

# Protein kinase A-mediated CREB phosphorylation is an oxidant-induced survival pathway in alveolar type II cells

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**Abstract** Oxidant stress plays a role in the pathogenesis of pulmonary diseases, including fibrotic lung disease and cancer. We previously found that hydrogen peroxide ( $H_2O_2$ ) initiates an increase in  $Ca^{2+}$ /cAMP-response element binding protein (CREB) phosphorylation in C10 alveolar type II cells that requires activation of extracellular regulated kinases 1/2 (ERK1/2). Here, we investigated the role of crosstalk between protein kinase A (PKA) and epidermal growth factor receptor (EGFR) in oxidant-induced signaling to ERK1/2 and CREB in C10 cells. Application of  $H_2O_2$  increased nuclear accumulation of PKA, and inhibition of PKA with H89 reduced oxidant-mediated phosphorylation of both CREB and ERK1/2. Single cell measurements of cAMP and redox status, using a FRET-based biosensor and a redox-sensitive GFP, respectively, indicated that  $H_2O_2$  increases production of cAMP that correlates with redox state. Inhibition of EGFR activity decreased both  $H_2O_2$ -induced CREB phosphorylation and translocation of PKA to the nucleus, suggesting that crosstalk between PKA and EGFR underlies the oxidant-induced CREB response.

Furthermore, knockdown of CREB expression using siRNA led to a decrease in bcl-2 and an increase in oxidant-induced apoptosis. Together these data reveal a novel role for crosstalk between PKA, ERK1/2 and CREB that mediates cell survival during oxidant stress.

**Keywords** Pulmonary epithelium · Lung fibrosis · Oxidative stress · FRET biosensor · Transcription factor

## Abbreviations

CRE	$Ca^{2+}$ /cAMP-response element
CBP300	CREB binding protein 300
CREB	$Ca^{2+}$ /cAMP-response element binding protein
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases 1/2
$H_2O_2$	Hydrogen peroxide
GO	Glucose oxidase
JNK	c-Jun amino terminal kinase
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
bcl-2	B-cell lymphoma-2
TNF $\alpha$	Tumor necrosis factor $\alpha$

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## Introduction

Lung epithelial cells, a target cell of inhaled oxidants, are subjected to a variety of environmental stresses, including oxidizing gases, particulates, and airborne microorganisms. Although the formation of oxidants is normally well regulated, excessive production may cause inflammation and cellular injury. Numerous studies suggest a role for oxidant

stress in the pathogenesis of pulmonary diseases, including asthma, pulmonary fibrosis, and cancer [1].

Reactive oxygen species (ROS) are involved in multiple physiological processes through their capacity to regulate the expression of several growth factor receptors, protein kinases and phosphatases. In alveolar type II lung epithelial cells, hydrogen peroxide ( $H_2O_2$ ) induces tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) [2] and leads to activation of protein kinase C (PKC) [3] and the mitogen activated protein kinases (MAPK) p38, c-Jun amino-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2) [4, 5].  $H_2O_2$  also activates the expression of transcription factors, including  $Ca^{2+}$ /cyclic AMP-response element binding protein (CREB) [6] and members of the activator protein (AP-1) family, c-jun and c-fos [7], resulting in proliferation [4] and apoptosis [6, 8].

CREB is a 43 kDa transcription factor belonging to the basic-leucine zipper (bZIP) family and is regulated by phosphorylation at serine residue 133 [9, 10]. Phosphorylation of CREB initiates recruitment of co-factors to the  $Ca^{2+}$ /cAMP-response element (CRE) that are necessary for transcriptional activation such as CREB-binding protein (CBP300) [11]. CREB activation is regulated by both  $Ca^{2+}$  and cAMP, which have been shown to regulate both ERK1/2- and protein kinase A (PKA)-mediated CREB phosphorylation [6, 12, 13]. Many genes important for regulation of proliferation and apoptosis, including c-fos and bcl-2, respectively, contain CREs in their promoter [11].

Our previous studies have shown that exposure of alveolar type II cells to bolus  $H_2O_2$  or asbestos fibers results in CREB activation that is dependent on ERK1/2 activation [6, 14]. Furthermore, we found that preventing CREB activation promoted cell survival and enhanced bcl-2 transcription, revealing a potential role for CREB in  $H_2O_2$ -mediated apoptosis [6]. Because of known crosstalk between PKA, ERK1/2, and CREB in many cell types and the dose-dependent effects of  $H_2O_2$ , the goal of this study was to determine their relative importance in oxidant-induced signaling. The effects of transient bolus  $H_2O_2$  as well as a peroxide generating system were tested to represent a range of oxidant concentrations that lead to cell proliferation and/or apoptosis. The data support the hypothesis that PKA and EGFR are central regulators of oxidant-induced ERK1/2 and CREB activation and demonstrate a link between PKA, EGFR, and CREB in cell survival following exposure to  $H_2O_2$  in alveolar type II cells.

## Materials and methods

### Cell culture and treatments

C10 cells, a contact-inhibited, non-transformed murine alveolar type II epithelial cell line [15], were grown in

CMRL 1066 medium supplemented with L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (FBS) (GIBCO BRL, Rockville, MD). Cells were grown to 90% confluence, and 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 to the medium at concentrations from 100  $\mu$ M to 300  $\mu$ M. Recombinant glucose oxidase (GO; 5 mU/ml, 15 mU/ml, or 30 mU/ml) (Roche, Indianapolis, IN) was used to provide low level fluxes of  $H_2O_2$  [16, 17]. Forskolin and epidermal growth factor (EGF) (Sigma, St. Louis, MO) (10  $\mu$ M and 100 ng/ml, respectively) were used as positive controls for induction of phospho-CREB and phospho-EGFR, respectively. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Calbiochem, LaJolla, CA) was used at 0.1  $\mu$ g/ml to induce apoptosis. Control cultures received medium without agents and were treated identically.

The ERK1/2 inhibitor, U0126 (10  $\mu$ M for 30 min prior to treatment), the EGFR tyrosine kinase inhibitor, Tyrphostin AG1478 (10  $\mu$ M for 1 h prior to treatment), and the antioxidant enzyme, catalase (1000 U/ml 1 h prior to treatment) were obtained from Calbiochem. The PKA inhibitor, H89 (10  $\mu$ M for 1 h pre-treatment) was obtained from Biomol (Plymouth Meeting, PA). N-acetyl-L-cysteine (NAC; 10 mM 16 h prior to treatment) and DL-Buthionine-(S,R)-sulfoximine (BSO; 10  $\mu$ M 16 h prior to treatment) were purchased from Sigma (St. Louis, MO).

