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Possible involvement of Janus kinase Jak2 in interferon-γ induction of nitric oxide synthase in rat glial cells

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Abstract

To clarify the induction pathway of inducible nitric oxide (NO) synthase in the brain, we examined the effects of interferon-γ and lipopolysaccharide on the induction of inducible NO synthase in glial cells cultured from neonatal rats, compared to those in the macrophage cell line RAW264.7 which was derived from Abelson leukemia virus-induced BALB/c lymphocytic lymphoma. NO synthase activity (NO_2^- accumulation) and 130 kDa protein of inducible NO synthase were induced 24 h after treatment with interferon-γ or lipopolysaccharide in both glial cells and RAW264.7 macrophages. These induction activities were inhibited by a tyrosine kinase inhibitor, herbimycin A. Immunoprecipitation assay using antibodies against Janus kinases, and the signal transducer and activator of transcription-1 (STAT1), revealed that interferon-γ induced tyrosine phosphorylation of the just another kinase-2 (Jak2) and STAT1α but did not induced the phosphorylation of Jak1, the non-receptor tyrosine kinase-2 (Tyk2) and STAT1β. Tyrosine phosphorylation of Jak2 and STAT1α induced by interferon-γ was also inhibited by herbimycin A, while lipopolysaccharide did not induce any tyrosine phosphorylation of Janus kinases and STAT1 at all. These results suggest that the interferon-γ-induced inducible NO synthase induction involves activation of Jak2-STAT1α pathway in both glial cells and macrophages.

Keywords: Nitric oxide (NO) synthase, inducible; Herbimycin A; Interferon-γ; Janus kinase; Signal transducer and activator of transcription-1 (STAT1); Microglia

1. Introduction

In the central nervous system (CNS), the concentration of several cytokines, e.g., interleukin-1, interferon-γ or tumor necrosis factor-α is increased with mechanical lesions. Recently, it is known that microglial cells secrete these cytokines in response to endotoxin stimulation (Benveniste, 1993; Banati et al., 1993). It should be noted that there are accumulations of microglial and reactive astroglial cells in the proximity of neurodegenerative sites in the brains of patients with Alzheimer's disease, Parkinson's disease and acquired immunodeficiency syndrome (Dickson et al., 1993; McGeer et al., 1993). These observations strongly suggest that the glial cells could play an active part as the immune system in the CNS and related to these neurodegeneration.

It is known recently that nitric oxide (NO) is synthesized in mammalian systems, i.e., the immune, cardiovascular and central nervous systems, where NO acts as a signaling or cytotoxic molecule (Nathan, 1992; Lowenstein and Snyder, 1992). NO is enzymatically formed from a terminal guanidino-nitrogen of L-arginine (L-Arg) by NO synthase that yield L-citrulline as a co-product (Knowles and Moncada, 1994). The current studies indicate that there are at least three isoforms, e.g., neuronal type (type I), inducible macrophage type (type II), and endothelial type (type III) (Schmidt, 1992). The neuronal and endothelial NO synthases are constitutively expressed, requiring Ca^{2+} and calmodulin for their activation, while inducible NO synthase is expressed only after several hours of exposure to some cytokines and bacterial endotoxins such as lipopolysaccharide in macrophages or hepatocytes (Nathan, 1992). The neuronal NO synthase in the brain has been characterized in detail (Bredt et al., 1991). Although recent papers reported that lipopolysaccharide and cytokine induce NOS in glial cells (Boje and Arora, 1992;

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Simmons and Murphy, 1992; Galea et al., 1992), it is not clear how inducible NO synthase is induced and activated. We previously reported that an activation of tyrosine kinase is possibly involved in lipopolysaccharide-induced expression of inducible NO synthase in glial cells (Nomura and Kitamura, 1993; Kitamura et al., 1996). In the present study, we further examined how inducible NO synthase are induced by interferon-γ and lipopolysaccharide in the glial cells, compared to those in macrophage cell line RAW264.7.

2. Materials and methods

2.1. Materials

Recombinant rat interferon-γ from Gibco BRL; lipopolysaccharide (*E. coli* serotype 055:B5) from Sigma; rabbit anti-inducible NO synthase antibody from affinity BioReagents; rabbit polyclonal antibodies against just another kinase-1 and -2 (Jak1 and Jak2) from Upstate Biotechnology; rabbit polyclonal antibody against non-receptor tyrosine kinase-2 (Tyk2) from Santa Cruz Biotechnology; mouse anti-phosphotyrosine antibody (PY-20) and rabbit polyclonal antibody against signal transducer and activator of transcription-1 (STAT1) from Transduction Lab.; protein A-Sepharose CL-4B from Pharmacia Biotech; mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) from Chemicon International; mouse monoclonal antibody against rat cluster of differentiation-11b (CD11b) (OX-42) from Serotec; enhanced chemiluminescent detection (ECL) kit from Amersham; herbimycin A from Wako Chemicals; and Vectastain Elite ABC kit from Vector Lab. were used. Rabbit anti-rat inducible NO synthase antibody for immunocytochemistry was a gift from H. Esumi (National Cancer Research Institute, Japan).

2.2. Preparation and culture of glial cells and RAW264.7 macrophages

Glial cells (mixture of astrocytes and microglia) were cultured from whole brains of 0–2-day-old Wistar rats and allowed to grow to confluency (10–14 days) in Dulbecco's modified Eagle medium (DMEM), 10% (vol/vol) fetal calf serum with 50 μg/ml of penicillin and 100 μg/ml of streptomycin. All cultured cells were kept at 37°C in 5% CO_2/95% air. Subsequently, glial cells were secondarily cultured for 7–10 days in 12-well plates (for the assay of NO_2^- accumulation and immunoblotting of anti-inducible NO synthase antibody), or 100 mm diameter dishes (for immunoprecipitation assay) and then used in the following experiments.

