EGFR-Activated Signaling and Actin Remodeling Regulate Cyclic Stretch–Induced NRF2-ARE Activation

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Cyclic stretch (CS) associated with mechanical ventilation (MV) can cause excessive alveolar and endothelial distention, resulting in lung injury and inflammation. Antioxidant enzymes (AOEs) play a major role in suppressing these effects. The transcription factor Nrf2, via the antioxidant response element (ARE), alleviates pulmonary toxicant- and oxidant-induced oxidative stress by up-regulating the expression of several AOEs. Although gene expression profiling has revealed the induction of AOEs in the lungs of rodents exposed to MV, the mechanisms by which mechanical forces, such as CS, regulate the activation of Nrf2-dependent ARE-transcriptional responses are poorly understood. To mimic mechanical stress associated with MV, we have cultured pulmonary alveolar epithelial and endothelial cells on collagen I-coated BioFlex plates and subjected them to CS. CS exposure stimulated ARE-driven transcriptional responses and subsequent AOE expression. Ectopic expression of a dominant-negative Nrf2 suppressed the CS-stimulated ARE-driven responses. Our findings suggest that actin remodeling is necessary but not sufficient for high-level CS-induced ARE activation in both epithelial and endothelial cells. We also found that inhibition of EGFR activity by a pharmacologic agent ablated the CS-induced ARE transcriptional response in both cell types. Additional studies revealed that amphiregulin, an EGFR ligand, regulates this process. We further demonstrated that the PI3K-Akt pathway acts as the downstream effector of EGFR and regulates CS-induced ARE-activation in an oxidative stress-dependent manner. Collectively, these novel findings suggest that EGFR-activated signaling and actin remodeling act in concert to regulate the CS-induced Nrf2-ARE transcriptional response and subsequent AOE expression.

Keywords: oxidative stress; MAP kinases; mechanical stress; antioxidant response element; lung

Mechanical ventilation (MV) and supplemental oxygen therapy (hyperoxia) have been widely used, often in combination, to treat patients with acute lung injury. MV can improve intrapulmonary shunting in patients with acute respiratory distress syndrome (ARDS). However, it can also cause excessive alveolar distention, resulting in lung injury and increased pulmonary vascular permeability as well as an increase in the production of pro-inflammatory mediators (1–3) known to be associated with production of reactive oxygen species (ROS), a condition generally referred to as oxidative stress. These events may then serve to initiate and/or potentiate an inflammatory response, leading to a vicious cycle of inflammation either locally or systemically (4, 5).

CLINICAL RELEVANCE

Our findings may have particular physiologic importance in clinical syndromes such as ventilator-induced lung injury where inflammation and oxidative stress (an imbalance between antioxidant and oxidant systems) are thought to play a preponderant role.

Recent studies have shown that cyclic stretch (CS) or mechanical stress in general can cause cell deformation, leading to alteration in the structure and function of a number of tissues, including the lung (6). Furthermore, *in vitro* and *in vivo* studies have shown that both the degree and pattern of CS are important in determining cell responses (4). CS has been shown to differentially regulate gene expression, in part through the activation of MAP kinase signaling in lung epithelial cells (4, 7). Preliminary results have demonstrated that administration of antioxidant decreased lung neutrophil influx in rats exposed to MV, indicating a role for oxidative stress in the development of ventilator-induced lung injury (8). Although these studies have suggested the involvement of both molecular and cellular alterations, the exact mechanisms involved in the pathogenesis of MV-induced lung injury remain unclear, and particularly the role of regulators of antioxidant enzymes (AOEs) and their mechanisms of activation in response to CS.

Emerging evidence indicates that the transcription factor Nrf2 acts as one of the “biosensors” that participate in cellular switching of the genetic program in response to various oxidative and toxic stimuli. Nrf2 binds to the DNA sequence 5'-TGACN NNGC-3', known as the antioxidant response element (ARE), and regulates the expression of a network of integrated AOEs involved in cellular detoxification process, thereby protecting cells from the deleterious effects of ROS (*see recent reviews in Refs. 9, 10*). We recently demonstrated that Nrf2-deficient mice are more susceptible than wild-type mice to inflammatory and hyperpermeability responses in response to hyperoxic exposure (11). Both basal and hyperoxia-inducible expression of mRNAs encoding several AOEs, such as glutathione peroxidase 2 (Gpx2), glutamate-cysteine ligase catalytic subunit (Gclc), and glutamate cysteine ligase modifier subunit (Gclm), are significantly lower in Nrf2-knockout mice than in wild-type mice (11, 12). Consistent with these findings, studies from other laboratories have shown an important role for Nrf2 in the regulation of AOE expression in response to various oxidative and cytotoxic insults in many cells and tissues (9, 10).

Gene expression profiling has demonstrated that MV modulates the expression of prototypical Nrf2 target genes, such as *Gclc* and *Gclm*, in the lungs of animals in various experimental models (13), further suggesting a role for Nrf2-dependent ARE-mediated transcriptional responses during MV. Because CS associated with conventional MV exacerbates lung injury and in-

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flammation, deciphering the mechanisms of CS-induced cellular responses, especially the induction of AOE, is critical to developing strategies aimed at minimizing MV-related stress. The upstream signaling pathways that control the activation of Nrf2 by CS remain unclear. We have therefore used *in vitro* studies to examine the mechanism of activation of the Nrf2-dependent ARE-mediated transcriptional response in pulmonary epithelial and endothelial cells subjected to CS. Here we report for the first time that actin remodeling and EGFR-activated PI3K-Akt signaling are necessary for the regulation of Nrf2-dependent ARE-mediated transcriptional responses elicited by CS. Moreover, we demonstrate that oxidative stress regulates this process, suggesting the existence of a regulatory feedback mechanism for ARE activation.

