Am J Physiol Lung Cell Mol Physiol 282: L1349–L1357, 2002.
First published January 18, 2002; 10.1152/ajplung.00338.2001.

Bleomycin upregulates expression of γ-glutamylcysteine synthetase in pulmonary artery endothelial cells

REGINA M. DAY,[1] YUICHIRO J. SUZUKI,[2] JULIE M. LUM,[1]
ALEXANDER C. WHITE,[1] AND BARRY L. FANBURG[1]
[1]*Pulmonary and Critical Care Division, Tupper Research Institute, New England Medical Center; and* [2]*Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts 02111*

Received 27 August 2001; accepted in final form 9 January 2002

Day, Regina M., Yuichiro J. Suzuki, Julie M. Lum, Alexander C. White, and Barry L. Fanburg. Bleomycin upregulates expression of γ-glutamylcysteine synthetase in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 282: L1349–L1357, 2002. First published January 18, 2002; 10.1152/ajplung.00338.2001.—The chemotherapeutic agent bleomycin induces pulmonary fibrosis through the generation of reactive oxygen species (ROS), which are thought to contribute to cellular damage and pulmonary injury. We hypothesized that bleomycin activates oxidative stress response pathways and regulates cellular glutathione (GSH). Bovine pulmonary artery endothelial cells exposed to bleomycin exhibit growth arrest and increased cellular GSH content. γ-Glutamylcysteine synthetase (γ-GCS) controls the key regulatory step in GSH synthesis, and Northern blots indicate that the γ-GCS catalytic subunit [γ-GCS heavy chain (γ-GCS_h)] is upregulated by bleomycin within 3 h. The promoter for human γ-GCS_h contains consensus sites for nuclear factor-κB (NF-κB) and the antioxidant response element (ARE), both of which are activated in response to oxidative stress. Electrophoretic mobility shift assays show that bleomycin activates the transcription factor NF-κB as well as the ARE-binding factors Nrf-1 and -2. Nrf-1 and -2 activation by bleomycin is inhibited by the ROS quenching agent *N*-acetylcysteine (NAC), but not by U-0126, a MEK1/2 inhibitor that blocks bleomycin-induced MAPK activation. In contrast, NF-κB activation by bleomycin is inhibited by U-0126, but not by NAC. NAC and U-0126 both inhibit bleomycin-induced upregulation of γ-GCS expression. These data suggest that bleomycin can activate oxidative stress response pathways and upregulate cellular GSH.

reactive oxygen species; Nrf-1 and -2; nuclear factor-κB; antioxidant response element; mitogen-activated protein kinase

BLEOMYCIN IS PRIMARILY USED for the treatment of testicular carcinoma, lymphoma, and squamous cell carcinoma, but its use as a chemotherapeutic agent is limited by adverse side effects, especially pulmonary injury and fibrosis. Because of this, bleomycin has been used to develop a rodent experimental model of pulmonary fibrosis that has allowed the study of changes in the cellular composition of the lung and the expression of specific proteins leading to fibrosis. Many changes identified in the bleomycin model system have also been observed in pulmonary fibrosis induced by other causes (2, 20, 21, 30). Treatment of rodents with bleomycin endotracheal instillation or subcutaneous injection results in initial pulmonary inflammation and a spike of epithelial/endothelial apoptosis (13, 30, 38, 46). This is followed by the proliferation of myofibroblasts (fibroblasts expressing α-smooth muscle actin) and sustained epithelial/endothelial apoptosis. The bleomycin model has allowed the identification of potentially critical changes in protein expression, including the induction of transforming growth factor-β1 (18, 26, 27, 29, 30). However, the signaling mechanism(s) inducing these changes is not well understood.

Bleomycin triggers apoptosis in growing cells by causing single- and double-stranded DNA breaks through the direct binding of bleomycin to DNA. This activity is dependent on oxygen and a bound ferrous ion. Bleomycin is also believed to produce reactive oxidative species (ROS), including superoxide, H_2O_2, and/or organoperoxides, which may also play a role in the toxicity of bleomycin (34). Several studies have shown that cells are partially protected from the cytotoxic effects of bleomycin by acute hypoxia (thus eliminating the oxygen source for ROS) or by the addition of superoxide dismutase enzyme or transfection with an expression vector for superoxide dismutase (thus reducing the level of ROS generated by bleomycin) (10, 34). In contrast, the detrimental effects of bleomycin can be intensified via reduction of the intrinsic cellular defenses against oxidative stress. Either buthionine sulfoximine (BSO)-induced depletion of cellular glutathione (GSH) or the lack of GSH *S*-transferase (a phase II detoxifying enzyme) results in hypersensitivity of cells to bleomycin-induced apoptosis (12, 31).

On the basis of studies indicating that bleomycin cytotoxicity may be modulated by oxidants and antioxidants, we hypothesized that bleomycin may regulate cellular defenses against oxidative stress. GSH is a

Address for reprint requests and other correspondence: R. M. Day, New England Medical Center, Pulmonary and Critical Care Division, NEMC #257, 750 Washington St., Boston, MA 02111 (E-mail: rday@lifespan.org).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ubiquitous sulfhydryl-containing tripeptide that serves as a primary biological defense against oxidative damage (6, 33, 37, 44). GSH, normally present in cells at millimolar levels, directly interacts with ROS and toxins during cellular detoxification (6, 37, 44). Adaptation of cells to oxidative stress can occur via the induction of antioxidant enzymes and increased cellular levels of GSH. The enzyme γ-glutamylcytsteine synthetase (γ-GCS) controls the rate-limiting step in GSH synthesis (16, 17, 24). This enzyme is composed of two subunits, a catalytic heavy chain (γ-GCS_h) and a regulatory light chain. Both γ-GCS subunits have been shown to be upregulated in response to oxidative stress, including ionizing radiation, ROS, heavy metals, phenolic antioxidants, and the GSH-depleting compound BSO (16, 17, 32, 33, 36, 41, 44). The regulation of the γ-GCS subunits by oxidative stress has been demonstrated in variety of species, including human, rat, and bovine (32, 33, 36, 44).

In the present study, we examined the effect of bleomycin on the regulation of GSH in bovine pulmonary artery endothelial cells (BPAEC). Our results indicate that bleomycin treatment increases total cellular levels of GSH and upregulates the level of γ-GCS_h mRNA. We also show that bleomycin activates the DNA binding activity of nuclear factor (NF)-κB and Nrf-1 and -2 transcription factors. These factors are known to be activated by oxidative stress and to regulate the expression of human γ-GCS_h (14–17, 24, 40, 41, 44, 45).

