Nitric Oxide Modulates the c-Jun N-terminal Kinase/Stress-Activated Protein Kinase Activity through Activating c-Jun N-Terminal Kinase Kinase

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ABSTRACT: Nitric oxide is a signaling molecule that has a broad range of physiological functions, including neurotransmission, macrophage activation, and vasodilation. The mechanism by which nitric oxide regulates signal transduction mediating diverse biological activities is not fully understood, however. Here, we demonstrate that nitric oxide induced the stimulation of c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) in intact cells. Exposure of cultured HEK293 cells to sodium nitroprusside, a nitric oxide releasing agent, resulted in the stimulation of JNK1 activity. The sodium nitroprusside-induced stimulation of JNK1 activity was abolished by treatment of cells with N-acetylcysteine. Nitric oxide production from HEK293 cells ectopically expressing nitric oxide synthases resulted in the stimulation of JNK1 activity, while JNK1 stimulation in nitric oxide synthase-overexpressing cells was abrogated by a nitric oxide synthase inhibitor, N^G-nitro-L-arginine. Furthermore, exposure of cells to sodium nitroprusside resulted in the stimulation of JNK kinase (JNKK1/SEK1). Taken together, our data suggest that nitric oxide modulates the JNK activity through activating JNKK1/SEK1.

Nitric oxide (NO)[1] is a small signaling molecule, which is generated with L-citrulline from L-arginine by NO synthase (NOS). Thus far, three isoforms of NOS have been identified and their genes have been cloned: neural NOS (nNOS or type 1 NOS), inducible NOS (iNOS or type 2 NOS), and endothelial NOS (eNOS or type 3 NOS) (*1*−*7*). NO is involved in a variety of physiological functions, including neurotransmission, activation of macrophages, and vasodilation. NO is also involved in many pathological events, such as some neurodegenerative diseases. The biochemical actions of NO include the activation of guanylyl cyclase by binding to heme (*8*), and S-nitrosylation of protein that may modify protein function (*9*). NO can also interact with superoxides to form highly reactive peroxynitrite that can attack various intracellular components (*10*). Thus, the biological activities of NO appear to be mediated by multiple effectors. Nonetheless, the mechanism by which NO modulates signal transduction mediating diverse cellular events remains unclear, although NO has been shown to affect the activities of some regulatory proteins, including type I adenylyl cyclase, G proteins and $p21^{ras}$ (*11*−*13*).

MAP kinase signaling pathway mediates a variety of biological events, including mitogenesis, differentiation, and development (*14*). The mammalian MAP kinase family includes extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 HOG1 kinase (*15*−*18*). ERKs are often activated in response to mitogens such as peptide growth factors (*14*). JNK, which is identical to stress-activated protein kinase (SAPK), is activated in response to a variety of cellular stresses, including DNA damage, heat schock, or proinflammatory cytokines such as tumor necrosis factor-α and interleukin 1-*β* (*16*, *17*). Similar to JNK, p38 kinase is also activated in response to various stresses (*18*). Each group of MAP kinases may mediate signal transduction for different cellular processes.

In the present study, we investigated the action of NO on the regulation of the JNK pathway. We observed that NO can induce stimulation of the JNK activity in intact cells. This effect of NO on the JNK signaling pathway may elucidate a molecular mechanism by which NO exerts its physiological functions in its target cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction. A rat brain nNOS cDNA in pBluescript SK (kindly provided by Dr. Solomon H. Snyder, Johns Hopkins University) was digested with *Xho*I and *Xba*I and inserted into the unique *Xho*I−*Xba*I sites of pcDNAI-neo expression vector (Invitrogen). A bovine endothelial NOS cDNA in pBluescript II SK (a generous gift from Dr. Thomas Michel, Harvard Medical School) was digested with *Hin*dIII and *Bam*HI. The *Hin*dIII/*Bam*HI fragment containing the complete coding sequence of eNOS was inserted into the *Hin*dIII−*Bam*HI sites of pcDNAI-neo vector.

Cell Culture, DNA Transfection, and Chemical Treatment. HEK293 cells were cultivated at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 95% air, 5% CO_2 incubator. For DNA transfections, cells were plated in 100 mm dishes

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; JNKK, JNK kinase; SEK, SAPK kinase; MAPKKK, MAP kinase kinase kinase; PAK, p21-activated kinase; ASK, apoptosis signal-regulating kinase; TAK, TGF*β*-activated kinase; GST, glutathione-S-transferase; SNP, sodium nitroprusside; NNA, N^G-nitro-L-arginine; PAGE, polyacrylamide gel electrophoresis; 8-bromo-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; NAC, *N*-acetylcysteine.