MATERIALS AND METHODS

Reagents

Horse radish peroxidase-conjugated secondary antibodies were obtained from Amersham GE (Piscataway, NJ). Native antibodies specific for amphiregulin (R&D Systems, Minneapolis, MN) and anti-ERK1/2 (Santa Cruz Biotech, Santa Cruz, CA) and phosphospecific anti-ERK1/2 and anti-Akt antibodies (Cell Signaling, Danvers, MA) were obtained from various commercial sources as indicated. The pharmacologic inhibitors AG1478 and LY 294,002 were obtained from Calbiochem (La Jolla, CA). Real-time PCR assays were purchased from Applied Biosystems (Foster City, CA).

Cell Culture and CS Exposures

A murine nonmalignant alveolar type II-like epithelial cell line, C10 (14), was cultured in CMRL medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Rat pulmonary microvascular endothelial cells (RPMECs) were cultured in RPMI medium supplemented with 10% FBS and antibiotics. Cells were seeded onto collagen I-coated BioFlex plates, and once confluence was reached, the medium was replaced with fresh complete medium 2 h before CS exposure. Plates were mounted onto the FX-4000T Flexercell Tension Plus system (Flexercell International, McKeesport, PA) equipped with a 25-mm BioFlex loading station. This system provides uniform radial and circumferential strain across a membrane surface along all radii (more details at <http://www.flexcellint.com>). Cells were subjected to 18% elongation at 24 cycles per minute for various time points. Cells grown on BioFlex plates and simultaneously placed in a cell culture incubator were considered as static controls.

Measurement of ROS

ROS production was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) as detailed elsewhere. In brief, cells were cultured and subjected to either static or cyclic stretch conditions for 15 min or 6 h. After the exposure, cells were rinsed two times with warm serum and phenol red-free MEM and loaded for 30 min with DCFH-DA (3 μ M) in MEM without phenol red. After treatment, the cells were rinsed and then incubated with phenol red-free MEM. Images were acquired on an inverted Nikon microscope (TE1200-S; Nikon Instruments, Melville, NY) outfitted with digital CCD camera. Five individual images were captured per well using Spot advanced software 4.1 (Diagnostic Instruments, Inc, Sterling Heights, MI). The total number of cells showing the oxidized dichlorofluorescein (DCF) staining were counted under a green fluorescence field and compared with the total number of cells observed under the phase-contrast field for five images per sample. Representative fields with % DCF staining are shown.

Measurement of Cellular GSH and GSSG Levels

Reduced and oxidized thiols were measured essentially as described elsewhere (15). For GSH measurement, 10 μ l of cell lysate was mixed with 80 μ l potassium phosphate-EDTA (1 mM, pH 8.0) and 10 μ l of *o*-phthalaldehyde (OPT, 1 mg/ml). After a 15-min incubation at room temperature, the fluorescence of the sample was read at 360 nm excitation and 465 nm emission using a fluorescence plate reader (HT7000;

Perkin-Elmer, Wellesley, MA). For determination of oxidized glutathione, 10 μ l of sample solution was mixed with 70 μ l of potassium phosphate-EDTA (1 mM, pH 8.0) and incubated with 4 μ l of 40 mM *N*-ethylmaleimide (which reduces the oxidized GSH) for 30 min. The reaction was stopped with 6 μ l of 0.1 N NaOH and incubated with 10 μ l of OPT (1 mg/ml) for 15 min, and the amount of fluorescence was measured as detailed above. The values are expressed as % oxidized glutathione over reduced glutathione for the respective samples, with the value for the static control group set at 100%.

F-Actin Staining

Immunofluorescent detection of F-actin was determined as described previously (16). C10 cells grown on coverslips were treated either with dimethyl sulfoxide (DMSO) or LA (1 μ M) for 30 min, fixed with cold methanol for 10 min, and permeabilized with PBS buffer containing 0.1% Triton X. Permeabilized cells were washed and blocked in 3% bovine serum albumin for 30 min. Cells were washed three times, and F-actin was stained using Texas Red X-phalloidin (1:200) and photographed.

Western Blot Analysis

Cells subjected to CS for various times were harvested and immunoblotted using native and phosphospecific antibodies as previously described (17). The blots were then visualized with the ECL Western blot detection system.

Transfections and Reporter Gene Analyses

Transient transfections were performed using the ARE Luciferase reporter construct (hereafter referred as ARE-Luc) as described previously (18). To normalize transfection efficiency between wells, the cells were cotransfected with 1 ng of the *Renilla* luciferase plasmid pRL-TK (Promega Corp., Madison, WI). After exposure to static conditions or CS for 5 h, cell extracts were assayed for firefly and *Renilla* luciferase activities using a dual luciferase kit (Promega Corp.). Firefly luciferase activity was normalized to that of *Renilla* luciferase.

Electrophoretic Mobility Shift Assays

Nuclear extracts were isolated according to the manufacturer's instructions, using an NE-PER nuclear and cytoplasmic extraction reagent kit (#78833) from Pierce Biotechnology (Rockford, IL), and electrophoretic mobility shift assays (EMSAs) were performed as described previously (19) using nuclear extracts (2.5 μ g) and a 32 P-labeled double-stranded ARE oligonucleotide probe. In super-shift assays, nuclear extracts were incubated on ice with 2 μ g of anti-Nrf2 antibody (sc-722X) and IgG (sc-2025) (Santa Cruz Biotechnology) for 2 h before addition of the probe.

