Neuronal nitric-oxide synthase (nNOS) is a constitutively expressed enzyme responsible for the production of nitric oxide (NO) from l-arginine and O2. Nitric oxide is an intra- and intercellular messenger that mediates a diversity of signaling pathways in target cells. In the absence of l-arginine, nNOS has been shown to generate superoxide (O2•−). Superoxide, either directly or through its dismutation to H2O2, is likewise believed to be a cell-signaling agent. Because nNOS can generate NO and O2•−, we examined the activation of cellular signal transduction pathways in nNOS-transfected cells grown in the presence or absence of l-arginine. Spin trapping/EPR spectroscopy confirmed that stimulated nNOS-transfected cells grown in an l-arginine environment secreted NO into the surrounding milieu. Production of NO blocked Ca2+ ionophore-induced activation of the ERK1/2 through a mechanism involving inhibition of the Ras G-protein and Raf-1 kinase. In contrast, ERK activation was largely unaffected in nNOS-transfected cells grown in l-arginine-free media. Inhibition of nNOS-generated NO with the competitive NOS inhibitor, Nω-nitro-l-arginine methyl ester, in cells grown in l-arginine restored ERK1/2 activation to levels similar to that found when nNOS was activated in l-arginine-free media. These findings indicate that nNOS can differentially regulate the ERK signal transduction pathway in a manner dependent on the presence of l-arginine and the production of NO.

Nitric-oxide synthases (NOS; EC 1.14.13.39) are heme-containing enzymes that catalyze the oxidation of l-arginine to nitric oxide (NO) and l-citrulline via the intermediate Nω-hydroxyl-l-arginine (1). There are three distinct isoforms of this enzyme that are regulated by distinct genes (2) as follows: a constitutive neuronal NOS (nNOS or NOS I) (3), a constitutive endothelial NOS (eNOS or NOS III) (4, 5), and an endothelin- or cytokine-inducible NOS (iNOS or NOS II) (6). These NOS isoforms contain an N-terminal oxidase domain with binding sites for l-arginine, tetrahydrobiopterin, and a C-terminal reductase domain with binding sites for FAD, FMN, and NADPH. These domains are connected by a Ca2+/calmodulin binding region (7).

Previously, it has been reported that nNOS will produce superoxide (O2•−) in the absence of l-arginine (8). More recently, eNOS and iNOS, like nNOS, have been found to generate O2•− (9–13). In the presence of l-arginine, however, nNOS generates NO and O2•−; the ratio of these free radicals is dependent upon the concentration of l-arginine (12). This makes NOS unique in that this enzyme can produce two free radicals, NO and O2•−, both of which exhibit unique biologic activities (14).

Not surprisingly, NO, secreted from the different isoforms, mediates a wide variety of regulatory functions. In the case of iNOS, a high level of NO production for prolonged periods is consistent with its role in host immunity (15). In contrast, the production of NO by eNOS and nNOS is at a much lower level, where NO is believed to be a transient cell-signaling agent (16). For example, NO is an activator of soluble guanylate cyclase and cGMP production, which is an important second messenger that mediates a wide variety of physiological functions (17). These include, for instance, the regulation of vascular tone, platelet aggregation, inflammation, neurotransmission, learning and memory, penile erection, gastric emptying, and hormone release (18). In the case of O2•−, where the enzyme, superoxide dismutase (SOD) accelerates the disproportionation of O2•− to H2O2 and O2 (19), cell signaling activity of O2•− were it to occur, is undoubtedly at unique cellular sites, distant from SOD (20).