### Western blot analysis

After C10 cells were exposed to agents as described above, the cells were washed twice with cold PBS and collected in 4X sample buffer (200  $\mu$ M Tris, pH 6.8, 4% SDS, 4 mg/ml bromophenol blue, 0.04%  $\beta$ -mercaptoethanol, 40% glycerol, 2  $\mu$ M pyronin-Y). The amount of protein in each sample was determined using the RC/DC protein assay (Bio-Rad). About 30  $\mu$ g of protein was separated by a 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed as described previously [6] using antibodies specific to total and phosphorylated CREB (1:1000; rabbit polyclonal anti-CREB, Cell Signaling Technologies, Danvers, MA; 1:500; rabbit polyclonal anti-phospho-CREB, Cell Signaling Technologies), total and phosphorylated ERK1/2 (1:1000; rabbit polyclonal anti-ERK1/2, Cell Signaling Technologies; 1:500; rabbit polyclonal anti-phospho-ERK1/2, Cell Signaling Technologies). Antibody binding was detected using horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc., West grove, PA), followed by chemiluminescence (Kirkgaard and Perry Laboratories, Gaithersburg, MD). QuantityOne (Bio-Rad,

Hercules, CA) was used to quantify band density, and intensity of phospho-CREB bands were normalized to the intensity of the corresponding total CREB bands.

#### Live cell imaging of cAMP and redox potential

C10 cells grown on glass coverslips in 6-well tissue culture dishes were transiently transfected with 8  $\mu\text{g}$  of a plasmid encoding a unimolecular fluorescence resonance energy transfer (FRET)-based cAMP biosensor, Epac1-camps (provided by Dr. Martin Lohse, University of Würzburg) [18, 19], or 1  $\mu\text{g}$  of a plasmid encoding a redox-sensitive green fluorescent protein (GFP), roGFP2 (a gift from Dr. James Remington, University of Oregon) [20], using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The transfection efficiency was approximately 50%.

Imaging experiments were conducted after 48–72 h of transfection, and the growth medium removed and replaced with Krebs-Ringer bicarbonate solution (KRB; 119 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$  or 10 mM HEPES-NaOH (pH 7.40), and 2 mM glucose). Coverslips were placed into a heated microperfusion chamber mounted on the specimen stage of an inverted fluorescence microscope (Nikon TE-2000U) equipped with a CARV spinning disk confocal system (Atto Bioscience Inc., Rockville, MD). Cells were continuously superfused with Krebs-Ringer bicarbonate solution (2–5 ml/min) at 37°C. Dithiothreitol (DTT) (Calbiochem, LaJolla, CA) was used to calibrate the redox minimum.

For imaging Epac1-camps FRET and roGFP2, cells were visualized with a Nikon Super Fluor 40 $\times$  objective. MetaFluor/MetaMorph software was used for image acquisition and analysis (Universal Imaging). The Epac1-camps EYFP excitation wavelength was 440 nm. Dual emission ratio imaging at 485 nm and 535 nm for Epac1-camps and dual excitation ratio imaging at 400 nm and 490 nm for roGFP2 were accomplished using a computer-controlled high speed filter wheel (Lambda 10-2 optical filter changer with rotation every 60–80 ms, Sutter Instrument Co., Novato, CA). For roGFP2, a 505DRLP dichroic mirror and an emission filter, 535DF25, were used. Images (50–250 ms exposure) were captured every 10 s with a 16-bit Cascade 650 digital camera (Roper Instruments, Trenton, NJ) and background-corrected by manual selection of background regions [19].

The ratio values were normalized to the average baseline values measured 1-min prior to application  $\text{H}_2\text{O}_2$ . Epac1-camps FRET decreases with increasing cAMP concentration, thus increased  $[\text{cAMP}]_c$  is correlated with an increase in the relative emission ratio of ECFP/EYFP (485/535) [18]. Data were thus expressed as relative ratio 485/535 for Epac1-camps and fold increase in relative ratio 400/490 for roGFP2.

#### Immunofluorescence in C10 cells

C10 cells were grown on glass coverslips for all experiments. After experimental exposures, immunofluorescence to detect the catalytic subunit of PKA $\alpha$  was performed as previously described [6, 21]. Briefly, cells were washed with phosphate buffered saline (PBS), fixed in 3.7% formaldehyde, permeabilized with  $-20^\circ\text{C}$  methanol and incubated with blocking solution containing 2% bovine serum albumin (BSA) in PBS. Cells were incubated with primary antibody (1:100; rabbit polyclonal PKA $\alpha$  catalytic subunit antibody, Santa Cruz, Santa Cruz, CA) diluted in 2% BSA plus 0.1% Triton X-100 in PBS (BSA/PBS-T) overnight at 4°C. Secondary antibody (1:400; AlexaFluor 568 goat-anti-rabbit IgG, Molecular Probes) diluted in BSA/PBS-T was applied for 1 h at room temperature (RT), and followed by incubation with nuclear counterstain, YOYO-1 iodide (Molecular Probes, Carlsbad, CA; 1:10,000), 1 unit/ml RNase, and 0.1% sodium azide in BSA/PBS-T for 30 min at RT. Coverslips were mounted onto slides with AquaPolyMount (Polysciences, Inc. Warrington, PA). For each sample, confocal images were collected in fluorescence modes using a Bio-Rad MRC1024ES confocal scanning laser microscope (Bio-Rad, Hercules, CA). For quantification, a nuclear mask was generated in Corel Photopaint using the YOYO-1 image template, and pixel intensities for PKA $\alpha$  were determined within the nuclear mask area as previously described [22].

#### Transient transfections with small interfering RNA (siRNA)

The siCONTROL non-targeting siRNA #2 and SMART-pool mouse CREB siRNA (100 nM; Dharmacon, Lafayette, CO) were transfected into C10 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. After 4 h in transfection media, FBS was added to 10% and cells were incubated for 24 h. Cells were then transferred to reduced serum media (0.5% FBS) for 24 h prior to treatments.

#### Real time quantitative PCR (RT qPCR)

Total RNA was extracted from C10 cells using the RNeasy<sup>TM</sup> PLUS protocol for total RNA isolation from animal cells (Qiagen, Valencia, CA). cDNA was reverse transcribed from 500 ng total RNA using an Omniscript<sup>TM</sup> Reverse Transcriptase RNase free DNase kit (Qiagen), with an oligo dT primer, according to the manufacturer's protocol. RT qPCR primers and probes for B-cell lymphoma-2 (bcl-2) and c-fos were obtained as Assays-on-Demand<sup>TM</sup> kits from Applied Biosystems (Foster City,

CA). PCR products were detected by TaqMan qPCR, as previously described [23]. Expression levels of target genes were determined using hypoxanthine-guanine phosphoribosyl transferase (*hprt*) as the internal standard. Samples were run in duplicate from 3 independent experiments and the comparative Ct (cycle threshold) method for relative quantity (RQ value) was used to calculate relative mRNA expression among samples.

#### Detection and quantification of apoptosis

Apoptosis was detected by measuring single-stranded DNA using Apostain<sup>TM</sup> as previously described [24]. Briefly, cell monolayers grown on glass coverslips were treated with 30 mU/ml GO for 24 h, then fixed in methanol for 24 h at  $-20^{\circ}\text{C}$ , boiled for 5 min in PBS containing 5 mM  $\text{MgCl}_2$ , and then immersed in ice-cold PBS for 10 min. Cells were blocked with 40% FBS and then incubated with Apostain F7-26 (Alexis Biochemicals, San Diego, CA, 10  $\mu\text{g}/\text{ml}$ ) followed by HRP-conjugated secondary antibody (goat anti-mouse IgM; Jackson Laboratories, West Grove, PA, 1:400). To visualize secondary antibody binding, the peroxidase substrate DAB (Sigma) was used. Coverslips were mounted onto slides with AquaPolyMount (Polysciences, Inc. Warrington, PA) for subsequent examination using bright field light microscopy. To determine the numbers of apoptotic cells and total cell numbers per field, 5 random fields were evaluated per experimental condition at x200 total magnification.