Real-Time PCR

TaqMan gene expression assays detecting mRNAs encoding mouse *Hmox1* (Mm00516005_m1), *Gpx2* (Mm00850074_g1), *Nqo1* (Mm00500822_g1), *Gclc* (Mm00802655_m1), *Gclm* (Mm00514996_m1), *Gapdh* (Mm99999915_g1), and *Actb* (Mm00607939_s1) were purchased from Applied Biosystems, and mRNA levels were quantified in triplicate according to the supplier's recommendations. The absolute values for each gene were normalized to that of *Gapdh* and/or *Actb*, and the relative value for the static or vehicle-treated control group was considered as equal to one arbitrary unit (AU).

Data Analysis

Data are expressed as the mean $\pm$ SE. Statistical significance was determined using *t* test and accepted at $P < 0.05$. All transfections were performed in triplicate, and each experiment was repeated at least twice. Data are presented as the mean luciferase activity $\pm$ SD ($n = 3$ –5) for a representative experiment. All Western blots and gel shift analyses were performed twice in duplicate. Real-time PCR was performed in triplicate ($n = 3$) and was repeated to obtain reproducible results. The statistical significance of the differences between groups was determined using Student's *t* test, and $P < 0.05$ was considered statistically significant.

RESULTS

The Nrf2 Transcription Factor Is Essential for the CS-Induced ARE-Mediated Transcriptional Response

MV has been shown to induce the expression of several antioxidant enzymes (AOEs), such as *Gpx2*, *Gclc*, and *txrmd*, in the lungs of various animal models (13, 20). To determine whether CS associated with MV modulates AOEs expression, murine C10 alveolar type II-like epithelial cells were grown on BioFlex plates and subjected to static conditions (control) or CS at 18% elongation (6). This exposure has been most frequently used by various laboratories to determine CS-induced pulmonary epithelial responses (21, 22). Total RNA was isolated from the cells, and *Gpx2* and *Gclc* expression was analyzed by real-time PCR. CS markedly up-regulated (~ 5 -fold) *Gpx2* mRNA levels, as compared with the static control group (Figure 1A). Similarly, CS significantly ($\sim 40\%$) stimulated *Gclc* mRNA expression over that of static control cells. This modest but significant increase in the expression level of *Gclc*, the first rate-limiting enzyme of GSH synthesis, is consistent with several previous studies (10).

To determine whether CS stimulates the ARE-mediated transcriptional response, cells were transfected with ARE reporter vector, along with a reference plasmid, and subjected to 18% elongation. The ARE-driven reporter expression was then analyzed as described in MATERIALS AND METHODS. CS markedly enhanced ARE-driven luciferase activity when compared with static controls at 6 h (~ 5 -fold compared with cells exposed to static conditions) and was further increased at 16 h of exposure (Figure 1B). To confirm the role of Nrf2 in mediating the ARE-driven transcriptional response, we have transiently transfected cells with the Nrf2 mutant along with the ARE reporter plasmid (Figure 1C). Ectopic expression of the Nrf2 mutant, which lacks the transcriptional activation domain (23), ablated CS-stimulated reporter activity. Collectively, these results suggest a prominent role for Nrf2 in regulating CS-induced ARE-mediated transcriptional responses.

Exposure to CS did not significantly alter *Nrf2* mRNA expression levels (data not shown). This result is consistent with several

other studies that have demonstrated that nuclear accumulation of Nrf2, rather than its transcriptional induction, plays a predominant role in up-regulating ARE-dependent gene expression by various oxidants (10). To further validate this notion, nuclear extracts were prepared from static and CS-stimulated cells, and Nrf2 binding to the putative ARE site was analyzed by EMSA to determine Nrf2 nuclear accumulation and activation. As shown in Figure 1D, the ARE oligo formed a strong protein complex with nuclear extracts isolated from both static (*lane 2*) and CS-stimulated cells (*lane 3*). However, the intensity of this complex was markedly ($> 50\%$) enhanced after CS stimulation (compare *lanes 2* and *3*, Figure 1D). The specificity of these complexes was analyzed by competition assays using the unlabeled probe. Incubation of nuclear extracts with unlabeled probe blocked the formation of DNA-protein complex (data not shown). Furthermore, to verify Nrf2 binding to the ARE probe, nuclear extracts were incubated with nonimmune IgG (*lanes 4* and *5*) or anti-Nrf2 antibodies (*lanes 6* and *7*) before the addition of the labeled probe. Preincubation with anti-Nrf2 antibodies markedly decreased the formation of protein-DNA complex in CS-stimulated (*lane 7*) cells, as compared with respective CS-stimulated samples incubated with IgG (*lane 5*). There was evidence of a basal level of protein binding to the ARE in static control cells. Collectively, these results suggest that Nrf2 accumulates in the nucleus, binds to the ARE, and up-regulates AOE gene expression in response to CS, which is consistent with the notion that Nrf2 is essential for both basal and CS-stimulated ARE-mediated gene transcription.

Actin Remodeling Is Necessary but Not Sufficient to Modulate the CS-Induced ARE-Mediated Transcriptional Response

Actin remodeling has been shown to play a key role in the modulation of transcription factor activation and subsequent gene transcription in both pulmonary endothelial and epithelial cells (24). To determine the contribution of actin remodeling to the CS-induced Nrf2-ARE transcriptional response, we used jasplakinolide, which promotes actin polymerization (25). Treatment of C10 cells cultured under static conditions with jasplakinolide had no significant effect on basal level luciferase activity.

