Herbimycin A suppresses NF-κB activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells

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Abstract Herbimycin A, a potent tyrosine kinase inhibitor, suppressed nitric oxide synthase (NOS) induced by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in C6 glial cells. LPS activated NF-κB, and this effect was inhibited by pretreatment with herbimycin A. In addition, IFN-γ activated the tyrosine protein kinase, JAK2, and tyrosine-phosphorylation by itself was also inhibited by herbimycin A. These results suggest that herbimycin A suppresses iNOS induction by inhibition of both NF-κB activation caused by LPS, and tyrosine-phosphorylation of JAK2 caused by IFN-γ in C6 glioma cells.

Key words: Nitric oxide synthase; Herbimycin A; NF-κB; JAK2; C6 glioma cell

1. Introduction

Recent reports have shown that nitric oxide (NO), a short-lived gaseous molecule, is a potent biological mediator in the vascular, immune, and nervous systems [1,2]. In particular, NO is believed to be involved in neurotoxicity after various neuronal stresses and in synaptic plasticity in the cerebellum and the hippocampus in the central nervous system (CNS) [3]. In the brain, a constitutively expressed calcium-dependent NO synthase (cNOS) is expressed in specific neuronal populations, where it can act as mentioned above. On the other hand, another type of NOS, inducible NOS (iNOS), is expressed in brain glial cells when induced by treatment with bacterial endotoxin (lipopolysaccharide, LPS) and/or cytokines [4–6].

However, the signal transduction system responsible for induction of iNOS is poorly understood. In the mouse macrophage cell line RAW 264.7, LPS activates the nuclear transcription factor NF-κB [7]. Moreover, same group also reported that NF-κB was necessary for LPS inducibility of the iNOS promoter in those cells [8]. In rat primary cultured astrocytes and rat C6-N glioma cells, genistein suppressed iNOS induction stimulated by LPS plus cytokines [9]. High concentrations of genistein (>15 μM), however, are known to inhibit not only tyrosine kinases but also other types of protein kinases such as protein kinase C (serine/threonine kinase) [10]. Thus, no target protein kinases of genistein have been identified in detail. To clarify the mechanism of iNOS expression stimulated by LPS plus IFN-γ in glial cells, we investigated the effects of herbimycin A, a potent selective tyrosine kinase inhibitor [11], on LPS plus IFN-γ-induced iNOS expression in an astrocyte cell line, rat C6 glioma cells. Our results indicate that inhibitory effects of herbimycin A on iNOS expression are due to the suppression of both NF-κB activation and tyrosine-phosphorylation of JAK2.

2. Materials and methods

2.1. Materials

Rat C6 glioma cells were obtained from the American Type Culture Collection (USA). The plasmid carrying cDNA sequence for iNOS (pTZ 18U) was a kind gift from Dr. H. Esumi [12]. Oligo(dT)-Latex was obtained from Takara Biomedicals (Japan). Protein G-Sepharose beads and poly(dI-dC) were obtained from Pharmacia Biotech (USA). Anti-phosphotyrosine antibody (4G10) and anti-JAK2 antiserum were obtained from Upstate Biotechnology Incorporated (USA). Anti-iNOS antibody was obtained from Transduction Laboratories (UK). NF-κB consensus oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), anti-NF-κB (p50) antibody and (p65) antibody were obtained from Santa Cruz Biotechnology Inc. (USA). LPS, IFN-γ, and herbimycin A were obtained from Sigma (USA), Gibco BRL (USA), and Wako Pure Chemical Industries Ltd. (Japan), respectively.

2.2. Cell culture

Rat C6 glioma cells were maintained in F-10 medium supplemented with 15% horse serum, 2.5% fetal calf serum, 50 μg/ml penicillin and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2.

2.3. Western blot analysis

Cells (6×10^5) were washed with 3 ml of ice-cold phosphate-buffered saline (PBS), harvested and sonicated for 5 s. The lysates were centrifuged at 15,000 rpm for 20 min at 4°C and supernatants were boiled with SDS sample buffer for 5 min. Samples were stored at −70°C until use.