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at a density of 2×10^6 cells/plate, grown overnight, and then tranfected with pcDNAI-neo vector, pcDNAI-neo containing a complete coding sequence of nNOS, or pcDNAI-neo containing eNOS cDNA by the calcium phosphate method (*19*). After 48 h of transfection, cultures were maintained in the complete medium containing G418 (500 μg/mL) to select neomycin-resistant cells. HEK293 cells expressing ectopic nNOS or eNOS protein were named as 293-nNOS or 293-eNOS, respectively. Heterogeneous populations of the stably transfected cells were used in this study in order to avoid any possible clonal variations. Microglial BV-2 cells were routinely cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. For an experiment, cultured BV-2 cells were rinsed twice with phosphate-buffered saline solution, and treated for 18 h with 10 μg/mL lipopolysaccharide in the absence or in the presence of 100 nM *S*-ethylisothiourea.

Protein Kinase Assay for JNK1 or ERK2. Confluent cells were harvested and lysed using buffer A containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Cell lysates were subjected to centrifugation at 12000*g* for 10 min at 4 °C. Soluble fraction was incubated 1 h at 4 °C with antibodies against JNK1 (PharMingen) or ERK2 (UBI). After the addition of protein G-agarose, the reaction mixtures were incubated for 1 h at 4 °C, and then subjected to microcentrifugation. The immunopellets were rinsed three times with buffer A, then twice with 20 mM Hepes, pH 7.4. Immunocomplex kinase assays were performed by incubating the immunopellets for 30 min at 30 °C with appropriate substrate proteins (2 μg) in 20 μL of the reaction buffer containing 0.2 mM sodium orthovanadate, 2 mM dithiothreitol, 10 mM $MgCl_2$, 2 μCi [γ^{32}P]ATP, and 20 mM Hepes, pH 7.4. The reaction was terminated by adding 5 μL of 5× sample buffer and heating the solution at 80 °C for 3 min. The reaction mixture was subjected to electrophoresis on 12% polyacrylamide gel. The phosphorylated substrates were visualized by autoradiography and quantified by densitometry. Glutathione-S-transferase (GST)-c-Jun, or myeline basic protein (MBP) was used as a substrate for JNK1, or ERK2, respectively. GST-fusion proteins were prepared as previously described (*20*). Protein concentrations were determined by the BCA method (Pierce) as described in the manufacturer's protocol.

Protein Kinase Assay for GST-SEK1. HEK293 cells were transiently transfected with an expression vector pEBG containing an insert encoding GST-SEK1 fusion protein (*21*). Transfected cells in culure were treated with indicated agents, harvested, and homogenized in phosphate buffer solution containing 1 mM PMSF, 5 μg/mL leupeptin, and 7 μg/mL aprotinin. Cell lysates were subjected to microcentrifugation at 4 °C for 10 min, and solubilized with 1% Triton X-100. The soluble fraction was applied to a glutathione-agarose resin, and GST-fusion proteins were prepared as previously described (*20*). For GST-SEK1 assay, GST-fusion proteins eluted from the resin were incubated for 30 min at 30 °C with 2 μg of recombinant SAPKβ in 20 μL of the reaction buffer containing 0.2 mM sodium orthovanadate, 2 mM dithiothreitol, 10 mM $MgCl_2$, 2 μCi [γ^{32}P]ATP, 20 mM Hepes, pH 7.4. The reaction was terminated, and the phosphorylated proteins were visualized by polyacrylamide gel electrophoresis and autoradiography, as described above for JNK1 assay.