Figure 1. CS stimulates an Nrf2-dependent ARE-mediated transcriptional response in pulmonary epithelial cells. (A) C10 cells were subjected to static conditions (St) or CS for 90 min. Total RNA was isolated, and the expression of the *Gclc* and *Gpx2* genes, the prototypical targets of Nrf2, was analyzed by real-time PCR. The values obtained for the static control group were normalized to one unit. Results are given as means $\pm$ SD ($n = 3$). * $P < 0.005$. (B) C10 cells were transfected with 400 ng of the ARE-Luc reporter construct, along with 5 ng of the pRL-TK reference plasmid. After 24 h, cell plates were exposed to CS for 6 or 16 h at 18% elongation, and reporter activity was analyzed as described in MATERIALS AND METHODS. The luciferase activity of samples exposed to static conditions (*open bars*) was normalized to one unit. Data shown are the means $\pm$ SD ($n = 3$) for cells from a representative experiment (* $P < 0.001$, # $P < 0.0005$). A similar qualitative result was obtained in two other independent experiments. (C) Cells were co-transfected with the ARE-Luc and pRL-TK plasmids

in the presence of a dominant-negative Nrf2 (dn-Nrf2) expression vector or a corresponding empty vector. Values are means $\pm$ SD ($n = 6$). *Open* and *solid bars* represent static and CS-stimulated cells, respectively (* $P < 0.001$). (D) Nuclear extracts (2.5 μ g) isolated from C10 cells exposed to static conditions (*lane 2*) or CS (*lane 3*) for 60 min were used in EMSAs with the 32 P-labeled consensus Nrf2 binding sequence, ARE, as a probe. In *lane 1*, the probe incubated without nuclear extract. To examine Nrf2 binding to the ARE, nuclear extracts were incubated with 2 μ g of IgG (*lanes 4* and *5*) or anti-Nrf2 antibody (α -Nrf2, *lanes 6* and *7*) for 2 h before the addition of the probe (*right panel*). The *open arrow* shows the position of the unbound free probe (FP). A representative autoradiogram from two independent experiments is shown.

Figure 2. Effect of actin-polymerizing agents on CS-stimulated ARE reporter activity in alveolar and endothelial cells. (A) C10 cells were incubated without (–) or with (+) jasplakinolide (Jasp, 2 μ M) for 1 h before CS stimulation for 6 h ($*P < 0.001$). Open and solid bars represent static and CS-stimulated cells, respectively. (B) C10 cells were stimulated without (–) or with (+) latrunculin (LA, 1 μ M), and the luciferase activity was analyzed. (C and D) RPMECs were transfected with ARE-Luc along with a reference plasmid and subjected to 18% elongation for 6 h, and luciferase activity was analyzed. (C) RPMECs were incubated without (–) or with (+) Jasplakinolide (Jasp, 2 μ M) before 18% CS. $*P < 0.001$ (D) The effect of latrunculin (LA, 1 μ M) on luciferase activity in RPMECs cultured

under static conditions. Data shown are means $\pm$ SD ($n = 6$). (E) The effect of latrunculin (LA) on CS-induced luciferase activity in C10 cells cultured under static conditions. The % increase in ARE-Luc activity produced by CS over vehicle-treated respective static control was considered as 100%. Data shown are means $\pm$ SD ($n = 4$). (F) C10 cells were treated with LA for 30 min and stained for F-actin using Texas Red X-phalloidin as detailed in MATERIALS AND METHODS. The vehicle DMSO was used as a control. Right panels, the nuclei were stained with DAPI.

However, pretreatment of the cells with this chemical ablated the CS-induced transcriptional response by the ARE (Figure 2A). We also performed analogous experiments using latrunculin (LA), which destabilizes cytoskeletal organization (Figure 2B). Contrary to our expectations, despite having a modest but significant effect, LA failed to significantly up-regulate ARE reporter activity, when compared with the vehicle-treated control group (Figure 2C). However, pretreatment of cells with latrunculin suppressed CS-induced ARE-driven reporter expression (Figure 2D). To confirm the cytoskeletal destabilization by latrunculin under our experimental conditions, we treated cells with latrunculin and F-actin was stained using Texas Red X-phalloidin as detailed in MATERIALS AND METHODS. Cells treated with vehicle control (DMSO) showed distinct actin distribution through out the cytoplasm (Figure 2E, top panel), whereas the F-actin staining was mainly focused around the periphery of the cells treated with latrunculin (bottom panel).

We next performed the experiments described above in rat pulmonary microvascular endothelial cells (RPMECs) which, like alveolar epithelial cells and as essential components of the alveolar-capillary units, can be subject to cyclical stretch during mechanical ventilation. Moreover, the mechanisms controlling the CS-stimulated ARE-mediated transcriptional response in these cells are poorly defined. Similar to alveolar epithelial cells, CS significantly stimulated ARE-driven luciferase activity in RPMEC as compared with static controls (Figure 2C, compare bars 1 and 2), an effect that was completely abrogated by treatment of the cells with jasplakinolide (bars 3 and 4). Jasplakinolide had no significant effect on basal level ARE activity (Figure 2C, compare bars 1 and 3). On the other hand, latrunculin had no significant effect on luciferase activity in RPMEC cultured under static conditions (Figure 2D). The lack of ability of either latrunculin or jasplakinolide to markedly stimulate the ARE-

driven transcriptional response above the basal level seen under static conditions, and the blunted response of CS-induced ARE-activation in the presence of an actin polymerization agent strongly suggest that actin remodeling is necessary but not sufficient to modulate ARE-mediated gene transcription.

