Peroxy nitrite Targets the Epidermal Growth Factor Receptor, Raf-1, and MEK Independently to Activate MAPK*

Received for publication, December 30, 1999, and in revised form, May 4, 2000
Published, JBC Papers in Press, May 8, 2000, DOI 10.1074/jbc.M910425199

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Activation of ERK-1 and -2 by H2O2 in a variety of cell types requires epidermal growth factor receptor (EGFR) phosphorylation. In this study, we investigated the activation of ERK by ONOO- in cultured rat lung myofibroblasts. Western blot analysis using anti-phospho-ERK antibodies along with an ERK kinase assay using the phosphorylated heat- and acid-stable protein (PHAS-1) substrate demonstrated that ERK activation peaked within 15 min after ONOO- treatment and was maximally activated with 100 μM ONOO-. Activation of ERK by ONOO- and H2O2 was blocked by the antioxidant N-acetyl-l-cysteine. Catalase blocked ERK activation by H2O2, but not by ONOO-, demonstrating that the effect of ONOO- was not due to the generation of H2O2. Both H2O2 and ONOO- induced phosphorylation of EGFR in Western blot experiments using an anti-phospho-EGFR antibody. However, the EGFR tyrosine kinase inhibitor AG1478 abolished ERK activation by H2O2, but not by ONOO-. Both H2O2 and ONOO- activated Raf-1. However, the Raf inhibitor forskolin blocked ERK activation by H2O2, but not by ONOO-. The MEK inhibitor PD98059 inhibited ERK activation by both H2O2 and ONOO-. Moreover, ONOO- or H2O2 caused a cytotoxic response of myofibroblasts that was prevented by preincubation with PD98059. In a cell-free kinase assay, ONOO- (but not H2O2) induced autophosphorylation and nitration of a glutathione S-transferase-MEK-1 fusion protein. Collectively, these data indicate that ONOO- activates EGFR and Raf-1, but these signaling intermediates are not required for ONOO- induced ERK activation. However, MEK-1 activation is required for ONOO- induced ERK activation in myofibroblasts. In contrast, H2O2-induced ERK activation is dependent on EGFR activation, which then leads to downstream Raf-1 and MEK-1 activation.

Inflammation following tissue injury is associated with increased generation of reactive oxygen species such as superoxide anion (O2-) and hydrogen peroxide (H2O2) (1). Moreover, nitric oxide (NO-) is synthesized by inflammatory cells and has the potential to react with O2- via a nearly diffusion-limited reaction to form peroxynitrite (ONOO-). These oxidants may serve several physiological or pathophysiological functions. For example, NO- is thought to play a major role in host defense, but is also presumed to contribute to tissue injury (2). H2O2 and O2- released into the extracellular environment by mononuclear cells during an oxidative burst may also play a role in immune defense (1). ONOO- is a potent cytotoxic species that has been proposed to contribute to the pathophysiology of a wide variety of inflammatory diseases. Although ONOO- is extremely short-lived at physiological pH (1-s half-life) (6), the formation of 3-nitrotyrosine by ONOO- reaction with tyrosyl residues serves as a stable marker or "footprint" (7–9). ONOO- not only affects tyrosine residues on proteins, but also may induce oxidative reaction products through modifications of cysteine, methionine, and tryptophan (4).

Increasing evidence supports the idea that oxidants serve as signaling intermediates required for receptor tyrosine kinase function and downstream activation of mitogen-activated protein kinases (MAPKs). In particular, H2O2 generated intracellularly following the binding of platelet-derived growth factor or epidermal growth factor (EGF) to their respective receptors appears to reversibly inhibit protein-tyrosine phosphatase activity, which is required for phosphorylation of receptor tyrosine kinases (10, 11). In the absence of EGF, exogenous H2O2 alone can cause phosphorylation of EGFR through reversible oxidative modification of cysteine residues, leading to downstream activation of a Raf/MEK/MAPK phosphorylation cascade (12–14). In contrast, ONOO- stimulates phosphorylation of EGFR tyrosine residues by causing irreversible dimerization of EGFR via dityrosine cross-links (15). More recently, several nitric oxide-related species, including ONOO-, have been reported to activate MAPKs (16–18), yet the mechanism of MAPK activation by ONOO- remains unclear.

