The Differential Expression of Apoptosis Factors in the Alveolar Epithelium Is Redox Sensitive and Requires NF-κB (RelA)-Selective Targeting

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Fetal alveolar type II (fATII) epithelial cells were used to evaluate the role of signaling factors involved in oxidative stress-induced programmed cell death (PCD; apoptosis). Bcl-2, an antiapoptotic proto-oncogene, showed maximum abundance in hypoxia and mild reoxygenation, but declined thereafter. The Bcl-2 counterpart, Bax, which promotes PCD, displayed an increasing logarithmic profile with ascending $\Delta p\text{O}_2$ regimen, such that the ratio of Bcl-2/Bax decreased as $p\text{O}_2$ increased. The expression of p53, a cell cycle regulator, paralleled Bax abundance. Pretreatment of fATII cells with L-buthionine-(*S,R*)-sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of glutathione (GSH), enhanced Bax and p53 expression over Bcl-2. The GSH analogue, γ-glutamylcysteinyl-ethyl ester, down-regulated Bax/ p53 abundance but restored that of Bcl-2, thereby increasing Bcl-2/Bax. The antioxidant and GSH precursor *N*-acetyl-L-cysteine favored Bcl-2 at the expense of Bax/ p53, whereas pyrrolidine dithiocarbamate induced Bax against Bcl-2, with mild effect on p53. Sulfasalazine, a potent and specific inhibitor of NF-κB, induced Bax at the expense of Bcl-2, in a p53-dependent manner. We conclude that the differential expression of signaling factors involved in PCD in the alveolar epithelium is redox-sensitive and mediated, at least in part, by a negative feedback mechanism transduced by NF-κB.   © 2000 Academic Press

Key Words: N-acetyl-L-cysteine; apoptosis; glutathione; NF-κB (RelA/p65); pyrrolidine dithiocarbamate; redox equilibrium; sulfasalazine.

Apoptosis, or programmed cell death, is a genetically controlled mechanism involved in development and homeostasis. This process is characterized by a variety of cellular changes including loss of membrane phospholipid asymmetry, chromatin condensation, mitochondrial swelling and DNA cleavage (1), and may be induced by stimuli as diverse as hyperthermia, growth factor withdrawal, chemotherapeutic agents, radiation, cytokines and oxidative stress (2, 3).

The biological pathways controlling cell fate are sequentially organized at the molecular level, with several regulatory proteins identified that activate or suppress this process (4, 5). The family of B-cell leukemia/ lymphoma-2 (Bcl-2) related proteins constitutes a class of apoptosis-regulatory gene products that act at the effector stage of cell death. Two functional classes of Bcl-2-related proteins have been identified that share highly conserved homology domains: (*i*) antiapoptotic, or antagonistic, members, including Bcl-2, which confer negative control in the pathways of cellular suicide machinery, and (*ii*) proapoptotic, or agonistic, members, including Bcl-associated x protein (Bax), which promote cell death by competing with Bcl-2 (6). For instance, gene ablation studies confirm that the balance between death promoting and repressing proteins contribute a critical checkpoint that determines a cell's susceptibility to an apoptotic stimulus (7). While Bax/ Bax homodimers induce apoptosis, Bcl-2/Bax heterodimer formation evokes a survival signal. Moreover, both Bcl-2 and Bax are transcriptional targets for the tumor suppresser protein, p53, which induces cell cycle arrest and apoptosis in response to DNA damage. The coordinate performance of these interacting molecules is, therefore, crucial for controlling life and death of the cell.

Bcl-2 has been shown to prevent apoptosis induced by diverse stimuli (8, 9), possibly by acting as an antioxidant (10). This hypothesis is consistent with the observation that *N*-acetyl-L-cysteine, an antioxidant thiol, prevents apoptosis in several cell models (10–13). Expression of Bcl-2, in addition, was shown to elevate intracellular GSH, favor a reduction equilibrium, and retard GSH extrusion in stress (14). Although the ob-

servation that Bcl-2 promotes nuclear uptake of GSH is unprecedented, GSH depletion and efflux occur at a very early stage of apoptosis, concomitant with up-regulation of Bax. Furthermore, the proapoptotic effects of p53 are associated with transcription of genes particularly involved in modulating the response of the cell to oxidative stress (15). It is evident, therefore, that alteration in redox equilibrium may have pleiotropic effects on susceptibility to DNA damage, gene transcription, and nuclear signal transduction in response to an apoptotic stimulus. This in essence may determine cellular response to stimulation and the subsequent onset of apoptosis, thereby giving an explanation for the multiplicity of the effects that agonists and antagonists have on the death machinery.

This study investigates *in vitro* the properties of glutathione and thiol-related agents in governing apoptosis in the foetal alveolar epithelium. To this end, we tested the hypotheses that (*i*) exposure to ascending ΔpO_2 regimen (oxyexcitation) triggers a signaling mechanism that induces the differential expression of apoptotic agonists and/or antagonists; (*ii*) selective inhibition of glutathione biosynthesis and subsequent intracellular depletion accelerates the onset of apoptosis by favoring Bax/p53 against Bcl-2; (*iii*) shifting redox equilibrium into a reduction state creates an environment that necessitates the balance of antagonists against potential agonists; and (*iv*) selective targeting of redox-sensitive transcription factor NF-κB draws an equilibrium in favor of apoptotic agonists, a pathway which is p53-dependent. We conclude that redox-mediated pathways are intimately linked to the expression of proteins which determine the potential for apoptosis in the perinatal epithelium.

MATERIALS AND METHODS

Chemicals and reagents. Unless specifically indicated, chemicals of the highest analytical grade were purchased from Sigma-Aldrich Co. All experimental procedures involving the use of live animals were reviewed and approved under the Animals Act legislation, 1986 (UK).

Epithelial primary cell cultures. Fetal alveolar type II (fATII) epithelial cells were isolated from the lungs of fetuses of pregnant rats at day 19 of gestation, essentially as reported elsewhere (16). Briefly, lung tissues were transferred into ice-cold HBSS and dissected followed by digestion and successive centrifugation repeats (420g at 4°C). fATII cells were harvested and grown (5×10^6) in polyester filters at 23 Torr for 24 h in serum-free PC-1 base medium, and maintained at this pO_2 until further analysis. The change in oxygen equilibrium from fetal (~23–30 Torr) to postnatal environments constitutes a potential signaling mechanism in the perinatal lung (17). The choice of this form of oxidative stress was based on its clinical relevance of the situation resembling the birth transition period and beyond (16). Shifts in pO_2 were re-created where cells were cultured at fetal alveolar pO_2 (23 Torr), followed by a control period at the same pO_2, or re-equilibrated to early postnatal alveolar pO_2 (100 Torr), mild (152 Torr) and severe hyperoxia (722 Torr) for 4 h at 37°C. In each case, the adenylate energy charge, an index of cell viability and competence, remained ≥0.7 and transepithelial

monolayer resistance was monitored constant at ≥250–300 Ωcm² throughout experiments.

