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Glutathione Levels and BAX Activation during Apoptosis Due to Oxidative Stress in Cells Expressing Wild-type and Mutant Cystic Fibrosis Transmembrane Conductance Regulator*

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Cystic fibrosis is characterized by chronic inflammation and an imbalance in the concentrations of alveolar and lung oxidants and antioxidants, which result in cell damage. Modifications in lung glutathione concentrations are recognized as a salient feature of inflammatory lung diseases such as cystic fibrosis, and glutathione plays a major role in protection against oxidative stress and is important in modulation of apoptosis. The cystic fibrosis transmembrane conductance regulator (CFTR) is permeable to Cl^-, larger organic ions, and reduced and oxidized forms of glutathione, and the ΔF508 CFTR mutation found in cystic fibrosis patients has been correlated with impaired glutathione transport in cystic fibrosis airway epithelia. Because intracellular glutathione protects against oxidative stress-induced apoptosis, we studied the susceptibility of epithelial cells (HeLa and IB3–1) expressing normal and mutant CFTR to apoptosis triggered by H_2O_2. We find that cells with normal CFTR are more sensitive to oxidative stress-induced apoptosis than cells expressing defective CFTR. In addition, sensitivity to apoptosis could be correlated with glutathione levels, because depletion of intracellular glutathione results in higher levels of apoptosis, and glutathione levels decreased faster in cells expressing normal CFTR than in cells with defective CFTR during incubation with H_2O_2. The pro-apoptotic BCL-2 family member, BAX, is also activated faster in cells expressing normal CFTR than in those with mutant CFTR under these conditions, and artificial glutathione depletion increases the extent of BAX activation. These results suggest that glutathione-dependent BAX activation in cells with normal CFTR represents an early step in oxidative stress-induced apoptosis of these cells.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] forms an ion channel that is permeable to Cl^- and other large organic anions (1–3). Mutations in the gene encoding CFTR are responsible for cystic fibrosis, whose pathology was thought to be due primarily to a decrease in Cl^- permeability through the CFTR (4). However, it is not clear how a change in Cl^- permeability could account for the large variety of symptoms observed during cystic fibrosis. The pathology associated with cystic fibrosis is maintained by repeated lung infections, mainly by *Pseudomonas aeruginosa*, which provokes inflammatory responses that lead to lung fibrosis and respiratory failure (5, 6). Pulmonary injury is related to the production of oxidants in the inflammatory environment that lead to necrosis of lung epithelial cells (3, 5), and infected epithelial cells expressing mutant CFTR are less sensitive to apoptosis than cells expressing normal CFTR (7). However, even in the absence of infection, inflammation is manifest in young children, and inflammatory mediators are maintained constitutively at high levels in cystic fibrosis patients (8–10). Because cells undergoing necrosis release debris that initiate inflammatory responses, it is thus likely that cells expressing mutant CFTR are also more resistant to apoptosis than normal cells in the absence of infection. Consistent with this view, large DNA fragments, typical of necrotic cells, are released in cystic fibrosis epithelia, thus increasing the viscosity of the mucus, and inhaled DNase I improves the respiratory condition of cystic fibrosis patients (11–15). In apoptotic cells, DNA is fragmented into small fragments and packaged into apoptotic bodies that are phagocytosed by neighboring cells, in a way that minimizes an inflammatory response.

Recently it was shown that both the anionic tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as the oxidized glutathione can permeate through the plasma membrane from the cytosol to the extracellular space via a CFTR-dependent mechanism (1, 16, 17) thus establishing a possible link between CFTR and antioxidant defenses of the lung. Glutathione is considered to be the most important water-soluble antioxidant within cells. It keeps other antioxidants such as β-carotenes, tocopherols, and ascorbate in their reduced form, and it functions in the reduction of hydrogen peroxide in reactions catalyzed by glutathione peroxidases (18). Glutathione is also an important antioxidant in the lung (19, 20), and the GSH

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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; BSO, DL-buthionine sulfoximine; GSH, glutathione; GSHee, glutathione ethyl ester; FCS, fetal calf serum; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PE, phycoerythrin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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concentration is greatly reduced in airway surface fluid of cystic fibrosis patients (21). These defects can be attributed directly to missing or defective CFTR channels, because the glutathione concentration in the lung epithelial lining fluid of CFTR-deficient mice is decreased by half, compared with that in wild-type mice (22). The oxidative stress that results from chronic inflammation in the lungs of cystic fibrosis patients could thus be exacerbated by the decrease in GSH levels.

Because glutathione effluxes through the CFTR directly (1, 16) or indirectly (17), and depletion of cytosolic glutathione contributes to apoptosis of lung epithelia and other tissues (23, 24), it has been proposed that the decreased ability of cells expressing mutant CFTR to secrete glutathione may result in their decreased ability to undergo apoptosis (3). Consistent with this possibility, glutathione is actively extruded during apoptosis of cells treated with reactive oxygen species (25).

