p38mapk and MEK1/2 inhibition contribute to cellular oxidant injury after hypoxia

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Powell, Charles S., Marcienne M. Wright, and Robert M. Jackson. p38mapk and MEK1/2 inhibition contribute to cellular oxidant injury after hypoxia. Am J Physiol Lung Cell Mol Physiol 286: L826–L833, 2004. First published December 12, 2003; 10.1152/ajplung.00119.2003.—Lung epithelial cells produce increased reactive oxygen species (ROS) after hypoxia exposure, and they are more susceptible after hypoxia to injury by agents that generate superoxide [O2•−; e.g., 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)]. Cellular GSH and MnSOD both decrease in hypoxic lung epithelial cells, altering the redox state. Because ROS participate in signaling pathways involved in cell death or survival, we tested the hypothesis that mitogen-activated protein kinases (MAPK) were involved in a protective response against cellular injury during reoxygenation. Human lung epithelial A549 cells were incubated in hypoxia (<1% O2; for 24 h) and then reoxygenated by return to air. p38mapk and MKK3 phosphorylation both decreased after hypoxia. During reoxygenation, cells were incubated with DMNQ (0–50 μM), a redox cycling quinone that produces O2•−. Hypoxia preexposure significantly increased epithelial cell lysis resulting from DMNQ. Addition of the p38mapk inhibitors SB-202190 or SB-203580 markedly increased cytotoxicity, as did the mitogen/extracellular signal-regulated kinase (MEK) 1/2 inhibitor PD-98059 (all 10 μM), suggesting a protective effect of downstream molecules activated by the kinases. Transfection of A549 cells with a dominant active MKK3 plasmid (MKK3[Glu]) partially inhibited cytosis resulting from DMNQ, whereas the inactive MKK3 plasmid (MKK3[Ala]) had less evident protective effects. Stress-related signaling pathways in epithelial cells are modulated by hypoxia and confer protection from reoxygenation, since hypoxia and chemical inhibition of p38mapk and MEK1/2 similarly increase cytosis resulting from O2•−.

Alveolar epithelium; hypoxia; reoxygenation; mitogen-activated protein kinase; mitogen/extracellular signal-regulated kinase

A number of protein kinase signaling pathways exist in lung epithelial and endothelial cells, where they play important roles in response to stress. Extracellular signal-regulated kinase (ERK), activated by the GTPase Ras, is involved in the kinase cascade leading to cell proliferation in response to growth factors, including EGF and PDGF (13). Activation of mitogen-activated protein kinases (MAPK) causes phosphorylation of nuclear transcription factors, resulting in stimulation of cell growth and proliferation (14, 41). c-Jun NH2-terminal kinase (JNK), a stress-activated protein kinase (SAPK), mediates signals in response to cytokines and environmental stresses such as ultraviolet radiation, osmotic shock, heat stress, and LPS (19). Hypoxia modulates both MAPK signaling and gene expression, and data in the literature strongly support a role for oxidant signaling in ischemia-reperfusion injury (3, 20, 31). We sought evidence that MAPK and mitogen/extracellular signal-regulated kinase (MEK) pathways are involved in protecting lung epithelial cells from oxidant injury during reoxygenation.

MAPK pathways are activated by a variety of tyrosine kinase receptor families or environmental stresses, including redox changes, some of which are transmitted through G protein-coupled receptors (13, 35, 37). MAPK kinase kinases (MAPKKks, including Raf1, MEK1, and MUK) activate dual specificity kinases (MAPKKPs, MKks, or MEKs) through serine and threonine phosphorylation (19). MAPKs are then activated by threonine and tyrosine phosphorylation by the MKks to stimulate various cellular responses. Activator protein-1 and nuclear factor (NF)-κB contain specific cysteine motifs that must be reduced to be active (8, 9). In some models of oxidant stress, JNK activation and p38mapk activation are associated with cell death by apoptosis. NF-κB activation, conversely, has been associated with cellular survival (17). Nuclear translocation of NF-κB can be activated by reactive oxygen species (ROS; and especially H2O2) and is associated with inflammation, proliferation, and survival (17, 32).

MAPK are involved in the cellular response to oxidant stress. A constitutively active form of Akt introduced in the lungs of mice by adenoviral gene transfer protects from hyperoxic pulmonary damage (23). Akt is a downstream effector of the phosphatidylinositol (PI)-3 kinase pathway, which itself has been implicated in hypoxia-induced protection of lung epithelial cells against hyperoxic injury (1). Lung reperfusion activates ERK, JNK, and p38mapk, which stimulate heme oxygenase-1 gene expression and lead to an apparently protective response (42, 43).

