Oxidant-Mediated cAMP Response Element Binding Protein Activation

Calcium Regulation and Role in Apoptosis of Lung Epithelial Cells

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Oxidant stress-mediated regulation of extracellular signal-regulated kinases (ERK1/2) is linked to pathologic outcomes in lung epithelium, yet a role for Ca^{2+} and Ca^{2+} /cAMP-response element binding protein (CREB) in ERK1/2 signaling has not been defined. In this study, we tested the hypotheses that oxidants induce Ca^{2+} -mediated phosphorylation of ERK and CREB, and that CREB is required for oxidant-induced proliferation and apoptosis. H_2O_2 initiated an influx of extracellular Ca^{2+} that was required for phosphorylation of both ERK and CREB in C10 lung epithelial cells. H_2O_2 -mediated CREB phosphorylation was sensitive to MEK inhibition, suggesting that crosstalk between Ca^{2+} , ERK, and CREB signaling pathways contributes to the oxidant-induced response. Reduction of CREB activity, using a dominant-negative CREB construct, inhibited *c-fos* steady-state mRNA levels, but unexpectedly enhanced *bcl-2* steady-state mRNA levels after H_2O_2 exposure. Whereas inhibition of CREB activity had no detectable effect on H_2O_2 stimulation of cell cycle, loss of CREB activity significantly reduced the number of cells undergoing apoptosis. These data support a novel communication between Ca^{2+} -ERK1/2 and CREB elicited by H_2O_2 , and further provide evidence that CREB is an important regulator of apoptosis in oxidant-mediated responses of lung epithelial cells.

Keywords: calcium signaling; lung pathology; mitogen-activated protein kinase; reactive oxygen species

Reactive oxygen species (ROS) play a significant role in the pathogenesis of lung diseases and are involved in multiple physiological processes through their capacity to regulate the expression of several protein kinases and phosphatases. In lung epithelial cells, the target cells of inhaled oxidants, H_2O_2 leads to the activation of Ser/Thr and Tyr protein kinases, including protein kinase C (1) and the mitogen-activated protein kinases (MAPK) p38, c-Jun amino-terminal kinase, and extracellular signal-regulated kinase 1 and 2 (ERK1/2) (2, 3). H_2O_2 also serves as an important signaling molecule to activate the expression of transcription factors, including members of the activator protein family, *c-jun* and *c-fos* (4), resulting in proliferation (2) and apoptosis (5) of lung epithelial cells.

Oxidant stress-mediated phosphorylation of ERK1/2 is believed to be integral to pathologic signaling in lung epithelium. Upon phosphorylation, ERK1/2 can translocate to the nucleus, where it induces *c-fos* and *c-jun*, leading to the activation of transcription factors intrinsic to proliferation and apoptosis (6). ERK1/2 also mediates the actions of both Ca^{2+} and growth

factors, resulting in activation of the Ca^{2+} /cAMP-response element binding protein (CREB) (7).

CREB is a 43 kD transcription factor belonging to the basic-leucine zipper family (7, 8). The function of CREB is regulated by the phosphorylation of ^{133}Ser , a serine at position 133. Phosphorylation, in turn, initiates the recruitment of cofactors to the Ca^{2+} /cAMP-response element (CRE) that are necessary for transcriptional activation, including CREB-binding protein (CBP) 300 (9). Multiple kinase signaling pathways contribute to the phosphorylation of CREB, including protein kinase A, Ca^{2+} /calmodulin-dependent kinase (CaMK), protein kinase C, and p90 ribosomal kinase (7, 10). Phosphorylation of CREB by ERK1/2 is proposed to be indirect through the phosphorylation of p90 ribosomal kinase (11).

CREB phosphorylation is important in the control of gene transcription driven by CREs. Many genes important for regulation of proliferation and apoptosis, including *c-fos* and *bcl-2*, respectively, contain CREs in their promoter (9). Although CREB has the capacity to regulate these gene targets (9), its role in oxidant-mediated signaling has not been assessed.