RAW264.7, a cloned murine macrophage cell line which was derived from Abelson leukemia virus-induced BALB/c lymphocytic lymphoma, was established by Raschke et al. (1978). We used RAW264.7 macrophages as the positive control of the induction of inducible NO synthase (Xie et al., 1993). In the present study, RAW264.7 (TIB 71; American Type Culture Collection) was cultured under the same condition as the glial cells were.

2.3. Treatment of glial cells and macrophages with interferon-γ and lipopolysaccharide

Glial cells and RAW264.7 macrophages were incubated with interferon-γ (10–1000 U/ml) and lipopolysaccharide (1 μg/ml) in the absence or presence of herbimycin A (1 μg/ml) for 24 h at 37°C. On the pretreatment of herbimycin A, the inhibitor was added into the culture 1 h before the incubation of interferon-γ and/or lipopolysaccharide. After these treatments, NO_2^- accumulation in culture media was assayed and cell lysate was subjected to immunoblot analysis using a rabbit anti-inducible NO synthase antibody (Affinity BioReagents). For immunoprecipitation assay, both cells were incubated with interferon-γ, lipopolysaccharide, and interferon-γ plus lipopolysaccharide in the absence or presence of herbimycin A (1 μg/ml) at 37°C for 10 min (with antibodies against Jak1, Jak2 and Tyk2), or for 15 min (with anti-STAT1 antibody).

2.4. Assay of NO_2^- accumulation in culture medium

The amount of NO_2^- accumulation in a culture medium of treated glial cells or RAW264.7 macrophages was measured spectrophotometrically. In brief, 120 μl of cell culture medium was mixed with 40 μl of Griess reagent (Green et al., 1982) and incubated at room temperature for 15 min, and then the absorbance at 540 nm was measured (DU-640 Spectrophotometer, Beckman). Fresh culture medium served as the blank in all of the experiments. Solutions of sodium nitrite diluted in culture media served as the standard. Statistical differences were analyzed by Student's t test.

2.5. Immunoprecipitation using antibodies against Janus kinases and STAT1

Glial cells and RAW264.7 macrophages were incubated with interferon-γ (1000 U/ml), lipopolysaccharide (1 μg/ml) and interferon-γ plus lipopolysaccharide at 37°C for 10 min, or for 15 min. Cells were scraped and lysed for 60 min in 1 ml of ice-cold lysis buffer: 50 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM sodium vanadate, 20 U/ml of aprotinin and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 15 000 $\times g$ for 10 min. Resulting supernatants were incubated with protein A-Sepharose (20 μl of 50% slurry) for 1 h at 4°C, and centrifuged at 1000 $\times g$ for 5 min. Antibody against Jak1 (3 μl), Jak2 (3 μl), Tyk2 (10 μl), or STAT1 (10 μl) was added to cell lysates thus obtained, and incubated for 20 min at 4°C. Protein A-Sepharose (50 μl of 50% slurry) was then added, and incubated overnight

at 4°C. Subsequently, the immunoprecipitates were washed three times in 1 ml of cold lysis buffer, resuspended in Laemmli's sample buffer: 50 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 5% 2-mercaptoethanol, 1% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. Immunoblot assay of antibodies against inducible NO synthase and phosphotyrosine

The lysates of glial cells and RAW264.7 macrophages treated with interferon-γ and/or lipopolysaccharide, and the immunoprecipitated proteins using antibodies against Janus kinases and STAT1 were dissolved in Laemmli's sample buffer and subjected to SDS-PAGE (8% polyacrylamide gels) (Laemmli, 1970). Western blotting was performed by transferring proteins from a slab gel to a sheet of polyvinylidene difluoride membrane (Bio-Rad Lab.) by electroelution at a constant voltage of 50 V for 2 h at 4°C. After the polyvinylidene difluoride membrane was incubated with Tris-buffered saline (pH 7.5) containing 0.3% Triton X-100 (TBS-T) and 5% dehydrated skim milk (Difco Lab.) to block nonspecific protein binding, the membrane was incubated with primary antibodies such as rabbit anti-inducible NO synthase antibody (diluted 1/1000) or mouse anti-phosphotyrosine antibody (diluted 1/1000), and a secondary antibody (horseradish peroxidase-linked antibodies against rabbit immunoglobulin (Ig) or mouse Ig, each diluted 1/1000). Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected by the enhanced chemiluminescence detection system (ECL kit, Amersham). The protein bands that cross reacted with antibodies could be detected in X ray films (X-Omat JB-1, Kodak) 5–30 s after the exposure. The protein band of inducible NO synthase on X ray film was scanned and densitometrically analyzed by a dual-wavelength flying spot scanner (CS-9000, Shimadzu). Prestained SDS-PAGE standards (Bio-Rad Lab.) was used as the molecular weight markers. Apparent molecular weights of myosin, β-galactosidase, phosphorylase *B*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were 205, 116.5, 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa, respectively according to the manufacture's instruction.

