Forskolin inhibits expression of inducible nitric oxide synthase mRNA via inhibiting the mitogen activated protein kinase in C6 cells

Je-Seong Won, Jin-Koo Lee, Hong-Won Suh*

Department of Pharmacology and Institute of Natural Medicine, Hallym University, 1 Okchun-Dong 1, Chunchon, Kangwon-Do, 200-702, South Korea

Accepted 30 January 2001

Abstract

This study has demonstrated the mechanism of protein kinase A (PKA)-dependent inhibition of astrocytic nitric oxide production and inducible NO synthase mRNA expression induced by lipopolysaccharide. In C6 glioma cells, the stimulation with lipopolysaccharide (LPS; 1 μg/ml) evoked increases of nitric oxide (NO) production, NO synthase (iNOS) mRNA expression, phosphorylation of p38 mitogen activated protein kinase (p-p38), and the activation of NFκB. LPS-induced NO production and iNOS mRNA expression were inhibited by the pretreatment with forskolin (FSK; 5 μM) in a dose-dependent manner, and which were reversed by PKA inhibition by compound H89. Furthermore, LPS-induced increases of p-p38, but not activation of NFκB, were also reduced by FSK and H89 reversed the FSK-induced inhibition response. The dose-dependent inhibition of NO production and iNOS mRNA expression by compound SB203580 (p38 inhibitor) suggests the participation of p38 in PKA-dependent inhibition of LPS-induced NO production and iNOS mRNA expression. However, the activation of NFκB by LPS also not affected by SB203580. Therefore, our results suggest that, in C6 glioma cells, LPS-induced NO production and iNOS gene expression may be regulated by PKA pathway through the reduction of activity of p38 kinase. This inhibitory role of PKA may not involve the activation of NFκB. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Signal transduction: gene expression

Keywords: Forskolin; Inducible nitric oxide synthase; p38 MAPK; Protein kinase A; Gene expression; C6 glioma cell

1. Introduction

Nitric oxide (NO) is a short-lived molecule that mediates a wide range of biologic effects, such as vasorelaxation [40], neuro-transmission [19], inhibition of platelet aggregation [42], and microbial and tumor cell killing [34]. In the brain, three genes encode NO synthase isoforms, with significant differences in their regulation. Neuronal and endothelial NOS are constitutively expressed in astrocytes and in subpopulation of neurons and predominantly regulated through intracellular calcium/calmodulin signals [6,7]. The third NOS, an inducible NOS (iNOS) which is present in macrophages and astrocytes, is regulated at the transcriptional level in response to stimuli (e.g. cytokine/lipopolysaccharide) [24,34]. Although NO produced by iNOS accounts for the bacterial and tumoridal properties of macrophages, it is of particular importance in the pathophysiology of several inflammatory neurological diseases, including demyelinating disorders (e.g. multiple sclerosis, experimental allergic encephalopathy, and X-adrenoleukodystrophy) and in ischemia and traumatic injuries associated with infiltrating macrophages and the production of proinflammatory cytokines [4,10,21,27,31,32]. Astrocytes in the healthy brain do not express iNOS; however, after ischemic, traumatic, neurotoxic, or inflammatory damage, the reactive astrocytes express iNOS in rodent and human [17,22,36–38]. Although NO derived from both astrocytes and macrophages has been believed to be involved in demyelinating disease and neuronal death during ischemia and trauma.
[4,31,32], little is known about the signal transduction pathway of iNOS induction in astrocytes.

Current studies have suggested that the inhibitors of tyrosine kinase suppress astroglial iNOS gene expression and NO production induced by cytokines or bacterial LPS [15,47]. Among tyrosine kinases, p38 mitogen activated protein (p38) kinase have suggested to mediate LPS or cytokine induced iNOS induction in primary rat and mouse astrocyte-enriched culture [2,11]. However, another MAP kinase, an extracellular signal regulated protein kinase (ERK), is reported to be involved selectively in iNOS gene expression in primary cultured rat astrocytes but not in mouse [2,11]. Whether this differential dependency of iNOS gene expression on ERK pathway due to the difference of signal transduction mechanisms between in rats and mice astrocytes, or microglia contamination in the primary culture has been not been well characterized.

