DUAL ROLE OF cAMP IN iNOS EXPRESSION IN GLIAL CELLS AND MACROPHAGES IS MEDIATED BY DIFFERENTIAL REGULATION OF p38-MAPK/ATF-2 ACTIVATION AND iNOS STABILITY

JE-SEONG WON,* 1 YEOG-BIN IM,* AVTAR K. SINGH,1,2 and INDERJIT SINGH*

*Developmental Neurogenetics, Department of Pediatrics and 1Department of Pathology, Medical University of South Carolina, 316 CB, Charleston, SC 29425, USA. 2Ralph H. Johnson V. A. Medical Center, Charleston, SC, USA

(Rceived 13 May 2004; Revised 24 August 2004; Accepted 26 August 2004)

Available online 15 September 2004

Abstract—We reported previously that cAMP analogues or cAMP synthesis activator (forskolin; FSK) inhibit lipopolysaccharide (LPS)-induced inducible nitric-oxide systase (iNOS) gene expression in astrocytes, while they enhance that in macrophages. Here, we report that the FSK-mediated inhibition of iNOS expression in C6 glial cells is due to its reduced transcriptional activity, while the FSK-mediated enhancement of iNOS expression in RAW264.7 macrophages is a result of increased stability of iNOS protein without transcriptional enhancement. The LPS/interferon-γ (IFN)-induced iNOS transcription was inhibited by FSK via inhibition of p38-MAPK/ATF-2 activity in glial cells while it was not affected in macrophages. In both cell types, proteasome activities were required for the spontaneous degradation of iNOS protein, and the inhibition of proteasome activity by MG132 after maximum increase of iNOS protein levels further enhanced iNOS protein induction by LPS/IFN, suggesting the involvement of proteasome in iNOS degradation. More importantly, the iNOS protein levels were equalized by the MG132 posttreatment in macrophages treated with LPS/IFN alone and along with FSK, and ubiquinated iNOS protein levels were reduced by FSK posttreatment, suggesting that the FSK-mediated inhibition of ubiquitination of iNOS protein and the following increased stability of iNOS protein are one of the mechanisms of cAMP-pathway-mediated enhancement of iNOS gene expression in macrophages. To our knowledge, this is the first evidence that cAMP regulates iNOS expression at the posttranslational level in macrophages. © 2004 Elsevier Inc. All rights reserved.

Keywords—ATF-2, p38-MAPK, iNOS, Proteasome, cAMP, Forskolin, Glial cells, Macrophages, Free radicals

INTRODUCTION

Nitric oxide (NO), a bioactive free radical, is involved in various physiological and pathological processes in many organ systems [1]. NO is enzymatically formed from L-arginine by nitric-oxide synthase (NOS). Basically, the NOS are classified into two groups. The constitutively expressed forms (neuronal NOS and endothelial NOS) are regulated predominantly at the posttranscriptional level by calmodulin in a calcium-dependent manner [2]. In contrast, the inducible form (iNOS), expressed in various cell types including smooth muscle cells, macrophages, keratinocytes, hepatocytes, and brain cells, is induced in response to a series of proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN), and bacterial lipopolysaccharide (LPS) [3–6]. Although NO produced from iNOS has many beneficial roles such as elimination of microorganisms, reduction of thrombosis, and improvement of blood supply in injured tissue, it also can cause tissue damage because it is a potent toxic compound especially when it reacts with reactive oxygen species (ROS), forming the highly reactive peroxynitrite.

Growing evidence suggests that signaling cascades of cAMP suppress various immune responses to minimize...
damage to the host. For example, cAMP inhibits the inflammatory process including the production of proinflammatory cytokines [7–9] and oxidative burst [10] in the central nervous system and peripheral immune system. However, an exceptional role of cAMP in the inhibition of the inflammatory process is the regulation of iNOS expression. Previously, we reported that compounds (forskolin (FSK), 8-bromo-cAMP, and $(S\_p)$-cAMP) that increase cAMP and activate protein kinase A (PKA) inhibit (LPS)- and/or cytokine-mediated production of NO and the expression of iNOS in astrocytes. However, in macrophages, the cAMP analogues stimulated the LPS- and/or cytokine-induced production of NO. In vitro, PKA had no effect on iNOS activity in LPS-treated astrocytes or macrophages, suggesting that PKA modulates the intracellular signaling events associated with cellular expression of iNOS enzyme rather than regulation of enzymatic activity in both cell types. Similar observations were reported in Kupffer cells [11], hepatocytes [12], and C6 rat glioma cells and primary rat astrocytes [13–18], where the increase in cAMP or its analogue negatively regulates the LPS/cytokine-mediated induction of iNOS and production of NO, while it enhances the expression of iNOS in glomerular mesangial cells [19], smooth muscle cells [20], cardiac myocytes [21], murine 3T3 fibroblasts [22], and macrophages [17].

