Signaling by eNOS through a superoxide-dependent p42/44 mitogen-activated protein kinase pathway

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Wang, Weihan, Shuimang Wang, Erfant V. Nishanian, Ana Del Pilar Cintron, Robert A. Wesley, and Robert L. Danner. Signaling by eNOS through a superoxide-dependent p42/44 mitogen-activated protein kinase pathway. Am J Physiol Cell Physiol 281: C544–C554, 2001.—Expression of endothelial nitric oxide synthase (eNOS) in transfected U-937 cells upregulates phorbol 12-myristate 13-acetate (PMA)-induced tumor necrosis factor-α (TNF-α) production through a superoxide (O2-) dependent mechanism. Because mitogen-activated protein kinases (MAPK) have been shown to participate in both reactive oxygen species signaling and TNF-α regulation, their possible role in eNOS-derived O2- signal transduction was examined. A redox-cycling agent, phenazine methosulfate, was found to both upregulate TNF-α (5.8 ± 1.0 fold; P = 0.01) and increase the phosphorylation state of p42/44 MAPK (3.1 ± 0.2 fold; P = 0.01) in PMA-differentiated U-937 cells. Although S-nitroso-N-acetylpenicillamine, a nitric oxide (NO) donor, also increased TNF-α production, NO exposure led to phosphorylation of p38 MAPK, not p42/44 MAPK. Upregulation of TNF-α production by eNOS transfection was associated with increases in activated p42/44 MAPK (P = 0.001), whereas levels of phosphorylated p38 MAPK were unaffected. Furthermore, cotransfection with Cu/Zn superoxide dismutase, which blocks TNF-α upregulation by eNOS, also abolished the effects on p42/44 MAPK. Expression of Gln361eNOS, a mutant that produces O2- but not NO, still resulted in p42/44 MAPK phosphorylation. In contrast, two NADPH binding site deletion mutants of eNOS that lack oxidase activity had no effect on p42/44 MAPK. Finally, PD-98059, a p42/44 MAPK pathway inhibitor, blocked TNF-α upregulation by eNOS (P = 0.02). Thus O2- produced by eNOS increases TNF-α production via a mechanism that involves p42/44 MAPK activation.

Signal transduction; tumor necrosis factor-α; reactive oxygen species; endothelial nitric oxide synthase

REACTIVE OXYGEN SPECIES (ROS) can function as important and specific signal transduction intermediates that regulate cell growth, differentiation, proliferation, and apoptosis (17, 20, 27, 28). Although there are many cellular sources of ROS, the molecular identities and precise origins of ROS involved in signal transduction events have largely remained obscure (13, 37). Several investigations have shown that endothelial nitric oxide synthase (eNOS), a NADPH oxidase, is capable of releasing oxygen intermediates in cell-free systems deficient in tetrahydrobiopterin (BH4) and Ca2+ (32, 38). This finding raises the provocative possibility of a dual role for this enzyme in both nitric oxide (NO) and ROS signal transduction. Notably, increased eNOS expression in endothelial cells has been associated with elevated ROS production (4, 9, 24). In vivo, the infusion of BH4 in hypercholesterolemic patients has been shown to restore endothelium-dependent vascular responses (26), supporting the concept that eNOS dysfunction, with impaired NO production and increased O2- formation, might occur in humans. During shear stress, the demonstrated coregulation of eNOS and superoxide dismutase (SOD) in the endothelium (12) might serve as a mechanism to reduce the prooxidant potential of eNOS and thereby to relax vessels without triggering oxidant-induced apoptosis.

Despite interest in the NADPH oxidase activity of eNOS and its potential importance in atherogenesis (24, 26), a number of technical obstacles have hindered the direct demonstration in intact cells of eNOS-derived O2- signaling. As noted above, O2- has many possible sources within cells (13, 37) and vigorously interacts with NO (16, 37). Furthermore, a specific mechanism that can switch eNOS from NO to O2- production has not yet been described. To circumvent these problems, we developed a model system in phorbol 12-myristate 13-acetate (PMA)-differentiated U-937 cells, a monoblastoid lineage. These cells lack soluble guanylate cyclase, thereby simplifying the study of cGMP-independent signaling by eNOS (34, 40). In addition, they can be transfected with wild-type and mutant versions of eNOS without confounding effects from endogenously expressed NOS isoforms (40). Finally, U-937 cells do not produce BH4 (25), an eNOS cofactor important in suppressing O2- production.

Using this intact cell model, we recently demonstrated that eNOS expression upregulates tumor ne-
cross factor-α (TNF-α) promoter activity and TNF-α production through a ROS-dependent signaling pathway (35). Signaling by eNOS-derived O₂⁻ was abolished by Cu/Zn SOD expression and by eNOS mutations that destroy its NADPH oxidase activity. Conversely, these eNOS effects were unaffected by N⁷-methyl-L-arginine (L-NMA), a NOS inhibitor, or by an eNOS mutation that disrupts NO formation but leaves NADPH oxidase activity intact. Finally, using a sensitive aconitase assay, eNOS upregulation of TNF-α production was directly linked to O₂⁻ release (35). Thus eNOS was shown to regulate TNF-α through a mechanism dependent on O₂⁻. Identifying distal components of this signaling pathway could serve as a useful signature for its activity in other cell types.