Transcription factors and signaling proteins may be activated or inactivated directly by reactive oxygen (ROS) and nitrogen (RNS) species or by a change in the redox couple GSSG-GSH (9). Small thiols and thiol-containing signaling proteins mediate many oxidant-induced effects (6). Such proteins contain cysteines that can be oxidized to a disulfide bond or to sulfenic acid (both of which can later be reduced) or sulfonic acid or sulfonic acid (neither of which can be reduced; see Ref. 7). Modification of thiols also can result in conformational changes that affect DNA binding or enzymatic activity. Thioredoxin reduces and activates transcription factors through the action of an enzyme, Ref1 (40).

Protein tyrosine phosphatases (PTP) can be inactivated by exogenous hydrogen peroxide (H2O2), but this appears cell-

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hypoxia, suggesting a functional role of the p38mapk pathway.

Our goals were 1) to find whether inhibition of p38mapk and MEK1/2 pathways influenced the extent of oxidant injury during reoxygenation, 2) to investigate whether hypoxia exposure affected expression of steady-state MAPK mRNA levels, 3) to find whether phosphorylation of MAPK, MKK, and JNK decreased in hypoxia, and 4) to determine whether repletion of MKK3 by transient transfection would reverse effects of hypoxia on oxidant injury.

We utilized a fully characterized cellular model of hypoxia-reoxygenation in which human A549 lung epithelial cells were incubated in hypoxia (<1% oxygen) for 0–24 h (22) and then exposed for 24 h in air to 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), a quinone that generates O$_2$ during redox cycling (28). Under these conditions, previously hypoxic A549 cells lysed much more extensively when exposed to DMNQ than cells incubated only in air. Cells were incubated with chemical inhibitors of MEK1/2 and p38mapk, and lactate dehydrogenase (LDH) release in response to DMNQ during reoxygenation was quantified. Cellular RNA was harvested for use in RNase protection assays, and protein was collected for Western blots after the 24-h hypoxia exposures. Steady-state MAPK mRNA levels did not change in hypoxia, whereas phosphorylation of both p38mapk and MKK3 decreased. Repletion of MKK3 by MKK3[Glut] transfection appeared to partially inhibit oxidant-induced cytolysis, suggesting a functional role for this kinase in the p38mapk pathway.

MATERIALS AND METHODS

Experimental design. The experimental design is summarized in Fig. 1. In these studies, we exposed human lung epithelial A549 cells to air (controls) or <1% oxygen (hypoxia) for 24 h. In some experiments, cells were incubated with chemical inhibitors of MEK1/2 [PD-98059 (2’-amino-3’-methoxystyryl)alcohol]; Calbiochem, San Diego, CA] and p38mapk MAPK [SB-203580 [4-(4-fluorophenyl)-2-(4-methylsulfonilphenyl)-5-(4-pyridyl)1H-imidazole] or SB-202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole]; both from Calbiochem]. Inhibitors were used at a concentration of 10 μM. The nominal intracellular IC$_{50}$ for each inhibitor is as follows: PD-98059 (2 μM); SB-203580 (600 nM), and SB-202190 (280–350 nM).

Cellular RNA was harvested for use in gene arrays and RNase protection assays, and protein was collected for Western blots after the 24-h hypoxia exposures. After hypoxia, cells were provided with fresh complete medium and then reoxygenated for 24 h by incubation in normoxia. Cytotoxicity resulting from DMNQ was assessed by measuring LDH release.

Cell cultures. A549 human papillary adenocarcinoma cells were obtained frozen at passage 78 from American Type Culture Collection (Manassas, VA). A549 cells are human peripheral airway epithelial cells that demonstrate polarity, microvilli on apical surfaces, and surfactant multilamellar bodies in the apical cytoplasm. The cells contain Clara cell and alveolar epithelial type II (ATII) cell granules, surfactant protein (SP)-A protein (~170 ng/mg protein), and SP-A and SP-B mRNAs (11). A549 cells express MnSOD protein that is regulated by both TNF-α and oxidants (39), exactly as in primary cultures of ATII cells. A549 cells share morphological and functional characteristics with lung epithelial cells, although they do not duplicate primary ATII cell cultures.

A549 cells were thawed and split 1:4 three times before use in experiments. Cells were cultured in DMEM-F-12 (50:50%) (800 Cellgro; MediaTech, Herndon, VA) with 10% FBS (Hyclone, Logan, UT) and 1% penicillin-streptomycin (MediaTech). Cells were grown nearly to confluence (>80% by visual estimate) in air-5% CO$_2$ and transferred by trypsinization to 100-mm-diameter culture dishes or 96-well plates (Falcon; Becton-Dickinson, Franklin Lakes, NJ).