Another strike feature of astroglial iNOS gene expression by LPS or cytokines is negative regulation by a cAMP-dependent protein kinase (PKA). In rodent astrocytes, several compounds (forskolin, 8-bromo-cAMP, and (Sp)-cAMP) that increase cAMP and activate PKA were found to inhibit LPS- and cytokine-mediated production of NO as well as the expression of iNOS, whereas compounds (H-89 and (Rp)-cAMP) that decrease PKA activity stimulated the production of NO and the expression of iNOS in rat primary astrocytes [36]. Although it has been shown by several groups that augmentation of intracellular cAMP blocks the signaling pathway from Ras to MAP kinase in cells such as fibroblasts and fat cells by phosphorylation of Raf (an upstream member of MAP kinase, such as ERK) [9,20,51], the detailed signal transduction mechanisms underlying PKA-dependent attenuations of LPS- or cytokines-induced iNOS gene expression and NO production have not been well characterized.

In addition to various signaling kinases, the present study also have focused on delineating the effect of PKA on of nuclear factor κB (NFκB). Currently, computer-assisted analysis of rodent iNOS promoter and in vitro transfection assay identified a potential site for the binding of NFκB [53]. NFκB is an ubiquitous transcriptional factor and a pleiotropic regulator of the inducible expression of many genes that encode proteins involved in the modulation of inflammatory and host defense process [44,50]. Activation of NFκB by cytokines or oxidative stress requires either the degradation of its cytoplasmic inhibitor IkB-α or proteolytic cleavage of p105 [3]. Free NFκB dimers translocate to the nucleus and activate target gene. Indeed, NFκB has been believed to be a most important transcriptional factor in the regulation of iNOS transcription in various cell types including astrocytes [14,30,39,45]. Given the importances of NFκB in iNOS gene expression, we set out to determine the effect of PKA activation on LPS-induced change of NFκB DNA binding activities and its constituent factors, such as p50, p52, p65, and c-Rel using gel shift and supershift analysis.

2. Materials and methods

2.1. C6 rat glioma cell cultures and serum starvation

C6 rat glioma cells obtained from Korean Cell Line Bank were maintained in DMEM/F-12 medium containing 10% fetal bovine serum and 2 mM gentamicine. At 70% confluency, the cells were incubated with serum free DMEM/F-12 medium for 24 h prior to the incubation with LPS and other chemicals.

2.2. Assay for NO synthesis

Synthesis of NO was determined by an assay of the culture supernant for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent [16] and incubate at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all experiment. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

2.3. Total cellular protein extraction and western blot analysis

After incubation in the presence or absence of different stimuli, cells were washed two times with cold Tris buffered saline (TBS; 20 mM Trizma base, and 137 mM NaCl, pH 7.5). Immediate after washing, cells were lysis with SDS lysis buffer (62.5 mM Trizma base, 2% w/v SDS, 10% glycerol) containing 0.1 mM Na₃VO₄, 3 mg/ml aprotonin, and 20 mM NaF. After brief sonication to shear DNA and reduce viscosity, the concentration of protein was determined with the detergent compatible protein assay reagent (Bio-Rad Laboratories, CA, USA) using bovine serum albumin (BSA) as the standard. After adding with dithiothreitol (5 mM) and bromophenol blue (0.1% w/v), the proteins were boiled, separated by electrophoresis in 10–12% polyacrylamide gels, and transferred onto a polyvinylidene difluoride membrane. The membranes were immunoblotted with antibodies against phospho-p38 (New England Biolabs) and phospho-ERK1/2 (New England Biolabs) and visualized with ECL-plus solution (Amersham Life Science Co., UK).