Cell harvesting, total/nuclear protein extraction, and Western analysis. Subcellular extracts were prepared as described previously (16). Total proteins (20–25 μg) were resolved over SDS–PAGE (7.5%) gels at RT, blotted onto nitrocellulose membrane, and nonspecific binding sites were subsequently blocked. Rabbit polyclonal IgG anti-Bcl-2 (N-19), polyclonal IgG anti-Bax (N-20), and mouse monoclonal IgG$_1$ anti-p53 (Pab-246) (Santa Cruz Biotechnology, USA) antibodies were used for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science, UK) was employed for secondary detection, followed by the addition of streptavidin-horseradish peroxidase conjugate and visualized on film by chemiluminescence. β-Actin standard was used as an internal reference for semiquantitative loading in parallel lanes for each variable. Nuclear extracts were analyzed for NF-κB (RelA/p65) DNA binding activity by electrophoretic mobility shift assay (EMSA), as described by us previously (16).

L-Buthionine-(S,R)-sulfoximine (BSO), N-acetyl-L-cysteine (NAC), γ-glutamylcysteinyl-ethyl ester (γ-GCE), pyrrolidine dithiocarbamate (PDTC), and sulfasalazine (SSA) pretreatments. Stock solutions of BSO (1125 μM), NAC (1125 mM), γ-GCE (1125 μM), PDTC (1125 μM) and SSA (25 mM) were prepared in sterile deionized water and stored at 4°C. Cells grown to confluence were pretreated at 23 Torr for 24 h at 37°C with BSO (50 μM), NAC (50 mM), γ-GCE (100 μM), PDTC (100 μM) or SSA (0.1, 1, 5 mM) before exposure to ascending ΔpO_2 regimen for additional 4 h. After each treatment, cells were washed with HBSS, followed by extraction and analysis.

Lactate dehydrogenase (LDH) release activity assay. A modification of the method by Bergmeyer (18) was used to determine LDH release activity. Supernatants were collected and cells extracted in a buffer containing (in mM): 20 HEPES (pH 7.5), 1.5 MgCl$_2$, 0.2 EDTA, 100 NaCl, 5 DTT, 1 PMSF, and 1.2 Na$_3$VO$_4$, briefly sonicated and centrifuged at 10,000g for 5 minutes at 4°C, and cytosolic extracts were transferred to -70°C. Samples (100 μl), β-NADH (100 μl of 1.2 mg/ml) and K$_3$PO$_4$ (800 μl of 50 mM (pH 7.5) buffer) were vortexed and 250-μl aliquots in triplicates were loaded onto a microtiter plate, and the reaction was initiated by the addition of 25 μl of sodium pyruvate (0.35 mg/ml). LDH activity was measured by the rate of consumption of pyruvate and β-NADH, where the decrease in optical density at 340 nm due to oxidation of β-NADH was followed for 5 minutes. The relative LDH release activity was determined according to the equation

LDH % Release Activity

$$= \frac{\text{Experimental Release} - \text{Background Release}}{\text{Total LDH Activity}} \times 100.$$

The ratio is defined in units · mg^{-1} protein, where a *unit* is the amount of enzyme which changes the O.D. of β-NADH at 340 nm and 25°C by 0.001 per minute. Results are expressed as % maximal activity relative to pO_2 = 23 Torr.

Quantification of DNA fragmentation. DNA fragmentation was assayed as reported elsewhere (19). Briefly, cells were resuspended in 250 μl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and lysed by addition of 250 μl cold buffer (5 mM Tris-HCl (pH 8.0), 2 mM EDTA and 0.5% (v/v) Triton X-100). Samples were incubated for 30 minutes at 4°C prior to centrifugation at 14,000g for 15 minutes to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 μl TE and precipitated with 500 μl of 10% trichloroacetic acid (TCA) at 4°C. Following addition of 300 μl 5% TCA, samples were boiled at 95°C for 15 minutes. DNA content of pellets and supernatants was measured using the diphenylamine reagent and the degree of fragmentation was determined as the ratio of [DNA]$_{supernatant}$/[DNA]$_{pellet}$. The variation in glutathione levels was spectrophotometrically determined in
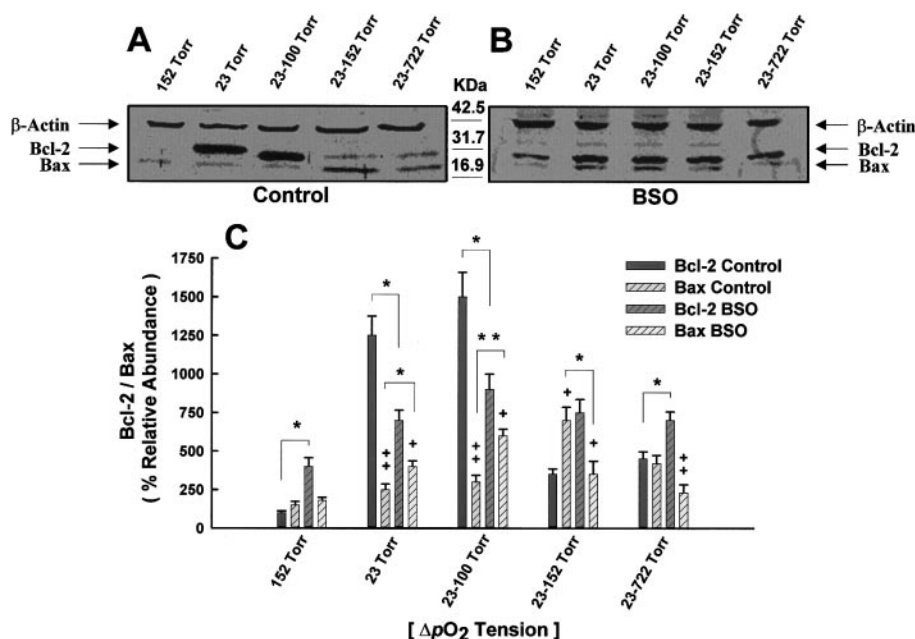
FIG. 1. The effect of oxyexcitation on signaling factors involved in apoptosis. (A) The abundance of Bcl-2 is maximal at fetal distal lung pO_2 and declines with ascending ΔpO_2 regimen, in contrast to Bax which shows increasing abundance with oxyexcitation. (B) BSO up-regulates both Bcl-2 and Bax regardless of pO_2. (C) Histogram analysis of the corresponding bands relative to β-actin (*$P < 0.05$, **$P < 0.01$, as compared to control at each pO_2; [+]$P < 0.05$, [++]$P < 0.01$, for [Bax] versus [Bcl-2], with or without BSO). $n = 4$, which represents the number of experiments performed with independent preparations.

neutralized samples as accounted previously (16). Correlation between reduced glutathione and DNA damage was subsequently carried out.

Assessment of mono- and oligonucleosomes in cytoplasmic fractions. This assay is based on a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using mouse mAbs directed against nucleosomes (Boehringer Mannheim, USA). Briefly, samples (20 μl) were transferred into precoated plates and covered with 80 μl of an immunoreagent, as per the manufacturer's instructions. Plates were thoroughly washed and substrate solution was added and left until the colour development was sufficient for photometric analysis at 405 nm. The specific enrichment factor (EF) of histone-associated DNA fragments was determined according to EF = mU of sample/mU of control (1 mU = 10^{-3} OD).

Statistical analysis. Experimental results are expressed as mean $\pm$ SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's test to determine significance of mean separation among treatments. The *a priori* level of significance at 95% confidence was considered at $P < 0.05$.

