The regulation of inducible nitric oxide synthase gene expression induced by lipopolysaccharide and tumor necrosis factor-α in C6 cells: involvement of AP-1 and NFκB

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

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

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Abstract

The roles of AP-1 and NFκB in the regulation of inducible nitric oxide synthase (iNOS) mRNA expression induced by the combination of lipopolysaccharide and tumor necrosis factor-α (LT) in C6 cells were examined in the present study. The iNOS mRNA level and NO release were increased by several cytokines alone or combination treatments at 24 hr. LT-induced iNOS mRNA level was maximally increased at 6 hr and maintained at higher level at least up to 24 hr. At 6 hr, iNOS protein level and NO release were also increased by LT. By western blot analysis, AP-1, such as Fra-1, Jun B, and phospho-CREB protein levels were increased by LT and translocation of NFκB p52 from the cytoplasm to the nucleus was increased. In addition, phosphorylations of MAPKs (ERK 1/2, p38, JNK 1/2) were increased by LT. LT-induced iNOS mRNA level was inhibited by PD98059 (MEK 1/2 inhibitor), SB203580 (p38 inhibitor), and cycloheximide (a protein synthesis blocker), indicating that the phosphorylation of ERK 1/2 and p38, and on-going protein synthesis are necessary for LT-induced iNOS expression. Electrophoretic mobility shift assay (EMSA) showed that AP-1 and NFκB DNA binding activities were increased at 6 hr and these AP-1 and NFκB DNA bands increased by LT were super-shifted when Fra-1, Jun B, or NFκB p50 antibody was coincubated. These findings strongly suggest that, in C6 cells, Fra-1, Jun B, NFκB p50, and NFκB p52 appear to be involved in the regulation of iNOS mRNA induced by LT.

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**Introduction**

Nitric oxide (NO) is a short-lived molecule that mediates a wide range of biologic effects. Its biological effects include vasorelaxation (Palmer et al., 1987), neuro-transmission (Garthwaite, 1991), inhibition of platelet aggregation (Radomski et al., 1987), as well as microbial and tumor cell killing (Nathan, 1992). The enzyme responsible for NO synthesis, nitric oxide synthase (NOS), converts L-arginine to L-citrulline and NO (Marletta, 1993). 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. Their activity is predominantly regulated via intracellular calcium/calmodulin signals in response to hormone or neurotransmitter stimulation (Bredt and Snyder, 1990; Busse and Mulsch, 1990). A third type of NOS [an inducible NOS (iNOS)], which presents in microglia and astrocytes, is regulated at the transcriptional level in response to cytokines and lipopolysaccharide (LPS), and does not require calcium for its activity (Jaffrey and Snyder, 1995; Nathan, 1992). Although NO produced by iNOS accounts for the bacterial and tumoricidal properties of microglia, it is a particular importance in the pathophysiologies of 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 microglia and the production of proinflammatory cytokines (Bo et al., 1994; Cross et al., 1994; Hooper et al., 1997; Koprowski et al., 1993; Merrill et al., 1993; Mitrovic et al., 1994). It is now increasingly clear that glial cells in the central nervous system also produce NO in response to the induction of iNOS by bacterial LPS and series of cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ). Astrocytes in the healthy brain do not express iNOS; however, after the ischemic, traumatic, neurotoxic, or inflammatory damages, the activated astrocytes express iNOS in rodent and human (Galea et al., 1992; Hu et al., 1995; Pahan et al., 1997a,b, 1998a). Although NO derived from both astrocytes and microglia has been believed to be involved in demyelinating disease and neuronal death during the ischemia and trauma (Bo et al., 1994; Merrill et al., 1993; Mitrovic et al., 1994), little is known about the signal transduction pathway involved in iNOS induction in astrocytes.

Previous studies have suggested that the inhibitors of tyrosine kinase suppress astroglial iNOS gene expression and NO production induced by cytokines or bacterial LPS (Feinstein et al., 1994b; Simmons and Murphy, 1994). Among tyrosine kinases, p38 mitogen activated protein kinase (MAPK) has been suggested to mediate LPS- or cytokine-induced iNOS expression in primary rat and mouse astrocyte-enriched cultures (Bhat et al., 1998; Da Silva et al., 1997). However, another MAPK, 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 (Bhat et al., 1998; Da Silva et al., 1997). This differential dependency of iNOS gene expression on ERK pathway may be due to the difference of signal transduction mechanisms between in rat and mouse astrocytes.