2.4. Isolation of total RNA and iNOS northern blot analysis

Total cellular RNA was extracted from primary cultured astrocytes using a rapid guanidine thiocyanate–water saturated phenol–chloroform extraction procedure and subsequent precipitation with acidic sodium acetate [8]. Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples
were subjected to spectrophotometric analysis at 260 and 280 nm. Total RNA (5 μg) was denatured and electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon hybond-N hybridization membrane sheets (Amersham, Buckinghamshire, UK). After baking for 1–2 h at 80°C, the membranes were prehybridized at 68°C for at least 1 h in prehybridization buffer (5×SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent). The digoxigenin (DIG)-labeled iNOS probes were added to prehybridization buffer containing 50% formamide. The membranes were incubated at 68°C in a shaking water bath, and washed twice for 10 min per wash in 2×SSC and 0.1% SDS at room temperature. Then, the membranes were washed twice for 15 min per wash with 0.1×SSC and 0.1% SDS. After equilibrating the membranes in Buffer I (100 mM maleic acid and 150 mM NaCl [pH 7.5]) for 1 min, the membranes were gently agitated in Buffer II (1% blocking reagent in Buffer I) for 30–60 min. The membranes were hybridized with the diluted anti-DIG-alkaline phosphatase [1:10 000 (75 mU/ml)] in Buffer II for 30 min. After washing the membranes twice for 15 min per wash in 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III (100 mM Tris–HCl [pH 9.5], 100 mM NaCl, and 50 mM MgCl₂) for 2 min. Diluted CSPD⁺⁺ (Boehringer Mannheim) (1:100 dilution in Buffer II) was spread over the surface of the membranes. After incubation of the membranes at 37°C for 15–20 min, the membranes were exposed to Hyperfilm-ECL (Amersham) for detection of the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterilized (Millipore) water, then the membranes were washed for overnight at 65°C in 50 mM Tris–HCl (pH 8.0), 50% dimethylformamide and 1% SDS to remove the hybridized probe and rehybridized to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle [12,49]. The cRNA probes for iNOS [18] and cyclophilin [12] were synthesized in vitro from linearized expression vectors with DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim).

2.5. Preparation of nuclear extracts and non-isotopic electrophoretic mobility–shift assay

Nuclear extract from stimulated or unstimulated C6 cells (1 x 10⁷ cells) were prepared using the method of Dignam et al. with slight modification [13]. Cells were harvested, washed twice with ice-cold TBS, and lysed in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 5000×g for 5 min. The pellet nuclei were washed with buffer A without Nonidet P-40, and resuspended in 40 μl of buffer B (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 15 000×g for 15 min. Supernatant containing the nuclear proteins were diluted with modified buffer C (20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol) and stored at −70°C until use. Nuclear proteins were used for the electrophoretic mobility shift assay for detection of NFκB DNA binding activity. The double strands of NFκB (5′-AGT-TGAGGG-GAC-TTT-CCC-AGG-C-3′) were annealed by incubating an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl at 95°C for 10 min, and then the mixture was allowed to cool to room temperature. DNA-protein binding reactions were carried out at room temperature for 20 min and reaction mixtures contained 10 μg of total protein, 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 specified probe labeled with DIG-ddUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Protein–DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% polyacrylamide (30:1, acrylamide:bisacrylamide) gels. Gels were run at room temperature in 50 mM Tris (pH 8.3), 0.38 M glycerine, and 2 mM EDTA, and electrophoblotted onto positively charged nylon membranes. The membranes were UV-crosslinked, washed with 0.3% Tween 20 in Buffer I, and hybridized with the diluted anti-DIG-alkaline phosphatase [1:10 000 (75 mU/ml)] in Buffer II for 30 min. After washing five times for 15 min with 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III (100 mM Tris–HCl [pH 9.5], 100 mM NaCl, and 50 mM MgCl₂) for 2 min. The method for detection of chemiluminescence was identical to the method used for the non-isotopic northern blot analysis. For supershift assay, 2 μg of antibodies against p50 (NFκB), p52 (NFκB), p65 (NFκB), and c–Rel were added into DNA–protein binding reaction mixture 1 h prior to adding DIG-labeled probes.