The mitogen-activated protein (MAP) kinase-signaling pathways are major mediators of extracellular signals. MAP kinases are serine/threonine kinases, which include the extracellular signal-regulated kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs) (21). The MAP kinase proteins are involved in many cellular functions such as proliferation, differentiation, migration, cell death, and inflammatory responses (22, 23). Activation of the MAP kinase pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases, which are coupled to the activation of Ras G-proteins through Src homology 2 domain-containing proteins, such as Shc and Grb2, and guanine nucleotide exchange factors such as SOS (23). The active GTP-bound form of Ras can activate kinases, such asraf-1 and other MEK kinase-1.
(MEKK1), which phosphorylate and activate MAP or ERK kinases (MEKs) required for MAP kinase activation (24). The MAP kinase family members regulate this diverse range of biological functions by phosphorylating transcription factors, other kinases, structural proteins, and other signaling proteins (23, 25).

Studies to date have investigated the cell-signaling pathways for NO/\(\text{H}_\text{18528}\) and O\(_2\) by exposing cells to exogenous sources of these free radicals (26). Although useful data have come from these experiments, little is known about how NOS-secreted NO/\(\text{H}_\text{18528}\) and O\(_2\) mediate cell-signaling pathways. We envision that by altering the free radical profile of nNOS, from a primary generator of NO/\(\text{H}_\text{18528}\) to a primary producer of O\(_2\), we could begin to address the seminal question: how are cell-signaling pathways regulated by nNOS generated NO/\(\text{H}_\text{18528}\) and/or O\(_2\)? Here we report on experiments that examine the nNOS regulation of MAP kinases, specifically ERK1/2, under conditions where nNOS is stimulated in the presence or absence of L-arginine in the cell media. We show that the selective production of NO by nNOS inhibits ERK activation by blocking Ras G-protein and Raf-1 kinase function. In contrast, stimulation of nNOS in the absence of L-arginine Medium, which is likely to generate O\(_2\), had little effect on Ca\(^{2+}\)/H\(\text{11001}\) ionophore-induced ERK activity. These findings demonstrate that nNOS has the capability to regulate differentially the ERK signal transduction pathway, depending on the presence or absence of L-arginine and the subsequent production of NO/\(\text{H}_\text{18528}\) and/or O\(_2\).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, LipofectAMINE, penicillin G/streptomycin solution, Geneticin®, and Dulbecco’s phosphate-buffered saline (PBS) were purchased from Invitrogen. Phenylmethylsulfonil fluoride, EDTA, EGTA, NADPH, N\(^{\text{nitro-L-arginine methyl ester}}\) (L-NAME), polyethylene glycol-superoxide dismutase (PEG-SOD), arginase, and poly-L-lysine were obtained from Sigma. The Ca\(^{2+}\)-ionophore, A23187, was purchased from Calbiochem. The L-arginine-depleted Medium was obtained from ICN (Irvine, CA). 2',5'-ADP-Sepharose was obtained from Amersham Biosciences. Ferrous sulfate was purchased from Mallinkrodt. Sodium N-methyl-N-glucamine dithiocarbamate (NaMGD) was prepared as de-

![Fig. 1. Spin trapping of NO' and O'\(_2\).](image1)

Schematic of the reaction of the spin traps ferro-N-methyl-N-glucamine dithiocarbamate (Fe(MGD)\(_2\)) and 2,5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) with NO' and O'\(_2\) respectively. The corresponding spin-trapped adducts are detected by EPR spectroscopy.

![Fig. 2. Representative EPR spectra derived from spin trapping of O'\(_2\) and NO' from isolated nNOS.](image2)

