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Hyperoxia Stimulates an Nrf2-ARE Transcriptional Response via ROS-EGFR-PI3K-Akt/ERK MAP Kinase Signaling in Pulmonary Epithelial Cells

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ABSTRACT

Nuclear factor erythroid 2-related factor (Nrf2) confers protection against cell death induced by hyperoxia and other proapoptotic stimuli. Because phosphoinositide-3-kinase (PI3K)/Akt signaling promotes cell survival, the significance of this pathway in mediating reactive oxygen species (ROS)-dependent hyperoxia-induced Nrf2 activation was investigated in the murine pulmonary epithelial cell line, C10. Inhibition of the PI3K pathway markedly attenuated hyperoxia-induced Nrf2 translocation and ARE (antioxidant response element)-mediated transcription. Consistent with this, hyperoxia markedly stimulated the activation of PI3K pathway, while an NADPH oxidase inhibitor and an antioxidant prevented such activation. The inhibition of Akt activity using a pharmacological inhibitor markedly attenuated Nrf2 translocation and ARE-driven expression. Moreover, overexpression of a dominant-negative Akt mutant attenuated the transcription, whereas a constitutively active mutant stimulated it. These results suggest that PI3K/Akt signaling regulates Nrf2 activation by hyperoxia. Inhibition of the PI3K pathway prevented hyperoxia-stimulated Akt and ERK1/2 kinase activation, which is critical for Nrf2-mediated transcription. Likewise, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, AG1478, blocked hyperoxia-stimulated Akt and ERK1/2 phosphorylation, Nrf2 nuclear accumulation, and ARE-driven transcription. Consistent with this result, an NADPH oxidase inhibitor blocked hyperoxia-stimulated EGFR phosphorylation, which was correlated with the attenuation of Akt and ERK activation. Collectively, our data suggest that EGFR-PI3K signaling through Akt and ERK kinases regulates ROS-dependent, hyperoxia-induced Nrf2 activation in pulmonary epithelial cells. *Antioxid. Redox Signal.* 8, 43–52.

INTRODUCTION

MANY PULMONARY DISEASES, such as acute respiratory distress syndrome (ARDS) and emphysema, require oxygen supplementation (also known as hyperoxia) to maintain adequate tissue oxygenation (28). However, experimental evidence obtained from animal models indicates that exposure to hyperoxia can result in lung injury, inflammation, edema, and epithelial and endothelial cell death. These pathological features are similar to those seen in ARDS patients (28). The damaging effects of hyperoxia are believed to be mediated by superoxide, hydroxyl radicals, and H_2O_2 products, collectively known as reactive oxygen species (ROS), that are formed by the incomplete reduction of oxygen (12). An increased expression of antioxidant enzymes (AOEs) and phase 2 detoxifying enzymes in lung epithelial cells has been shown to play a protective role(s) against the damage caused by ROS generated by hyperoxic insult (13). Some of these enzymes include superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase (NQO-1). The protective roles of these enzymes in the pathogenesis of oxidative

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lung damage have been demonstrated *in vivo* using genetic models (reviewed in Refs. 13, 21), further underscoring the idea that expression and regulation of AOEs in response to oxidant stress plays a significant role in pulmonary defense mechanisms. However, the specific upstream cellular signal transduction pathway(s) and the downstream terminal effector(s) controlling the induction of AOEs by hyperoxia and/or other proapoptotic stimuli remain unclear.

The accumulated evidence clearly demonstrates that the rapid induction of AOEs expression in response to oxidant and toxic insults is mainly mediated by the antioxidant response element (ARE). Consistent with this notion, one or more functional ARE sites, with a consensus sequence 5'-TGACNNNGC-3', are commonly found in the regulatory regions (promoter and/or enhancers) of detoxifying enzymes, such as NQO1, HO-1, and GSTs (33). Emerging evidence obtained from both *in vivo* and *in vitro* studies strongly supports a critical role for nuclear factor erythroid 2-related factor (Nrf2), a b-Zip transcription factor, in mediating the induction of several AOEs in response to a variety of stimuli (33). It is clearly established that in unstressed cells Nrf2 is predominantly localized in the cytoplasm in association with Keap1, an actin-binding cytoskeletal protein (31). Oxidant or toxic insults disrupt the sequestration of Nrf2 by Keap1, thereby triggering the translocation of Nrf2 from the cytoplasm to the nucleus (31), where it binds to the ARE and regulates transcription. However, the upstream signals that control the translocation of Nrf2 from the cytoplasm to the nucleus appear to vary according to the inducer and /or cell type (31).

We have previously shown that Nrf2 plays a key role in the lung inflammation, injury, and repair induced by oxidants such as hyperoxia and bleomycin (5, 6). For example, Nrf2-deficient mice are more susceptible than wild-type mice to inflammatory and hyperpermeability responses to hyperoxia exposure. The inducible mRNA levels of NQO1, GST-Ya, and HO-1 are significantly lower in Nrf2-knockout mice than in wild-type mice (4, 5), suggesting that Nrf2 regulates the induction of several AOEs in response to hyperoxia. To better understand the mechanisms controlling the activation of Nrf2 in the lung in response to oxidants, we have utilized an *in vitro* culture model of murine lung alveolar type-II like epithelial cells, which are known to be the primary targets of injury caused by hyperoxia exposure (36). Using this system, we have recently shown that NADPH oxidase and ROS mediated signaling control both the translocation of Nrf2 from the cytoplasm to the nucleus and subsequent ARE-mediated transcription (34).