Several efforts were made to identify the mechanism of cAMP-mediated regulation of iNOS gene expression. We previously reported that the cAMP signaling pathway inhibits iNOS gene expression induced by LPS via inhibition of p38-MAPK without altering NFκB activity in C6 rat glioma cells [18]. This observation was further supported by Bhat and colleagues [23]. Additionally, they suggested the possible involvement of CCAAT/enhancer-binding protein (C/EBP) and ATF-2 as effector transcription factors in cAMP-mediated regulation of iNOS expression. However, the exact mechanism of the differential effect of cAMP in iNOS gene expression (induction vs inhibition) in astrocytes and macrophages is not understood at present.

This study was designed to demonstrate the mechanism of cAMP-mediated regulation of iNOS expression in glial cells and macrophages. We report that cAMP inhibited the LPS/IFN-mediated increase in iNOS mRNA and protein expression in glial cells, while it enhanced LPS/IFN-mediated increase in NO production and iNOS protein levels without altering the iNOS mRNA level in macrophages. MAP kinase (p38) and transcription factor ATF-2 were involved in the induction of iNOS. cAMP attenuated the phosphorylation of p38-MAPK and ATF-2 in glial cells but had no effect in macrophages. Moreover, in macrophages, cAMP did not alter LPS/IFN-induced increase in iNOS mRNA level, but it enhanced LPS/IFN-mediated increase in iNOS protein level by enhancing the iNOS protein stability by blocking the ubiquitination of iNOS protein.

**EXPERIMENTAL PROCEDURES**

**C6 glioma cell cultures**

C6 rat glioma cells and RAW264.7 murine macrophages were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L of glucose, 10% fetal bovine serum (FBS), and 10 µg/ml gentamicin. All the cultured cells were maintained at 37°C in 5% CO2/95% air. At 80% confluency, the cells were incubated with serum-free DMEM medium overnight prior to the incubation with LPS/IFN and other chemicals.

**Assay for NO production and induction of iNOS enzyme**

C6 rat glioma cells and RAW264.7 murine macrophages were cultured in 12-well plastic tissue culture plates. After the appropriate treatment, production of NO was determined by assay of the culture supernatant for nitrite [24]. Briefly, 100 µl of culture supernatant was allowed to react with 100 µl of Griess reagent. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO2 in fresh media. For iNOS protein, the cells were washed with cold Tris-buffered saline (TBS; 20 mM Trizma base, 137 mM NaCl, pH 7.5) and lysed in 1× SDS sample loading buffer (62.5 mM Trizma base, 2% w/v SDS, 10% glycerol), followed by sonication and centrifugation at 15,000g for 5 min. The supernatant was used for the iNOS Western immunoblot assay.

**Western blot analysis**

The protein concentration of samples was determined with the detergent-compatible protein assay reagent (Bio-Rad Laboratories, CA) using bovine serum albumin (BSA) as the standard. Samples were boiled for 3 min with 0.1 volume of 10% β-mercaptoethanol and 0.5% bromophenol blue mix; 50 µg of total cellular protein or 10 µg of nuclear extract was resolved by electrophoresis in 8 or 12% polyacrylamide gels, electrotransferred to polyvinylidene difluoride (PVDF) filter, and blocked with PVDF buffer [1-Block (Tropix, MA), 10 mM Trizma base (pH 7.4), 1% Tween 20, 150 mM NaCl]. After incubation with antiserum against iNOS (BD PharMingen, CA), C/EBPβ, C/EBPδ, c-Jun, or JunD (all from Santa Cruz Biotech) in PVDF buffer for 2 h at room temperature, the filters were washed three times with PVDF buffer and then incubated with anti-rabbit...
donkey IgG-horseradish peroxidase conjugate (1:5000) for 1 h. The membranes were autoradiographed using ECL-plus (Amersham Pharmacia Biotech) after washing with PVDF buffer.

Isolation of total RNA and Northern blot assay

C6 rat glioma cells and RAW264.7 murine macrophages were cultured in six-well tissue culture plates. After the appropriate treatment, total cellular RNA was extracted using a rapid guanidine thiocyanate–water-saturated phenol/chloroform extraction procedure and subsequent precipitation with acidic sodium acetate; 5 μg of total RNA was denatured and electrophoresed on 1% agarose–formaldehyde gels and transferred onto positively charged nylon hybridization membrane sheets (Amersham Pharmacia Biotech, NJ). After UV-cross-linking, the membranes were incubated at 68°C in prehybridization buffer (5× standard saline citrate (SSC), 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent (Roche Molecular Biochemicals, IN). The digoxigenin (DIG)-labeled iNOS probes were added to prehybridization buffer and incubated overnight. The membranes were washed three times in 2× SSC and 0.1% SDS at room temperature, and in 0.1× SSC and 0.1% SDS at 68°C. Membranes were equilibrated in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) and then blocked in maleic acid buffer containing 1% blocking reagent. The chemiluminescent autoradiography detection was performed as suggested by the manufacturer (Roche Molecular Biochemicals) using alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Molecular Biochemicals) and CSPD (Disodium 3-(3,5-dichlorosalicylamide-2-yl)phenyl phosphate, Roche Molecular Biochemicals).