EXPERIMENTAL METHODS

Reagents. Bleomycin (Blenoxane) was from Mead Johnson (Princeton, NJ). The mitogen-activated protein kinase/extracellular signal-regulated kinase [MAPK/ERK (MEK)] 1/2 inhibitor U-0126 was purchased from New England Biolabs (Beverly, MA). Other reagents are described below.

Cell culture and bleomycin treatment. BPAEC were obtained from freshly slaughtered calves as previously described (4). *Passage 3–8* cells were used for all experiments. Rat pulmonary microvascular endothelial cells (RPMEC) were a gift of Dr. Una Ryan (Avant Immunotherapeutics, Needham, MA) (3); these cells were used as a control in Fig. 2. Both BPAEC and RPMEC were cultured in RPMI 1640 (GIBCO-BRL, Rockville, MD) with antibiotics (penicillin and streptomycin), fungisone, and 10% fetal bovine serum in 5% CO_2 at 37°C in a humidified atmosphere. For cell culture treatment, bleomycin was dissolved in 0.9% NaCl and added to the culture medium.

Propidium iodide staining and fluorescence activated cell sorting. Propidium iodide and fluorescence-activated cell sorting (FACS) were used to determine cellular apoptosis. Cells (~3–7.5 × 10^5) were washed twice with phosphate-buffered saline (PBS) at 25°C and trypsinized. Cells were then pelleted by centrifugation and washed twice on ice with cold PBS. After the last wash, we resuspended cell pellets in 100 μl of cold PBS, and added 1 ml of cold 80% ethanol dropwise while vortexing on a low setting. Ethanol-treated cells were stored at 4°C for at least 4 h and up to 1 wk. Before FACS analysis, cells were pelleted at 4°C, resuspended in 0.5 ml of propidium iodide solution [0.05 mg/ml RNase A in PBS containing 5% (wt/vol) propidium iodide], and incubated for 30 min on ice in the dark. Cells were then analyzed on a Becton-Dickinson FACS Calibur (Franklin Lakes, NJ) cell sorter.

Cell counts and preparation for GSH assays. Cell counts and GSH assays were performed as described (43). Culture dishes were rinsed twice with PBS at 25°C and incubated for 3–5 min with 1.0 ml of trypsin-EDTA. The cells were rapidly suspended and placed on ice. The cellular suspension (0.1 ml) was removed, diluted, and counted on a ZM Coulter Counter (Coulter Electronics, Hialeah, FL). Of the remaining suspension, 0.8 ml was treated with 0.1 ml of 1% perchloric acid. Lysates were sonicated on ice for 10 s and centrifuged at 14,000 *g* at 4°C for 20 min. Aliquots were stored at −20°C for GSH assays.

GSH assay. GSH assays were performed as specified (40). Frozen perchloric acid-treated supernatants were thawed on ice and sonicated for 10 s on ice. The pH was adjusted to 7.0 with 0.3 M potassium hydroxide-3-(*N*-morpholino)propanesulfonic acid (MOPS). The sonicate was then centrifuged at 14,000 *g* at 4°C for 20 min. The supernatant was assayed for total cellular GSH by the spectrophotometric Tietze method (1). Briefly, the sum of the oxidized and reduced forms of GSH was determined using a kinetic assay in which GSH or GSH disulfide and GSH reductase reduce 5,5′-dithiobis(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoate (TNB). The formation of TNB was followed spectrophotometrically at 412 nm. Each assay was individually calibrated with standard GSH, and the concentration of each sample was adjusted by dilution to ensure that the reaction rate was on the linear portion of the standard curve. Cellular GSH levels were expressed as nanomoles per 10^6 cells. For the assay, brewer's yeast GSH reductase, β-NADPH, and GSH disulfide were obtained from Sigma (St. Louis, MO).

RNA purification and Northern blot. RNA was purified using Trizol (GIBCO-BRL) according to the manufacturer's instructions; RNA concentrations were determined by absorbance at 260 nm. RNA (10 μg) was denatured in a loading buffer [0.4 M MOPS, pH 7.0, 2.5% formaldehyde, 67% formamide, 0.2 μg ethidium bromide, 1.2 mM EDTA, 6.7 mM Na acetate, and 13% dye (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol FF)] for 15 min at 65°C. Denatured RNA was run in a 0.9% (wt/vol) denaturing agarose gel (0.2 M MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, and 1.8% formaldehyde) with running buffer (0.2 M MOPS, pH 7.0, 5 mM Na acetate, 1 mM EDTA, and 1.8% formaldehyde). RNA was transferred to a Zeta Probe blotting membrane (Bio-Rad, Hercules, CA) using capillary blotting in 1× SSC (20× SSC = 3 M NaCl, 3 mM sodium citrate, pH 7.0). Blots were baked at 80°C for 2 h under vacuum and prehybridized in ExpressHyb (Clontech, Palo Alto, CA) for 30 min at 68°C under shaking. The 2 × 10^6 counts·min^{-1}·ml^{-1} of labeled, denatured probe were then added with denatured salmon sperm DNA (100 ng/ml) to the prehybridized blot. Hybridization was performed at 68°C overnight with agitation. Blots were washed at 37°C; the first wash was performed in triplicate (2× SSC and 0.05% SDS) for 15 min each; the second wash was done twice (1× SSC and 0.1% SDS) for 15 min each at 50°C. The blot was then exposed to Kodak X-OMAT AR film (NEN, Boston, MA) at −80°C using intensifying screens.

The probe for human γ-GCS_h was the gift of Dr. Takahito Kondo (Nagasaki University School of Medicine, Nagasaki, Japan); this probe hybridizes strongly to human, mouse, and rat γ-GCS_h mRNA (16). To produce labeled probes, we incubated 10–20 ng of the excised γ-GCS_h cDNA with 1× hexanucleotide mix (Boehringer Mannheim, Indianapolis, IN), 0.05 mM dATP, dTTP, and dGTP, and 50 μCi α-[^{32}P]dCTP for 1 h at 37°C. The labeled probe was purified from unincor-

porated α-[^{32}P]dCTP using Sephadex G-50 Quick Spin columns (Boehringer Mannheim).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as described by Garner and Revzin (11). To prepare nuclear extracts, cells were washed in PBS and incubated in 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM tetrasodium pyrophosphate for 15 min at 4°C. (Octylphenoxy)polyethoxyethanol (Igepal CA-630, Sigma) was then added at a final concentration of 0.6%(vol/vol). Samples were vortexed and centrifuged. Pelleted nuclei were resuspended in extraction buffer [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, and 1% (vol/vol) glycerol], then mixed vigorously for 20 min, and centrifuged for 5 min. The supernatants were harvested, and protein concentrations were determined (22).