A possible role for mitogen-activated protein kinases (MAPK) in eNOS-derived O₂⁻ signaling is suggested by the participation of these enzymes in both ROS signaling events (15, 18, 27, 28) and regulation of TNF-α responses (2, 20, 31). The MAPK family is a conserved network of signal transduction enzymes that are activated by phosphorylation and can be translocated to the nucleus (7). Upon activation, MAPK phosphorylates serine and threonine residues in target proteins, typically other kinases and transcription factors. By utilizing task-related signaling complexes, and through both kinase-dependent and kinase-independent functions, relatively few MAPK components can be differentially activated, thereby resulting in a much larger number of pathway-specific signaling events (7, 23). Here we investigate the relationship between ROS-dependent signal transduction initiated by eNOS and MAPK activation in PMA-differentiated U-937 cells. Furthermore, the ROS and NO specificity of MAPK responses is examined in these cells.

METHODS

Reagents. Anti-eNOS antibodies were purchased from Transduction Labs and Research Diagnostics. The enhanced chemiluminescence Western blotting detection system was obtained from Amersham Pharmacia Biotech. Polyvinylidene difluoride (PVDF) membranes and 4-20% Tris-glycine gels were obtained from Novex. Phospho-specific antibody against phospho-p38 MAPK was obtained from Promega or Santa Cruz Biotechnology. Phospho-specific antibody against phospho-p42/44 MAPK and phospho-nonspecific antibodies against p38 MAPK and p42/44 MAPK were all obtained from New England Biolabs. S-nitroso-N-acetylpenicillamine (SNAP), hygromycin B, and PD-98059 [2'-amino-3'-methoxyphenoxy]anaphthen-4-one] were purchased from Calbiochem. Phenazine methosulfate, N-formyl-methionyl-leucyl-phenylalanine (fMLP), tert-butyl hydroperoxide, and hydrogen peroxide (H₂O₂) were obtained from Sigma Aldrich.

Expression vectors. Construction of the plasmids used in this investigation has been described previously (35, 40). Briefly, human eNOS expression vector was constructed by insertion of human eNOS cDNA (4 kb) into the pCEP4 eukaryotic expression vector (Invitrogen). A mutant eNOS cDNA that results in a Glu-to-Gln substitution at position 361 (5) was cut from pGEM-3Z with EcoRI and ligated into pBluescript SK(−). A HindIII/Not I fragment containing the entire open reading frame of eNOS was then excised from pBluescript SK(+) and ligated into pCEP4 to create Gln³⁶¹eNOS. NADPH binding site deletion mutants of eNOS, d(NADPH)eNOS and d(NADPH)2eNOS, were constructed by digesting eNOS and Gln³⁶¹eNOS cDNA with XhoI, thus removing 98 amino acids from the COOH-terminal end of eNOS that contain the NADPH-adenine binding site sequence. Human Cu/Zn SOD cDNA [American Type Culture Collection (ATCC)] was excised from pSP64 vector at HindIII/BamHI sites and ligated into pCEP4. For these pCEP4 constructs, transcription of the inserted sequence of interest is driven by the cytomegalovirus immediate early enhancer/promoter. The pCEP4 plasmid contains a hygromycin B-resistance gene that allows selection. All of the expression vectors were partially sequenced to confirm the correct sequence and orientation.

Transfection, selection, and eNOS expression. U-937 cells (ATCC) were grown in RPMI 1640 complete medium containing HEPES (25 mM), endotoxin-free fetal calf serum (10%), L-glutamine (2 mM), and antibiotics at 37°C in a humidified incubator with 5% CO₂. Empty pCEP4 (control vector) or vectors overexpressing human eNOS, Gln³⁶¹eNOS, d(NADPH)eNOS, d(NADPH)²eNOS, and human Cu/Zn SOD were transfected into cells by electroporation, as previously described (35, 40). Briefly, U-937 cells (5 x 10⁶) were suspended in 260 μl of RPMI 1640 complete medium. Cells were electroporated (240 V, 960 μs) with an Electro Cell Manipulator 600 (BTX). After electroporation, cells were grown in RPMI complete medium for 3 days and then selected with hygromycin B (275 U/ml) for at least 2 wk. The expression and activity of eNOS in transfected cells was measured using a [l-¹⁴C]arginine to l-¹⁴C]citrulline conversion assay, as described previously (39, 40). In addition, expression of eNOS and mutant eNOS protein was demonstrated by Western blot with the use of antibody directed at either the COOH-terminal (Transduction Labs) or NH₂-terminal (Research Diagnostics) portion of eNOS.