Air and hypoxia exposures. When cells reached 80% confluence, they were incubated in air-5% CO$_2$ or 95% N$_2$-5% CO$_2$ (hypoxia) for 24 h. Cells were reoxygenated by return to air-5% CO$_2$. Hypoxia exposures were done in a Flexiglas enclosure (33 × 33 × 33 cm; PlasLabs, Lansing, MI) kept at 37°C in a tissue culture incubator (Fisher Isotemp model 230D). Periodic inflow of 95% N$_2$-5% CO$_2$ gas controlled by a proportional flow oxygen controller (Pro Ox 110; Biospheric, Redfield, NY) maintained hypoxia. The oxygen controller monitored the percentage of oxygen within the chamber and kept it at the set level of ±0.1%. Medium oxygen and CO$_2$ tensions and pH were measured with a clinical blood gas instrument. In some experiments, oxidant stress was imposed by addition of DMNQ or the vehicle (DMSO) in phenol red-free DMEM-F-12 (50:50%) containing 1% FBS during reoxygenation (28).

LDH assay. After experiments, 96-well plates were centrifuged at 250 g for 4 min. Control wells were lysed with detergent to measure maximum LDH release (100%). Cell culture supernatant (50 μl) was added to wells of an enzymatic assay plate (CytoTox96 NonRadioactive Cytotoxicity Assay; Promega, Madison, WI). The substrate mix (50 μl NAD$^+$ diaphorase, lactate, and a tetrazolium salt) was added and incubated for 30 min. Reaction was stopped by addition of 1 M acetic acid. Absorbance, which is proportional to LDH activity, was read in an ELISA reader (Bio-Rad 3550 Microplate Reader). LDH release was expressed as the percentage of total LDH release.
ysis was assessed by release of LDH (Cytotox96 assay) after 24 h reoxyegenation (22).

R\textit{N}a\textit{s}e protection assays. Target RNA was prepared from A549 cell total RNA using a Qiagen RNeasy kit (Qiagen, Valencia, CA). Integrity of the RNA (2 μg/lane) was tested on a 1% agarose formaldehyde mini gel run at 120 V.

DNA template sets [human (h) MAPK (catalog no. 559471) and hMKK (catalog no. 559953) from BD Biosciences Pharmingen] were used for T7-directed synthesis of antisense RNA probes. The hMAPK probes included ERK1 (430 nucleotides protected), ERK2 (388), ERK3 (349), ERK2 rel (313), ERK5 (280), p38 \textit{y} (250), p38 \textit{b} (223), p38b (199), p38 \textit{b} (178), L32 (112), and GAPDH (96). The hMKK probes included MEK1 (364 nucleotides protected), MEK2 (322), MEK3 (286), MEK4 (253), MEK5 (226), MEK6 (202), MEK7 (181), L32 (113), and GAPDH (96).

In vitro transcription was done using 1 μl Rnasin, 1 μl GACU mix, 2 μl DTT, 1 μl 5× transcription buffer, 1 μl MAPK or MEKK template, 10 μl \textit{[}\textit{32}P\textit{]}UTP, and 1 μl T7 RNA polymerase. The mixture was incubated at 37°C for 1 h and at 37°C for 30 min after addition of 2 μl DNase. Reaction templates were run using 26 μl EDTA, 25 μl Tris-saturated phenol (lower phase), 25 μl chloroform-isoamyl alcohol (24:1), and 4 μl yeast tRNA (to assist precipitation).

Labeled probes were hybridized to target RNA in 1× hybridization buffer in a dry bath at 90°C initially and then at 56°C overnight. R\textit{N}a\textit{s}e protection assays were incubated for 45 min at 30°C and then treated with proteinase K. Phenol-chloroform extraction was done using 65 μl Tris-saturated phenol (lower phase) and 65 μl chloroform-isoamyl alcohol. The aqueous layer was transferred to new tubes containing 120 μl of 4 M (NH\textsubscript{4})\textsubscript{2}OAc. RNA was precipitated with ethanol. For gel analysis, 5 μl of 1× gel-loading buffer were used to samples. Five percent PAGE was done, and autoradiography was used to visualize mRNAs of interest.

Each R\textit{N}a\textit{s}e protection assay was repeated on three separate occasions (i.e., 3 independent air or hypoxia exposures). Bands were quantified by densitometry, and the absolute and L32 normalized signals were compared and averaged to estimate message expression.