Nrf2 confers protection against hyperoxia-induced cell death (34) and against proapoptotic stimuli such as Fas (30) and tunicamycin (9). It is well established that phosphoinositide-3-kinase (PI3K) signaling regulates cell survival (3), and a critical contribution of this pathway in providing protection against lung epithelial cell death induced by variety of stimuli has been demonstrated. For example, the overexpression of a constitutive active form of Akt in pulmonary epithelial cells provides protection against hyperoxia-induced cell death *in vivo* (22). Thus, we have hypothesized that this pathway acts on one of the downstream effectors of NADPH oxidase and contributes to the Nrf2 activation in response to hyperoxia. Here, we report that the PI3K pathway regulates both Akt and ERK1/2 MAP kinase activation and subsequent Nrf2-ARE mediated transcription in pulmonary epithelial cells in response to hyperoxic insult. Moreover, the epidermal growth factor receptor (EGFR) is apparently involved in this process.

MATERIALS AND METHODS

Reagents and Plasmids

Akt inhibitor II (cat # 124008) and Akt inhibitor IV (cat # 124011), AG 1478 and LY294002 were obtained from Calbiochem (San Diego, CA). Diphenyleneiodonium (DPI) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, MO). The phospho-specific antibodies for EGFR (Tyr 845, cat # 2231S), Akt (Serine 473), GSK-3b (Serine 9), ERK1/2 and their respective unphosphorylated antibodies were obtained from Cell Signaling Technology (Beverly, MA). The expression vectors coding for a kinase-dead (K1791A) dominant-negative Akt (dn-Akt) (16) or constitutively active myristoylated form of Akt (ca-Akt) (19) were generously provided by Anke Klippel (Atugen AG, Berlin). ARE reporter luciferase construct (ARE-Luc), which contains three copies of ARE fused upstream of the HO-1 minimal promoter (1) was kindly provided by Jawed Alam (Alton Ochsner Clinic, Foundation, New Orleans, LA).

Cell culture and exposure

C10, a murine alveolar type II-like epithelial cell line (24) (obtained from Al Malkinson, University of Colorado Health Sciences Center, Denver), was grown in MEM supplemented with 10% fetal bovine serum and antibiotics. Before exposure to hyperoxia, the culture medium was changed to serum-free medium. After 3 h the cells were exposed to hyperoxia using a ProOx (BioSpherix, Ltd., Redfield, NY) chamber as described previously (34). As a control, cells were exposed to room air.

Immunocytochemistry

C10 cells were grown to 80% confluence in chamber slides (Lab-Tek II, Nalgene, Rochester, NY), then exposed to room air or hyperoxia for 1 h, and immunocytochemical analysis was performed using primary anti-Nrf2 antibody (SC-13032), followed by FITC-labeled secondary antibody (SC-2365) (both obtained from Santa Cruz Biotech Inc, Santa Cruz, CA) as described elsewhere (34). Slides were mounted using DAPI (H-1500, Vector Labs, Burlingame, CA), and the subcellular localization of Nrf2 was observed using a fluorescent microscope (Eclipse TE2000-S, Nikon, Melville, NY) and images were captured. For inhibitor studies, cells were treated with pharmacological inhibitor or vehicle for 30 min before exposure.

Western blot analysis

Cells were exposed to hyperoxia in the presence or absence of the indicated inhibitors, and total protein was extracted in MAP kinase lysis buffer and immunoblotted as described previously (34). Comparable quantities of protein (~40 μg) were separated on SDS-PAGE gels and probed with anti-Akt and anti-ERK antibodies using a standard protocol.

Transfections and reporter gene analyses

Cells were transfected with 100 ng of ARE-Luc along with the *Renilla* luciferase plasmid pRL-TK (5 ng, Promega, Madi-

son, WI), to monitor transfection efficiency between wells. Cell extracts were assayed for firefly and *Renilla* luciferase activities using a dual luciferase kit (Promega). Firefly luciferase activity was normalized to that of *Renilla* luciferase. All transfections were performed in quadruplicate, and each experiment was repeated at least twice. Data are presented as the mean luciferase activity ± SE ($n = 4$) of a representative experiment. The statistical significance of the differences between groups was determined using Student's *t*-test, and a $p < 0.05$ value was considered statistically significant.

Electrophoretic mobility shift assays (EMSAs)

Cells were exposed to room air or hyperoxia for 90 min, nuclear extracts were isolated in the presence of the proteasome inhibitor MG-132 (10 μ*M*), and EMSA was carried out with a ^{32}P-labeled double-stranded oligo ARE probe as described previously (34).