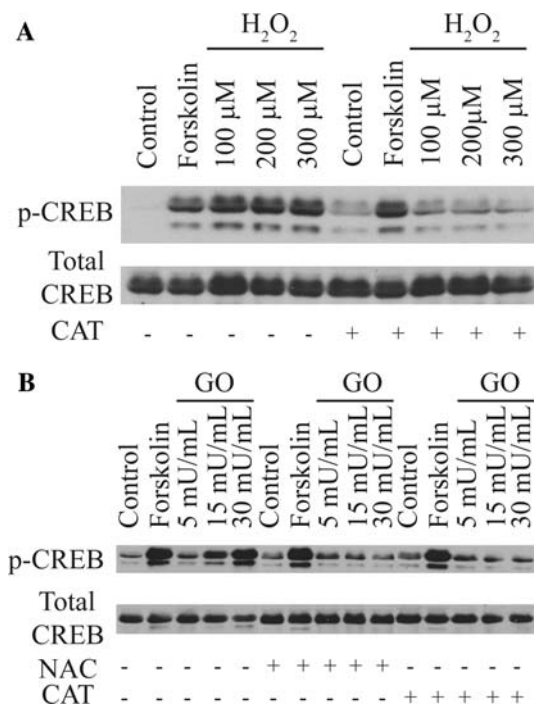
#### Statistical analysis

Statistical analyses were performed using non-normalized data, and pair-wise comparisons between treatment groups were achieved using Student's *t*-test or Mann–Whitney Rank Sum Test method for unequal variances. ANOVA (Holm–Sidak method) was used for multiple comparisons. Differences were considered statistically significant at  $P < 0.05$ .

## Results

### CREB is phosphorylated after exposure to $\text{H}_2\text{O}_2$ and glucose oxidase

We have previously shown that  $\text{H}_2\text{O}_2$  causes CREB phosphorylation in alveolar type II cells [6]. Here, we further show by Western blot that the CREB response to bolus addition of  $\text{H}_2\text{O}_2$  (Fig. 1a) and  $\text{H}_2\text{O}_2$  generated by glucose oxidase (GO) (Fig. 1b) is reduced when pretreated with the antioxidants NAC or catalase at effective concentrations documented previously in C10 cells [25].

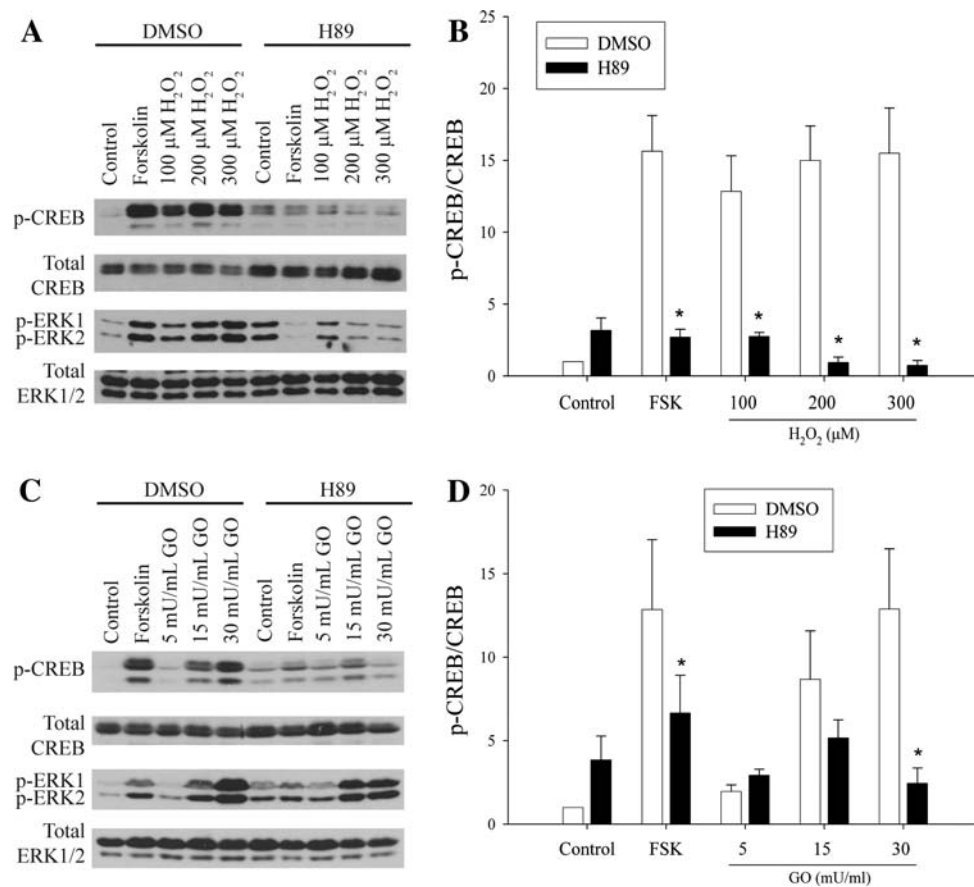


**Fig. 1** Bolus and glucose oxidase generated  $\text{H}_2\text{O}_2$  mediate CREB phosphorylation in an oxidant-dependent manner. **(a)** C10 cells, pre-incubated with 1,000 U/ml catalase (CAT) for 1 h, were treated with 10  $\mu\text{M}$  forskolin or 100, 200, or 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 10 min, and then analyzed by Western blot analysis for phospho-CREB (p-CREB). **(b)** C10 cells, pre-incubated with 10 mM NAC for 16 h or 1,000 U/ml catalase (CAT) for 1 h, were treated with 10  $\mu\text{M}$  forskolin or 5, 15, or 30 mU/ml glucose oxidase (GO) for 4 h, and then analyzed by Western blot analysis for phospho-CREB (p-CREB). An antibody recognizing total CREB was used as a control for protein loading in A and B. Data are representative of 3 separate experiments

### $\text{H}_2\text{O}_2$ -induced CREB phosphorylation is decreased after the reduction of PKA activity

Because PKA is an important regulator of CREB [9], we investigated its role in  $\text{H}_2\text{O}_2$ -induced CREB phosphorylation. Cells were examined after exposure to lower concentrations previously associated with expression of cyclin D (100  $\mu\text{M}$ ) and higher concentrations known to induce apoptosis (300  $\mu\text{M}$ ) [6, 26]. Exposure to  $\text{H}_2\text{O}_2$  led to CREB phosphorylation in a concentration-dependent manner that was significantly decreased after reduction of PKA activity using the specific inhibitor H89 (Fig. 2a, b). The pattern of CREB phosphorylation in response to  $\text{H}_2\text{O}_2$  closely paralleled that seen in response to activation of PKA by forskolin. H89 also reduced  $\text{H}_2\text{O}_2$ -mediated ERK1/2 activation, suggesting that PKA may regulate CREB activation directly or indirectly through ERK1/2-mediated CREB activation.

