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C-Jun N-Terminal Kinase (JNK) Regulation of iNOS Expression in Glial Cells: Predominant Role of JNK1 Isoform

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ABSTRACT

The mitogen-activated protein kinases (MAPKs) play a central role in mediating the activation and transcriptional responses of diverse cells, including glia. c-Jun N-terminal kinase (JNK), a member of the MAPK family, is activated by a variety of stress and proinflammatory signals and in turn phosphorylates its downstream substrates including nuclear factors, leading to transcriptional activation of target genes. There are at least three subtypes of JNK (i.e., JNKs 1–3) that may play isoform-specific roles. This study examined the role of JNK isoforms in the induction of inducible nitric oxide synthase (iNOS) in astrocytes in response to lipopolysachharide (LPS) and interferon (IFN)-γ. While an inhibitor of the JNK pathway (SP600125) inhibited iNOS expression, ectopic expression of a constitutively active form of MEKK1 (MAPK/ERK kinase kinase-1), an upstream activator of JNK, led to an induction of co-transfected iNOS promoter activity and, in the presence of LPS, to an enhanced expression of iNOS. RNA knockdown studies with JNK subtype-specific short-interfering RNA (siRNA), indicated that JNK1- but not JNK2- nor JNK3-specific siRNA, interfered with LPS/IFNγ induction of iNOS. It is concluded that, of the three JNK forms, JNK1 is the major mediator of iNOS induction and perhaps, inflammatory signaling in general, in glial cells. *Antioxid. Redox Signal.* 8, 903–909.

INTRODUCTION

THE BRAIN EXPRESSION OF INDUCIBLE NITRIC OXIDE synthase (iNOS) forms an important aspect of neuroinflammation associated with conditions of brain injury, infection, ischemic stroke, and a variety of neurodegenerative and neuroinflammatory diseases (6, 10, 11, 21, 27). The enzyme, which can generate toxic levels of NO, is mainly expressed by glial cells (astrocytes and microglia) activated under the above conditions. A number of factors, both endogenous and exogenous, have been identified that induce glial expression of iNOS including microbial products such as bacterial lipopolysaccharide (LPS), cytokines such as interferon-γ (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin 1-beta (IL-1β), and certain "disease-specific" abnormal proteins including β-amyloid, HIV-specific gp41 and HIV-Tat, and prion protein (23, 26).

The induction process involves an activation of intracellular signaling pathways, prominently NFκB and members of the mitogen-activated protein kinase (MAPK) pathways, that are induced in response to activating signals (26). Previous studies, primarily using a pharmacological approach, have indicated the roles played by each of the major MAPKs (i.e., extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK) in iNOS induction in glia as well as other immune cells. While the role of p38 MAPK has been the subject of extensive investigation with regard to its proinflammatory signaling, including the expression of iNOS, information available regarding the JNK pathway has been sparse. Further, since the kinase comes in several isoforms, it would be desirable to determine isoform-specific roles, if any, of the kinase in cellular responses, and in particular, in specific gene expression such as that of iNOS.

The three main subtypes of JNK (JNK1, JNK2, and JNK3) are encoded by three different genes (14). Alternative splicing results in a total of about 10 isoforms. JNKs 1 and 2 are expressed ubiquitously, whereas JNK3 is expressed mainly in the brain, heart, and testis (20). There is evidence that JNKs

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1–3 may have specific roles *in vivo* (1, 9, 17, 24), and in the brain, specific pathological roles played by the JNK isoforms have been described (7) including the critical role of neural-specific JNK3 in ischemic apoptosis (19).

Previously, we had shown that JNK is potently activated in glial cells in response to the inflammogen bacterial lipopolysaccharide (LPS) and proinflammatory cytokines (30, 31), combinations of which also induce iNOS. In the present study, we have used this experimental paradigm coupled with multiple strategies including transfection of cells with a constitutively active form of the upstream JNK activator [MAPK/ERK kinase kinase-1 (MEKK1)] pharmacological inhibition of JNKs, and most importantly, RNA interference to show that the JNK pathway plays an important role in the induction of iNOS in glial cells. The use of siRNAs targeted against the three isoforms of JNKs has further allowed us to conclude that JNK1 predominantly mediates the induction of iNOS and the cytokine, TNFα, in LPS-treated astrocytes.