To address directly the link between glutathione transport by CFTR and apoptosis, we have measured apoptosis of epithelial cells expressing mutant and normal CFTR after exposure to hydrogen peroxide. We have also measured the concentration of glutathione in the same cells as a function of time during oxidative stress and have studied the effects of glutathione depletion on apoptosis of the cells. Finally, because metabolic or oxidative stress activates or induces expression of the pro-apoptotic BCL-2 family member BAX (26–29), we also studied activation of BAX in the different cells exposed to hydrogen peroxide and the effect of glutathione depletion on BAX activation. Our results support the view that cells expressing normal CFTR are more sensitive to oxidative stress-induced apoptosis than cells expressing mutant CFTR because of enhanced glutathione depletion in the normal cells. In addition, we propose that glutathione depletion results in BAX activation, which is responsible for the subsequent apoptosis of normal cells.

EXPERIMENTAL PROCEDURES

Cells and Materials—HeLa cells were from the American Type Culture Collection (Manassas, VA). HeLa cells stably transfected with the plasmid alone (pTracer), the wild-type CFTR construct (pTCFwt), the CFTR ΔF508 mutation (pTCFΔF508), and the CFTR G551D mutation (pTCFG551D) were prepared as previously described (30). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 250 μg/ml Zeocin (all reagents were from Invitrogen) in an incubator at 37 °C and 5% CO_2. The starting cell lines were regularly maintained in Plasmocin (Invitrogen).

IB3–1 CF airway epithelial cells (31) were grown in airway epithelial cell growth medium KIT from PromoCell (Heidelberg, Germany) containing the Supplement Pack without epinephrine-250 and RA-50 and supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Measurement of Apoptosis—HeLa cells at 70% confluency were incubated with H_2O_2 at the indicated concentrations for different times by diluting the H_2O_2 directly into the cell culture medium. Both adherent cells and cells in supernatant were collected, washed, and prepared for cytofluorimetry. Annexin V-PE (BD PharMingen) was used to identify apoptotic cells, following the manufacturer's instructions (32). The cells were washed and transferred into 12- × 75-mm Falcon 2052 FACS tubes (Becton Dickinson, San Jose, CA), and data from 10,000 HeLa cells were collected on a FACScan flow cytometer (Becton Dickinson) with an argon laser tuned to 488 nm. Cell death was quantified using CellQuest software.

Transient Lipofection of IB3–1 CF Cells—Cells were plated at a density of 2.5×10^5 cells in 60-mm Petri dishes 24 h before transfection with the pTracer plasmid, the wild-type CFTR construct (pTCFwt), or pTCFΔF508 using the LipofectAMINE Plus reagent from Invitrogen (San Diego, CA), following the manufacturer's instructions. Cells in each Petri dish were transfected with 500 μl of cell culture medium, 2 μg of plasmid DNA, 8 μl of Plus reagent, and 15 μl of LipofectAMINE without FCS or antibiotics. After transfecting the cells for 4 h at 37 °C, 2 ml of cell culture medium containing 20% FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin was added to each dish. Transfection of IB3–1 CF cells under these conditions yielded 40–55% positively transfected cells, as determined by cytofluorimetry measurement of the green fluorescent protein (GFP) fluorescence. Twenty-four hours after transfection cells were incubated with the indicated concentrations of H_2O_2 for an additional 24 h. Apoptosis was measured by cytofluorimetry using annexin V-PE, as above, but gating only on GFP-positive cells.

Measurement of Intracellular Glutathione—A quantitative determination of the total intracellular glutathione was performed as described by Tietze (33). Confluent HeLa cell monolayers in six-well plates were collected in 0.5 ml of PBS, 1 mM EDTA. Glutathione extraction was performed by treating the cell suspension with 0.2 equivalent volume of a 5% sulfosalicylic acid solution on ice for 15 min and assaying the supernatant reactions after centrifugation at 12,000 rpm in an Eppendorf 5415C centrifuge (stored at 4 °C until use). After glutathione extraction, the protein concentration was determined by the Bradford assay (Bio-Rad protein assay).

Samples were diluted 10-fold in a 100 mM sodium phosphate, 1 mM EDTA buffer solution at pH 7.5, and 50 μl per well were transferred to a 96-well microplate. The following freshly prepared reagents were then mixed at room temperature: 2.8 ml of 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 3.75 ml of 1 mM NADPH, 5.85 ml of 100 mM sodium phosphate, 1 mM EDTA buffer, and 20 units of glutathione reductase (reaction mixture). A range of reduced glutathione concentrations was prepared on the same day in 100 mM sodium phosphate, 1 mM EDTA, pH 7.5, with 0.02 equivalent volumes of 5% sulfosalicylic acid, and was used for calibration by transferring 50 μl per well to a 96-well microplate. One hundred microliters of reaction mixture was immediately added to each well containing sample or standard, and the microplate was placed on the microtiter plate reader using a 420-nm filter. The samples were mixed for 5 s, and absorbance was read for 5 min. Samples were compared with the calibration curve to determine the glutathione concentration in each well.

