Differential Activation of Mitogen-activated Protein Kinases by Nitric Oxide-related Species

Harry M. Lander†, Andrew T. J. acovina, Roger J. Davis§, and James M. Taura
From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021 and §Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Many studies have identified nitric oxide (NO) and related chemical species (NOx) as having critical roles in neurotransmission, vasoregulation, and cellular signaling. Previous work in this laboratory has focused on elucidating the mechanism of NOx signaling in cells. We have demonstrated that NOx-induced activation of the guanine nucleotide-binding protein p21ras leads to nuclear translocation of the transcription factor NFκB. Here, we investigated whether intermediary signaling elements, namely the mitogen-activated protein (MAP) kinases, are involved in mediating NOx signaling. We found that NOx activates the extracellular signal-regulated kinase (ERK), p38, and c-jun NH2-terminal kinase (JNK) subgroups of MAP kinases in human J urkat T cells. JNK was found to be 100-fold more sensitive to NOx stimulation than p38 and ERK. In addition, the activation of JNK and p38 by NOx was more rapid than ERK activation. Depletion of intracellular glutathione augmented the NOx-induced increase in kinase activity. Furthermore, endogenous NO, generated from NO synthase, activated ERK, and NOx-induced MAP kinase activation was effectively blocked by the farnesyl transferase inhibitor α-hydroxyfarnesylphosphonic acid. These data support the hypothesis that critical signaling kinases, such as ERK, p38, and JNK, are activated by NO-related species and thus participate in NO signal transduction. These findings establish a role for multiple MAP kinase signaling pathways in the cellular response to NOx.

Our previous work focused on identifying the signaling cascade responsible for the positive regulatory effects of nitric oxide (NO) and related chemical species (NOx) on human peripheral blood mononuclear cells (1, 2). NOx was found to activate human lymphocytes, as evidenced by increased glucose uptake, tumor necrosis factor α secretion, and nuclear translocation of the transcription factor NFκB. Further studies demonstrated that NOx directly activates p21ras, leading to downstream events such as the activation of NFκB (3). We have also identified p21ras as a general signaling target of reactive free radicals and suggested that it may be a cellular sensor for redox stress (4). In those studies, reactive oxygen species were shown to signal downstream of p21ras by activation of the extracellular signal-regulated kinases (ERKs). Here, we examined whether activation of low molecular weight G proteins such as p21ras, Rac1, and Cdc42 by NOx also leads to activation of one or more of the mitogen-activated protein (MAP) kinase family members.

The three identified mammalian MAP kinase subgroups include ERK (5), c-jun NH2-terminal kinase (JNK; Refs. 6–8), and the recently identified p38 MAP kinase (9–13). The well studied ERK group is typically activated by growth factors via a p21ras-dependent signal transduction pathway (5, 9, 14). JNK and p38 MAP kinases respond to proinflammatory cytokines and environmental stress, although it is not clear whether this occurs via common or parallel pathways (5). The signal transduction pathways leading to ERK, JNK, and p38 kinase activation are biochemically and functionally distinct (7, 9). Nevertheless, some extracellular stimuli can activate all of the MAP kinases simultaneously (5). In the present study, we attempt to further clarify the NOx-signaling pathway downstream of low molecular weight G proteins such as p21ras by exploring the effects of NOx-related chemical species on the ERK, p38, and JNK subgroups of MAP kinase.

**EXPERIMENTAL PROCEDURES**

Materials—A saturated phosphate-buffered saline solution containing 1.25 mM NO (Matheson gas) was prepared as described (3). This solution also contains higher oxides of NO (NOx), S-nitroso-N-acetylpenicillamine (SNAP), and S-nitroso-N-acetylpenicillamine; ERK, extracellular signal-regulated kinase; JNK, c-jun NH2-terminal kinase; MAP, mitogen-activated protein; BSO, l-buthionine-(SR)-sulfomycin; GST, glutathione S-transferase.

| 19705 |
Nitric Oxide Signaling

Fig. 1. Effect of NO-generating compounds and NOx gas on ERK1 and ERK2 activity. Jurkat T cells were pretreated for 16 h in serum-free RPMI 1640 medium with or without 100 μM BSO. Cells were treated for 10 min in a 37°C and 5% CO2 incubator with the indicated concentrations of SNAP or NOx-saturated phosphate-buffered saline. MAP kinase assays were performed as described under “Experimental Procedures” using 1 μg of myelin basic protein as substrate. Data points represent the means from at least three separate experiments, each performed in duplicate. Standard deviations were less than 5% in all cases.