MATERIALS AND METHODS

Materials

Calf serum (CS), Dulbecco's modified Eagle medium (DMEM), and RPMI 1640 were from GIBCO-BRL (Grand Island, NY). IFNγ was obtained from Peprotech (Rocky Hill, NJ). The c-Jun N-terminal kinase (JNK) inhibitor SP600125 was from Calbiochem (La Jolla, CA). Oligonucleotides were ordered from SigmaGenosys (Fisher Scientific, Suwanee, GA). Acrylamide and other reagents used for SDS-PAGE were purchased from BioRad (Carlsbad, CA). JNK isoform-specific antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Murine-Moloney Leukemia Virus reverse transcriptase (MMLV–RT) was from New England BioLabs (Beverly, MA). LPS, antibiotic-antimycotic mixture and unless otherwise noted, all other chemicals were from Sigma (St. Louis, MO). The transfection reagent, "Polyfect" was from Qiagen (Valencia, CA). Pregnant Sprague–Dawley rats were procured from Harlan Sprague–Dawley (Indianapolis, IN). pcDNA3–ΔMEKK1 plasmid was a gift from Dr. Roger Davis (University of Massachusetts).

Cell culture

The primary cultures of astrocytes were prepared from cerebral hemispheres dissected from newborn rat pups and used for the experiments as described previously (3, 25). These cells were cultured in DMEM supplemented with 10% CS and an antibiotic–antimycotic mixture. Stock cultures maintained in 75 cm² flasks were subcultured as needed into 6-well and 24-well plates. C-6 glial cells were maintained as described (4).

Transient transfections and luciferase assay

Transfections were carried out using "Polyfect" (Qiagen) according to standard protocol with minor modifications. Briefly, plasmid DNA (0.3 μg/well of a 24-well plate) was diluted in serum-free DMEM (60 μl) and 2.5 μl of Polyfect was added. The mixture was incubated at room temperature for 15 min and then diluted with DMEM containing serum (0.5 ml) before adding to the cultures (at 60%–70% confluency). Following incubation for 3–4 h, the medium was changed to remove the complexes. In some experiments, the kinase inhibitor, SP600125, was added at this time.

Luciferase assay was performed using a kit from Promega (Madison, WI) as described before (4). Briefly, cells in 24-well plates transfected with a iNOS promoter construct cloned into the Promega pGL3 vector (iNOS-luciferase) (4) were lysed by adding 100 μl of lysis buffer, sonicated for 10 s, and centrifuged in a tabletop microcentrifuge. An aliquot (40 μl) was mixed with 20 μl of assay reagent, vortexed, and read immediately in a Berthold luminometer.

Nitrite production

Nitrite production was assayed as described before (4), by mixing 100 μl of culture medium with 100 μl of Griess reagent [1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine and 2.5% phosphoric acid]. Absorption at 570 nm was read in a microplate reader using $NaNO_2$ as the standard.

Immunoblots

Cell lysates containing equal amounts of protein were electrophoresed through 10% SDS-polyacrylamide gels and transferred to PVDF membranes (3, 4). The membranes were blocked with 5% BSA in TBST (20 m*M* Tris, pH 7.5, 137 m*M* NaCl, 0.1% Tween-20) and incubated with TBST-1% BSA containing primary antibodies at dilutions of 1:1,000 to 1:5,000 overnight. After washing three times in TBST, they were incubated in anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish-peroxidase (HRP) at dilutions of 1:5,000 to 1:7,500 in TBST–1% BSA. Membranes were washed four times in TBST for 5 min each. Detection was done using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) and used for RT–PCR as described before (4, 25). Briefly, an aliquot (1 μg) of the RNA was reverse-transcribed by MMLV-RT using oligo-dT as the primer. The reaction was performed at 42°C for 60 min in a 20 μl volume. The product was diluted to 100 μl with water and an aliquot (1 μl) was used as the template for PCR. The primers used for amplifying iNOS, TNFα, and GAPDH sequences were as reported before (25).