Oligonucleotides containing the sense and antisense antioxidant response element (ARE) consensus sequence were 5′-TCACAGTGACTCAGCAGAATC-3′ and 5′-GATTCTGCTGAGTCA-CTGTGA-3′, respectively. [The ARE consensus sequence is underlined; the ARE sequence used is identical to ARE4 of the human γ-GCS promoter (25, 42).] Oligos were made 5 μM in H_2O, heated to 80°C for 20 min, and allowed to anneal by cooling slowly to ambient temperature. The double-stranded oligonucleotide probe containing consensus κB sequence was 5′-GAT CCG AGG GGA CTT TCC GCT GGG GAC TTT CCA GG-3′. To perform EMSA, we incubated binding reaction mixtures containing 2 μg protein of nuclear extract, 1 μg poly(dI-dC)·poly(dI-dC), and ^{32}P-labeled double-stranded ARE or κB oligonucleotide in 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, and 20 mM Tris·HCl (pH 7.5) for 20 min at 25°C. Electrophoresis of samples through a native 6% polyacrylamide gel (acrylamide-bis, 29:1) was followed by autoradiography. Supershift experiments were performed by incubating 2 μg Nrf-1, Nrf-2, c-Jun, c-Fos, NF-κB p65, or NF-κB p50 antibody (Santa Cruz Biotech) in the binding reaction mixture for 1 h at 4°C before the addition of the ^{32}P-labeled oligonucleotide probe to start the binding reaction. All experiments were repeated at least three times.

Western blot analysis. To prepare lysates, cells were washed in PBS and solubilized with 50 mM HEPES solution (pH 7.4) containing 1% (vol/vol) Triton X-100, 4 mM EDTA, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysate was cleared by centrifugation at 4°C for 15 min. Protein concentrations in the supernatant were determined as above (22). Cell lysates (10 μg protein) were electrophoresed through reducing (5% β-mercaptoethanol) SDS polyacrylamide-bis gels (10%) and electroblotted onto nitrocellulose membranes. After the transfer, membranes were blocked in 5% milk in Tween- and Tris-buffered saline (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and then blotted with the antibody for the NF-κB p65 subunit and IκBα (Santa Cruz Biotech). Levels of proteins and phosphoproteins were detected with horseradish peroxidase-linked secondary antibodies and ECL System (Amersham Life Science, Arlington Heights, IL). Western blots were repeated at least three times.

Statistics. Statistical comparisons were performed using a Student's *t*-test for unpaired samples and a two-way analysis of variance for multiple comparisons. Statistical significance was determined at $P < 0.05$.

RESULTS

Bleomycin upregulates cellular GSH and the γ-GCS gene. Bleomycin has been shown to induce growth arrest, apoptosis, and necrosis in a number of cells, including immortalized cells and primary cancer cells (31, 34). We first determined doses of bleomycin that induce growth arrest with limited cell death in BPAEC. Cells were placed in RPMI medium containing 0.1% FBS for 24 h and then exposed to increasing doses of bleomycin for 24 h. Cell numbers indicated that 10 μg/ml of bleomycin induced high levels of growth arrest (Fig. 1*A*), which was associated with visible cell death (data not shown). Levels of 1.0 and 0.1 μg/ml of bleomycin induced lower levels of growth arrest. Propidium iodide staining and FACS analysis showed that 1.0 μg/ml of bleomycin induced only 8.8% (± 1.6 SD) apoptosis, compared with 5.8% (± 1.7 SD) apoptosis in control cells ($n = 3$).

Bleomycin is believed to induce oxidative stress on cells by the generation of ROS through a bound ferrous ion. GSH is a primary constituent for protecting cells against oxidative stress, and its formation can be upregulated by ROS-producing compounds (6, 32, 33, 44).

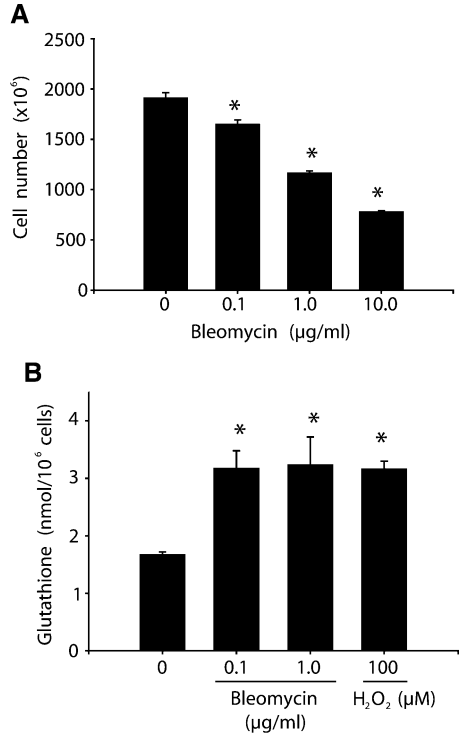

Fig. 1. Bleomycin causes growth arrest in bovine pulmonary artery endothelial cells (BPAEC) and upregulates cellular levels of glutathione (GSH). *A*: BPAEC were plated at 4×10^4 cells/35 mm dish for 48 h before being placed in RPMI-0.1% fetal bovine serum (FBS) for 24 h and then exposed for 24 h to the presence or absence of bleomycin before counting. Under these conditions, BPAEC continue to grow slowly for up to 72 h in the absence of serum. Values represent means ± SE, $n = 5$. *Significantly different from the control value without bleomycin treatment. *B*: BPAEC were plated at 4×10^4 cells/35-mm dish for 48 h before being placed in RPMI-0.1% FBS for 24 h and then exposed to bleomycin or H_2O_2. Total GSH levels were measured after 18 h of treatment. Values represent means ± SE, $n = 4$. *Significantly different from the control.

We analyzed total cellular GSH to determine whether bleomycin was able to regulate GSH formation. A spectrophotometric assay for total cellular GSH showed that both 0.1 and 1.0 μg/ml of bleomycin caused approximately twofold increases within 18 h compared with control (Fig. 1*B*). These results were comparable with the twofold increase in total cellular GSH induced by an 18-h treatment with 100 μM H_2O_2, used as a positive control (43).