Western blots. Cells (~2 × 10\textsuperscript{7}) were harvested and washed with PBS. Cells were sonicated for 10 s and centrifuged at 750 g for 10 min at 4°C. Protein concentration of the supernatant was assayed using Bio-Rad reagent (Life Science Research, Hercules, CA).

Protein samples (50 μg) were diluted with 4× SDS sample loading buffer and denatured for 5 min at 100°C. Protein samples were loaded on 12% SDS-PAGE gels. Gels were run at 20 V/cm after the dye front had moved into the separating gel. Gels ran until bromphenol blue reached the bottom of resolving gels. Gels were then placed in the staining buffer for 30 min at room temperature.

Proteins were transferred electrophoretically from gels to polyvinylidene difluoride (PVDF) membranes for 1 h with cooling. Membranes were washed with TTBS (10 mM Tris-HCl/150 mM NaCl/0.05% Tween 20, pH 7.4). PVDF membranes were blocked with 5% nonfat milk for 60 min. Membranes were washed four times with TTBS for 5 min each time. PVDF membranes were incubated with serum containing rabbit polyclonal IgG (1:2,000 dilution) for 1 h at room temperature [p38[H-147], 7c-7149; MEK-3[l-20], sc-960; p-MEK-3/6(B-9), sc-8407; or p-ERK, sc7383; all from Santa Cruz Biotechnology (Santa Cruz, CA); anti-c-Jun from Upstate Biotechnology (Lake Placid, NY); anti-p38\textit{mapk} phosphospecific from Calbiochem]. PVDF membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:2,500) for 1 h at room temperature. Membranes were soaked in chemiluminescent substrate solution (KPL; Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 60 s and exposed to photographic film.

Each Western blot was repeated on three separate occasions (3 independent air or hypoxia exposures). Bands were quantified by densitometry, and the absolute and β-actin normalized signals were compared and averaged to estimate phosphoprotein expression.

RESULTS

\textit{Cytotoxicity and inhibitor studies.} Lung epithelial cells were incubated in air or hypoxia for 24 h with MAPK and MEK inhibitors at 10 μM. LDH release was assayed in 96-well plates after exposure to 0 to 50 μM DMNQ for 24 h. Hypoxia preexposure significantly increased cell lysis by DMNQ. Addition of the p38\textit{mapk} and MEK1/2 inhibitors (SB-203580, SB-202190, or PD-98059) at 10 μM markedly increased cell lysis by DMNQ, especially in cells preexposed to hypoxia. Therefore, inhibition of p38\textit{mapk} and MEK1/2 was assessed with Western blots using the rabbit polyclonal IgG antibody to MKK3 described above (MEK-3[I-20], sc-960; Santa Cruz Biotechnology).

\textit{Data analysis.} Data were expressed and shown as arithmetic means ± SE, unless indicated. Multiple comparisons were by two-way or three-way ANOVA, as required by the design, followed by Bonferroni’s procedure for multiple comparisons. Statistical calculations were done with SigmaStat statistical software version 2.03 (Jandel Scientific Software, San Rafael, CA).

Inactive and constitutively active MKK3 transfections. Dominant active MKK3 [MKK3(Glu)] and inactive MKK3 [MKK3(Ala)] plasmid vectors were obtained from Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA; see Ref. 33). Flag-MKK3 was subcloned into pRc/RSV (Invitrogen, Carlsbad, CA) at \textit{Hind} III and Spe I restriction sites. Plasmid DNA was amplified in DH5α Escherichia coli and purified using QIAGEN EndoFree Plasmid Maxi kits (Qiagen).

The day before transfection, 2 × 10\textsuperscript{4} cells/well were seeded in 100 μl complete medium. Cells were transfected with the empty control vector pcDNA3.0 (Invitrogen) to determine the effects of transfection per se. Cells were incubated at 37°C in a CO\textsubscript{2} incubator until 80% confluent. For each well, 0.2–0.4 μg DNA was diluted in 25 μl Opti-MEM I Reduced Serum Medium (Invitrogen) and mixed gently. Simultaneously, 0.5–5 μl lipofectamine (Invitrogen) were diluted in 25 μl Opti-MEM and mixed. Diluted DNA and diluted lipofectamine reagent were mixed and incubated at room temperature for 30 min to form DNA-liposome complexes.