Similar to results with bolus addition of  $\text{H}_2\text{O}_2$ ,  $\text{H}_2\text{O}_2$  generated by GO stimulated CREB phosphorylation in a dose-dependent manner and the phosphorylation was



**Fig. 2** H<sub>2</sub>O<sub>2</sub>-mediated CREB activation is inhibited by the PKA inhibitor, H89. (a) C10 cells, pre-incubated with 10 μM H89 for 1 h, were treated with 10 μM forskolin or indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min, and then analyzed by Western blot analysis for phospho-CREB (p-CREB), total CREB, phospho-ERK1/2 (p-ERK1/2) and total ERK1/2. (b) Quantification of p-CREB band intensities from A corrected using the corresponding CREB band intensity and normalized to the untreated control. Data represent 4 separate experiments; \**P* < 0.05 when compared with corresponding condition

without H89. (c) C10 cells, pre-incubated with 10 μM H89 for 1 h, were treated with 10 μM forskolin or indicated concentrations of glucose oxidase (GO) for 4 h, and then analyzed by Western blot for phospho-CREB (p-CREB), total CREB, phospho-ERK1/2 (p-ERK1/2), and total ERK1/2. (d) Quantification of p-CREB band intensities from C corrected using the corresponding CREB band intensity and normalized to the untreated control. Data represent mean ± SEM from 4 separate experiments; \**P* < 0.05 when compared with corresponding condition without H89

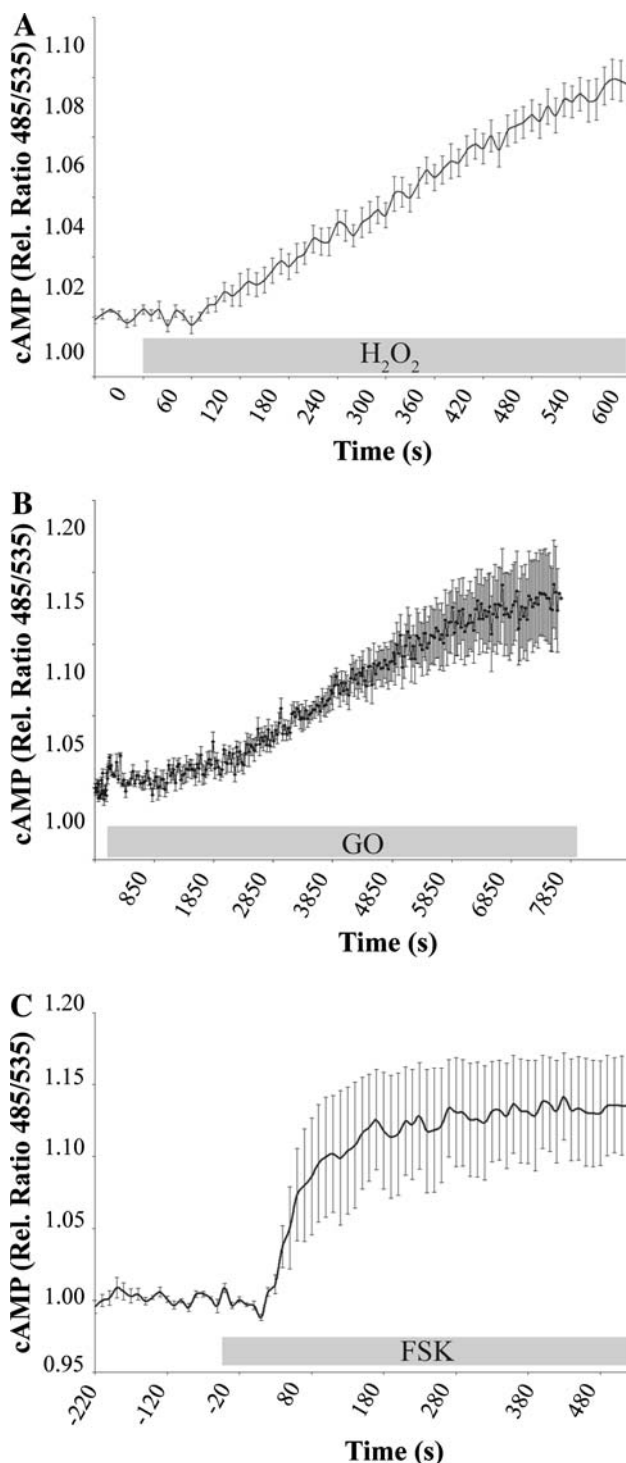
significantly reduced by PKA inhibition at higher concentrations of GO (Fig. 2c, d). However, unlike bolus addition, H<sub>2</sub>O<sub>2</sub> generated by GO led to a dose-dependent increase in ERK1/2 phosphorylation that was marginally sensitive to inhibition of PKA activity, suggesting that the duration of the stimulus may cause differences in signaling patterns.

#### H<sub>2</sub>O<sub>2</sub> stimulates cAMP production in C10 cells

Single cell imaging was utilized to determine whether H<sub>2</sub>O<sub>2</sub> exerts a direct effect on upstream regulation of PKA through production of cAMP. Epac1-camps, a FRET-based cAMP biosensor, was expressed in C10 cells. Epac1-camps senses changes in cytoplasmic cAMP levels ([cAMP]<sub>c</sub>) through a cAMP-dependent conformational change resulting in reduced FRET between ECFP (485 emission) and EYFP (535 emission) [18, 19]. Exposure to H<sub>2</sub>O<sub>2</sub> caused a

monophasic increase in [cAMP]<sub>c</sub> (Fig. 3a) that was comparable to the response elicited by direct activation of adenylyl cyclase with forskolin, but with a slower rise time (Fig. 3c). H<sub>2</sub>O<sub>2</sub> generated by GO stimulated a rise in [cAMP]<sub>c</sub> similar in magnitude to bolus addition of H<sub>2</sub>O<sub>2</sub>, but with a longer lag-time presumably due to the slower generation of oxidant (Fig. 3b).

Single cell imaging was also used to measure redox status and correlate cAMP levels with redox status. The redox sensitive GFP variant, roGFP2, was expressed in C10 cells. Oxidation is detected by roGFP through a change in GFP excitation from 400 nm and 490 nm [20]. The time course of H<sub>2</sub>O<sub>2</sub>-mediated cAMP production correlated well with the level of intracellular oxidation measured by roGFP2 (Fig. 4a). Upon removal of H<sub>2</sub>O<sub>2</sub>, the redox state returned to baseline approximately 36 min after the initial exposure (Fig. 4b).