A, isolated nNOS (92 \(\mu\)g of protein) containing NADPH (1 mM) was incubated with CaCl\(_2\) (2 mM), calmodulin (100 \(\mu\)g/ml), and DEPMPO (20 mM). Superoxide was spin-trapped in the absence of L-arginine. Hyperfine splitting constants for DEPMPO-OOH (Fig. 1) are \(A_N = 13.4\) G, \(A_H = 11.9\) G, and \(A_p = 52.5\) G. B, same conditions as in A except O'\(_2\) was spin-trapped in the presence of l-arginine (100 \(\mu\)m). C, same conditions as in A except CaCl\(_2\) and calmodulin were not included in the reaction mixture. Receiver gain was \(5 \times 10^3\). D, same conditions as in A except Fe(MGD)\(_2\) (1 mM) was substituted in place of DEPMPO. Nitric oxide was spin-trapped with the addition of l-arginine (100 \(\mu\)m). Hyperfine splitting constant for ferro-N-methyl-N-glucamine dithiocarbamate was \(A_N = 14.9\) G, and the receiver gain was \(1.25 \times 10^4\).
washed three times with cold PBS, containing 0.9 mM CaCl$_2$ and 5.5 mM
70 non-transfected cells, cells were cultured in DMEM for 2 days until
containing all other amino acids except L-arginine for 24 h as described
Ca$^2+$ ionophore (A23187) to activate nNOS. The receiver gain was $10.0 \times 10^3$.

Cell transfections were done as described previously (29) using 1 μg
of plasmids expressing constitutively active versions of MKK1 (a gift
from Dr. Natalie Ahn, University of Colorado), Raf-I (BXB-Raf-I, a gift
from Dr. Ulf Rapp, University of Wurzburg, Germany), and H-Ras (V12)
(gift from Dr. Melanie Cobb, University of Texas, Southwestern).

Cell Culture—Human embryonic nNOS-transfected kidney 293 cells
(abbreviated 293 cells) were obtained from Dr. Yong Xia at The Johns
Hopkins University. These cells were cultured in DMEM supplemented
with 10% heat-inactivated fetal bovine serum and antibiotics penicillin
G (50 μg/ml) and streptomycin (50 μg/ml) at 37 °C in a 5% CO$_2$, 95%
air-humidified incubator. Cells were selected by DMEM containing 500
μg/ml Geneticin® at every five passages. Non-transfected 293 cells
were treated with Ca$^2+$ ionophore for 15 min to 6 h. Cultured cells were
stimulated with or without A23187 (CaI) for 15–180 min. Cell lysates were collected and immunoblotted for active ERK1/2 (ppERK)
and total ERK2 for a protein loading control. Data are representative of
total ERK2 for a protein loading control. Data are representative of
four independent experiments.

Free radical production inhibits ERK activation by Ca$^2+$ ionophore. Non-transfected control (A) and nOS-expressing 293 cells (B) grown in the presence or absence of L-arginine (L-ARG) for 24 h were stimulated with or without A23187 (CaI) for 15–180 min. Cell lysates were collected and immunoblotted for active ERK1/2 (ppERK)
and total ERK2 for a protein loading control. Data are representative of
four independent experiments.

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FIG. 3. EPR spectrum of NO$^\cdot$ generation in the presence of L-arginine in cultured cells. EPR spectrum ferro-N,N-dimethyl-p-glucamine dithiocarbamate. A. 293-expressing nNOS cells cultured in media containing L-arginine. B. 293-expressing nNOS cells cultured in media without L-arginine for 24 h. Cells cultured in the presence or absence of L-arginine were treated with 1 μM Ca$^{2+}$ ionophore (A23187) to activate nNOS.

FIG. 4. Free radical production inhibits ERK activation by Ca$^{2+}$ ionophore. Non-transfected control (A) and nOS-expressing 293 cells (B) grown in the presence or absence of L-arginine (L-ARG) for 24 h were stimulated with or without A23187 (CaI) for 15–180 min. Cell lysates were collected and immunoblotted for active ERK1/2 (ppERK) and total ERK2 for a protein loading control. Data are representative of four independent experiments.

FIG. 5. Free radical production has no effect on JNK activation by Ca$^{2+}$ ionophore. Non-transfected control (A) and nOS-expressing 293 cells (B) grown in the presence or absence of L-arginine (L-ARG) for 24 h were stimulated with or without A23187 (CaI) for 15–180 min. Cell lysates were collected and immunoblotted for active phosphorylated JNK (pJNK) and total ERK2 for a protein loading control. Data are representative of four independent experiments. Cells exposed to UV light (302 nm) for 15 s were used as a positive control for JNK activation as in A.

