Macrophages Survive Hyperoxia via Prolonged ERK Activation Due to Phosphatase Down-regulation*

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Macrophages exposed to hyperoxia in the lung continue to survive for prolonged periods. We previously reported (Nyunoya, T., Powers, L. S., Yarovinsky, T. O., Butler, N. S., Monick, M. M., and Hunninghake, G. W. (2003) J. Biol. Chem. 278, 36099–36106) that hyperoxia induces cell cycle arrest and sustained extracellular signal-related kinase (ERK) activity in macrophages. In this study, we determined the mechanisms of hyperoxia-induced ERK activation and how ERK activity plays a pro-survival role in hyperoxia-exposed cells. Inhibition of ERK activity decreased survival of hyperoxia-exposed macrophages. This was due, at least in part, to down-regulation of the pro-apoptotic Bcl-2 family member, BimEL. In determining the mechanism of ERK activation by hyperoxia, we found that ERK activation was not associated with hyperoxia-induced activation of the upstream ERK kinase mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2. When we examined the ability of whole cell lysates from hyperoxia-exposed cells to dephosphorylate purified phosphorylated ERK, we found decreased ERK-directed phosphatase activity. Two particular ERK-directed phosphatases (protein phosphatase 2A and MAPK phosphatase-3) demonstrated decreased activity in hyperoxia-exposed cells. Moreover, whole cell lysates from normoxia-exposed cells depleted of PP2A or MAPK phosphatase-3 were also less able to dephosphorylate ERK. These data demonstrate that, in hyperoxia-exposed macrophages, sustained activation of ERK due to phosphatase down-regulation permits macrophage survival via effects on the balance between pro- and anti-apoptotic Bcl-2 family proteins.

Supplemental oxygen therapy is indispensable for patients with respiratory failure, even though high concentrations of inspired oxygen can cause tracheobronchitis, acute lung injury, and subsequent pulmonary fibrosis. Hyperoxia can induce cellular damage, in part, through increased production of reactive oxygen species (1). The effect of hyperoxia has been extensively evaluated in epithelial cells (2–7) and endothelial cells (8, 9); however, little is known about the effect of hyperoxia on macrophages. Macrophages in the lung are at ~13% oxygen under normal conditions. Under conditions of supplemental oxygen exposure (acutely ill patients), macrophages can be exposed to up to 90% oxygen. It is known that macrophages exposed to one type of oxidant stress, cigarette smoke, survive for prolonged periods of time in the lung (10). The prolonged life of the macrophages of smokers compared with our previous observations on the cell cycle effects of hyperoxia (hyperoxia induces macrophage cell cycle arrest accompanied by induction of p21CIP1 and activation of the retinoblastoma protein (11)) suggests the activation of one or more survival pathways by hyperoxia. Macrophages, in vitro, survive hyperoxia for a prolonged period of time, and this is associated with sustained activation of extracellular signal-regulated kinases (ERKs) (11). In this study, we pursued the hypothesis that hyperoxia-linked down-regulation of ERK-directed phosphatases leads to prolonged ERK activation and increased ERK-dependent survival.

ERK can be activated via stimulation of receptor tyrosine kinases, G-protein-coupled receptors, and integrin receptors (12). ERK can also be activated in response to redox changes in the cell through still unidentified mechanisms (13). ERK is activated by dual phosphorylation of tyrosine and threonine residues by mitogen-activated protein kinase kinase 1/2 (MEK1/2). The duration of ERK phosphorylation affects the biological responses in cells. Short duration of ERK activation (as seen with lipopolysaccharide (LPS)) does not alter the cell cycle, whereas prolonged ERK activation regulates cell proliferation (14).