Computer-assisted analysis of rodent iNOS promoter and in vitro transfection assay has identified potential cytokine-responsive transcriptional elements. These include activator protein-1 (AP-1), nuclear factor kappa B (NFκB), cAMP response element (CRE), CAAT box/enhancer binding protein (C/EBP), γ-interferon (IFN) activating site (GAS), IFN stimulated response element (ISRE), and IFN regulatory factors (IRFs) (Zhang et al., 1998). Among these elements, AP-1 and NFκB are ubiquitous transcriptional factors and pleiotropic regulators of the inducible expression of many genes that encode proteins involved in the modulation of inflammatory and host defense process (Angel and Karin, 1991; Schreck
et al., 1991; Thanos and Maniatis, 1995). AP-1 is encoded by a set of genes called “immediate early genes” whose transcription is rapidly induced independently of de novo protein synthesis, following cell stimulation. Indeed, in human lung carcinoma cell line, a transient transfection assay has suggested that AP-1 activation is important for the induction of iNOS transcription.

NFκB family, which includes p50, p52, RelA (p65), RelB, c-Rel, v-Rel and dorsal and Dif proteins (Baldwin, 1996), is sequestered in the cytoplasm through its binding with its inhibitors, p105 or IkB-like proteins in resting state (Blackwell and Christman, 1997). Activation of NFκB by cytokines or oxidative stress requires either the degradation of its cytoplasmic inhibitor IkB-alpha or proteolytic cleavage of p105 (Blackwell and Christman, 1997). Free NFκB dimers translocate to the nucleus and activate the target gene, such as iNOS. Indeed, NFκB has been believed to be the most important transcriptional factor in the regulation of iNOS transcription in various cell types including astrocytes (Eberhardt et al., 1994; Lowenstein and Snyder, 1992; Pahan et al., 1998b; Sherman et al., 1993).

Therefore, we now report that the inhibition of p38 and ERK1/2 attenuates combination of LPS plus TNF-α (LT)-induced iNOS gene expression and NO production in C6 rat glioma cells. We examined the possible involvement of AP-1 (Fra-1 and Jun B) or NFκB (p50 and p52) in the regulation of LT-induced iNOS gene expression.

Methods

Drugs and Chemicals

LPS and CHX were purchased from sigma chemicals (MI, USA). PD98059 was purchased from New England Biolabs Inc. (MA, USA). SB203580 was purchased from Calbiochem (CA, USA). TNF-α, IFN-γ, and IL-1β were purchased from R and D systems (Woongbee Meditech, Korea).

C6 glioma cell culture

C6 rat glioma cells obtained from Korean Cell Line Bank (Seoul) were maintained in DMEM/F-12 medium containing 10% fetal bovine serum and 2 mM gentamicin. Cells were plated on 25 cm² culture flask (Falcon, Franklin, NJ). The cultures were incubated at 37 °C in 5% CO₂, and after 1 day, the medium was completely changed to a fresh culture medium. At 70% confluency, the cells were incubated with serum free DMEM/F-12 medium (DMEM-base medium for nitrite assay) for 24 hrs prior to the incubation with LPS and other chemicals.

Nitrite assay

Synthesis of NO was determined by an assay of the culture supernatant 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 (Sigma Chemicals) (Feinstein et al., 1994a) and incubated at the 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 the experiments. Nitrite concentration was calculated from a standard curve derived from the reaction of NaNO₂ in the assay.
Isolation of total RNA and proteins

Total cellular RNA was extracted from C6 rat glioma cells using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction procedure and subsequent precipitation with acidic sodium acetate (Chomczynski and Sacchi, 1987). Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted twice with an equal volume of sterilized (Millipore) water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer (6 M guanidium chloride, 20 mM Tris-HCl [pH 8.0], and 1 mM EDTA). The protein samples were dialyzed against a renaturing buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl₂, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol) at 4 °C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin (BSA) as the standard.

Preparation of DIG-labeled cRNA probes

The cRNA probes for iNOS (Galea et al., 1994) and cyclophilin (Danielson et al., 1988) were synthesized in vitro from linearized expression vectors which contained SP6 or T7 viral promoter. One µg of linearized plasmid was mixed with RNA labelling mixture that containing ATP, CTP, GTP and Dig-labeled-UTP, transcription buffer, and SP6 or T7 RNA polymerase. After incubation at 37 °C for 2 hrs, the mixture was co-incubated with DNase I (RNase free) at 37 °C for 15 min, precipitated in ethanol containing lithium chloride at −70 °C for 30 min, and washed with 70% chilled ethanol.