FIG. 6. Inhibition of NO$^\cdot$ production with L-arginine analogue partially restores ERK activity. Cells grown in the presence or absence of L-arginine (L-ARG) were pretreated with or without L-NAME for 24 h and then activated by treatment with A23187 (CaI) for 15 min. Active ERK (ppERK1/2, upper panels) was detected by immunoblotting in non-transfected control (A) and nOS-expressing 293 cells (B). Lower panels in A and B show total ERK2 protein levels. Data are representative of three independent experiments.

X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH
8.0, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40 using a rubber scraper. Cells were lysed and
centrifuged at 15,000 rpm to remove insoluble material.

Isolation of nNOS from 293 Cells—Neuronal nitric-oxide synthase, from nNOS-transfected 293 cells, was isolated following the method described in the literature (31) with some modification as reported previously (8). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (32).

Spin Trapping of NO$^\cdot$ from Isolated nNOS and nNOS-transfected
Human Kidney Cells by an Iron Chelate—The iron chelate, ferro-N-
methyl-L-glucamid dithiocarbamate (33), was used as the spin trap for NO. This chelate was freshly prepared by mixing a stock solution of ferrous sulfate and NaMGD in a molar ratio of 1:5 in distilled water, anaerobically, prior to each experiment (33). Spin trapping of NO from isolated nNOS was performed by mixing all the components described in the legends for Figs. 2 and 3 to a final volume of 0.3 ml. The solution was then mixed in an EPR quartz flat cell and inserted into the cavity of an EPR spectrometer (model E109, Varian Associates, Palo Alto, CA). The EPR spectrum shown in Fig. 2D was obtained at room temperature, 10 min after commencing the reaction. Instrument settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; sweep time, 12.5 G/min; and response time, 1 s. The receiver gain is given in the legend of Fig. 2.

Nitric oxide was spin-trapped from nNOS-transfected cells. In this case, Fe(MGD)$_2$ (1 mM, final concentration, prepared as described above) was added to the Ca$^{2+}$ ionophore, A23187, activated cells (20 $\times$ 10$^6$ cells) (Fig. 3). In an independent series of studies, arginase (15 units/ml) was added to the culture media 24 h prior to experimentation. Ca$^{2+}$ ionophore was added to cells for 1 h, and 500 $\mu$l of media was used to perform the Griess reaction.

**Immunoblot Analysis**—Cell protein lysates were diluted with an equal volume 2× SDS-PAGE loading buffer and heated to 100 °C for 2 min. Total protein (20 $\mu$g) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The immobilized proteins were then blocked in Tris-buffered saline (TBS-Tween) (50 mM Tris, pH 7.5, 0.15 mM NaCl, and 0.1% Tween 20) and 5% nonfat dry milk. Following an overnight incubation at 4 °C with the following primary antibodies, ppERK (catalog number M8159 Sigma), ERK2 (C-14), pJNK (G-7), and PARP (H-250) purchased from Santa Cruz Biotech (Santa Cruz, CA), the membranes were washed several times with TBS-Tween. The horseradish peroxidase-conjugated secondary antibody was diluted 1:10,000 in TBS-Tween and incubated with the membrane for 45 min at room temperature. Following washes with TBS-Tween, proteins were detected using ECL purchased from PerkinElmer Life Sciences.

**PEG-SOD Enhancement of Measured NO**—PEG-SOD (30 units/ml) was added to nNOS-293 cells in the presence of l-arginine 24 h before experimentation. Ca$^{2+}$ ionophore was added to cells for 1 h, and 500 $\mu$l of media was used to perform the Griess reaction.