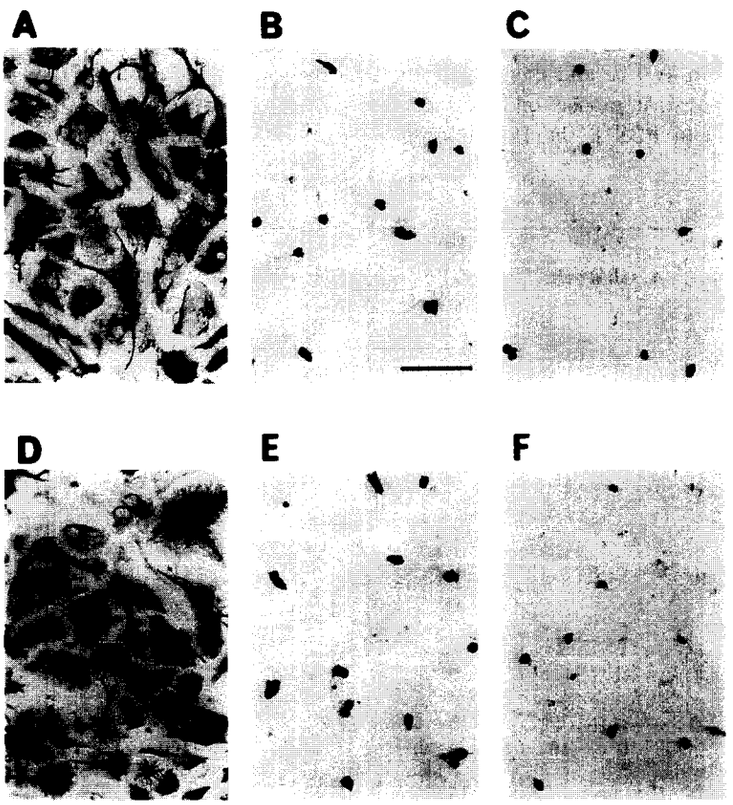

Fig. 1. Immunoreactivities of antibodies against glial fibrillary acidic protein (GFAP), cluster of differentiation-11b (CD11b) and just another kinase-2 (Jak2) in rat glial cells. Glial cells, which were prepared from rat brains at neonatal 0–2 days as described in Section 2, were subsequently cultured for 7 days in 12-well plates. After these cultures were incubated without (A, B, C) or with 1 μg/ml of herbimycin A (D, E, F) for 24 h, cells were fixed and then immunostained by antibodies against GFAP (A, D), CD11b (B, E), and Jak2 (C, F). Bar (in B) = 50 μm.

2.7. Stripping anti-phosphotyrosine antibody from immunoblot membranes and reprobing with antibodies against Janus kinases or STAT1

To clarify whether tyrosine phosphorylated proteins were Janus kinases or STAT1, anti-phosphotyrosine antibody were stripped from the blotting membranes in a stripping buffer composed of 62.5 mM Tris-HCl (pH 6.7) containing 2% SDS and 100 mM 2-mercaptoethanol at 50°C for 30 min and the membranes were further washed three times with TBS-T, then with TBS-T containing 5% skim milk overnight at 4°C. Subsequently, each membrane was further incubated with primary antibody (each diluted 1/1000) against Jak1, Jak2, Tyk2 or STAT1, respectively. Horseradish peroxidase-linked anti-rabbit Ig antibodies were then incubated, and horseradish peroxidase-labeled antibodies were detected by ECL kit.

2.8. Immunocytochemistry in rat glial cells

Immunocytochemistry was performed according to the modification of the avidin-biotin peroxidase procedure. In brief, the treated glial cells were rinsed in phosphate-buffered saline (PBS) and then fixed by adding 4% paraformaldehyde in PBS to the dishes for 1 h at room temperature. After rinsing with PBS containing 0.3% Triton X-100 (PBS-T), the fixed cells were incubated with primary antibodies against GFAP, CD11b, Jak2, and inducible NO synthase for 10 h at 4°C, and then incubated with biotinylated antibody against mouse IgG or rabbit IgG (1:1000, Vector Lab.) in PBS-T for 1 h at room temperature. Subsequently, The cells were incubated with the avidin-biotin-peroxidase complex (diluted 1/4000, Vector Lab.) in PBS-T for 1 h at room temperature. After each incubation, all cells were rinsed several times with PBS-T. The labeling was revealed by an incubation with 50 mM Tris-HCl buffer (pH 7.6) containing 0.02% 3,3′-diaminobenzidine, 0.0045% hydrogen peroxide and 0.6% nickel ammonium sulfate for 5–10 min.

3. Results

3.1. NO synthase activity induced by interferon-γ and lipopolysaccharide in cultured glial cells and RAW264.7 macrophages

The glial cells were prepared from rat brains at neonatal 0–2 days. Over 90% of the cells in cultured cells were astrocytes (positive to anti-GFAP antibody), and approximately 7% were microglia (positive to anti-CD11b antibody) (Fig. 1A and Fig. 1B, respectively). The immunostaining of anti-Jak2 antibody was similar the staining by anti-CD11b antibody (Fig. 1C), suggesting Jak2 exists mainly microglia. In these cultured glial cells, microglia were almost all amoeboid type but ramified type cells were not observed (Fig. 1B).

Using these cultures, we examined effects of interferon-γ and lipopolysaccharide on NO_2^- accumulation and expression of inducible NO synthase, compared to those in RAW264.7 macrophages. Fig. 2 shows that rat interferon-γ (10–1000 U/ml) alone induced concentration-dependent

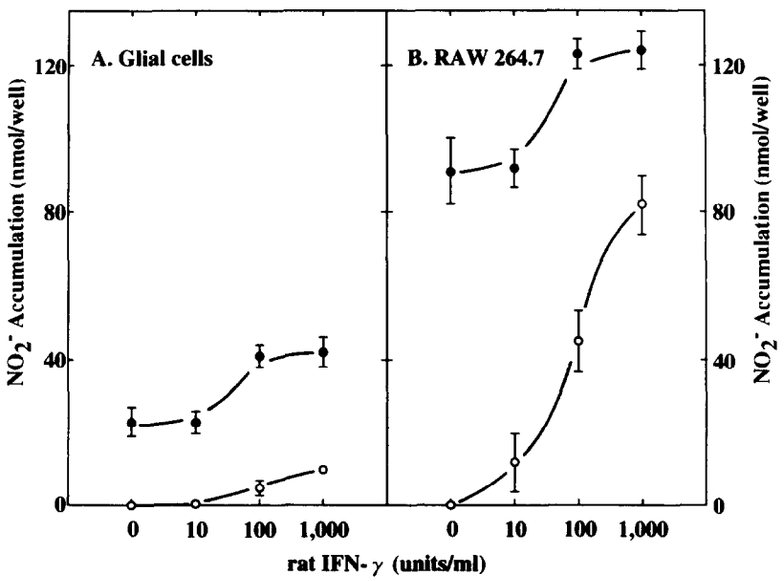

Fig. 2. NO_2^- accumulation induced by interferon-γ and lipopolysaccharide in culture media of glial cells and RAW264.7 macrophages. Glial cells (A) and RAW264.7 macrophages (B) were cultured in 12-well plates. These glial cells were treated with recombinant rat interferon-γ (0, 10, 100, 1000 U/ml) in the absence (○) or presence of 1 μg/ml of lipopolysaccharide (●) for 24 h. After treatment, NO_2^- concentration in each cultured medium was measured spectrophotometrically based on the Griess reaction at 540 nm, with sodium nitrite as the standard. Each value is the mean ± S.E. of 6–9 determinations.