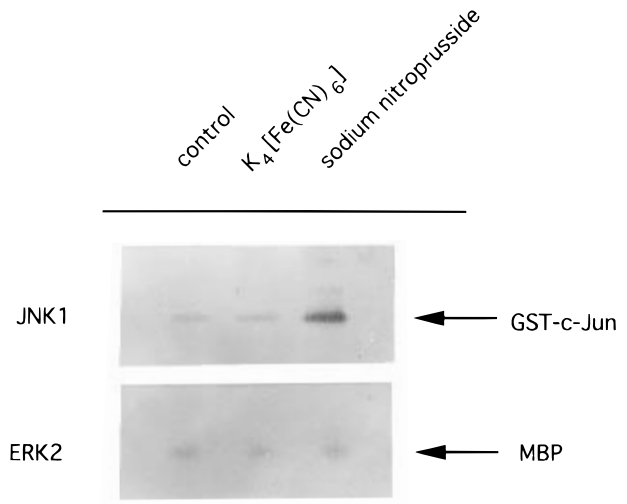

FIGURE 1: Effects of sodium nitroprusside on JNK1 and ERK2 in cultured cells. Confluent cultures of HEK293 cells were exposed to 500 μM SNP or 500 μM $K_4[Fe(CN)_6]$ for 1 h at 37 °C. When indicated, cells were irradiated with ultraviolet light (40 J m^{-2}) and incubated for 1 h at 37 °C. Cultured cells were harvested and lysed using buffer A. JNK1 or ERK2 activity was isolated by immunoprecipitation using an appropriate antibody, and the catalytic activities of JNK1 and ERK2 in the immunopellets were assayed for phosphorylating GST-c-Jun, or myelin basic protein, respectively, as described in the Experimental Procedures. The reaction mixture was analyzed by PAGE on 12% polyacrylamide gel, and autoradiography.

Protein Kinase Assay for PAK. HEK293 cells were harvested, lysed using buffer A, and immunoprecipitated with a rabbit polyclonal anti-PAK p65 antibody (Santa Cruz Biotechnology). The immunocomplex kinase assay was performed by measuring the autophosphorylation of PAK, as previously described (*22*). Autophosphorylation of PAK was visualized by polyacrylamide gel electrophoresis and autoradiography.

Measurement of NO Formation. NO generation was determined using the Griess method (Promega) that measures nitrite (NO_2^-), a stable breakdown product of NO, as described in the manufacturer's protocol.

RESULTS

In order to test possible effects of NO on JNK in intact cells, we examined enzymatic activity of JNK in HEK293 cells after exposing cells to sodium nitroprusside (SNP), a NO releasing agent. SNP spontaneously releases NO in solution with ferrocyanide as a byproduct (*23*). SNP is widely used to investigate the actions of NO on the functions of many proteins (*24*−*28*). Exposure of cells to SNP resulted in the stimulation of JNK1 activity as determined by phosphorylation of a substrate protein, GST-c-Jun (Figure 1A). SNP-induced JNK1 activation was dependent upon concentrations of SNP we used (data not shown). To rule out the possibility that ferrocyanide generated from SNP might affect JNK1 activity, we also examined JNK1 activity in cells treated with $K_4[Fe(CN)_6]$ alone. $K_4[Fe(CN)_6]$ did not affect JNK1 activity (Figure 1A). These data strongly suggest that NO released from SNP may be responsible for the stimulation of JNK in SNP-exposed cells. The SNP-induced stimulation of JNK1 activity was also observed in other cell lines including HeLa and mouse L929 cells (data not shown). In comparison, SNP did not affect the activity of ERK2 MAP kinase in HEK293 cells.