The enzyme γ-GCS controls the key regulatory step in the production of cellular GSH (6, 35, 44). Northern blot analysis shows that γ-GCS_h mRNA levels are upregulated by bleomycin in BPAEC. Figure 2*A* is a representative time course in which γ-GCS_h mRNA is increased by 30 min in response to 1 μg/ml of bleomycin. BSO, an agent known to induce γ-GCS_h expression, was used as a positive control (44). Our probe for γ-GCS_h is based on rat cDNA, but the bovine size, sequence, and, therefore, complementarity to rat γ-GCS_h are not known. The rat mRNA was included as a positive control for the position of γ-GCS_h (Fig. 2*A*). Densitometry of the representative data showed that within 30 min, bleomycin caused a 1.5-fold increase in γ-GCS_h mRNA levels, whereas at 3–6 h bleomycin induced a 3–3.5-fold increase (Fig. 2*C*). BSO exposure for 3 and 6 h induced a 2.5- and 2-fold increase in γ-GCS_h mRNA, respectively.

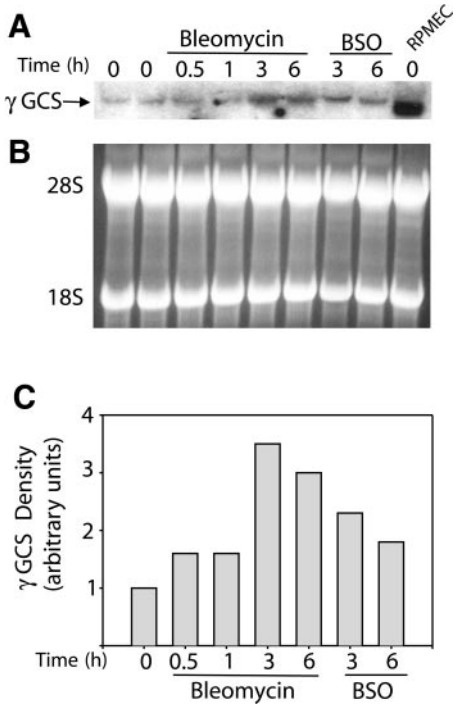

Fig. 2. Bleomycin induces increased expression of γ-glutamylcysteine synthetase (γ-GCS). BPAEC were grown to confluence and treated with bleomycin (1.0 μg/ml) or buthionine sulfoximine (BSO, 100 μM) for the indicated times before RNA was harvested. *A*: Northern blot for γ-GCS. Rat pulmonary microvascular endothelial cell (RPMEC) RNA was used as a positive control for the position of γ-GCS in the bovine samples. *B*: ethidium bromide staining of the agarose gel of bovine and rat RNAs. The positions of 28S and 18S rRNA are indicated. *C*: densitometry of a Northern blot for γ-GCS. Representative data are shown.

Bleomycin activates NF-κB and the ARE-binding transcription factors Nrf-1 and -2. Studies of human γ-GCS_h gene expression have identified two promoter *cis* elements that are believed to primarily regulate its transcription: the κB element and the ARE (6, 16, 17, 37, 44). Increased expression of γ-GCS_h in response to ionizing radiation, which produces physical and chemical damage in addition to inducing the formation of ROS, is dependent on NF-κB activation (16). However, regulation of γ-GCS_h by xenobiotics and ROS-generating compounds is believed to occur through the activation of proteins that bind to the ARE (17, 37, 44). Because bleomycin induces physical damage to cellular proteins and DNA while at the same time producing ROS, we investigated the activation of DNA binding to both κB and ARE.

A time course of bleomycin treatment showed an increase in DNA-binding activity of NF-κB within 1 h as monitored by EMSA (Fig. 3*A*). Densitometric analysis showed that bleomycin induced an approximately fourfold increase in NF-κB activation in 2 h. This increase in NF-κB activity persisted for at least 24 h (data not shown). NF-κB is activated in coordination with the degradation of its inhibitory subunit, IκB. Western blots show that tumor necrosis factor-α induces rapid degradation of IκB-α within 30 min in BPAEC, whereas bleomycin causes a more gradual degradation (Fig. 3*B*). Western blots of NF-κB p65 subunit were performed as a control, showing that levels of the transcription factor are unchanged by bleomycin treatment (Fig. 3*B*). Supershift analysis of the NF-κB band showed that both the p65 and p50 subunits are involved in the complex activated by bleomycin (Fig. 3*C*).

A number of factors are believed to bind ARE and affect downstream transcription; these include the NF-E2-related factors (Nrf) 1 and 2, the Jun protein family (c-Jun, Jun-B, and Jun-D), c-Fos, and the Maf protein family (c-Maf, hMaf, MafG, and MafK) (7, 8, 14, 17, 23, 40, 41, 44). Factor binding to the ARE increases with bleomycin treatment of BPAEC. EMSA experiments detected a >10-fold increase in the binding of factors to a consensus oligo for ARE, which was detected within 30 min of treatment with bleomycin and persisted for at least 24 h (Fig. 4).

We performed supershift analysis to determine which protein(s) was activated in BPAEC by bleomycin. Preincubation of the nuclear extract with an antibodies specific for Nrf-1 and -2 decreases the density of the shifted ^{32}P-labeled ARE band (Fig. 5*A*). Additionally, the Nrf-2 antibody causes the appearance of an ARE-containing band with increased electrophoretic mobility. These data indicate that the original EMSA band contains Nrf-1 and Nrf-2. Our results further suggest that the faster mobility EMSA band, which forms in the presence of the Nrf-2 antibody, contains another protein bound to the ARE. We have labeled this band ARE + X. Importantly, the antibodies directed against Nrf-1 or -2 do not form complexes directly with the ARE in the absence of nuclear extract (Fig. 5*A*, *lanes 2* and *3*).