Nuclear extraction

Nuclear extracts from C6 rat glioma cells and RAW264.7 murine macrophages (1 × 10⁷ cells) were prepared using a previously published method [25] with slight modification. Cells were harvested, washed twice with ice-cold TBS, and lysed in 400 μl of buffer A containing 10 mM KCl, 2 mM MgCl₂, 0.5 mM dithiothreitol, EDTA-free Complete protease inhibitor (Roche Molecular Biochemicals), and 0.1% Nonidet P-40 in 10 mM Hepes, pH 7.9, for 10 min on ice. After centrifugation at 5000g, the pelleted nuclei were washed with buffer A without Nonidet P-40 and resuspended in 40 μl of buffer B containing 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitor cocktail (Sigma) in 20 mM Hepes, pH 7.9, for 30 min on ice. The lysates were centrifuged at 15,000g for 15 min and the supernatants containing the nuclear proteins were stored at −70°C until use.

Electrophoretic mobility shift assay

Ten micrograms of nuclear proteins was used for the electrophoretic mobility shift assay for detection of NFkB and CREB DNA binding activities. DNA–protein binding reactions were performed at room temperature for 20 min in 10 mM Trizma base (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1 μg poly(dI-dC), 5% (v/v) glycerol, and approximately 0.3 pmol of NFkB or C/EBP (both from Santa Cruz Biotech) labeled with DIG-ddUTP using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). For the supershift assay 2 μg of antibody was added in protein–DNA binding mixture. Protein–DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels at room temperature in 50 mM Tris, pH 8.3, 0.38 M glycine, 2 mM EDTA, and electroblotted onto positively charged nylon membranes. The chemiluminescence detection method for DIG-labeled probes is identical to the method used for the nonisotopic Northern blot analysis.

Vectors, transient transfections, and reporter gene assay

Expression vectors encoding wild type C/EBPα and β (pBluescript-MSV-C/EBPα and β) were a gift from Dr. Steven L. McKnight (Tularik, San Francisco, CA). Expression vector coding wild type c-Jun (pCMV-c-Jun) was a gift from Dr. Hongtao Liu. Expression vector coding dominant negative p38-MAPK (pCMV-Flag-p38-agf) was a gift from Dr. Roger Davis. Expression vector coding dominant negative ATF-2 (pcDNA3-dn-ATF2) was gift from Dr. Frank Bier. The pGL3 (Promega) containing −3.2 kb of 5′-flanking region of rat iNOS gene (pGL3/−3.2iNOS) was a gift from Dr. Hangfang Zhang. The β-B-reporter construct (pNFkB-TA-Luc) was purchased from Clontech (CA). C6 rat glioma cells and RAW264.7 murine macrophages (3 × 10⁵ cells/well) were cultured in six-well plates for 36 h before the transfection. Transfection was performed with 2 μg of plasmid DNA and 8 μl of FuGene transfection reagent (Roche Molecular Biochemicals) for C6 rat glioma cells or 10 μl of Superfect Transfection Reagent (Qiagen, Germany) for RAW264.7 murine macrophages. One day after transfection, the cells were placed in serum-free media overnight. Following appropriate treatment, the cells were washed with phosphate-buffered saline (PBS) and lysed with 200 μl of lysis buffer (40 mM Tricine, pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM MgSO₄, 5 mM dithiothreitol, and 1% Triton X-100). Following incubation at room temperature for 15 min with occasional vortexing, the samples were centrifuged.
The luciferase and β-galactosidase activities were measured by using luciferase assay kit (Stratagene, CA) and β-gal assay kit (Invitrogen, CA), respectively. The emitted light and optical absorbance were measured using Spectra Max/Gemini XG fluorometer and SpectraMax 190 spectrophotometer (Molecular Devices, CA).

