Bcl-2 Suppresses Sarcoplasmic/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase Expression in Cystic Fibrosis Airways
Role in Oxidant-mediated Cell Death

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Rationale: Modulation of the activity of sarcoendoplasmic reticulum calcium ATPase (SERCA) can profoundly affect Ca\(^{2+}\) homeostasis. Although altered calcium homeostasis is a characteristic of cystic fibrosis (CF), the role of SERCA is unknown.

Objectives: This study provides a comprehensive investigation of expression and activity of SERCA in CF airway epithelium. A detailed study of the mechanisms underlying SERCA changes and its consequences was also undertaken.

Methods: Lung tissue samples (bronchus and bronchiole) from subjects with and without CF were evaluated by immunohistochemistry. Protein and mRNA expression in primary non-CF and CF cells was determined by Western and Northern blots.

Measurements and Main Results: SERCA2 expression was decreased in bronchial and bronchiolar epithelia of subjects with CF. SERCA2 expression in lysates of polarized tracheobronchial epithelial cells from subjects with CF was decreased by 67% as compared with those from subjects without CF. Several non-CF and CF airway epithelial cell lines were also probed. SERCA2 expression and activity were consistently decreased in CF cell lines. Adenoviral expression of mutant F508 cystic fibrosis transmembrane regulator gene (CFTR), inhibition of CFTR function pharmacologically (CFTRinh172), or stable expression of antisense oligonucleotides to inhibit CFTR expression caused decreased SERCA2 expression. In CF cells, SERCA2 interacted with Bcl-2, leading to its displacement from caveolae-related domains of endoplasmic reticulum membrane, as demonstrated in sucrose density gradient centrifugation and immunoprecipitation studies. Knockdown of SERCA2 using siRNA enhanced epithelial cell death due to ozone, hydrogen peroxide, and TNF-α.

Conclusions: Reduced SERCA2 expression may alter calcium signaling and apoptosis in CF. These findings decrease the likelihood of therapeutic benefit of SERCA inhibition in CF.

Keywords: cystic fibrosis; SERCA2; pulmonary epithelium; ER

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane regulator gene (CFTR), an epithelial anion channel, resulting in the common autosomal recessive disease. Relentless progressive lung infection and excessive secondary inflammation remains the principal cause of death in CF. The most frequent CF-associated mutation, accounting for about 70% of CF alleles, is deletion of phenylalanine 508 (ΔF508 CFTR). ΔF508 CFTR has reduced chloride channel activity, impaired processing, and decreased stability at the cell surface. Abnormal processing leads to its retention in the endoplasmic reticulum (ER) and rapid intracellular degradation. For these reasons, ΔF508 CFTR fails to function as a cAMP-activated Cl\(^{-}\) channel (1). Previous reports have indicated that sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitors can decrease calcium concentrations within the ER and thereby interfere with the ability of calcium-dependent chaperone proteins to retain misfolded ΔF508 CFTR within the ER (2). These investigators have suggested that blockade of this chaperone interaction by the use of SERCA inhibitors allows misfolded ΔF508 CFTR to escape the ER, reach the cell surface, and function as a Cl\(^{-}\) channel (3). These findings precipitated a remarkable number of investigations and resulted in several papers, indicating that SERCA pump inhibitors like curcumin and/or thapsigargin can (4–9) or cannot (10–13) enhance ΔF508 CFTR trafficking to the plasma membrane and apical epithelial chloride transport.

The release of calcium ions from ER regulates essential cellular functions, including secretion, contraction, gene transcription, and survival (14). Because SERCA is responsible for (re)loading of ER calcium after such signaling events, its function can be important at the level of the whole organ and organism. For example, SERCA2 is the only SERCA isoform expressed in cardiac muscle, and decreased SERCA2 expression is a key event in congestive heart failure (15). Its importance is further demonstrated by the lethal phenotype of SERCA2 knockout mice and the loss of calcium regulation in cells with only one copy of the SERCA2 gene (16, 17). In CF, release of ER calcium by activation of purinergic receptors...
can allow activation of Ca\(^{2+}\)-activated chloride channels, a principal, albeit partial, compensatory mechanism for impaired chloride secretion in CF that could diminish excessive sodium reabsorption by ENaC (18). Hence, ER Ca\(^{2+}\) stores can have direct importance in the adaptation of CF epithelium.