TNF-α production. Untransfected naive or transfected U-937 cells (1 x 10⁷ cells each), as indicated by experiment, were suspended in RPMI 1640 complete medium containing PMA (100 nM) for 48 h to induce differentiation and TNF-α production. After differentiation, adhered cells were removed by incubation with 1 mM EDTA in Hank’s balanced salt solution without Ca²⁺ or Mg²⁺. Cells were then washed, resuspended in RPMI 1640 complete medium, counted, tested for viability by trypsin blue exclusion (∼90% viable for all experiments), and plated into 24-well plates at 5 x 10⁶ cells/ml. For some experiments, as indicated, these final incubations occurred in the absence or presence of increasing concentrations of one of the following: a redox cycling agent (phenazine methosulfate), a membrane-permeant oxidant (tert-butyl hydroperoxide), authentic H₂O₂, a NO donor (SNAP), or a p42/44 MAPK inhibitor (PD-98059). Cell-free supernatants were collected after 22 h of incubation at 37°C in 5% CO₂, and then assayed for TNF-α production with an enzyme-linked immunosorbent assay, according to the manufacturer’s protocol (R&D Systems).

Ferricytochrome c assay for O₂⁻. The formation of superoxide by PMA-differentiated U-937 cells was determined as previously described (10, 29), with minor modifications, by SOD-inhibitable reduction of ferricytochrome c (horse heart type VI; Sigma). PMA-differentiated U-937 cells were washed twice in Hank’s balanced salt solution without phenol red, Ca²⁺, or Mg²⁺. The cells (5 x 10⁶) were incubated for 1 h at 37°C in 5% CO₂ in a 1-mL solution of Hank’s balanced salt solution with Ca²⁺ and Mg²⁺ containing 80 μM ferricytochrome c, 2 mM CaCl₂, and 20 μM catalase. SOD (80 μg/ml) was added to control tubes. O₂⁻ production was initi-
ated with the addition of 10⁻⁶ M fMLP. The reaction was stopped by placing the tubes in a cold water bath. Tubes were then centrifuged at 12,000 g for 10 min, the supernatant was then transferred to cuvettes, and the absorbance was read at 550 nm in a spectrophotometer (Molecular Devices). Blanks were prepared by using reaction mixtures without cells, and these measurements were subtracted as background.

MAPK phosphorylation. Again, U-937 cells, either untransfected or transfected, were PMA (100 nM) differentiated for 48 h and washed with PBS. For the oxidant stress and NO donor experiments, respectively, these PMA-differentiated cells (~1 × 10⁷ cells/flask) were then incubated in RPMI 1640 complete medium in the presence of phenazine methosulfate or fMLP for 30 min, tert-butyl hydroperoxide or H₂O₂ for 10 min, or SNAP for 15 min. For each agent, MAPK phosphorylation-time curves (data not shown) had been performed, and optimal incubation times were selected for these studies. In all experiments, cells for MAPK phosphorylation determination were removed from plates with a cell scraper and then lysed with ice-cold lysis buffer containing 50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals). Solubilized proteins were centrifuged at 10,000 g in a microcentrifuge at 4°C for 10 min, and supernatant protein concentration was quantified by bicinchoninic acid assay (Pierce). These protein preparations (20 μg) were then separated on 4–20% Tris-glycine gels by SDS-PAGE and transferred to PVDF membranes at 30 V for 2 h. Membranes were blocked overnight with PBS containing 5% nonfat dry milk and 0.05% Tween 20 at 4°C. The blots were incubated for 1 h with primary antibody. After incubation with secondary antibody, phosphorylated and nonphosphorylated forms of MAPK were detected by enhanced chemiluminescence. Results were arbitrarily quantitated by using laser densitometry and expressed as relative change from control values.

MAPK activity. Lysates of U-937 cells (500 μg total protein) were incubated with rabbit anti-p42/44 antibody overnight at 4°C and then incubated with protein A-Sepharose beads (20 μg) for 1.5 h at 4°C with gentle rocking to immunoprecipitate the protein. The beads were washed three times with 500 μl of kinase buffer (25 mM Tris, pH 7.5, 7.5 mM glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, and 10 mM MgCl₂), and then an in vitro kinase assay was performed using the transcription factor Elk-1 as a substrate. Phosphorylated Elk-1 protein was then detected by Western blot with the use of an anti-phospho-Elk-1 antibody according to the manufacturer's instructions (New England Biolabs).