MAPKs are a family of serine/threonine kinases that regulate a diversity of cellular activities. Three major classes have been described: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) also known as stress-activated protein kinases), and p38 MAPK (reviewed in Ref. 19). JNKs and p38 MAPKs mediate signals in response to cytokines and environmental stress, whereas the ERK subtypes are classically recognized as key transducers in the signaling cascade mediating cell proliferation in response to growth factors such as platelet-derived growth factor and EGF. Two major isoforms of ERK, p44 (ERK-1) and p42 (ERK-2), have been identified in...
mammalian systems. A major pathway involved in ERK-1 and -2 phosphorylation in a variety of cell types requires the sequential activation of Raf and MEK (20, 21). It is becoming increasingly clear that the ERK pathway, like those of p38 and JNK, is activated by environmental stress, including reactive oxygen species such as H₂O₂ (12).

In this study, we have investigated the mechanism of ONOO⁻–induced ERK-1 and -2 activation. Similar to previous studies on the mechanism of H₂O₂–induced ERK activation, we found that ONOO⁻ activates EGFR, Raf-1, and MEK-1. However, unlike H₂O₂, which requires EGFR phosphorylation to initiate a downstream signaling cascade for activation of ERK, we report that ONOO⁻ induces autophosphorylation of EGFR, Raf-1, and MEK-1 independently.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary passage rat pulmonary myofibroblasts were isolated and characterized as described previously (22). Immunohistochemical analysis demonstrated these cells to be positive for markers of smooth muscle cells (α-smooth muscle actin and desmin) and fibroblasts (vimentin), indicating a myofibroblast phenotype (23). Following the isolation procedure, aliquots of cells (1 × 10⁶/ml) were stored in liquid nitrogen. Cells (1 × 10⁶) were thawed from the liquid nitrogen and plated in a 175-cm² flask in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium supplemented with L-glutamine and fungizone. After reaching confluence, the cells were liberated with trypsin, replated on 100-mm dishes, and grown to confluence in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium. At this point, the cells were designated as passage 2. Cells were then rendered quiescent for 24 h with serum-free defined medium (SFDM) consisting of Ham’s F-12 medium supplemented with 0.25% bovine serum albumin and an insulin/transferrin/selenium mixture (Roche Molecular Biochemicals) for the experiments described below.

**Oxidant Treatments**— Peroxynitrite was synthesized from acidic nitrite and hydrogen peroxide (Upstate Biotechnology, Inc., Lake Placid, NY). Stocks of ONOO⁻ were stored in 1.2 N NaOH at −80 °C. Prior to experimentation, ONOO⁻ was quantitated spectrophotometrically (extinction coefficient at 302 nm = 1670 M⁻¹ cm⁻¹) (24). Cells in SFDM were washed with phosphate-buffered saline (PBS) and then equilibrated in PBS for 5 min. ONOO⁻ was delivered as a single bolus (e.g. 10 µl of 100 mM stock delivered in 1 ml to give a final concentration of 1 µM ONOO⁻) to one side of the dish while rapidly swirling the medium to assure optimal exposure of the cells to ONOO⁻ before decomposition (15, 25). In control experiments, ONOO⁻ was prediluted for 1 min in an equivalent volume of PBS prior to adding to the cells. This “delayed” PBS solution contained completely decomposed ONOO⁻ as determined spectrophotometrically, but still contained contaminants such as nitrite and hydrogen peroxide. In other control experiments, cells were treated with equal volumes of 1.2 N NaOH (vehicle control). PBS, rather than SFDM, was used as the medium for ONOO⁻ treatments to avoid interfering reactions of ONOO⁻ with media constituents.

**Western Blot Analysis**—Confluent cell monolayers on 100-mm dishes were growth-arrested in serum-free medium for 24 h prior to treatment with ONOO⁻, H₂O₂, or metabolic inhibitors. Cells were washed twice with PBS, scraped, resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml pepstatin, 200 µg/ml Na₃VO₄, and 0.2 mM benzamidine) and clarified by centrifugation at 13,000 rpm for 10 min. Thirty µg of protein/sample was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked for 2 h at 25 °C with 5% nonfat milk in PBS buffer (20 mM Tris, 500 mM NaCl, and 0.01% Tween 20). The membrane was then incubated overnight at 4 °C with an appropriate dilution of anti-phospho-ERK or anti-ERK (New England Biolabs Inc.), anti-phospho-MEK-1 or anti-MEK-1 (New England Biolabs Inc.), or anti-phospho-EGFR or anti-EGFR (Upstate Biotechnology, Inc.) polyclonal antibody at 4 °C overnight, followed by incubation for 2 h with a 1:2000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody. The immunoblot signal was visualized through enhanced chemiluminescence.