Synthesis and transfection of siRNA targeted against JNK isoforms

mRNA sequences of JNK1, JNK2, and JNK3 were downloaded from Pubmed (www.ncbi.nih.gov) and inserted into the siRNA design tool at Ambion's website (www.ambion.com) which generates possible target sequences for siRNAs based on position in the mRNA, GC content, and other parameters. From the possible targets, three each were selected for JNK1, JNK2, and JNK3. The sequences and the

positions of the first nucleotide in the mRNA sequences were JNK1: 5′-AAAGATGTTGCAATCAAGA-3′ (position 146), 5′-AAGATTCTTGATTTTGGACTG-3′ (position 496), and 5′-AAAGAACTGATATACAAGGAG-3′ (position 1057); JNK2: 5′-AATAAATGTTGCTGCTGTCAAGAA-3′ (position 147), 5′-AACTTTATGATGACTCCCTAT-3′ (position 535), and 5′-AATTTACAAAGAAGTGATGGA-3′ (position 1065); JNK: 5′-AATAGTTTGTGCTGCGTATGA-3′ (position 114), 5′-AAGTCTGATTGCACACTGAAA-3′ (position 478), and 5′-AAGCAATTGGATGAAAGGGAG-3′ (position 1018).

DNA oligonucleotide templates corresponding to the targets were ordered from Sigma-Genosys. RNAs were transcribed from these templates using the *Silencer* siRNA construction kit (Ambion), annealed to form double-stranded siRNA, and purified using the above kit according to the instructions provided. siRNAs against GAPDH were transcribed using templates provided with the kit and used as a control. Fluorescein- and rhodamine-tagged control siRNAs with no known homology to mammalian DNAs were obtained from Qiagen and used as negative controls and to determine transfection efficiencies of siRNAs in different cells.

siRNAs were transfected using Transmessenger reagent (Qiagen). Cells were grown to 70%–80% confluency in 24-well or 6-well plates. siRNAs (0.4–0.8 μg/well for 24-well plates and 2–4 μg/well for 6-well plates) were mixed using equal amounts of the three siRNAs for a given JNK subtype. An aliquot (100 μl/well) of buffer "EC-R" was pipetted into a 1.5 ml sterile Eppendorf tube and 0.8–8 μl of "Condenser-R" (always twice the amount of siRNA v/w) was added and mixed. The siRNA was then added and vortexed for 10 s. The sample was incubated for 5 min and a volume of transmessenger equal to the volume of condenser-R was added. Following another 5-min incubation, cells were washed once with sterile PBS and overlaid with serum-free antibiotic-free media (0.4 ml for 24-well and 0.9 ml for 6-well) followed by the transfection mixture. Cells were incubated overnight up to 24 h, and the media was changed to regular medium containing serum and antibiotics. No cell death was observed with this long incubation.

Data analysis

Experiments were performed at least three times. The data are expressed as mean ± standard error of mean. Statistical analysis of the data was performed by one-way ANOVA. *p* values lower than 0.05 were considered significant.

RESULTS

Inhibition of LPS/IFN-γ induced iNOS expression in rat primary astrocytes by SP600125, a JNK inhibitor

SP600125, a pharmacological inhibitor of the JNK pathway (2), was used to determine if JNK activation is involved in the induction of iNOS in astrocytes treated with LPS and IFNγ. Cultures of astrocytes, pre-exposed (30 min) to increasing concentrations of SP600125 were treated with a combination of LPS and IFNγ. After 2 days, the levels of nitrite in the culture medium were measured, as described in Methods. The cell extracts were used for immunoblot analysis of iNOS protein using anti-iNOS antibodies. As shown in Fig. 1, there was a concentration-dependent inhibition of nitrite production (A) and iNOS expression (B) by the JNK inhibitor.