A potential mechanism to induce sustained activation of ERK is a decrease in phosphatase activity, because ERK is inactivated by phosphatases. The specific removal of phosphate from either the tyrosine or the threonine is enough to eliminate or decrease enzyme activity (15, 16). There are several types of known phosphatases that regulate ERK, including serine/threonine phosphatases, such as PP2A (17), dual-specificity phosphatases, such as mitogen-activated protein kinase phosphatases (MKPs) (18, 19), protein/tyrosine phosphatases (PTPs), such as streptomycin-enriched phosphatase (STEP) (20), and hematopoietic PTP (HePTP) (21). Under most conditions, ERK activation is brief, because PP2A, a constitutively expressed

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‡ The abbreviations used are: ERK, extracellular signal-regulated kinase; BH, Bcl-2 homology; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; MEK, MAPK kinase; PP2A, protein phosphatase 2A; PTP, protein/tyrosine phosphatase; HePTP, hematopoietic PTP; STEP, streptomycin-enriched phosphatase.
phosphatase is already present in the complex at base line and rapidly switches off ERK activity by removing a phosphate from the threonine residue (17). Another means of ERK inactivation occurs via induction and activation of one or more MKP's leading to dual dephosphorylation of ERK (22). MKP's have unique specificities toward their MAPK substrates and distinct subcellular localizations. MKP-1 is located in the nucleus and preferentially inactivates p38 MAPK, c-Jun N-terminal kinase, and ERK (in that order) (23). In contrast, MKP-3 is located in the cytoplasm and preferentially dephosphorylates ERK's (19, 24, 25).

Both MKP's and PTP's have catalytic site cysteines that, when oxidized, inactivate the phosphatases (26–28). The serine/threonine phosphatase PP2A has also been shown to be negatively regulated by redox modulation of a cysteine (29, 30).

Because of this known redox-linked negative regulation of phosphatases, we hypothesized that hyperoxia induces prolonged ERK activation via inactivation of one or more phosphatases involved in ERK regulation.

Bcl-2 family members are essential regulators of apoptosis. The Bcl-2 family consists of two classes: anti-apoptotic members, such as Bcl-2 and Bcl-xL that protect cells from apoptosis, and pro-apoptotic members that trigger or sensitize for apoptosis. More than 20 pro-apoptotic Bcl-2 family proteins have been identified in mammals and can be divided into two subgroups by the number of Bcl-2 homology (BH) domains that they contain (31). Pro-apoptotic Bcl-2 family members include Bax and Bak with 2–3 BH domains and other proteins identified as BH3-only proteins, including Bim and Bad. The BH3-only proteins are crucial for initiation of apoptosis. In contrast, Bax/Bak-like proteins are further downstream effectors in the cell death program. The BH-3-only protein Bim is expressed in various cells, including macrophages, and is strictly regulated at both the transcriptional and post-transcriptional level (31). Bim is a major apoptotic signal following withdrawal of survival factors. Bim−/− mice show a marked accumulation of monocytes, granulocytes, and lymphocytes (32). Moreover, Bim−/− lymphocytes are resistant to cytokine withdrawal in culture (33). ERK can bind and promote phosphorylation of Bim, targeting it for degradation via the proteosome (34). Luciano et al. (35) reports that ERK-dependent degradation of Bim protects leukemia cells from Imatinib-induced apoptosis (35). However, the role of Bim regulation in cell survival under hyperoxia has not been studied.

In this study, we showed that hyperoxia induces sustained ERK phosphorylation leading to a pro-survival change in the balance between pro- and anti-apoptotic Bcl-2 family members. The prolonged ERK activity is not associated with an increase in activity of upstream kinases. Rather, hyperoxia induces a sustained decrease in total ERK-directed phosphatase activity.

In vitro ERK Dephosphorylation Assay—In vitro ERK dephosphorylation in cell lysates was evaluated using a chemiluminescent substrate (and ECL or ECL Plus). An autoradiograph was obtained using the primary antibodies or anti-ERK antibodies. Immunoreactive bands were developed using a chemiluminescent detection system (ECL, ECL Plus). Anti-ERK antibodies were observed with exposure times of 10–20 s.

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Experimental Procedures

Reagents and Antibodies—Reagents and antibodies were obtained from Sigma, American Type Culture Collection, Santa Cruz Biotechnology, and Cell Signaling. Antibodies were obtained from various sources.

Cell Culture and Cell Viability Assay—RAW 264.7 cells (TIB-71, American Type Culture Collection) were maintained at 37 °C in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum and gentamicin (40 μg/ml). The cells were subcultured every 2–3 days. Experiments were performed in six-well (35 mm) Costar tissue culture plates or regular tissue culture dishes (100 mm) under normoxia (O2 = 21%) or hyperoxia (O2 = 95%) at the starting cell density of 5 × 104/ml. The percentage of viable cells in each group at 24 h was determined by the trypan blue assay.