2.6. Statistical analysis

All values shown in the figures of NO production and iNOS mRNA level are expressed as mean±standard error of mean (S.E.M.) of n determinations, obtained on at least three independent experiment days. The expression of iNOS mRNA level was quantified with the Bio-profile Bio-1D application (Vilber-Lourmat, France). 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.

3. Results

3.1. Activator of PKA (FSK) inhibits the expression of iNOS mRNA and production of NO induced by LPS in C6 rat glioma cells

We investigated the effect of forskolin (FSK) on the induction of iNOS and production of NO in C6 rat glioma cells in serum-free DMEM/F12 media. As shown in Fig. 1A, 1 μg/ml of bacterial LPS induced the iNOS mRNA level significantly at 3 h, and maximal increase of iNOS mRNA level (about 11 fold) was detected at 6 h after LPS treatment. To investigate the effect of FSK on LPS-induced iNOS mRNA expression, serial diluted FSK (0.1, 1, and 10 μM) was pretreated 20 min prior to 6 h LPS stimulation. Fig. 1B shows that LPS-induced iNOS mRNA level was dose-dependently attenuated by FSK. The significant attenuation was detected at a concentration of 1 μM of FSK. FSK-dependent attenuation of LPS-induced iNOS mRNA level was reversed by the pretreatment with H89 (20 μM; PKA inhibitor) 20 min prior to FSK (1 μM) treatment, suggesting that the effect of FSK on iNOS mRNA accumulation is PKA specific. A similar pattern is also represented in NO accumulation. As shown in Fig. 1C, LPS induced NO production was dose-dependently inhibited by the pretreatment with FSK. Furthermore, the inhibitory action of FSK against LPS-induced NO production also significantly reversed by the pretreatment with H89 (20 μM).

3.2. Activator of PKA (FSK) inhibits the activation of MAP kinases induced by LPS in C6 rat glioma cells

According to previous studies that the kinase activities of ERKs [1,5] and p38 [43] correlated their dual phosphorylation at specific sites (Tyr202/Tyr204 in ERK1/2 and Tyr180/Tyr182 in p38), the activities of these three MAP kinases were indirectly monitored using antibodies recognizing dual phosphorylation sites. As shown in Fig. 2A, heavy quantity of dual phosphorylated ERK1/2 (p-ERK1/2) levels were detected in resting cells, and which were not affected by 1 μg/ml of LPS at indexed time point (0.5, 1, 3, 6, 12, and 24 h). In contrast to p-ERK1/2, dual phosphorylated p38 (p-p38) markedly induced 0.5–1 h after LPS treatment, and which was returned to the basal level 12 after LPS treatment.

To investigate the effect of PKA activation on the LPS-stimulated or unstimulated p-ERK1/2 and p-p38 levels, concentration dependency of FSK-induced inhibitory action was examined. As shown in Fig. 2B, in the presence of 0.1 μM of FSK, the basal p-ERK1/2 level and LPS-induced p-p38 began to reduce, and were further attenuated by 1 and 10 μM of FSK pretreatment. Furthermore, FSK-induced inhibition response was also reversed by H89 pretreatment (Fig. 2C).

3.3. Involvement of MAP kinases in LPS-induced iNOS transcription and NO production

To investigate the possible involvement of the ERK1/2 and p-p38 in the regulation of iNOS transcription and NO production, SB203580, a specific inhibitor of p38 [29], and PD98059, a specific inhibitor of MEK1/2 which is upstream kinases of ERK1/2 [41], were pretreated 20 min prior to LPS treatment. SB 203580 treatment caused a reduction of reduced LPS-induced iNOS mRNA level and NO production in a concentration-dependent manner (Fig. 3A and B). At a concentration of 1 μM, SB203580 showed a significant attenuation of LPS-induced increase of iNOS mRNA level was detected, while 0.1 μM of SB203580 was sufficient to reduce significantly the LPS-stimulated NO production. In contrast to SB203580, PD98059 (0.1, 1, and 10 μM) did affect neither LPS-induced increase in iNOS mRNA expression nor NO production.