Effects of a constitutively active form of a JNK upstream kinase on glial iNOS expression

JNK is activated in a kinase cascade involving several upstream kinases (MKKKs), including MLKs, MEKK1, and MEKK5 that act via the intermediate kinases (MKK4 and MKK7) (12, 24). A truncated form of MEKK1 (ΔMEKK1) has been shown to act as a constitutively active form that when expressed in cells, potently induces the activation of JNKs and JNK-mediated cellular responses (29). We used this paradigm to determine the role of JNK activation on iNOS gene expression in glial cells. C-6 glial cells were co-transfected with an iNOS promoter-reporter construct (iNOS-luc) and either pcDNA3 (empty vector) or pcDNA3-ΔMEKK1. Cells were harvested 48 h posttransfection and assayed for luciferase activity. MEKK1 caused a marked increase in luciferase activity in a time and dose-dependent manner (Figs. 2A and B). Active MEKK1 overexpression also resulted in an activation of JNK as determined by immunoblots performed using antibodies directed against p-JNKs (Fig. 2C).

The expression of ΔMEKK1 also induced the activity of the co-transfected iNOS promoter in primary astrocytes as shown in Figure 3A. This induction was subject to inhibition

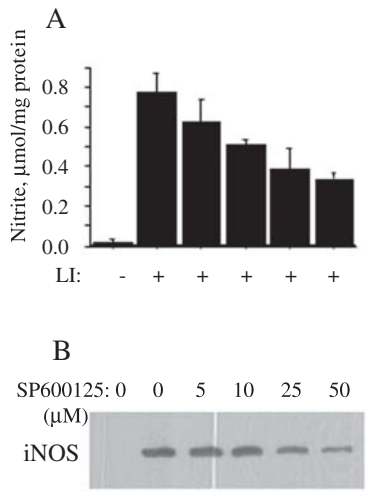

FIG. 1. Inhibition of LPS and IFNγ induced iNOS expression in primary astrocytes by SP600125. Cultures of primary astrocytes were incubated with increasing concentrations SP600125, for 30 min, after which LPS (200 ng/ml) and IFNγ (10 ng/ml) were added and the cultures incubated for 2 days. (**A**) Nitrite in the culture medium was measured as described under Methods. Values are expressed as means ± S.D. of triplicate determinations. (**B**) The cell extracts were subjected to Western blot using anti-iNOS antibodies.

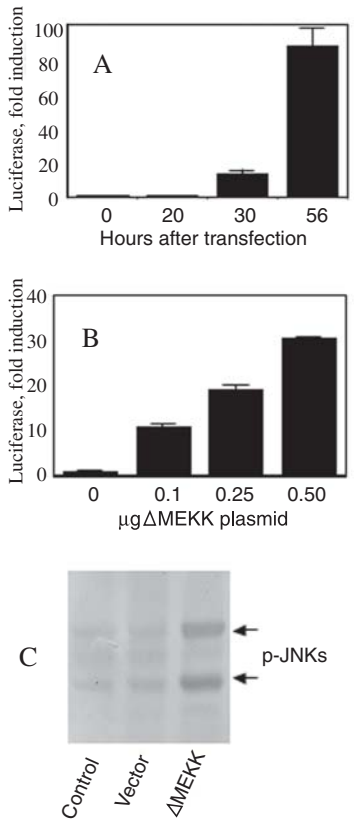

FIG. 2. Induction of iNOS-luciferase activity by ectopic expression of an active form of JNK-activating upstream kinase, ΔMEKK1. (**A**) C6 cells were co-transfected with iNOS-luciferase and either pcDNA3 (empty vector) or pcDNA3-ΔMEKK1 and at indicated times after transfection, the cells were harvested and assayed for luciferase activity. (**B**) Sets of cultures were also transfected with increasing amounts of ΔMEKK1 and after 48 h, processed for luciferase activity. (**C**) Cell extracts transfected for 24 h with ΔMEKK1 and the empty vector were analyzed by immunoblot for activated (phosphorylated) JNK using phospho-JNK specific antibodies.