While complexes were forming, medium was aspirated and cells were rinsed with Opti-MEM. After 30 min incubation, Opti-MEM was added to the complex solution, and diluted complex solution was layered on seeded cells. Cells were incubated with complexes for 5 h at 37°C. After incubation, 0.05 ml growth medium containing 10% FBS was added to each well. Complete medium was replaced 24 h after the start of transfection before incubation in air or hypoxia. After 24 h of hypoxia or air exposures, DMNQ was added to cells at concentrations of 0, 50, 100, and 250 μM to provide oxidant stress. Cells were incubated for an additional 24 h at 37°C in air. Cytotoxicity assays were done after the 24-h incubation using the CytoTox96 NonRadioactive Cytotoxicity Assay (Promega). Transfection efficiency and expression were confirmed with Western blots using the rabbit polyclonal IgG antibody to MKK3 described above (MEK-3[I-20], sc-960; Santa Cruz Biotechnology).

Data analysis. Data are expressed and shown as arithmetic means ± SE, unless indicated. Multiple comparisons were by two-way or three-way ANOVA, as required by the design, followed by Bonferroni’s procedure for multiple comparisons. Statistical calculations were done with SigmaStat statistical software version 2.03 (Jandel Scientific Software, San Rafael, CA).
Fig. 2. p38\textsuperscript{mapk} and MEK1/2 inhibitors increase DMNQ cytotoxicity after hypoxia. A549 cells were incubated in air or hypoxia for 24 h with p38\textsuperscript{mapk} (SB-202 or SB-203) or MEK1/2 (PD-980) inhibitors at 10 µM. LDH release in response to oxidant stress during reoxygenation was assayed in 96-well plates after exposure to 0–50 µM DMNQ. Hypoxia preexposure significantly increased cytolysis by DMNQ. p38\textsuperscript{mapk} or MEK1/2 inhibition further increased cell lysis by DMNQ, especially in cells preexposed to hypoxia. Hypoxia caused greater cell lysis in 25 and 50 µM DMNQ concentration groups compared with corresponding air-exposed cells \(P < 0.001\) by simple main effects tests (3-way ANOVA). *\(P < 0.05\) compared with the respective air-exposed groups (2-way ANOVA); **\(P < 0.05\) compared with vehicle-treated cells at the same DMNQ concentration (2-way ANOVA); \(n = 12\) for each inhibitor dose in each condition. At each DMNQ concentration, bars (left to right) are vehicle air, SB-202 air, SB-203 air, PD-980 air, vehicle hypoxia, SB-202 hypoxia, SB-203 hypoxia, PD-980 hypoxia.

cells. However, the extent of p38 phosphorylation was on average significantly less after hypoxia (relative to native p38\textsuperscript{mapk} protein), demonstrating decreased activation of the p38\textsuperscript{mapk}. MKK3 protein did not change after 24 h of hypoxia. MKK3 was significantly less phosphorylated after hypoxia (relative to native MKK3 protein), indicating inhibition of activation of MKK3. These data are summarized in Fig. 3.

**MKK3 dominant active and inactive transfection studies.** Lipid-mediated transfection itself decreased the degree of cytolysis resulting from DMNQ, although the effect depended on the DMNQ concentration (compared with the data in Fig. 2). Therefore, transfection per se, possibly because of changes in lipid composition of cell membranes, mitigates oxidant injury resulting from DMNQ and likely results in underestimation of the effects of the dominant negative MKK3[Ala] on cellular injury. Western blots confirmed that dominant active and dominant negative transfected cells expressed protein reactive with the polyclonal MKK3 antibody (data not shown). Both MKK3[Glu] and MKK3[Ala] themselves have a protective effect compared with pcDNA3.0, possibly because of misdirected overexpression of the MKK3 proteins.

Despite the above considerations, the degree of injury is detectably and significantly higher with the inactive MKK3[Ala] vector. Thus it is clear that the dominant active vector, MKK3[Glu], partially inhibited cytolysis resulting from DMNQ compared with the inactive vector, MKK3[Ala], over a range of DMNQ concentrations. MKK3[Glu] transfection significantly \(P < 0.001\) inhibited cytolysis (compared with MKK3[Ala]) in air control A549 cells at 50, 100, and 250 µM DMNQ concentrations. MKK3[Glu] also significantly \(P < 0.001\) inhibited cytolysis (compared with MKK3[Ala]) of 24 h hypoxia-preexposed cells at 100 and 250 µM DMNQ concentrations.

These data are summarized in Fig. 4.