Isolation of Whole Cell Extracts—Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40) supplemented with Complete protease inhibitors (Roche Applied Science) and 1× phosphatase inhibitors (Calbiochem, La Jolla, CA). The lysates were then sonicated for 20 s, centrifuged for 10 min at 4 °C, and then centrifuged at 4 °C for 15 min. Protein was quantified using a protein measurement kit (Protein Assay Kit 500–0006, Bio-Rad). Cell lysates were stored at −70 °C until use.

Western Blot Analysis—Western blot analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed on whole cell lysates. Protein (30–80 μg) was mixed 1:2 with 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue, and 1.25× M Tris, pH 6.8; all from Sigma) heated to 95 °C for 5 min, and fractionated on a 10 or 12.5% SDS-polyacrylamide gel run at 100 V for 90 min. Cell lysates were transferred to nitrocellulose membranes (Amersham Biosciences) by semidy transfer (Bio-Rad) at 20 V for 45 min. Equal loading of the protein groups on the blots was evaluated using the primary antibodies or anti-β actin or Ponceau S (Sigma). The membranes were blocked with Tris-buffered saline with 0.1% Tween 20 for 1 h, washed, and then incubated with the primary antibody overnight. The blots were washed four times with Tris-buffered saline with Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies. Immuneoreactive bands were developed using a chemiluminescent substrate (and ECL or ECL Plus). An autoradiograph was obtained using the exposure times of 10–20 s.
Eppendorf tubes in ice and were used as PP2A- or MKP-3-immunodepleted cell lysates. PP2A- or MKP-3-immunodepleted cell lysates (20 μg) were incubated with recombinant pERK (0.1 μg) at 37 °C for 20 min in phosphatase buffer and were boiled in 2× sample buffer for 5 min. The ERK-directed phosphatase activity in PP2A- or MKP-3-immunodepleted cell lysates was measured by Western blotting using anti-pERK antibodies.

Statistical Analysis—The results were expressed as the mean ± S.E. The number of cells in each condition at various time points was compared using a Student’s unpaired t test.

RESULTS

Macrophages Survive Hyperoxia via Sustained ERK Activation—Our initial studies showed that RAW 264.7 cells (macrophages) do not die for up to 48 h under hyperoxia. We found that hyperoxia did not alter the activity of protein kinase B (Akt), a transducer of multiple survival signals. In contrast, we found that hyperoxia induces sustained ERK activation (11). Previous studies have shown that ERK activity can play both a pro- (38–40) and an anti-apoptotic role (34, 35, 41), depending on the cell type and context. To directly evaluate the role of hyperoxia-induced ERK activation on cell survival in macrophages, we performed the following experiments. Macrophages were cultured in standard tissue culture plates, with or without U0126, a MEK inhibitor (the ERK upstream kinase, 10 μM), under conditions of normoxia (21% O₂) or hyperoxia (95% O₂ and 5% CO₂). Western blot analysis was performed for phosphorylation of ERK. Equal loading was determined by stripping the blot and reprobing with antibodies to total ERK2. Western blotting data are representative of three experiments.

Hyperoxia Down-regulates a Pro-apoptotic Bcl-2 Family Protein, BimEL, in an ERK-dependent Manner—The Bcl-2 family members are key regulators of cell survival and death. However, little is known about the effects of hyperoxia on the Bcl-2 family proteins in macrophages. To evaluate this, macrophages were cultured in conditions of normoxia (21% O₂) or hyperoxia (95% O₂ and 5% CO₂) for 24 h. Western blot analysis was performed for a total amount of BimEL. Equal loading was determined by stripping the blot and reprobing with antibodies to β-actin. Western blotting data are representative of three experiments.