RESULTS

Analysis of Bcl-2 and Bax expression with ascending ΔpO_2 regimen (oxyexcitation) and BSO. The Bcl-2 expression profile in the alveolar epithelium is maximal in hypoxia (23 and 23 $\rightarrow$ 100 Torr), and gradually declines with oxyexcitation (Fig. 1A). Bax abundance increases $\approx$3-fold, such that the ratio of Bcl-2/Bax decreases as pO_2 increases (Fig. 1A). Glutathione depletion by BSO increases the abundance of Bcl-2 and Bax at all oxygen tensions (Fig. 1B). Histogram analysis of the corresponding bands relative to β-actin is shown in Fig. 1C.

Analysis of p53 expression with oxyexcitation and BSO. p53 abundance is increased with ascending pO_2, in comparison to cultures maintained constant at 23 or 152 Torr (Fig. 2A). Pretreatment with BSO induces the expression of p53 (Fig. 2B), such that the ratio $p53_{BSO}/p53_{control}$ is elevated $\approx$threefold, as shown in Fig. 2C.

Effect of NAC on p53, Bcl-2, and Bax expression. The antioxidant and glutathione (GSH) precursor, NAC, induced the expression of Bcl-2 at all oxygen tensions (Fig. 3A). In contrast, NAC reduced the expression of Bax and p53, whose abundance was depressed with oxyexcitation (Fig. 3A), such that the ratio Bcl-2/Bax increases $\approx$4- to 5-fold as pO_2 increases (Fig. 3B).

Effect of γ-GCE on p53, Bcl-2, and Bax expression. The cell-permeant glutathione pro-drug, γ-GCE, mimicked the effects of NAC. γ-GCE is rapidly de-esterified intracellularly by esterases thus serving as an effective delivery of GSH (20). γ-GCE induced the accumulation of Bcl-2, at the expense of Bax and p53 (Fig. 4A), thereby increasing Bcl-2/Bax and Bcl-2/p53 ratios $\approx$5- to 7-fold (Fig. 4B).

Effect of PDTC on p53, Bcl-2 and Bax expression. PDTC is an antioxidant/pro-oxidant molecule, which elevates oxidized disulfide glutathione (GSSG) at the expense of GSH (Haddad *et al.,* unpublished observations). PDTC favors the balance of Bax against Bcl-2,
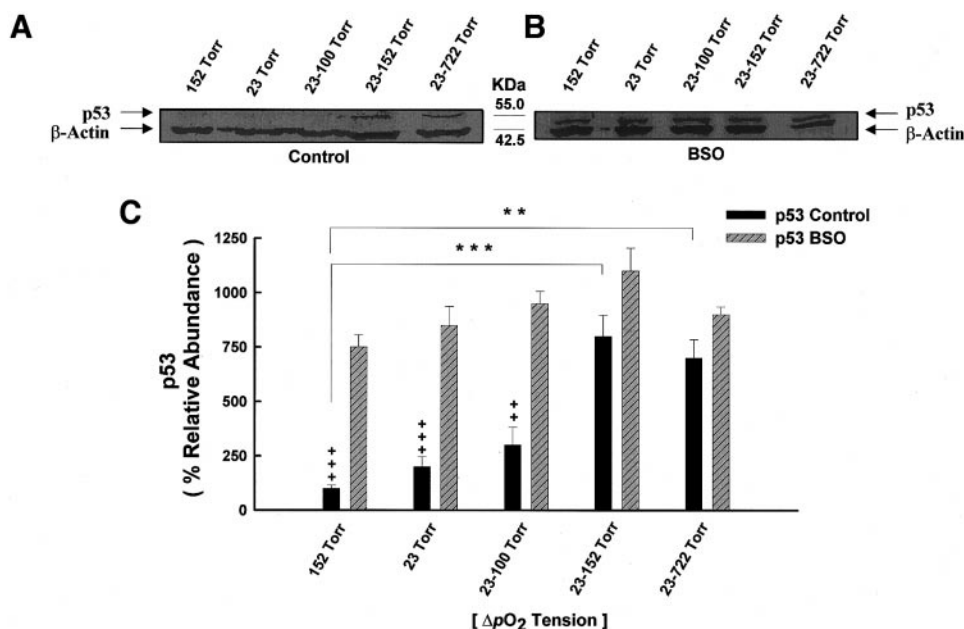
FIG. 2. The effect of oxyexcitation on p53 expression. (A) The expression of p53 shows increasing abundance with ascending ΔpO_2 regimen. (B) BSO up-regulates p53 at all oxygen tensions. (C) Histogram analysis of the corresponding bands relative to β-actin (**$P < 0.01$, ***$P < 0.001$, as compared to control (152 Torr); ++$P < 0.01$, +++$P < 0.001$, as compared to BSO at each pO_2). $n = 4$, which represents the number of experiments performed with independent preparations.

with no prominent effect on p53 abundance (Fig. 5A). Histogram analysis of PDTC effect is shown in Fig. 5B.

Modulatory role of SSA on redox-sensitive transcription factor NF-κB and subsequent regulation of apoptotic pathways. SSA, a potent and specific inhibitor of NF-κB (21), up-regulates Bax and p53, and down-regulates Bcl-2 accumulation (Fig. 6A). Histogram analysis of the abundance of these factors is shown in Fig. 6B. The dose-response relationship for SSA-dependent inhibition of NF-κB activation with oxyexcitation is shown in Fig. 6C.

Analysis of the potency of thiol modulating agents. As shown in Table 1, the relative potency of thiols is given in reference to Bcl-2 abundance. In contrast to BSO, PDTC and SSA, which lowers Bcl-2, NAC and its mimetic γ-GCE favors the balance of Bcl-2 against Bax and p53. The effect of thiols on LDH release activity is shown in Table 2, where NAC has a protective effect against necrotic death.

Analysis of DNA fragmentation and correlation with GSH profile. DNA fragmentation was not detectable at either static pO_2 (23 and 152 Torr; Baseline) or 23 → 100 Torr shift (Fig. 7A), but increased with oxyexcitation. The profile of the effects of thiol modulating agents on DNA fragmentation is shown in Fig. 7B. Regression correlation for GSH variations with time on DNA fragmentation pool is given in Fig. 7C, showing an inverse relationship. DNA strand breakage had a significant inverse correlation with intracellular gluta-

thione in hyperoxic shifts, making it a suitable marker to include when protective strategies are assessed.

Detection of nucleosomal fragments and the effect of thiol modulating agents. Consistent with the DNA fragmentation assay was the appearance of nucleosomal fragments in the cytosol with ascending pO_2 shifts (Table 3). BSO increased the formation of mono- and oligonucleosomes at 23 → 100 and 23 → 152 Torr, although variably comparable to SSA but not to PDTC. In contrast, NAC lowered the formation of fragmented nucleosomes induced by oxyexcitation and/or by the effects of hydrogen peroxide (H_2O_2) and xanthine/xanthine oxidase (X/XO) (Table 3), hydroxyl radical (·OH) and superoxide anion (O_2^-) generating systems, respectively.