**PHAS-1 Kinase Assay**—ERK activity in cell lysates was measured as described previously (26) by phosphorylation of PHAS-1, a substrate for ERK (27). Briefly, confluent cell monolayers on 100-mm dishes were growth-arrested in serum-free medium for 24 h, treated with ONOO⁻, chilled on ice, washed twice with PBS, and scraped with 800 µl of lysis buffer. ERK was immunoprecipitated by incubating 200 µl of lysate with 2 µg of anti-ERK antibody (Santa Cruz Biotechnology, Inc.) for 2 h and then adding 20 µl of protein A-agarose (Santa Cruz Biotechnology, Inc.). After an overnight incubation at 0–4 °C with end-over-end mixing, the immune complex was recovered by centrifugation and washed three times with lysis buffer and once with 250 mM HEPES (pH 7.4), 10 mM MnCl₂, and 200 µM NaN₃. Immunoprecipitates were used in a MAPK assay kit (Stratagene). The ERK pellets were resuspended in Stratagene reaction buffer containing 120 µg of PHAS-1 substrate along with 3–5 µCi of [γ-³²P]ATP in a final volume of 180 µl. Kinase reactions took place for 30 min at room temperature and were stopped by adding 4× SDS-polyacrylamide gel electrophoresis reducing sample buffer and boiling for 10 min. ERK-PHAS samples were resolved on SDS-polyacrylamide gels, dried, and autoradiographed.

**Raf-1 Kinase Assay**—Raf-1 kinase activity was measured by immunoprecipitation with an anti-Raf-1 antibody followed by a phosphorylation assay using bacterially expressed, kinase-inactive MEK-1 (Upstate Biotechnology, Inc.) as the substrate. The lysis buffer consisted of 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% bovine serum albumin, aprotinin, 20 µg/ml leupeptin, 20 µg/ml pepstatin, 200 µg/ml Na₃VO₄, and 0.2 mM benzamidine. Lysates were incubated with antibody for 90 min at 4 °C, and protein A-agarose beads were then added for an additional 30 min to immunoprecipitate Raf-1. Pellets were washed and resuspended in 20 µl of kinase buffer (20 mM HEPES (pH 7.0), 10 mM MnCl₂, 20 µg/ml aprotinin, and 200 µg/ml NaN₃) containing 1 ng of kinase-inactive MEK-1 and 5 µCi of [γ-³²P]ATP. Recombinant non-activated MEK-1 resolved on SDS-polyacrylamide gels, dried, and autoradiographed.

**Cell-free MEK-1 Phosphorylation Assay**—This assay was a modification of a previously reported method (21). Recombinant non-activated GST-MEK-1 (0.5 µg; Upstate Biotechnology, Inc.) was diluted in 20 µl of assay dilution buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, and 1 mM diethylthiocarbamate) supplemented with a magnesium/ATP mixture (75 mM magnesium chloride and 500 µM unlabeled ATP). An increasing concentration of ONOO⁻ (1–1000 µM) was added to the solution and incubated for 15 min at 30 °C. The reaction was stopped by the addition of polyacrylamide gel electrophoresis sample buffer containing 2-mercaptoethanol. Activated GST-MEK-1 (Upstate Biotechnology, Inc.) was loaded on gels and separated by electrophoresis. As an immunoprecipitate of MEK-1 was incubated with non-activated GST-MEK-1. Phosphorylation of GST-MEK-1 was detected by Western blotting using an anti-phospho-MEK-1 antibody. Western blots were stripped and reblotted using an anti-nitrotyrosine antibody (Upstate Biotechnology, Inc.).