EGFR-Activated Signaling Is Essential for CS-Induced Nrf2/ARE-Mediated Transcription

Because actin remodeling is not sufficient to stimulate CS-induced Nrf2-dependent ARE-mediated transcription, we next examined the involvement of other potential mechanisms. We focused our studies on EGFR-activated signaling, as it regulates downstream effector pathways (e.g., PI3K and MAP kinases) that activate various transcription factors in response to specific stimuli (26, 27). Furthermore, several studies have shown that EGFR acts as a biosensor to transduce signals from mechanical forces in various cell types, including pulmonary epithelial cells (28–33). To determine whether this receptor-activated signaling modulates CS-induced Nrf2-ARE activation, CS-stimulated ARE-Luc activity was analyzed in the presence or absence of AG1478, a pharmacologic agent that specifically blocks EGFR tyrosine kinase activity. As demonstrated in Figure 3A, CS markedly stimulated luciferase activity in vehicle-treated cells, an effect that was abrogated by pretreatment of the cells with AG1478. In separate experiments, and consistent with previous other reports (13, 20), we had observed high-level expression of amphiregulin (AREG, a known activator of EGFR) in the lungs of mice subjected to MV (data not shown). AREG has also been shown to play a role in the transactivation of EGFR by compressive stress in lung epithelial cells (34). Based on these observations, we used anti-AREG antibodies to determine their effect on modulating the EGFR-dependent Nrf2-ARE transcriptional response induced by CS. Incubation of cells with anti-AREG

Figure 3. Effect of EGFR inhibition and anti-AREG on CS-stimulated ARE activation. (A) Cells transfected with ARE-Luc were treated with DMSO or AG1478 (10 μ M) for 40 min before CS stimulation ($*P < 0.001$, $**P < 0.05$). (B) Cells transfected with ARE-Luc were incubated with IgG or anti-AREG antibody (2 μ g/ml) for 40 min before CS exposure. Values are means $\pm$ SD ($n = 4$) from a representative experiment of three independent experiments ($*P < 0.001$). Open and closed bars represent static and CS-stimulated cells, respectively. In C and D, experiments similar to above were performed in RPMECs. Results are from a representative experiment, which was repeated twice ($n = 3$). $*P < 0.0002$ and $**P < 0.004$. Open and solid bars represent static conditions and CS-stimulated cells, respectively.

antibodies obliterated CS-induced luciferase activity driven by the ARE, as compared with IgG-incubated control cells (Figure 3B). Analogous experiments performed in RPMEC demonstrated that CS-stimulated luciferase activity was markedly attenuated in the presence of AG1478 (Figure 3C), suggesting that EGFR-activated signaling is essential for the Nrf2-ARE transcriptional response in endothelial cells. Incubation of cells with anti-AREG antibodies (Figure 3D, bars 3 and 4) also significantly attenuated the CS-induced transcriptional response driven by the ARE, while IgG had no effect (Figure 3D, bars 1 and 2). We found that treatment of cells with recombinant AREG alone only modestly stimulated ARE-driven reporter expression when compared with vehicle-treated control cells (data not shown). Collectively, these results demonstrate that AREG-dependent EGFR-activated signaling, in addition to actin remodeling, is essential for the CS-induced ARE transcriptional response in alveolar epithelial and endothelial cells.

The PI3K-Akt Pathway Regulates CS-Induced Nrf2-Dependent Transcription

PI3K and ERK MAP kinases act as major downstream effectors of EGFR-activated signaling. A role for Akt and ERK signaling has been demonstrated in mechanical force-induced cellular responses (5, 7, 31). We examined the involvement of these kinases in the regulation of CS-stimulated ARE transcriptional response. Pretreatment of C10 cells with the PI3K inhibitor LY294002 (Figure 4A) almost completely inhibited CS-induced luciferase activity (bar 4) as compared with vehicle-treated CS-exposed cells (bar 2). In contrast, the MEK-ERK pathway-specific inhibitor PD98059 had no significant effect (Figure 4B). This result

Figure 4. PI3K-dependent signaling mediates CS-induced Nrf2-ARE-mediated gene transcription. (A) C10 cells transfected with ARE-Luc were treated either with DMSO or LY294002 (10 μ M) and then exposed to static conditions or CS for 6 h ($*P < 0.001$, $#P < 0.05$). (B) C10 cells were treated either with DMSO or PD98059 (20 μ M) for 40 min and then exposed to CS or static conditions ($*P < 0.001$). (C) C10 cells were transfected with ARE-Luc and pRL-TK along with an empty vector or dn-Akt mutant expression vector. Cells were exposed to CS, and the luciferase activity was analyzed ($*P < 0.001$, $**P < 0.05$). Open and solid bars represent static and CS-stimulated cells, respectively. Values are means $\pm$ SD ($n = 4$).

suggests that the PI3K pathway regulates the CS-induced ARE transcriptional response, whereas ERK signaling plays a minimal role in this process. We next examined the role of Akt, a major effector of PI3K, using a dominant-negative kinase-deficient form of Akt bearing the K179A mutation (35). Ectopic expression of a dominant Akt mutant significantly reduced CS-stimulated luciferase activity, whereas the empty vector (control) had no effect (Figure 4C). Together, these results suggest a prominent role for the PI3K-Akt pathway in regulating the Nrf2-ARE transcriptional response in alveolar epithelial cells.

