in LPS-treated macrophages [Donnelly et al., 1995; Herrero et al., 2003; Hu et al., 2006]; however, the mechanism and its effects remain unclear. Our results (Figs. 3 and 4) reveal that GSK-3-mediated IL-10 downregulation is, at least in part, contributing to the IFN-γ-induced synergistic effects on LPS-induced iNOS/NO biosynthesis. In addition, inhibiting GSK-3 causes IL-10 upregulation via a mechanism involving the CREB-mediated pathway (Fig. 5). These findings are in agreement with recent reports [Hu et al., 2006, 2007] that IFN-γ synergizes with TLRs-regulated cytokine production, including TNF-α, IL-6, and IL-12, by suppressing TLR-induced feedback inhibition, partly mediated by IL-10, and by a mechanism that depends on GSK-3 regulation of CREB and AP-1. Previous reports [Martin et al., 2005; Woodgett and Ohashi, 2005] and our studies [Huang et al., unpublished work] also demonstrate that GSK-3 is a negative regulator of IL-10 production under LPS signaling. In IFN-γ/LPS-stimulated cells, the cross-talk between IFN-γ- and LPS-mediated signaling depends on, at least in part, GSK-3.

Our results (Figs. 3–5) and previous studies [Martin et al., 2005; Woodgett and Ohashi, 2005; Hu et al., 2006, 2007] show that inhibiting GSK-3 using inhibitors and GSK-3 siRNA causes IL-10 upregulation via a mechanism involving the CREB-mediated pathway. CREB, a downstream substrate of GSK-3, is indispensable for IL-10 gene expression [Moore et al., 2001; Martin et al., 2005; Woodgett and Ohashi, 2005; Hu et al., 2006, 2007]. Previous studies found that GSK-3 directly phosphorylated CREB at serine 129 and downregulated its bioactivity of DNA binding [Fiol et al., 1994].

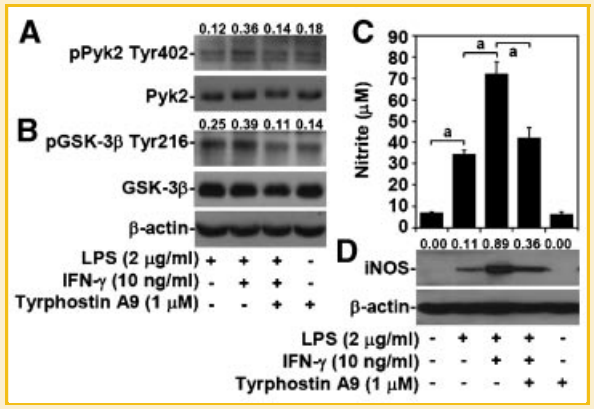

Fig. 6. The effects of protein tyrosine kinase Pyk2 on GSK-3 phosphorylation at tyrosine 216 and iNOS/NO biosynthesis in IFN-γ/LPS-stimulated macrophages. Mouse RAW264.7 cells (5×10^5) were pre-treated with the indicated doses of Pyk2 inhibitor tyrphostin A9 for 30 min. After the cells had been treated with the indicated doses of LPS or IFN-γ/LPS for 0.5 h, we used Western blotting to determine Pyk2 (Tyr402; A) and GSK-3β (Tyr216; B) phosphorylation. Relative proteins and β-actin were the internal controls. The ratio of phosphorylated protein to total protein is shown. Data shown are representative of three individual experiments. We used Griess reagent and Western blotting to determine NO (C) and iNOS (D) protein expression for the 6-h treatment, respectively. β-Actin was the internal control. The ratio of iNOS to β-actin is shown. Data shown are representative of three individual experiments. The data are means ± SD obtained from three individual cultures. $^aP < 0.05$.

Thus, activated GSK-3 may cause CREB deactivation followed by IL-10 inhibition [Martin et al., 2005; Woodgett and Ohashi, 2005]. Furthermore, we also found that inhibiting GSK-3 increased CREB phosphorylation at serine 133 (Fig. 5), an active site of CREB, and nuclear translocation (data not shown). These results are in agreement with previous reports [Grimes and Jope, 2001] that inhibiting GSK-3 causes the higher phosphorylation of CREB at serine 133 and increases its DNA binding activity. The effects of IFN-γ on IL-10 inhibition are caused by GSK-3-mediated CREB deactivation.

The mechanisms of IFN-γ-induced GSK-3 activation are still unclear. The involvement of GSK-3 activation under LPS and IFN-γ signaling are currently under investigation [Hu et al., 2006; Hu et al., 2007; Tsai et al., unpublished work]. Activation of GSK-3 is generally regulated by PI3K/Akt and protein phosphatase (PP) such as PP1 and PP2A [Frame and Cohen, 2001; Jope and Johnson, 2004]. Meanwhile, our unpublished work demonstrate that cells treated with PP inhibitor okadaic acid show a decrease in iNOS/NO biosynthesis [Tsai et al., unpublished work]. An earlier report showed that expression of iNOS mRNA induced by LPS, IFN-γ, and LPS plus IFN-γ requires PP1/PP2A activities [Dong et al., 1995]. We hypothesize that IFN-γ-activated PP1/PP2A negatively regulates Akt-mediated GSK-3 inactivation. Importantly, protein tyrosine kinase Pyk2 is also involved in GSK-3 activation by directly phosphorylating GSK-3β at tyrosine 216, an active site of GSK-3β [Frame and Cohen, 2001; Hartigan et al., 2001; Jope and Johnson, 2004; Sayas et al., 2006]. It has recently been demonstrated that IFN causes CaMKII-mediated Pyk2 activation [Wang et al., 2008] and this is critical for enhancing Janus activated kinase and signal transducer and activator of transcription activation. Our results (Fig. 6) show that inhibiting Pyk2 blocks IFN-γ/LPS-induced GSK-3β phosphorylation at tyrosine 216 and iNOS/NO biosynthesis. In addition to PP1/PP2A activation, we hypothesize the potential mechanism of GSK-3 activation through IFN-γ-activated Pyk2. While GSK-3 is activated, it may be involved in the negative regulation on CREB activity and IL-10 expression and may then contribute to IFN-γ-synergized with LPS-induced iNOS/NO biosynthesis.

Production of NO is both beneficial and harmful to the host, depending on pathogenesis and actual state of a disease [Kirkeboen and Strand, 1999]. Since IFN-γ synergizes with LPS to induce NO production, this process plays an essential immunomodulation role in response to the early stage of microbial infection. In septic patients with MOF/MODS, excessive NO production may contribute to the systemic inflammation and cellular cytotoxicity [Kirkeboen and Strand, 1999]. Despite the failure in a previous clinical trial of using NOS inhibitor to treat septic patients [Takala et al., 1997], the strategies of downregulating NOS activity may still be useful to inhibit the excessive inflammation caused by NO [Kirkeboen and Strand, 1999; Zidek, 2001]. Our results (Figs. 3 and 4) demonstrate that inhibiting GSK-3 significantly blocked LPS-, IFN-γ-, and IFN-γ/LPS-induced iNOS/NO biosynthesis. Therefore, with the importance of GSK-3 in regulating these signaling pathways, it is therefore suggested as a possibly potent target of anti-inflammation.

In conclusion, our results demonstrate the mechanisms by which IFN-γ synergizes with LPS to induce iNOS/NO biosynthesis through a mechanism involving GSK-3-inhibited CREB and IL-10. The activation of GSK-3 by IFN-γ is, at least in part, tyrosine kinase Pyk-2-dependent. To inhibit the excessive production of NO in cytokine/LPS-induced inflammation, GSK-3 may be a potential therapeutic target.

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