NO_2^- accumulation after 24 h in cultured media of both glial cells and RAW264.7 macrophages. The NO_2^- accumulation in RAW264.7 cells had approximately 5-fold larger maximal activity in glial cells. Human interferon-γ also concentration-dependently induced NO_2^- accumulation in both cells, but human interferon-γ required 10-fold concentration of rat interferon-γ, and the accumulation was lower than that by rat interferon-γ (data not shown).

In addition, LPS (1 μg/ml) alone induced NO_2^- accumulation and enhanced interferon-γ-induced accumulation in both glial cells and RAW264.7 macrophages (Fig. 2). Although 1 μg/ml of lipopolysaccharide for 24 h induced half-maximum of NO_2^- accumulation and expression of inducible NO synthase in glial cells, the stimulation sometimes induced the maximal activities and morphological changes of astrocytes. The reason of these differences is not clear. Since the sensitivity of lipopolysaccharide might be different in each culture, autocrinic or paracrinic production of several cytokines, which was sometimes produced by lipopolysaccharide, might cause the enhancement of inducible NO synthase expression and the morphological changes in glial cells.

130 kDa protein of inducible NO synthase was also expressed by interferon-γ or lipopolysaccharide (1 μg/ml) alone in glial cells and RAW264.7 macrophages (Fig. 3). In addition, lipopolysaccharide enhanced interferon-γ-induced expression (Fig. 3). In addition, the immunostaining

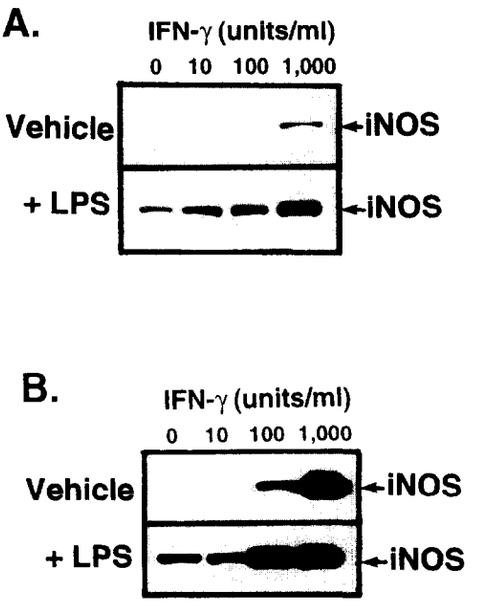

Fig. 3. Expression of 130 kDa protein of inducible NO synthase induced by interferon-γ and lipopolysaccharide in glial cells and RAW264.7 macrophages. Glial cells (A) and RAW264.7 macrophages (B) were treated with rat interferon-γ (0, 10, 100, 1000 U/ml) in the absence (Vehicle, upper panel) or presence of 1 μg/ml of lipopolysaccharide (+LPS, lower panel) for 24 h. After treatment, cells were scraped and lysed. Subsequently, each sample was subjected to immunoblotting of anti-inducible NO synthase antibody. Arrows indicate 130 kDa proteins.

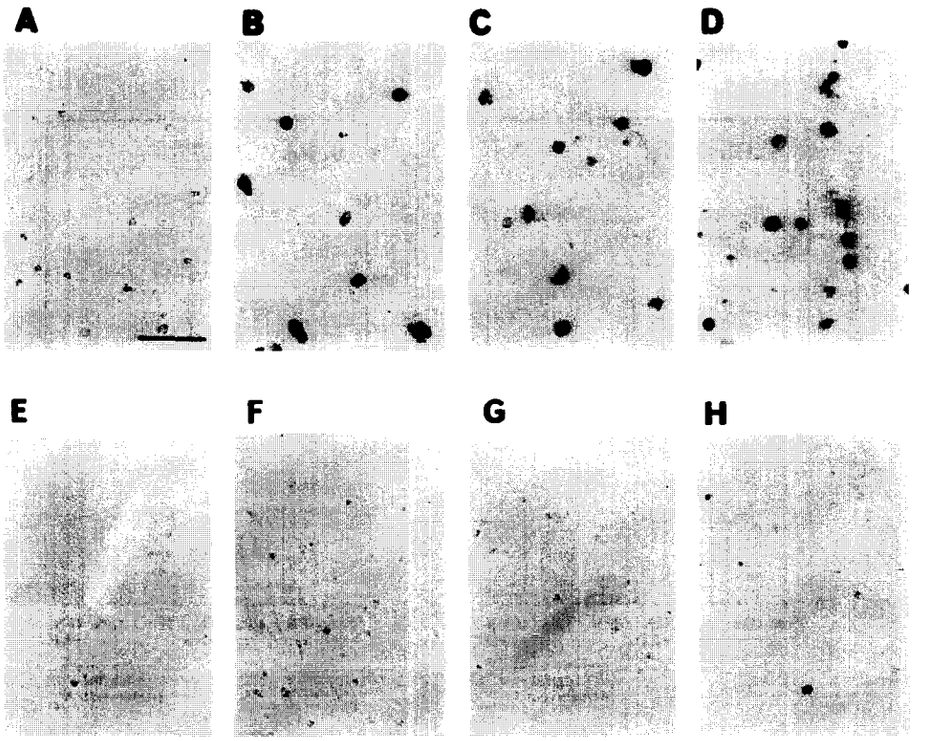

Fig. 4. Immunoreactivity of anti-rat inducible NO synthase antibody in glial cells. Cultured glial cells were treated with vehicle (A, E), 1000 U/ml of rat interferon-γ (B, F), 1 μg/ml of lipopolysaccharide (C, G) and 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide (D, H), in the absence (A, B, C, D) or presence of 1 μg/ml of herbimycin A (E, F, G, H) for 24 h. Subsequently, treated cells were fixed and then immunostained by anti-rat inducible NO synthase antibody. Bar (in A) = 50 μm.

of anti-rat inducible NO synthase antibody in the glial cells, which were treated with interferon-γ (1000 U/ml), lipopolysaccharide (1 μg/ml) and interferon-γ plus lipopolysaccharide for 24 h (Fig. 4), was similar to the immunostaining of antibodies against CD11b and Jak2 (Fig. 1B and Fig. 1C). Treatment with interferon-γ (1000 U/ml) plus lipopolysaccharide (1 μg/ml) induced strong immunostaining of anti-inducible NO synthase antibody, and the number of immunostained cells was increased (Fig. 4D).