There is little information regarding the role of Ca^{2+} and Ca^{2+} -mediated transcription in oxidant-mediated signaling pathways in lung epithelium, an initial target of inhaled oxidants. Because of known crosstalk between ERK1/2, Ca^{2+} , and CREB in other cell types, the goal of this study was to determine their relative importance in H_2O_2 -induced signaling in lung epithelial cells. In the present study, we found that H_2O_2 exposure resulted in Ca^{2+} influx that was required for phosphorylation of both ERK1/2 and CREB in lung epithelial cells. Reduction of CREB activity, by introducing a dominant-negative CREB construct, led to a decrease in apoptosis corresponding with enhanced *bcl-2* transcription. These data suggest signaling crosstalk between Ca^{2+} , ERK1/2, and CREB in lung epithelial cells, and reveal an important role for CREB in H_2O_2 -mediated apoptosis.

MATERIALS AND METHODS

Cell Culture and Treatment

C10 cells, a contact-inhibited, nontransformed murine alveolar type II epithelial cell line (12), were grown in CMRL 1066 medium supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 $\mu\text{g}/\text{ml}$), and 10% FBS (GIBCO BRL, Rockville, MD). Cells were grown to near confluence, then complete medium was replaced with CMRL 1066 medium supplemented with L-glutamine, penicillin/streptomycin, and 0.5% FBS for 48 h before exposure to agents.

H_2O_2 (Sigma, St. Louis, MO) was added as a bolus to the medium at the indicated concentrations. Where indicated, recombinant glucose oxidase (15 mU/ml) (Roche, Indianapolis, IN) was used to provide low-level fluxes of H_2O_2 (13). Forskolin and ATP (Sigma) (10 μM for 10 min) were used as positive controls for induction of phospho-CREB and phospho-ERK1/2, respectively. Control cultures received medium without agents and were treated identically.

The MAP or ERK kinase (MEK) inhibitor, U0126 (10 μM), and CaMKII inhibitor, KN93 (30 μM), or its inactive isomer, KN92 (30 μM), were obtained from Calbiochem (La Jolla, CA). Inhibitors were added 30 min before H_2O_2 . For "low Ca^{2+} " conditions, cells were maintained

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in low-calcium HEPES-buffered saline solution (10 mM HEPES [pH 7.4], 6 mM KCl, 140 mM NaCl, 100 μ M CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, 1 mM EGTA, effectively 100 nM Ca^{2+}) 5 min before experiments. All experiments were performed in triplicate.

Stable Dominant-Negative CREB C10 Lung Epithelial Cells

A ^{133}Ser phosphorylation mutant CREB construct (CREB133) (BD Biosciences, Palo Alto, CA) was ligated into pCMV-Tag5a (Stratagene, La Jolla, CA) through a BamHI restriction site. A total of 8 μ g of CREB133 or pCMV-Tag5a were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions for creating stable cell lines. Transfected cells were selected using 0.5 mg/ml geneticin (GIBCO BRL) for 14 d. The presence of the dominant-negative construct was confirmed by immunofluorescence and Western blot analysis.

Ca^{2+} Imaging Using Fluorescence Resonance Energy Transfer Constructs

Cells were grown on glass coverslips. After 48 h in low serum medium, 8 μ g of the cameleon fluorescence resonance energy transfer (FRET) construct, YC2.1, in pRSETB (a gift from Roger Tsien, University of California at San Diego), was transfected into the cells using Lipofectamine 2000, following the manufacturer's instructions. After 4 h, the medium was replaced with low-serum medium and cells were incubated for 24 h before use in experiments.