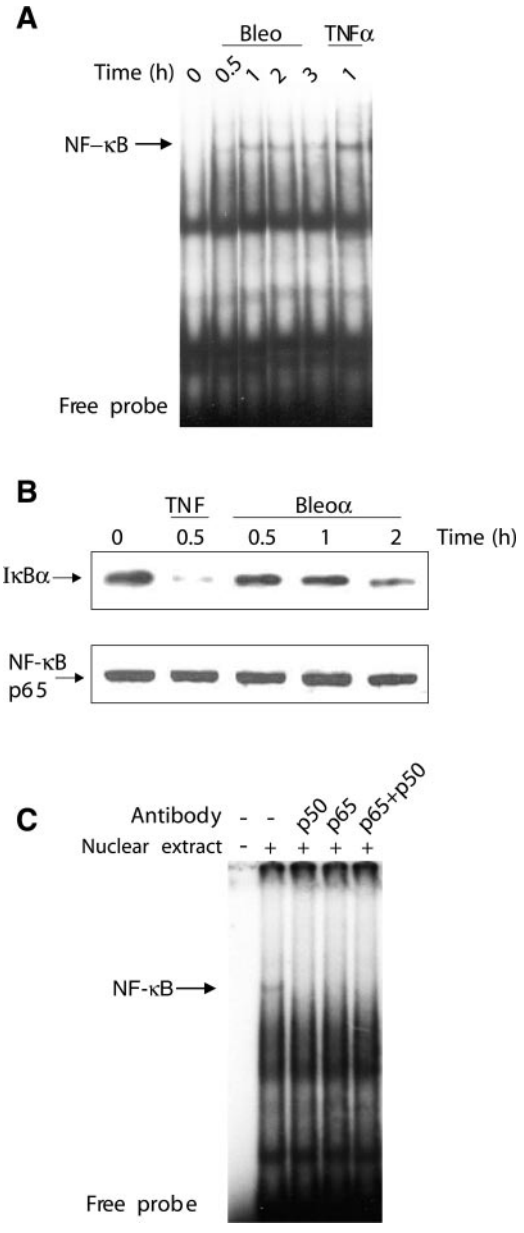

Fig. 3. Bleomycin activates DNA-binding by nuclear factor (NF)-κB and degradation of IκB. *A*: electrophoretic mobility shift assay (EMSA) of protein binding to the κB consensus oligonucleotides. Confluent BPAEC were treated with 1 μg/ml bleomycin (Bleo) or 1 nM tumor necrosis factor (TNF)-α for the indicated times before purification of nuclear extracts. EMSA experiments were performed as described in EXPERIMENTAL METHODS. EMSAs were repeated at least 3 times. The position of NF-κB is indicated. *B*: Western blots of cell lysates after treatment of BPAEC with 1 μg/ml bleomycin or 1 nM TNF-α for the indicated times. *Top*: blotting with antibody directed against IκBα. *Bottom*: same samples blotted using an antibody directed against NF-κB p65 subunit. *C*: supershifts of NF-κB p65 and p50 subunits. Confluent BPAEC were treated with 1 μg/ml bleomycin for 2 h. Nuclear extracts were incubated with 1 μg of antibody against NF-κB p65, NF-κB p50, or both before EMSA was performed. Bleo, bleomycin.

Previous reports have suggested that Nrf-2 may heterodimerize with c-Jun, JunD, MafG, and MafK; these proteins contain DNA-binding domains and are capable of directly binding the ARE (7, 15, 23, 44). An antibody directed against c-Jun did not result in any change in the ARE-containing band, suggesting that c-Jun is not activated by bleomycin and is not involved in Nrf-2 binding to the ARE (Fig. 5*B*). Antibodies directed against c-Fos also caused no change in the ARE binding pattern; this factor is believed to be involved in the inhibition of transcription downstream of ARE (40).

Bleomycin activates ARE-binding via ROS and NF-κB via MAPK. Both ARE and NF-κB site binding factors are known to be activated through a variety of signaling pathways (7, 8, 14, 28, 40, 41, 44, 45). Both the overexpression of the ERK1-MAPK and the induction of ROS activate NF-κB (16, 24, 28). Studies have shown that Nrf-1 and -2 are activated in response to ROS, and the inhibition of ERK1/2-MAPK blocks activation of ARE-binding factors by xenobiotic compounds (17, 40, 44, 45). We previously reported that bleomycin activates the ERK1/2 family of MAPKs (5), and it is widely believed that bleomycin produces ROS through its bound ferrous ion (10, 13, 34, 39). Thus we investigated whether one or both of these mechanisms downstream of bleomycin lead to the activation of factors that bind ARE and/or the NF-κB site.

BPAEC were pretreated for 30 min with *N*-acetylcysteine (NAC), an antioxidant, or U-0126, an inhibitor of MEK1/2 (the kinase upstream of ERK1/2-MAPK). Cells were then treated with ± 1.0 μg/ml of bleomycin for 30 min, and nuclear extracts were prepared. EMSA shows that whereas NAC inhibited bleomycin activation of ARE-binding factors, U-0126 has no effect on the activation (Fig. 6*A*). In contrast, U-0126 blocks bleomycin-induced NF-κB activation, whereas NAC has little or no effect (Fig. 6*B*). Pretreatment of cells with either NAC or U-0126 for 30 min blocks bleomycin upregulation of γ-GCS_h mRNA as determined by Northern blot analysis (Fig. 6*C*).

Fig. 4. Bleomycin activates transcription factor binding to the antioxidant response element (ARE) consensus sequence. Confluent BPAEC were treated with 1 μg/ml bleomycin for the indicated times. Nuclear extracts were incubated with the consensus oligonucleotide for ARE. The position of ARE is indicated with an arrow. EMSA experiments were repeated at least 3 times.