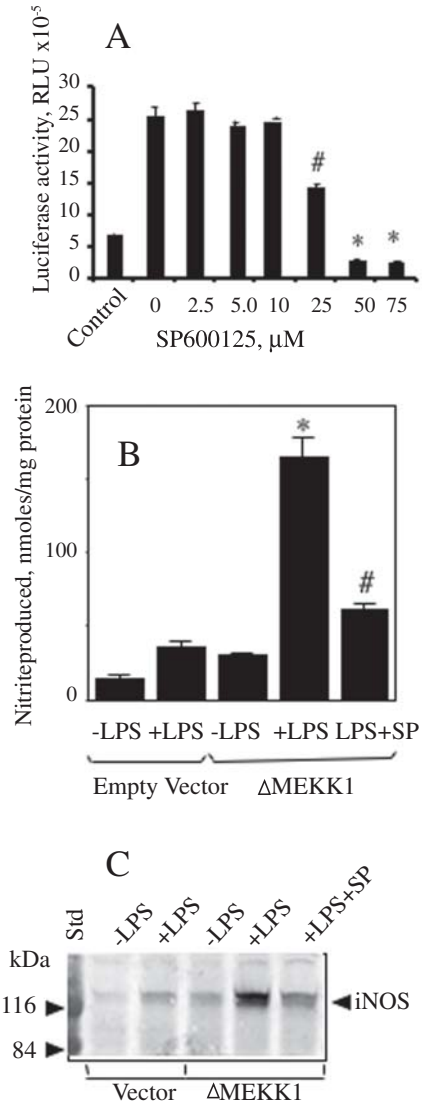

FIG. 3. Induction of iNOS expression by ΔMEKK1 in primary astrocytes. Subconfluent cultures of primary astrocytes in 24-well plates were co-transfected with iNOS-luciferase and ΔMEKK1 as in Fig. 2, and at the time of medium change (4 h posttransfection), treated in triplicate, with increasing concentrations of SP600125. (**A**) After 48 h, the cells were harvested and assayed for luciferase activity. #$p < 0.05$ compared to ΔMEKK1; *$p < 0.01$ compared to ΔMEKK1. In a parallel set of experiments, (**B**) triplicate cultures transfected with either the empty vector or ΔMEKK1 were treated with LPS (200 ng/ml) with or without SP600125 (50 µ*M*) and after 48 h, the medium was assayed for nitrite levels and the cell extracts analyzed by immunoblot for iNOS protein. *$p < 0.01$ compared to pcDNA/LPS. ΔMEKK1. #$p < 0.01$ compared to ΔMEKK1/LPS.

by SP600125. Interestingly, ΔMEKK1 overexpression in astrocyte cultures, (but not in C-6 glia, data not shown), led to a marked enhancement of LPS-induced NO production and this induction was suppressed by the JNK inhibitor, SP600125 (Fig. 3B).

Isoform specificities of JNKs for iNOS induction, an siRNA approach

We next investigated the isoform-specific roles of JNK in the induction of iNOS expression in astrocyte cultures using RNA interference technique. The cultures were transfected with siRNAs made against JNK-1, JNK-2, and JNK-3, as well as GAPDH (control) and the cell extracts subjected to Western blotting using isoform-specific anti-JNK antibodies. As can be seen from Fig. 4, the siRNAs were found to preferentially suppress the synthesis of the respective JNK isoforms.