**RESULTS**

Free Radical Detection in NOS-expressing 293 Cells—The objective of these studies was to investigate the response of the ERK1/2 MAP kinase-signaling pathway to stimulated nNOS expressed in 293 cells grown in the presence or absence of l-arginine. Before such studies can be undertaken, however, we verified the production of NO and O$_2^\bullet$ by using nNOS, isolated from nNOS-expressing 293 cells. In the absence of l-arginine, O$_2^\bullet$ accepts an electron from nNOS, resulting in the generation of O$_2$^−, which was identified by its reaction with DEPMPO, affording DEPMPO-OOH (Figs. 1 and 2A). When l-arginine is present, however, there is a binding of the guanidino nitrogen in an ordered position near the heme (36), which allows the oxidation of this amino acid to proceed to L-citrulline and NO$^\bullet$.

We have demonstrated previously that as the concentration of l-arginine increases, the amount of spin-trapped O$_2^\bullet$ diminishes (12). This finding is illustrated in Fig. 2A and B, where in the presence of l-arginine (100 $\mu$m) nNOS-generated O$_2^\bullet$ was markedly decreased (Fig. 2B). As a control, when CaCl$_2$ and calmod-
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Ulin were not included in the reaction mixture, O$_2^\cdot$ was not generated from the inactive nNOS, as no EPR spectrum corresponding to DEPMPO-OOH was observed (Figs. 1 and 2C). When l-arginine (100 μM) was added to a competent nNOS in the presence of Fe(MGD)$_2$ (1 min), a three-line EPR spectrum corresponding to NO-Fe(MGD)$_2$ was observed (Figs. 1 and 2D); the EPR spectrum of which is typical of NO-Fe(chelates) (37).

When nNOS-transfected 293 cells (20 × 10^6 cells), cultured in media containing l-arginine, were treated with the Ca$^{2+}$- ionophore A23187 to activate nNOS in the presence of Fe(MGD)$_2$, as described previously (38), the three-line EPR spectrum of NO-Fe(MGD)$_2$ was recorded (Fig. 3A). In the absence of A23187, NO$^\cdot$ was not spin-trapped (data not shown). Similarly, with non-transfected 293 cells that have been treated with A23187, NO$^\cdot$ was again not spin-trapped (data not shown). These data demonstrate that the activation of nNOS in these nNOS-transfected 293 cells by the Ca$^{2+}$- ionophore A23187 was responsible for the generation of NO$^\cdot$, which was detected extracellularly (33).

Next, nNOS-transfected 293 cells were cultured in an l-arginine-free media for 24 h to diminish the intracellular pool of this amino acid, as described in the literature (38). As expected, NO$^\cdot$ was not spin-trapped upon activation of these cells with A23187 (Fig. 3B). In addition, we were unable to spin trap NO$^\cdot$ in cells that were cultured in normal media to which arginase (15 units/ml) was added 24 h prior to experimentation (34) (data not shown).

The Fe(chelate) used in the experiments described above to spin trap NO$^\cdot$ is ionic. Although it may bind to the surface of these cells, due to the alcohol groups on the NaMGD (Fig. 1), there is no evidence that this iron-chelate diffuses into cells (33). Because NO$^\cdot$ is produced intracellularly by stimulated nNOS-transfected 293 cells, a portion of the generated NO$^\cdot$ must have diffused into the surrounding milieu where it was spin-trapped by Fe(MGD)$_2$. The ability to spin trap NO$^\cdot$ at a site distal from its evolution is not unexpected. The neutrality of this free radical and its slow reaction with O$_2$ in buffers allow NO$^\cdot$ to readily diffuse through membranes to targets (39), in this case, the spin trap Fe(MGD)$_2$.

It has been reported that upon treatment of nNOS-transfected 293 cells cultured in l-arginine-free media for 24 h with A23187, O$_2^\cdot$ was spin-trapped extracellularly by 5,5-dimethyl-1-pyrroline-N-oxide (38). In our hands, however, when we treated nNOS-transfected 293 cells cultured in l-arginine-free media for 24 h with A23187, we were unable to spin trap O$_2^\cdot$ with 5,5-dimethyl-1-pyrroline-N-oxide or with even more robust spin traps, DEPMPO and 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide. These findings are not surprising considering this is one of the few reports (38) of O$_2^\cdot$ spin trapping in an extracellular milieu from an intracellular site where an enzyme generated this free radical. Nevertheless, based on the isolated nNOS experiments described, we believe that activation of nNOS-expressing 293 cells will, depending on the availability of l-arginine, generate NO$^\cdot$ and O$_2^\cdot$.