3.2. Inhibitory effects of herbimycin A on NO_2^- accumulation and 130 kDa protein of inducible NO synthase induced by interferon-γ and lipopolysaccharide in glial cells and RAW264.7 macrophages

We further examined effects of a tyrosine kinase inhibitor, herbimycin A, on the expression of inducible NO synthase by interferon-γ and lipopolysaccharide. Glial cells and RAW264.7 macrophages were incubated with interferon-γ (1000 U/ml), lipopolysaccharide (1 μg/ml) and interferon-γ plus lipopolysaccharide in the absence or presence of 1 μg/ml herbimycin A. After these treatments for 24 h, NO_2^- accumulation in the culture media was assayed (Fig. 5) and cell lysates were analyzed by immunoblotting of anti-inducible NO synthase antibody (Fig. 6). Herbimycin A inhibited significantly NO_2^- accumulation by interferon-γ, lipopolysaccharide and inter-

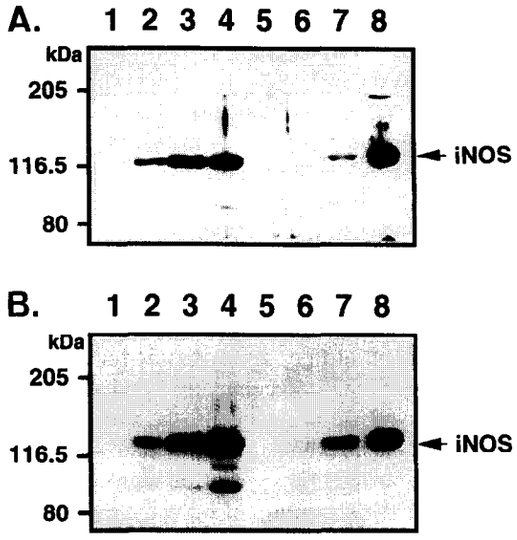

Fig. 6. Effects of herbimycin A on expression of 130 kDa protein of inducible NO synthase induced by interferon-γ and lipopolysaccharide in glial cells and RAW264.7 macrophages. Glial cells (A) and RAW264.7 macrophages (B) were treated with vehicle (lane 1), 1000 U/ml of rat interferon-γ (lanes 2 and 5), 1 μg/ml of lipopolysaccharide (lanes 3 and 6) and 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide (lanes 4 and 7) in the absence (lanes 1, 2, 3 and 4) or presence of 1 μg/ml of herbimycin A (lanes 5, 6 and 7) for 24 h. After treatment, cells were scraped and lysed. Subsequently, each sample was subjected to immunoblotting of anti-inducible NO synthase antibody. 20 ng of purified inducible NO synthase was used as positive control (lane 8). Arrows indicate 130 kDa protein of inducible NO synthase.

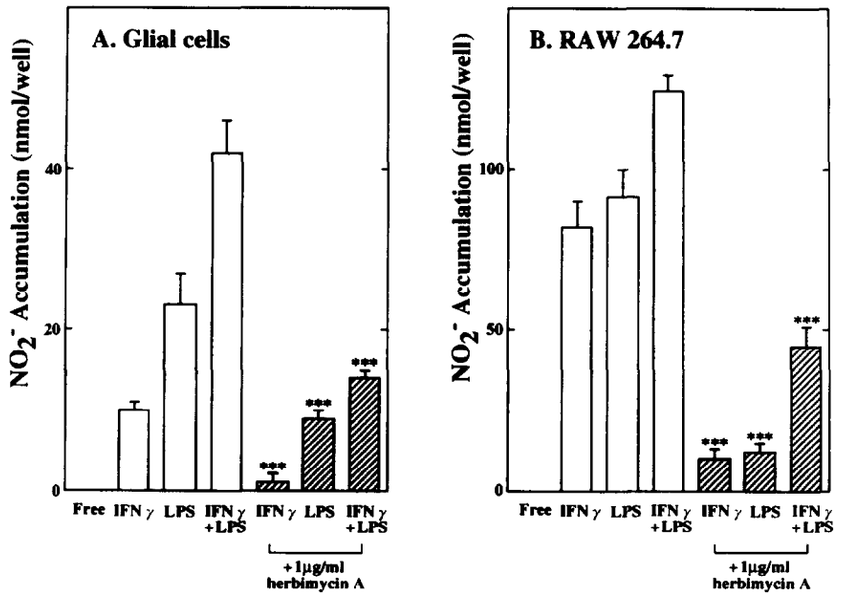

Fig. 5. Effects of herbimycin A on NO_2^- accumulation induced by interferon-γ and lipopolysaccharide in culture media of glial cells and RAW264.7 macrophages. Glial cells (A) and RAW264.7 macrophages (B) were cultured in 12-well plates. These cells were treated with 1000 U/ml of rat interferon-γ, 1 μg/ml of lipopolysaccharide and 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide in the absence (open column) or presence of 1 μg/ml of herbimycin A (hatched column) for 24 h. After treatment, NO_2^- concentration in each cultured medium was measured. Each value is the mean ± S.E. of 6–9 determinations. Significances: *** $P < 0.001$ vs. each activity in the absence of herbimycin A.

feron-γ plus lipopolysaccharide in glial cells and RAW264.7 macrophages (Fig. 5). In addition, expression of 130 kDa protein of inducible NO synthase was also inhibited by herbimycin A in both cells (Fig. 6). By densitometrical analysis of protein band of inducible NO synthase in immunoblot of glial cells (Fig. 6A), expression of inducible NO synthase by interferon-γ (lane 2), lipopolysaccharide (lane 3), or interferon-γ plus lipopolysaccharide (lane 4) was inhibited by 100% (lane 5), 100% (lane 6), or 85% (lane 7) by herbimycin A, respectively. Also, in RAW264.7 macrophages (Fig. 6B), the expression by interferon-γ (lane 2), lipopolysaccharide (lane 3), or interferon-γ plus lipopolysaccharide (lane 4) was inhibited by 100% (lane 5), 94% (lane 6), or 79% (lane 7) by herbimycin A, respectively.