RESULTS

Inhibition of the PI3K pathway blocks Nrf2 nuclear accumulation and ARE-mediated transcription

To examine whether the PI3K pathway regulates Nrf2-ARE signaling, cells were treated without or with LY294002 prior to hyperoxic exposure. Hyperoxia-induced nuclear accumulation, DNA binding, and functional activities of Nrf2 were then assessed by immunofluorescent staining, EMSA, and transient reporter assays, respectively. As expected, immunostaining analysis revealed the presence of Nrf2 antigen predominantly in the cytoplasm of unstimulated cells (Fig. 1A, top left panel). Exposure to hyperoxia caused an accumulation of Nrf2 antigen in the nucleus (top right panel). However, treatment of cells with LY294002 markedly diminished hyperoxia-enhanced Nrf2 accumulation in the nucleus (bottom right panel), as compared with DMSO-treated, hyperoxia-exposed cells (top right panel).

To further verify this result, the nuclear accumulation of Nrf2 was monitored by EMSA with a ^{32}P-labeled consensus ARE probe, using nuclear extracts isolated from cells exposed to room air or hyperoxia for 90 min (Fig. 1B). As we previously reported (34), the ARE probe formed AP-1 and Nrf2 protein complexes, designated I and II. Consistent with our immunostaining data, LY294002 significantly blocked the hyperoxia-stimulated Nrf2-protein specific complex I (compare lanes 2 and 4, bottom band), whereas it had a minimal effect on the AP-1 protein complex II (compare lanes 2 and 4, top band). The specificity of formation of these complexes was assessed by both mutational analysis and supershift assays using AP-1- and Nrf2-specific antibodies as previously reported (34). These results suggested that the PI3K pathway modulates the nuclear accumulation of Nrf2 in response to hyperoxia.

To further confirm the nuclear accumulation of Nrf2 that we detected by immunocytochemical staining and EMSA, we analyzed the effect of LY294002 on hyperoxia-stimulated ARE-mediated transcription using transient transfection of a reporter construct, ARE-Luc. ARE-Luc contains sufficient information to mediate Nrf2-dependent gene transcription (1) and is frequently used to study ARE-mediated transcriptional

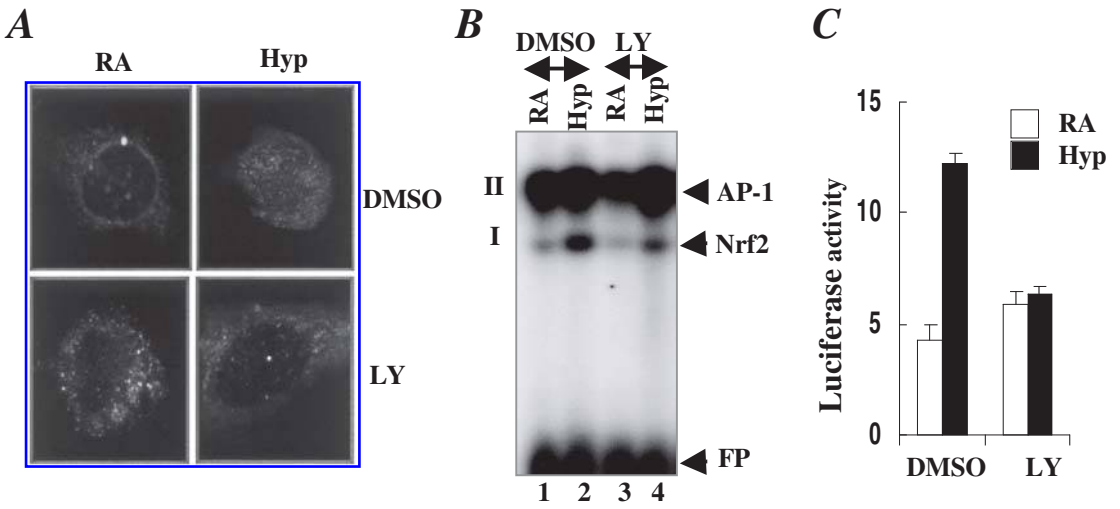

FIG. 1. A PI3K pathway-specific inhibitor blocks hyperoxia-stimulated Nrf2 activation. (**A**) Cells were treated with LY294002 (10 μ*M*) or DMSO (vehicle) for 30 min and exposed to room air (RA) or hyperoxia (Hyp) for 60 min. After the exposure, cells were fixed and the subcellular localization of Nrf2 was visualized by immunocytochemical staining. (**B**) Nuclear extracts isolated from C10 cells exposed to RA or Hyp for 180 min were incubated with the consensus Nrf2 binding sequence, ARE. The positions of the AP-1- and Nrf2-based DNA-protein complexes are indicated by the *arrow* and *arrowhead*, respectively. FP indicates the position of free probe. A representative autoradiogram from two independent experiments is shown. (**C**) Cells were transfected with 100 ng of ARE-Luc reporter together with the 5 ng of pRL-TK reference plasmid, and luciferase activity was analyzed after exposure to RA or Hyp for 5 h. The normalized luciferase activity of samples exposed to RA was considered equal to one unit. Values represent mean ± SE ($n = 4$) of a representative experiment.

responses. Cells were exposed to hyperoxia in the absence or presence of LY294002, and Luc activity was analyzed as described in Materials and Methods. Consistent with our previous result (34), hyperoxia significantly stimulated the luciferase activity (> 2.5-fold), as compared to room air-exposed controls (Fig. 1C). In striking contrast, LY294002 blocked this effect. Collectively, these results (Fig. 1A–C) strongly indicate that the PI3K pathway contributes to Nrf2-ARE signaling in pulmonary epithelial cells in response to hyperoxia.