Depletion of Intracellular Glutathione—DL-Buthionine sulfoximine (BSO) and glutathione ethyl ester (GSHee) were from Sigma Chemical Co. For depletion of intracellular glutathione, an HeLa cell monolayer at 50% confluence was pretreated with 200 μM BSO for 24 h before incubating with H_2O_2 in the presence of the same concentration of BSO (34). For reversal of glutathione depletion, the cells were treated with 200 μM BSO and 3 mM GSHee for 24 h, changing the medium every 12 h, and then with H_2O_2 in the presence of the same concentrations of BSO and GSHee (34).

Quantification of BAX Activation by Cytofluorimetry—A monolayer of HeLa cells confluent at 70% was plated in six-well plates and incubated for the indicated times with H_2O_2 in culture medium. The cells were then collected with PBS, 1 mM EDTA, and washed twice with PBS by centrifugation. The pellet was resuspended in 50 μl of 2 μg/ml BAX rabbit polyclonal IgG (BAX N-20, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS, 1% BSA, 0.05% saponin for 30 min, then washed with PBS and resuspended in 50 μl of PE-conjugated anti-rabbit polyclonal IgG (1:200 dilution; from Molecular Probes, Eugene, OR) in PBS, 1% BSA, 0.05% saponin for 20 min. Samples were washed and suspended in PBS, transferred into 12- × 75-mm Falcon 2052 FACS tubes, and analyzed on the FACScan flow cytometer and CellQuest software, as above.

Visualization of BAX Activation by Confocal Microscopy—HeLa cells were cultured on microscope slides in six-well plates. After treatment with 90 μM H_2O_2 for 8 h, the supernatant was removed and cells were fixed with 4% paraformaldehyde (35). Fixation was stopped by adding the same volume of 50 mM NH_4Cl in PBS for 20 min. Slides were washed in PBS and incubated with 2 μg/ml BAX rabbit polyclonal IgG in PBS, 1% BSA, 0.05% saponin for 45 min, then washed with PBS and incubated with 10 μg/ml of fluorescein isothiocyanate-labeled anti-rabbit polyclonal IgG (Immunotech) in PBS, 0.05% saponin for 15 min. A monoclonal antibody against mitochondrial hsp70 (Affinity Bioreagents, Golden, CO) was used at a dilution of 1:100, followed by a Cy3-conjugated second antibody at a 1:100 dilution. The microscope slides were rinsed in PBS, air-dried, and mounted with Dako mounting medium. Samples were examined with a Leica confocal microscope (Institut Jacques Monod, Paris), and images were analyzed with Adobe Photoshop software.

RESULTS

Sensitivity to Oxidative Stress-induced Apoptosis of HeLa Epithelial Cells Expressing Mutant and Normal CFTR—The CFTR contains several functional domains, including hydrophobic regions thought to interact with membranes, a regula-

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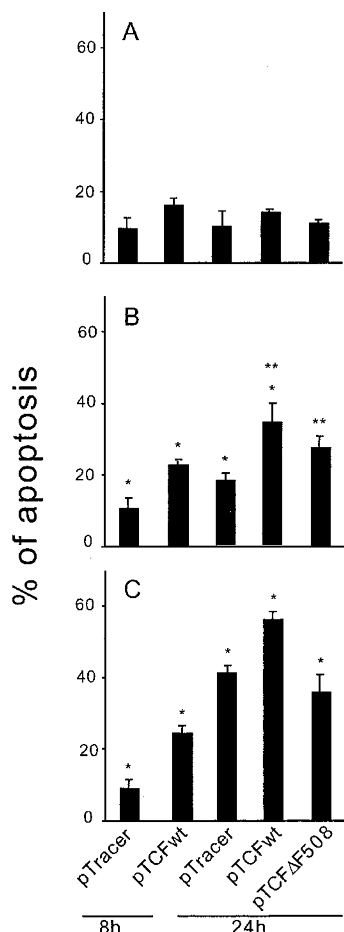

FIG. 1. **Apoptosis of HeLa epithelial cells expressing wild-type or mutated CFTR after treatment with H_2O_2.** *A*, cells transfected with plasmid alone (*pTracer*), the wild-type CFTR construct (*wt*) or the CFTR ΔF508 construct were plated at 70% confluence and cultured in an incubator at 37 °C and 5% CO_2 for 8 or 24 h. *B*, the same cells were treated with 90 μM H_2O_2 in the incubator, and apoptosis was measured 8 or 24 h later, as indicated. *, $p < 0.01$; **, $p < 0.1$, for pTCFwt compared with pTracer at 8 h, or pTCFwt compared with pTracer or pTCFΔF508 at 24 h. *C*, the same cells were incubated with 900 μM H_2O_2 in the incubator, and apoptosis was measured 8 or 24 h later. *, $p < 0.01$, for pTCFwt compared with pTracer at 8 h, or pTCFwt compared with pTracer or pTCFΔF508 at 24 h. Apoptosis was measured by cytofluorimetry with annexin V-PE staining, as described under "Experimental Procedures." The experiments were performed on at least two separate days, and the values represent the mean and S.D. of separate measurements.