In addition, herbimycin A inhibited immunoreactivity of anti-inducible NO synthase antibody and decreased the number of the immunopositive cells (Fig. 4F, G and H). However, treatment with herbimycin A for 24 h did not change the immunoreactivity of antibodies against GFAP, CD11b, or Jak2 (Fig. 1D, E and F). It is suggested that herbimycin A-induced inhibition of inducible NO synthase expression was not caused by the cell toxicity.

3.3. Effects of interferon-γ and lipopolysaccharide on tyrosine phosphorylation in Janus kinases in glial cells and RAW264.7 macrophages

To clarify whether the expression of inducible NO synthase is involved in the activation of tyrosine kinases, we further examined immunoprecipitation of antibodies against Janus kinases and immunoblotting of anti-phosphotyrosine antibody in glial cells and macrophages. Glial cells and RAW264.7 macrophages were incubated with interferon-γ (1000 U/ml), lipopolysaccharide (1 μg/ml) and interferon-γ plus lipopolysaccharide for 10 min. After these treatments, cells were scraped, lysed and immunoprecipitated by antibodies against Jak1, Jak2 and Tyk2. Subsequently, each immunoprecipitated sample was subjected to immunoblot using anti-phosphotyrosine antibody. To confirm that tyrosine phosphorylated protein which was immunoprecipitated by each antibody is identified as each kinase, anti-phosphotyrosine antibody was stripped from these membranes, washed, and further subjected to immunoblotting using each antibody against Janus kinase. Fig. 7B shows that interferon-γ alone and interferon-γ plus lipopolysaccharide induced tyrosine phosphorylation of Jak2 (130 kDa protein) in glial cells but lipopolysaccharide alone did not induce the phosphorylation. In addition, herbimycin A inhibited tyrosine phosphorylation of Jak2 by interferon-γ (Fig. 7B, lane 5). On the other hand, the tyrosine phosphorylation of Jak1 (130 kDa protein) and Tyk2 (134 kDa protein) was not detected in any treatments (Fig. 7, A and C). Similar results were also obtained in RAW264.7 macrophages (Fig. 8).

By immunocytochemistry, anti-Jak2 antibody mainly immunostained microglial cells, and the immunoreactivity

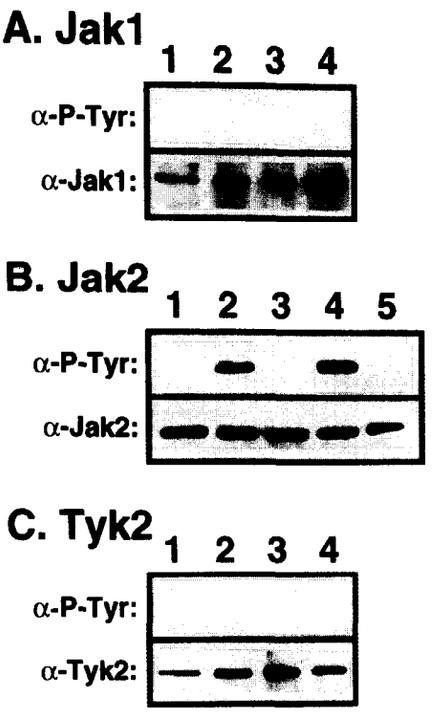

Fig. 7. Effects of interferon-γ and lipopolysaccharide on tyrosine phosphorylation in Janus kinases in glial cells. Glial cells were treated with vehicle (lane 1), 1000 U/ml of rat interferon-γ (lane 2), 1 μg/ml of lipopolysaccharide (lane 3), 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide (lane 4), and 1000 U/ml of interferon-γ plus 1 μg/ml of herbimycin A (lane 5) for 10 min. After treatment, cells were scraped and lysed. After each sample was subjected to immunoprecipitation using antibodies against Jak1 (A), Jak2 (B) and Tyk2 (C), immunoblotting of anti-phosphotyrosine antibody (α-P-Tyr, upper panel) was performed. Subsequently, bound antibodies were stripped from immunoblotting membranes, and then the membranes were further subjected to immunoblotting using antibodies against Jak1 (α-Jak1), Jak2 (α-Jak2) and Tyk2 (α-Tyk2).

was not changed by the treatment with herbimycin A for 24 h (Fig. 1C and F).

3.4. Effects of interferon-γ and lipopolysaccharide on tyrosine phosphorylation in STAT1 in glial cells and RAW264.7 macrophages

Recently, approximately 1500 base pair fragment from the 5′-flanking region of inducible NO synthase gene is cloned from mouse genomic cosmid library (Xie et al., 1993; Lowenstein et al., 1993). It is suggested that the promoter region of mouse inducible NO synthase gene involves the interferon-γ activation site (GAS), nuclear factor κB (NF-κB)-binding site, etc. In addition, it is known that GAS is activated by interferon-γ-activated factor (GAF), which is homodimer of tyrosine phosphorylated signal transducer and activator of transcription-1α (STAT1α, p91) (Darnell et al., 1994). From these observations, we further examined whether STAT1 was also tyrosine phosphorylated by interferon-γ or lipopolysaccharide.