3.4. Effect of FSK, SB203580, and PD98059 on LPS-induced NFkB and their DNA binding activity

As shown in Fig. 4A, LPS began to increase NFkB DNA binding activity 0.5 h after stimulation. This enhancement was further increased until 6 h and began to decrease 12 h after LPS treatment. In a competition study, LPS-induced increase of NFkB DNA binding activity was not affected by unlabelled AP-1 and CRE, but completely inhibited by unlabelled NFkB DNA oligomer, suggesting that this DNA binding activity was NFkB sequence specific. In addition, antibodies against p50 and p65, but not p52 and c-Rel, attenuated LPS-induced increase of NFkB DNA binding activity, suggesting that LPS-induced NFkB DNA binding activity is mainly composed of p50 and p65 in C6 rat glioma cells.

To investigate the involvement of NFkB on LPS-induced iNOS gene expression, we examined the effect of FSK, SB203580, and PD98059 on LPS-induced NFkB DNA binding activity. As shown in Fig. 4B, FSK (0.1, 1, and 10 μM), SB203580 (0.1, 1, and 10 μM), or PD98059 (0.1, 1, and 10 μM) did not alter the increase of NFkB DNA binding activity induced by LPS at 1 h after stimulation.

4. Discussion

The similarity of properties of C6 rat glioma cells (rat type I astrocyte-derivative cell line) and astrocytes in iNOS gene expression suggests that C6 cells can be used as a model for the study of astrocyte iNOS [16,36,46]. The
Fig. 1. Activator of PKA (forskolin) inhibits the expression of inducible nitric oxide synthase mRNA and the production of nitric oxide in lipopolysaccharide-induced C6 rat glioma cells. (A) At the indexed time points (0.5, 1, 3, 6, 12, and 24 h) after lipopolysaccharide (LPS; 1 µg/ml) stimulation, the level of inducible nitric oxide synthase (iNOS) mRNA was examined using Northern blot analysis. At 6 h after LPS stimulation, the concentration dependent effect of forskolin (FSK; 0.1, 1, and 10 µM) on LPS-induced (B) iNOS mRNA level and (C) nitrite level in culture supernatant and reversed effect of H89 (10 µM) were examined using the methods as described in Material and methods. FSK was pretreated 20 min prior to LPS treatment. H89 was pretreated 20 min prior to FSK (1 µM) treatment. The unregulated mRNA level of cyclophilin (CPN) was used for normalizing the increase in iNOS mRNA level in Northern blot analysis. The vertical bar in each panel indicates the standard error of mean (*, P<0.05; **, P<0.01; ***, P<0.001 compared to the control group; +, P<0.05; ++, P<0.01; ++++, P<0.001 compared to LPS-stimulated group; ###, P<0.01; ####, P<0.001 compared to FSK-LPS group).

study related to NO and iNOS in which C6 cell line culture also provide an advantage that was brought by avoidance of the possible contributions and interactions with other cell types such as microglia, oligodendroglia, macrophages, and fibroblasts in primary astrocyte cultures. Therefore, in the present study, we have demonstrated that
the signal transduction pathway of PKA-dependent inhibition of NO production and iNOS mRNA expression induced by LPS using C6 rat glioma cells.