DISCUSSION

The regulation of apoptotic pathways and the possible involvement of redox-sensitive transcription factors in the perinatal lung epithelium are not well characterized. The present investigation highlights *in vitro* the significance of thiol regulation of signaling factors governing alveolar cell death in oxidative stress. Specifically, we show that: (*i*) signaling factors in apoptosis are differentially expressed with ascending ΔpO_2 shifts; (*ii*) selective inhibition of γ-GCS, the rate-limiting enzyme in the biosynthesis of GSH, up-regulates apoptotic agonists and induces DNA frag-
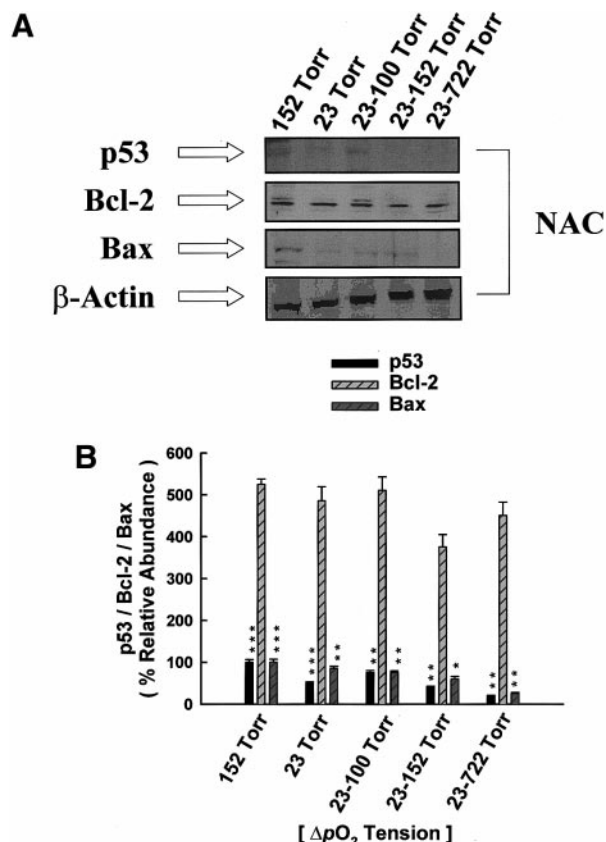
FIG. 3. Effect of NAC on apoptotic factors. (A) The expression of Bcl-2 shows increasing abundance with ascending ΔpO_2 regimen in response to NAC, in contrast to Bax and p53 whose abundance was completely obliterated. (B) Histogram analysis of the corresponding bands, relative to β-actin (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, as compared to [Bcl-2]). $n = 4$, which represents the number of experiments performed with independent preparations.

mentation; (*iii*) replenishing intracellular GSH by NAC favors the up-regulation of antagonists, an effect mimicked by γ-GCE; (*iv*) PDTC, a nonselective blocker of NF-κB and a precursor of GSSG, draws an equilibrium in favor of Bax independent of significant change in p53 expression; and (*v*) selective and irreversible inhibition of NF-κB suppresses Bcl-2 and induces Bax in a p53-dependent pathway.

Exposure to oxyexcitation triggers a signaling mechanism in the perinatal epithelium (16). The mechanics of this process centers on the activation of redox-sensitive transcription factors, which modulate the pattern, and determine the specificity of, the molecular response to oxidative stress. The scenario behind a differential up-regulation of apoptosis agonists and/or antagonists is largely affected by shifting fATII cells from hypoxia to ascending ΔpO_2 regimen. Bcl-2, an antiapoptotic proto-oncogene, showed accumulating abundance under hypoxic conditions, but declined with oxyexcitation, whereas Bax, Bcl-2 counterpart, followed an opposite expression scheme. Although Bcl-2

intervention in reducing the onset of apoptosis in response to oxyexcitation might reflect its antioxidant properties (10), Bcl-2 can protect cells from inevitable death even under anaerobic conditions (9), suggesting that the production of reactive oxygen species (ROS) is not necessarily a prerequisite for mediating apoptosis. The observation that the oxyexcitation pathway favors the balance of Bax against Bcl-2 strongly suggests the involvement of an oxygen signaling-dependent mechanism leading to suppression of Bcl-2. The likely pathway implicated in this respect could be involving cell cycle arrest through the activation of p53. This is consistent with the hypothesis that hyperoxic injury in mammalian cells is characterized by a complex but reproducible pattern of lung injury and repair during which the alveolar surface is damaged, denuded and repopulated by fATII epithelial cells (22). It is therefore reasonable to postulate that chemioxyexcitation (ROS/ ΔpO_2) triggers a process ultimately leading to suppression of Bcl-2 and up-regulation of Bax, possibly through a p53 linked pathway. This postulate is fur-
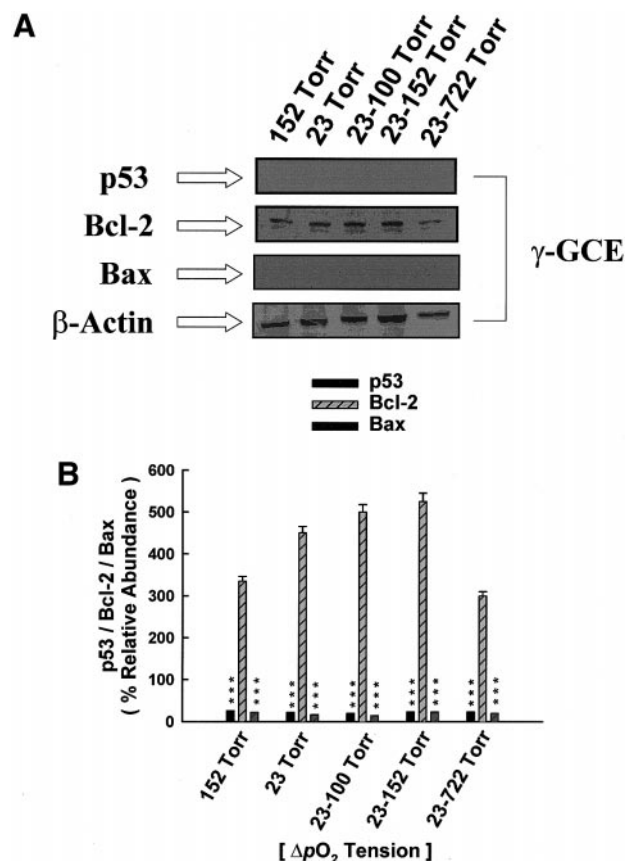

FIG. 4. Effect of γ-GCE on apoptotic factors. (A) The expression of Bcl-2 shows increasing abundance with ascending ΔpO_2 regimen in response to γ-GCE, in contrast to Bax and p53 whose abundance was completely obliterated. (B) Histogram analysis of the corresponding bands, relative to β-actin (***$P < 0.01$, as compared to [Bcl-2]). $n = 4$, which represents the number of experiments performed with independent preparations.
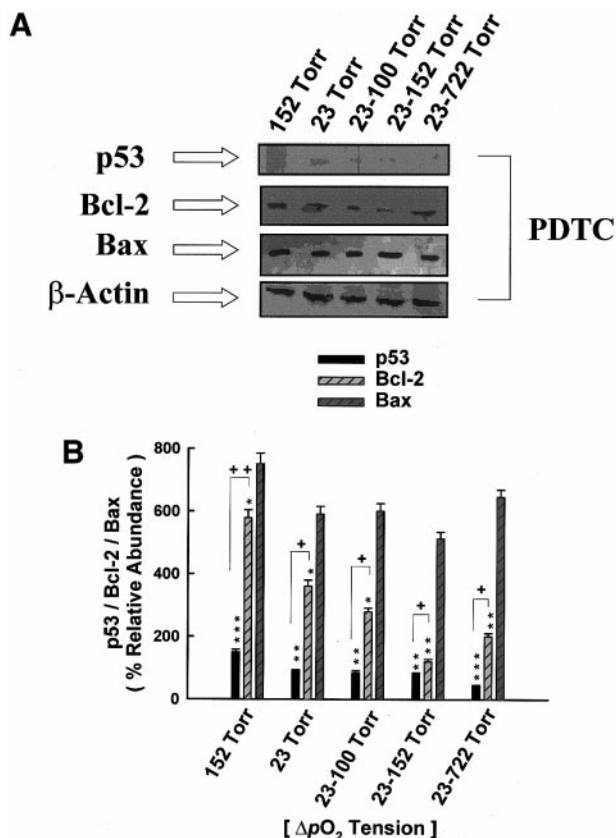
FIG. 5. Effect of PDTC on apoptotic factors. (A) The expression of Bax, but not p53, shows increasing abundance with ascending ΔpO_2 regimen in response to PDTC, in contrast to Bcl-2 whose abundance was decreased. (B) Histogram analysis of the corresponding bands, relative to β-actin (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to [Bax]; $^+P < 0.01$, $^{++}P < 0.001$, as compared to [p53]). $n = 4$, which represents the number of experiments performed with independent preparations.