Inhibition of EGFR Activity Blocks CS-Stimulated PI3K Signaling and Subsequent ARE-Mediated AOE Expression

We next examined whether EGFR-activated signaling regulates CS-stimulated PI3K-Akt activation in the alveolar epithelial cell line C10. We first assessed the activation of the PI3K-Akt and MEK1/2-ERK1/2 pathways using phospho-specific Akt and ERK1/2 antibodies, respectively (Figure 5A). CS exposure markedly stimulated both Akt and ERK1/2 MAP kinase phosphorylation, an effect that was markedly attenuated by pretreatment of cells with AG1478 (Figure 5A, compare lanes 2 and 4). As anticipated, the PI3K inhibitor LY294002 completely blocked Akt activation (Figure 5B, compare lanes 2 and 4). Interestingly, inhibition of the PI3K pathway completely blocked CS-stimulated ERK1/2 phosphorylation. These data, together with results shown in Figure 4B, suggest that CS-induced ERK activation is controlled by PI3K pathway. These results also indicate that ERK1/2 signaling only minimally contributes to CS-stimulated ARE-mediated transcription (Figure 4B).

To further confirm a role for the EGFR-activated PI3K-Akt pathway in regulating Nrf2 activation by CS, we performed EMSA assays to examine whether EGFR and PI3K inhibitors affected the binding of Nrf2 to the ARE (Figure 5C). As reported above (Figure 1D), incubation of nuclear extracts isolated from both static and CS-exposed nuclear extracts with the ARE probe yielded protein complexes (arrow in Figure 5C). The protein complex formation was stronger in CS-exposed cells. Pretreatment of cells either with the EGFR inhibitor or PI3K inhibitor markedly reduced CS-stimulated protein binding to the ARE, as compared with DMSO treatment (Figure 5C). This result is consistent with the inhibitory effects of EGFR and PI3K inhibitors on CS-stimulated Nrf2-ARE transcriptional response reported above (Figures 4 and 5).

Figure 5. Effect of AG1478 and LY294002 on CS-stimulated expression of kinases and ARE activation in alveolar epithelial cells. (A) C10 cells were pretreated with DMSO or AG1478 (5 μ M) for 40 min and subsequently exposed to static conditions (–) or CS at 18% elongation (+) for 15 min. The activation of Akt and ERK signaling was detected by immunoblotting using native and phospho-specific antibodies as indicated. Results are representative of three independent experiments. (B) The effect of LY294002 (5 μ M) on Akt and ERK phosphorylation in static (–) or CS (+)-exposed cells. (C) Cells were pretreated with DMSO (lanes 3 and 4), AG1478 (AG, lanes 5 and 6), or LY294002 (LY, lanes 7 and 8) for 40 min. Nuclear extracts were isolated from cells exposed to static conditions (–) or CS (+) for 60 min, and EMSA was performed using the ARE probe. Extracts were also incubated with a 50-fold excess of unlabeled ARE oligo (CC) before the addition of the labeled probe. Solid and open arrows represent the position of Nrf2 and free probe (FP), respectively. A representative autoradiogram from two independent experiments is shown. (D) RNA was isolated from cells that had been treated with either DMSO or LY294002 (LY) for 40 min before stimulation with CS for 90 min. Cells cultured under static conditions and treated with DMSO or LY were used as a control. *Gclc* mRNA expression was analyzed by real-time PCR. The percent increase in *Gclc* expression produced by CS over vehicle-treated static control was considered as 100%.

To further confirm the role of the PI3K–Akt pathway in regulating Nrf2-dependent ARE-mediated gene expression, the effect of LY294002 on CS-stimulated *Gclc* expression was analyzed by real-time PCR. We chose this gene because it is a prototypical target of Nrf2 and plays a key role in GSH synthesis. Consistent with the results presented above (Figure 1), CS strongly stimulated *Gclc* mRNA expression, as compared with static control exposure (Figure 5D). However, pretreatment of cells with LY294002 ablated the induction of *Gclc* expression by CS. Collectively, these results further confirm a critical role for the PI3K–Akt pathway in regulating CS-stimulated, Nrf2-dependent, ARE-mediated AOE gene expression.

Oxidative Stress–Mediated Signaling Regulates CS-Induced ARE-Mediated Transcription

ROS generated in response to oxidants or stressful stimuli have been implicated in the activation of various transcription factors,

including Nrf2 in lung epithelial cells (18). To examine the role of ROS, C10 cells were treated with or without the antioxidant N-acetyl-cysteine (NAC), and CS-stimulated ARE-driven luciferase activity was analyzed. As shown in Figure 6A, NAC diminished the CS-stimulated ARE transcriptional response, suggesting a role for ROS in this process. To examine whether ROS-dependent signaling is required for PI3K–Akt activation, the effects of NAC on CS-stimulated PI3K–Akt signaling were analyzed. Immunoblotting experiments revealed that activation of the PI3K–Akt pathway after exposure to CS (Figure 6B) was inhibited in the presence of NAC, consistent with the notion that CS activates the PI3K–Akt pathway in an ROS-dependent manner. To examine whether oxidative stress–mediated signaling regulates CS-induced EGFR, cell extracts were exposed to CS for 15 min, and the receptor phosphorylation was measured by immunoblot analysis using phospho-specific EGFR antibodies (Figure 6C). Exposure to CS stimulated the phosphorylation of EGFR (lane 1), as compared with static controls (lane 2). However, treatment of cells with NAC markedly attenuated such activation (lane 4). To monitor equal protein loading, the immunoblot was subsequently probed with anti-actin antibodies.