![Fig. 1. Macrophages survive hyperoxia via sustained ERK activation. A, RAW264.7 cells were cultured under normoxia (21% O₂) with or without U0126 (10 nM) and hyperoxia (95% O₂ and 5% CO₂) at the starting cell density of 0.5 × 10⁶/ml. Western blot analysis was performed for phosphorylation of ERK. Equal loading was determined by stripping the blot and reprobing with antibodies to total ERK2. Western blotting data are representative of three experiments. B, RAW264.7 cells were treated as described for A. The cell viability assay was performed with trypan blue staining. Data are expressed as mean ± S.E. for three independent experiments. Normoxia without U0126 compared with hyperoxia with U0126 is significantly different, p < 0.05.](http://www.jbc.org/figure1)

![Fig. 2. Hyperoxia down-regulates a pro-apoptotic Bcl-2 family protein, BimEL, in an ERK-dependent manner. A, RAW264.7 cells were cultured in conditions of normoxia (21% O₂) or hyperoxia (95% O₂ and 5% CO₂). Western blot analysis was performed for the total amount of BimEL and Bcl-xL at 24 h. Equal loading was determined by stripping the blot and reprobing with antibodies to β-actin. Western blotting data are representative of three experiments. B, RAW264.7 cells were cultured with or without U0126 (10 nM) in conditions of normoxia (21% O₂) or hyperoxia (95% O₂ and 5% CO₂) for 24 h. Western blot analysis was performed for a total amount of BimEL. Equal loading was determined by stripping the blot and reprobing with antibodies to β-actin.](http://www.jbc.org/figure2)
O₂) or hyperoxia (95% O₂) in standard tissue culture plates. Western blot analysis was performed for total BimEL and phosphorylation of ERK at 24 h. Hyperoxia decreased BimEL protein levels, and the effect was dependent on ERK activity (Fig. 2B). These results suggest that hyperoxia down-regulates BimEL protein in an ERK-dependent manner. We next addressed the mechanism of extended ERK activation in hyperoxia-exposed cells.

Hyperoxia-induced ERK Activation Is Not Associated with Increased Activation of the Upstream ERK Kinase MEK1/2 but with Down-regulation of ERK-directed Phosphatases—ERK activity is regulated by the balance between upstream kinase activity and downstream phosphatase activity. We determined whether the increased activity of ERK induced by hyperoxia was due to activation of upstream kinases in the ERK pathway. We used LPS as a positive control for activation of upstream kinases. Macrophages were cultured under conditions of normoxia (21% O₂) or hyperoxia (95% O₂) for 24 h. Western blot analysis was performed to determine whether there was activation (phosphorylation) of MEK1/2. LPS induced phosphorylation of MEK1/2 at 15 min, but hyperoxia did not increase MEK activity for up to 24 h (Fig. 3A). This demonstrates that hyperoxia-induced ERK activation is not linked to increased activity of upstream kinases. Hyperoxia does not increase MEK activity, but there is still a potential role for the base-line MEK activity in ERK activation by hyperoxia. To address this question, macrophages were cultured under conditions of normoxia (21% O₂) or hyperoxia (95% O₂) with or without U0126 (10 μM), a MEK inhibitor. Western blot analysis was performed to determine whether there was base-line activation (phosphorylation) of ERK that could be inhibited by U0126. MEK inhibition blocked ERK phosphorylation in both normoxia and hyperoxia (Fig. 3B).

These data indicate that base-line MEK activity is necessary for hyperoxia-induced ERK activation. However, the lack of increased MEK activity with hyperoxia suggests an alternative mechanism for the increase in ERK activity. One possibility is that hyperoxia decreases the constitutive activity of the phosphatases involved in ERK regulation. Thus, we postulated that hyperoxia decreases dephosphorylation of ERK in exposed macrophages. The following experiments address this hypothesis.

To investigate the effect of hyperoxia on total macrophage phosphatase activity, an in vitro ERK dephosphorylation assay was performed. Macrophages were cultured in normoxia (21% O₂) or hyperoxia (95% O₂) for 24 h. Whole cell lysates were obtained and incubated with recombinant pERK protein at 37 °C for 20 min. Total phosphatase activity for pERK in conditions of normoxia and hyperoxia were measured by Western blot analysis using antibodies to pERK.