FRET Ca^{2+} imaging was performed as described previously (14). Coverslips were placed into an SA-NIK chamber (Warner Instruments, Hamden, CT) on a Nikon Diaphot 200 inverted microscope (Nikon, Melville, NY). Agents were perfused at room temperature into the chamber containing HEPES-buffered saline solution (10 mM HEPES, 6 mM KCl, 140 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose). ATP was used as a positive control, as it has been previously shown to stimulate an increase in Ca^{2+} levels in epithelial cells (14). Ionomycin (10 μ M; Calbiochem) and EGTA (2 mmol/l) were used to determine the maximum and minimum values of intracellular Ca^{2+} , respectively. To record Ca^{2+} -induced FRET, cells were excited at a wavelength of 440 nm, and the emission ratio of 535/480 nm was recorded every 10 s with an acquisition time of 200 ms. A neutral density filter of 1.0 nm was used. Images were captured using a Hamamatsu camera (Hamamatsu, Bridgewater, NJ) and analyzed with Metafluor Imaging System 3.0 software (Universal Imaging Corporation, West Chester, PA). Data were normalized by subtracting out background from both 535 and 480 wavelengths and then dividing 535 by 480 to obtain the emission ratio.

Detection of Phospho-CREB, Phospho-ERK, and Ki-67 by Immunofluorescence

C10 cells were grown on glass coverslips for all experiments. After experimental exposures, immunofluorescence to detect phospho-CREB, phospho-ERK, and Ki-67, a marker of cell cycle progression, was performed as previously described (2, 10). Briefly, cells were washed with PBS, fixed in 3.7% paraformaldehyde, permeabilized with -20°C methanol, and incubated with blocking solution containing 2% BSA in PBS. Cells were incubated with primary antibody (rabbit polyclonal phospho-p44/42 MAPK or rabbit polyclonal phospho-CREB [Cell Signaling Technologies, Beverly, MA], 1:250, or monoclonal rat Ki-67 [Dako, Carpinteria, CA], 1:50) diluted in 2% BSA plus 0.1% Triton X-100 in PBS (BSA/PBS-T) for 1 h at 37°C . Secondary antibody (Cy3-conjugated goat anti-rabbit IgG, 1:500, or AlexaFluor568-conjugated goat anti-rat IgG [Jackson Laboratories, West Grove, PA], 1:400), diluted in BSA/PBS-T, was applied for 1 h at 37°C , followed by incubation with nuclear counterstain, YOYO-1 iodide (1:10,000; Molecular Probes, Carlsbad, CA) and 1 unit/ml RNase in BSA/PBS-T for 30 min at 37°C . Coverslips were mounted onto slides with AquaPolyMount (Polysciences Inc., Warrington, PA). For each sample, confocal images were collected in fluorescence modes using a MRC1024ES confocal scanning laser microscope (Bio-Rad, Hercules, CA).

Western Blot Analysis for Phospho-ERK1/2

After C10 cells were exposed to agents as described previously here, the medium was aspirated and the cells were washed twice with cold

wash buffer (250 mM sucrose, 25 mM Tris [pH 7.4], 2.5 mM MgOAc , and 2 mM DTT), collected in lysis buffer (250 mM sucrose, 25 mM Tris [pH 7.4], 2.5 mM MgOAc , 2.5 mM EGTA, 2 mM DTT, 1 mM NaVO_3 , 1 mM $\text{Na}_4\text{O}_7\text{P}_2$, 10 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 mM PMSF), sonicated $5\times$ for 5 s, and centrifuged at $28,000 \times g$ for 3 min to remove debris. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). A total of 20 μ g of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed as described previously (2). In brief, transfer blots were blocked in Tris-buffered saline containing 5% nonfat powdered milk (Sigma), then incubated with antibodies specific to total and phosphorylated ERK1/2 (rabbit polyclonal total or phospho-p44/42 MAPK [Cell Signaling Technology], 1:1000). Antibody binding was detected using a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by chemiluminescence (Kirkgaard and Perry Laboratories, Gaithersburg, MD). QuantityOne (Bio-Rad) was used to quantify band density. Band densities were normalized to total ERK.

Western Blot Analysis for Phospho-CREB

After C10 cells were exposed to agents as described previously here, the medium was aspirated, and the cells were washed twice with cold PBS and collected in 4X sample buffer (200 μ M Tris [pH 6.8], 4% SDS, 4 mg/ml β -mercaptoethanol, 40% glycerol, 2 μ M pyronin-Y). The amount of protein in each sample was determined using the RC/DC protein assay (Bio-Rad). A total of 20 μ g of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed as described previously here, using antibodies specific to total and phosphorylated CREB (rabbit polyclonal CREB, 1:1000, and phospho-CREB, 1:500; Cell Signaling Technology). QuantityOne was used to quantify band density, and values were normalized to total CREB.