Non-isotopic Northern blot analysis

Ten microgram of total RNA were denatured in 65 °C. The denatured RNA samples were electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon hybond-N hybridization membrane sheets (Amersham, Buckinghamshire, England). After UV cross-linking, the membranes were pre-hybridized at 68 °C for at least 1 hr in prehybridization buffer containing 50% formamide. The DIG-labeled iNOS probe was added to prehybridization buffer. The membranes were incubated overnight at 68 °C. Following hybridization, the membrane was washed in washing solution (0.1 × SSC and 0.1% SDS). The membranes were gently equilibrated in Buffer I (100 mM maleic acid [pH 7.5] and 150 mM NaCl) and agitated in Buffer II (1% blocking reagent in Buffer I) for 30–60 min. The membranes were incubated with the diluted anti-DIG-alkaline phosphatase (1:10,000). After washing the membranes in 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in alkaline buffer. Diluted CSPD (Boehringer Mannheim) (1:100 dilution) 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, 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 (Danielson et al., 1988; Takahashi et al., 1989).

**Western blot analysis**

Cellular protein (50 μg) was mixed with an equal volume of SDS loading buffer (20% glycerol, 100 mM tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue), boiled for 3 min, and separated by electrophoresis in 12% polyacrylamide gels as described (Laemmli, 1970). A pre-stained rainbow protein mixture (Amersham Co., Arlington Heights, IL) was used as the molecular weight standard. Proteins were transferred from acrylamide gel onto polyvinylidene difluoride filters (PDVF; Amersham Pharmacia Biotech, England) according to the previous published procedures (Towbin et al., 1979). Electro-transferred to PDVF were first blocked with blocking buffer (3% skim milk, 1% BSA, 10 mM Trizma base [pH 8.0], and 150 mM NaCl) and then incubated with antisera against Fra-1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), Jun B (1:1000) (Santa Cruz Biotechnology), iNOS (1:1000) (Santa Cruz Biotechnology), CREB (1:1000) (New England Biolabs, Beverly, MA), phospho-CREB (1:1000) (New England Biolabs), p38MAPK/phospho-p38MAPK (1:1000) (New England Biolabs), JNK/phospho-JNK (1:1000) (New England Biolabs), and ERK/phospho-ERK (1:1000) (New England Biolabs) in blocking buffer for 4 h at room temperature. Filters were then washed with Tris-buffered saline containing 0.3% Tween-20 (TBST) for 5 min and then incubated with the goat anti-rabbit IgG-horseradish peroxidase conjugate (1:5000) in blocking buffer at room temperature for 1 hr. After washing the filters with TBST, ECL-plus solution (Amersham Life Science Co., England) was added. Then, the membranes were exposed to Hyperfilm-MP (Amersham) for detection of light emission.

**Preparation of nuclear extracts and non-isotopic electrophoretic mobility-shift assay**

Nuclear extract from stimulated or unstimulated C6 cells (1 × 10^7 cells) were prepared using the method of Dignam et al. (1983) with slight modification. The AP-1 (5'-CGC-TTG-AGT-CTGCGG-GAA-3') and NFκB (5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3') (Santa Cruz Biotechnology) were annealed by incubation 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 the room temperature. The DNA-binding assay was performed according to the instructions in the manual provided with the DIG-Gel Shift Kit (Boehringer Mannheim). Binding reactions were carried out at the room temperature for 20 min and reaction mixtures contained 30 μg of nuclear protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween 20, 50 mg/ml poly (dl-dC), 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 gels. Gels were run at a constant voltage (8 V/cm), and electro-blotted onto positively charged nylon membranes. The membranes were baked at 80 °C for 15 min, washed with 0.3% Tween 20 in Buffer I, and hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000) in Buffer II for 30 min. After two washes for 15 min with 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in alkaline buffer for 2 min. The method for detection of chemiluminescence was identical to the method used for the non-isotopic Northern blot analysis.
Statistical Analysis

The expression of iNOS mRNA induced by drugs was quantified with the Bio-profil Bio-1D application (Vilber-Lourmat, France), and expressed as the percentage of the control iNOS mRNA levels. A Student’s paired \( t \)-test was used to compare the relative abundance of mRNA in the experimental and control groups.