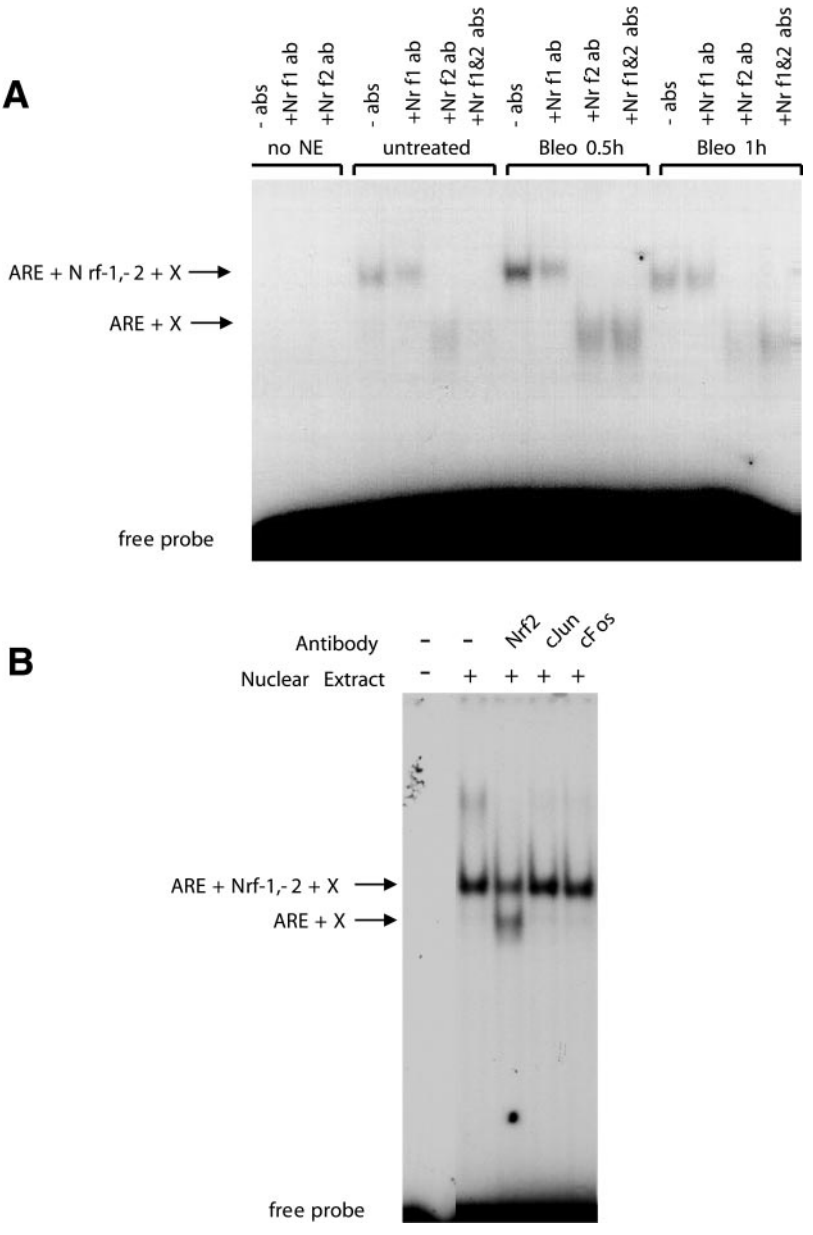

Fig. 5. Bleomycin activates binding of Nrf-1 and -2, but not c-Jun or c-Fos, to the ARE consensus sequence. Confluent BPAEC were treated with 1 μg/ml bleomycin (Bleo) for the indicated times before preparation of nuclear extracts. *A*: EMSAs were performed in the absence and the presence of antibodies (abs) for Nrf-1 and/or Nrf-2 with and without nuclear extract (NE). *Top* arrow: the position of bands containing ARE bound to Nrf-1 and -2 together with an unknown protein(s) X; *bottom* arrow: the position of ARE bound to unknown protein X. *B*: EMSAs were performed in the absence and the presence of antibodies for Nrf-2, c-Jun, or c-Fos. Supershift experiments were repeated at least 3 times.

DISCUSSION

The chemotherapeutic agent bleomycin can induce pulmonary fibrosis, thus limiting its application in cancer treatment in humans. This side effect has been utilized in an animal model system to identify cellular and molecular changes that occur during the development and progression of pulmonary fibrosis (10, 14, 18, 20, 21, 26, 27, 29, 30, 34, 38). Despite extensive work demonstrating the changes in mRNA and protein expression that occur during bleomycin-induced fibrosis, comparatively little is known about the mechanism(s) of these events. The cytotoxic effects of bleomycin are modulated by antioxidants, and we hypothesized that bleomycin regulates cellular defenses against oxidative stress.

The present study indicates that in endothelial cell culture, treatment with sublethal levels of bleomycin (0.01–1.0 μg/ml) for 18 h causes increased total cellular GSH. We also demonstrated that in 30 min, bleomycin increases the level of γ-GCS_h mRNA, the catalytic subunit of γ-GCS that controls the key regulatory step in GSH synthesis. Karam et al. (19) previously reported a ~50% decrease in the level of total GSH in alveolar T2 cells isolated from Wistar rats treated for 7 or 14 days with bleomycin. This was accompanied by a slight decrease in γ-GCS enzyme levels at 7 days. Further experiments are required to determine whether the increase in cellular GSH as observed in the present study would also occur in vivo after acute exposure to bleomycin.

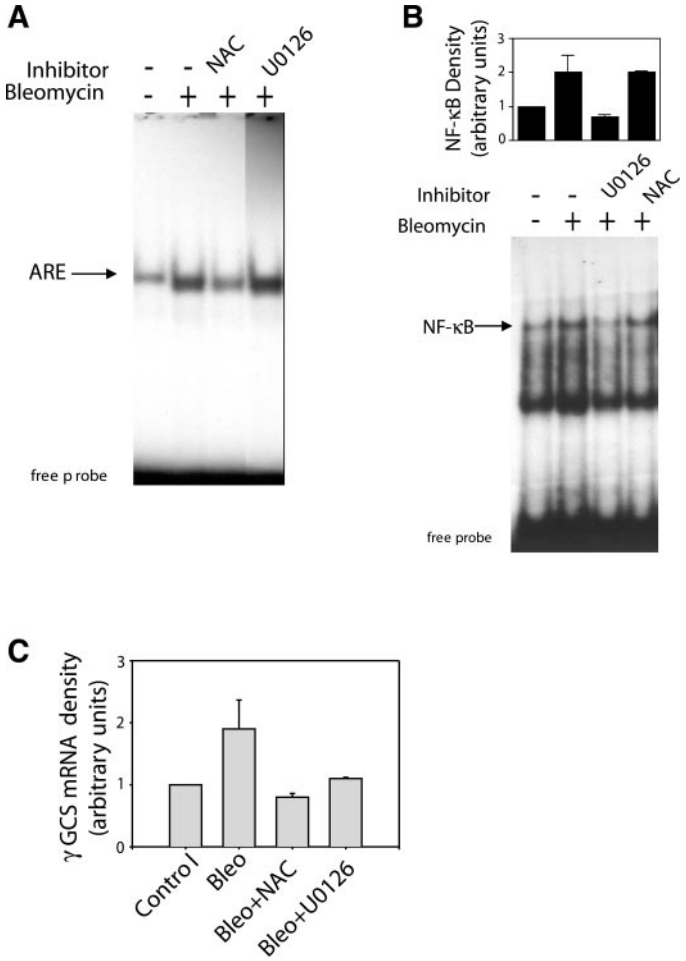

Fig. 6. Bleomycin activation of ARE-binding proteins is dependent upon reactive oxygen species (ROS) generation, but activation of NF-κB is dependent upon extracellular signal-regulated kinase 1/2-mitogen-activated protein kinase (ERK1/2-MAPK). Confluent BPAEC were pretreated with 10 μg/ml U-0126 or 20 mM *N*-acetylcysteine (NAC) for 30 min, then treated with 1 μg/ml bleomycin for 30 min before preparation of nuclear extracts. EMSA experiments were performed as described. *A*: EMSA using ARE consensus oligonucleotide. *B*: EMSA using NF-κB consensus oligonucleotide. Positions of specific bands are indicated. EMSAs with inhibitors were repeated at least 3 times. Densitometry of the NF-κB band is shown above EMSA. *C*: total cellular RNA was extracted, and Northern blot analysis was performed as described for γ-GCS heavy chain (γ-GCS_h). Densitometric quantitation of γ-GCS_h mRNA was performed, and control levels were normalized between experiments. Data show the mean value (n = 3, ±SE).