ther supported by the report of Lowe *et al.* (23) where p53$^{-/-}$ cell line transfection with a p53 mutant was shown to lower the threshold for inducing apoptosis following oxidative challenge. This coheres with the proposal that p53 acts as a sensor of cellular damage no matter how undetectable it might be, and that this process falls under tight regulation of redox responsive transcription factors (24).

It is now widely accepted that the process of apoptosis, which is characterized by specific morphologic and biochemical properties, is redox-modulated (25). These unique properties, as differentiated from necrosis, may fall into three distinct phases termed *initiation, commitment,* and *execution* (26). During *commitment,* ROS increase concomitant with lowering GSH levels were reported even in systems where various stimuli were used that do not in themselves induce the formation of intracellular ROS (27). Selective inhibition of γ-GCS, the rate-limiting enzyme in the biosynthesis of GSH, has been shown to up-regulate Bax and p53 regardless of pO_2 shift direc-

tion. We have previously reported that BSO depletes intracellular GSH by $\geq 80\%$ (16) and up-regulates the production of ROS (Haddad *et al.,* unpublished observations). Since GSH depletion is an important trigger of the apoptotic pathway, it's very likely that ROS are involved in BSO-mediated induction of Bax and p53. Unexpectedly, however, BSO also induced the accumulation of Bcl-2 in a manner independent of pO_2, although redox disequilibrium seems to be in favor of apoptosis, consistent with other observations (28, 29). Moreover, apoptosis cannot be induced in cells in which a reduced environment is constantly maintained, consistent with the observations that implementing high GSH levels and/or preventing its extrusion increases resistance to apoptosis (28, 30). Conversely, cells undergoing apoptosis extrude GSH, indicating that glutathione loss during this process is not a consequence of oxidative stress (31). We therefore postulate that loss of cellular GSH content might cause oxidative stress, possibly by inducing the formation of ROS, thereby altering redox potential in favor of an oxidation equilibrium. This subsequently leads to up-regulation of downstream agonists of apoptosis and cell cycle arrest, however, we note that GSH depletion alone is not necessarily sufficient to evoke commitment to apoptosis, as BSO is also induces the accumulation of Bcl-2.

To further support the notion that replenishing intracellular GSH affects the commitment to and/or execution of apoptosis, we investigated the potential role of NAC as a glutathione precursor. As expected, NAC obliterated the appearance of either Bax or p53 in response to oxyexcitation, and induced Bcl-2 accumulation, in agreement with another observation (19). Effective pharmacological intervention by NAC in alveolar cells may indicate modulation of redox equilibrium in association with oxyexcitation. NAC is well known as a thiol antioxidant which, after uptake, deacylation, and biochemical conversion to GSH, may function as a redox buffer and/or effective ROS scavenger (32). A possible mechanism by which NAC blocks apoptosis is by down-regulating the activation of caspases which are linked to the final executive pathway of cell death (33), a concept that has to be ascertained in the alveolar epithelium.

The cell-permeable glutathione pro-drug γ-GCE was shown to be potently effective, as NAC, in mitigating the apoptotic response. γ-GCE is rapidly de-esterified by intracellular esterases, thereby serving as an effective delivery agent for glutathione, which is a peptide incapable of crossing membranes in its native form (20). Exogenous or endogenous GSH, therefore, may feed into one of the well-characterized pathways of glutathione metabolism (34). Since γ-GCE is able to suppress the formation of intracellular ROS, the antioxidant/scavenging effects of this molecule are likely to contribute to protective effects in oxidative
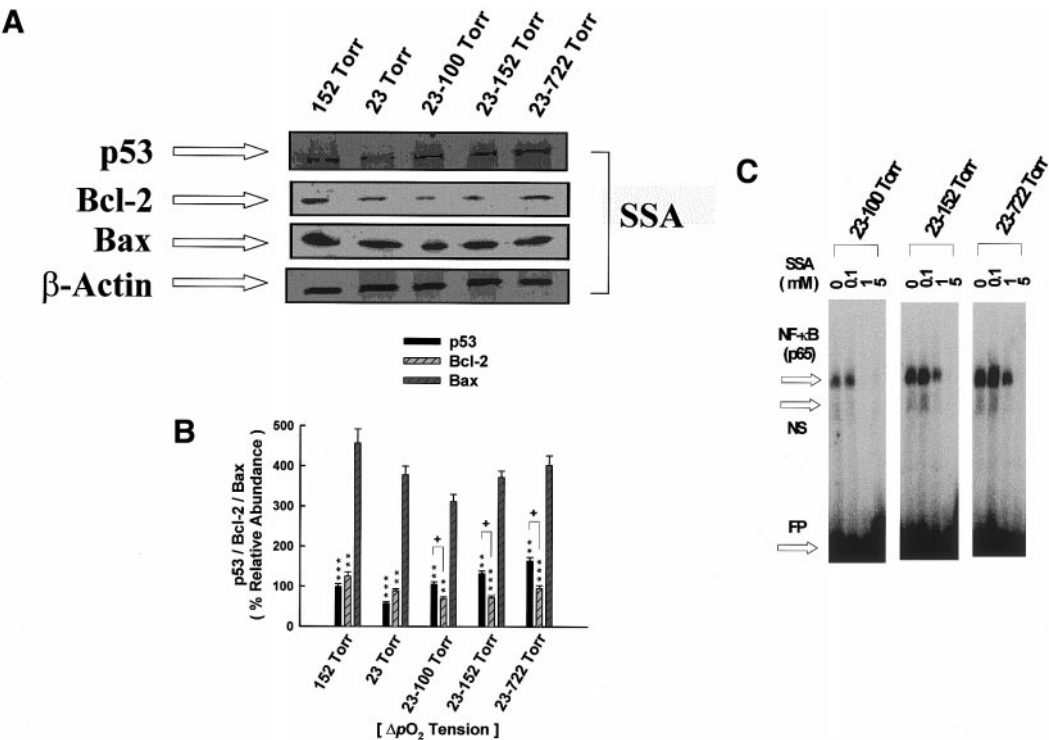
FIG. 6. Effect of selective inhibition of NF-κB on apoptotic signaling factors. (A) The expression of Bax and p53 shows increasing abundance with ascending $\Delta p O_2$ regimen in response to SSA, in contrast to Bcl-2 whose abundance was decreased. (B) Histogram analysis of the corresponding bands, relative to β-actin. (C) The dose-response curve of the SSA-dependent inhibition of NF-κB activation (*NS*, nonspecific) (**$P < 0.01$, ***$P < 0.001$, as compared to [Bax]; +$P < 0.01$, as compared to [Bcl-2]). $n = 4$, which represents the number of experiments performed with independent preparations.

stress. Taken together in hand with the cyto-protective effects of NAC, it is evident that inhibition of apoptosis in response to oxyexcitation is dependent on NAC/γ-GCE ability to suppress Bax and p53 in equilibrium favoring a reduction environment through induction of Bcl-2.