**DISCUSSION**

**Summary of key findings.** We have reported enhanced vulnerability of previously hypoxic lung epithelial cells to exogenous oxidant stresses (22). Data resulting from new experiments herein reveal that 1) inhibition of p38\textsuperscript{mapk} and MEK1/2 intensifies posthypoxic cytolysis resulting from oxidant stress (DMNQ), 2) steady-state mRNA levels for specific MAPKs and MEKs do not change in hypoxia, 3) phosphorylation of p38\textsuperscript{mapk} and MKK3 consistently decreases (relative to the native protein) in hypoxia, and 4) transfection with a dominant

| Table 1. Expression of MAPK and MEK mRNAs after hypoxia |
|-----------------|-----------------|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        | \(n=4\)        |
| ERK1            | ERK2            | ERK3            | p38-\(\gamma\) | p38-\(\beta\) | p38-\(\delta\) | MEK1           | MEK2           | MEK3           | MEK4           | MEK5           | MEK6           | MEK7           |
| 0.85            | 0.88            | 0.86            | 0.81           | 0.76           | 0.75           | 0.99           | 0.95           | 0.92           | 0.86           | 0.84           | 0.86           | 0.94           | 0.93           |
| ±0.04           | ±0.03           | ±0.03           | ±0.03          | ±0.04          | ±0.04          | ±0.15          | ±0.12          | ±0.12          | ±0.08          | ±0.09          | ±0.09          | ±0.10          |
| 0.88            | 0.91            | 0.87            | 0.83           | 0.78           | 0.77           | 1.07           | 1.00           | 1.04           | 1.00           | 0.97           | 0.99           | 1.02           | 1.03           |
| ±0.01           | ±0.03           | ±0.02           | ±0.03          | ±0.07          | ±0.07          | ±0.10          | ±0.08          | ±0.10          | ±0.09          | ±0.06          | ±0.08          | ±0.07          | ±0.11          |

Data are means ± SE of densitometer readings in arbitrary units compared with the densitometer reading for L32 in the same lane; \(n\), no. of animals. See text for description of data analysis. ERK, extracellular signal-regulated kinase; MEK, mitogen/extracellular signal-regulated kinase. No differences in mRNA expression resulting from hypoxia were detected by 2-way ANOVA.
active MKK3 vector partially ameliorates DMNQ-induced oxidant injury.

Hypoxia, thus, clearly modulates MAPK signaling, which modulates oxidant injury. Data in the literature also strongly support a role for oxidant signaling in ischemia-reperfusion injury (14, 20, 31). Our results provide further, novel evidence that MAPK and MEK pathways are involved in protecting lung epithelial cells from oxidant injury during reoxygenation because MAPK and MEK inhibition significantly increased DMNQ-induced cytolysis. Hypoxia also appears to decrease protein kinase activation, which then exacerbates reoxygenation injury. We did not compare the degree of phosphorylation after 24 h reoxygenation in normoxia with that of air controls, so it is possible that reoxygenation could foster MAPK phosphorylation in general and minimize reoxygenation injury.

In these studies, exposure to normoxia after 24 h hypoxia did not cause release of LDH from A549 cells. That is consistent with previous observations (21) and is not the result of insensitivity of the LDH assay. We previously sought apoptosis in both A549 and H441 cells using the same hypoxia-reoxygenation regimen. No increased annexin V staining or DNA laddering occurred after hypoxia-reoxygenation. Both calcein AM/ethidium bromide and propidium iodide staining failed to reveal any increase in necrotic cells in the absence of an exogenous oxidant stress, and cellular ATP content did not decrease significantly. Therefore, hypoxia (as opposed to anoxia) does not cause epithelial cell death during reoxygenation. We have not tested hyperoxia during reoxygenation, which presumably would increase mitochondrial O2 production and exacerbate oxidant stress.

DMNQ, a redox cycling quinone, was used as a model of exogenous oxidant stress, as during inflammation. DMNQ is not accumulated in cells, as it is reduced and reoxidized but not otherwise metabolized (28). Micromolar concentrations of DMNQ such as those used here are quite low compared, e.g., with paraquat, which accumulates in cells by active transport. Because cells vary in their antioxidant defenses, toxic concentrations of DMNQ vary. The quantity of O2 and H2O2 produced by DMNQ is buffered by proteins and antioxidants present in tissue culture medium.