CS Exposure Causes Antioxidant and Oxidant Imbalance in Lung Epithelial Cells

To examine whether the ROS generated by CS can tilt the balance between antioxidant and oxidant status, we measured ROS generation, GSH levels, and AOE expression in cells under static conditions or after exposure to two different levels of CS, 5% and 18%. As anticipated, cells cultured under static conditions showed very low or undetectable levels of ROS, as measured by the DCFDA fluorescence method (Figure 7A). However, cells exposed to 5% (left top panel) or 18% CS (right top panel) for 15 min showed high levels of DCF staining relative to those of static cultures. The %DCF staining was slightly higher in cells exposed to 18% CS as compared with 5% CS. There was a significant increase in ROS production in cells subjected to 5% or 18% CS for 6 h. However, the %DCF staining was markedly higher in cells exposed to 18% elongation (Figure 7B, bar 6) than those exposed to 5% CS (bar 5), suggesting that cyclic strain enhances ROS levels in lung epithelial cells in a dose-dependent manner. To determine whether an increase in ROS production correlates with the AOE-mediated cytoprotective response, we measured the expression levels of antioxidant gene expression in cell cultures under these identical conditions. Quantitative analysis revealed that the induction of AOE was differentially regulated by CS (Figure 7C). The induction of *Gclc* and *Hmox1* by 18% CS was markedly higher than that for 5% CS. However, the inducible expression of *Gclm* was comparable for 5% and 18% CS exposure. We found a lack of induction of *Nqo1* by 18% CS, as compared with 5% CS. Collectively, these results suggest the existence of an imbalance between ROS levels and antioxidant expression in lung epithelial cells in response to CS. To confirm this notion, as a readout of the antioxidant imbalance, we analyzed the levels of reduced and oxidized GSH concentrations in cell cultures subjected to static conditions or 5% or 18% CS (Figure 7D). The increased level of GSH oxidation in 18% CS exposed cell cultures (bar 3) was significantly higher than that by 5% CS (bar 2) and static control (bar 1) groups. Collectively, these results suggest that CS at higher levels causes an imbalance between antioxidant and oxidant levels.

DISCUSSION

The induction of cytoprotective AOE in response to oxidant and stressful stimuli is critical for cellular detoxification. Emerging

Figure 6. Effect of oxidative stress on CS-induced ARE-dependent transcription. (A) C10 cells transfected with ARE-Luc were treated with DMSO or NAC (10 mM) before CS exposure, and luciferase activity was analyzed. * $P < 0.001$. Open and solid bars represent static and CS-stimulated cells, respectively. (B) C10 cells were treated without or with NAC for 40 min and then subjected to CS exposure (+) for 15 min and immunoblotted with native and phosphospecific Akt and ERK1/2 antibodies. Cells cultured under static conditions (–) were used as a control. Results shown are from a representative experiment, which was repeated twice. (C) Cell extracts (as in B) were blotted and probed with phosphospecific EGFR (Tyr-1068) antibodies. To demonstrate equal protein loading, the membrane was probed with actin antibodies. The results shown are from a representative experiment.

evidence suggests a role for antioxidant and oxidant imbalance in the exacerbation of lung injury and inflammation caused by various injurious insults, including MV. Because exposure to CS results in the generation of oxidative stress (21, 22, 36), understanding the mechanisms regulating CS-induced cellular responses, especially antioxidant gene expression, is of considerable clinical significance, not only in alleviating the side effects of mechanical forces but also for the development of new therapeutic strategies.

Using an *in vitro* model of pulmonary epithelial and endothelial cells exposed to 18% elongation, which mimics mechanical stress induced by MV with high tidal volume (37), we have for the first time demonstrated that CS stimulates the induction of cytoprotective AOE, such as Gpx2 and Gclc. By ectopic

expression of the Nrf2 mutant and using mouse embryonic cells lacking the Nrf2 gene (data not shown), we have also demonstrated that Nrf2 is critical for high-level CS-stimulated ARE-mediated transcriptional responses. The present data obtained in an *in vitro* cell culture model are consistent with the results that have been obtained in intact lungs subjected to MV. For example, several studies have reported high-level expression of AOE, such as Gpx2 and Gclc, in the intact lungs of mice subjected to high tidal volume of MV (13, 20), a response that is absent in Nrf2-deficient mice (data not shown). Collectively, these results underscore a crucial role for Nrf2 in cellular protection against oxidative stress induced by CS.

Actin remodeling plays a critical role in transforming a mechanical signal into a biological response (38). Several studies

Figure 7. CS-induced oxidative stress and antioxidant gene expression in lung epithelial cells. (A) C10 cells were cultured to confluence and subjected to different levels of cyclic strain (CS), either 5% or 18% elongation for 15 or 360 min. To detect ROS, cells were stained with DCFH-DA, washed, and photographed (see MATERIALS AND METHODS for details). Representative figures of DCF stained samples are shown. The static control group showed a very little DCF staining (pictures not shown). (B) The total number of DCF-stained cells from five images per sample ($n = 2$) observed under green fluorescence were compared with the number of total cells observed under phase contrast from the respective slide. (C) RNA was isolated from cells that had

been subjected to static conditions or exposed to 5% or 18% CS for 6 h. Gene expression was analyzed by real-time PCR using gene-specific assays. The expression levels of two house keeping genes, actin and Gapdh, were used to normalize candidate gene expression. The values obtained for the static control group were normalized to one unit. Results are given as means $\pm$ SD ($n = 3$). * $P < 0.05$. (D) GSSG/GSH ratio of static, 5% and 18% CS exposure samples was determined as detailed in MATERIALS AND METHODS. The values obtained for the static control group were normalized to 100%. * $P < 0.05$.