Detection of ubiquitin-conjugated iNOS protein

For the detection of ubiquitin-conjugated iNOS protein in RAW264.7 murine macrophages, the cells were cultured in a 100-mm tissue culture dish. After the appropriate treatment, the cells were washed with ice-cold PBS and lysed in 50 mM Hepes (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10 mM N-ethylmaleimide, and EDTA-free Complete protease inhibitor (Roche Molecular Biochemicals); 500 μg of total cell lysate was incubated with polyubiquitin affinity beads (Calbiochem, CA) for 3 h. After the washing with lysis buffer three times, the beads were boiled in SDS sample loading buffer, and supernatant was applied to 8% SDS–PAGE. The ubiquitin-conjugated iNOS protein was detected by Western blot using anti-iNOS antibody.

Assay of proteasome activity

To measure the proteasome activity in RAW264.7 murine macrophages, the cells were cultured in six-well tissue culture plates. After the appropriate treatment, the cells were washed with ice-cold PBS and lysed in 50 mM Tris–HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN₃, 1 mM pepstatin A, 3 μM leupeptin, and 0.2% 3-(3-cholamidopropyl)dimethylammonio]propanesulfonic acid, 1 μM peptatin A, 3 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride; 50 μg of total cell lysate was incubated in 150 μl of reaction buffer [50 mM Tris–HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN₃, 50 μM Suc-LLVY-AMC] at 37°C for 5 min in the presence or absence of MG-132. Proteolysis of the fluorescent probe (Suc-LLVY-AMC) was monitored using a Spectra Max/Gemini XG (Molecular Devices) with filter settings of 360 nm for excitation and 470 nm for emission for 1 h with 5-min intervals. The subtracted V max values from V max of MG-132-treated sample were taken as proteasome activity.

Statistical analysis

All values shown in the figures are expressed as means ± standard error of mean (SE) of n determinations, obtained on at least three independent experiments. The results were examined by one- and two-way ANOVA; individual group means were then compared with the Bonferroni test. A p value of less than 0.05 was considered significant.

RESULTS

FSK differentially regulates the iNOS protein expression in glial cells and macrophages

We previously reported that cAMP analogues or FSK, an activator of cAMP synthesis, differentially regulate the expression of iNOS and production of NO in astrocytes and macrophages in culture [17]. In the present study, to evaluate the role of cAMP signaling pathway in LPS/IFN-mediated expression of iNOS gene, the effects of FSK (100 μM) on LPS/IFN-mediated NO production and iNOS enzyme level were examined. As shown in Figs. 1A and 1B, LPS/IFN-mediated induction of iNOS protein levels and production of NO, measured at 24 h after LPS/IFN treatment, were completely abolished by FSK in C6 rat glioma cells. On the other hand, FSK further enhanced the LPS/IFN-mediated induction of iNOS protein level and NO production in RAW264.7 macrophages.

To understand the mechanism(s) of transcriptional regulation of iNOS in both cell lines, the effect of FSK on iNOS mRNA expression with respect to time was
examined. In C6 rat glioma cells, the maximum increase in iNOS mRNA level was observed at 6 h after LPS/IFN treatment, and it was completely inhibited by pretreatment with FSK at all time points tested (Fig. 1C). In RAW264.7 macrophages, LPS/IFN-induced expression of iNOS mRNA was at the highest level 9 h after treatment. However, FSK had no effect on iNOS mRNA level induced by LPS/IFN at all time points (Fig. 1C). These results indicate that in macrophages the enhanced LPS/IFN-mediated increase in iNOS protein level by FSK may not be due to the enhanced induction of iNOS mRNA expression.

C/EBP, NFκB, and AP-1 are not involved in cAMP-mediated differential regulation of iNOS expression in glial cells and macrophages

Previously, it has been suggested that the C/EBP-response element in iNOS promoter/enhancer is critical for cAMP-mediated gene induction via interaction with C/EBPs and CREB in rat mesangial cells [26]. To evaluate the involvement of C/EBP in iNOS gene regulation, wild type C/EBPα and C/EBPβ were cotransfected with −3.2 kb iNOS promoter/enhancer-luciferase construct. As shown in Fig. 2A (left panel), C/EBPα up-regulated the iNOS promoter activity, while C/EBPβ down-regulated the iNOS promoter activity in glial cells. Moreover, the FSK further enhanced the LPS-mediated increase in C/EBPα protein level and C/EBP DNA binding activity (Fig. 2A, right panel), suggesting that C/EBP may not be involved in the cAMP-mediated regulation of iNOS transcription. We have previously reported that NFκB may not be involved in cAMP-mediated regulation of iNOS gene expression in C6 rat glioma cells [18]. Consistent with these results, FSK failed to affect LPS/IFN-mediated increase in NFκB DNA binding activity in glial cells and macrophages (Fig. 2B, right panel). Moreover, FSK did not alter LPS/IFN-mediated increase in NFκB transactivity in either cell line (Fig. 2B, left panel), further supporting the conclusion that NFκB is not involved in cAMP-mediated regulation of iNOS gene expression. The involvement of AP-1 in iNOS gene expression seems to depend on the cell type. It was reported to act as a negative regulator in J774A.1 murine macrophage cell line [27] and as a positive regulator in a human epithelial cell line [28]. In the present study, we observed that the over-expression of wild type c-Jun, a key factor for the formation of functional AP-1 complex, negatively regulated the −3.2 kb iNOS promoter/enhancer activity in C6 rat glioma cells (Fig. 2C, left panel). In addition, FSK down-regulated c-Jun and JunD protein levels induced by LPS/IFN (Fig. 2C, right panel), indicating that c-Jun is not involved in cAMP-mediated differential regulation of iNOS gene expression.