RNase Protection Assay

After C10 cells were exposed to agents as described previously, total RNA was prepared and quantitated as described by Shukla and colleagues (15). Steady-state mRNA levels of *cdk5*, *c-fos*, *PCNA*, *cyclin D1*, and *bcl-2*, the ribosomal probe *L32*, and glyceraldehyde-3-phosphate dehydrogenase were examined with a RiboQuant multiprobe RNase protection assay system and a custom-made multiprobe template set (Pharmingen, San Diego, CA) according to the manufacturer's protocol. Autoradiograms were developed and quantitated with a Bio-Rad phosphorimager. Results were normalized to the expression of the housekeeping gene, *L32*.

Detection and Quantitation of Apoptosis

Detection of apoptosis was performed as previously described (16). Briefly, cell monolayers grown on glass coverslips were treated with 300 μ M H_2O_2 for 4, 8, or 24 h, then fixed in methanol for 24 h at -20°C , boiled for 5 min in PBS containing 5 mM MgCl_2 , and then immersed in ice-cold PBS for 10 min. Cells were blocked with 40% FBS and then incubated with Apostain F7-26 (10 μ g/ml; Alexis Biochemicals, San Diego, CA) followed by AlexaFluor568-conjugated secondary antibody (goat anti-mouse IgM, 1:400; Jackson Laboratories, West Grove, PA). Nuclei were counterstained with YOYO-1 iodide (1:10,000; Molecular Probes) and 1 unit/ml RNase in BSA/PBS-T for 30 min at room temperature. Coverslips were mounted onto slides with AquaPolyMount (Polysciences). For each sample, confocal images were collected in fluorescence modes using a Bio-Rad MRC1024ES confocal scanning laser microscope. To determine the number of apoptotic cells and total number of cells per field, five random fields were evaluated per experimental condition.

Statistical Analysis

Differences between treatment groups were assessed by two-way ANOVA in a randomized block design. The analyses were based on non-normalized data, and pair-wise comparisons between treatment groups were performed using Fisher's least significant difference procedure. Differences were considered statistically significant at $P < 0.05$.

RESULTS

H₂O₂ Initiates a Rise in Cytoplasmic Calcium that Is Prevented by Reducing Extracellular Calcium

To measure calcium signaling in lung epithelial cells, the FRET-based chameleon Ca²⁺ indicator, YC2.1, was expressed in C10 cells. YC2.1 senses changes in intracellular Ca²⁺ levels through a Ca²⁺-dependent conformational change, resulting in FRET between yellow fluorescent protein (YFP) (480 nm emission) and cyan fluorescent protein (CFP) (535 nm emission) that can be detected by live cell fluorescence imaging (14). Intracellular Ca²⁺ levels are thus directly correlated to the 535/480 FRET ratio.

ATP stimulation resulted in a rapid and transient rise in intracellular Ca²⁺ levels. H₂O₂ stimulation resulted in a response that was consistent but less pronounced (Figures 1A and 1C).

Figure 1. H₂O₂ initiates a rise in cytoplasmic calcium that requires extracellular Ca²⁺. C10 cells were transfected with the fluorescence resonance energy transfer (FRET)-based Ca²⁺ indicator, YC2.1, and the emission ratio of 535/480 nm (yellow fluorescent protein/cyan fluorescent protein [YFP/CFP]) was acquired every 10 s. (A) Pseudocolor images of the FRET emission ratio recorded live from a single cell expressing YC2.1. The color range represents a ratio of 535/480 from 1.0 (low Ca²⁺) to 1.5 (high Ca²⁺). ATP was used at 10 μ M. Time 0 represents the addition of 200 μ M H₂O₂. (B) Time-lapse images of the Ca²⁺ responses as in (A) using buffer containing low Ca²⁺ (100 nM). (C) Percent basal ratio (YFP/CFP) plot of the Ca²⁺ response from the live emission recording of a representative cell. Treatments were added to the bath as indicated by the lines on the plot. (D) Percent basal ratio (YFP/CFP) plot of the Ca²⁺ responses as in (C) using buffer containing low Ca²⁺ (100 nM).