For functional analysis, sets of astrocyte cultures were transfected with three sets directed against the JNK isoforms and after 24 h, following the repeat transfection, the cultures were treated with a combination of LPS and IFNγ. Nitrite levels were measured after an additional 24 h. While there was no significant difference in nitrite production between mock-transfected and GAPDH-siRNA transfected cultures, the cultures transfected with JNK siRNAs showed that among the JNK siRNAs, JNK-1 siRNA inhibited nitrite production

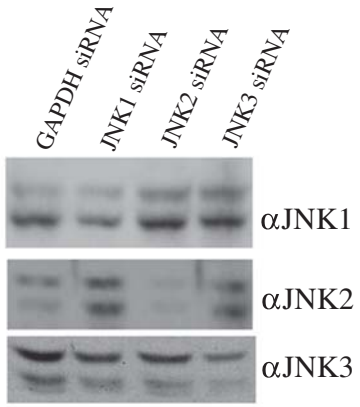

FIG. 4. RNAsi knockdown of JNK isoforms. Primary rat astrocytes were transfected with siRNAs targeted against JNK1, JNK2, and JNK3, as described under Methods. Control cultures were transfected with siRNAs directed against GAPDH. At the end of 60 h, the cultures were re-transfected with the respective siRNAs. After 24 h, the cells were harvested and the cell extracts subjected to Western blot analysis using isoform-specific anti-JNK antibodies.

by 46%, followed by siJNK-3 (22%), and siJNK2 (18%) (Fig. 5). Western blot analysis of iNOS protein performed on the cell lysates showed changes that corresponded to the levels of nitrite produced (Fig. 5, lower panel).

In another set of experiments, primary astrocytes were transfected with the three JNK and GAPDH siRNAs, as above, treated with a combination of LPS and IFNγ for 6 h, followed by total RNA extraction for determining iNOS mRNA levels by RT–PCR analysis. The results included in Fig. 6 show that siRNA-mediated knockdown of JNK1, in particular, results in a substantial inhibition of iNOS and TNFα mRNA expression.

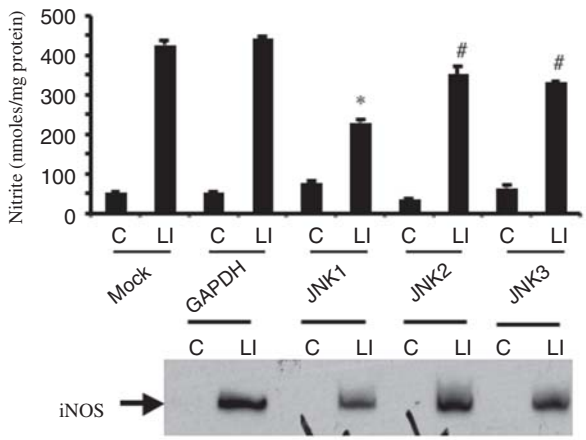

FIG. 5. Effect of siRNA knockdown of JNK isoforms on iNOS expression. Primary astrocytes, transfected as above with siRNAs targeted against JNK1, JNK2, JNK3, and GAPDH, were treated with a combination of LPS (200 ng/ml) and IFNγ (10 ng/ml). Nitrite levels in the culture medium were measured after 24 h. The cell extracts were used for Western blot analysis of iNOS protein. *$p < 0.01$ compared to GAPDH siRNA. #$p < 0.05$ compared to GAPDH siRNA.

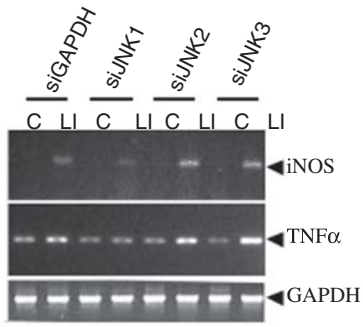

FIG. 6. RT–PCR analysis of JNK siRNA inhibition of iNOS and TNFα expression. Primary astrocytes were transfected with siRNAs targeted against JNK isoforms and GAPDH as in Figure 5 and treated with a combination of LPS/IFNγ for 6 h before extracting for total RNA. RT-PCR analysis for iNOS and TNFα was performed using specific primer sets as described under Methods.