(16). 100–150 μg of total crude extract was immunoprecipitated with 5 μg of either the antibody-Sepharose conjugates (anti-ERK1 and anti-ERK2) or 5 μg of antibody coupled to protein A-Sepharose (anti-JNK and anti-p38). After 1 h at 4°C, the beads were washed three times with lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl2, 25 mM β-glycerophosphate, 1 mM NaVO3, and 2 mM dithiothreitol). Immune complex kinase assays were performed at 37°C for 15 min in a total volume of 30 μl of kinase buffer containing the immunoprecipitate, 1 μg of substrate, 16 μM unlabeled ATP, and 0.67 μCi of [γ-32P]ATP (6000 Ci/mmol, 10 μCi/ml). The reaction was terminated with Laemmli buffer containing 200 mM dithiothreitol. Samples were boiled and resolved on a 15% sodium dodecyl sulfate-polyacrylamide gel. The dried gel was subjected to PhosphorImager analysis (Molecular Dynamics).

RESULTS

Effect of NOx Gas, NO-generating Compounds, and Endogenous NO on ERK1 and ERK2 Activity—We examined whether NOx delivered as a gas-saturated solution or from decomposition of a NO donor compound could stimulate ERK activity in human Jurkat T cells. To test this hypothesis, we treated Jurkat T cells with various concentrations of NOx-saturated phosphate-buffered saline or SNAP in the presence and absence of l-buthionine-(SR)-sulfoximine (BSO). BSO, a selective inhibitor of γ-glutamylcysteine synthetase, blocks de novo synthesis of glutathione (17), a vital intracellular antioxidant. We have previously used BSO to demonstrate a redox-sensitive signaling pathway in cells (4). In the presence of BSO, NOx, and SNAP increased ERK1 and ERK2 activity 2-3 fold in a concentration-dependent manner (Fig. 1). We found that untreated Jurkat cells possessed a measurable amount of basal ERK activity. It is possible that stress-activated mechanisms arising from cell culture may be responsible for the basal activity of ERK. We have previously found a similar amplitude of ERK activation by reactive oxygen species in these cells (4). It is also possible that nonphysiological depletion of cellular glutathione (as accomplished with BSO treatment) results in cell damage, leading to enzymes that are susceptible to activation by NOx-related species.

NOx gas elicited a peak response at a concentration range of 0.3-1 μM. The NO-generating compound SNAP required 300-1000 μM to produce similar levels of ERK activation. The disparity in the activating concentrations of NOx gas versus SNAP is likely due to their different chemical natures. In the cell, NO can exist as several different redox species (18), each with distinctive chemical properties. The relative abundance of these redox forms may explain the concentration-dependent variations in ERK activation seen between NOx and the various NO-generating compounds. Other factors that influence the relative potencies of exogenous NOx donors include decomposition rates, donor transport, and donor stability (18).

Non-BSO-treated cells showed no increase in ERK activity when stimulated with SNAP and required a higher activating concentration when stimulated with NOx-related species (Fig. 1). Our previous work has demonstrated that depletion of the intracellular antioxidant glutathione resulted in enhanced oxygen free radical signaling through p21ras, MAP kinase, and NFκB (4).

To explore the physiological relevance of our studies using exogenous NO donors, we induced NO synthesis in human umbilical vein endothelial cells by treating with l-arginine and interferon-γ (19). We found that overnight induction of nitric oxide synthase did not yield detectable ERK1 and ERK2 activation (Fig. 2, open bars), although NO was produced (19). To determine whether a pulse of endogenous NO could activate ERK1 and ERK2, we induced nitric oxide synthase overnight in the presence of a competitive inhibitor, N′-methyl-l-arginine. This was followed by a 15-min pulse of substrate, l-arginine. Under these conditions, ERK1 and ERK2 activity was dramatically increased (Fig. 2, hatched bars).

Human umbilical vein endothelial cells were either untreated or treated for 16 h with l-arginosaccharide (LPS, 30 μM) and interferon-γ (IFN-γ, 50 ng/ml) or N′-methyl-l-arginine (NMA; 3 mM). Then, indicated samples were treated with l-arginine (ARG, 10 mM) for 15 min and assayed for ERK activity. Data represent the means and standard deviations (bars) from three separate experiments.

Fig. 2. Effect of endogenous NO on ERK1 and ERK2 activity. Human umbilical vein endothelial cells were either untreated or treated for 16 h with l-arginosaccharide (LPS, 30 μM) and interferon-γ (IFN-γ, 50 ng/ml) or N′-methyl-l-arginine (NMA; 3 mM). Then, indicated samples were treated with l-arginine (ARG, 10 mM) for 15 min and assayed for ERK activity. Data represent the means and standard deviations (bars) from three separate experiments.