After incubation of glial cells and RAW264.7 macrophages with interferon-γ (1000 U/ml), lipopoly-

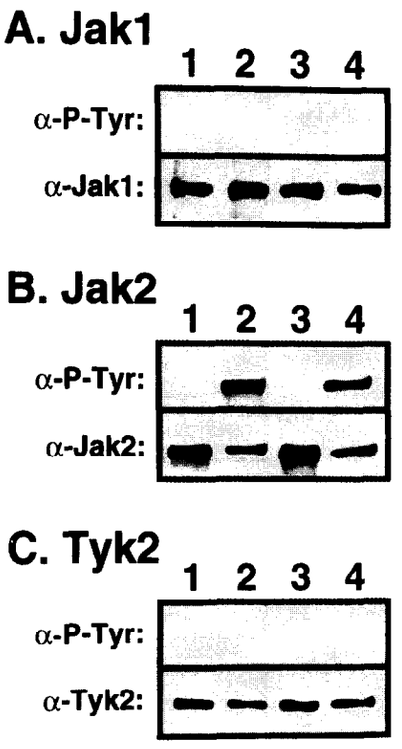

Fig. 8. Effects of interferon-γ and lipopolysaccharide on tyrosine phosphorylation in Janus kinases in RAW264.7 macrophages. RAW264.7 macrophages were treated with vehicle (lane 1), 1000 U/ml of rat interferon-γ (lane 2), 1 μg/ml of lipopolysaccharide (lane 3) and 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide (lane 4) for 10 min. After treatment, cells were scraped and lysed. After each sample was subjected to immunoprecipitation using antibodies against Jak1 (A), Jak2 (B) and Tyk2 (C), immunoblotting of anti-phosphotyrosine antibody (α-P-Tyr, upper panel) was performed. Subsequently, bound antibodies were stripped from immunoblotting membranes, and then the membranes were further subjected to immunoblotting using antibodies against Jak1 (α-Jak1), Jak2 (α-Jak2) and Tyk2 (α-Tyk2).

saccharide (1 μg/ml) and interferon-γ plus lipopolysaccharide for 15 min, the cell lysates were immunoprecipitated by anti-STAT1 antibody. Although anti-STAT1 antibody immunoprecipitated both p91 (STAT1α) and p84 (STAT1β), only STAT1α (p91) resulted in tyrosine phosphorylation by interferon-γ alone and interferon-γ plus lipopolysaccharide in glial cells (Fig. 9A, lanes 2 and 4). In addition, herbimycin A inhibited interferon-γ-induced tyrosine phosphorylation of STAT1α (Fig. 9A, lane 5). On the other hand, the tyrosine phosphorylation of STAT1β (p84) was not detected in any treatments. Similar results were also obtained in RAW264.7 macrophages (Fig. 9B).

4. Discussion

Recently, several laboratories reported that several endotoxins and cytokines induced inducible NO synthase in glial cells (Boje and Arora, 1992; Simmons and Murphy, 1992; Galea et al., 1992). The activation was caused by the de novo synthesis of inducible NO synthase proteins with a molecular size of 130 kDa in glial cells of rat brain. Lipopolysaccharide injection in vivo also induced inducible NO synthase activities in peripheral tissues, e.g., liver, lung, and spleen (Salter et al., 1991; Oguchi et al., 1992). In addition, recent papers suggested that glial inducible NO synthase mRNA expressed in glial cells seems to identify inducible NO synthase mRNAs expressed in hepatocytes and macrophages (Wood et al., 1993; Adachi et al., 1993; Hokari et al., 1994). Previously, we reported that after glial cells were treated with lipopolysaccharide, the expression of 130 kDa protein of inducible NO synthase began at 5 h and reached a peak at 24 h and that NO_2^- accumulation in culture media was linearly accumulated from 6 h to 48 h in the presence of L-Arg (Nomura and Kitamura, 1993; Kitamura et al., 1996). Since calmodulin was constitutively expressed and bound with glial inducible NO synthase even in the absence of Ca^{2+}, calmodulin seems to be a subunit of inducible NO synthase in glial cells. Thus, NO production in glial cells is simply stimulated by the synthesis of inducible NO synthase proteins and is not regulated by intracellular Ca^{2+} concentration (Kitamura et al., 1996). In the present study, we further examined how inducible NO synthase proteins were induced by interferon-γ and lipopolysaccharide.

In glial cells, rat interferon-γ alone induced NO_2^- accumulation and expression of 130 kDa protein of inducible NO synthase after 24 h. Lipopolysaccharide enhanced rat interferon-γ-induced NO_2^- accumulation and expression of

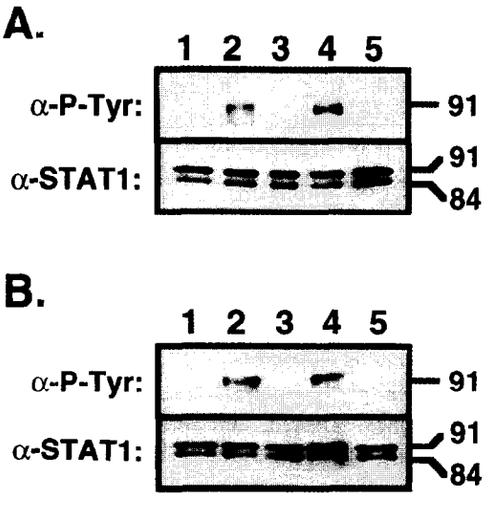

Fig. 9. Effects of interferon-γ and lipopolysaccharide on tyrosine phosphorylation in STAT1 in glial cells and RAW264.7 macrophages. Glial cells (A) and RAW264.7 macrophages (B) were treated with vehicle (lane 1), 1000 U/ml of rat interferon-γ (lane 2), 1 μg/ml of lipopolysaccharide (lane 3), 1000 U/ml of interferon-γ plus 1 μg/ml of lipopolysaccharide (lane 4), and 1000 U/ml of interferon-γ plus 1 μg/ml of herbimycin A (lane 5) for 15 min. After treatment, cells were scraped and lysed. After each sample was subjected to immunoprecipitation using anti-STAT1 antibody, immunoblotting of anti-phosphotyrosine antibody (α-P-Tyr, upper panel) was performed. Subsequently, bound antibodies were stripped from immunoblotting membranes, and then the membranes were further subjected to immunoblotting using anti-STAT1 antibody (α-STAT1, bottom panel), showing p91 (STAT1α) and p84 (STAT1β).