To determine the role of extracellular calcium in the H₂O₂-mediated Ca²⁺ response, cells were bathed in low-Ca²⁺ buffer (100 nM), and then intracellular Ca²⁺ concentration was monitored after the addition of ATP and H₂O₂. Although the ATP response was not dependent on extracellular Ca²⁺ levels, the H₂O₂-mediated Ca²⁺ signal was abolished by lowering extracellular Ca²⁺ (Figures 1B and 1D), suggesting that H₂O₂-mediated Ca²⁺ signaling requires entry of extracellular Ca²⁺.

H₂O₂ Stimulates Phosphorylation of CREB that Requires Extracellular Ca²⁺ and Is Regulated by ERK1/2

We have previously shown that H₂O₂ leads to ERK1/2 phosphorylation in lung epithelial cells (2). To determine if the observed Ca²⁺ influx stimulated by H₂O₂ is required for the activation of ERK1/2, phospho-ERK1/2 levels were detected by Western blot and immunofluorescence. The level of phospho-ERK1/2 increased in response to H₂O₂ when compared with control (100 ± 11.90 versus 658.27 ± 91.71 ; $n = 3$), and the response was reduced by lowering extracellular Ca²⁺ (658.27 ± 91.71 versus 348.96 ± 51.22 ; $n = 3$) (Figures 2A and 2B).

Because Ca²⁺ and ERK are important regulators of the transcription factor, CREB (7, 17), we investigated the possibility that H₂O₂ mediates CREB phosphorylation, using Western blot and immunofluorescence analyses. Exposure to bolus

Figure 2. Extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation stimulated by H₂O₂ is dependent on Ca²⁺. (A, B) C10 cells were treated with 10 μ M forskolin, 10 μ M ATP, or 200 μ M H₂O₂ for 10 min in buffer containing normal (2 mM) or low (100 nM) Ca²⁺ and analyzed by Western blot (A) or immunofluorescence (B) using an antibody specific for phospho-ERK1/2 (p-ERK1/2). An antibody recognizing total ERK was used as a control for protein loading in (A).

addition of H_2O_2 led to CREB phosphorylation in a dose- and time-dependent manner, with maximal activation occurring at 200 μM H_2O_2 (Figure 3A). Similar results were observed when glucose oxidase was used as the H_2O_2 -generating system (data not shown). The induction of phospho-CREB was significantly reduced by lowering extracellular Ca^{2+} (281.82 ± 16.07 versus 155.09 ± 22.47 ; $P < 0.05$, $n = 3$) (Figure 3B, C), and the pattern of CREB phosphorylation in response to H_2O_2 closely paralleled that seen in response to activation of protein kinase A using forskolin.

To determine the contributions of ERK1/2 and CaMKII in the observed H_2O_2 -induced CREB phosphorylation, cells were evaluated with or without pretreatment with the MEK inhibitor, U0126, or the CaMKII inhibitor, KN93. H_2O_2 -mediated CREB activation was partially inhibited by U0126 at 100, 200, and 300 μM concentrations of H_2O_2 (83.23 ± 13.41 versus 44.08 ± 11.95 , 104.64 ± 10.61 versus 66.32 ± 10.44 , and 100.40 ± 7.17 versus 61.81 ± 9.77 , respectively; $n = 5$) (Figure 4A). KN93 did not affect H_2O_2 -mediated CREB activation (199.45 ± 18.72 versus 165.92 ± 29.37 ; $n = 4$) (Figure 4B), but did inhibit H_2O_2 -mediated phospho-CaMKII activity (data not shown). Taken together, these data suggest that H_2O_2 -mediated CREB phosphorylation is partially mediated by the MAPK/ERK pathway and is independent of CaMKII function.