Hyperoxia stimulates the PI3K/Akt pathway in lung epithelial cells

To determine whether hyperoxia stimulates PI3K signaling, C10 cells were exposed to hyperoxia for 0–180 min, and the activation of Akt and GSK-3β, major downstream effectors of PI3K, was monitored by western blotting using the corresponding phospho-specific antibodies (Fig. 2). Hyperoxia markedly stimulated phosphorylation of Akt (~4-fold) as early as 30 min, and this increase persisted through 180 min (upper panel). The activation of Akt was correlated with an increase (~ 3- to 5-fold) in the phosphorylated levels of GSK-3β, a downstream target of Akt (lower panel). These results suggest that hyperoxia stimulates the activation of P13K/Akt signaling in lung epithelial cells. A similar result was obtained in the human pulmonary epithelial cell line A549 (data not shown). To confirm that PI3K signaling mediates Akt activation, we treated cells with a PI3K pathway-specific inhibitor, LY294002, prior to exposure and the cellular lysates were extracted and reacted with phospho-specific Akt antibodies in western blot assays. As anticipated, LY294002 completely blocked both basal and hyperoxia-stimulated Akt phosphorylation (Fig. 2B).

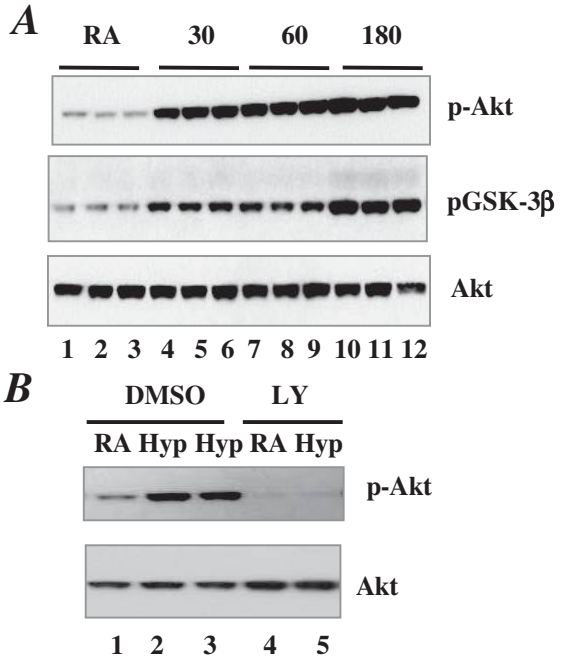

FIG. 2. Hyperoxia stimulates PI3K/Akt signaling in C10 cells. (**A**) Cells were exposed to room air (RA) or hyperoxia (Hyp) for 30–180 min and harvested in MAP kinase lysis buffer. A comparable quantity of protein (~40 μg) was separated on SDS-PAGE gels and reacted in Western blotting assays with phospho-specific anti-Akt and anti-GSK-3β antibodies. The membrane was stripped and probed with anti-Akt antibodies. (**B**) Cells were treated with LY294002 for 30 min prior to RA or Hyp exposure. After a 30-min exposure, cells were lysed, and Western blotting was performed using anti-Akt antibodies.

Akt regulates hyperoxia-induced Nrf2/ARE-mediated transcription

To examine the effector mechanism of PI3K that regulates hyperoxia-stimulated ARE-mediated transcription, we have mainly focused on Akt, which promotes cell survival. We analyzed the effects of Akt using the known pharmacological inhibitors, Akt inhibitor II and Akt inhibitor IV. Akt inhibitor II specifically inhibits the activation of Akt without affecting other upstream or downstream kinases (18). Akt inhibitor IV inhibits phosphorylation/activation of Akt by targeting its immediate upstream kinase, PDK-1, but not PI3K (15). The effects of these inhibitors on hyperoxia-stimulated ARE-mediated transcription were analyzed by transient transfection analysis. Treatment of cells with Akt inhibitor II and IV markedly suppressed hyperoxia-enhanced ARE-Luc activity (Fig. 3A). To further validate these results, cells were transfected with dn-Akt or ca-Akt, and the reporter expression stimulated by hyperoxia was analyzed. Overexpression of the Akt mutant (Fig. 3B, dn-Akt) markedly inhibited hyperoxia-induced ARE-transcription, while the expression of active mutant (Fig. 3B, ca-Akt) alone markedly (~2-fold) stimulated the transcription. Treatment of cells with Akt inhibitor IV diminished hyperoxia-enhanced Nrf2 accumulation, as detected by immunocytochemical staining, in the nucleus (Fig. 3C, right panel), as compared with DMSO-treated, hyperoxia-exposed cells (Fig. 3C, left panel). As anticipated, Nrf2 antigen was mostly localized in the cytoplasm in the room air-exposed cells treated with Akt inhibitor IV and DMSO (data not shown). These observations suggests that Akt controls the Nrf2–ARE mediated transcriptional response in pulmonary epithelial cells.