**NO$^\cdot$ Inhibits ERK Activation**—The activation of the ERK1/2 MAP kinase signal pathway was investigated in nNOS-expressing 293 cells. Previous reports (40) suggest that free radicals have the ability to regulate biological responses through activation of MAP kinase-signaling pathways. ERK1/2 activation was measured by immunoblotting for the active dually phosphorylated forms of ERK1/2. A23187 stimulated ERK1/2 activation in controls as described in the literature (41) (Fig. 4A). In contrast, A23187 treatment of nNOS-expressing 293 cells grown in the presence of l-arginine resulted in no ERK activation (Fig. 4A). However, when these cells were cultured in the absence of l-arginine in the media, followed by A23187 stimulation of nNOS, a slight decrease in ERK1/2 activation was shown (Fig. 4B). These data suggest that nNOS generation of NO$^\cdot$ yields an inhibitory affect on Ca$^{2+}$ ionophore-induced ERK1/2 activity. Previously, it has been shown that NO$^\cdot$ donors or bacterial toxins can activate MAP kinases, JNK and p38 (42, 43). Therefore, the effects of free radical formation on the JNK MAP kinase pathway were examined. Ca$^{2+}$- ionophore stimulation of nNOS in cells grown with or without l-arginine had no apparent effect on JNK activity (Fig. 5).

The specificity of the nNOS-secreted NO$^\cdot$ inhibition of ERK was verified using the l-arginine inhibitor N$^\cdot$ nitro-l-arginine methyl ester (l-NNAME), which prevents the production of NO$^\cdot$ and partially blocks nNOS production of O$_2^\cdot$ (8). We found that treatment of cells with l-NNAME restored the ERK activity that was inhibited by NO$^\cdot$ (Fig. 6). These results indicate that NO$^\cdot$ blocks ERK1/2 activation and probably functions as a more potent free radical inhibitor of ERK1/2 than O$_2^\cdot$.

**NO$^\cdot$ Stimulates an Apoptotic Response**—Previous studies (44) have suggested that NO$^\cdot$ may promote an apoptotic response. We next examined whether generation of NO$^\cdot$ by NOS activation promoted apoptosis by examining proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), which is a substrate for caspasas during early stages of apoptosis. Cells producing NO$^\cdot$ increased the formation of the cleaved (85 kDa) form of PARP (Fig. 7A). However, nNOS stimulation, in the absence of l-arginine, had no effect on PARP cleavage (Fig. 7A). To show this response was due to NO$^\cdot$, treatment with l-NNAME completely reversed NO$^\cdot$-mediated PARP cleavage (Fig. 8A). l-NNAME inhibition of PARP cleavage and restoration of Ca$^{2+}$-ionophore induced ERK activity (Fig. 6), suggesting that NO$^\cdot$ enhances apoptotic events through a mechanism involving decreased ERK activity.