Implication of the p38 mapk pathway. Because the MAPKs and MEKs are demonstrated to play a role in protecting cells from oxidant stress, kinases or transcription factors downstream in the signaling cascade are possibly involved in the protective response. Inhibition of p38 mapk and MEK1/2 using SB-203580, SB-202190, or PD-98059 closely mimicked effects of hypoxia. The p38 mapk pathway is involved in adaptation to hypoxia and in protective responses against oxidant stress induced by carbon monoxide (43). The p38 mapk signal

Fig. 3. p38 phosphorylation decreases in hypoxia. Total MKK3 protein content did not change (bottom left). MKK3 appeared less strongly phosphorylated after hypoxia (top left), indicating inhibition of activation. p38 is detectable in both air and hypoxic cells (bottom right). The extent of phosphorylation is consistently less in hypoxia, demonstrating decreased activation after 24-h hypoxia exposure (top right). Densitometer readings (n = 3) for the nonphosphorylated proteins were as follows: MKK3 air 194 ± 8; MKK3 hypoxia 196 ± 8; p38 air 145 ± 22; p38 hypoxia 143 ± 20. *P < 0.05 compared with the nonphosphorylated kinase protein. A, air; H, hypoxia.

Fig. 4. Dominant active MKK3(Glu) transfection partially inhibits cytolysis resulting from DMNQ. The dominant active vector, MKK3(Glu), partially inhibited cytolysis resulting from DMNQ compared with the dominant negative vector, MKK3(Ala), over a range of DMNQ concentrations from 50 to 250 μM. MKK3(Glu) transfection significantly (P < 0.001) inhibited cytolysis in air control A549 cells at 50, 100, and 250 μM DMNQ concentrations. MKK3(Glu) significantly (P < 0.001) inhibited cytolysis of 24 h hypoxia-preexposed cells at 100 and 250 μM DMNQ concentrations. *P < 0.001 compared with MKK3(Glu) transfected cells in the same exposure group by 2-way ANOVA and Bonferroni’s procedure. ‡P < 0.05 compared with MKK3(Ala) transfected cells in the same exposure group. †P < 0.05 compared with MKK3(Glu) transfected cells in the same exposure group. air-G, air control A549 cells; hypox-G, hypoxia-preexposed A549 cells; air-A, air control, MKK3(Ala) transfected cells; air-C, air control, pcDNA 3.0 transfected cells; hypox-A, hypoxia-preexposed, MKK3(Ala) transfected cells; hypox-C, hypoxia-preexposed, pcDNA 3.0 transfected cells; n = 18 for each vector in each condition (results of 3 experiments pooled).
transduction pathway causes a selective increase in immediate early gene expression. Transcription products regulated by the p38mapk pathway, such as activating transcription factor 2 (ATF-2) and Ets-like protein 1 (Elk-1), conceivably could be involved in the protective response (34, 36). Inhibition of upstream kinases (p38mapk and MKK3) by hypoxia could decrease ATF-2 and Elk-1 activation and possibly limit c-fos transcription (19). p38mapk phosphorylates ATF-2 on threonines-69 and -71 and causes increased transcriptional activation. ERK-mediated phosphorylation of a Ser/Thr cluster at the carboxy terminus activates Elk-1. Activated Elk-1 binds to the cAMP response element (34). cAMP also has a protective effect in ischemia-reperfusion lung injury models (4). The JNK signal transduction pathway also activates ATF-2, but JNK is not implicated in our model.

**ROS and signaling in hypoxia-reoxygenation.** ROS and RNS are involved in a number of intracellular signaling events during ischemia-reperfusion. ROS interact with a number of molecular targets in reoxygenated cells, including pathways leading to activation of MAPKs that mediate proliferation. SAPKs implicated in apoptosis, the NF-kB pathway, and several caspases (14, 15, 29, 35). T cell receptor cross-linking by antigen causes rapid production of both O2•− and H2O2 (5). ROS or RNS produced during reoxygenation can themselves activate the SAPKs, including JNK and p38mapk (8, 9). Induction of c-jun/c-fos (activator protein-1) expression during hypoxia occurs before poly(ADP) ribose polymerase activation and histone H1 ADP-ribosylation (2), which contribute to apoptotic cell death (26). ERK1/2 signaling may be involved in both inhibition and induction of apoptosis. For example, the GTPases p21 Harvey-Ras (Ha-Ras) and Kirsten-Ras (Ki-Ras), respectively, decrease or increase tolerance to oxidant stress (H2O2; see Ref. 3). Likewise, the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1 regulate the restoration of DNA synthesis after hypoxic mouse embryo fibroblasts are returned to normoxia (12).

In rat pulmonary artery cells, hypoxia appears to activate JNK, and activation of p38mapk and ERK are associated with hypoxia-induced vascular remodeling (16). Inhibition of H2O2 production in reperfused rat kidneys prevented JNK phosphorylation and promoted ERK activation (18). Reoxygenation-induced apoptosis of human kidney cells can be inhibited by an antisense oligonucleotide targeted to JNK-1 (10). However, in our model, c-Jun phosphorylation does not occur, and the mode of cell death is predominately necrotic (22), rather than apoptotic. Decreased MKK and MAPK phosphorylation in hypoxia in our model may account for the observed differences.