Fig. 1. Effect of a redox-cycling agent, phenazine methosulfate, on tumor necrosis factor-α (TNF-α) production and mitogen-activated protein kinase (MAPK) phosphorylation. A: TNF-α production in phorbol 12-myristate 13-acetate (PMA)-differentiated U-937 cells after phenazine methosulfate treatment. Cells (5 × 10⁷/ml) were treated with different doses of phenazine methosulfate in 24-well plates for 22 h. Supernatants were harvested for TNF-α determinations. Values are means ± SE; n = 3 experiments. B: effect of phenazine methosulfate on p42/44 MAPK phosphorylation. PMA-differentiated U-937 cells were washed and treated with different doses of phenazine methosulfate for 30 min. Cells were then lysed and blotted for measurement of phosphorylated (p42/44 MAPK) and total p42/44 MAPK. Each blot shown is 1 of 3 independent experiments that gave similar results. Across these experiments, phenazine methosulfate (2 μM) produced a 3.1 ± 0.2-fold increase in phosphorylated p42/44 MAPK (P = 0.01) as determined by densitometry. Values are means ± SE; n = 3 experiments. C: effect of phenazine methosulfate on p38 MAPK phosphorylation. Experimental conditions are identical to those described for B. Phenazine methosulfate produced a small, 0.9 ± 0.02-fold decrease in phosphorylated p38 MAPK (P = 0.15) as determined by densitometry. Values are means ± SE; n = 3 experiments.
Statistics. Data are shown as means ± SE, and differences were considered significant where two-tailed \( P < 0.05 \). Paired comparisons were made using \( t \)-tests. Escalating dose experiments were analyzed by computing nonparametric Sen-Theil estimates of dose-response slopes and then performing one-sample \( t \)-tests to determine whether these derived slopes were different from 0. Experiments involving multiple comparisons were subjected to an initial analysis of variance (ANOVA) followed by post hoc Fisher’s least significant difference tests of all individual comparisons. To determine if the response to PD-98059, a p42/44 MAPK inhibitor, was different in eNOS compared with control vector transfectants, a repeated-measures ANOVA was computed, with the comparison evaluated from the transfectant/dose interaction term.

RESULTS

Effects of a redox-cycling agent, phenazine methosulfate, on TNF-α production and MAPK phosphorylation. In a previous study we found that eNOS expression increased TNF-α production and promoter activity in PMA-differentiated U-937 cells (35). L-NMA, an inhibitor of eNOS that blocks NO production but not the NADPH oxidase activity of the enzyme, had no effect on TNF-α upregulation by eNOS. Phenazine methosulfate, a redox-cycling agent that has been used to generate intracellular \( \text{O}_2^\cdot \), was found to simulate the effect of eNOS. Furthermore, cotransfection of eNOS with SOD prevented TNF-α upregulation (35). To determine intermediate signaling steps in the regulation of TNF-α by eNOS-derived \( \text{O}_2^\cdot \), we first directly examined the effect of phenazine methosulfate on the activation of MAPK pathways. Again, phenazine methosulfate, an agent that had mimicked eNOS transfection, dose-dependently increased TNF-α production (\( P = 0.01 \)) in PMA-differentiated U-937 cells (Fig. 1A). This effect on TNF-α regulation was associated with a concomitant, dose-dependent change in the phosphorylation state of p42/44 MAPK (Fig. 1B). In contrast to p42/44 MAPK, the phosphorylation state of p38 MAPK in PMA-differentiated U-937 cells, was not altered by phenazine methosulfate exposure (Fig. 1C). These data closely resemble the effects of eNOS-derived \( \text{O}_2^\cdot \) (see Effect of eNOS expression on p42/44 MAPK and p38 MAPK activation: role of superoxide) and suggest that induction of TNF-α production by \( \text{O}_2^\cdot \) in U-937 cells might utilize downstream activation of p42/44 MAPK, but not p38 MAPK, as an intermediary step.

Effect of fMLP-stimulated respiratory burst on MAPK phosphorylation. To determine the possible effects of enzymatically generated \( \text{O}_2^\cdot \) on MAPK phosphorylation, we used fMLP to activate endogenous Fig. 2. Effect of \( N \)-formyl-methionyl-leucyl-phenylalanine (fMLP) on superoxide (\( \text{O}_2^\cdot \)) production and MAPK phosphorylation. A: \( \text{O}_2^\cdot \) production in PMA-differentiated U-937 cells after fMLP treatment. Cells (5 \( \times \) 10^6/ml) were activated with fMLP (10^-6 M), and \( \text{O}_2^\cdot \) was measured by a ferricytochrome \( c \) assay over 90 min. Values are means ± SE; \( n = 3 \) experiments. SOD, superoxide dismutase. B: effect of fMLP on p42/44 MAPK phosphorylation. PMA-differentiated U-937 cells were washed and exposed to different doses of fMLP for 30 min. Cells were then lysed and blotted for measurement of phosphorylated and total p42/44 MAPK. Each blot shown is 1 of 5 independent experiments that gave similar results. Across these experiments fMLP (10^-6 M) produced a 2.6 ± 0.5-fold increase in phosphorylated p42/44 MAPK (\( P = 0.01 \)) as determined by densitometry. Values are means ± SE; \( n = 5 \) experiments. C: effect of fMLP on p38 MAPK phosphorylation. Experimental conditions are identical to those described in B. fMLP produced a small, 1.2 ± 0.28-fold increase in phosphorylated p38 MAPK (\( P = 0.37 \)) as determined by densitometry. Values are means ± SE; \( n = 5 \) experiments.
phagocyte-type NADPH oxidase in PMA-differentiated U-937 cells. First, fMLP was shown to trigger a respiratory burst in PMA-differentiated U-937 cells with the release of $O_2$ as measured by a standard ferricytochrome $c$ assay (Fig. 2A; $P = 0.05$ compared with SOD control). Cell activation by fMLP was accompanied by a dose-dependent increase in p42/44 MAPK phosphorylation (Fig. 2B; 2.6 ± 0.5-fold increase for fMLP 10$^{-6}$ M; $P = 0.01$). Conversely, in these PMA-differentiated U-937 cells, p38 MAPK was not significantly activated (Fig. 2C; $P = 0.37$). Notably, cell activation by fMLP is a complex event that involves multiple signal transduction pathways (11, 22). Determining in PMA-differentiated U-937 cells whether fMLP-induced p42/44 MAPK activation is $O_2$ dependent is beyond the focus of the current investigation. However, our results do show in these cells that fMLP triggers a respiratory burst and produces a pattern of MAPK activation similar to that produced by phenazine methosulfate and eNOS (see Effect of eNOS expression on p42/44 MAPK and p38 MAPK activation: role of superoxide).