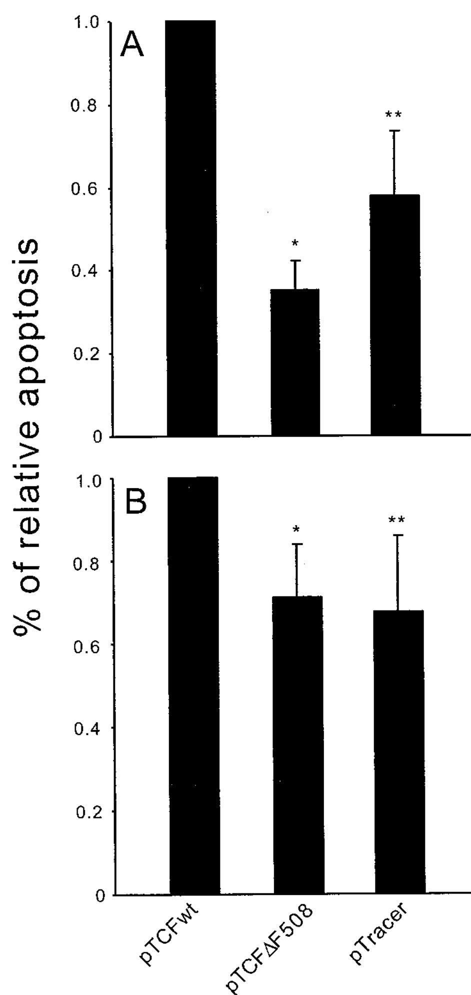

FIG. 2. **Apoptosis of human airway epithelial cells expressing wild-type or mutated CFTR after treatment with H_2O_2.** *A*, cells transfected transiently with plasmid alone (*pTracer*), the wild-type CFTR construct (*wt*), or the CFTR ΔF508 construct were incubated with 90 μM H_2O_2, and apoptosis was measured 24 h later. *, $p < 0.001$; **, $p < 0.01$, for pTCFwt compared with pTCFΔF508 or pTracer. *B*, the same cells were incubated with 300 μM H_2O_2, and apoptosis was measured 24 h later. *, $p < 0.01$; **, $p < 0.02$, for pTCFwt compared with pTCFΔF508 or pTracer. There was not a significant difference between pTCFΔF508 and pTracer. Apoptosis was measured by cytofluorimetry, as described under "Experimental Procedures." The experiment was performed on three separate days, and the values represent the mean and S.D. from the three experiments. For each experiment, the highest value of apoptosis (for pTCFwt) was defined as 100 (15% apoptosis for *A*, and 41% for *B*), and the other values were normalized with respect to the value for pTCFwt.

tory region with several protein kinase substrate sites, and two ATP-binding domains. The most common CFTR mutation associated with cystic fibrosis leads to omission of a phenylalanine residue (Phe-508) in a nucleotide binding region (36). We therefore studied apoptosis induced by H_2O_2 in epithelial (HeLa) cell lines stably transfected with the plasmid (pTracer), the wild-type CFTR construct (pTCFwt), and the CFTR ΔF508 mutation (pTCFΔF508).

All cell lines showed a similar level of apoptosis after a 24-h incubation in cell culture medium, but there was a significant increase in apoptosis after treatment with 90 or 900 μM H_2O_2 for 24 h (Fig. 1). Little enhancement of apoptosis due to 90 μM H_2O_2 was observed after 8 h, but wild-type cells displayed higher levels of H_2O_2-induced apoptosis than pTracer or ΔF508 after treatment with 90 μM H_2O_2 for 24 h. The same tendency, although at higher levels, was observed when the different cell lines were exposed to 900 μM H_2O_2 for 8 h or 24 h (Fig. 1). Thus, HeLa cells expressing normal CFTR are more sensitive to oxidative stress-induced apoptosis than either cells expressing plasmid alone or cells expressing the ΔF508 mutation.

Sensitivity to Oxidative Stress-induced Apoptosis of Airway Epithelial Cells Expressing Mutant and Normal CFTR—Sensitivity to H_2O_2-induced apoptosis was also measured in a human airway epithelial cells from a cystic fibrosis patient (IB3–1) (31) that were transiently transfected with pTracer, pTCFwt, or pTCFΔF508. This CF bronchial epithelial cell line was incubated with 90 or 300 μM H_2O_2, and apoptosis was measured by cytofluorimetry using annexin V-PE staining, as above for HeLa cells. Because the transfected cells also expressed GFP, apoptosis was measured only for the GFP-positive cells. After treatment with 90 μM H_2O_2, IB3–1 cells expressing normal CFTR were significantly more sensitive to apoptosis than cells expressing mutant CFTR (Fig. 2). There was also a higher sensitivity of the CFTR-expressing cells to