Reduction of CREB Activity Inhibits *c-fos* Steady-State mRNA Levels, Enhances *bcl-2* Steady-State mRNA Levels, and Significantly Reduces the Number of Cells Undergoing Apoptosis after H_2O_2 Exposure

CREB has the capacity to regulate multiple genes involved in proliferation and apoptosis (9). To examine the role of CREB in H_2O_2 -mediated proliferation and apoptosis, stable lines of C10 cells were generated that express a CREB phosphorylation mutant construct (CREB133) or empty vector (Tag5a). A mutation in ^{133}Ser results in inactivation of CREB transcriptional activity (18). Expression of CREB133 resulted in an elevation of CREB expression, but did not change the forskolin response of endogenous CREB (Figure 5). Thus, as expected, the ratio of phospho-CREB/CREB is greatly reduced by the mutant (from 5.571 to 1.851 in response to forskolin), leading to inhibition of active dimer formation.

An RNase protection assay was used to measure the effect of CREB inhibition on H_2O_2 -mediated induction of select CRE-containing genes, including *c-fos* and *bcl-2*. Exposure of cells to 100 μM H_2O_2 , a concentration leading to proliferation at early time points (data not shown), led to an increase in *c-fos* steady-state mRNA levels that was reduced by expression of CREB133 (10.12 ± 0.108 versus 1.00 ± 0.023 ; $P < 0.05$, $n = 3$) (Figure 6). Exposure of cells to 300 μM H_2O_2 , a concentration resulting in apoptosis after 24 h (2), resulted in a small but consistent increase in the steady-state mRNA level of *bcl-2* only in cells expressing CREB133 cells (Figure 6), suggesting that CREB may exert negative regulation of *bcl-2* in response to high concentrations of oxidants.

To assess whether CREB activity is necessary for H_2O_2 -induced proliferation, C10 cells stably transfected with Tag5a or CREB133 treated with 100 μM H_2O_2 were examined with the proliferation marker, Ki-67. After exposure to H_2O_2 for 1 h, both Tag5a and CREB133 cells showed a significant increase in the percentage of Ki-67-positive cells ($3.72 \pm 2.11\%$ versus $11.69 \pm 3.24\%$ and $1.22 \pm 1.21\%$ versus $8.74 \pm 1.49\%$, respectively; $P < 0.05$, $n = 3$). Although CREB133 cells exhibited a trend toward less proliferation when compared with Tag5a cells, it was not significant, suggesting that the reduction of CREB activity by CREB133 was not sufficient to inhibit H_2O_2 -mediated proliferation.

Figure 3. H_2O_2 mediates cAMP response element binding protein (CREB) phosphorylation in a dose- and time-dependent manner. (A) Protein expression of phospho-CREB was determined using Western blot analysis after C10 cells were exposed to H_2O_2 at the indicated dose and time points. The graph represents data normalized to the control response. $^{\#}P < 0.05$ at 15 min time point when compared with control; $^{*}P < 0.05$ at all time points when compared with control. H_2O_2 induces CREB phosphorylation in a Ca^{2+} -dependent manner. (B, C) C10 cells were treated with 10 μM forskolin or 200 μM H_2O_2 for 10 min in normal (2 mM) or low (100 nM) Ca^{2+} buffer and analyzed by Western blot (B) or immunofluorescence (C) using an antibody specific for phospho-CREB (p-CREB). An antibody recognizing total CREB was used as a control for protein loading in (B).

Figure 4. H_2O_2 -mediated CREB activation is inhibited by the MEK inhibitor, U0126, but is unaffected by the CaMKII inhibitor, KN93. (A) C10 cells, preincubated with 10 μM U0126 for 30 min, were treated with 10 μM forskolin or 100, 200, or 300 μM H_2O_2 for 10 min, and then analyzed by Western blot for p-CREB, total CREB, phospho-ERK1/2 (p-ERK1/2), and total ERK1/2. (B) C10 cells were preincubated with 30 μM KN93 or its inactive isomer, KN92, for 30 min, and then treated with 10 μM forskolin or 200 μM H_2O_2 for 10 min. p-CREB was then detected by Western blot. An antibody recognizing total CREB was used as a control for protein loading in A and B.