Results

**iNOS gene expression induced by LPS plus TNF-\( \alpha \) (LT) in C6 cells**

NO production and the regulation of iNOS mRNA level in rat C6 glioma cells were examined after the treatment with LPS (500 ng/ml) plus TNF-\( \alpha \) (20 ng/ml). As shown in Fig. 1A, a combination of LPS and TNF-\( \alpha \) stimulated NO production in C6 rat glioma cells. In Northern blot analysis, increased iNOS mRNA level by combination treatment was in line with NO production in 24 hr (Fig. 1B). The combination of LPS and TNF-\( \alpha \) showed an additive effect for NO production in C6 glioma cells (Fig. 1A).

In C6 glioma cells, iNOS mRNA level began to increase at 1 hr, reached a maximum at 6 hr, and maintained for up to 24 hr after the treatment with LPS (500 ng/ml) plus TNF-\( \alpha \) (20 ng/ml) (Fig. 2A).

![Figure 1](image-url)

Fig. 1. Effects of LPS and TNF-\( \alpha \) on iNOS mRNA expression and NO production in C6 glioma cells. (A) Nitrite concentrations were measured in culture media using the Griess reaction at 24 hr after combination treatment of LPS and TNF-\( \alpha \). (B) Northern blot analysis was performed at 24 hr after the combination treatment of LPS and TNF-\( \alpha \). Final concentration was as follows: LPS, 500 ng/ml; TNF-\( \alpha \), 20 ng/ml. The vertical bars in graphs indicate the S.E.M. (**\( P < 0.001 \); compared to the control group, \( n = 3 \) independent experiments).
a short time-course study, western blot analysis using antibody against iNOS (130 kDa) protein was carried out and showed that the iNOS protein level was increased in a time-dependent manner (Fig. 2B). Furthermore, NO production was increased in a time-dependent manner in LPS plus TNF-α-treated C6 glioma cells (Fig. 2C).

**Effect of LPS plus TNF-α on the phosphorylation of MAPKs protein and several transcription factors**

To examine the phosphorylation of MAPKs for the stimulation of LPS plus TNF-α, western blot analysis using antibodies against ERK 1/2 (p44/p42 MAPK), p38 MAPK, and JNK 1/2 (p46/p54...
MAPK) proteins and against their phosphorylated proteins were performed (Fig. 3). ERK 1/2, p38 MAPK, and JNK1/2 proteins were not affected by the stimulation of LPS plus TNF-α (data not shown). However, phospho-ERK 1/2 began to increase at 10 min and was increased at a maximum at 1 hr and returned to the basal level at 6 hr after the LPS plus TNF-α treatment. Phosphorylated p38 protein level began to increase at 10 min and reached a peak at 3 hr. At last, phosphorylated JNK 1/2 was increased at 10 min.

Western blot analysis using antibodies against AP-1 proteins, such as, Fra-1 (35 kDa), Jun B (39 kDa) and CREB/phospho-CREB (45 kDa) proteins was performed. Fra-1 and Jun B proto-oncoproteins were remarkably increased 3–6 hrs after the stimulation with LPS plus TNF-α (Fig. 3). In contrast to the increase of Fra-1 and Jun B proteins, Fra-2 and Jun D proteins were not affected by LPS plus TNF-α treatment (data not shown). Total CREB protein level was not affected by LPS plus TNF-α treatment. However, phospho-CREB protein level was increased at 1 hr and was returned to the control level at 3–6 hr (Fig. 3).

**Effects of PD98059, SB203580, and cycloheximide on LT-induced iNOS mRNA level**

To examine the possible involvement of phospho-ERK 1/2 and phospho-p38 in the regulation of iNOS expression, PD98059, a specific inhibitor of MEK1/2, which is upstream kinases of ERK1/2
(Payne et al., 1991) and SB203580, a specific inhibitor of p38 (Lee et al., 1994), were pretreated 30 min prior to the combined LPS (500 ng/ml) and TNF-α (20 ng/ml) treatment. PD98059 (10 µM) partially inhibited the increased iNOS mRNA level induced by LPS plus TNF-α stimulation at 3–6 hrs (Fig. 4A). As shown in Fig. 4B, SB203580 (10 µM) caused a complete inhibition of increased iNOS mRNA level induced by LPS plus TNF-α stimulation at 3–6 hrs (Fig. 4B). The dose of inhibitors was chosen on the basis of preliminary studies and a previous publication (Won et al., 2001).