In response to oxidative or toxic stress, expression of the human γ-GCS_h is positively regulated at the level of transcription by factors binding to both ARE and NF-κB promoter elements (16, 17, 40, 41, 44). Our EMSA results indicate that bleomycin activates both ARE-binding factors and NF-κB in BPAEC within 30 min. Although the sequence of the bovine γ-GCS_h promoter has not been reported, Shi et al. (32, 33) found that endogenous bovine γGCS_h mRNA is increased in BPAEC in response to quinone-induced oxidative stress, similar to findings in other species, including human (44). Therefore, in BPAEC, the induced increase of γ-GCS_h mRNA by bleomycin is consistent with a mechanism involving the induction of ARE-binding factors and NF-κB.

A number of proteins have been shown to bind the ARE element, including Nrf-1 and -2 (members of the cap 'n' collar subfamily of basic region-leucine zipper transcription factors), members of the Jun family (c-Jun, JunB, and JunD), and several small Maf family proteins (c-Maf, hMaf, MafG, and MafK) (7, 8, 14, 17, 23, 40, 41, 44). Supershift experiments using antibodies directed against Nrf-1 or Nrf-2 caused a reduction in the level of the ARE-containing band. However, neither the Nrf-1 nor -2 antibodies caused the appearance of a supershifted species, suggesting that these antibodies dissociate Nrf proteins from a complex with ARE. Inclusion of the antibody against Nrf-2 caused both the reduction of the original ARE-containing band and the appearance of a band with increased electrophoretic mobility. The induction of a band with faster mobility suggests that complexes with Nrf-2 contain at least one additional protein (X) and that the new, faster complex contains the ARE probe bound to X. The identity of X is currently unknown. Other laboratories have shown that Nrf-2 forms heterodimers with small Maf proteins, including MafG (7, 23, 44, 45) and Jun proteins (44). Supershift experiments were performed using antibodies against c-Jun and c-Fos, but these antibodies did not alter the mobility of the ARE complex, indicating that these proteins are not activated by bleomycin.

Nrf proteins have been shown to be activated downstream of protein kinase C and the MAPKs ERK1/2 and stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) (14, 44, 45). In BPAEC, bleomycin activates p38/HOG1 (unpublished results) and ERK1/2 (5), but not SAPK/JNK (unpublished results). Our current data suggest that bleomycin activates Nrf-1 and -2 through a mechanism independent of ERK1/2-MAPK, as the MEK inhibitor U-0126 failed to block bleomycin induction. Our data showing the inhibition Nrf activation by NAC suggest that ROS act as second messengers downstream of bleomycin.

In contrast to Nrf-1 and -2 activation by bleomycin, NF-κB activation is blocked by U-0126 but not by the ROS-quenching agent NAC, suggesting that the ERK1/2-MAPK pathway is involved. NF-κB activation has been shown to occur in response to the overexpression of ERK1-MAPK (9, 28).

In summary, our results indicate that bleomycin upregulates GSH and γ-GCS through the activation of both ROS- and MAPK-dependent pathways in a mechanism that likely involves the induction of ARE-binding factors and NF-κB. The use of bleomycin in animals remains the most useful model system for the study of pulmonary fibrosis, and the understanding of mechanisms of bleomycin signal transduction pathways should help in developing therapeutic strategies.

We thank Drs. Amy Simon and Daniel Rotiz for technical help.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-42376 and the United States Department of Agriculture (58-1950-9-001). R. Day is a recipient of an American Heart Association National Center Scientist Development Award and an American Lung Association Research Grant.