Samples were subjected to 7.5% SDS-PAGE followed by transfer on nitrocellulose filters at 100 V for 1 h at 4°C. Filters were then blocked with TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 1% bovine serum albumin (BSA) for 1 h and incubated with the first antibody in TBST for 1 h at room temperature. After three washes with TBST, filters were incubated with the secondary antibody (anti-mouse IgG-horseradish peroxidase conjugate, 1:2000 dilution in TBST) for 45 min. After three washes with TBST, antibody-reactive bands were revealed by chemiluminescent detection (ECL Western detection kit; Amersham International).

2.4. RNA isolation and Northern blot analysis

Total cellular RNA was prepared from 1.2×10^7 cells using the guanidinium-cesium chloride method as described previously [13]. Poly(A)$^+$ RNA was isolated by the oligo(dT)-Latex method [14]. Five μg of poly(A)$^+$ RNA was denatured by heating at 55°C for 15 min in 2.2 M formaldehyde, 50% (v/v) formamide, electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and then transferred on nitrocellurose filters by capillary action using 20 × SSC (1 × SSC is 0.015 M sodium citrate buffer, pH 7.0, containing 0.15 M NaCl). The filters were prehybridized for 5 h at 40°C in a solution containing 50% formamide, 50 mM NaP_i, 5 × SSC, 1 × Denhardt's solution and 60 μg/ml salmon sperm DNA, and then incubated for 16 h at 42°C in the same solution (except for 5 μg/ml salmon sperm DNA) with ^{32}P-labeled DNA probe specific for iNOS (a 700 bp DNA fragment including the

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5′ portion of the cloned rat liver inducible-type NO synthase cDNA [12]) labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit (Takara, Japan). Following washing of the filters twice at 68°C in 2 × SSC, 0.1% SDS for 30 min and twice at 68°C in 1 × SSC, 0.05% SDS for 30 min, autoradiography was performed by exposure for 16 h to imaging plates and visualized on a FUJIX BAStation (Fuji Photo Film Co., Japan).

2.5. Electrophoretic mobility shift assays (EMSA) and antibody inhibition

Nuclear extracts were prepared using a slight modification of the method described previously [15]. Briefly, 3 × 10^6 cells were harvested, washed once with 2 ml of ice-cold PBS and resuspended in 400 ml of buffer A consisting of 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES, pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 20 U/ml aprotinin. After incubation for 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% and vigorously vortex mixed for 10 s. Nuclei were precipitated and resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 U/ml aprotinin) and vigorously vortex mixed for 15 min at 4°C. Lysates were centrifuged at 15,000 rpm for 20 min at 4°C and supernatants containing the nuclear proteins were transferred into new vials. The protein concentrations of extracts were measured using a Bio-Rad protein assay kit.

Electrophoretic mobility shift assays [16] were performed by incubating 7.5 μg of nuclear extracts with 2 μg of poly(dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mg/ml BSA, 0.05% NP-40, 5% glycerol) (20 μl final volume) for 30 min at 4°C. Then, end-labeled double-stranded oligonucleotide probe (50,000 cpm/0.3 ng) was added and the reaction mixture was incubated for 15 min at room temperature. For supershift assay with specific antibodies against NF-κB, nuclear extracts were preincubated with 1 μg of each antibody for 2 h at 4°C before addition of end-labeled double-stranded oligonucleotide probe was added [17]. The samples were separated by 5% native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25 × Tris-borate-EDTA).

2.6. Immunoprecipitation

Cells (3 × 10^6) were scraped into 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 0.5% Triton X-100 and 20 U/ml aprotinin) and broken by passage through a 21-gauge needle. The lysates were sonicated for 5 s and centrifuged at 15,000 rpm for 20 min at 4°C. The supernatants were precleaned with 50 ml of protein G-Sepharose beads (50% slurry) for 1 h at 4°C. After removal of nonspecific immune complex, 4 μl of anti-JAK2 antiserum was added, and the mixture was incubated for 4 h at 4°C. Immune complexes were precipitated with 30 μl of protein G-Sepharose beads (50% slurry) and washed five times with 1 ml of ice-cold lysis buffer. Bound proteins were eluted by heating the samples with 60 ml of 2 × SDS sample buffer for 5 min at 95°C. Samples were stored at −70°C until use.