Hyperoxia Decreases PP2A and MKP-3 Activity for ERK Dephosphorylation—It is known that oxidant stimuli, such as hydrogen peroxide, decrease activity of some phosphatases, resulting in ERK activation (29, 42, 43). To identify the specific phosphatases responsible for hyperoxia-induced ERK activation, we performed the following experiments. Macrophages...
FIG. 4. Hyperoxia decreases PP2A and MKP-3 activity for ERK dephosphorylation. A, RAW264.7 cells were cultured in normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) for 24 h. Immunoprecipitation was performed by incubation of the whole cell lysates with antibodies for PP2A or MKP-3. Immunoprecipitated PP2A or MKP-3 was incubated with recombinant pERK in phosphatase buffer at 37 °C for 20 min. PP2A or MKP-3 activity for pERK was measured by Western blotting using antibodies to pERK. The amount of PP2A or MKP-3 was measured by stripping the blot and reprobing with antibodies to PP2A or MKP-3. These data are representative of three experiments. B and C, RAW264.7 cells were cultured in normoxia (21% O2). Immunoprecipitation was performed as described for A. PP2A- or MKP-3-immunodepleted supernatants were incubated with recombinant pERK in phosphatase buffer at 37 °C for 20 min. Each activity for pERK in PP2A- or MKP-3-immunodepleted lysates in conditions of normoxia was measured by Western blotting using antibodies to pERK. The amount of PP2A or MKP-3 was measured by stripping the blot and reprobing with antibodies to PP2A or MKP-3. These data are representative of three experiments. D, RAW264.7 cells were cultured in conditions of normoxia (21% O2) with or without LPS (1 µg/ml) or hyperoxia (95% O2 and 5% CO2). Western blot analysis was performed for the total amount of MKP-1, MKP-3, and PP2A in the whole cell lysates. Equal loading was determined by stripping the blot and reprobing with antibodies to β actin. Western blotting data are representative of three experiments.
were cultured in normoxia (21% O₂) or hyperoxia (95% O₂) for 24 h. Whole cell lysates were obtained and PP2A, MKP-3, STEP, and HePTP activity were immunoprecipitated and used in an in vitro ERK dephosphorylation assay. Macrophage exposure to hyperoxia decreased the ability of immunoprecipitated PP2A and MKP-3 to dephosphorylate ERK (Fig. 4A). However, hyperoxia did not decrease the ability of STEP or HePTP to dephosphorylate ERK (data not shown). These results suggest that a decrease in PP2A and MKP-3 activity contribute, at least in part, to the sustained ERK activation in hyperoxia-exposed macrophages.

We then evaluated whether PP2A and MKP-3 modulate base-line ERK activity in normoxia. For these studies, macrophages were cultured in normoxia (21% O₂) for 24 h. Whole cell lysates were obtained, and immunoprecipitation was performed using anti-PP2A or anti-MKP-3 antibodies. Instead of keeping the immunoprecipitated PP2A and MKP-3, the beads were discarded and the depleted lysates used in an in vitro ERK dephosphorylation assay. Both PP2A- and MKP-3-immune depleted lysates were less able to dephosphorylate ERK (Fig. 4, B and C). This demonstrates that PP2A and MKP-3 play a role in dampening ERK activity in normoxic macrophages.

One reason for the transient activation of ERK by LPS is that LPS-induced ERK leads to increased MKP-1 protein and autocrine dephosphorylation of ERK (44). To investigate possible MKP-1, PP2A, and MKP-3 total protein changes by hyperoxia, we cultured macrophages in normoxia (21% O₂), with or without LPS, or hyperoxia (95% O₂). Western blot analysis was performed for total MKP-1, MKP-3, and PP2A. LPS induced accumulation of MKP-1 at 60 min, but hyperoxia induced a trivial amount of MKP-1. Both LPS and hyperoxia slightly decreased the amount of MKP-3. The amount of PP2A protein did not change under conditions of hyperoxia for 24 h or with LPS (Fig. 4D). These data suggest that hyperoxia-induced ERK is not shut off by MKP protein amount changes and that hyperoxia-induced down-regulation of PP2A and MKP-3 activity is not due to loss of phosphatase proteins.