![Fig. 2.](image-url)

**Fig. 2.** C/EBP, NFκB, and AP-1 are not involved in cAMP-mediated differential regulation of iNOS expression in C6 glioma cells and RAW264.7 macrophages. To examine the involvement of C/EBPα, C/EBPβ, and c-Jun in differential regulation of iNOS gene by forskolin in C6 rat glioma cells and RAW264.7 macrophages, C6 rat glioma cells were cotransfected with wild type C/EBPα or C/EBPβ (A), wild type c-Jun (C) expression vectors, and −3.2 kb 5′-flanking region of iNOS gene linked with luciferase gene. Twenty-four hours after lipopolysaccharide (LPS) plus interferon-γ (IFN) treatment, the cells were lysed and the activity of luciferase was measured by standard protocol. To identify the effect of forskolin (FSK; 100 μM) on LPS/IFN-mediated activation of NFκB transactvity, C6 rat glioma cells and RAW264.7 macrophages were transfected with pNFκB-B-TA-Luc, and the activity of luciferase was measured 24 h after drug treatment (B). To identify the effects of FSK on LPS/IFN-mediated C/EBP (A) or NFκB (B) DNA binding activity, C/EBPα or C/EBPβ protein expression, and c-Jun or JunD protein expression, 10 μg of nuclear extracts was used for gel shift or Western immunoblot assay. For the stimulation of C6 rat glioma cells and RAW264.7 macrophages, 1 μg/ml of LPS plus 10 unit/ml of IFN and 50 ng/ml of LPS plus 0.2 unit of IFN, respectively, were used. FSK or dimethyl sulfoxide as a vehicle (VHC) was pretreated 0.5 h prior to LPS/IFN treatment. The vertical bar in each panel indicates the standard error of mean (** p < 0.01, *** p < 0.001 compared to control group; p < 0.05, **** p < 0.01 compared to LPS/IFN-treated group).**

p38-MAPK/ATF-2 mediates FSK-mediated differential regulation of iNOS mRNA expression in glial cells and macrophages

Previously, we reported the involvement of p38-MAPK in cAMP-mediated inhibition of iNOS gene expression [18]. To evaluate the role of p38-MAPK in the differential regulation of iNOS gene expression in C6
rat glioma cells and RAW264.7 macrophages, the effect of FSK on p38-MAPK activation and its involvement in iNOS gene expression was examined. As shown in Fig. 3A, FSK reduced the LPS/IFN-mediated phosphorylation of p38-MAPK in C6 rat glioma cells. Similar to the iNOS mRNA level, FSK did not alter the LPS/IFN-mediated p38-MAPK phosphorylation in RAW264.7 macrophages. However, SB203580 (an inhibitor of p38-MAPK) pretreatment or over-expression of dominant negative p38-MAPK reduced the LPS/IFN-mediated induction of iNOS protein and mRNA expression or promoter activity of iNOS gene in both cell types (Figs. 3B and 3C). The reduction of iNOS mRNA by inhibition of p38-MAPK and the inhibition of iNOS promoter activity by dominant negative p38-MAPK indicate the involvement of p38-MAPK in iNOS gene expression in both of these cell types. However, the observed reduction of iNOS protein in glial cells but not in RAW264.7 cells indicates that differential regulation of iNOS protein levels by the cAMP signaling pathway may be via differential regulation of p38-MAPK in glial cells and macrophages.

Since the transcriptional factor ATF-2 is regulated mainly by p38-MAPK, its positive role in cAMP-mediated differential regulation of iNOS in C6 rat glioma cells and RAW264.7 macrophages was examined. The increase in LPS/IFN-mediated phosphorylated ATF-2 levels was reduced by pretreatment with FSK in C6 rat glioma cells (Fig. 4A). On the other hand, similar to iNOS mRNA levels and p38-MAPK phosphorylation, FSK had no effect on LPS/IFN-mediated phosphorylation of ATF-2 in RAW264.7 macrophages. The over-expression of dominant negative ATF-2 reduced the LPS/IFN-mediated activation of iNOS promoter/enhancer reporter gene activity in both cell lines, suggesting that ATF-2 is involved in iNOS gene regulation in both cell lines (Fig. 4B). The observed differential regulation of ATF-2 activation by FSK may be one of the transcription factors responsible for differential regulation of iNOS gene expression by cAMP in glial and macrophage cell lines.