As was stated earlier, nNOS generates NO$^\cdot$ and O$_2^\cdot$. Reaction of these two free radicals produces peroxynitrite (ONOO$^-$), which, with a rate constant of $19 \times 10^9$ M$^{-1}$ s$^{-1}$ (45), is faster than the reaction of O$_2^\cdot$ with SOD at $2 \times 10^9$ M$^{-1}$ s$^{-1}$ (46). Therefore, we surmised that by scavenging NOS-generated O$_2^\cdot$ through enhancement of SOD, cellular levels of NO$^\cdot$ would increase, resulting in an increase in PARP cleavage as depicted in Figs. 7 and 8. Verification for this approach came from studies with isolated nNOS in which inclusion of SOD enhanced the spin trapping of NO$^\cdot$ (47). To increase cellular levels of SOD, we used PEG-SOD, which we have shown previously is taken up by cultured endothelial cells, and thereby impacted O$_2^\cdot$-dependent reactions (48). Therefore, NOS-expressing 293 cells were incubated with PEG-SOD (30 units/ml) for 24 h prior to experimentation. After 1 h of exposure to A23187, the concentration of nitrite, a measure of NO$^\cdot$ production by the stimulated nNOS-containing cells in the culture media, was estimated. The concentration of nitrite increased from 2.5 μM for control cells to 4.1 μM for PEG-SOD-treated cells. Not surprisingly, PEG-SOD-treated nNOS-expressing 293 cells grown in l-arginine culture media enhanced PARP cleavage, as compared with control cells (Fig. 9). This is likely due to an increase in the available NO$^\cdot$ for cell signaling. In contrast, in nNOS-expressing 293 cells cultured in l-arginine-free media containing PEG-SOD, neither resulted in any measurable production of nitrite nor change in biological activity (data not shown).

**NO$^\cdot$ Inhibits ERK Activity by Targeting Ras and Raf-1**—Mechanisms of NO$^\cdot$-mediated inhibition of ERK proteins were further examined in nNOS-expressing cells transfected with constitutively active mutants of MKK1, Raf-1, and H-Ras. We postulated that intracellular production of NO$^\cdot$ could directly influence signaling proteins involved in regulating ERK. NOS-expressing 293 cells transfected with constitutively active mutants of MKK1, Raf-1, or H-Ras were treated with or without...
A23187 as described above, and ERK activation was examined by immunoblotting. In the absence of A23187 treatment, all of the constitutively active mutants caused a robust activation of ERK (Fig. 10A). In contrast, A23187 treatment caused strong inhibition of Ras- or Raf-1-mediated ERK activation but had no effect on ERK activated by active MKK1 (Fig. 10A). However, when cells transfected with the constitutively active MKK1, Raf-1, or Ras mutants were grown in l-arginine-depleted media, ERK activity was not affected after stimulation with A23187 (Fig. 10B). These results suggest that NO can block ERK activation by directly inhibiting Ras or Raf-1 activity.

**DISCUSSION**

The data presented here indicate that NO’ is a potent inhibitor of Ca²⁺ ionophore-mediated ERK activation. The ERK pathway can be activated by elevating intracellular Ca²⁺ with Ca²⁺ ionophores, membrane depolarization, or increased extracellular calcium (49–52). Thus, activation by Ca²⁺ ionophore in cells expressing NOS is an appropriate model for examining signaling pathways specifically regulated by NO’ and O₂⁻. Although ERK activity was slightly decreased when nNOS-expressing 293 cells were grown under conditions where l-argi-
nNOS

Low [L-Arginine]

O$_2^-$

High [L-Arginine]

Ras

NO$
$

Raf

M KK1/2

Apoptosis Response

ERK1/2

Fig. 11. Proposed schematic of nNOS-mediated regulation of the ERK-signaling pathway. Depending on the availability of l-arginine substrate, nNOS is capable of producing two free radicals that may differentially regulate cell-signaling pathways and physiological functions. Under conditions of cellular levels of l-arginine levels, nNOS-generated NO$^-$ inhibits ERK activation through a mechanism involving direct targeting of Ras and Raf-1. Inhibition of ERK signaling may sensitize cells to undergo an apoptotic response. Under situations where low cellular levels of l-arginine are present, nNOS generates primarily O$_2^-$, which has little effect on ERK pathway activation and may allow other physiological events to occur, such as cell growth, proliferation, differentiation, or an immune response.

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released from S-nitrosoglutathione also showed no activation of p38 in rat cardiac myocytes (57). In neuronal cell models, activation of ERK proteins is linked to protection from neuronal cell death (29). Similarly, treatment with PD98059, a synthetic MEK inhibitor, enhanced apoptosis, suggesting a protective role for the ERK pathway in response to death stimuli (30).