Recent studies have provide the evidence that cAMP induces the expression of iNOS in LPS- and cytokine-stimulated glomerular mesangial cells [33], smooth muscle cells [26], cardiac myocytes [23], murine 3T3 fibroblasts [25], and peritoneal macrophages [48]. The increase in cAMP as a result of inhibition of phosphodiesterase (an enzyme that degrades cAMP), an exogenous supply of cAMP derivatives, or compounds that enhance intracellular level of cAMP cause induction of iNOS. Contrary to these results, we have observed that FSK, a compound increasing intracellular levels of cAMP, negatively and dose-dependently regulate the expression of iNOS and NO production in LPS-stimulated C6 cells (Fig. 1B). In addition, a reversal effect of H89 against the inhibitory action of FSK in the expression of iNOS mRNA level and NO production suggests that the inhibitory action of FSK mainly exerts through a PKA pathway in LPS-stimulated C6 cells. This result is in line with several studies in that the inhibition of LPS- and cytokine-mediated iNOS gene expression were inhibited by compounds, such as FSK, 8-bromo-cAMP, and (Sp)-cAMP, in primary cultured astrocytes [36]. Furthermore, our data are consistent with a report published by Pahan et al., where FSK also inhibits the NO production induced by a combinatorial treatment with LPS and tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), or interleukin-1β (IL-1β) in C6 rat glioma cells. In spite of this result that the increase in intracellular cAMP negatively regulate the expression of astrocyte iNOS gene expression in contrast to other cell types, such as glomerular mesangial cells, smooth muscle cells, cardiac myocytes, fibroblasts, and macrophages, the mechanisms underlying diverse role of cAMP depend on cell types has been currently unveiled.

One approach to characterize the PKA-evoked inhibition of LPS-induced iNOS gene expression is a possible role of PKA in the regulation of MAP kinase activation. In a recent study, the selective inhibitors of p38 and ERK1/2 dramatically reduces the gene expression of iNOS and NO production induced by LPS or cytokine in cultured murine astrocyte-enriched culture [11], although there are controversies in involvement of ERK1/2 in the regulation of LPS-induced iNOS gene expression [2]. In the present study, we found that LPS induces the active phosphorylated form of p38, and which were effectively inhibited by FSK in the presence of LPS. Although the current studies have suggested that PKA-dependent ERK1/2 inactivation is the PKA-mediated phosphorylation of Raf-1 (an upstream member of ERK1/2 pathway) which decreases its affinity for Ras and prevents its action [9,20,51], the PKA-dependent inhibition of p38 activity has been recently unveiled in C6 rat glioma cells as well as other cell types. The exact signal transduction mechanism underlying FSK-evoked inhibition of the activities of p38 as well as ERK1/2 in C6 and astrocytes should be further determined.

To investigate whether the decreased activities of p38 and ERK1/2 by FSK affect LPS-induced iNOS gene expression, we examined the effect of SB203580 (a
Fig. 3. Involvement of MAP kinases in lipopolysaccharide-induced inducible nitric oxide synthase transcription and nitric oxide production in C6 rat glioma cells. At 6 h after lipopolysaccharide (LPS; 1 μg/ml) stimulation, the concentration dependent effect of (A) SB203580 (0.1, 1, and 10 μM) or (B) PD98059 (0.1, 1, and 10 μM) on LPS-induced inducible nitric oxide synthase (iNOS) mRNA level and nitrite level in culture supernatant were examined using the methods as described in Material and methods. SB203580 and PD98059 were pretreated 20 min prior to LPS treatment. The unregulated mRNA level of cyclophilin (CPN) was used for normalizing the increase in iNOS mRNA level in Northern blot analysis. The vertical bar in each panel indicates the standard error of mean (*, P < 0.05; **, P < 0.01; ***, P < 0.001 compared to the control group; +, P < 0.05, ++, P < 0.01, ++++, P < 0.001 compared to LPS-stimulated group).