DISCUSSION

JNK and other MAP kinase pathways play important roles in cytoplasmic to nuclear signal transduction, mediating transcriptional responses to extracellular stimuli. JNK is potently activated in glial cells in response to different stimuli, including LPS, proinflammatory cytokines (TNFα, IL-1β) as well as other stress stimuli, resulting in inflammatory and apoptotic responses. The present study has focused on the role of JNK in the induction of iNOS, a prototype inflammatory mediator. We used three experimental strategies: use of a pharmacological inhibitor (SP600125) of the pathway, ectopic expression of an active upstream kinase, and siRNA-mediated knockdown of JNK isoforms. The results obtained while confirming the role of JNK signaling in LPS-induced iNOS expression in astrocytes, further demonstrate an isoform-specific regulation (i.e., a predominant role of JNK1 over JNK2 and JNK3).

We showed previously that the strategy of ectopic expression of active kinases can be used to implicate the role of MAP kinases in inflammatory cell signaling by demonstrating that transfection of glia with active forms of MKK3 and MKK6, the two upstream kinases in the p38 MAPK pathway, and TGFβ-activated kinase (TAK1), an upstream MKKK that activates JNK, p38MAPK as well as NFκB, results in transcriptional activation of iNOS (4, 5). In the present study, we used a similar approach and tested the consequence of overexpression of a truncated, constitutively active form of MEKK1, which is known to potently activate the JNK pathway, on iNOS expression in astrocytes. The results presented showed that the active MEKK1 markedly induced the activity of iNOS promoter and potentiated LPS induction of the enzyme in a JNK-dependent manner.

Two previous studies have shown contrasting results with regard to the role of JNK in iNOS expression in human astrocytes. Thus, Hua *et al*. (16) showed that transfection of astrocytes with a dominant negative JNK prevented the induction of iNOS by a combination of IL-1β and IFNγ, while a later study by Jana *et al*., (18) showed a negative role of JNK under the same conditions (treatments) with

the use of a peptide inhibitor of JNK. However, the latter study did show that the JNK inhibitor suppressed transcriptional activation of iNOS in parallel to an inhibition of AP-1 response when the cultures were responding to IL-1β alone. Neither of these reports analyzed isoform-specific effects of JNK activation. In another study investigating JNK-mediated inflammatory signaling in microglia, Waetzig *et al*. (28) found that the JNK inhibitor, SP600125, attenuated LPS-induced expression of TNFα, IL-6, MCP-1, and COX-2. An analysis of the nuclear accumulation of JNK isoforms revealed that the amount of total JNK1 declined while JNK2, which is known to bind c-Jun 5–10 times stronger than JNK1 (14), increased and the weakly expressed JNK3 did not vary. However, the significance of these findings is unclear.

Our finding that JNK1 isoform plays a primary role in glial inflammatory response (i.e., expression of iNOS and TNFα), is in agreement with other recent publications that have indicated a predominant role for JNK1 in mediating inflammatory/apoptotic responses to diverse extracellular stimuli in different cell types, including lung cancer cells exposed to UV irradiation (8), cardiac myocytes under ischemia–reoxygenation (15), β-amyloid mediated stabilization of p53 and induction of apoptosis in cortical neurons (13), and mouse fibroblasts treated with TNFα (22). Liu *et al*. (22) further suggest that JNK1 is the main isoform that is activated by extracellular stimuli, while JNK2 functions to block JNK1-mediated signaling and responses by acting as a competing substrate for the upstream kinases. It follows that specific targeting of JNK1 may represent an important strategy to inhibit inflammatory and apoptotic responses in a variety of cells, including glia.

Finally, with respect to the mechanisms underlying isoform-specific roles of JNK, the cellular responses mediated by the kinase isoforms may perhaps be determined by substrate specificities of these kinases. The JNKs phosphorylate their downstream substrates (c-Jun, Elk-1, and ATF-2), leading ultimately to regulation of gene expression. Therefore, any differential regulation of these target factors due to their preferences for the kinase isoforms will translate into fine-tuning of transcriptional responses. Specific regulation is also possible at the posttranscriptional level mediated by preferred cytoplasmic substrates of the kinase isoforms.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ERK, extracellular signal-regulated kinase; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEKK1, MAPK/ERK kinase kinase-1; NO, nitric oxide; siRNA, short interfering RNA.

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