Fig. 4. Effect of PD98059 (A), SB203580 (B), and cycloheximide (C) on LPS plus TNF-α treated C6 glioma cells. Northern blot analysis was performed at specific time points (3 and 6 hr) in the cells treated with control medium, LPS (500 ng/ml) plus TNF-α (20 ng/ml), PD98059 (10 µM), SB203580 (10 µM), or cycloheximide (CHX; 15 µM), LPS plus TNF-α and PD98059, SB203580 or CHX together. PD98059, SB203580, and CHX were pre-treated for 30 min prior to the incubation of LPS plus TNF-α. The vertical bars in graphs indicate the S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001; compared to the control group, +P < 0.05, ++P < 0.01 and +++P < 0.001; compared to the LPS plus TNF-α-treated group, n = 3 independent experiments).
To investigate whether on-going protein synthesis is required for LPS plus TNF-α-induced iNOS mRNA expression, protein synthesis blocker, cycloheximide (CHX) was pretreated prior to 30 min the LPS plus TNF-α treatment. iNOS mRNA level induced by LPS plus TNF-α treatment was inhibited by CHX at 3–6 hr (Fig. 4C).

Fig. 5. Translocation of NFκB p52 from cytoplasm to nucleus in LPS plus TNF-α treated C6 glioma cells. Twenty micrograms of nuclear, cytosolic and total proteins, which were extracted at 30 min, 1 hr, and 3 hr after drug treatment were used for determination of NFκB p52. A polyclonal antiserum against NFκB p52 was used at a 1:500 dilution.

Fig. 6. AP-1 and NFκB DNA binding activities in LPS plus TNF-α treated C6 glioma cells. At 6 hr after LPS (500 ng/ml) plus TNF-α (20 ng/ml) stimulation, the AP-1 (A) and NFκB (B) DNA binding activities were examined using gel shift analysis as described in MATERIAL AND METHODS. For competition or supershift/attenuation assay, 50 X unlabelled AP-1 DNA oligomers or two μg of specific antiserum against c-Fos, c-Jun, Fra-1, or Jun B was added to each reaction. For NFκB DNA binding activity, 50 X unlabelled NFκB DNA oligomers or two μg of specific antiserum against p50, p52, c-Rel, or GR was added to each reaction.
LPS plus TNF-α induces the translocation of NFκB p52

To elucidate the involvement of NFκB activation on iNOS mRNA expression induced by LPS plus TNF-α treatment, we carried out western blot analysis from cytoplasm and nuclear proteins. As shown in Fig. 5, NFκB p52 protein level was increased in nuclear fraction and decreased in cytosolic fraction at 0.5–3 hr. The protein level of total NFκB p52 was not changed by LPS plus TNF-α treatment.

Effect of LPS plus TNF-α on the AP-1 and NFκB DNA binding activities

To investigate the possible involvement of AP-1 or NFκB transcriptional factors in the regulation of LPS plus TNF-α induced iNOS mRNA expression, the electrophoretic mobility shift assay was carried out. In a quantitative study, DNA binding activities of AP-1 and NFκB were increased at all time points (3 and 6 hr)(Data not shown). To examine the transcriptional factors involve in AP-1 and NFκB DNA binding activities induced by LPS plus TNF-α treatment, we performed self-competition and supershift assays using the various antibodies. In the self-competition study, AP-1 DNA binding activity induced by LPS plus TNF-α treatment was decreased by unlabelled AP-1 DNA oligomer (cold AP-1), suggesting that this DNA binding activity was AP-1 sequence specific (Fig. 6A). In a supershift study, antibodies against Fra-1 and Jun B supershifted LT-induced AP-1 DNA binding activity (Fig. 6A). NFκB DNA binding activity induced by LPS plus TNF-α treatment was also decreased by unlabelled NFκB DNA oligomer (cold NFκB) (Fig. 6B). In a supershift study, antibody against p50 supershifted NFκB DNA binding activity induced by LPS plus TNF-α treatment (Fig. 6B).

Discussion

The major findings in the present study are: (1) iNOS mRNA expression induced by LPS plus TNF-α treatment might be mediated via the activation of ERK 1/2 and p38 and (2) new protein synthesis require in iNOS mRNA expression induced by LPS plus TNF-α treatment. (3) iNOS mRNA expression induced by LPS plus TNF-α treatment is also mediated by the activation AP-1 (Fra-1 and Jun B) and NFκB p50 transcription factors.