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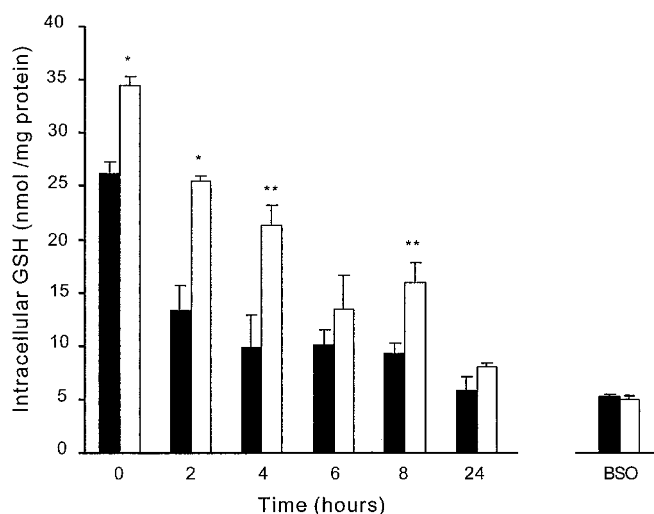

FIG. 3. **Concentration of intracellular glutathione in cells without CFTR or cells expressing wild-type CFTR after incubation with H_2O_2 or BSO.** The intracellular glutathione concentration in HeLa cells expressing wild-type CFTR (*black bars*) or no CFTR (*white bars*) was measured as a function of time, after addition of 90 μM H_2O_2. As a control for intracellular glutathione depletion, the cells were also preincubated with BSO for 24 h in the absence of H_2O_2. *, $p < 0.02$; **, $p < 0.05$, for pTCFwt compared with pTracer; the values at the other time points are not significantly different. The glutathione concentration was measured as described under "Experimental Procedures." The experiment was performed on three separate days, and one representative experiment out of three is shown. The values represent the mean and S.D. of two separate measurements.

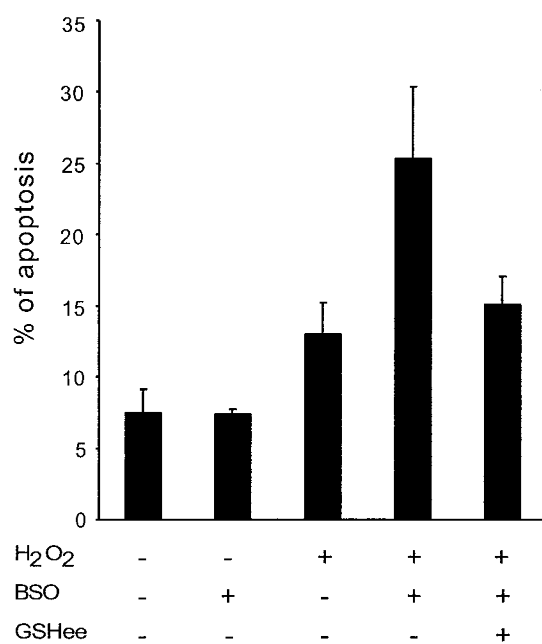

FIG. 4. **Effect of intracellular glutathione concentration on apoptosis of epithelial cells.** HeLa cells expressing wild-type CFTR were incubated with BSO alone; 90 μM H_2O_2 alone; BSO and 90 μM H_2O_2; or BSO, GSHee, and 90 μM H_2O_2. BSO by itself had no effect on the level of apoptosis but enhanced the level of apoptosis of cells treated with H_2O_2 for 24 h; the effect of BSO could be reversed by GSHee. $p < 0.01$ for all points. Apoptosis was measured by cytofluorimetry, as described under "Experimental Procedures." The experiment was performed on two separate days, and the values represent the mean and S.D. of three separate measurements.

apoptosis induced by 300 μM H_2O_2, but the difference between wild-type and mutant CFTR became smaller.

Effect of Glutathione Levels on Oxidative Stress-induced Apoptosis in Cells Expressing Mutant and Normal CFTR—Glutathione protects cells against oxidative damage and other types of toxicity that could lead to apoptosis (18). Moreover, H_2O_2 induces apoptosis in many cell types, including epithelial cells, and H_2O_2-mediated apoptosis is inhibited by intracellular glutathione (37–42). To evaluate whether glutathione levels could correlate with sensitivity to H_2O_2-induced apoptosis in our cells, the relative glutathione concentration was measured for the HeLa cell lines transfected with plasmid (pTracer) or expressing wild-type CFTR. The basal glutathione concentration was reproducibly lower in cells expressing normal CFTR than vector alone, and glutathione levels decreased faster in wild-type than control cells after treatment with 90 μM H_2O_2 (Fig. 3), suggesting that wild-type cells lose intracellular glutathione due to CFTR-dependent transport during oxidative stress.

Glutathione is synthesized by γ-glutamylcysteine synthetase and glutathione synthetase. Buthionine sulfoximine (BSO) is an irreversible inhibitor of γ-glutamylcysteine synthetase and is often used to inhibit glutathione synthesis (18). A large decrease in the intracellular concentration of glutathione was observed after treatment of HeLa cells with BSO, in the absence of H_2O_2 (Fig. 3). Because the glutathione depletion by BSO was equally effective in cells expressing wild-type CFTR and no CFTR, the effect of BSO was then tested on the sensitivity to H_2O_2-induced apoptosis of wild-type cells. BSO treatment by itself had no effect on the level of spontaneous apoptosis, but it increased dramatically the extent of apoptosis after incubation with H_2O_2 (Fig. 4). In addition, the effect was reversible, because it could be counteracted by the presence of GSHee, which prevents BSO-dependent glutathione depletion. Cells expressing wild-type CFTR are, therefore, more sensitive to H_2O_2-induced apoptosis at least in part due to the lower intracellular glutathione concentration in wild-type cells. Likewise, the sensitivity to apoptosis could be exacerbated through artificial depletion of intracellular glutathione.