Inhibition of oxidative stress suppresses hyperoxia-stimulated PI3K-Akt signaling

Oxidative stress generated *via* NADPH oxidase in response to hyperoxia has been implicated in various cellular responses, including the activation of various transcription factors (21, 27, 35). To examine the role of ROS in the activation PI3K pathway, C10 cells were treated with or without the NADPH oxidase inhibitor diphenyleneiodonium (DPI) prior to hyperoxic exposure, and the phosphorylation of the Akt, a major downstream target of PI3K, was analyzed by Western blotting with phospho-specific antibodies (Fig. 4). As expected, hyperoxia stimulated Akt phosphorylation (compare lanes 1 and 2); however, treatment of cells with DPI markedly suppressed the activation (compare lanes 4 and 2). Supplementation of cells with an antioxidant, *N*-acetyl-L-cysteine (NAC), had a similar effect on hyperoxia-enhanced Akt activation (compare lanes 9 and 10 with lanes 6 and 7). These results suggest that

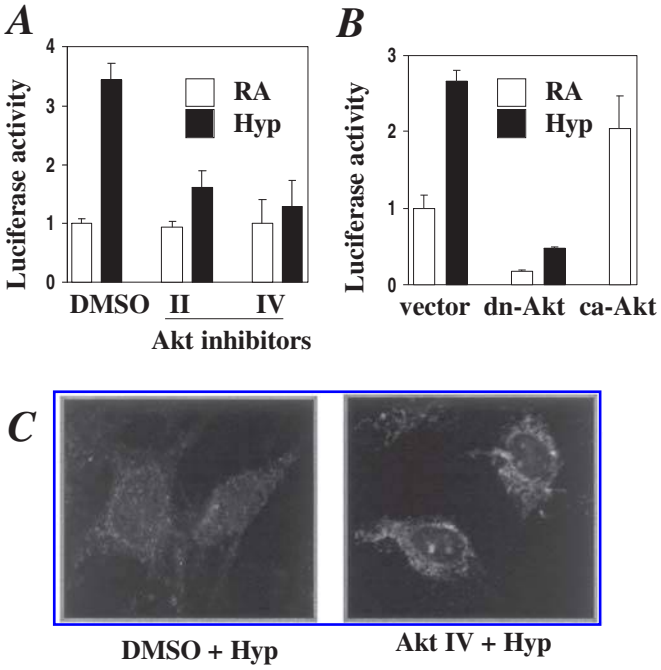

FIG. 3. Akt regulates hyperoxia-stimulated ARE transcription. (**A**) Cells were transfected with the ARE reporter and pRL-TK plasmid, then treated with the Akt-specific pharmacological inhibitors II (50 μ*M*) and IV (10 μ*M*) for 30 min. DMSO was used as a vehicle control. Cells were exposed to RA or Hyp, and luciferase activity was analyzed. (**B**) Cells were transfected with the ARE reporter (100 ng) together with a pRL-TK plasmid in the presence or absence of 100 ng of a dominant (dn-Akt) or active (ca-Akt) Akt mutant expression vector. Empty vector (vector) was used as control. (**C**) Cells were treated with Akt inhibitor IV (10 μ*M*) or DMSO (vehicle) for 30 min and exposed to hyperoxia (Hyp) or room air (data not shown) for 60 min. Subcellular localization of Nrf2 was visualized by immunocytochemical analysis.

ROS, generated through the activation of NADPH oxidase, play a role in mediating hyperoxia-induced PI3K/Akt signaling in pulmonary epithelial cells.

The PI3K pathway controls the activation of ERK1/2 pathway by hyperoxia

In our previous report (34) we showed a critical role for ERK1/2 MAP kinase signaling in mediating hyperoxia-stimulated Nrf2 activation and the subsequent ARE-driven transcriptional response. Given that PI3K signaling also controls ARE-mediated transcription we have now asked whether this pathway regulates ERK1/2 MAP kinase activation. As shown in Fig. 5A, pretreatment of cells with LY294002 (2 or 10 μ*M*) markedly attenuated hyperoxia-stimulated ERK1/2 phosphorylation. As expected, LY completely abrogated Akt activation stimulated by hyperoxia. A similar result was obtained when we examined the effect of LY on ERK1/2 and Akt phosphorylation stimulated by H_2O_2 (an oxidant) (Fig. 5B). Taken together, these results suggest that the PI3K pathway regulates oxidant-stimulated ERK1/2 activation in pulmonary epithelial cells.

The EGFR controls the activation of Akt and ERK MAP kinase signaling and subsequent ARE-reporter expression

The EGFR is known to play a central role in mediating the activation of Ras-MEK-ERK MAP kinase and PI3K-Akt signaling in response to oxidative stress induced by a variety of stimuli. To address whether it acts as the upstream activator of ERK and PI3K signaling, cells were treated without or with AG1478 (which specifically blocks the tyrosine kinase activity of the EGFR) and exposed to room air or hyperoxia. Cell lysates were prepared and separated on SDS-PAGE, and membranes were probed with phospho-specific ERK and Akt antibodies. As shown in Fig. 6A, pretreatment of cells with AG1478 markedly attenuated hyperoxia-stimulated ERK and Akt phosphorylation (compare lanes 2 and lane 4).