**Fig. 3**  $\text{H}_2\text{O}_2$  stimulates an increase in cAMP levels. C10 cells were transiently transfected with the cAMP FRET indicator Epac1-camps and then exposed to  $10\ \mu\text{M}$   $250\ \mu\text{M}$   $\text{H}_2\text{O}_2$  (a),  $15\ \text{mU/ml}$  glucose oxidase (GO) (b), or  $10\ \mu\text{M}$  forskolin (FSK) (c) for the times indicated. Data (means  $\pm$  SEM) are expressed as the relative ratio (485/535) of the cAMP response from the live emission recording of a representative cell. Grey bars indicate time of addition and length of exposure. Results are representative of  $\geq 9$ , 5, and 4 cells (A, B and C, respectively)

EGFR tyrosine kinase activity is important for signaling from  $\text{H}_2\text{O}_2$  to PKA and CREB

It has been shown that the protein tyrosine activity of EGFR is important for EGF-mediated stimulation of adenylyl cyclase [27]. Thus to determine the contributions of EGFR in the observed  $\text{H}_2\text{O}_2$ -induced CREB phosphorylation, cells were evaluated with or without pretreatment with the specific EGFR tyrosine kinase inhibitor, AG1478. Application of AG1478 significantly reduced CREB phosphorylation in response to bolus addition  $\text{H}_2\text{O}_2$  and to  $\text{H}_2\text{O}_2$  generated by higher concentrations of GO (Fig. 5), suggesting that EGFR activity is involved in  $\text{H}_2\text{O}_2$ -induced CREB phosphorylation. As expected, reduction of EGFR activity also inhibited  $\text{H}_2\text{O}_2$ -induced ERK phosphorylation (Fig. 5).

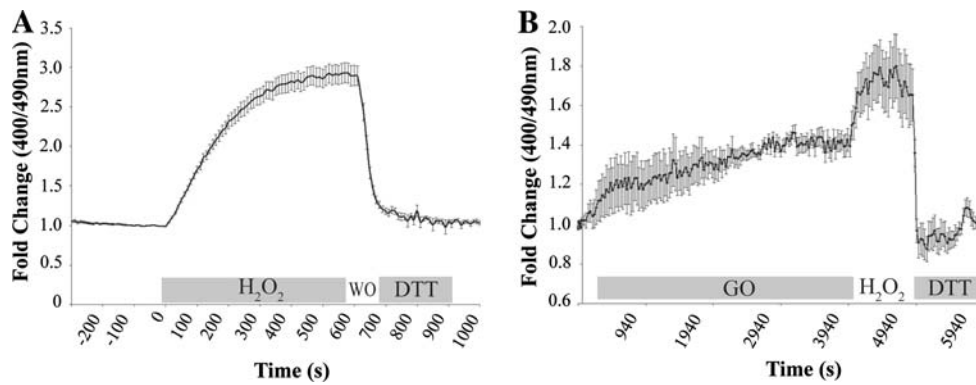
Immunofluorescence evaluation of activated PKA translocation to the nucleus was used to more directly test the effects of  $\text{H}_2\text{O}_2$  on PKA activation and to further evaluate a role for EGFR. Exposure to  $\text{H}_2\text{O}_2$  led to an increase in the PKA free catalytic subunit that was significant in the nucleus at  $300\ \mu\text{M}$  and similar to the response to forskolin. Inhibition of EGFR tyrosine kinase activity with AG1478 prevented the  $\text{H}_2\text{O}_2$ -induced increases at both  $200\ \mu\text{M}$  and  $300\ \mu\text{M}$  (Fig. 6).

Direct activation of CREB by PKA is a minor pathway in EGF-induced CREB phosphorylation

Because of the known crosstalk between EGFR, ERK1/2, and PKA, we examined the role of PKA and ERK1/2 in forskolin- and EGF-induced CREB activation. Treatment with both forskolin and EGF resulted in a significant increase in CREB phosphorylation when compared to control cells (Fig. 7). Inhibition of PKA activity with H89 significantly decreased forskolin-induced CREB phosphorylation, but only blunted EGF-induced CREB phosphorylation. Inhibition of ERK1/2 phosphorylation with the MEK inhibitor, U0126, also considerably decreased CREB phosphorylation. The inhibitory effects of H89 and U0126 were not additive towards either forskolin- or EGF-induced CREB phosphorylation, suggesting that direct activation of CREB through PKA may play a minor role in the activation of CREB. Furthermore, both forskolin and EGF induced ERK1/2 phosphorylation, but only forskolin-mediated CREB phosphorylation was inhibited by H89, suggesting that PKA regulates CREB indirectly through the MAPK/ERK1/2 pathway.

Knockdown of CREB using siRNA results in loss of oxidant-induced c-fos and bcl-2 transcription

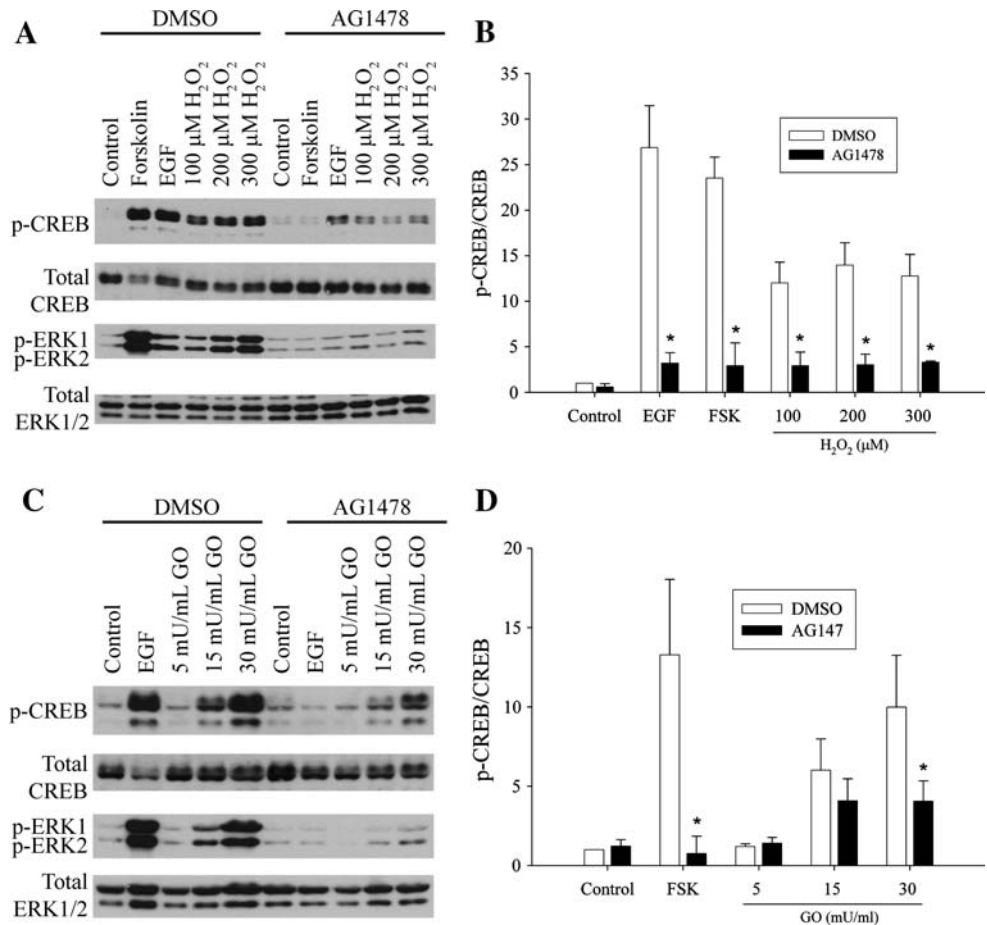
To more directly assess the role of CREB in oxidant-mediated responses, an siRNA approach was used to reduce levels of CREB within C10 cells. Transfection with siCREB resulted