Effect of tert-butyl hydroperoxide and $H_2O_2$ on TNF-$\alpha$ production and MAPK phosphorylation. We have previously associated eNOS production of $O_2$ but not NO, with TNF-$\alpha$ upregulation in U-937 cells (35). Because $O_2$ can be converted into other ROS and, in particular, $H_2O_2$, which participates in important signal transduction events (15, 20, 27, 28), we examined the effects of tert-butyl hydroperoxide, a membrane-permeant oxidant, and authentic $H_2O_2$ on TNF-$\alpha$ production and MAPK activation in PMA-differentiated U-937 cells. TNF-$\alpha$ production was not upregulated by tert-butyl-hydroperoxide (Fig. 3A; $P = 0.057$ for a downregulatory effect). However, tert-butyl-hydroperoxide markedly increased p38 MAPK phosphorylation (Fig. 3C; $P = 0.02$) and had a modest effect on p42/44 MAPK phosphorylation (Fig. 3B; $P = 0.052$). Interestingly, $H_2O_2$ also strongly increased p38 MAPK phosphorylation (Fig. 4C; $P = 0.002$) while showing only a minor effect on p42/44 MAPK activation that did not reach statistical significance (Fig. 4B; $P = 0.09$). However, in contrast to tert-butyl hydroperoxide, $H_2O_2$ significantly upregulated TNF-$\alpha$ (Fig. 4A; $P = 0.01$). Notably, the pattern of MAPK phosphorylation produced by either tert-butyl hydroperoxide or $H_2O_2$ differed from that seen with phenazine methosulfate or eNOS.
with eNOS expression (see Effect of eNOS expression on p42/44 MAPK and p38 MAPK activation: role of superoxide).

Effect of a NO donor, SNAP, on TNF-α production and MAPK phosphorylation. We have shown previously that inducible NOS (iNOS) and NO donors can upregulate TNF-α production in PMA-differentiated U-937 cells through an unusual cAMP-dependent mechanism (34, 40). Because the main function of eNOS is to produce NO, MAPK activation by a NO donor, SNAP, was investigated to determine whether its MAPK pathway effects could be discerned from those associated with \( \text{O}_2^\text{2} \) in PMA-differentiated U-937 cells. As previously shown in other studies, NO dose-dependently augmented TNF-α production (Fig. 5A). Although SNAP had no effect on the phosphorylation state of p42/44 MAPK (Fig. 5B), it intensely increased the amount of phosphorylated p38 MAPK in a dose-dependent manner (Fig. 5C). Total p42/44 MAPK and p38 MAPK were not changed by NO exposure. These results suggest that eNOS-derived NO and eNOS-derived \( \text{O}_2^\text{2} \) might be expected to differentially activate MAPK pathways and, therefore, may be distinguishable from each other on the basis of their respective downstream signal transduction events.

Effect of eNOS expression on p42/44 MAPK and p38 MAPK activation: role of superoxide. In our previous investigation, eNOS upregulation of TNF-α production was abolished by Cu/Zn SOD cotransfection (35), so we examined here the effect of SOD on MAPK phosphorylation induced by eNOS expression. U-937 transfectants expressing eNOS demonstrated increased intracellular levels of phosphorylated p42/44 MAPK compared with control vector transfected cells (Fig. 6A; \( P < 0.001 \)). Furthermore, this increment in p42/44 MAPK phosphorylation was substantially abolished by coexpression of Cu/Zn SOD (compared with control vector, \( P = 0.5 \)). Total p42/44 MAPK was not altered by any transfections. As shown in Fig. 6B, eNOS expression, as well as eNOS/SOD cotransfection, had no effect on p38 phosphorylation (\( P = 0.5 \)), suggesting that p38 MAPK plays no significant role in eNOS-induced TNF-α upregulation. Figure 6C shows that eNOS and SOD were expressed in the transfectants used in these experiments. These data, combined with the experimental results obtained with the use of various oxidants and a NO donor, suggest that p42/44 MAPK activation in eNOS-transfected U-937 cells is dependent on the release of superoxide.