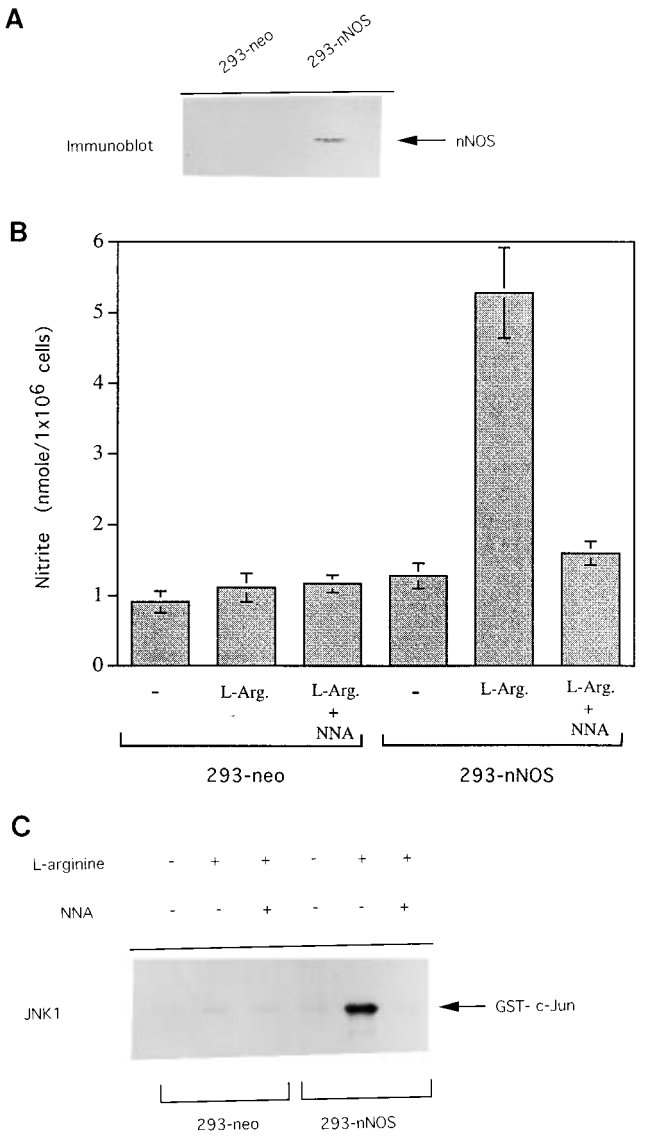

FIGURE 2: Effect of NO generation on JNK1 activity in nNOS-expressing cells. (A) Immunoblot analysis of the expression of nNOS in either 293-neo control cells or 293-nNOS cells. (B) Either 293-neo or 293-nNOS cells were incubated for 1 h at 37 °C in complete medium without or with 20 mM L-arginine (L-Arg.). When indicated, 100 μM NNA was added to culture medium 30 min prior to exposure of cells to L-arginine. One hundred microliters of culture medium in each cultured dish was collected and examined for NO release by measuring nitrite formation (Griess method), as described in the Experimental Procedures. (C) Cells were treated with either 20 mM L-arginine or 20 mM L-arginine plus 100 μM NNA, as for panel B. Treated cells were lysed and examined for JNK activity by the immunocomplex kinase assay, as described in the Experimental Procedures.

To further test the effect of NO on JNK activity in intact cells, we transfected HEK293 cells with a mammalian expression vector encoding nNOS, and named the nNOS-transfected cells as 293-nNOS. Expression of nNOS protein in 293-nNOS cells was confirmed by immunoblot analysis (Figure 2A). nNOS protein was not detected in 293-neo control cells. Incubation of nNOS-expressing cells in culture medium containing 20 mM L-arginine resulted in an increase in NO production in these cells (Figure 2B). The NO production from nNOS-expressing cells by adding L-arginine was almost completely abolished by 100 μM N^G-nitro-L-arginine (NNA), a potent reversible inhibitor of NOS (Figure 2B). In order to test whether endogenous NO produced by

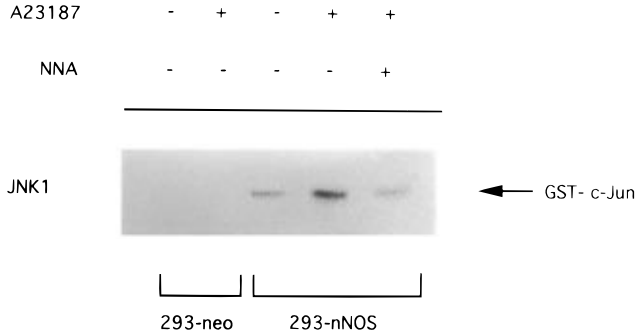

FIGURE 3: Effect of ionophore A23187 on JNK1 activity in nNOS-expressing cells. Either control 293-neo or 293-nNOS cells were exposed to 20 μM ionophore A23187 for 1 h at 37 °C without or with 100 μM NNA, and the immunocomplex JNK1 assay was performed, as for Figure 2.

nNOS could induce the stimulation of JNK activity, cells in culture were incubated with L-arginine for 1 h and cellular level of JNK1 activity was determined. As shown in Figure 2C, JNK1 activity was markedly enhanced after treatment of nNOS-expressing cells with 20 mM L-arginine. Furthermore, NNA completely blocked the L-arginine-dependent stimulation of JNK1 activity in nNOS-expressing cells. In contrast, neither L-arginine nor NNA affected JNK1 activity in 293-neo control cells (Figure 2C). Calcium ion is the major intracellular signal for activating nNOS activity (*29*). We, therefore, examined whether an increase in intracellular Ca^{2+} could induce the JNK1 stimulation in nNOS-expressing cells. As shown in Figure 3, treatment of nNOS-expressing cells with ionophore A23187 resulted in the stimulation of JNK1 activity. The A23187-induced stimulation of JNK1 activity was prevented by NNA in nNOS-expressing cells. In contrast, A23187 did not affect JNK1 activity in control 293-neo cells. Taken together, these data strongly suggest that NO production may induce the JNK1 stimulation in nNOS-expressing cells. Similar results were observed in the experiments using 293 cells that stably expressed recombinant eNOS (data not shown).