BAX Activation in Cells Expressing Mutant and Normal CFTR—We then explored the possibility that enhanced depletion of glutathione in CFTR-expressing cells could cause BAX to be activated faster than in cells with defective CFTR. The state of BAX activation was detected by immunofluorescence and cytofluorimetry using an antibody that is specific for an N-terminal epitope that becomes exposed during activation of BAX and its translocation from the cytosol to mitochondria (28, 43).

In wild-type cells in the absence of H_2O_2 treatment, there was only faint labeling of BAX, suggesting that the BAX was present mostly in the cytosol. Following incubation with 90 μM H_2O_2 for 8 h, the fluorescence labeling of BAX became more intense, as expected for BAX activation, and most of the protein was localized on organelle structures that expressed a mitochondrial heat-shock protein (Fig. 5). Thus, oxidative stress causes BAX to become activated in epithelial cells and to translocate to mitochondria.

The ability of H_2O_2 to activate BAX was then tested in pTracer, wild-type CFTR cells, ΔF508, and cells expressing another CFTR mutation, G551D. The G551D mutation is less common than ΔF508, but it traffics normally to the plasma membrane, unlike ΔF508, most of which is processed improperly and remains within the cell (44). There was no significant increase in BAX activation in any of the cell lines after a 2-h incubation with 90 μM H_2O_2, but a large increase in BAX activation was already observed in wild-type cells after 4 h (Fig. 6). Some BAX activation, reaching 20%, was measured in all cell types after 8 h of H_2O_2 treatment, at which time 40% of wild-type cells contained activated BAX. Oxidative stress is therefore able to activate BAX in epithelial cells, and BAX is activated faster in cells expressing normal CFTR than in cells expressing defective CFTR.

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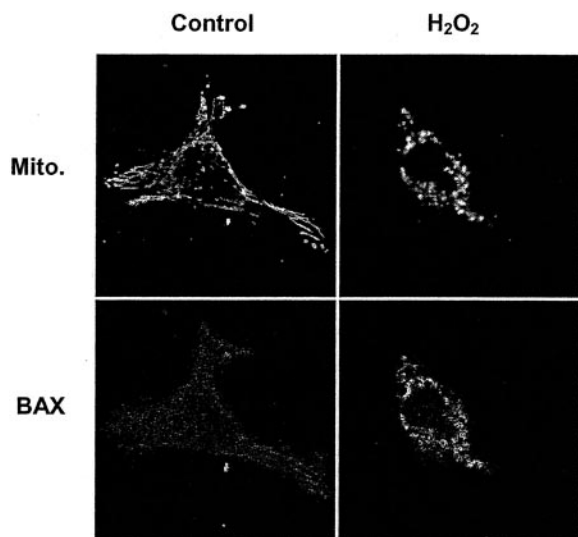

FIG. 5. **BAX activation and distribution in epithelial cells expressing wild-type CFTR after treatment with H_2O_2.** Subcellular localization of BAX in HeLa cells expressing wild-type CFTR, and the same cells treated with 90 μM H_2O_2 for 8 h. Weak BAX staining in the cytosol is observed in untreated cells, while staining becomes more intense and distributes to mitochondria after treatment with H_2O_2. The antibody against BAX (N-20) recognizes an amino-terminal domain of BAX that is exposed during BAX activation. BAX activation and distribution were visualized by confocal microscopy, as described under "Experimental Procedures."

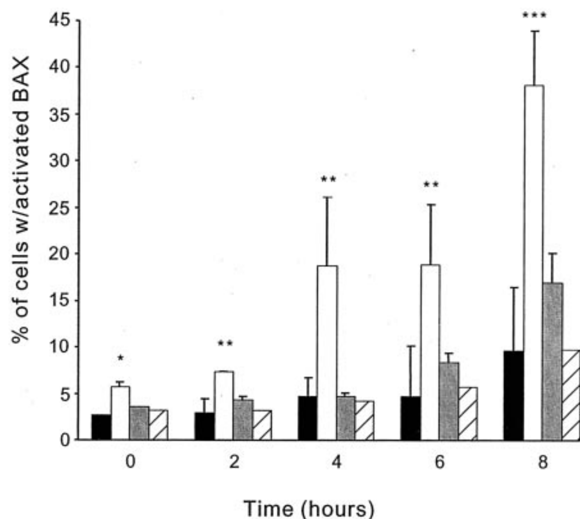

FIG. 6. **BAX activation in cells expressing wild-type or mutated CFTR as a function of time after treatment with H_2O_2.** BAX activation in HeLa cells stably transfected with plasmid (*black bars*), wild-type CFTR (*white bars*), ΔF508 CFTR (*gray bars*), or G551D CFTR (*striped bars*) was measured as a function of time after addition of 90 μM H_2O_2. *, $p < 0.05$; **, $p < 0.02$; and ***, $p < 0.01$, for pTCFwt compared with pTCFΔF508 for each time point. BAX activation was measured with the N-20 antibody by cytofluorimetry, as described under "Experimental Procedures." The experiment was performed on two separate days, and the values represent the mean and S.D. of three separate measurements.