**Fig. 4** Redox status correlates with cAMP level in cells responding to H<sub>2</sub>O<sub>2</sub>. C10 cells were transiently transfected with the redox indicator, roGFP2 and then exposed to 250 μM H<sub>2</sub>O<sub>2</sub> for 10 min followed by 1 mM DTT (a) or 15 mU/ml glucose oxidase (GO) for 65 min, followed by 200 μM H<sub>2</sub>O<sub>2</sub>, and then 10 mM DTT (b). Data

(means ± SEM) are expressed as the fold change in the excitation ratio at 400/490 nm normalized to the average base-line ratio values measured 1 min before stimulation. Results are averages of ≥23 and 11 cells (A and B, respectively). Grey bars indicate time of addition and length of exposure, and WO indicates wash out with KREBs buffer

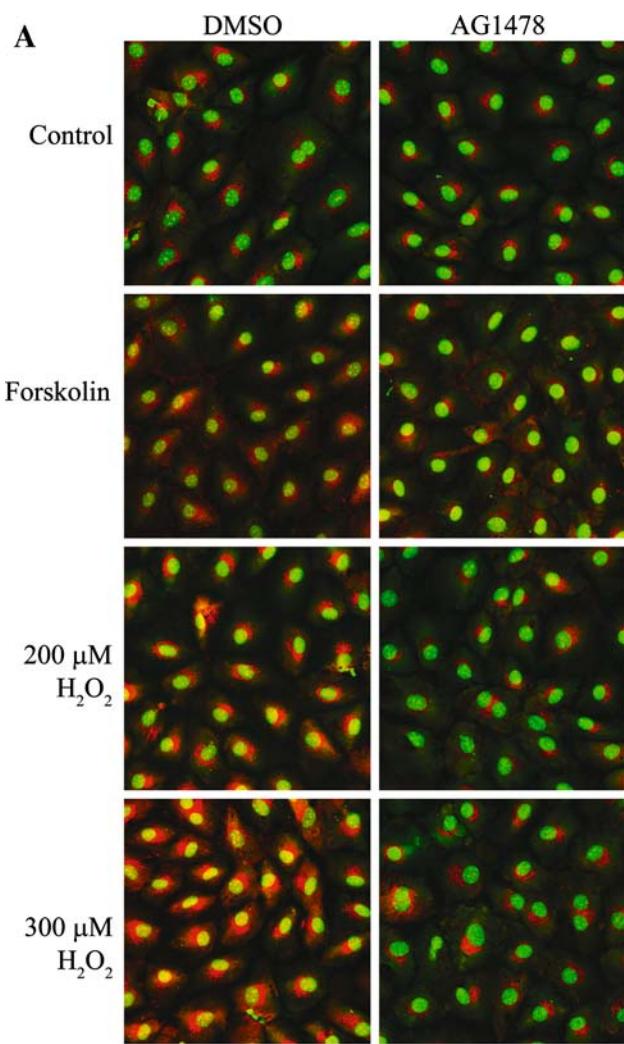
**Fig. 5** Reduction of EGFR tyrosine kinase activity leads to the inhibition of both H<sub>2</sub>O<sub>2</sub>-induced CREB and ERK1/2. C10 cells, pre-incubated with the EGFR tyrosine kinase inhibitor AG1478 (10 μM) for 1 h, were treated with 10 μM forskolin or 100 ng/ml EGF for 5 min, the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min (a) or glucose oxidase (GO) for 4 h (e), and then analyzed by Western blot analysis for phospho-CREB (p-CREB), total CREB, phospho-ERK1/2 (p-ERK1/2), and total ERK1/2. (b, d) Quantification of p-CREB band intensities from A and C respectively corrected using the corresponding CREB band intensity and normalized to the untreated control. Data represent mean ± SEM of 3 separate experiments; \*P < 0.05 when compared with corresponding condition without AG1478



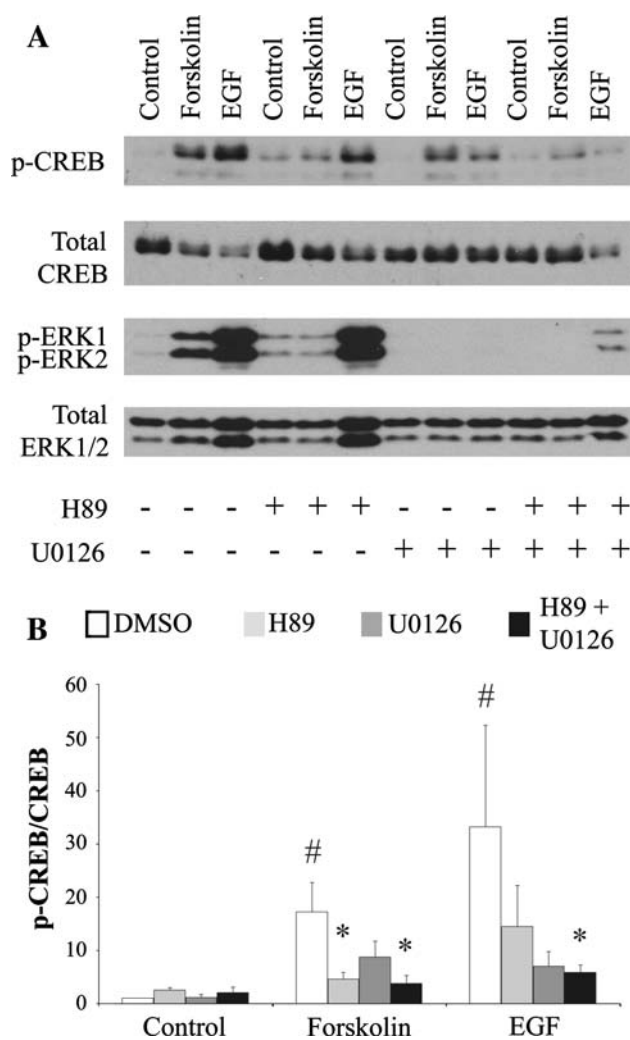
in greater than 80% knockdown of both CREB mRNA and protein levels (Fig. 8a, b). CRE-containing genes previously shown to be upregulated by H<sub>2</sub>O<sub>2</sub> including bcl-2 and c-fos were found to be dose-dependently induced by GO. Induction was significantly reduced in cells transfected with siCREB (Fig. 8c, d), suggesting that CREB is essential for the transcriptional response of these genes to H<sub>2</sub>O<sub>2</sub>.