Fig. 4. Effect of \( \text{H}_2\text{O}_2 \) on TNF-α production and MAPK phosphorylation. A: TNF-α production in PMA-differentiated U-937 cells after \( \text{H}_2\text{O}_2 \) treatment. Cells (5 × 10⁵/ml) were treated with different doses of \( \text{H}_2\text{O}_2 \) in 24-well plates for 22 h. Supernatants were harvested for TNF-α determinations. Values are means ± SE; \( n = 4 \) experiments. B: effect of \( \text{H}_2\text{O}_2 \) on p42/44 MAPK phosphorylation. PMA-differentiated U-937 cells were washed and treated with different doses of \( \text{H}_2\text{O}_2 \) for 10 min. Cells were then lysed and blotted for measurement of phosphorylated and total p42/44 MAPK. Each blot shown is 1 of 5 independent experiments that gave similar results. Across these experiments, \( \text{H}_2\text{O}_2 \) (200 μM) produced a small, 1.6 ± 0.23-fold increase in phosphorylated p42/44 MAPK (\( P = 0.09 \)) as determined by densitometry. Values are means ± SE; \( n = 5 \) experiments. C: effect of \( \text{H}_2\text{O}_2 \) on p38 MAPK phosphorylation. Experimental conditions are identical to those described for B. \( \text{H}_2\text{O}_2 \) produced a 7.0 ± 0.6-fold increase in phosphorylated p38 MAPK (\( P = 0.002 \)) as determined by densitometry. Values are means ± SE; \( n = 5 \) experiments.
Effect of mutating the L-arginine binding site or deleting the NADPH binding site of eNOS on p42/44 MAPK and p38 MAPK phosphorylation. Previous work has shown that the mutant enzyme Gln361eNOS is unable to produce NO but retains its NADPH oxidase activity and the ability to upregulate TNF-α (5, 35), whereas eNOS mutants lacking an adenosine NADPH binding site, d(NADPH)eNOS and d(NADPH)Gln361 eNOS, have neither NADPH oxidase activity nor the ability to upregulate TNF-α (35). Consistent with these previous results, we found (Fig. 7A) that both eNOS and Gln361eNOS similarly increased the phosphorylation state of p42/44 MAPK ($P < 0.01$), but NADPH binding site deletion mutants of eNOS had no effect on p42/44 MAPK phosphorylation compared with control vector-transfected cells ($P = 0.54$). In contrast, neither eNOS nor any of its mutant forms affected p38 MAPK phosphorylation (Fig. 7B). These results further suggest that superoxide generated by the NADPH oxidase activity of eNOS is essential to its ability to increase the phosphorylation state of p42/44 MAPK and, as previously shown (35), to upregulate TNF-α production.

Effect of eNOS expression on p42/44 MAPK activity and effect of PD-98059, a p42/44 MAPK pathway inhibitor, on eNOS-induced TNF-α upregulation. To confirm that p42/44 MAPK phosphorylation reflects changes in enzymatic activity, we performed an assay for p42/44 MAPK activity. A specific downstream target of p42/44 MAPK, the transcription factor Elk-1, served as a substrate for measuring p42/44 MAPK activity. Similar to our results obtained by examining p42/44 MAPK phosphorylation, eNOS expression compared with control vector transfection (Fig. 8A) resulted in the upregulation of p42/44 MAPK activity ($P = 0.001$). Finally, we used PD-98059, an inhibitor of p42/44 MAPK phosphorylation, to test whether the p42/44 MAPK pathway participates in TNF-α upregulation by eNOS. As shown in Fig. 8B, PD-98059 dose-dependently blocked the eNOS-dependent upregulation of TNF-α ($P = 0.02$ for interaction between eNOS expression and the effect of PD-98059). The inhibitory effect of PD-98059 was significantly greater in eNOS cells than in control vector transfectants, suggesting...

Fig. 5. Effect of a nitric oxide (NO) donor, S-nitroso-N-acetylpenicillamine (SNAP), on TNF-α production and p42/44 and p38 MAPK phosphorylation. A: TNF-α production in PMA-differentiated U-937 cells after SNAP treatment. Cells ($5 \times 10^5$/ml) were treated with different doses of SNAP in 24-well plates for 22 h. Supernatants were harvested for TNF-α determinations. Values are means ± SE; $n = 3$ experiments. B: effect of SNAP on p42/44 phosphorylation. PMA-differentiated U-937 cells were washed and treated with different doses of SNAP for 15 min. Cells were then lysed and blotted for measurement of phosphorylated and total p42/44 MAPK. Each blot shown is 1 of 3 independent experiments that gave similar results. Across these experiments, SNAP (500 µM) produced no effect on phosphorylated p42/44 MAPK (1.0 ± 0.08 fold; $P = 0.99$) as determined by densitometry. Values are means ± SE; $n = 3$ experiments. C: effect of SNAP on p38 MAPK phosphorylation. Experimental conditions are identical to those described for B. SNAP produced a 5.4 ± 1.1-fold increase in phosphorylated p38 MAPK ($P = 0.049$) as determined by densitometry. Values are means ± SE; $n = 3$ experiments.
that TNF-α upregulation in PMA-differentiated U-937 cells is mediated by activation of the p42/44 MAPK pathway.