Consistent with this result, Western blot analysis revealed activation of the EGFR by hyperoxia as early as 10 min after exposure to hyperoxia (Fig. 6B, compare lanes 1 and 2), but not in the presence of DPI (lanes 3 and 4), indicating that hyperoxia induces EGFR activation in an NADPH oxidase-dependent manner. The inhibition of EGFR activation also

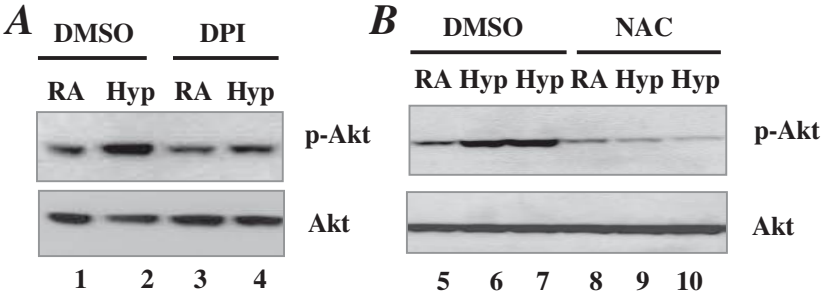

FIG. 4. An NADPH oxidase inhibitor (DPI) and antioxidant (NAC) suppress hyperoxia-stimulated PI3K/Akt signaling. Cells were treated with either DMSO or 10 μ*M* DPI (**A**) or 10 m*M* NAC (**B**) for 30 min prior to the exposure to hyperoxia or room air, and Akt activation was analyzed by Western blotting using the antibodies indicated.

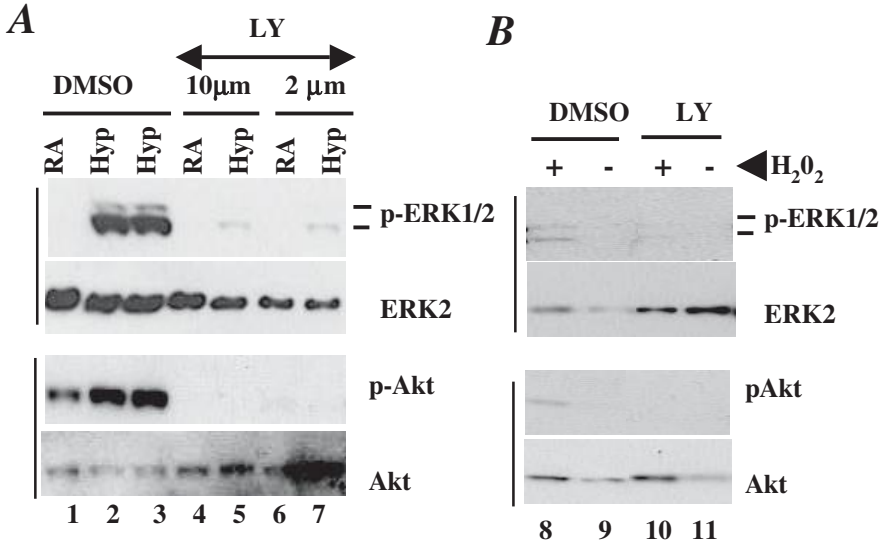

FIG. 5. Inhibition of the PI3K pathway suppresses hyperoxia-stimulated ERK1/2 phosphorylation. (**A**) C10 cells were treated with DMSO or LY294002 (LY, 2 and 10 μ*M*) for 30 min and then exposed to room air (RA) or hyperoxia (Hyp) for 30 min. ERK1/2 and Akt activation was analyzed by Western blotting using phospho-specific anti-ERK-1/2 and anti-Akt antibodies. The membranes were stripped and probed with anti-ERK2 and anti-Akt antibodies, respectively. Results shown are from a representative experiment that was repeated at least twice. (**B**) The effect of LY on H_2O_2-stimulated ERK and Akt phosphorylation. Cells were serum-starved for 1 h and then treated with 500 μ*M* H_2O_2 (+) or water (−) for 30 min, and ERK1/2 and Akt phosphorylation was analyzed.

caused an attenuation of both Akt and ERK phosphorylation, suggesting that this receptor regulates the PI3K and ERK pathways in response to hyperoxia. To verify this result at the level of ARE-mediated transcription, cells were transfected with the ARE-Luc construct, and the reporter expression in response to hyperoxia was analyzed in the absence or presence of AG1478 (Fig. 6C). As expected, hyperoxia markedly stimulated luciferase activity, while AG1478 abrogated this effect. Immunocytochemical staining analysis revealed that AG1478 (Fig. 6D, right panel) prevents the hyperoxia enhanced nuclear accumulation of Nrf2, as compared with DMSO treated control (Fig. 6D, left panel). As expected, Nrf2 antigen was mostly localized in the cytoplasm of room air exposed cells treated with AG178 and DMSO (data not shown). Collectively, these observations strongly support a prominent role for EGFR-dependent signaling in mediating the hyperoxia-stimulated ARE transcriptional response in pulmonary epithelial cells.

DISCUSSION

Our current findings establish, for the first time, a link between the anti-apoptotic PI3K-Akt pathway and both hyperoxia-induced Nrf2 activation and subsequent ARE-mediated gene transcription in pulmonary epithelial cells. Given that an Nrf2 deficiency enhances the cell death, and its overexpression confers cellular protection against proapoptotic stimuli (9, 30) that include hyperoxia (34), it is likely that this transcription factor acts as one of the important downstream effectors of PI3K pathway to regulate the expression of genes that contribute to cell survival following hyperoxic insult.