Effect of Depletion of Glutathione Levels on BAX Activation—The possible correlation between glutathione levels and BAX activation was then investigated by measuring BAX activation by cytofluorimetry in wild-type cells treated with 90 μM H_2O_2 for 6 h. In cells depleted of glutathione by BSO, oxidative stress induced BAX activation to a higher extent than in the absence of depletion, and the enhancement of apoptosis by BSO could be reversed by GSHee (Fig. 7). Intracellular glutathione concentrations therefore control the ex-

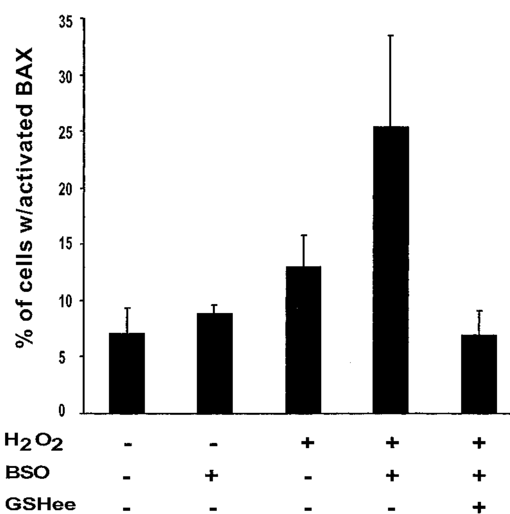

FIG. 7. **Effect of intracellular glutathione concentration on BAX activation in epithelial cells.** HeLa cells expressing wild-type CFTR were incubated with BSO; 90 μM H_2O_2; BSO and 90 μM H_2O_2; or BSO, GSHee, and 90 μM H_2O_2, as indicated in the *x*-axis legend. BSO by itself had no effect on the level of BAX activation but enhanced the extent of activation in cells treated with H_2O_2 for 6 h; the effect of BSO could be reversed by GSHee. $p < 0.02$ for cells treated with H_2O_2, compared with control untreated cells; to cells treated with H_2O_2 and BSO; or to cells treated with H_2O_2, BSO, and GSHee. BAX activation was measured by cytofluorimetry, as described under "Experimental Procedures." The experiment was performed on two separate days, and the values represent the mean and S.D. of three separate measurements.

tent to which H_2O_2 promotes BAX activation in epithelial cells. Because there is significant BAX activation in H_2O_2-treated cells within 4 h (Fig. 6), at which time there is still only a low level of apoptosis (Fig. 1), these results suggest that glutathione-dependent BAX activation precedes apoptosis of these cells during oxidative stress.

DISCUSSION

Cystic fibrosis was originally thought to be due to CFTR mutations that impair Cl^- channel activity. However, additional CFTR functions modified by the mutations were later identified, affecting notably Cl^--coupled HCO_3^- transport (45–47), and CFTR may also transport ions and molecules other than Cl^-, including both the oxidized and reduced forms of glutathione (1, 16).

CFTR-dependent Cl^--coupled HCO_3^- transport could potentially modify cytosolic and extracellular pH, and CFTR mutations have been associated with defects in tissue acidification (45). Cytosolic acidification is also an early step in many pathways of apoptosis (28, 29, 48–50), suggesting that CFTR mutations could affect cell sensitivity to apoptosis. A number of reports are in fact consistent with the view that inappropriate apoptosis due to decreased cytosolic acidification may contribute to the symptoms of cystic fibrosis (51–53). However, contradictory results were also obtained (54, 55).

Thus, it was first reported that mammary epithelial cells (C127) expressing normal CFTR are more sensitive to apoptosis due to treatment with the protein synthesis inhibitor, cycloheximide, than cells expressing mutant (ΔF508) CFTR, and that the cytosol is acidified to a larger extent in cells expressing normal CFTR (53). The mechanism of the apoptosis is not clear, because cycloheximide can both induce and inhibit apoptosis (56) and could influence synthesis and intracellular transport of CFTR. However, apoptosis of cells expressing mutant CFTR is enhanced by treatment with the weak organic acid, propionic acid, without affecting apoptosis of cycloheximide-treated cells with normal CFTR (53), suggesting that acidification could

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play a role in this process. The same laboratory subsequently found that the CFTR affects proliferation but not apoptosis of epithelial cells lining the gastrointestinal tract in mice, and that CFTR does not affect the susceptibility of C127 cell lines to UV irradiation-induced apoptosis (55). The effects of CFTR on apoptosis or proliferation of airway epithelial cells in the mice were not reported, and the possible link between oxidative stress and CFTR-dependent apoptosis was not investigated (55). Finally, an older study (51) suggests that mutant CFTR may, on the contrary, increase the number of cells with fragmented DNA in the lung, as determined by *in situ* terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) of DNA in tissues from cystic fibrosis and control patients. Only two out of fourteen cystic fibrosis patients had the ΔF508/ΔF508 mutation (51), and a larger group of patients should therefore be studied to determine conclusively if this mutation has an effect on cell death in humans. Nonetheless, as both apoptotic and necrotic cells contain free DNA ends that can be labeled with the terminal deoxynucleotidyltransferase (57–59), many if not all of the TUNEL-positive cells observed in the human lung tissues could have been necrotic.