have shown that actin remodeling regulates the activation of various intracellular signaling pathways and effector transcription factors that control gene transcription (39). We found that treatment of cells with a pharmacologic agent that promotes actin polymerization blocked CS-induced ARE-mediated gene transcription in both epithelial and endothelial cells (Figures 2 and 3). In contrast, studies with latrunculin B, which disrupts microfilament organization, had a minimal effect on ARE-mediated transcriptional response in both cell types (Figures 2 and 3). These results imply that actin remodeling is necessary but not sufficient to enhance high level CS-induced ARE-mediated gene transcription. We found that EGFR-activated signaling is also essential for the CS-stimulated transcriptional response induced by the ARE. Thus, it appears that EGFR-activated signaling and actin remodeling may act in concert to stimulate AOE expression to provide protection against CS-induced oxidative stress (21, 22).

CS-induced EGFR-mediated signaling and subsequent MAP kinase activation have been demonstrated in various cell types, including cardiomyocytes (40), bladder smooth muscle cells (41), vascular smooth muscle (32), and lung epithelial cells (42), suggesting that EGFR serves as a mechanosensor regulating various cellular processes. We now demonstrate that the inhibition of EGFR activity ablates transcription from the ARE in both epithelial and endothelial cells (Figures 4 and 5). Because transcription from the ARE is vital to alleviating the oxidative stress induced by CS, it is likely that EGFRs act as sensors to regulate CS-induced initial cellular responses that converge on Nrf2. We have demonstrated that anti-AREG antibodies completely blocked the CS-stimulated Nrf2-ARE-mediated transcriptional response in both cell types (Figure 3). Consistent with our results, several studies have shown high-level AREG expression in the lungs of rodents subjected to MV and in experimental bronchopulmonary dysplasia induced by prolonged oxidative stress (13, 43). The fact that EGFR is a known target of AREG and that EGFR inhibition also blocks ARE activation strongly suggests a predominant role for AREG in regulating Nrf2-dependent ARE-mediated gene transcription induced by CS in lung cells.

Both the PI3K–Akt and MEK–ERK pathways act as effectors of EGFR-activated signaling and regulate various cellular processes induced by mitogens and toxicants. A role for these effectors in MV-induced lung injury has been suggested. For example, inhibition of the PI3K pathway prevents both MV-induced lung cell activation and enhanced cytokine expression, in part via the modulation of NF- κ B activity in both alveolar epithelial cells and macrophages (7). A role for EGFR-activated ERK MAP kinase in stretch-induced fetal epithelial cell differentiation (29) and adipocyte differentiation (44) has been reported. In the present study, we have shown that CS stimulates both PI3K–Akt and MEK–ERK pathways in an EGFR-dependent manner (Figure 5), while the inhibition of PI3K–Akt pathway or ectopic expression of dominant-negative Akt strongly suppresses CS-induced ARE-mediated transcription. Our findings have also revealed that inhibition of the PI3K pathway suppresses CS-induced ERK phosphorylation. However, the inhibition of MEK1/2–ERK1/2 pathway had no significant effect on CS-induced ARE-driven transcription, suggesting a minimal role for this signaling pathway in this process. In contrast, we have previously shown an important role for ERK signaling in regulating hyperoxia-induced ARE-mediated transcriptional response in the same cell type (17). This strongly suggests that Nrf2-dependent ARE-mediated response is controlled by different downstream effectors in a stimulus-specific manner in epithelial cells. We have shown that the PI3K inhibitor markedly reduces the binding of Nrf2 to the ARE, as well as ARE transcription and Gclc expression. Thus, it appears that PI3K–Akt signaling regu-

lates CS-induced Nrf2-ARE-mediated transcription, thereby suppressing the effects of oxidative stress associated with CS. Recently, Healy and coworkers (45) have demonstrated the involvement of the PI3K pathway in shear stress-induced ARE-regulated transcriptional responses. Collectively, these results underscore a role for PI3K-dependent signaling in regulating mechanical force-induced AOE expression.

Previous studies have shown increased ROS production in response to CS in several cell types and as early as 30 min after exposure to CS in lung epithelial cells, suggesting that ROS might contribute to the onset of MV-induced lung injury (21, 22). We have demonstrated that supplementation of cells with an antioxidant, NAC, prevents the activation of PI3K/Akt and ERK signaling and subsequent ARE-mediated gene transcription by CS (Figures 6A and 6B). A similar result was obtained with DPI (data not shown), which inhibits the generation of ROS by potentially blocking NADPH oxidase activity and other flavoproteins that regulate ROS production. Supplementation of cells with NAC before exposure blocked CS-induced EGFR phosphorylation, suggesting that ROS-mediated signaling regulates EGFR activation in response to CS. Importantly, we found that deregulation of AOE enzyme expression (Figure 7C) in cells subjected to a higher level of CS (18%) was correlated with an increased oxidation of GSH to GSSG, when compared with that seen for static and 5% CS samples (Figure 7D). We therefore speculate that CS-induced ROS play a key role in activating early cellular signaling that involve Nrf2-ARE-mediated gene transcription, thereby suppressing the side effects of ROS generated by CS. Prolonged or higher magnitude of CS exposure may lead to an imbalance between antioxidant and oxidants, thereby stimulating acute inflammation and lung injury.

In summary, our data demonstrate that EGFR-activated pathways and actin remodeling act in concert to activate Nrf2-dependent ARE-mediated AOE transcription, thereby providing cellular protection against oxidative stress induced by CS.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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