The MAP kinase pathways have been linked to mediating many of the physiological responses to NO$. For example, NO$^-$ regulation of matrix metalloproteinases proteins, including MMP-1, during inflammatory and angiogenic responses may require MAP kinase proteins (58). In addition, vascular endothelial cells treated with an NO$^-$ donor showed increased MMP-13 expression through a mechanism requiring the ERK pathway (59). Although it has been suggested that JNK MAP kinase activation protected against NO$^-$ donor-induced apoptosis in cardiac myocytes (57), we did not observe JNK activation by NO$^-$ in our system. Others report that treatment of cells with the NO$^-$ donor, sodium nitroprusside, enhances the tumor necrosis factor-related apoptosis-inducing ligand, initiating the tumor necrosis factor-related apoptosis-inducing ligand apoptosis death pathway, which includes increases in cytochrome c release, caspase-3 activation, and enhanced PARP cleavage (60). Similarly, our data also demonstrate an increase in PARP cleavage following stimulation of intracellular NO$^-$ production (Figs. 7 and 8).

There may be a number of physiological conditions where intracellular l-arginine levels may vary and affect the production of NO$^-$-generated NO$^-$ and O$_2^-$ As stated above, scavenging of O$_2^-$ by inclusion of SOD in a tissue bath prolonged the vasodilatory action of aortic rings (54, 55). Similarly, l-arginine can be classified as a semi-essential amino acid, where endogenous synthesis may be insufficient as in situations of accelerated tissue growth after infection (61). Progressive loss of kidney function, hypertension, and hypertensive nephrosclerosis occurs in a Dahl/Rapp salt-sensitive rat model and can be reversed following l-arginine treatment through a mechanism that likely involves NO$^-$-mediated vascular smooth muscle cell relaxation and subsequent decreased blood pressure (62). l-Arginine may also enhance the immune response in patients with breast cancer by enhancing the mitogenic responses of peripheral blood lymphocytes (63). It remains to be determined whether variable cellular levels of l-arginine, with changes in NO$^-$ and O$_2^-$ production, utilize MAP kinase-signaling pathways in these examples.

Based on our findings, the following schematic of how free radicals produced from nNOS regulate the ERK signal transduction pathway is proposed (Fig. 11). In the presence of normal cellular levels of l-arginine, nNOS generates primarily NO$, which will lead to an inhibition of the ERK1/2 pathway by direct targeting of Ras and Raf-1 and increase cell susceptibility to an apoptotic response. Under conditions of low l-arginine levels, nNOS produces predominantly O$_2^-$, which has little effect on ERK1/2 activation and may be involved in other biological responses either as O$_2^-$ or as its dismutation product H$_2$O$_2$.

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REFERENCES
1. Moncada, S., and Higgs, A. (1993) N. Engl. J. Med. 329, 2002–2012
2. Förstermann, U., Schmidt, H. H., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M., and Murad, F. (1991) Biochem. Pharmacol. 42, 1849–1857
3. Xu, W., Gorman, P., Sheer, D., Bates, G., Kishimoto, J., Lizhi, L., and Emsen, P. (1995) Cyto. Genet. Cell Genet. 64, 62–63
4. Robinson, L. J., Werenowicz, S., Morton, C. C., and Michel, T. (1994) Genomics 19, 350–357
5. Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schoppert, K. T. (1993) J. Biol. Chem. 268, 17478–17488
6. Chartrain, N. A., Geller, D. A., Kotsy, P. P., Sitrin, N. F., Nussler, A. K.,
Nitric Oxide Inhibition of ERK1/2 Activity in Cells Expressing Neuronal Nitric-oxide Synthase
Kimberly W. Raines, Guan-Liang Cao, Supatra Porsuphatana, Pei Tsai, Gerald M. Rosen and Paul Shapiro

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