DISCUSSION

Recently, we demonstrated in PMA-differentiated U-937 cells that eNOS could regulate TNF-α transcription and production through a signal transduction pathway dependent on the release of O₂ and, importantly, completely independent of NO (35). However, downstream events in this signaling pathway have not been identified. Therefore, distal components of this putative O₂-dependent pathway have not been shown to be distinct from those initiated by NO, the principal product of eNOS under homeostatic conditions. We now demonstrate that eNOS-generated O₂ leads to activation of p42/44 and p38 MAPK. eNOS expression (bottom gel) is shown in the various transfectants with the use of an antibody against the NH₂-terminal portion of human eNOS (Research Diagnostics). Human endothelial cell lysate served as a positive control.

Fig. 6. Phosphorylation of p42/44 and p38 MAPK in endothelial nitric oxide synthase (eNOS)-transfected U-937 cells with or without SOD cotransfection. A: phosphorylation of p42/44 MAPK in various transfectants after PMA differentiation. Densitometry data from blots were normalized to control vector values. Values are means ± SE; n = 3 experiments. B: phosphorylation of p38 MAPK in various transfectants after PMA differentiation. Densitometry data from blots were normalized to control vector values. Representative blots for phosphorylated and total p42/44 MAPK are shown. Values are means ± SE; n = 3 experiments. C: SOD and eNOS expression in cells used for experiments in A and B. Purified bovine SOD (Sigma) and human endothelial cell lysate served as positive controls for human Cu/Zn SOD and eNOS, respectively. Gels were immunoblotted with antibody directed at human Cu/Zn SOD (Research Diagnostics) or with antibody against the COOH-terminal portion of human eNOS (Transduction Labs).

Fig. 7. Phosphorylation of p42/44 MAPK and p38 MAPK in wild-type eNOS- and mutant eNOS-transfected U-937 cells. A: phosphorylation of p42/44 MAPK in various transfectants after PMA differentiation. Densitometry data from blots were normalized to control vector values. Values are means ± SE; n = 5 experiments. B: representative blots for phosphorylated and total p42/44 and p38 MAPK. eNOS expression (bottom gel) is shown in the various transfectants with the use of an antibody against the NH₂-terminal portion of human eNOS (Research Diagnostics). Human endothelial cell lysate served as a positive control.

that TNF-α upregulation in PMA-differentiated U-937 cells is mediated by activation of the p42/44 MAPK pathway.

DISCUSSION

Recently, we demonstrated in PMA-differentiated U-937 cells that eNOS could regulate TNF-α transcription and production through a signal transduction pathway dependent on the release of O₂ and, importantly, completely independent of NO (35). However, downstream events in this signaling pathway have not been identified. Therefore, distal components of this putative O₂-dependent pathway have not been shown to be distinct from those initiated by NO, the principal product of eNOS under homeostatic conditions. We now demonstrate that eNOS-generated O₂ leads to activation of p42/44 MAPK, an event that is pivotal to its subsequent upregulation of TNF-α. Signaling by eNOS through this pathway is blocked by Cu/Zn SOD coexpression and by deletion of the NADPH binding site of eNOS, but not by a l-arginine binding site mutation that only abolishes NO production. tert-Butyl hydroperoxide and H₂O₂ produce patterns of MAPK activation dissimilar to that seen with phenazine
methosulfate or eNOS expression. Importantly, NO, the primary product of eNOS that has its own upregulatory effects on TNF-α (30, 34, 40), was shown to activate p38 MAPK, not p42/44 MAPK.

ROS, once thought of as metabolic by-products and toxins, are now recognized as essential signal transduction intermediates in the regulation of cell proliferation, differentiation, and apoptosis (13, 17, 20, 27, 28, 37). Although ROS signaling pathways have been associated previously with MAPK activation in many cell types, the enzymatic sources and the molecular identity of the ROS involved in these regulatory events have often remained obscure (13, 37). The finding that eNOS-derived O2− and NO produce different MAPK activation signatures may provide a tool for differentiating between these two eNOS-based signaling pathways in other cells.