130 kDa protein of inducible NO synthase. In addition, inducible NO synthase-expressing cells were mainly microglial cells (Fig. 4). On the other hand, NO_2^- accumulation in culture medium of glial cells was lower than that of RAW264.7 macrophages (Fig. 2). Expression of inducible NO synthase induced by interferon-γ in the glial cells was also lower than that in macrophages (Fig. 3). Although these differences between glial cells and macrophages were not clear, the sensitivities of interferon-γ and lipopolysaccharide in glial cells were lower than those in macrophages.

Herbimycin A (a tyrosine kinase inhibitor) inhibited significantly NO_2^- accumulation, expression of 130 kDa protein of inducible NO synthase and immunostaining of anti-inducible NO synthase antibody by a treatment with interferon-γ, lipopolysaccharide and interferon-γ plus lipopolysaccharide in both glial cells and RAW264.7 macrophages. Recent papers indicated that several tyrosine kinase inhibitors such as tyrphostins, genistein or herbimycin A suppressed lipopolysaccharide-induced toxicity (Novogrodsky et al., 1994) and the induction of inducible NO synthase (Feinstein et al., 1994; Nishiya et al., 1995; Kitamura et al., 1996). These observations suggested that the induction of inducible NO synthase is mediated by activation of tyrosine kinases in both glial cells and macrophages. Tyrosine kinases are known as Janus kinase family, Src family, mitogen-activated protein (MAP) kinase kinase (MAPKK) family and receptor-linked tyrosine kinases. In the present study, we focused whether the Janus kinases such as Jak1, Jak2 and Tyk2 are involved in the expression of inducible NO synthase in glial cells.

Interferon-γ alone and interferon-γ plus lipopolysaccharide induced tyrosine phosphorylation of one of Janus kinases, Jak2 (130 kDa proteins), in both glial cells and macrophages. However, tyrosine phosphorylation of Jak1 and Tyk2 was not detected. On the other hand, lipopolysaccharide alone did not induce the phosphorylation of Janus kinases at all. Although a recent paper suggested that interferon-γ receptors activate both Jak1 and Jak2 (Müller et al., 1993; Igarashi et al., 1994), the present study indicated that the activation of Jak2 is mainly involved in interferon-γ-induced expression of inducible NO synthase in glial cells and RAW264.7 macrophages. In fact, Jak2 constitutively existed in microglial cells (Fig. 1C), and interferon-γ induced inducible NO synthase mainly in microglial cells (Fig. 4B). Furthermore, recent papers indicated that the promoter region of mouse inducible NO synthase gene involves the interferon-γ activation site (GAS) (Xie et al., 1993) and that GAS is activated by tyrosine phosphorylated signal transducer and activator of transcription-1α (STAT1α, p91) (Darnell et al., 1994). Fig. 9 shows that interferon-γ induced tyrosine phosphorylation of STAT1α (p91) but did not that of STAT1β (p84). In addition, herbimycin A inhibited the phosphorylation in both rat glial cells and mouse macrophages. These results suggested that the promoter region of rat inducible NO synthase gene was similar to that of mouse gene. From these observations, it is suggested that interferon-γ-induced expression of inducible NO synthase in glial cells may involve the pathway of interferon-γ receptor, Jak2 and STAT1α (GAF) in rat microglial cells.

Although lipopolysaccharide-induced expression of inducible NO synthase was inhibited by herbimycin A, the present results suggested that this expression did not seem to be induced through the activation of Janus kinases. We previously reported that lipopolysaccharide-induced expression of inducible NO synthase was not inhibited by inhibitors for protein kinase C (staurosporine) and cAMP-dependent protein kinase (KT-5720) and that lipopolysaccharide induced tyrosine phosphorylation of 120 kDa proteins in glial cells (Kitamura et al., 1996). Although the transmembrane control of lipopolysaccharide stimulation in detail is still unclear, recent papers indicated that: (i) lipopolysaccharide binds to CD14 and CD14 couples to Src family Lyn in human monocytes (Štefanová et al., 1993); (ii) lipopolysaccharide induces tyrosine phosphorylation of MAP kinases and activation of MAP kinases in RAW264.7 macrophages (Weinstein et al., 1992); (iii) tyrosine phosphorylation of MAP kinases is catalyzed by MAPKK (Nishida and Gotoh, 1993); and (iv) the translocation of NF-κB into nucleus is necessary for lipopolysaccharide-induced induction of inducible NO synthase in RAW264.7 macrophages (Xie et al., 1994). In addition, herbimycin A inhibited tyrosine phosphorylation of MAP kinases (Weinstein et al., 1992) and translocation of NF-κB (Nishiya et al., 1995). These observations suggest that inhibitory effects of herbimycin A on lipopolysaccharide-induced expression of inducible NO synthase in both glial cells and macrophages may be mediated by inhibition of other tyrosine kinases such as Lyn, MAPKK, or unidentified tyrosine kinases which regulate NF-κB activation. The intracellular mechanism of lipopolysaccharide-induced expression of inducible NO synthase requires for further investigations.

In conclusion, we have shown here that inducible NO synthase protein was induced by interferon-γ and lipopolysaccharide and the induction was predominantly occurred in microglial cells. Both induction was inhibited by herbimycin A. Interferon-γ induced tyrosine phosphorylation of Jak2 and STAT1α, and both tyrosine phosphorylation was inhibited by herbimycin A. These results suggest that the interferon-γ-induced induction of inducible NO synthase involves activation of Jak2-STAT1α pathway in both glial cells and macrophages. On the other hand, lipopolysaccharide-induced expression was mediated by other tyrosine kinases.

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