3. Results

3.1. Herbimycin A inhibits the LPS- plus IFN-γ-stimulated iNOS induction in C6 glioma cells

We observed that accumulation of NO_2^-, a breakdown product of NO produced by LPS- plus IFN-γ-induced iNOS, was considerably decreased by pretreatment with herbimycin A, a potent protein tyrosine kinase inhibitor [11], in C6 glioma cells. To examine whether herbimycin A inhibits the LPS- plus IFN-γ-stimulated iNOS induction at transcription and translation stages, cytosolic fraction and poly(A)$^+$ RNA were prepared from C6 glioma cells pretreated with herbimycin A prior to LPS- plus IFN-γ stimulation and subjected to Western blot and Northern blot analysis, respectively. Herbimycin A suppressed the induction of both 130 kDa iNOS protein (Fig. 1A) and mRNA (Fig. 1B) in a concentration-dependent manner with a half -maximal effect at approximately 0.3 μg/ml.

3.2. Herbimycin A inhibits LPS-induced NF-κB activation in C6 glioma cells

CD14, the only molecule that has been demonstrated definitively to be involved in LPS-initiated cellular activation, is a 53 kDa glycosylphosphatidylinositol-linked antigen present on macrophages and monocytes [18] which mediates LPS stimulation of the translocation of the transcription factor NF-κB into the nucleus. To investigate the signal transduction system inducing NOS, we next studied whether LPS also mediates NF-κB activation in C6 glioma cells as found in macrophages and neutrophils. C6 glioma cells were treated with 10 mg/ml LPS for increasing periods of time. Nuclear extracts were prepared as described in section 2 and samples were subjected to EMSA using a DNA probe containing the NF-κB-binding element. NF-κB was detected in C6 glioma cell nuclear extracts