Despite the abundant research into potential effects of SERCA inhibitors on ΔF508 CFTR trafficking and function, the expression of SERCA isoforms in CF and non-CF airway epithelium has not been systematically evaluated. Herein, we report that expression of the principal lung isoform, SERCA2, was consistently down-regulated in CF airways, primary cultures of polarized CF airway epithelial cells, in response to genetic or pharmacologic inhibition of wild-type CFTR expression or function and upon expression of ΔF508 CFTR in non-CF epithelium. Even though the low-affinity SERCA3 isoform was up-regulated, total SERCA activity was decreased. In CF cells, SERCA2 was associated with Bcl-2 in ER membranes, and SERCA/Bcl-2 interaction was previously reported to cause inactivation and displacement of SERCA from membrane microdomains, referred to as caveolae-related domains (CRDs) (19, 20). Knockdown of SERCA2 using siRNA revealed that it is required for the survival of airway epithelial cells during oxidative stress. Not only do these findings indicate additional reasons why the strategy of SERCA inhibition should not be of benefit in CF, but they also have potential implications for altered calcium homeostasis and epithelial cell response in the disease pathogenesis. Some of the results of these studies have been previously reported in the form of abstracts (21, 22).

**MATERIALS AND METHODS**

**Cell Culture**

Primary human bronchial epithelial cells (HBEs) were obtained from non-CF and CF lungs under a protocol and consent form approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Epithelial cells were removed from the lower trachea and bronchi by protease XIV digestion, and cells were plated in bronchial epithelium growth medium on collagen-coated dishes as described previously (23). Approximately 5 × 10^5 passage 2 cells were seeded onto 12-mm-diameter type VI collagen (Sigma, St. Louis, MO)-coated Millicell CM inserts (0.4 μM pore size) (Millipore Corporation, Bedford, MA) and, after confluence on Days 4 and 5, were maintained at an air–liquid interface (ALI). Additional primary human bronchial epithelial cells were isolated in our laboratory from donor airway tissues obtained from the National Disease Research Interchange, with approval of National Jewish Institutional Committee for the Protection of the Rights of Human Subjects. The airway epithelial cell lines used were IB3–1 and a “corrected” cystic fibrosis (CF) cell line that was derived from IB3–1 cells stably transfected with wild-type CFTR (C-38) (ATCC, Manassas, VA). IB3–1 and C-38 cells were grown in LHC-8 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin/streptomycin. CFBE41o- (CF41o-) and CFBE45o- (CF45o-) and a wild-type airway epithelial cell line, 16HBE14o- (16HBEo-), were provided by Prof. D. Gruenert (California Pacific Medical Center Research Institute, University of California at San Francisco). These cell lines were cultured in Eagle’s minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, i-glutamine, and penicillin/streptomycin at 37°C under 5% CO\(_2\). 16HBEo- cells with stable expression of sense (16HBE-S) and antisense CFTR (16HBE-AS) oligonucleotides were cultured as described previously (24). Minimally transformed primary bronchial epithelial UNCN3T from the lab of Dr. Randall was also used (25). All experiments comparing primary non-CF and CF cells were performed with polarized cultures grown simultaneously and matched for passage number, the number of cells plated, and days in culture.

**Adenoviral Transduction**

Transduction of wild-type CFTR, mutated CFTR (ΔF508), and GFP-encoding adenoviral vectors was performed as described previously (26). The vectors (H5’ 0.040CMVGFP-CFTR and H5’ 0.040CMVGFP-ΔF508) were provided by Vector Core at the University of Pennsylvania as described elsewhere (27). The recombinant viruses were added to the cell cultures (multiplicity of infection, 10:1) on Day 3 for 17 hours. The transduction efficiency was estimated by observing green fluorescence of adenoviral GFP-transduced cells.

**SERCA2 Gene Silencing Using siRNA**

Predesigned human ‘SMARTPOOL’ SERCA2 siRNAs were purchased from Dharmacon (Lafayette, CO). Primary airway epithelial cells not at ALI were transfected with 50 nM siRNA using DharmaFECT2 siRNA transfection reagent (Dharmacon) according to the manufacturer’s instructions. Silencer negative control siRNA was used as a nonspecific control. Transfection of siRNA in primary human airway epithelial cells has previously been described (28).