We have recently demonstrated an important contribution of oxidative stress-dependent ERK signaling in mediating the hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells (34). In the present study, we show that supplementation of cells with an NADPH oxidase inhibitor or an antioxidant prevents the activation of PI3K/Akt signaling by hyperoxia (Fig. 4). These results were clearly correlated with an attenuation of the both Akt and ERK1/2 MAP kinase activation, which play key roles in cellular responses against oxidative stress. Consistent with these findings, inhibition of the PI3K pathway blocked both the activation of Akt and ERK kinases (Fig. 5) and the subsequent Nrf2-ARE mediated gene transcription (Fig. 1). Based on these observations, we propose that PI3K acts as one of the downstream effectors of NADPH oxidase and regulates the activation of Akt and ERK kinases. Blocking the PI3K pathway significantly attenuated ERK phosphorylation stimulated by hyperoxia under our experimental conditions (Fig. 5A). Likewise, a PI3K pathway inhibitor had a similar effect on H_2O_2-induced ERK activation (Fig. 5B). A requirement for PI3K pathway for ERK activation by several growth factors has been documented in other cell systems. For example, it has been shown that inhibition of PI3K pathway by wortmannin blocks the activation of the ERK pathway in L6 rat skeletal muscle cells, apparently at the level between Ras and Raf (7). Similarly, incubation of hepatic stellate cells with wortmannin attenuates PDGF-stimulated ERK activity at the level of the upstream kinase cascade that leads to ERK activation (25). It is unclear whether a similar

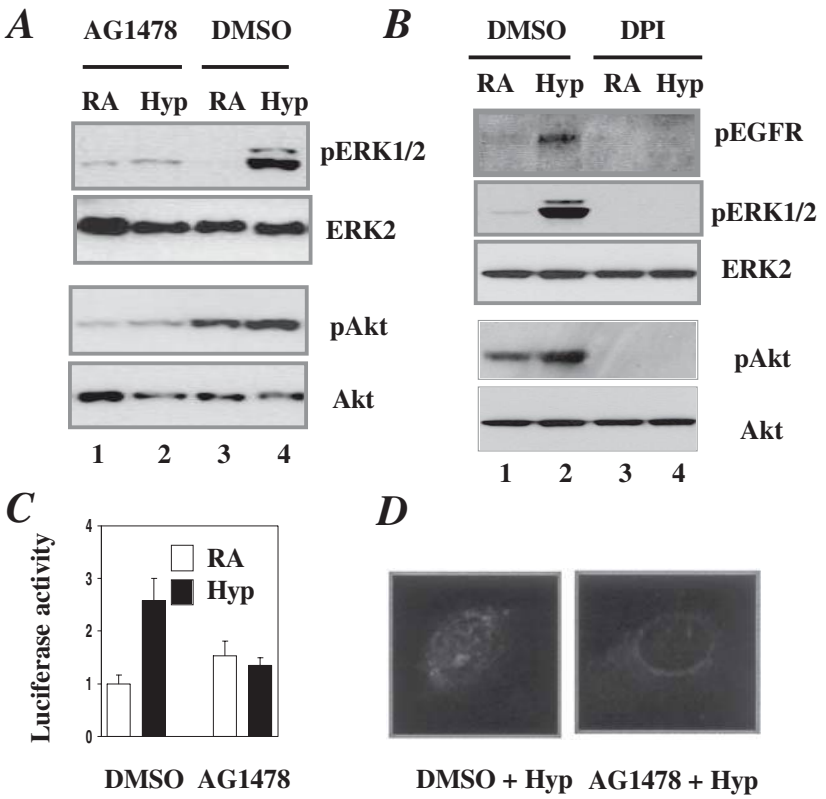

FIG. 6. The EGFR tyrosine kinase-specific inhibitor AG1478 blocks hyperoxia-induced Akt and ERK1/2 phosphorylation. (**A**) Cells were treated with DMSO or AG1478 (10 μ*M*) for 30 min and then exposed to hyperoxia for 30 min. (**B**) To determine whether ROS is required for EGFR activation, cells were treated with DMSO or DPI (10 μ*M*) for 30 min and then exposed to hyperoxia for 10 min. The phosphorylation of ERK1/2, Akt, and EGFR was analyzed by Western blotting. (**C**) The effect of AG1478 on hyperoxia-stimulated ARE-mediated transcription. ARE reporter expression was analyzed as in panel C of Fig. 1. (**D**) The effect of AG1478 (10 μ*M*) or DMSO (vehicle) on hyperoxia (Hyp)-induced nuclear accumulation of Nrf2. Immunocytochemical analysis was performed as detailed in Materials and Methods.

mechanism or a totally different mechanism is involved in mediating PI3K-dependent ERK activation under our experimental conditions.