TABLE 1

Comparison of the Potency of Thiol-Modulating Agents on Agonist and Antagonist Members Involved
in the Alveolar Apoptotic Pathway as Determined by the Effective Ratio R^a

$\Delta p O_2$ (Torr)	Control	BSO	NAC	γ-GCE	PDTC	SSA
			p53			
152	1.00	0.53 ↓	5.25 ↑	13.4 ↑	3.85 ↑	2.12 ↑
23	6.25‡	0.82 ↓	9.70 ↑	22.5 ↑	4.00 ↓	2.05 ↓
23 → 100	5.00‡	0.95 ↓	6.80 ↑	26.3 ↑	4.00 ↓	2.00 ↓
23 → 152	0.44*	0.68	9.38 ↑	22.8 ↑	1.52 ↑	2.25 ↑
23 → 722	0.65*	0.77	22.5 ↑	13.6 ↑	2.21 ↑	6.00 ↑
			Bax			
152	0.67	2.22 ↑	5.25 ↑	16.8 ↑	0.77	0.27 ↓
23	5.00‡	1.75 ↓	5.64 ↑	28.1 ↑	0.61 ↓	0.23 ↓
23 → 100	5.00‡	1.50 ↓	6.80 ↑	35.7 ↑	0.57 ↓	0.22 ↓
23 → 152	0.50	2.14 ↑	6.25 ↑	25.0 ↑	0.30 ↓	0.19 ↓
23 → 722	1.07*	3.04 ↑	18.0 ↑	16.7 ↑	0.14 ↓	0.24 ↓

[a] The effective ratio (R) is defined as the Bcl-2/p53 or Bcl-2/Bax ratios, where $R \geq 1.00$ refers to higher abundance and $R \leq 1.00$ to lower abundance of Bcl-2 relative to either p53 or Bax at any given $p O_2$. *$P < 0.05$, ‡$P < 0.001$, as compared to 152 Torr. ↑ ↓, [Bcl-2] is higher and lower, respectively, as compared to control.

TABLE 2

Analysis of LDH Release Activity, a Marker of Necrotic Cell Death, in Response
to Oxyexcitation and Thiol-Modulating Agents at 4 h and 37°C

ΔpO_2 (Torr)	Control	BSO	NAC	γ-GCE	PDTC	SSA
152	3.58 ± 0.25	3.62 ± 0.17	3.45 ± 0.15	3.22 ± 0.08	3.50 ± 0.21	3.63 ± 0.14
23	3.04 ± 0.12	3.10 ± 0.15	3.00 ± 0.10	3.13 ± 0.14	3.12 ± 0.15	3.14 ± 0.12
23 → 100	5.50 ± 1.24	5.25 ± 1.12	5.12 ± 1.56	5.21 ± 1.54	5.32 ± 1.47	6.05 ± 1.86
23 → 152	7.23 ± 1.56	7.58 ± 1.28	6.79 ± 1.35	7.25 ± 1.10	8.15 ± 1.50	8.48 ± 1.71
23 → 722	12.23 ± 2.37*	13.36 ± 2.40*	8.12 ± 1.88*$^\phi$	7.00 ± 0.78*$^\phi$	12.82 ± 1.73*	12.98 ± 1.75*

Note. Data are means ± SEM. *$P < 0.05$, as compared to 23 and 152 Torr; $^\phi P < 0.05$, as compared to control. $n = 4$, which represents the number of experiments performed with independent preparations.

Recent studies have demonstrated that NF-κB activation and subsequent nuclear translocation has an essential role in preventing cells from entering apoptosis mediated by cytokines, irradiation, chemotherapeutic agents and other stimuli (35, 36). Support for this model is provided with the finding that overexpression of a dominant-negative form of IκB-α, an inhibitor of NF-κB, in transgenic mice and several cell lines promoted apoptosis *in vitro* (37, 38). In addition, experiments with RelA$^{-/-}$ (p65$^{-/-}$) mice show that they are more susceptible to agents stimulating apoptosis than the wild-type (39). This suggested that a probable in-

volvement of NF-κB in apoptotic pathways in the alveolar epithelium could also confer protective strategies against oxidative challenge. We approached this theory from two different, although closely related perspectives; firstly, we investigated the effect of nonspecific inhibition of NF-κB using PDTC as an antioxidant/pro-oxidant agent which elevates GSSG at the expense of GSH (Haddad *et al.,* unpublished observations); and secondly, we studied the effect of SSA, a selective NF-κB inhibitor (21), and determined whether these two pathways are diverging at an endpoint where the execution of apoptosis is finally acti-
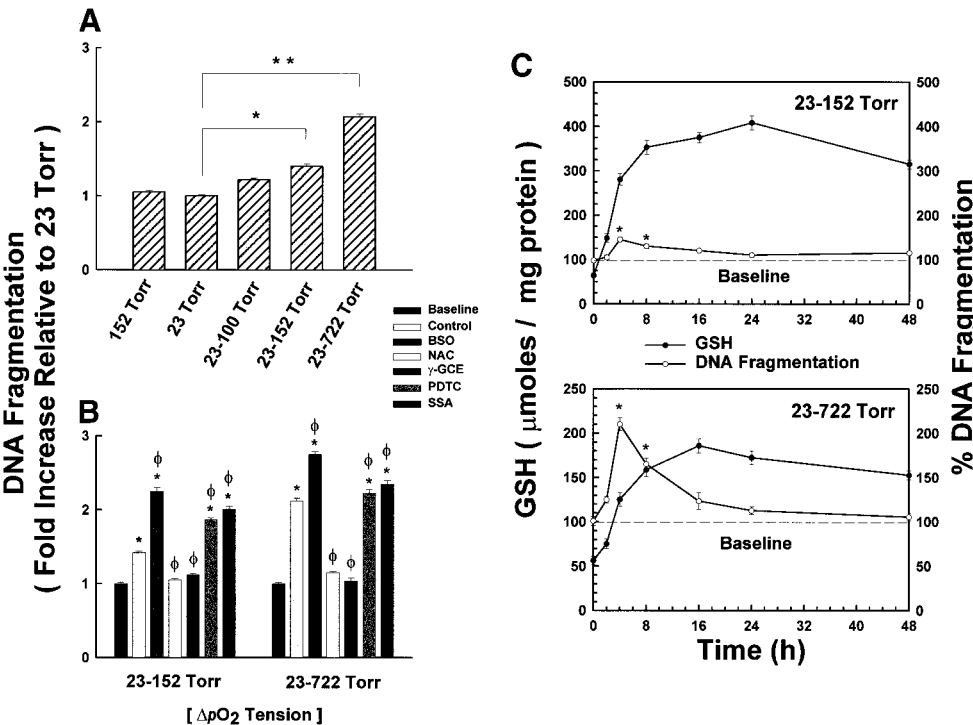

FIG. 7.   Correlation between the levels of GSH and degree of DNA laddering. (A) Analysis of DNA fragmentation with oxyexcitation (*$P < 0.05$, **$P < 0.01$, as compared to 23 Torr). (B) The effect of thiol modulating agents on DNA fragmentation at 23 → 152 and 23 → 722 Torr shifts (*$P < 0.05$, as compared to baseline; $^\phi P < 0.05$, as compared to control at each pO_2). (C) Regression analysis for GSH variations with time on DNA fragmentation pool and the inverse correlation (*$P < 0.05$, as compared to baseline marked by the dotted line at 100%). $n = 4$, which represents the number of experiments performed with independent preparations.