The PI-3 kinase pathway activates protein kinase (PK) C, which activates MEKs (25). Hypoxic PKC and PI-3 kinase inhibition could conceivably block phosphorylation of MAPK. In a model of chemical hypoxia of human endothelial cells, chelating Ca2+, and PKG, PKC or p38mapk inhibition blocked increased permeability increases (30).

Although our results show a clear downregulation of MAPK and MEK phosphorylation by hypoxia, oxidative stress has the potential to activate kinases through inhibition of PTPs. Redox signaling often occurs through reversible inhibition of PTPs. The active site of PTP is susceptible to H2O2 that oxidizes the thiolate to sulfenic acid, which inhibits phosphatase activity. The sulfenic acid intermediate rapidly reacts with GSH to produce a mixed disulfide that is also inactive; however, a second molecule of GSH can then react with the mixed disulfide to yield GSSG and the original active PTP (9).

**Mechanisms accounting for increased oxidant stress.** Decreased activation of MKK3 is important because this kinase is upstream from p38mapk and ATF-2, both of which are implicated in cell survival (19, 41). Decreased MKK3 phosphorylation could be the result of decreased activity or expression of MKKK-1–3, its upstream activator. MKK3 is a specific activator of p38mapk; it does not activate JNK and ERK signaling pathways (33). The p38mapk signaling cascade is involved in the response to oxidant stress. Cytokines and environmental stresses normally strongly activate p38mapk. Inhibition of p38mapk is linked to decreased glutathione content and increased cell death during oxidant stress. Therefore, hypoxia-induced loss of p38mapk activation may be another mechanism to explain decreased GSH and increased oxidant stress observed in our model.

p38mapk also phosphorylates and activates heat shock protein (hsp) 27. The hsp27 regulates cellular GSH levels, which depend on the state of hsp27 oligomerization (24). Hsp27 also interferes with the mitochondrial pathway of caspase-dependant cell death by preventing cytochrome-mediated interaction of apa1 with procaspase-9.

**MKK3 transfections.** Inhibition of MEK1/2 results in significant neuroprotection from brain reperfusion (38). The dominant interfering mutant of MKK3 has been shown to inhibit posthypoxic neuronal cell death, as have SB-203580 and SB-202190 (44). These observations clearly implicate the p38mapk pathway in neuronal cell death after hypoxic-ischemic insults. Transient transfection with dominant active (MKK3(3Glut)) vector that expresses constitutively active MKK3 partially inhibits epithelial cytology (compared with the inactive vector) resulting from DMNQ. We used higher concentrations of DMNQ, because transfection with lipofectamine itself appeared to protect from oxidant stress, possibly by changing lipid composition of the cell membrane. Despite this, the protective effect of MKK3(3Glut), the dominant active vector, was statistically significant compared with the dominant negative vector (33). These results are consistent with the notion that the hypoxia-induced decrease in MKK3 expression we observed is physiologically important. Transfection with the dominant active vector could conceivably enhance activation of downstream kinases or nuclear transcription factors otherwise inactivated by hypoxia.

In these studies, we demonstrate involvement of MAPK and MEK pathways in protection of lung epithelial cells from oxidant injury. Such findings are consistent with observations in rat pulmonary artery endothelial cells, in which CO increases p38mapk activation during anoxia-reoxygenation; activation of the p38mapk appears to have an antiapoptotic effect on caspase-3. CO appears to enhance p38mapk activation in anoxia-reoxygenation and protects lung endothelial cells. A p38mapk dominant negative vector and SB-203580 inhibit the protective effect of CO (44).

These experiments are physiologically and clinically relevant because ischemia-reperfusion lung injury is a major cause of morbidity and mortality after lung transplantation (4). The syndrome is characterized by alveolar injury, edema, and hypoxemia. It can cause primary graft failure and may be associated with long-term graft dysfunction. Oxidative stress is
a key component of ischemia-reperfusion (anoxia-reoxygenation) lung injury (4).

Our data provide a novel mechanistic explanation by which lung tissue hypoxia, as occurs after lung harvest, leads to inactivation of key MAPKs and MEKs that protect cells from oxidant stress. Strategies to prevent MAPK and MEK inactivation (e.g., PTP inhibition with vanadate), or to replete specific MAPKs and MEKs (e.g., graft transfection with MKK3(Glu)), could enhance tissue preservation and improve outcome of lung transplants.

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