**[³²H]Thymidine Incorporation Assay**—Confluent rat lung myofibroblasts on 24-well tissue culture plates were rendered quiescent in SFDM for 24 h; then the medium was switched to PBS, and ONOO⁻ (1–1000 µM) was added to the medium with constant swirling. After 15 min, the medium was removed, and fresh SFDM containing 5 µCi/ml [³²H]thymidine (Amersham Pharmacia Biotech) was added back. After 24 h at 37 °C with 5% CO₂ and humidified air, the cells were rinsed three times with SFDM and chilled on ice for 30 min. The medium was removed, and 5% trichloroacetic acid (0.5 ml/well) was added for 10 min. After removing the trichloroacetic acid, the cells were washed three times with ice-cold water and solubilized with a solution of 1% Triton X-100 and 0.1% NaOH. Radioactivity was measured in a liquid scintillation counter.

**Cytotoxicity Assay**—Cytotoxicity was measured by the MTT cell viability assay (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

**RESULTS**

ONOO⁻ Activation of ERK Requires Immediate Addition to Cell Cultures—Since ONOO⁻ has an extremely short half-life at neutral pH (∼1 s) (6), we assessed ERK activation in cultures of pulmonary myofibroblasts that received ONOO⁻ immediately as compared with cell cultures that received ONOO⁻ that had first been allowed to incubate (i.e. delayed) in PBS for 1 min at pH 7.4. The immediate addition strategy was performed by delivering a single bolus of the ONOO⁻ stock against the side of the dish while swirling the PBS solution to allow for mixing. Direct addition resulted in strong ERK activation, whereas the delay of ONOO⁻ in PBS for 1 min caused no activation of ERK (Fig. 1). This experiment was essential for demonstrating that ERK activation was due to ONOO⁻ and not to trace amounts of H₂O₂, which is relatively stable at pH 7.4.
ERK and total ERK after the immediate addition of ONOO⁻ absence of cells and then adding the 1 mM ONOO⁻ kinase assay using the PHAS-1 substrate (Fig. 2). In these experiments, Western blotting with an antibody that detects unphosphorylated ERK was used to show that ERK protein levels did not change appreciably as a consequence of ONOO⁻ treatment. H₂O₂ also maximally activated ERK at 100 µM (data not shown). The concentrations of ONOO⁻ or H₂O₂ required to activate ERK in our study were within the micromolar range reported by other investigators for activation of cellular signaling pathways.

Effect of Antioxidants on ONOO⁻- and H₂O₂-induced ERK Activation—To determine if ONOO⁻-induced ERK activation was due to the presence of H₂O₂, cells were preincubated with catalase (3000 units/ml) for 1 h and then treated with either ONOO⁻ or H₂O₂ for 15 or 30 min. H₂O₂-induced ERK activation was completely inhibited by catalase, whereas ONOO⁻-induced ERK activation was not affected (Fig. 3). N-Acetyl-l-cysteine has been reported to block the effects of both H₂O₂ (11) and ONOO⁻ (28–30). Pretreatment of cells with 50 mM N-acetyl-l-cysteine for 1 h prior to adding oxidants inhibited both ONOO⁻- and H₂O₂-induced ERK activation (Fig. 3).

EGFR Phosphorylation Is Required for H₂O₂-induced ERK Activation, but Not for ONOO⁻-induced ERK Activation—We observed that EGF, ONOO⁻, and H₂O₂ induced phosphorylation of EGFR as determined by Western blotting using an antibody specific for phospho-EGFR (Fig. 4A). Moreover, the EGFR tyrosine kinase inhibitor AG1478 (31) abolished phosphorylation of EGFR induced by all of these agents (Fig. 4A). However, although AG1478 significantly inhibited ERK activation induced by EGF and H₂O₂, AG1478 did not significantly inhibit ERK activation induced by ONOO⁻ (Fig. 4, B and C).

ERK Activation by H₂O₂ Is Dependent on Raf-1, yet ONOO⁻-induced ERK Activation Is Not Raf-dependent—Using a Raf-1 kinase assay that utilized MEK-1 as the substrate, we observed that both H₂O₂ and ONOO⁻ stimulated Raf-1-dependent phosphorylation of MEK-1 (Fig. 5A). Furthermore, activation of Raf-1 by both of these oxidants was blocked by pretreatment with forskolin (Fig. 5A). Pretreatment of cells with forskolin prior to the addition of oxidant significantly blocked H₂O₂-induced ERK activation in phospho-ERK Western blot assays, yet forskolin did not affect ONOO⁻-induced ERK activation (Fig. 5, B and C).