FSK differentially regulates the turnover of iNOS protein in glial cells and macrophages

To elucidate the mechanism of increase in macrophage iNOS protein level by FSK without increase in iNOS mRNA, the time course effect of FSK on iNOS protein levels was examined (Fig. 5A). In both cell types, iNOS protein levels were maximally increased at 12 h after the stimulation with LPS/IFN and then reduced spontaneously. In C6 rat glioma cells, the iNOS protein levels induced by LPS/IFN were reduced by FSK at all time points similar to the level of NO production and iNOS mRNA levels (Fig. 5A). In macrophages, although the iNOS protein level induced by LPS/IFN was not increased by FSK at 12 h, FSK delayed the spontaneous decrease in LPS/IFN-mediated increase in iNOS protein levels after 24 h (Fig. 5A), suggesting that cAMP may decrease the rate of turnover of iNOS protein in macrophages.

To evaluate the effect of FSK-mediated delayed decrease in iNOS protein on NO production in macrophages, the time course of effect of FSK on LPS/IFN-mediated NO production was also examined. In C6 rat glioma cells, LPS/IFN-mediated increase in NO produc-
tion was inhibited by FSK at all time points tested (0.33- to 0.21-fold) (Fig. 5B). However, in the macrophages, FSK increased the LPS/IFN-mediated NO production 1.17-fold at 9 h to 1.8- to 2.0-fold at 24 and 48 h, respectively (Fig. 5B). These results suggest that FSK-mediated inhibition of spontaneous turnover of iNOS protein may be responsible for FSK-mediated enhanced production of NO.

FSK inhibits the ubiquitin/proteasome-mediated iNOS protein degradation in macrophages

To investigate the possible role of FSK in proteasome-mediated iNOS protein degradation in macrophages, the effects of FSK on proteasome activity and ubiquitination of iNOS protein were examined. FSK inhibited the proteasome activity (28%) in untreated control and in LPS/IFN-treated macrophage cells (Fig. 6B). Furthermore, to examine the effect of FSK on the ubiquitination of iNOS protein, the macrophages were incubated with LPS/IFN and FSK for 14 h and ubiquitinated iNOS protein was isolated using polyubiquitin affinity beads.

Fig. 4. ATF-2 mediates FSK-mediated differential regulation of iNOS mRNA expression in C6 glioma cells and RAW264.7 macrophages. (A) The effect of forskolin (FSK) on lipopolysaccharide (LPS)/interferon-γ (IFN)-mediated increase in phosphorylation of ATF-2 was examined. C6 rat glioma cells and RAW264.7 macrophages were stimulated with 1 μg/ml of LPS plus 10 unit/ml of IFN and 50 ng/ml of LPS plus 0.2 unit of IFN, respectively. Forskolin (FSK) or dimethyl sulfoxide as a vehicle (VHC) pretreatment was done 0.5 h prior to LPS/IFN treatment. A half hour after LPS/IFN treatment, the nuclear proteins were extracted and 10 μg of nuclear protein was used for the detection of level of phospho-ATF-2. (B) To confirm the involvement of ATF-2 in iNOS gene expression, cells were cotransfected with dominant negative (DN) ATF-2 and ~3.2 kb 5'-flanking region of iNOS gene linked with luciferase gene. Twenty-four hours after LPS/IFN treatment, the cells were lysed and the activity of luciferase was measured by standard protocol. The vertical bar in each panel indicates the standard error of mean (**p < 0.01, ***p < 0.001 compared to control group; ^p < 0.05, ~p < 0.01 compared to LPS/IFN-treated group).

Fig. 5. FSK differentially regulates LPS/IFN-mediated iNOS protein expression in C6 glioma cells and RAW264.7 macrophages. The time course effect of forskolin (FSK; 100 μM) on the iNOS protein levels (A) and nitric oxide (NO) production (B) were compared after lipopolysaccharide (LPS)/IFN treatment in C6 rat glioma cells and RAW264.7 macrophages. To stimulate iNOS expression, combinations of LPS plus IFN were used in C6 rat glioma cells (1 μg LPS + 10 unit IFN/ml) and RAW264.7 macrophages (50 ng LPS + 0.2 unit IFN/ml). FSK or dimethyl sulfoxide as a vehicle (VHC) pretreatment was done 0.5 h prior to LPS/IFN treatment; 50 μg of total protein was used for the detection of iNOS protein level. For Western immunoblot assay of iNOS protein, the macrophages were incubated with LPS/IFN and FSK for 14 h and ubiquitinated iNOS protein was isolated using polyubiquitin affinity beads.