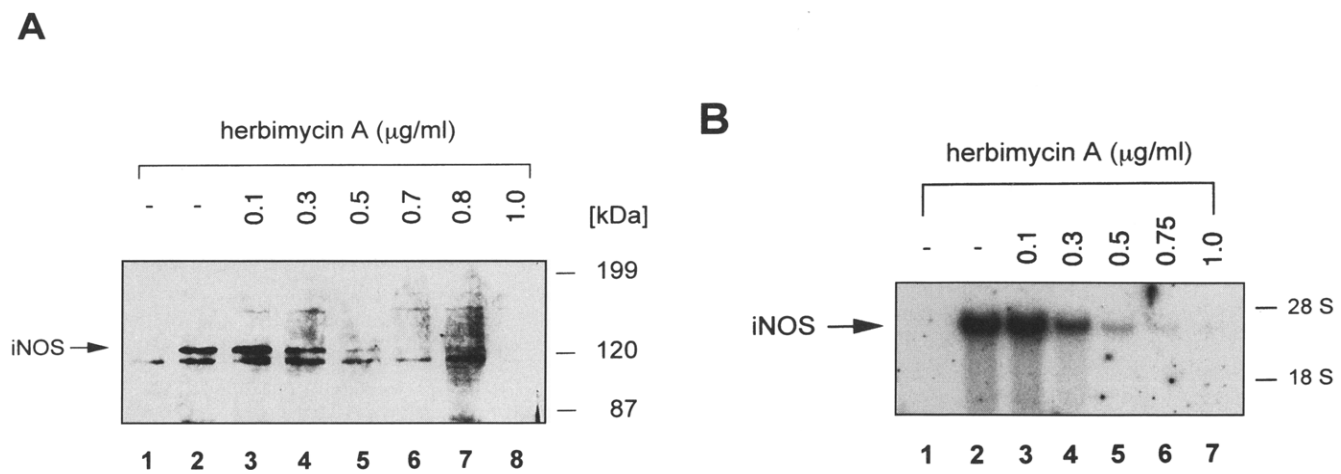

Fig. 1. Inhibition of LPS- plus IFN-γ-stimulated iNOS induction by herbimycin A. (A) Effect of herbimycin A on iNOS protein levels. 6 × 10^5 cells were pretreated for 12 h with herbimycin A at the indicated concentrations followed by stimulation with 10 μg/ml LPS plus 250 U/ml IFN-γ for 36 h (lane 2–8). iNOS protein levels were then determined by Western blot analysis as described in section 2. (B) Effect of herbimycin A on iNOS mRNA levels. mRNA was isolated from 1.2 × 10^7 cells pretreated for 12 h with herbimycin A at the indicated concentrations followed by stimulation with 10 μg/ml LPS plus 250 U/ml IFN-γ for 24 h (lane 2–7) and determined by Northern blot analysis as described in section 2.