Direct evidence for a link between CFTR and pH-dependent apoptosis was recently obtained with fibroblasts expressing transfected CFTR, which are significantly more sensitive to lovastatin-mediated apoptosis than untransfected controls (52). Lovastatin treatment causes cytosolic pH to decrease, and blocking intracellular acidification through overexpression of a Na^+/H^+ exchanger or increasing extracellular pH inhibits the enhancement of apoptosis induced by CFTR. It was proposed that CFTR enhances apoptosis through modulation of the C^-/HCO_3^- exchanger activity (52).

In line with the observations from other laboratories, we find that the cytosolic pH becomes acidic faster in cells expressing normal CFTR than in cells expressing mutant CFTR after treatment with H_2O_2.[2] In the present report, we evaluate the possibility that CFTR-dependent glutathione transport could contribute to differential sensitivity to apoptosis due to oxidative stress. Synthesis of reduced glutathione takes place in the cytoplasm, which contains glutathione concentrations of 1–10 mM (60). Faster extrusion of glutathione in cells expressing normal CFTR could therefore account for their higher sensitivity to apoptosis, compared with cells expressing defective or missing CFTR.

For both HeLa epithelial cells and human airway epithelial cells from a cystic fibrosis patient (IB3–1), we find that cells expressing normal CFTR display a significantly higher sensitivity to apoptosis due to H_2O_2 treatment than cells expressing no CFTR or cells expressing the ΔF508 mutation, the most common mutation of the CFTR in cystic fibrosis patients. At the same time, cells treated with H_2O_2 become depleted of cytosolic glutathione, and cells displaying normal CFTR are depleted faster than cells expressing mutant or no CFTR. Glutathione depletion in these cells correlates with the onset of apoptosis, because depleting glutathione further with BSO renders all the cells more sensitive to H_2O_2-induced apoptosis; inhibiting the depletion with GSHee restores the original sensitivity.

We then studied the mechanism whereby lower glutathione concentrations could lead to more apoptosis in normal cells. Apoptosis can be triggered via ligation of surface receptors such as Fas (61), but it can also be initiated from within the cell due to metabolic or oxidative stress. Stress-induced apoptosis is under the control of a number of molecules, including the BCL-2 family of proteins, which can either promote or inhibit apoptosis (62). The BCL-2 group (*e.g.* BCL-2, BCL-x_L, and BCL-w) promotes cell survival, whereas the BAX group (*e.g.* BAX, BAK, BAD, and BID) stimulates apoptosis. BAX exhibits a cytosolic location before the cell receives an apoptotic stimulus, and it translocates from the cytosol to mitochondria during apoptosis (28, 63). Activation of BAX causes release of cytochrome *c* from the mitochondria (64), which associates with Apaf-1 and procaspase-9 in the cytosol, resulting in activation of caspase-9 and, subsequently, in activation of caspase-3. This effector caspase then degrades cytosolic, nuclear, and cytoskeletal proteins, activates a caspase-dependent nuclease, and is responsible for many of the morphological and biochemical features of apoptosis (65–67).

It has been shown that cytosolic depletion of glutathione, a common event in damage-induced apoptosis, is necessary and sufficient to induce cytochrome *c* release (23), which would therefore lead to caspase-9 and caspase-3 activation, and finally cell death. We find that a downstream effect of glutathione depletion is BAX activation, which is found in the cytosol of untreated cells but translocates to the mitochondria after treatment with H_2O_2. In cells expressing normal CFTR, BAX is activated significantly faster than in cells expressing mutant CFTR after treatment with H_2O_2. BSO increases the extent of BAX activation in cells treated with H_2O_2, and the effect is reversed by GSHee, suggesting that BAX activation represents an early step in oxidation-induced apoptosis. Because BAX may also be activated by cytosolic pH changes (28, 29, 43), it is thus likely that both decreased cytosol acidification and slower glutathione depletion may result in the higher resistance to apoptosis observed in cells expressing defective or missing CFTR. Taken together, these results suggest that slower glutathione export and, consequently, delayed cell death of cells expressing mutant CFTR could contribute to the chronic inflammation observed in cystic fibrosis patients.

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Glutathione Levels and BAX Activation during Apoptosis Due to Oxidative Stress in Cells Expressing Wild-type and Mutant Cystic Fibrosis Transmembrane Conductance Regulator

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