REFERENCES

1. **Akerboom TPM and Sies H.** Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. *Methods Enzymol* 77: 373–382, 1981.
2. **Borzone G, Moreno R, Urrea R, Meneses M, Oyarzun M, and Lisboa C.** Bleomycin-induced chronic lung damage does not resemble human idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 163: 1648–1653, 2001.
3. **Cote CG, Yu FS, Zulueta JJ, Vostka RJ, and Hassoun PM.** Regulation of intracellular xanthine oxidase by endothelial-derived nitric oxide. *Am J Physiol Lung Cell Mol Physiol* 271: L869–L874, 1996.
4. **Dasarathy Y, Lanzillo JJ, and Fanburg BL.** Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors. *Am J Physiol Lung Cell Mol Physiol* 263: L645–L649, 1992.
5. **Day RM, Yongzhen Y, Suzuki YJ, Stevens J, Pathi R, Perlmutter A, Fanburg BL, and Lanzillo JJ.** Bleomycin upregulates gene expression of angiotensin converting enzyme via MAP kinase and Egr-1 transcription factor. *Am J Respir Cell Mol Biol* 25: 613–619, 2001.
6. **Deneke SM and Fanburg BL.** Regulation of cellular glutathione. *Am J Physiol Lung Cell Mol Physiol* 257: L163–L173, 1989.
7. **Dhakshinamoorthy S and Jaiswal AK.** Small Maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:quinone oxidoreductase1 gene. *J Biol Chem* 275: 40134–40141, 2000.
8. **Favreau LV and Pickett CB.** Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. *J Biol Chem* 268: 19875–19881, 1993.
9. **Finkel T.** Oxygen radicals and signaling. *Curr Opin Cell Biol* 10: 248–253, 1998.
10. **Galvan L, Huang CH, Prestayko AW, Stout JT, Evans JE, and Crooke ST.** Inhibition of bleomycin-induced DNA breakage by superoxide dismutase. *Cancer Res* 41: 5103–5106, 1981.
11. **Garner MM and Revzin A.** A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res* 9: 3047–3060, 1981.
12. **Giaccia AJ, Lewis AD, Denko NC, Cholon A, Evans JW, Waldren CA, Stamato TD, and Brown JM.** The hypersensitivity of the Chinese hamster ovary variant BL-10 to bleomycin killing is due to a lack of glutathione *S*-transferase-α activity. *Cancer Res* 51: 4463–4469, 1991.
13. **Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, and Hara N.** Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Cell Mol Biol* 16: 19–101, 1997.
14. **Huang H-C, Nguyen T, and Pickett CB.** Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor. *Proc Natl Acad Sci USA* 97: 12475–12480, 2000.
15. **Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, and Yamamoto M.** Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 275: 16023–16029, 2000.
16. **Iwanaga M, Mori K, Iida T, Urata Y, Matsuo T, Yasunaga A, Shibata S, and Kondo T.** Nuclear factor kappa B dependent induction of gamma glutamylcysteine synthetase by ionizing radiation in T98G human glioblastoma cells. *Free Radic Biol Med* 24: 1256–1268, 1998.
17. **Jeyapaul J and Jaiswal AK.** Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of γ-glutamylcysteine synthetase heavy subunit gene. *Biochem Pharmacol* 59: 1433–1439, 2000.
18. **Kaminski N, Allarc JD, Pittet JF, Zuo F, Griffiths MJD, Morris D, Huang X, Sheppard D, and Heller RA.** Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. *Proc Natl Acad Sci USA* 97: 1778–1783, 2000.
19. **Karam H, Hurbain-Kosmath I, and Housset B.** Antioxidant activity in alveolar epithelial type 2 cells of rats during the development of bleomycin injury. *Cell Biol Toxicol* 14: 13–22, 1998.
20. **King SL, Lichtler AC, Rowe DW, Xie R, Long GL, Absher MP, and Cutroneo KR.** Bleomycin stimulates pro-α1(I) collagen promoter through TFG β response element by intracellular and extracellular signaling. *J Biol Chem* 269: 13156–13161, 1994.
21. **Koslowski R, Knoch K-P, and Wenzel K-W.** Proteinases and proteinase inhibitors during the development of pulmonary fibrosis in rat. *Clin Chim Acta* 271: 45–56, 1998.
22. **Lowry OH, Rosenburg NJ, Farr AL, and Randall RJ.** Protein measurement with Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.
23. **Marini MG, Chan K, Casula L, Kan YW, Cao A, and Moi P.** HMAP, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem* 272: 16490–16497, 1997.
24. **Meyer M, Schreck R, and Baeuerle PA.** H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J* 12: 2005–2025, 1993.
25. **Mulcahy RT, Wartman MA, Bailey HH, and Gipp JJ.** Constitutive and b-naphthoflavone-induced expression of the human γ-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* 272: 7445–7454, 1997.
26. **Mutasaers SE, Foster ML, Chambers RC, Laurent GJ, and McAnulty RJ.** Increased endothelin-1 and its localization during the development of bleomycin-induced pulmonary fibrosis in rats. *Am J Respir Cell Mol Biol* 18: 611–619, 1998.
27. **Ortiz LA, Lasky J, Hamilton RF Jr, Holian A, Hoyle GW, Banks W, Peschon JJ, Brody AR, Lungarella G, and Friedman M.** Expression of TNF and the necessity of TNF receptors in bleomycin-induced lung injury in mice. *Exp Lung Res* 24: 721–743, 1998.
28. **Park J-H and Levitt L.** Overexpression of mitogen-activated protein kinase (ERK1) enhances T-Cell cytokine gene expression: role of AP1-NF-AT, and NF-KB. *Blood* 82: 2470–2477, 1993.
29. **Phan SH, Garaee-Kermani M, Wolber F, and Ryan US.** Stimulation of rat endothelial cell transforming growth factor-β production by bleomycin. *J Clin Invest* 87: 148–154, 1991.
30. **Phan SH and Kunkel SL.** Lung cytokine production in bleomycin-induce pulmonary fibrosis. *Exp Lung Res* 18: 29–43, 1992.
31. **Rosso A, Mitchell JB, McPherson S, and Friedman N.** Alteration of bleomycin cytotoxicity by glutathione depletion or elevation. *Int J Radiat Oncol Biol Phys* 10: 1675–1678, 1984.
32. **Shi MM, Iwamoto T, and Forman HJ.** γ-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. *Am J Physiol Lung Cell Mol Physiol* 267: L414–L421, 1994.
33. **Shi MM, Kugelman A, Iwamoto T, Tian L, and Forman HJ.** Quinone-induced oxidative stress elevates glutathione and induces γ-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J Biol Chem* 269: 26512–26517, 1994.
34. **Sikic BI.** Biochemical and cellular determinants of bleomycin cytotoxicity. *Cancer Surv* 5: 81–91, 1986.
35. **Suzuki YJ, Forman HJ, and Sevanian A.** Oxidants as stimulators of signal transduction. *Free Radic Biol Med* 22: 269–285, 1997.
36. **Tian L, Shi MM, and Forman HJ.** Increased transcription of the regulatory subunit of γ-glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. *Arch Biochem Biophys* 342: 126–133, 1997.
37. **Tsan M-F, Danis EH, del Vecchio PJ, and Rosano CL.** Enhancement of intracellular glutathione protects endothelial

cells against oxidant damage. *Biochem Biophys Res Commun* 127: 270–276, 1985.
38. **Vaccaro CA, Brody JS, and Snider GL.** Alveolar wall basement membranes in bleomycin-induced pulmonary fibrosis. *Am Rev Respir Dis* 132: 905–912, 1985.
39. **Venkatesan N, Punithavathi V, and Chandrakasan G.** Curcumin protects bleomycin-induced lung injury in rats. *Life Sci* 61: PL51–PL58, 1997.
40. **Venugopal R and Jaiswal AK.** Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc Natl Acad Sci USA* 93: 14960–14965, 1996.
41. **Venugopal R and Jaiswal AK.** Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* 17: 3145–3156, 1998.
42. **Wang B and Williamson G.** Detection of a nuclear protein which binds specifically to the antioxidant responsive element (ARE) of the human NAD(P)H:quinone oxidoreductase gene. *Biochim Biophys Acta* 1219: 645–652, 1994.
43. **White AC, Das SK, and Fanburg BL.** Reduction of glutathione is associated with growth restriction and enlargement of bovine pulmonary artery endothelial cells produced by transforming growth factor-β_1. *Am J Respir Cell Mol Biol* 6: 364–368, 1992.
44. **Wild AC and Mulcahy RT.** Regulation of the γ-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. *Free Radic Res* 32: 281–301, 2000.
45. **Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, and Kong A-NT.** Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *J Biol Chem* 274: 27545–27552, 1999.
46. **Zhang H-Y, Gharaee-Kermani M, Zhang K, Kaminol S, and Phan SH.** Lung fibroblast α-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 148: 527–537, 1996.