NO exerts many of its biological functions by forming free radicals such as peroxynitrite, or activating gualylyl cyclase that generates cGMP (*30*). We, therefore, examined whether either cGMP or free radical formation could mediate the stimulatory function of NO on JNK pathway. JNK1 activity was not affected by treatment of cells with 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP), a cell-permeable cGMP analog (data not shown). In contrast, N-acetylcysteine (NAC), an antioxidant and an effective free-radical scavenger (*31*), abolished the SNP-induced stimulation of JNK in cultured cells (Figure 4). JNK does not appear to be a direct target of the NO action, however, because SNP does not affect JNK1 activity *in vitro* (data not shown). It implies that NO might activate a target protein upstream of JNK in the JNK signaling pathway. JNK1 can be activated by an upstream protein kinase, SEK1/JNKK1, which, in turn, can be activated by MAPKKK such as MEKK1, MEKK3, MEKK4, TAK, or ASK1 (*21*, *32*−*38*). We, therefore, decided to test whether NO could affect the activity of SEK1. We transiently transfected HEK293 cells with an expression vector encoding GST-SEK1 fusion protein, and then examined whether SNP could affect the enzymatic activity of GST-SEK1. Exposure of transfected cells to SNP resulted in the stimulation of GST-SEK1 activity for phosphorylating SAPK/JNK, indicating that NO could induce the stimulation

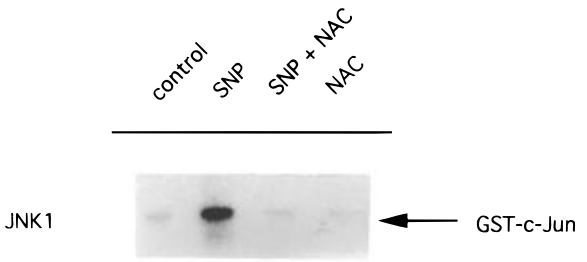

FIGURE 4: *N*-Acetylcysteine abolishes the SNP-induced stimulation of JNK1 activity in cultured cells. HEK293 cells in culture were preincubated for 90 min with 30 mM *N*-acetylcysteine (NAC), followed by exposure of cells to 500 μM SNP for 1 h at 37 °C. Cells were lysed, immunoprecipitated, and assayed for JNK1 activity, as for Figure 1.

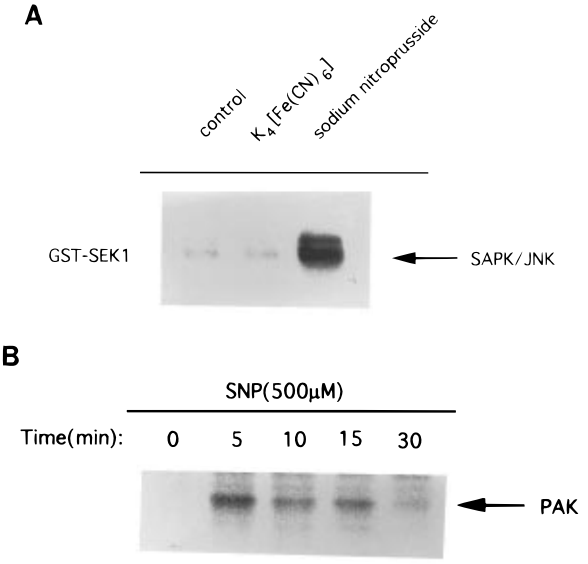

FIGURE 5: Effect of SNP on SEK1 and PAK activities. (A) HEK293 cells were transiently transfected with pEBG encoding GST-SEK1 fusion protein. After 2 days of transfection, cells were exposed to either 500 μM SNP or 500 μM $K_4[Fe(CN)_6]$ for 15 min at 37 °C, then harvested and lysed using buffer A. GST-SEK1 protein in cell lysates was isolated using glutathione-agarose beads, and examined for the catalytic activity of SEK1, as described in the Experimental Procedures. (B) HEK293 cells were exposed to 500 μM SNP at 37 °C for indicated times, then harvested, and lysed using buffer A. Cell lysates were immunoprecipitated with a rabbit polyclonal anti-PAK antibody. Immunocomplex kinase assay was performed by measuring autophosphorylation of PAK, as described in the Experimental Procedures.