![Fig. 2. Effect of endogenous NO on ERK1 and ERK2 activity.](image-url)
Nitric Oxide Signaling

FIG. 3. Effect of SNP on JNK and p38 activities. Jurkat T cells were pretreated for 18 h in serum-free RPMI 1640 medium. Samples were treated for 10 min in a 37 °C and 5% CO₂ incubator with the indicated concentrations of SNP. JNK or p38 assays were performed as described under “Experimental Procedures” using 1 μg of the GST-Jun-17 or GST-ATF-2 fusion proteins as substrates for JNK and p38, respectively. Data points represent the means from three separate experiments, each performed in duplicate. Standard deviations were less than 10% in all cases.

FIG. 4. Kinetics of activation of MAP kinases in response to H₂O₂, SNP, and NOx gas. Jurkat T cells were pretreated for 18 h in serum-free RPMI 1640 medium. Samples were treated for 2, 5, 10, 30, and 60 min in a 37 °C and 5% CO₂ incubator with 100 μM H₂O₂, 300 μM SNP, or 100 nM NOx. MAP kinase assays were performed as described under “Experimental Procedures” using 1 μg of myelin basic protein and GST-ATF-2 or GST-Jun fusion proteins as substrates for ERK, p38, and JNK, respectively. Data points represent the means from three separate experiments, each performed in duplicate. Standard deviations were less than 5% in all cases.

...eration effect we have previously observed (3). Together, the data suggest that NOx-induced redox stress may activate the ERK kinases and that this signal may be modulated by cellular antioxidants, similar to reactive oxygen. Furthermore, these data indicate that endogenous NO can trigger ERK activation and thus a downstream cellular response.

Effect of the NOx-generating Compound Sodium Nitroprusside on p38 and JNK Activities—Two other members of the MAP kinase family are JNK and p38. We examined whether these kinases, which are known to be activated by environmental stress (6, 9), could also be activated by NO-related species. We treated serum-starved Jurkat cells with various concentrations of sodium nitroprusside (SNP) and observed 2-fold activation of JNK and p38 at concentrations of 3 and 300 μM, respectively (Fig. 3). The concentration-response curves for both MAP kinases were biphasic. At high concentrations of SNP, we observed an inhibitory effect, resulting in activities approaching basal levels. We have previously observed an inhibitory component of NOx action in many of the systems we examined (2, 3). It is likely that at high concentrations, the toxic and nonspecific effects of NOx are observed.

JNK and p38 have been found to respond to environmental stress and proinflammatory cytokines with strikingly similar dose- and time-dependent kinetics (9). The organization of the signal transduction pathways linking these two kinases is not clear. JNK and p38 may be regulated by parallel or shared mechanisms. However, our data indicate that either NO and related species activate these kinases through different mechanisms, or they are differentially susceptible to redox activation.

Activation Kinetics of MAP Kinases in Response to H₂O₂, Nitroprusside, and NOx Gas—We next studied the kinetics of ERK, p38, and JNK activation in response to SNP, NOx, and H₂O₂, which generates a hydroxyl radical in the presence of cellular iron (20) (Fig. 4). Serum-starved cells were treated with a single concentration for 2, 5, 10, 30, or 60 min, and the relative MAP kinase activities were analyzed. ERK1 and ERK2 showed a 3.5-fold increase in activity after a 10-min treatment with H₂O₂, whereas a 2-min SNP treatment produced a peak increase of 2.5-fold. We did not observe a defined peak of activation by NO-related species. Instead, ERK activity seemed to rise slowly as the time of exposure to NOx was increased. It is possible that the maximal peak occurred before our 2-min time point. Alternatively, this difference in activation kinetics by SNP and NOx may reflect differential sensitivities to NO species. SNP is known to release the nitrosonium ion (NO⁺), an efficient nitrosating species (18). In contrast, our NO gas-saturated solution contains authentic NO as well as other higher oxides. Thus, several upstream activators that are differentially susceptible to activation by reactive nitrogen intermediates are likely to be involved in NOx-mediated MAP kinase activation. Peak increases in p38 occurred at 10 min with H₂O₂ and 5 min in cells treated with NOx or SNP. JNK showed peak activity after 5 min for all three compounds assayed. From these experiments, we conclude that SNP and NOx-related species rapidly induce the activation of the three MAP kinases studied. The effects of the NOx donors took place more rapidly than those attributed to the oxidant H₂O₂.