**Phosphatases Are Responsible for ERK Dephosphorylation**

Hyperoxia decreases ERK-directed phosphatase activity, including activity of PP2A and MKP-3. Our data demonstrates that hyperoxia decreases activity of both PP2A and MKP-3. We do not know that these are the only phosphatases involved in hyperoxia-regulated ERK activity. To investigate the effects of global phosphatase regulation of ERK activity in normoxic and hyperoxic macrophages, macrophages were cultured in normoxia (21% O₂) or hyperoxia (95% O₂) for 24 h. Whole cell lysates were obtained and an in vitro ERK dephosphorylation assay performed. Okadaic acid (3 μM), a serine/threonine phosphatase inhibitor (3 μM inhibits both PP2A and PP1 serine/threonine phosphatases), and/or sodium orthovanadate (20 mM), a PTP inhibitor, were added to the in vitro dephosphorylation assay. As before, ERK-directed phosphatase activity was measured by Western blot analysis using an anti-phospho-ERK antibody. As shown in Fig. 5, hyperoxia decreased the total phosphatase activity directed toward ERK. Inhibition of serine/threonine phosphatase or PTP significantly decreased the ability of whole cell lysates to dephosphorylate ERK in both normoxia and hyperoxia. (The PTP inhibitory effect is more significant than the serine/threonine phosphatase inhibitory effect.) Furthermore, there was an additional inhibitory effect of ERK-directed phosphatase activity with combinations of both inhibitors (Fig. 5). Thus, serine/threonine phosphatases, PTPs, and MKPs (MKPs are also inhibited by sodium orthovanadate) are likely to be involved in ERK dephosphorylation. These data are consistent with the view that hyperoxia decreases some phosphatases other than PP2A and MKP-3.

**DISCUSSION**

We have previously shown in macrophages that hyperoxia induces sustained activation of ERK. In this study, we showed that hyperoxia-induced activation of ERK is crucial for cell survival. In examining how hyperoxia is activating ERK, we found that increased ERK activity is not due to increased activation of the upstream kinases MEK1/2. Hyperoxia-induced ERK activation was associated with a decrease in ERK-
directed phosphatase activity, including PP2A and MKP-3. Hyperoxia-induced ERK activation was related to cell survival. Hyperoxia induced down-regulation of the pro-apoptotic BH3-only protein BimEL in an ERK-dependent manner. These data show that in macrophages, hyperoxia permits cell survival, in part, via sustained activation of ERK, resulting in down-regulation of BimEL. This information complements prior studies (43, 45), which have found that oxidant stimuli inhibit the activity of a variety of phosphatases, including those with the potential to regulate ERK activity.

Human alveolar macrophages proliferate at very low levels and survive for several months in normal lungs. Under some conditions of oxidant stress, like cigarette smoking, macrophages can live up to 2 years (10). This is accompanied by increased expression of Bcl-xL, an anti-apoptotic protein (46), which we also found was increased by hyperoxia.

The most proximal upstream kinase to ERK is MEK, which activates ERK by phosphorylating a key motif, TEY (threonine 202/tyrosine 204) (12). Inactivation of ERK occurs via dephosphorylation of these same key residues. A number of phosphatases have been linked to ERK inactivation, including the serine/threonine phosphatase PP2A, a number of tyrosine phosphatases, such as HePTP and STEP, and the family of dual specificity phosphatases, MKPs. Although it is known that PP2A is regulated by phosphorylation and methylation of the catalytic subunit, some studies indicate that PP2A may be regulated by redox modification of cysteine residues in the same way that PTPs are inactivated by oxidant modification of a cysteine in the catalytic domain (29, 30). Other studies, using brain, cultured T cells, and fibroblasts have shown that the oxidant stress hydrogen peroxide can increase ERK activity via a decrease in phosphatase activity, including PP2A and protein/tyrosine phosphatases (29, 42, 43). Inhibition of PP2A by okadaic acid, an inhibitor of protein serine/threonine phosphatase, can result in activation of ERK (47, 48). Our study showed that prolonged exposure of hyperoxia could trigger sustained ERK activation by a similar mechanism.

In contrast to rapid inactivation of ERK by PP2A, the delayed inactivation of ERK is mediated, at least in part, by MKPs, dual specificity phosphatases (22). Li et al. (49) reported that oxidant stimuli, such as arsenite- and hydrogen peroxide-induced MKP-1 is partially dependent on p38 MAPK. However, we found that hyperoxia does not increase the amount of MKP-1. Interestingly, we also found that hyperoxia does not activate p38 in contrast to hydrogen peroxide. The differential contribution and interaction of p38 and ERK by different oxidant stimuli might determine the amount of MKP-1 induction.