TABLE 3

Determination of Cytolplasmic Mono- and Oligonucleosome Histone-Associated DNA Fragments (μU/ml)
and the Effect of Thiol Modulating Agents at 24 h and 37°C

Treatment	ΔpO_2 (Torr)				
	152	23	$23 \to 100$	$23 \to 152$	$23 \to 722$
Control	2.7 ± 0.12	2.8 ± 0.23	2.9 ± 0.18	$3.2 \pm 0.15^*$	$3.5 \pm 0.24^*$
H_2O_2	2.6 ± 0.14	2.7 ± 0.15	2.2 ± 0.27	$4.0 \pm 0.28^{*\phi}$	$3.1 \pm 0.20^*$
X/XO	2.8 ± 0.10	2.9 ± 0.17	2.4 ± 0.15	2.6 ± 0.12	2.7 ± 0.11
BSO	2.9 ± 0.15	2.7 ± 0.16	$3.6 \pm 0.17^{*\phi}$	$3.6 \pm 0.12^{*\phi}$	2.5 ± 0.10
BSO + H_2O_2	3.0 ± 0.12	2.3 ± 0.09	2.5 ± 0.12	3.0 ± 0.15	2.3 ± 0.13
BSO + X/XO	2.7 ± 0.10	2.7 ± 0.12	2.6 ± 0.11	$3.1 \pm 0.17^*$	2.2 ± 0.18
NAC	$2.0 \pm 0.14^{\phi}$	$2.3 \pm 0.18^{\phi}$	$2.1 \pm 0.12^{\phi}$	$2.0 \pm 0.11^{\phi}$	$2.6 \pm 0.19^{\phi}$
NAC + H_2O_2	$1.5 \pm 0.07^{\phi}$	$2.3 \pm 0.14^{\phi}$	$3.5 \pm 0.17^{\phi}$	$2.5 \pm 0.13^{\phi}$	$2.1 \pm 0.21^{\phi}$
NAC + X/XO	$2.4 \pm 0.11^{\phi}$	$1.1 \pm 0.09^{*\phi}$	$0.8 \pm 0.05^{*\phi}$	$0.8 \pm 0.04^{*\phi}$	$0.9 \pm 0.04^{*\phi}$
γ-GCE	2.7 ± 0.10	3.1 ± 0.10	2.9 ± 0.15	3.0 ± 0.15	2.7 ± 0.12
PDTC	2.9 ± 0.12	2.9 ± 0.20	3.1 ± 0.18	3.1 ± 0.15	3.0 ± 0.14
SSA	2.8 ± 0.11	2.8 ± 0.12	3.0 ± 0.14	$3.2 \pm 0.12^*$	$3.2 \pm 0.17^*$

Note. Data are means $\pm$ SEM. H_2O_2 (250 μM); Xanthine (X, 100 μM); Xanthine Oxidase (XO, 2 mU/ml). $^*P < 0.05$, as compared to 23 and 152 Torr, $^{\phi}P < 0.05$, as compared to control. $n = 4$, which represents the number of experiments performed with independent preparations.

vated by regulating downstream effectors. As expected, both inhibitors favored the balance of Bax against Bcl-2, but more importantly the observation that the former appears to be mediating apoptosis in a p53-independent manner, in contrast to the latter where a p53-mediated pathway is involved.

PDTC has been proven to be a potent, although a nonselective, inhibitor of NF-κB in the alveolar epithelium. PDTC has the potential to decrease the GSH/GSSG ratio by elevating GSSG due to oxidation of GSH. It is possible, therefore, that GSSG might drive the formation of an oxidation equilibrium that renders NF-κB inactive, and subsequently triggers a signaling mechanism for the up-regulation of Bax independent of p53. This phenomenon is consistent with the observation that PDTC can act as a pro-oxidant that induces p53 cysteine residue oxidation, correlated with depression of mRNA accumulation
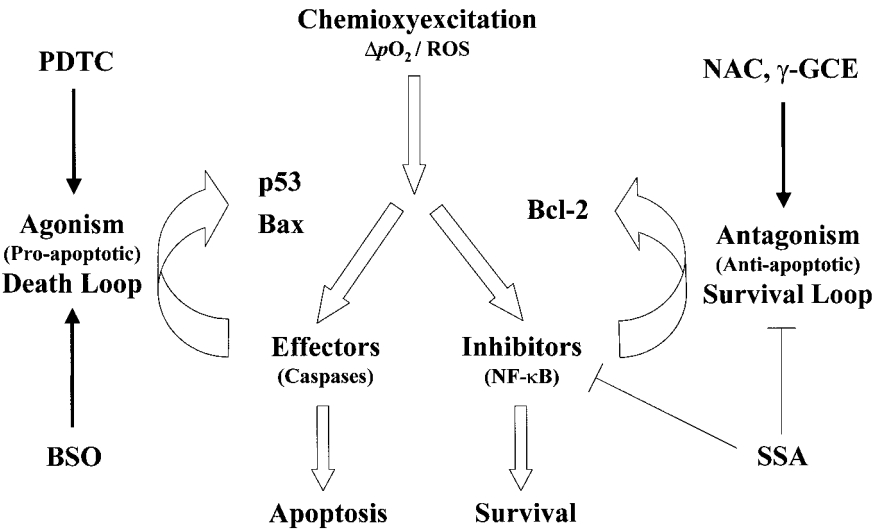

FIG. 8. Schematic model for apoptosis pathways in the perinatal alveolar epithelium. Exposure to ascending ΔpO_2 regimen and reactive oxygen species (ROS), a process referred to as chemioxyexcitation, triggers a signaling mechanism that could lead to either apoptosis or activation of NF-κB. NF-κB conveys a protective effect by amplifying Bcl-2 through the antagonism (antiapoptotic) survival loop. Whereas thiols such as *N*-acetyl-L-cysteine (NAC) and γ-glutamylcysteinyl-ethyl ester (γ-GCE) promote the survival loop, sulfasalazine (SSA), a selective inhibitor of NF-κB, confers negative regulation favoring apoptosis. Glutathione depletion by L-buthionine-(*S,R*)-sulfoximine (BSO) up-regulates the expression of Bax and p53 through activation of agonism (proapoptotic) death loop, an effect which is mimicked by pyrrolidine dithiocarbamate (PDTC). The likely implicated executioners of apoptosis are the caspases, the final effectors in the death machinery.

and activation of downstream functions (40). On the other hand, SSA favors a p53-dependent induction of Bax, in accord with other observations (41, 42), suggesting that selective targeting of RelA render the epithelium more sensitive to oxyexcitation. Although NF-κB activation is submaximal under mild hyperoxic conditions and increases with ascending $\Delta p\mathrm{O}_2$ regimen (16), the pathways proposed for SSA-mediated apoptosis under hypoxia are not validated. In a model of alveolar cells we have previously shown that the activation state of this factor at 23 Torr is minimal, but still detectable (16). The question is how selective inhibition of NF-κB by SSA similarly up-regulates Bax and p53 under both hypoxic and hyperoxic conditions? The explanation for this discrepancy is most probably provided by the observation that NF-κB has the potential to transduce hypoxic signals by regulating and facilitating the rapid control of hypoxic genes (43, 44). We speculate that SSA-mediated apoptosis is dependent on the activation of a secondary mediator within the signaling pathway from exposure to oxyexcitation to initiation/commitment to cell death. The most likely candidate in this respect is p53, thereby providing unequivocal evidence in mediating SSA but not PDTC-evoked up-regulation of Bax against Bcl-2. This strongly suggests that NF-κB conveys an antiapoptotic protective function in the alveolar epithelium, implicating that its induction/activation is critical to defending the integrity of cells following oxidant injury, presumably by regulating downstream genes that are particularly involved in rescuing the cell. Pathways mediating apoptosis in the perinatal alveolar epithelium are schematized (Fig. 8).