selective inhibitor of p38) and PD98059 (a selective inhibitor of MEK1/2, a upstreamal kinase of ERK1/2) on LPS-induced iNOS mRNA expression and NO production. As shown in Fig. 3A and B, SB203580, but not PD98059, effectively inhibited the LPS-induced iNOS mRNA expression and NO production. This observation does not accord with the result in primary cultured rat glial cells, where PD98059 also negatively regulates LPS-induced iNOS mRNA expression [2]. However, our result is in line with a study using in mouse astrocytes, in which p38, but not ERK1/2, was involved in LPS-induced iNOS gene expression in mouse astrocytes [11], and further supported by a recent study where the inhibition of INF-γ-activated ERK1/2 by PD98059 or by Ras dominant negative expression did not affect iNOS induction in C6 glioma cells [35]. Therefore, our result suggests that FSK-evoked inhibitions against iNOS mRNA expression and NO production induced by LPS are probably mediated through blocking a p38, rather than ERK1/2, pathway in C6 rat glioma cells. However, the reason for this different role of ERKs in the regulation of LPS-induced NO production and iNOS mRNA expression in the different cell types has been not currently understood.

Identification of the DNA binding site for NFκB in the promoter region of iNOS [52,53], and inhibition of iNOS induction by inhibitors of NFκB activation have established an essential role of NFκB activation in the induction of iNOS [28,37,39]. Suppression of NFκB and inhibition of iNOS gene expression by inhibitors of tyrosine kinase in different cell types suggested the possible involvement of tyrosine phosphorylation in the activation of NFκB and the induction of iNOS. In the present study, although LPS induced NFκB in C6 cells, we found FSK-evoked attenuation of iNOS mRNA and NO production induced by LPS was not correlated with NFκB DNA binding activity. Our result is not consistent with the result in primary cultured...
Fig. 4. Effect of forskolin, SB203580, and PD98059 on lipopolysaccharide-induced NFκB DNA binding activity. (A) At the indexed time points (0.5, 1, 3, 6, 12, and 24 h) after lipopolysaccharide (LPS; 1 μg/ml) stimulation, the NFκB DNA binding activity was examined using gel shift analysis as described in Material and methods. For cross/self competition assay or supershift/attenuation assay, 50×unlabelled AP-1, CRE, and NFκB DNA oligomers or 2 μg of specific antiserum against p50, p52, p65 or c-Rel, was added to each reaction. (B) At 3 h after LPS stimulation, the concentration dependent effect of forskolin (FSK; 0.1, 1, and 10 μM), SB203580 (0.1, 1, and 10 μM), or PD98059 (0.1, 1, and 10 μM) on LPS-induced NFκB DNA binding activity were examined. FSK, SB203580, or PD98059 was pretreated 20 min prior to LPS treatment.

In summary, in the present study, we have demonstrated that PKA activation-dependent inhibition in LPS-induced NFκB activity. Although the molecular basis of the discrepancy of the effect of FSK on LPS-induced NFκB DNA binding activity between in C6 glioma cells and primary rat astrocytes currently unknown, it may be due to the differential signal transduction mechanisms between in cancer cell line and primary cells. In addition to FSK, SB203580 and PD98059 also did not affect the NFκB DNA binding activity induced by LPS, suggesting that neither p38 nor ERK1/2 affect NFκB activation. Our result is supported by a report that p38 does not regulate the activation of NFκB in primary cultured mouse astrocytes [11]. Therefore, our results suggest that NFκB activation appears not to be involved in PKA activation- or p38 inhibition-dependent attenuation of NO production and iNOS mRNA expression. However, NFκB may represent a second independent pathway for iNOS induction, since NFκB has been reported to be an important factor for iNOS transcription in many cell types including C6 cells and astrocytes [14,30,39,45].

In summary, in the present study, we have demonstrated that PKA activation-dependent inhibition in LPS-induced iNOS mRNA expression and NO production in C6 rat glioma cells may involve the inhibition of LPS-induced p38. In addition, these inhibitory actions of PKA activation may not be involved in the attenuation of LPS-induced NFκB activity. PKA and NFκB may lie on two distinct pathways as shown by the present finding that PKA activation and p38 inhibition did not interfere with activation of NFκB.

Acknowledgements

This research was supported by the grant from Korea Research Foundation (1998-001-F00334) and Hallym Academy of Sciences in Hallym University (2000).

References