To determine whether CREB activation is essential for H_2O_2 -induced apoptosis, C10 cells stably transfected with Tag5a or CREB133 were treated with 300 μM H_2O_2 and apoptotic cells were identified by Apostain. H_2O_2 induced a significant increase in the percentage of cells undergoing apoptosis compared with the control in both Tag5a and CREB133 cells ($44.8 \pm 8.1\%$ versus $3.8 \pm 1.8\%$ and $16.9 \pm 3.7\%$ versus $0.5 \pm 0.4\%$; $P < 0.05$, $n = 3$) (Figures 7A and 7B). However, CREB133 cells had significantly lower levels of apoptosis at 24 h than the Tag5a cells ($16.9 \pm 3.7\%$ versus $44.8 \pm 8.1\%$; $P < 0.05$, $n = 3$) (Figures 7A and 7B), supporting a role for CREB in the regulation of apoptosis induced by H_2O_2 .

DISCUSSION

Lung epithelial cells are exposed to ROS through direct inhalation, through the production of oxidants during inflammation, or through by-products generated during cell metabolism. Excessive production of oxidants plays a role in the pathogenesis of many pulmonary diseases, including asthma, pulmonary fibrosis, and cancers in lung epithelial cells (19). Here, we present the first evidence for a link between oxidants and apoptosis through

Figure 5. Expression of CREB133 mutant results in a decreased p-CREB/CREB ratio. (A) C10 cells stably transfected with CREB133 or empty vector (Tag5a) were treated with 10 μM forskolin for 10 min, where indicated, and then analyzed by Western blot for p-CREB, total CREB, and β -actin. (B) Quantification of p-CREB in (A) normalized to β -actin. (C) Quantification of CREB in (A) normalized to β -actin.

Ca^{2+} -mediated activation of ERK1/2 and the CREB transcription factor in lung epithelial cells.

An increase in ROS has been linked to effects on Ca^{2+} signaling in primary macrophages (20), vascular endothelium, and smooth muscle cells (21). However, a relationship between ROS and Ca^{2+} signaling has not been described previously in lung epithelial cells. Using a FRET-based Ca^{2+} indicator, we examined the effects of ROS on intracellular Ca^{2+} signals in single lung epithelial cells. We show that H_2O_2 elicits a transient increase in the intracellular Ca^{2+} level. The dependence of the H_2O_2 -mediated Ca^{2+} signal on extracellular Ca^{2+} suggests that entry of Ca^{2+} is necessary, and that the signal is not likely generated through release of intracellular Ca^{2+} stores (22).

We have previously demonstrated that ERK1/2 is transiently phosphorylated in response to ROS in lung epithelial cells (2). Here, we show that H_2O_2 -mediated ERK1/2 activation is partially regulated by Ca^{2+} , indicating that Ca^{2+} is important for downstream signaling by ERK1/2 in cells responding to oxidants.

Figure 6. Reduction of CREB activity using a CREB phosphorylation mutant leads to inhibition of *c-fos* steady-state mRNA levels, but enhanced *bcl-2* steady-state mRNA levels after exposure to H₂O₂ at the indicated times and doses. (A) C10 cells stably transfected with CREB133 or empty vector (Tag5a) were treated with H₂O₂ at the indicated dose and time and then analyzed by RNase protection assay using a custom-made template of genes indicated on left. (B) Quantification of *c-fos* and *bcl-2* transcription in response to H₂O₂. **P* < 0.05 when compared with control.

Changes in intracellular Ca²⁺ levels have been shown to regulate the MAPK/ERK pathway through upstream activation of Ras in PC12 cells and primary neurons (17, 23). Rusanescu and colleagues (24) demonstrated that increased intracellular Ca²⁺ levels induced Shc phosphorylation, which ultimately leads to the activation of Ras. Ca²⁺ has also been implicated in regulating Ras by promoting enhanced GTPase activity directly or indirectly through calmodulin (23).