The present study has demonstrated the involvement of Akt in hyperoxia-induced ARE-transcription. The pharmacological inhibitors of Akt and its genetic mutant markedly attenuated hyperoxia-stimulated ARE transcription (Fig. 3). PI3K-Akt signaling has also been shown to regulate Nrf2-ARE-mediated transcription in other cell types, such as neuronal (20, 26, 32) and hepatoma cells (14). Although these studies and the present study unequivocally demonstrate an important contribution of Akt to Nrf2-ARE signaling, the precise mechanism by which Akt controls Nrf2 activation remains unclear, as this transcription factor apparently lacks a consensus serine-threonine Akt phosphorylation site (20, 26, 32). This fact argues against the modulation of Nrf2 activation by Akt via phosphorylation. We therefore speculate that Akt probably modulates the Nrf2-dependent transcriptional response indirectly, possibly by post-translational modification of Nrf2 abundance. Consistent with this hypothesis are reports that both ubiquitin ligase-dependent (8, 11, 17, 41) and proteasome-dependent (37, 40) mechanisms apparently regulate the constitutive steady-state levels of Nrf2, which has a short half-life of ~30 min.

Several recent reports support a role for Akt in the modulation of various transcription factors via ubiquitin- and proteasome-dependent pathways. For example, the PI3K-Akt pathway has been shown to regulate proteasomal degradation of the FoxO1 transcriptional regulator (2). This pathway has also been shown to prevent the expression of ubiquitin ligases by inhibiting FOXO transcription factors in muscle cells (38). Akt also regulates the stabilization of the HIF-1α transcription factor via the induction of heat shock proteins (42). Since both Akt and ERK regulate the hyperoxia-stimulated Nrf2-dependent transcriptional response, we propose that these two signaling pathways uniquely contribute to Nrf2 activation at two different levels: ERK directly modulates Nrf2 through phosphorylation, while Akt may play role in the modulation of the stability of this transcription factor, probably via proteasome- and/or ubiquitin-dependent mechanisms, thereby potentiating ARE-mediated transcription in response to hyperoxia. Alternatively, it is possible that Akt might activate other kinase(s) that regulate Nrf2 activity directly. It has been shown that Akt controls barrier function and disruption in mitochondria (23), which play a critical role in the generation of oxidative stress. Thus, it is possible that Akt regulates the generation of ROS, which control Nrf2 activation through hyperoxia. Further investigation, however, is necessary to address these possibilities.

The data presented here (Fig. 6) also shed some light on the involvement of EGFR-mediated signaling in the activation of both the ERK and PI3K pathways and ARE-mediated transcription. The EGFR plays a central role in various cellular processes following a variety of oxidant and toxic insults (10,

29). Consistent with these observations, DPI blocked the activation of this receptor by hyperoxia, suggesting a role for ROS in this process. We found that blocking EGFR tyrosine kinase activity suppressed hyperoxia-stimulated Akt phosphorylation, suggesting that this receptor regulates the PI3K pathway in addition to the classical Ras/Raf/ERK signaling axis (10, 29). In agreement with our results, Wang and coworkers (39) have demonstrated a requirement for EGFR for H_2O_2-stimulated Akt activity, which confers protection against oxidative-stress induced apoptosis, in multiple cell types. The exact mechanism by which hyperoxia activates the EGFR through ROS requires detailed investigation.

In summary, based on our previous (34) and current findings, we now propose a model (Fig. 7) depicting the signaling pathways controlling the activation of Nrf2 and subsequent ARE-mediated transcription by hyperoxia in pulmonary epithelial cells. In this model, hyperoxia activates NADPH oxidase, causing the generation of ROS, which subsequently triggers EGFR activation. Activation of the EGFR leads to PI3K kinase activation, which subsequently regulates ERK and Akt activation, respectively. These processes converge at the Nrf2:Keap1 complex in the cytoplasm, ultimately leading to the translocation of Nrf2 to the nucleus, where it regulates ARE-mediated transcription.

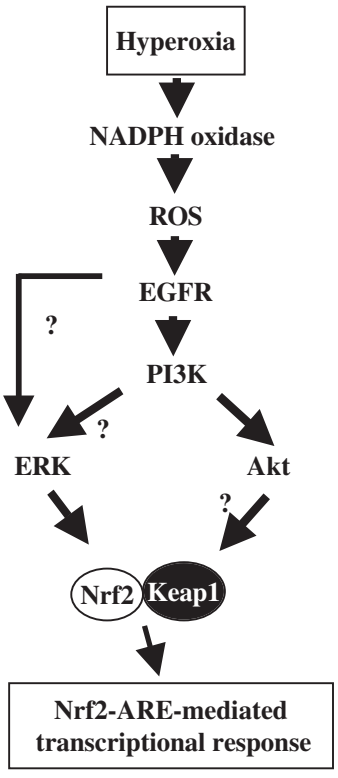

FIG. 7. Model depicting EGFR-PI3K-mediated, hyperoxia-induced Nrf2-ARE signaling in pulmonary epithelial cells. Shown are the positions of EGFR and PI3K in the proposed pathway, in which their stimulation by hyperoxia activates Nrf2-ARE signaling. *Arrows* with a question mark indicate mechanisms that are unknown. ROS, reactive oxygen species.

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ABBREVIATIONS

ARE, antioxidant response element; DPI, diphenyleneiodonium; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein (MAP) kinase; NAC, *N*-acetyl-L-cysteine; Nrf2, nuclear factor erythroid 2-related factor; PI3K, phosphoinositide-3-kinase; ROS, reactive oxygen species.

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