In contrast to MKP-1, little is known about the oxidant regulation of MKP-3. What is known about MKP-3 regulation is that ERK activation by serum promotes the degradation of MKP-3 through phosphorylation of its serine residues (50). In this study, hyperoxia did not significantly decrease the amount of MKP-3, and our data suggest that hyperoxia decreases the function of MKP-3, probably through oxidation of essential cysteine residues. We also evaluated other PTPs known to regulate ERK, such as STEP and HePTP, but hyperoxia did not change their amounts or functions. However, we cannot exclude the possibility that other phosphatases play a role in hyperoxia-induced ERK. Our data suggests that sustained ERK activity in hyperoxia-exposed cells is because of a down-regulation of two or more phosphatases (Fig. 6). There is baseline MEK activity in both normoxia/hypoxia (both of which for the purposes of this paper are defined as non-oxygen-supplemented levels of tissue PO2) and hyperoxia. In conditions of normoxia/hypoxia, ERK-directed phosphatases are active enough to dephosphorylate ERK. In contrast, hyperoxia-down regulates ERK-directed phosphatases, resulting in sustained and increased ERK activity.

Other potential candidates for a role in hyperoxia-induced cell survival include Akt and NFkB. In animal studies, extrinsic Akt expression prolongs the survival under hyperoxia (51). In epithelial cells, hyperoxia activates NFkB (52); however, this has not been evaluated in macrophages under conditions of hyperoxia. We evaluated these potential survival pathways in macrophages. Hyperoxia did not alter Akt phosphorylation and only transiently increased DNA binding activity of NFkB (data not shown). Thus, at least in macrophages, Akt and NFkB are unlikely to play a major role in cell survival under conditions of hyperoxia.

Members of the Bcl-2 family are essential regulators of apoptosis. The balance between pro- and anti-apoptotic family members is a determining factor in mitochondria-dependent apoptosis (53). Although reactive oxygen species accumulation is widely thought to be responsible for the cell death following exposure to hyperoxia, the mode of hyperoxia-induced cell death, cell necrosis, or apoptosis has been controversial. Several studies have evaluated the effects of hyperoxia on Bcl-2 family members and the role of Bcl-2 family proteins in cell survival under hyperoxia. Bucellato et al. (54) reported that hyperoxia-induced reactive oxygen species accumulation results in activation of Bax, a pro-apoptotic protein leading to cell death. The overexpression of Bcl-xL protected mouse lung epithelial cells under hyperoxia by antagonizing Bax. Budinger et al. (55) reported that murine embryonic fibroblasts from Bax(-/-) and Bak (-/-) mice are resistant to hyperoxia-induced cell death. Thus, the balance of pro-apoptotic and anti-apoptotic proteins of Bcl-2 family members may play an essential role in determining cell survival or death in hyperoxic conditions.

The cross-talk between ERK and BimEL, a pro-apoptotic protein, has been evaluated in other systems. In chronic myelogenous leukemia cells of the cell line K562, ERK activation induced specific phosphorylation of BimEL on serine 69, leading to degradation and subsequent cell survival (35). In our study, we found that hyperoxia decreases BimEL, a pro-apoptotic protein, in an ERK-dependent manner. This is consistent with an alteration in the balance between pro- and anti-apoptotic Bcl-2 family members by hyperoxia in favor of cell survival (we also found an increase in Bcl-xL, an anti-apoptotic protein, by hyperoxia). These data demonstrate that hyperoxia-induced ERK activation alters the balance of the Bcl-2 family proteins in macrophages in favor of anti-apoptosis by decreasing BimEL and increasing Bcl-xL.

As a composite, we showed that hyperoxia induces sustained ERK activation, which maintains macrophage cell viability. We also showed that hyperoxia decreases the pro-apoptotic protein

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**Fig. 6. A proposed mechanism for hyperoxia-induced ERK activation.** There is a base-line MEK activity regardless of concentrations of oxygen. In hypoxia (or non-lung tissue levels of oxygen), ERK-directed phosphatases are active enough to dephosphorylate ERK. In contrast, hyperoxia down-regulates ERK-directed phosphatases, resulting in sustained activation of ERK.
BimEL in an ERK-dependent manner. The mechanism of sustained ERK activation by hyperoxia appears to be down-regulation of phosphatase activity, including PP2A and MKP-3.

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