Recent studies show that H₂O₂ regulates the activity of CREB in astrocytes (25), glial cells (26), macrophages (27), and smooth muscle cells (28). The present study indicates that H₂O₂ also serves to activate CREB in lung epithelial cells. In addition, similar to H₂O₂-mediated ERK phosphorylation, CREB phosphorylation in response to H₂O₂ is regulated by extracellular Ca²⁺.

Ca²⁺ signaling has been implicated in the control of many cellular processes, including the activation of the CREB transcription factor (7). Although the CaMK pathway is often thought to be the primary Ca²⁺-mediated activator of CREB (29), we found that CREB phosphorylation after H₂O₂ exposure was not affected by blocking CaMKII activity, but was significantly reduced by inhibiting ERK1/2 signaling. These data are similar to the finding that H₂O₂ leads to CREB activation through the activation of ERK1/2 and p38 in vascular smooth muscle cells (28). Taken together, these data suggest that Ca²⁺ may be an important regulator of CREB through the ERK1/2 signaling pathway in lung epithelium exposed to oxidants.

CRE sequences are found in the promoters of many genes related to gene transcription, growth factors, and cell cycle (9). Modulation of *c-fos* is partially Ca²⁺- and CREB-dependent, suggesting that altered Ca²⁺ can affect expression of genes that contain a CRE (8). The mechanisms by which Ca²⁺-dependent signaling pathways regulate lung epithelial cell gene transcription

are not yet known. We show that reduction of CREB activity inhibits *c-fos* steady-state mRNA levels after H₂O₂ exposure, indicating that CREB plays an essential role in the regulation of oxidant-induced *c-fos* steady-state mRNA levels. Although c-Fos has been implicated in the signal transduction between ROS and proliferation (30), our data indicate that CREB activity does not play a significant role in H₂O₂-induced proliferation. In contrast to vascular smooth muscle cells, these data indicate that although CREB regulates *c-fos* steady-state mRNA levels, CREB activity is not necessary for H₂O₂-mediated proliferation in lung epithelial cells (31, 32).

Inhibition of CREB function resulted in the desensitization of lung epithelial cells to induction of apoptosis after extended exposure to high concentrations of H₂O₂. A small, but consistent increase in *bcl-2* transcript levels, when CREB function was suppressed, was also observed. These data thus support a role for CREB in the promotion of apoptosis after oxidant exposure. Although the observed effect on apoptosis is likely due to loss of CREB function, expression of the dominant-negative mutant has the potential to disrupt other signaling pathways not detected by the assays employed. These results are in contrast to findings in both neurons and vascular smooth muscle cells, where CREB plays a necessary role in growth factor-induced survival by stimulating expression of Bcl-2 through CREB. Furthermore, in those cells, reduction of CREB activity induces apoptosis, which can be rescued by overexpression of Bcl-2 (9, 33). The reasons for the discrepancies between lung epithelial cells, neurons, and smooth muscle cells are not clear, but in light of the multiple signaling pathways that regulate CREB, it is not surprising that different cell types and stimuli could lead to different physiologic outcomes.

In context of the findings of others, our data indicate that responses of lung epithelial cells to ROS include signaling

Figure 7. Reduction of CREB activity significantly reduces the number of cells undergoing apoptosis after H_2O_2 exposure. (A) C10 cells stably transfected with CREB133 or empty vector (Tag5a) were treated with 300 μM H_2O_2 for the indicated times and analyzed by immunofluorescence using an antibody specific for single-stranded DNA (Apostain). Nuclei were stained with YOYO-1. (B) Quantification of percent apoptosis (number of Apostain-positive cells/total number of cells) in response to H_2O_2 . * $P < 0.05$ when compared with control; # $P < 0.05$ when compared with Tag5a, 24 h, 300 μM H_2O_2 .

through Ca^{2+} - and ERK-dependent activation of CREB. Furthermore, subsequent changes in CREB-dependent gene expression play an integral role in oxidant-induced apoptosis. These data thus reveal an important role for CREB in oxidant-mediated signaling pathways in lung epithelial cells, and suggest a potential role for CREB in the development of pathologies related to oxidant-mediated lung injury.

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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