For many ROS signaling events, H2O2 is considered a more attractive intermediate than its more toxic precursor, O2− (13, 17, 20, 27, 28, 37). H2O2 is metabolized directly into water and oxygen by catalase and glutathione peroxidase. In contrast, O2−, a potentially injurious by-product of many cellular reactions, can lead to the formation of potent cytotoxins such as hydroxyl anion and peroxynitrite (16, 21, 36). Notably, production of these undesirable radicals is controlled by the conversion of O2− into less toxic H2O2 by SOD, a ubiquitous family of antioxidant enzymes (20). Therefore, it is interesting that eNOS expression produces a pattern of MAPK activation (p42/44 MAPK phosphorylation) similar to that caused by a redox-cycling agent used to generate intracellular O2−, but distinctly different from H2O2 exposure. H2O2 produced a strong p38 MAPK phosphorylation signal and a relatively weaker p42/44 MAPK effect. Furthermore, Cu/Zn SOD, an enzyme that converts O2− into H2O2, completely abolished the effects of eNOS on p42/44 MAPK and TNF-α. Collectively, these results suggest in PMA-differentiated U-937 cells that eNOS-derived O2−, rather than its downstream metabolite H2O2, may be required for TNF-α upregulation. However, H2O2 is well known to be a potent signal transduction intermediate in many experimental systems and, as shown here, clearly affects MAPK pathways and TNF-α production in PMA-differentiated U-937 cells. Therefore, the conversion of eNOS-derived O2− into H2O2 could still be an important signaling pathway, particularly for the regulation of genes other than TNF-α or in other cell types. As stated by Manna et al. (20), activations of redox-dependent signaling pathways are complex events that likely reflect the total cellular balance of opposing prooxidant and antioxidant factors rather than the presence of a single reactive intermediate. This concept suggests that the coregulation of eNOS and SOD in endothelial cells, as described by Dimmeler et al. (12), could act as a counterregulatory mechanism that serves to maximize the vasodilatory and antiadherence properties of eNOS-derived NO while suppressing the prooxidant potential of eNOS expression.

Not only did we find that eNOS expression and O2− production were both linked to p42/p44 (ERK1/2) phosphorylation, but, importantly, inhibition of p42/44 MAPK was shown to block eNOS-induced TNF-α upregulation. Conversely, even though NO exposure was
associated with phosphorylation of p38 MAPK, SB-203580 [(4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole), a p38 MAPK inhibitor, had no effect on NO-induced upregulation of TNF-α in concentrations up to 20 μM (experiment not shown). In previous investigations, we showed that TNF-α up-regulation by NO and iNOS expression occurs through a cAMP-dependent signaling pathway (34). NO inhibits basal adenylate cyclase activity and lowers intracellular cAMP concentrations, which in turn decreases cAMP-dependent protein kinase (PKA) activity. Notably, NO-induced upregulation of TNF-α is mimicked by PKA inhibitors and abolished by cell-permeable cAMP analogs (34). This effect of NO on TNF-α has been associated with decreased binding of Sp1 to the TNF-α promoter, which results in upregulation of TNF-α promoter activity (33). Therefore, although NO exposure leads to the phosphorylation of p38 MAPK, it appears that this effect may be tangential to TNF-α upregulation. Interestingly, PKA, whose activity is decreased by NO through its cAMP-lowering effects (33, 34), is known in some cells to phosphorylate Raf-1, thereby blocking its interaction with Ras and subsequent activation of MAPK (8, 14). The binding of Raf-1 with Ras, which arguably might be increased by NO inhibition of the cAMP/PKA pathway, leads to MAPK kinase activation. Thus the effects of NO on cAMP could independently lead to both TNF-α upregulation and p38 MAPK phosphorylation.

In summary, this investigation has identified p42/44 MAPK kinase as a downstream target of eNOS-derived O2·− signal transduction. Importantly, this pathway appears to be distinct from the MAPK effects of either H2O2 or NO. The results suggest that eNOS-derived O2·− and NO produce distinguishably divergent patterns of MAPK kinase activation that may serve as valuable markers for differentiating between the potential duality of eNOS-generated signals. Furthermore, the ability of eNOS to activate ERK1/2, a major cell growth and proliferative-response pathway (7), through the release of O2·−, suggests that, under pathological conditions, eNOS may have proatherosclerotic effects and may contribute directly to endothelial dysfunction. This postulated role for eNOS may be limited to abnormal vascular states in diseases such as septic shock and atherosclerosis. The primary cellular role of eNOS is to produce NO, a molecule associated with antiproliferative and antiatherogenic properties. It is notable that during experimentally created shear-stress, an atherogenic insult, the expression of both eNOS and SOD increases in normal vasculature in a coregulatory manner (12). This response might serve to maximize the beneficial effects of NO under these conditions, while minimizing the proliferative and potentially toxic effects of eNOS-generated O2·−. However, it is important to point out the artificial nature of the heterologous model system we have investigated. Bernier et al. (3) showed in endothelial cells that membrane-bound eNOS can complex with other proteins including Raf-1, Akt, and, importantly, p42/44 MAPK. They demonstrated that p42/44 MAPK activation led to eNOS phosphorylation, an inactivating event. Whether eNOS-derived O2·− might trigger this response in endothelial cells by activating p42/44 MAPK is highly speculative and requires further investigation.

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