Fig. 6A, MG132 treatment enhanced the in situ levels of iNOS protein in both cell lines, suggesting that spontaneous elimination of iNOS protein may be mediated by the proteasome. Furthermore, MG132 treatment abolished the difference in iNOS protein levels in LPS/IFN- and FSK+LPS/IFN-treated macrophages. These results suggest that cAMP regulates the iNOS protein stability via regulation of proteasome-mediated pathway.
As shown in Fig. 6C, LPS/IFN increased the ubiquitinated iNOS protein level, while FSK treatment reduced the levels of ubiquitinated iNOS protein. These results indicate that FSK delays the spontaneous iNOS protein degradation via inhibition of ubiquitination of iNOS protein. Therefore, reduced activity of proteasome and reduced ubiquitination of iNOS protein by FSK may play roles in the increased stability of iNOS protein by FSK in macrophages.

**DISCUSSION**

As summarized in Fig. 7, the data presented here demonstrate that the differential regulation of iNOS expression in macrophages and glial cells by cAMP is due to the selective inhibition of p38-MAPK/ATF-2 activation and iNOS stability. The differential regulation of iNOS expression in macrophages and glial cells by cAMP is due to the selective inhibition of p38-MAPK/ATF-2 activation in glial cells and to cAMP-mediated iNOS protein stabilization in macrophages. These conclusions are based on the following observations. (1) The LPS/IFN-induced increases in iNOS protein level and NO production were inhibited in glial cells but enhanced in macrophages by FSK, an activator of adenylyl-cyclase. (2) In glial cells, FSK inhibited the LPS/IFN-induced increase in iNOS mRNA expression via blocking p38-MAPK/ATF-2 activations. On the other hand, in macrophages, FSK had no effect on LPS/IFN-mediated increases in iNOS mRNA level or p38-MAPK/ATF-2 activation. (3) Rather than induction of iNOS mRNA expression, FSK increased the iNOS protein levels by inhibition of spontaneous ligation of ubiquitin to iNOS protein. To our understanding, this is first the report to document the involvement of cAMP pathway in posttranslational regulation of in situ level of iNOS protein.
Studies from our laboratory [17,18] and others [14–16,23] have previously reported an inhibitory effect of cAMP on LPS- or cytokine-mediated NO release and iNOS gene expression in glial cells. These studies are consistent with our observations in the present study that FSK inhibits the LPS/IFN-mediated induction of iNOS mRNA, protein, and NO production in glial cells. However, there is some controversy on the regulation of iNOS gene expression by cAMP pathway in macrophages. In RAW264.7 cells and rat peritoneal macrophages, cAMP-increasing reagents or cAMP analogues (FSK, cholera toxin, dibutyryl-cAMP, and 8-bromo-cAMP) further enhanced the LPS-mediated NO production and iNOS protein expression [17,30,31]. Conversely, in one study 8-Bromo-cAMP was reported to inhibit IFN-induced iNOS mRNA and protein induction in RAW264.7 macrophages [32]. In this study, we also observed FSK-enhanced NO production and iNOS protein expression after 24-h LPS treatment in RAW264.7 macrophages. The use of different stimuli (i.e., IFN vs LPS) and/or different levels of the intracellular level of cAMP or its analogue may account for these observed differences in levels of iNOS protein.

Previously, Eberhardt and colleagues [26] demonstrated that a region between bp −277 and −111 bearing a C/EBP response element is critical for cAMP-mediated iNOS gene induction via interaction with C/EBPβ and δ in rat mesangial cells. We previously confirmed the critical role of C/EBP response element in iNOS promoter in the induction of iNOS gene in glial cells [33]. Furthermore, we also observed that LPS-mediated increase in C/EBP DNA binding activity is mainly due to C/EBPβ. Consistent with these results, we also observed in this study that C/EBPβ, but not C/EBPβ, positively regulates iNOS gene expression. However, even with the decrease in iNOS expression, C/EBP DNA binding activity and C/EBPβ protein expression were further enhanced by cAMP in glial cells, suggesting that transcription factors other than C/EBP may be involved in cAMP-mediated inhibition of iNOS gene expression in glial cells.