Effect of Farnesyl Transferase Inhibition on the Activation of MAP Kinases in Response to H₂O₂, Nitroprusside, and NOx Gas—Previous work done in this laboratory (3, 4) demonstrated the activation of a low molecular weight G protein, most
likely p21ras, in the NOx-induced nuclear translocation of NFkB. In this study, we have found that ERK, p38, and JNK are activated by NOx and NO-generating compounds. We next studied the role of farnesylated G proteins in the NOx-induced activation of ERK, p38, and JNK. p21ras has been described as a molecular switch that directs incoming signals from the cell surface toward their appropriate transduction pathways (21). In addition to p21ras, the related low molecular weight G proteins Ras1 and Cdc42 have been shown to be critical in regulating the activation of JNK and p38 signaling (22-26). These proteins must undergo a series of posttranslational modifications before they can localize at the cytoplasmic face of the plasma membrane. An important posttranslational modification is the addition of a farnesyl group to the carboxyl-terminal end of the newly synthesized G protein. This step is catalyzed by the enzyme farnesyl transferase, which can be blocked by \( \alpha \)-hydroxyfarnesylphosphonic acid (27, 28). Thus, if not localized to the plasma membrane, these G proteins cannot activate their effector.

We pretreated Jurkat cells with the farnesyl transferase inhibitor for 24 h and then treated them for 10 min with SNP, NOx, or \( H_2O_2 \) (Fig. 5). In the absence of inhibitor, SNP, NOx, and \( H_2O_2 \) increased MAP kinase activity by 1.75-, 1.6-, and 2-fold, respectively. The addition of the inhibitor markedly reduced the ability of these stimuli to activate the three MAP kinases (Fig. 5). These experiments clearly suggest that the MAP kinase response to NOx requires a farnesylated upstream signaling component such as p21ras, Ras1, and/or Cdc42.

**DISCUSSION**

Previous work in this laboratory demonstrated a requirement for functional p21ras in activation of NFkB by NOx (3). We also found that reactive oxygen intermediates signaled through a similar mechanism and thus generalized our observations on signal transduction by NOx to include reactive oxygen species (4). In those studies, we found that reactive oxygen signals through p21ras, MAP kinase, and NFkB. We concluded from these studies that p21ras is directly targeted by reactive free radicals and may be a cellular sensor for redox status (4).

ERK, JNK, and p38 play critical intermediary roles in mediating signal transduction from the membrane to the nucleus (5, 7). Therefore, we investigated whether these signaling enzymes were activated by NOx and the NOx-generating compounds SNP and SNAP in human T cells. The molecular distinction between the different MAP kinase pathways lies in the substrate specificities of the MAP kinase kinases. There are six MAP kinase kinases in mammalian cells. MEK1, MEK2, and MEK5 activate ERK-related MAP kinases (29, 30); MKK3 and M KK6 activate p38 (31, 32); and M KK4 activates JNK and p38 (31, 33, 34). These MAP kinase kinases are themselves activated by receptor tyrosine kinases and other mechanisms. Our findings suggest that NO-related species and reactive oxygen intermediates activate the MAP kinases through upstream activation, most likely by activation of a low molecular weight G protein such as p21ras, Ras1, or Cdc42 (22-26). The time course of ERK activation by NOx was slow compared with the rapid effects of NOx on JNK and p38. This differential activation suggests that not all signals go through p21ras and that other related G proteins may be involved.

JNK activation by UV light has been shown to be potentiated by the activation of p21ras (6). This finding demonstrates that JNK signals via at least one p21ras-dependent pathway. Because the effects of UV irradiation often mimic those of oxidative stress, it was postulated that the UV-induced activation of JNK may be initiated by oxidative stress signaling through an unknown redox sensor. We have previously suggested p21ras as a redox sensor in the free radical-induced activation of NFkB (4). The present study describes the activation of JNK by some of the same reactive species. It is possible that p21ras or other low molecular weight G proteins (such as Rac1 and CDC42) serve similar roles in JNK activation.

NO production in many cell types (35). The effect of NO on cells depends on its local concentration, the redox status of its immediate environment, and the susceptibility of target sites for modification (18, 36). Reactive nitrogen and oxygen overproduction is often associated with the synthesis of destructive species, such as superoxide, peroxyxinitrate, and nitrosionium ion. When these reactive species overwhelm the cell's antioxidant defenses, the resulting redox stress can cause lipid peroxidation, membrane damage, DNA breakage, and enzyme inactivation (20). Our study suggests that cellular glutathione levels regulate a low molecular weight G protein-dependent nuclear signaling pathway in which the MAP kinases play an important role. Thus, the ability of NO to trigger some signaling events within the cell, such as ERK, JNK, and p38 kinase activation, as shown herein, will likely be revealed under conditions in which cellular glutathione levels are depleted.
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