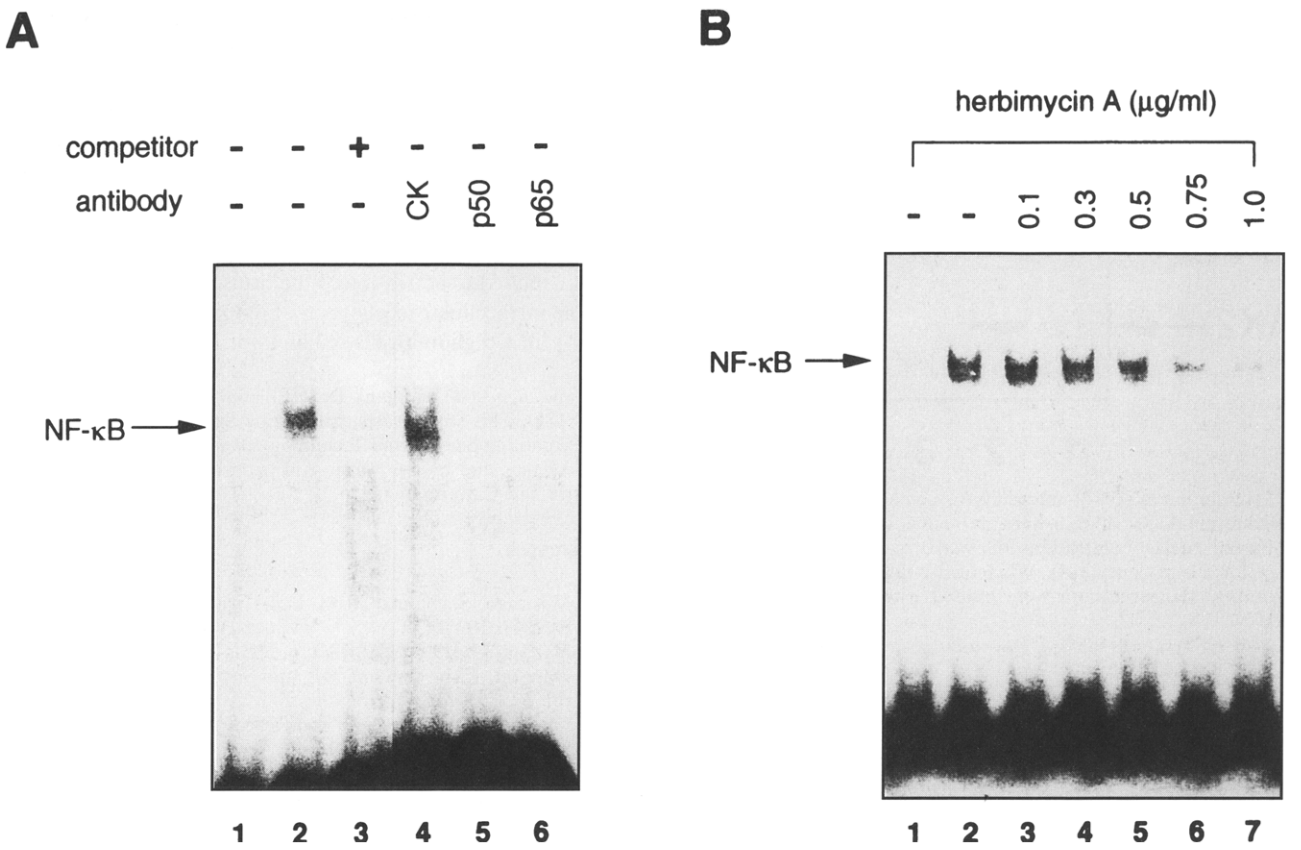

Fig 2 (A) Identification of nuclear NF-κB complexes. 3×10^6 cells were treated with 10 μg/ml LPS for 1 h (lanes 2–6) before preparing nuclear extracts for analysis by EMSA. Nuclear extracts were incubated in the absence (lane 1 and 2) or presence (lanes 5 and 6) of 1 mg of specific antibodies to each NF-κB component and a molar excess of unlabeled oligonucleotide probe (70-fold with probe, lane 3). CK (lane 4) represents control normal rabbit serum. (B) Inhibition of LPS-induced NF-κB activation by herbimycin A. C6 cells were pretreated for 12 h with herbimycin A at the indicated concentrations followed by incubation with 10 μg/ml LPS for 1 h. Then, nuclear extracts were prepared and analyzed by EMSA

within 15 min reaching maximal levels within 60 min following exposure to LPS (data not shown). Peak activation of C6 glioma cells resulted in an approximately 20-fold increase in nuclear NF-κB levels as determined by scanning densitometry (lanes 1 and 2, Fig. 2A). In addition, LPS-stimulated NF-κB complexes dramatically disappeared when the nuclear extract was co-incubated with non-radiolabeled DNA (lanes 2 and 3). The identity of the complexes was determined using anti-NF-κB p50 and p65 antibodies. Control rabbit IgG had no effect on LPS- stimulated NF-κB complexes in EMSA (lane 4). However, anti- p50 and p65 antibodies completely eliminated the complexes generated with C6 glioma nuclear extracts (lanes 5 and 6).

Moreover, to examine whether herbimycin A inhibits the LPS-induced NF-κB activation, nuclear extracts were prepared from C6 cells pretreated with herbimycin A prior to LPS stimulation and subjected to EMSA. As shown in Fig. 2B, herbimycin A inhibited LPS-activated NF-κB localization in nuclei in a concentration-dependent manner with a half-maximal effect at approximately 0.5 μg/ml.

3.3 Herbimycin A inhibits the IFN-γ-stimulated tyrosine-phosphorylation of JAK2 in C6 glioma cells

Another stimulator, IFN-γ, binds to its own cell-surface receptors and then causes activation of the associated protein kinases such as JAKs [19]. Firstly, to examine whether IFN-γ phosphorylates tyrosine residues of JAKs in C6 glioma cells, we performed immunoprecipitation using antibodies against JAK1, JAK2, and Tyk2. Subsequently, the immune complexes were subjected to Western blot analysis using an anti-phosphotyrosine antibody. In the quiescent state, tyrosine-phosphorylation of JAK1, JAK2, or Tyk2 were not detectable. IFN-γ specifically stimulated tyrosine-phosphorylation of JAK2, and slightly stimulated tyrosine-phosphorylation of JAK1, but not of Tyk2 (data not shown). Next, to examine whether herbimycin A inhibits IFN-γ-stimulated tyrosine-phosphorylation of JAK2, we pretreated C6 glioma cells with herbimycin A prior to IFN-γ stimulation and performed immunoprecipitation using an antibody against JAK2. The tyrosine-phosphorylation of JAK2 was suppressed by pretreatment with 0.5 μg/ml herbimycin A (Fig. 3).

4. Discussion

4.1. LPS stimulates NF-κB activation via tyrosine kinase

It has been shown that LPS binds to the cell-surface receptor CD14. Stefanova et al. [20] reported that tyrosine protein kinase $p53/56^{lyn}$ could be co-immunoprecipitated with CD14 using anti-CD14 antibodies in human monocytes. On the other hand, Delude et al. [21] reported that CD14-mediated translocation of NF-κB stimulated by LPS did not require tyrosine kinase activity in CHO-K1 cells transfected with human CD14.