In the present study, we demonstrated signaling pathways for NO production and iNOS expression induced by LT in C6 rat glioma cells. A combination of LPS and TNF-α increases NO production via iNOS expression at 24 hr in C6 glioma cells. This finding is in line with the several previous studies using the same cells (Feinstein et al., 1994a), as well as the primary cultured rat astrocytes (Simmons and Murphy, 1994). Furthermore, the necessity to stimulate cells with combined cytokines such as TNF-α plus IL-1β or IFN-γ or with cytokines combined with LPS to induce iNOS expression has been reported in several cell types such as fibroblasts (Farivar and Brecher, 1996) and endothelial cells (Kanno et al., 1994), although a single cytokine can induce iNOS expression in some other cell lines including hepatocytes (Geller et al., 1993), islet cells (Heitmeier et al., 1997), and vascular smooth muscle cells (Koide et al., 1993). Thus, in C6 rat glioma cells, co-stimulation with LPS and TNF-α was required to derive iNOS expression.

The exposure of C6 gliaoma cells to LPS plus TNF-α significantly increased the level of iNOS protein level and NO production at 3–6 hr after treatment. These results suggest that iNOS expression induced by LPS plus TNF-α treatment and its protein synthesis simultaneously generate. We have found for the fist time that AP-1 transcription factors (such as Fra-1 and Jun B) and phosphorylated CREB were
increased by LPS plus TNF-α treatment in C6 glioma cells. Induction of these transcription factors may cause an induction of iNOS mRNA through interacting with AP-1 or CRE and AP-1 or CRE DNA binding domains located in iNOS promoter regions. As shown in Fig. 6A, AP-1 DNA binding activity was increased by LPS plus TNF-α treatment. In a supershifted assay, supershifted bands observed in the cells co-incubated with antibody against Fra-1 or Jun B. These results are supported by several studies that promoter region of iNOS gene contains AP-1, NFκB, CRE, C/EBP, GAS, ISRE, and IRFs (Zhang et al., 1998; Angel and Karin, 1991; Schreck et al., 1991; Thanos and Maniatis, 1995). This contention is supported by the finding that AP-1 transcription factor is a complex composed of fos and jun proto-oncogene families, which need to dimerize to promote the binding of the complex to the AP-1 recognition site and transcriptional activation of the target gene (Hunter and Karin, 1992).

We also found that translocation of p52 protein from the cytosol into nucleus was increased by LPS plus TNF-α treatment. Furthermore, NFκB DNA binding activity also was increased by LPS plus TNF-α and the supershifted band observed in the cells co-treated with antibody against NFκB p50. These results indicate that NFκB p50 and p52 may be important for the regulation of iNOS mRNA expression induced by LPS plus TNF-α treatment. Recently, Nomura (2001) has reviewed that NFκB activation and IkB-α dynamism are involved in iNOS and chemokine induction in astroglial cells. However, the involvement of NFκB p52 in iNOS mRNA expression observed in the present study is a new finding.

The roles of p38, JNK, and ERK must be considered in the regulation of iNOS expression, since those kinases are shown to be activated by LPS plus TNF-α treatment with a slightly different kinetics as revealed in the present study. The extent and kinetics of p38 as well as ERK and JNK kinase activation did not provide the evidence for any additive or enhancing effects by LPS plus TNF-α treatment. This result is in line with other study that p38 mediates iNOS mRNA expression in mouse astrocytes (Da Silva et al., 1997). Furthermore, iNOS mRNA expression induced by LPS plus TNF-α treatment was inhibited by PD98059 and SB203580 at 3–6 hrs (Fig. 4A and B). These results suggest that ERK1/2 and p38 pathways may be important for the regulation of iNOS mRNA expression in C6 glioma cells. Recently, we have reported that the LPS-induced NO production and iNOS mRNA expression are inhibited dose-dependently by SB203580, but not PD98059 (Won et al., 2001). In the present study, SB203580 completely blocked the iNOS mRNA expression induced by LPS plus TNF-α while PD98059 showed a partial inhibition. It is suggested that p38 pathway may be more important for the modulation of iNOS expression than ERK1/2 pathway.

To investigate whether on-going protein synthesis is required for LPS plus TNF-α-induced iNOS mRNA expression, protein synthesis blocker, CHX was pretreated. LPS plus TNF-α-induced iNOS mRNA expression was blocked by CHX pretreatment. This result suggests that iNOS mRNA expression induced by LPS plus TNF-α treatment requires on-going protein synthesis.

In summary, the present study clearly shows that iNOS mRNA expression appears to be increased via activating p38 and ERK1/2 MAPKs in LPS plus TNF-α-treated C6 rat glioma cells. Activations of Fra-1, Jun B, phospho-CREB, and NFκB p50 and p52 proteins may serve as transcription factors for the upregulation of iNOS mRNA expression when C6 rat glioma cells are stimulated with LPS and TNF-α.

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References