Activation of ERK by ONOO⁻ and H₂O₂ Requires MEK—Pretreatment of cells with the MEK inhibitor PD98059 (50 µM) (32) abolished activation of ERK induced by either ONOO⁻ or H₂O₂ in phospho-ERK Western blot experiments (Fig. 6). Accordingly, the MEK inhibitor blocked the phosphorylation of MEK-1 by either ONOO⁻ or H₂O₂ in Western blot experiments using an anti-phospho-MEK-1 antibody.

ONOO⁻ Causes Nitration and Autophosphorylation of Recombinant GST-MEK-1 Fusion Protein in a Cell-free System—To further investigate the mechanism through which ONOO⁻ phosphorylates MEK-1, we treated recombinant non-activated GST-MEK-1 with ONOO⁻ in a buffer solution containing a magnesium/ATP mixture and performed Western blotting using an anti-phospho-MEK-1 antibody or an anti-nitrotyrosine antibody. As shown in Fig. 7, ONOO⁻ induced phosphorylation of GST-MEK-1. Moreover, ONOO⁻ caused a concentration-dependent increase in nitrotyrosine formation on GST-MEK-1 (Fig. 7). These experiments provide, for the first time, evidence of a direct biochemical modification of MEK-1 by ONOO⁻ that results in autophosphorylation. In parallel experiments, H₂O₂ did not phosphorylate GST-MEK-1 in this cell-free system (data not shown). Non-activated Raf-1 was not commercially available to perform a similar type of

Furthermore, this experiment ruled out the formation of stable nitrating agents that could potentially be formed by the reaction of ONOO⁻ with components of the cell culture medium. The addition of the same volume of 1.2 M NaOH alone to PBS (10 µl/1 ml), either immediately or delayed, did not cause ERK activation (data not shown).

ONOO⁻ Activates ERK in a Time- and Concentration-dependent Manner—Treatment of cells with 1 mM ONOO⁻ activated ERK maximally within 15 min as determined by Western blotting using an anti-phospho-ERK antibody or by an ERK kinase assay using the PHAS-1 substrate (Fig. 2A). Phospho-

**Fig. 1.** Immediate addition of ONOO⁻ is required to activate ERK-1 and ERK-2. A, representative Western blot results for phospho-ERK and total ERK after the immediate addition of ONOO⁻ versus addition of ONOO⁻ to cultures after 1 min in PBS (pH 7.4) to allow for ONOO⁻ degradation. Myofibroblasts were rendered quiescent for 24 h in SFDM and then switched to PBS (pH 7.4) for the addition of ONOO⁻ to a final concentration of 1 mM. Immediate addition was performed by delivering a bolus of the ONOO⁻ stock in 1.2 M NaOH directly against the side of the dish and immediately mixing the PBS solution overlaying the cells. Delayed addition was performed by adding the same amount of the ONOO⁻ stock in 1.2 M NaOH to PBS for 1 min in the absence of cells and then adding the 1 mM ONOO⁻ solution to the cells. After 15 min, the cell lysates were harvested and assayed for ERK activation by Western blot analysis using an anti-phospho-ERK antibody. The data shown are typical of four separate experiments. B, densitometric analysis of ERK activation from four separate experiments. The relative level of ERK activation was determined by densitometric scanning of the phospho-ERK bands and normalized to the ERK signal. ***, p < 0.01 as compared with no addition.
Activation of MAPK by Peroxynitrite

Fig. 2. ONOO\textsuperscript{−} activates ERK-1 and ERK-2 in a time- and concentration-dependent manner. A, myofibroblasts were rendered quiescent for 24 h in SFDM and then switched to PBS and exposed to 1 mM ONOO\textsuperscript{−} for the indicated time points. Cell lysates were collected for Western blotting using an antibody specific for phosphorylated ERK (upper panel) or an antibody against total ERK (middle panel). ERK activity was measured by kinase assay following immunoprecipitation of ERK and using PHAS-1 as a substrate (lower panel). B, cells were exposed to an increasing concentration of ONOO\textsuperscript{−} for 15 min prior to collecting cell lysates for phospho-ERK and ERK Western blotting (upper and middle panels, respectively) or immunoprecipitation of ERK followed by PHAS-1 kinase assay (lower panel).