Knockdown of CREB significantly increases the number of cells undergoing apoptosis following exposure to glucose oxidase

Recently, we have shown that reduction of CREB activity, by introducing a phosphorylation-null CREB construct, led to a decrease in apoptosis [6]. To resolve whether CREB is



**Fig. 6** Reduction of EGFR tyrosine kinase activity inhibits the H<sub>2</sub>O<sub>2</sub>-induced nuclear translocation of activated PKA. C10 cells, pre-incubated with 10 μM AG1478 for 1 h, were exposed to 10 μM forskolin and H<sub>2</sub>O<sub>2</sub> for the indicated concentrations for 10 min. (a) The PKA free catalytic subunit (red) was detected by immunofluorescence and nuclei were stained with YOYO-1 (green). (b) Quantification of PKA immunofluorescence pixel intensity within nuclei; \**P* < 0.05 compared to control, <sup>#</sup>*P* < 0.05 compared to treatment, n = 3 experiments, 5 fields/experiment



**Fig. 7** Crosstalk between PKA and ERK1/2 signaling is important in regulating CREB phosphorylation. (a) C10 cells, pre-incubated with 10 μM H89, U0126, or H89 and U0126 for 1 h, were treated with 10 μM forskolin for 10 min or 100 ng/ml EGF for 5 min, and then analyzed by Western blot analysis for phospho-CREB (p-CREB), total CREB, phospho-ERK1/2 (p-ERK1/2), and total ERK1/2. (b) Quantification of phospho-CREB in A corrected using CREB intensities and phospho-ERK1/2 in A corrected using ERK1/2 intensities; \**P* < 0.05 for DMSO treatment compared to AG1478 treatment; <sup>#</sup>*P* < 0.05 for DMSO control compared to treatment. Data represent mean ± SEM of 3 separate experiments

essential for H<sub>2</sub>O<sub>2</sub>-induced apoptosis, C10 cells transfected with scrambled siRNA (siControl) or siCREB were treated with 30 μM GO for 8 h and apoptotic cells were identified by Apostain (Fig. 9). H<sub>2</sub>O<sub>2</sub> generated from GO induced a significant increase in the percentage of cells undergoing apoptosis compared to the control in both siControl and siCREB cells. Cells expressing siCREB had significantly higher levels of apoptosis than the siControl cells in both the untreated condition and after GO exposure, supporting a role for CREB in the regulation of apoptosis induced by H<sub>2</sub>O<sub>2</sub>. Interestingly, the apoptotic



**Fig. 8** Knockdown of CREB using siRNA results in loss of oxidant-induced c-fos and bcl-2 transcription. (a) C10 cells were transfected with 100 nM scrambled siRNA (siControl) or siCREB followed by Western blot analysis to detect CREB.  $\beta$ -actin was used as a loading control. (b) Cells were transfected as in A, followed by treatment with 5 or 15 mU/ml glucose oxidase (GO) for 6 h. Total RNA was extracted and cDNA was assessed by RT qPCR to quantify CREB, bcl-2 and c-fos mRNA levels. Shown are relative quantity (RQ) values normalized to the siControl sample.  $^{\#}P < 0.05$  when compared to untreated siCon;  $^*P < 0.05$  when compared to treatment siCon

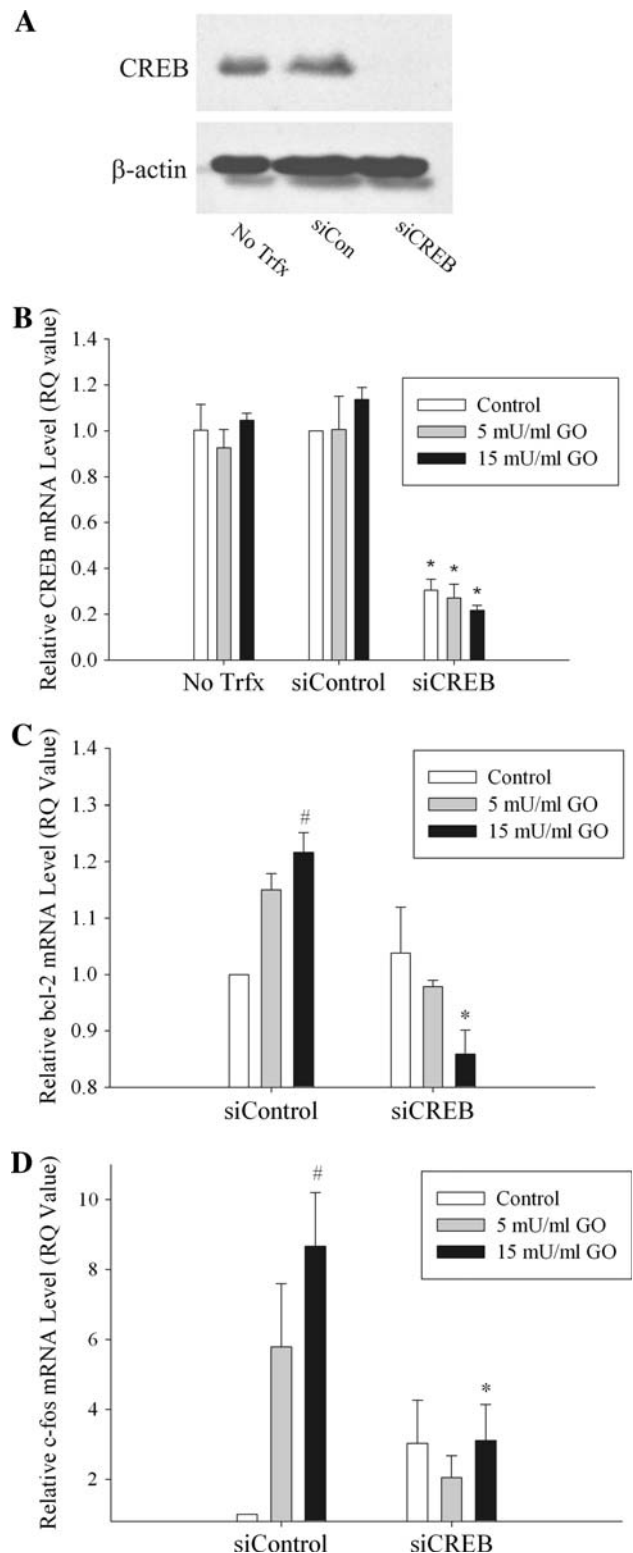
enhancing effect was not selective for  $H_2O_2$ -mediated apoptosis, as siCREB also enhanced the apoptotic effect of TNF $\alpha$ . Taken together, these results suggest that CREB plays an important role in the cellular response to oxidant stress including upregulation of survival genes and promoting cell survival.

## Discussion

Exposure to various environmental stresses, such as oxidative gases, metals, and particulates, initiates multiple physiological processes in alveolar type II cells. Oxidants have been implicated in the pathogenesis of lung cancer, pulmonary fibrosis, and asthma through their capacity to regulate an intricate network of protein kinases. Yet the relationship between oxidants and the pathogenesis of lung disease is unclear, primarily because of the lack of understanding of the mechanisms by which oxidants function in both normal physiological and disease states. In this study, we show for the first time that  $H_2O_2$  exposure leads to an increase in cytoplasmic cAMP levels, followed by PKA-dependent CREB and ERK1/2 phosphorylation in alveolar type II cells. Furthermore, we show that EGFR activity is necessary for  $H_2O_2$ -stimulated nuclear accumulation of activated PKA and for PKA-mediated CREB phosphorylation. Together these data suggest that PKA is a central hub for interplay between EGFR, ERK1/2, and CREB signaling pathways activated by oxidant stress.