of SEK1 activity (Figure 5A). It is not clear yet which form(s) of MAPKKK might be involved in the NO-induced stimulation of SEK1. Cellular activity of either MEKK1 or ASK1 was not affected after treatment of cells with 500 μM SNP (data not shown). Interestingly, treatment of cells to SNP resulted in the activation of p21-activated kinase (PAK) (Figure 5B). These data suggest that PAK might be involved in the NO-induced activation of the JNK pathway. PAK is a component of the JNK signaling pathway upstream of MAPKKK, and it has been suggested to mediate Rac1- or Cdc42-induced activation of the JNK pathway (*39*−*43*).

In this study, we have demonstrated that the stimulation of JNK1 activity can be induced by a NO-releasing agent or in NOS-transfected cells. We next examined whether JNK1 activity could be stimulated by induction of NO generation in microglial BV-2 cells. NOS activity in microglial cells can be induced when microglial cells are exposed to bacterial endotoxin, lipopolysaccharide (*44*). In our study, JNK1

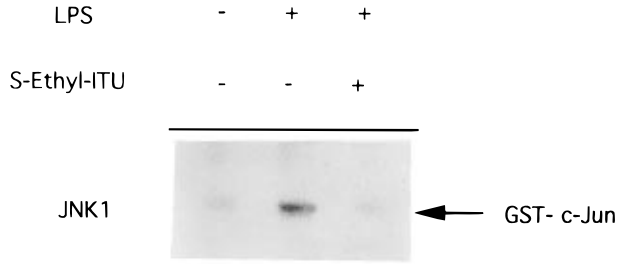

FIGURE 6: Effect of lipopolysaccharide on JNK1 in microglial BV-2 cells. Microglial BV-2 cells were exposed to 10 μg/mL lipopolysaccharide (LPS) for 18 h in serum-free DMEM without or with 100 nM *S*-ethylisothiourea (*S*-ethyl-ITU). Cells were harvested, lysed, and assayed for JNK1 activity, as described in the Experimental Procedures.

activity in microglial BV-2 cells was stimulated after exposure of cells to 10 μg/mL lipopolysaccharide (Figure 6). Furthermore, S-ethylisothiourea, a NOS inhibitor (*45*), blocked the lipopolysaccharide-induced stimulation of JNK1 activity in BV-2 cells.

DISCUSSION

NO has diverse biological activities, including neurotransmission, vasodilation, and cytotoxicity, in many tisssues (*30*). It may exert those actions through various signaling pathways. NO has been reported to modulate the functions of some regulatory proteins, including $p21^{ras}$, G proteins, and type I adenylyl cyclase (*11*−*13*). Intracellular signaling cascades downstream from NO production are not fully understood, however. Our findings in this study demonstrate that NO can induce the stimulation of JNK activity. Similar results were also reported very recently in human Jurkat T cells (*46*). Our data in this study further suggest that PAK may mediate the NO-induced JNK signaling pathway.

NO can induce activation of transcription factors such as AP-1 and NF-kB (*12*, *47*). AP-1 is a heterodimer of c-Fos and c-Jun. c-Jun is one of the major substrates of JNK. JNK phosphorylates c-Jun on serine-63 and serine-73 (*48*), and the phosphorylation of c-Jun enhances the transcriptional activity of AP-1 (*15*). Thus, the activation of AP-1 appears to be a major function of JNK. Our findings in the present study suggest that the JNK pathway may mediate the intracellular signal for the NO-induced AP-1 activation.

Excessive NO may cause cell death (*49*). The mechanism for NO-induced cell death is not clear, although DNA damage and activation of poly-ADP-ribose synthase have been proposed as a mechanism for NO-induced neuronal death (*50*). Interestingly, JNK plays a critical role in the regulation of cell death (*51*−*53*). The stimulation of JNK was prerequisite for cell death that occurred under various conditions, and a blockage of the JNK activation prevented cell death. It is, therefore, plausible that the stimulation of JNK may contribute to the cytotoxic action of NO on its target tissues.

We demonstrated in the present study that NO induced the stimulation of JNK through SEK/JNKK in intact cells. The regulation of the JNK signaling pathway by NO and NOS may be important for better understanding a mechanism by which NO exerts diverse functions in both normal physiological events and pathological states, including some neurodegenerative diseases.

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