![Western blot and kinase assay images for ONOO\textsuperscript{−} effects on ERK activation.](image)

Fig. 3. Effect of antioxidants N-acetyl-L-cysteine and catalase on ONOO\textsuperscript{−} and H\textsubscript{2}O\textsubscript{2}-induced ERK-1 and ERK-2 activation. Confuent quiescent cultures of rat pulmonary myofibroblasts were incubated with N-acetyl-L-cysteine (NAC; 50 mM) or catalase (3000 units/ml) and then treated with 1 mM ONOO\textsuperscript{−} (upper panel) or H\textsubscript{2}O\textsubscript{2} (lower panel) for 15 or 30 min prior to collecting cell lysates for Western blotting using an anti-phospho-ERK antibody.

![Western blot images for antioxidant effects on ERK activation.](image)

DISCUSSION

In this study, we have investigated possible differences in the mechanism of ERK activation by H\textsubscript{2}O\textsubscript{2} and ONOO\textsuperscript{−} in vitro using primary passage rat pulmonary myofibroblasts. ONOO\textsuperscript{−} was found to be a potent activator of ERK and also caused phosphorylation of EGFR, Raf-1, and MEK-1. Although these results initially suggested a mechanism of MAPK activation similar to that stimulated by H\textsubscript{2}O\textsubscript{2}, we observed that inhibitors of EGFR (AG1478) and Raf-1 (forskolin) did not block ONOO\textsuperscript{−}-induced MAPK activation. Since H\textsubscript{2}O\textsubscript{2} requires EGFR to activate MAPK, these data suggested a different mechanism of ONOO\textsuperscript{−} induced MAPK activation. Since H\textsubscript{2}O\textsubscript{2} was found to be a potent activator of ERK and also caused phosphorylation of EGFR, Raf-1, and MEK-1. Although these results initially suggested a mechanism of MAPK activation similar to that stimulated by H\textsubscript{2}O\textsubscript{2}, we observed that inhibitors of EGFR (AG1478) and Raf-1 (forskolin) did not block ONOO\textsuperscript{−}-induced MAPK activation. Since H\textsubscript{2}O\textsubscript{2} requires EGFR to activate MAPK, these data suggested a different mechanism of MAPK activation by ONOO\textsuperscript{−}. We further explored the mechanism of activation and found that ONOO\textsuperscript{−}, but not H\textsubscript{2}O\textsubscript{2}, caused nitration and autophosphorylation of recombinant GST-MEK-1 fusion protein in a cell-free system. Additionally, phosphorylated Raf-1 was co-immunoprecipitated with an anti-nitrotyrosine antibody from lysates of ONOO\textsuperscript{−}-treated cells. Collectively, these observations indicate that nitration of Raf-1 and MEK-1 causes autophosphorylation.

In our experiments, it was essential to test the possibility that H\textsubscript{2}O\textsubscript{2} was contributing to the activation of ERK since our preparations of ONOO\textsuperscript{−} were synthesized from acidified nitrite and H\textsubscript{2}O\textsubscript{2}. Thus, these stocks of ONOO\textsuperscript{−} could contain trace amounts of H\textsubscript{2}O\textsubscript{2}. Moreover, a recent study by Kirsch and de Groot (33) demonstrated that H\textsubscript{2}O\textsubscript{2} can be formed by the reaction of ONOO\textsuperscript{−} with nicotinamide nucleotides. We excluded the possible contribution of H\textsubscript{2}O\textsubscript{2} by demonstrating that ONOO\textsuperscript{−}-induced phosphorylation of ERK required the immediate addition of ONOO\textsuperscript{−} to cell cultures due to the rapid degradation of...
this oxidant at neutral pH (Fig. 1). Premixing the ONOO\(^-\) in PBS (pH 7.4) for 1 h resulted in a complete loss of ERK-phosphorylating activity. In contrast, this premixing delay did not diminish the activity of H\(_2\)O\(_2\) in activating ERK. However, to definitively exclude the possibility that trace levels of H\(_2\)O\(_2\) present in the preparations of ONOO\(^-\) were mediating activation of ERK, catalase was used to eliminate H\(_2\)O\(_2\) as has been reported previously (10, 11). Pretreatment of cells with catalase abolished activation of ERK by H\(_2\)O\(_2\), but did not inhibit ERK activation by ONOO\(^-\) (Fig. 3). These data confirmed that activation of ERK following treatment with ONOO\(^-\) was not due to the presence of H\(_2\)O\(_2\).