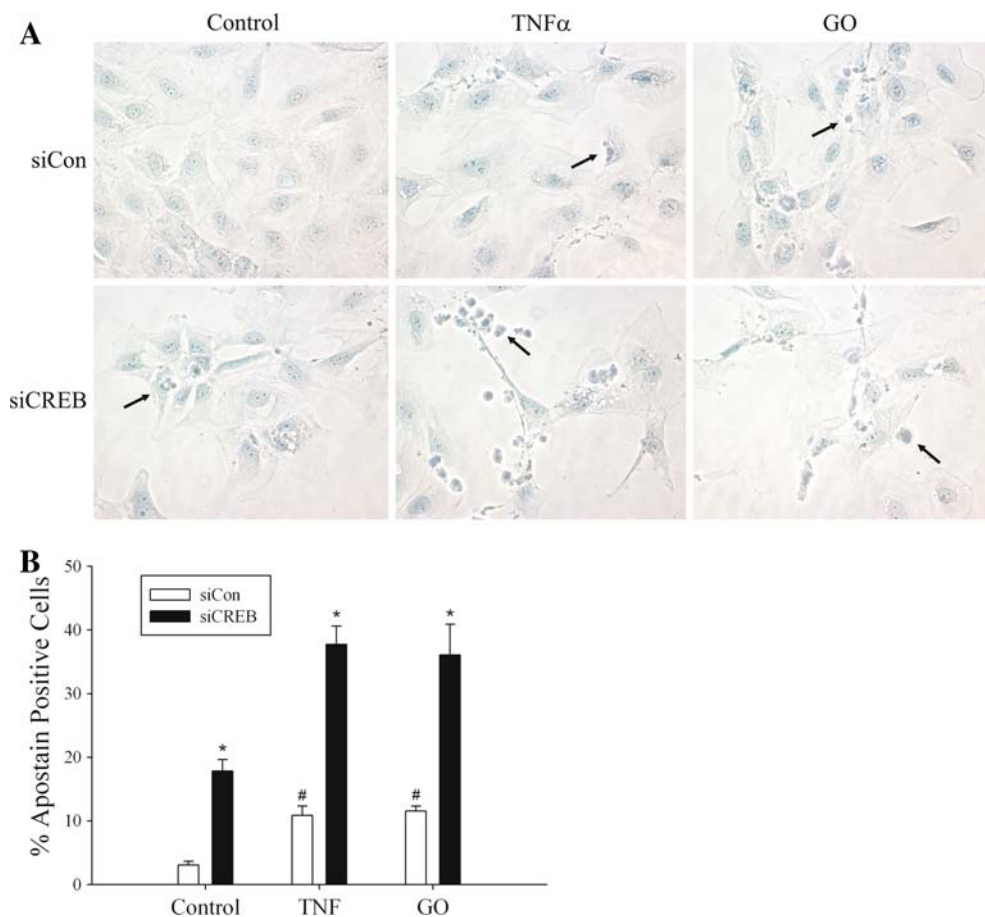
Several studies have shown crosstalk between PKA and ERK1/2 signaling pathways. This crosstalk appears to be cell type specific, since an increase in cAMP-dependent PKA activation stimulates ERK1/2 in some cells, but suppresses it in others [28–30]. We have previously demonstrated that  $H_2O_2$  exposure leads to ERK1/2-dependent CREB phosphorylation in C10 alveolar type II cells [6]. Studies here have dissected the underlying mechanisms to reveal that  $H_2O_2$ -induces cAMP formation and PKA activation that are important for both ERK1/2 and CREB phosphorylation.

EGFR is overexpressed and activated in response to epithelial injury [31]. Its activation is believed to play an integral role in the cellular responses of lung epithelium to injury and oxidant stress, possibly by signal amplification



through MAPK phosphorylation cascades. Oxidative modification of a reduced cysteine residue in the EGFR reversibly affects its activation [32, 33]. Moreover, Goldkorn and colleagues have shown that in alveolar type II cells,  $H_2O_2$  induces EGFR phosphorylation on tyrosine

**Fig. 9** CREB is protective against oxidant-induced apoptosis. **(a)** C10 cells, transfected with siControl or siCREB, were exposed to 30 mU/ml glucose oxidase (GO) or 0.1  $\mu$ g/ml tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) for 8 h followed by identification of apoptotic cells using Apostain. **(b)** Quantification of % Apostain positive cells was determined by scoring the average of 5 fields/condition; \* $P < 0.05$  when compared to untreated siCon; # $P < 0.05$  when compared to treatment siCon. Data represent mean  $\pm$  SEM of 4 separate experiments. Bar = 100  $\mu$ m



residues [2]. These findings suggest that even though there is no specific receptor for oxidants, their signaling may be efficiently transduced through interactions with the EGFR.

In the present study, we show that EGFR activity is necessary for both activation of PKA nuclear translocation and subsequent CREB phosphorylation in response to H<sub>2</sub>O<sub>2</sub>. Mitogenic signals transmitted through the EGFR have been shown to involve PKA-dependent signaling cascades. Tyrosine phosphorylation of the EGFR requires an increase in cAMP levels and PKA function in many, but not all cell types [34, 35]. In addition, tyrosine kinase activity of EGFR is required for growth factor stimulation of adenylyl cyclase activity, which leads to the activation of PKA [27, 36]. PKA activity has also been shown to promote downstream Raf-1 function and subsequent ERK activation [37]. Crosstalk has also been seen from EGFR to adenylyl cyclase activity and PKA in airway epithelial cells via beta adrenergic receptors [38, 39]. Thus, communication between EGFR and PKA is potentially bi-directional.

The H<sub>2</sub>O<sub>2</sub>-mediated generation of cAMP and the CREB activation response to H<sub>2</sub>O<sub>2</sub> increased with oxidant concentration but, with minor exception, were not dependent on whether oxidant was added by bolus or generated by glucose/glucose oxidase. Although reduction of the CREB

response by PKA or EGFR inhibition was only significant for the higher oxidant concentrations, a trend was observed with lower concentrations. While it is difficult to accurately predict the physiological significance of these oxidant levels, when put in context with previous findings, these observations suggest that CREB signaling through PKA and EGFR could participate in signaling that affects both oxidant-mediated proliferation and apoptosis.

Previously we demonstrated that expression of phosphorylation-incompetent CREB paradoxically increases bcl-2 transcription and reduces apoptosis following bolus H<sub>2</sub>O<sub>2</sub> exposure [6]. Findings here show that siRNA-mediated knockdown of CREB results in a loss of bcl-2 mRNA and an increase in apoptosis in cells responding to an H<sub>2</sub>O<sub>2</sub> generating system. The difference is likely due to the method of CREB inhibition, in that the dominant-negative CREB may have altered chromatin interactions with other transcription factors or caused quenching of the response. Putting these results in context with the findings of others, we propose that CREB activation through PKA signaling serves as a key survival pathway in cells responding to oxidant stress. PKA- and CREB-mediated gene expression may thus be important in regulating oxidant-mediated apoptosis resulting from lung pathologies, and future

experiments will explore the importance of these signaling pathways in promoting changes in gene expression following lung epithelial cell injury or oxidant exposure.

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