Cytokine-mediated transcriptional induction of the human iNOS gene requires both AP-1- and NFκB-binding sites [34]. Disruption of human iNOS promoter at AP-1 (at −5115) or NFκB (at −115 and −8283) sites reduces promoter activity. JunD and Fra-2 were identified in the heterodimers that bound to upstream and downstream AP-1 sites in the 8.3-kb hiNOS promoter [28,34]. NFκB is also essential for rodent iNOS gene expression, whereas, in contrast to humans, AP-1 has a negative role in rodent iNOS gene regulation [35,36]. In the present study, FSK inhibited expression of c-Jun and JunD. However, over-expression of wild type c-Jun down-regulated the iNOS promoter activity. Taken together, the lack of effect of FSK on NFκB in both cell types and inhibition of LPS/IFN-induced expression of c-Jun and JunD proteins indicates that NFκB or AP-1 may not play a role in FSK-mediated differential regulation of iNOS expression.

Since FSK was previously reported to down-regulate iNOS gene expression via inhibition of p38-MAPK in glial cells, and ATF-2 was recognized as the downstream target of p38-MAPK in glial cells, and macrophages, and other cell types [23,37,38], we examined the possible role of p38-MAPK/ATF-2 in differential regulation of LPS/IFN-mediated induction of iNOS in glial cells and macrophages. Similar to the observed induction of iNOS, FSK inhibited the LPS/IFN-mediated p38-MAPK and ATF-2 activations in glial cells, but FSK had no effect in macrophages. Since we observed the inhibition of iNOS promoter activity by dominant negative p38-MAPK and ATF-2, these results suggest the possible involvement of p38/ATF-2 in the differential regulation of iNOS mRNA expression in glial cells and macrophages. However, the mechanism of selective inhibition of p38-MAPK in glial cells is not known at this time.

In this study we have demonstrated that the stability of iNOS protein induced by LPS/IFN is increased by FSK via inhibition of ubiquitination. This conclusion is consistent with the observed increased degradation of iNOS protein in transforming growth-factor-β-treated mouse peritoneal macrophages [39], glucocorticoid-mediated suppression of iNOS expression in IL-1β-treated rat glomerular mesangial cells [39], and IFN-treated RAW264.7 macrophages [40]. Moreover, recent studies by Musial and Eissa [41] suggested that iNOS is primarily regulated by the proteasome pathway in RAW264.7 and HEK293 cells. Consistent with proteasomal degradation, iNOS was found to be ubiquitinated, and the formation of ubiquitin conjugates was critical for its degradation [42]. Although these studies suggest the essential role of the proteasome or other proteolytic pathway in the spontaneous elimination of iNOS protein, the detailed regulatory mechanism of proteasome-mediated iNOS degradation is not understood. In mammalian cells, ubiquitination can be modulated by phosphorylation of certain sites on a protein. For example, the degradation of IκB is enhanced by its phosphorylation on specific serine residues by IKK [43]. In this study, we observed that LPS/IFN increased the phosphorylation of serine and threonine residues on iNOS protein (data not shown). However, we were not able to observe reduction of the total level of phospho-serine or -threonine on iNOS protein by FSK (data not shown). These results suggest that the phosphorylation of specific sites rather than the degree of phosphorylation on serines and threonines may be important for the regulation of iNOS ubiquitination. Previous studies reported that the regulation of proteo-
lytic degradation of iNOS appears to be important in the development of salt-induced hypertension in Dahl/Rapp rats. An inbred strain of Dahl/Rapp rats that are susceptible to salt-induced hypertension have a serine\(^{714}\) to proline mutation in iNOS, whereas Dahl/Rapp rats that are resistant to salt-induced hypertension do not. This mutation in iNOS does not change the enzyme activity but confers to the mutated protein a shorter half-life than the wild-type iNOS when transfected into COS cells\([44]\). Furthermore, this increased turnover can be largely prevented by specific proteasome inhibitor (clasto-lactacystin \(\beta\)-lactone), implicating a role of the proteasome in this process. However, it remains to be seen whether serine\(^{714}\) phosphorylation is involved in cAMP-mediated signaling cascade. In addition to ubiquitin/proteasome-mediated proteolysis, dexamethasone-induced degradation of IFN-induced iNOS protein was reported to be mediated by calpain in RAW264.7 cells\([40]\). Since recent reports suggested that mammalian calpain is up-regulated by TNF\([45]\), and negatively regulated by PKA\([46]\), it suggests possible involvement of the PKA pathway in the inhibition of spontaneous degradation of iNOS protein via other proteolytic pathways. Therefore, the involvement of other proteolytic pathways should be also examined in future studies. The finding of cAMP-mediated regulation of iNOS stability provides another target for therapeutics in the regulation of iNOS enzyme expression in inflammatory disease conditions.

Acknowledgments — The authors are thankful to Dr. Anne G. Gilg for proofreading of the manuscript and Ms. Joyce Bryan and Ms. Hope Terry for laboratory assistance and secretarial assistance, respectively. These studies were supported by grants from National Institute of Health (NS-22576, NS-34741, NS-37766, and NS-40810).

REFERENCES