H\(_2\)O\(_2\)-induced ERK activation has been reported to require phosphorylation of EGFR (12, 13). For this reason, we investigated the role of EGFR in ONOO\(^-\)-induced ERK activation. Using a Western blot technique with an antibody specific for the phosphorylated form of EGFR, we showed that ONOO\(^-\), as well as EGF and H\(_2\)O\(_2\), strongly induced phosphorylation of EGFR within 5 min (Fig. 4). We then employed the EGFR-specific tyrphostin analog AG1478, which has been reported to

![Diagram](image-url)
Activation of MAPK by Peroxynitrite

Fig. 6. The MEK inhibitor PD98059 blocks ONOO−-and H2O2-induced ERK activation. Confluent quiescent cultures of rat pulmonary myofibroblasts were pretreated with 50 μM PD98059 or vehicle control (Me2SO) 1 h prior to treatment with 1 mM ONOO− or H2O2. After 15 or 30 min, cell lysates were collected for Western blot analysis. A, Western blot analysis of phospho-MEK-1 and MEK-1. B, representative Western blots of phospho-ERK and ERK. C, relative levels of ERK activation determined by densitometric scanning of the phospho-ERK bands and normalized to the ERK signal. PD98059 significantly inhibited ONOO−- and H2O2-induced ERK activation. Data are expressed as the means ± S.E. of three experiments. **, p < 0.01 (significant inhibition of ERK activation by PD98059 as compared with corresponding ONOO− or H2O2 treatments in the absence of PD98059).

Fig. 7. Western blot analysis showing phosphorylation and nitration of GST-MEK-1 fusion protein by ONOO− in a cell-free system. GST-MEK-1 was treated with ONOO− in PBS supplemented with the magnesium/ATP mixture and incubated for 15 min prior to stopping the reaction with SDS-polyacrylamide gel electrophoresis sample buffer. Active MEK-1 was loaded as a positive control. Western blot analysis was performed using an anti-phospho-MEK-1 antibody (upper panel), an anti-MEK-1 antibody raised against non-activated MEK-1 (middle panel), or an anti-nitrotyrosine antibody (lower panel).

block the tyrosine kinase activity of EGFR on pulmonary myofibroblasts (31). EGFR activation induced by ONOO− was completely blocked by a 1-h pretreatment with AG1478. Despite the complete block of ONOO−-induced EGFR phosphorylation by AG1478, ONOO−-induced ERK activation was not affected by AG1478. In contrast, H2O2-induced ERK activation was completely blocked by AG1478, indicating that phosphorylation of EGFR is required for further downstream signaling of MAPK by H2O2. These findings show that ONOO−, like H2O2, is capable of activating ERK via EGFR phosphorylation. However, unlike H2O2, ONOO− can also bypass blockade of EGFR and directly activate Raf and MEK to cause ERK phosphorylation.

Both H2O2 and ONOO− caused Raf-1 activation, and this was blocked by pretreatment of cells with forskolin (Fig. 5). H2O2-induced ERK activation was significantly inhibited by forskolin, yet activation of ERK by ONOO− was not affected by forskolin. These findings indicate that ONOO− can bypass the forskolin-mediated Raf-1 blockade and activate a signaling intermediate downstream of Raf-1. The most likely signaling intermediate that interfaces Raf-1 to ERK signaling is MEK (21). The MEK inhibitor almost completely inhibited phosphorylation of MEK and ERK by either ONOO− or H2O2 (Fig. 6). This observation suggests that ERK is not activated directly by ONOO−, but instead requires MEK.