The present study provides evidence that the differential expression of signaling factors mediating apoptosis in the alveolar epithelium is responsive to redox disequilibrium. Although the chemioxyexcitation pathway is governed by Bax/p53 up-regulation against Bcl-2, thiol regulation of these factors is largely p53-independent. Selective inhibition of the redox-sensitive transcription factor NF-κB shows the antiapoptotic potential following oxidative challenge, through activation of a survival loop. Crosstalk among cell death factors in the developing lung constitutes a potential target for therapeutic intervention in the treatment of neonatal respiratory distresses such as bronchopulmonary dysplasia, commonly witnessed in premature infants receiving clinical oxygen therapy.

ACKNOWLEDGMENTS

REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26,** 239–57.

2. Lin, K.-T., Xue, J.-Y., Nomen, M., Spur, B., and Wong, P. Y.-K. (1995) *J. Biol. Chem.* **270,** 16487–16490.

3. Gerschenson, L. E., and Rotello, R. J. (1992) *FASEB J.* **6,** 2450–2455.

4. Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz, R. J., Evan, G. I., and Guild, B. C. (1995) *Nature* **374,** 733–736.

5. Williams, G. T., and Smith, C. A. (1993) *Cell* **74,** 777–779.

6. Chao, D. T., and Korsmeyer, S. J. (1998) *Annu. Rev. Immunol.* **16,** 395–419.

7. Tsujimoto, Y. (1998) *Genes Cells* **3,** 697–707.

8. Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **75,** 241–251.

9. Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993) *Nature* **361,** 365–369.

10. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) *Science* **262,** 1274–1277.

11. Sandstrom, P. A., Mannie, M. D., and Buttke, T. M. (1994) *J. Leukocyte Biol.* **55,** 221–226.

12. Rothstein, J. D., Bristol, L. A., Hosler, B., Brown, R. H. Jr., and Kuncl, R. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4155–4159.

13. Abello, P. A., Fidler, S. A., Bulkley, G. B., and Buchman, T. G. (1994) *Arch. Surg.* **129,** 134–140.

14. Meredith, M. J., Cusick, C. L., Soltaninassab, S., Sekhar, K. S., Lu, S., and Freeman, M. L. (1998) *Biochem. Biophys. Res. Commun.* **248,** 458–463.

15. Polyak, K., Yong, X., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) *Nature* **389,** 300–305.

16. Haddad, J. J. E., and Land, S. C. (2000) *Am. J. Physiol.* **278,** L492–L503.

17. Cotton, R. B. (1998) Fetal and Neonatal Physiology, 2nd ed., Saunders, PA.

18. Bergmeyer, H. U. (1974) *in* Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), pp. 736–743, Verlag Chemie, Berlin.

19. Brockhaus, F., and Brüne, B. (1998) *Exp. Cell Res.* **238,** 33–41.

20. Campbell, E. B., and Griffith, O. W. (1989) *Anal. Biochem.* **183,** 21–25.

21. Wahl, C., Liptay, S., Adler, G., and Schmid, R. M. (1998) *J. Clin. Invest.* **101,** 1163–1174.

22. Tryka, F. H., Witschi, H., Gosslee, D. G., McArthur, A. H., and Clapp, N. K. (1986) *Am. Rev. Respir. Dis.* **133,** 1055–1059.

23. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) *Nature* **362,** 847–849.

24. Lane, D. P. (1992) *Nature* **358,** 15.

25. Hall, A. G. (1999) *Eur. J. Clin. Invest.* **29,** 238–245.

26. Kroemer, G., Petit, P., Zamzami, N., Vayssière, J. L., and Mignotte, B. (1995) *FASEB J.* **9,** 1277–1287.

27. Bustamante, J., Tovar, A., Montero, G., and Boveris, A. (1997) *Arch. Biophem. Biophys.* **337,** 4612–4619.

28. Boggs, S. E., McCormick, T. S., and Lapetina, E. G. (1998) *Biochem. Biophys. Res. Commun.* **217,** 229–233.

29. Celli, A., Que, F. G., Gores, G. J., and LaRusso, N. F. (1998) *Am. J. Physiol.* **275,** G749–G757.

30. Ghibelli, L., Fanelli, C., Rotilio, G., Lafavia, E., Coppola, S., Colussi, C., Civitareale, P., and Ciriolo, M. R. (1998) *FASEB J.* **12,** 479–486.

31. Ghibelli, L., Coppola, S., Rotilio, G., Lafavia, E., Maresca, V., and Ciriolo, M. R. (1995) *Biochem. Biophys. Res. Commun.* **216,** 313–320.

32. Bernard, G. R. (1991) *Am. J. Med.* **91(3C),** 54S–59S.

33. Cohen, G. M. (1997) *Biochem. J.* **326,** 1–16.

34. Meister, A. (1988) *J. Biol. Chem.* **263,** 17205–17208.

35. Bellas, R. E., FitzGerald, M. J., Fausto, N., and Sonenshein, G. E. (1997) *Am. J. Pathol.* **151,** 891–896.

36. Hettmann, T., DiDonato, J., Karin, M., and Leiden, J. M. (1999) *J. Exp. Med.* **189,** 145–157.

37. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–782.

38. Liu, Z.-G., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576.

39. Boothby, M. R., Mora, A. L., Scherer, D. C., Brockman, J. A., and Ballard, D. W. (1997) *J. Exp. Med.* **185,** 1897–1907.

40. Wu, H. H., and Momand, J. (1998) *J. Biol. Chem.* **273,** 18898–18905.

41. Kawai, H., Yamada, Y., Tatsuka, M., Niwa, O., Yamamoto, K., and Suzuki, F. (1999) *Cancer Res.* **59,** 6038–6041.

42. Liptay, S., Bachem, M., Kacker, G., Alder, G., Debatin, K. M., Schmid, R. M. (1999) *Br. J. Pharmacol.* **128,** 1361–1369.

43. Koong, A. C., Chen, K. Y., and Giaccia, A. J. (1994) *Cancer Res.* **54,** 1425–1430.

44. Schmedtje, J. F., Ji, Y. S., and Liu, W. (1997) *J. Biol. Chem.* **272,** 601–608.

45. Haddad, J. J. E., Olver, R. E., Murphie, H. M., and Land, S. C. (2000) *FASEB J.,* in press. [Abstract]