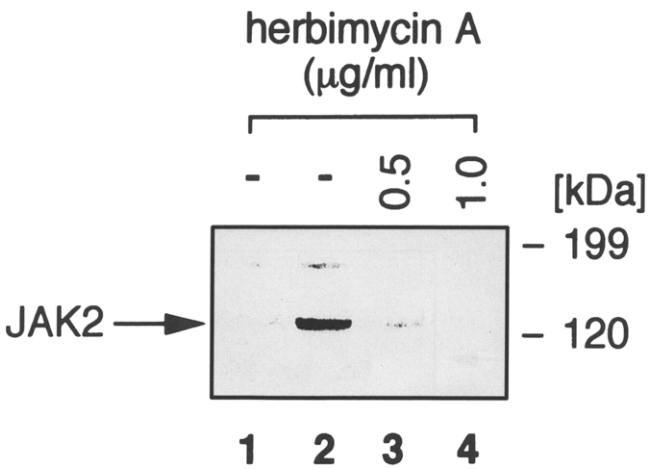

Fig. 3. Inhibition of IFN-γ-induced tyrosine-phosphorylation of JAK2 by herbimycin A. 3×10^6 cells were pretreated for 12 h with herbimycin A at the indicated concentrations followed by incubation with 250 U/ml IFN-γ for 5 min (lanes 2–4). Whole cell lysates immunoprecipitated with anti- JAK2 antiserum were blotted with anti-phosphotyrosine antibody.

Thus, the signal transduction system of LPS is still unclear Anderson et al. [22] reported that NF-κB activation was regulated by both tyrosine kinases and phosphatase in human T-cell lines. The tyrosine kinases involved in intracellular signaling pathways following LPS stimulation are not always located in membranes. The present results indicate that even a lower concentration of herbimycin A can inhibit NF-κB activation stimulated by LPS in C6 glioma cells (Fig. 2B). This inhibition may be due to inhibition of: (1) receptor-associated tyrosine kinases such as *lyn*; or (2) unidentified tyrosine kinases which regulate NF-κB activation. Although further studies are needed to investigate the precise mechanism of LPS-stimulated NF-κB activation, it should be noted that NF-κB activation is sensitive to a low concentration of herbimycin A in C6 glioma cells.

4.2. *IFN-γ stimulates JAK2 and slightly stimulates JAK1, but not Tyk2*

It has been shown that IFN-γ stimulates the receptor-associated tyrosine protein kinases JAK1 and JAK2 in several cell types [19]. In the present study, we demonstrated that IFN-γ-induced tyrosine-phosphorylation of JAK2 was more stimulated than that of JAK1 in C6 glioma cells. Moreover, LPS exerted no effect on phosphorylation of these kinases (unpublished data). JAK2 is known to cause tyrosine-phosphorylation of the substrate STAT1α (p91) [23]. Tyrosine-phosphorylated STAT1α can translocate to the nucleus from the cytosol and acts as a transcriptional factor [23]. Indeed, recognition sites for IFN-γ as well as NF-κB are present in the promoter of mouse iNOS [8]. Hence, inhibition of JAK2 by herbimycin A may bring about a lack of the transcription factor, STAT1α, which is needed for iNOS mRNA expression. We confirmed that IFN-γ stimulated tyrosine-phosphorylation of both JAK2 and STAT1α in rat primary glial cells (unpublished data).

4.3. *Conclusion*

The results presented here show that herbimycin A inhibits LPS- plus IFN-γ-stimulated induction of NOS in C6 glioma cells. Feinstein et al. [9] reported previously that LPS or the cytokines IL-1β, IFN-γ, or TNF-α alone did not induce NOS expression, but combination of LPS and each cytokine significantly stimulated the induction of NOS at the protein level in C6 glioma cells [8]. The signal transduction system involved in iNOS induction has not been investigated previously in primary cultured glial cells or astrocytes, C6 glioma cells. Our findings suggest the possibility that dual signal pathways are required to induce NOS in C6 glioma cells; i.e. LPS-stimulated NF-κB activation and IFN-γ-stimulated tyrosine-phosphorylation of JAK2 related to activation of the transcription factor STAT1α.

The intracellular mechanism of iNOS induction by LPS plus IFN-γ in C6 glioma cells remains for further investigation.

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