Our findings demonstrate important differences in the mechanisms of H2O2- and ONOO−-induced signal transduction involving MAPKs. The effect of H2O2 on MAPK activation is dependent on the phosphorylation of EGFR, whereas ONOO− is capable of bypassing EGFR and Raf-1 blockades to activate MAPK. Although H2O2 can be a source of cellular stress generated from exogenous environmental insult, it is becoming increasingly clear that intracellular H2O2 functions as a signaling molecule in normal physiological processes to regulate the phosphorylation of receptor tyrosine kinases such as EGFR and the platelet-derived growth factor receptor (10, 11). The effect of H2O2 on EGFR activation to trigger a MAPK signaling cascade appears to be due to the quenching of protein-tyrosine phosphatase activity (11, 34). In contrast, ONOO− appears to activate EGFR via nitration of tyrosine residues, which results in receptor dimerization and autophosphorylation (15). We have also observed that ONOO− treatment of rat pulmonary myofibroblasts causes nitration and autophosphorylation of EGFR and the platelet-derived growth factor receptor.2

We provide evidence for the first time that ONOO− directly activates MEK (i.e. autophosphorylation) in a cell-free system that excluded other proteins (Fig. 7). H2O2 did not activate MEK-1 in the cell-free system. As has been shown for ONOO−-induced autophosphorylation of EGFR (15), it is likely that a nitration reaction is responsible for autophosphorylation of MEK-1 by ONOO−, and we showed that GST-MEK-1 was nitrated in a concentration-dependent manner that correlated with the amount of ONOO− that was required to induce autophosphorylation (Fig. 7). We excluded the possible contribution of phosphatase inhibition as a mechanism of ONOO−-induced

2 P. Zhang and J. C. Bonner, unpublished observation.
MEK-1 activation since the cell-free phosphorylation assay excluded phosphatases.

We addressed the biological relevance of ONOO- induced MAPK activation. Since mitogenesis is the paradigm of EGFR activation and ONOO- activated EGFR, we first investigated the effect of ONOO- on DNA synthesis using a [3H]thymidine incorporation assay. Although EGF was a potent stimulator of mitogenesis in rat lung myofibroblasts, ONOO- caused a concentration-dependent decrease in mitogenesis (Fig. 8), and this was due to a reduction in cell survival as determined by the MTT cytotoxicity assay (Fig. 9). Our finding that the MEK inhibitor PD98059 prevented ONOO- and H2O2-induced cytotoxicity was surprising since activation of the ERK pathway is necessary for proliferative and cell survival responses (35). Indeed, both EGF-induced DNA synthesis and ONOO- induced cytotoxicity in myofibroblasts were blocked by PD98059.

Thus, in the same cell type, MEK is critical for both proliferative and cytotoxic responses, depending on the stimulus. Other investigators have made observations similar to ours in other systems. Bhat and Zhang (36) reported that PD98059 protected oligodendrocytes from the cytotoxic effects of H2O2. Jimenez et al. (37) showed that asbestos- and H2O2-induced pleural mesothelial cell death was abrogated by the MEK inhibitor PD98059. The mechanism through which MEK or ERK mediates cytotoxicity in response to ONOO- or other oxidants is unclear. One possibility is that activation of ERK along with simultaneous activation of other kinases (e.g. p38 or JNK) could lead to a cell death response, whereas EGF does not activate these other kinases in myofibroblasts. It is known that oxidants, including H2O2 and ONOO-, activate all three families of MAPKs (12, 18). Thus, we suggest that activation of the ERK pathway by ONOO- or H2O2 may be required (but not sufficient) for a cytotoxic response.

In summary, we report that ONOO- independently activates components of the MAPK signaling cascade that involves EGFR, Raf-1, and MEK-1. Although H2O2-induced ERK activation was completely blocked at the level of EGFR phosphorylation, ONOO- could bypass blockade of either EGFR tyrosine kinase or Raf-1 kinase and cause ERK activation. ONOO- caused nitration and autophosphorylation of GST-MEK-1 fusion protein in a cell-free system, suggesting that formation of nitrotyrosine activates MEK-1. These findings indicate that the mechanism of MAPK activation by ONOO- is fundamentally different from that of H2O2-induced MAPK activation.

**Acknowledgments**—We thank Drs. Ron Mason and Thomas Eling (NIEHS) for helpful comments during the preparation of this manuscript. Special thanks are due to Dr. Ron Mason for invaluable input during the course of the study. We are grateful to Annette Rice and Benita Parker for isolation and maintenance of rat pulmonary myofibroblasts.

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