**Statistical Analysis**

All statistical calculations were performed with JMP and SAS software (SAS Institute, Cary, NC). Means were compared by two-tailed t test for comparison between two groups or one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons for analyses involving three or more groups. \( P < 0.05 \) was considered significant.

**RESULTS**

**Expression of SERCA2 in CF Airway Cell Lines**

SERCA2 expression was determined by Western blot in non-CF and CF cell lines. Expression of SERCA2 protein was decreased in two CF cell lines (CF41o- and CF45o-) as compared...
with the non-CF 16HBEo- cells (Figure 1A). Quantitation of SERCA2/β-actin intensity yielded 3.51 ± 0.27, 1.76 ± 0.23, and 2.59 ± 0.29 arbitrary units for 16HBEo-, CF41o-, and CF45o-cells, respectively. A statistically significant decrease in SERCA2 protein was observed in IB3–1 cells versus C-38 cells (0.22 ± 0.02 vs. 1.24 ± 0.01 SERCA2/β-actin intensity units, respectively; figure not shown) and in JME/CF15 versus Calu-3 cells (0.38 ± 0.01 vs. 0.62 ± 0.01 SERCA2/β-actin intensity units, respectively; figure not shown). Decreased SERCA2 mRNA was also observed (Figure 1B) in CF41o- and CF45o- cells relative to that found in non-CF 16HBEo- cells.

Experiments were also performed to immunolocalize SERCA2. The results indicated diminished SERCA2 expression in CF cells relative to non-CF cells (Figure 1C). In non-CF cells, SERCA2 was located in ER, as demonstrated by the enhanced “merged” staining for the ER marker protein disulfide isomerase (PDI) and SERCA2 in these cells, whereas CF cells demonstrated decreased overall SERCA2 staining intensity, preservation of ER staining for PDI, and diminished merged signal for PDI and SERCA2 co-staining.

ER Density and SERCA2 Protein and Activity in CF Cells and Their Microsomal Fractions

The decreased expression of SERCA2 in CF cells was not caused by diminished ER mass in these cells. ER density was quantified...
SERCA2 Expression in Primary Polarized Non-CF and CF Airway Epithelial Cells

SERCA2 expression was also estimated in ALI cultures of differentiated primary non-CF (14 donors) and CF (eight donors) cells using immunohistochemistry and Western blot analysis. Using either technique, expression of SERCA2 in epithelial cells varied from donor to donor (demographics provided in Table E1 in the online supplement), despite comparable passage number and days in culture (Figures 3A and 3B). However, mean SERCA2 protein expression (Western blot) was decreased by 67% in airway epithelial cells from CF donors relative to non-CF (Figure 3C). We also analyzed SERCA2 expression at 7 days of culture, a stage at which cells are undifferentiated, and at 30 days of culture, at which time cells are more differentiated (30), in primary non-CF and CF airway epithelial cells from four different donors to determine if the extent of differentiation could affect SERCA2 expression. CF airway epithelial cells had decreased SERCA2 expression at 7 days and at 30 days of culture as compared with non-CF cells (data not shown). Thus, SERCA2 protein expression in primary polarized CF airway epithelial cells differed from the non-CF cells to a similar extent as seen in CF versus non-CF cell lines.

SERCA2 Expression in Proximal and Distal Airways from Human CF and Non-CF Lungs

Immunohistochemistry was used to estimate SERCA2 expression in lung tissue from subjects with and without CF. SERCA2 staining was predominantly localized to airway epithelium and to smooth muscle cells in airway and arterial/arteriolar walls. SERCA2 staining was decreased in bronchial and bronchiolar epithelium of subjects with CF compared with subjects without CF (Figures 4A–4D). CF airway epithelium also differed from non-CF airway epithelium in that the extent of mucus cell hyperplasia was greater in the former than in the latter. Quantitation of SERCA2 staining (excluding the mucus-containing regions) revealed a significant decrease in bronchial and bronchiolar epithelium of subjects with CF compared with subjects without CF (Figures 4E and 4F). The demographics of the individuals with and without CF are provided in Table E2.

SERCA3 expression in CF and non-CF airway epithelial cells and cell lines

SERCA3 is the only other known SERCA isoform expressed in lung. It differs from SERCA2 in that it has a low affinity for calcium. By contrast to SERCA2, SERCA3 protein expression was increased in both CF cell lines, CF41o- and CF45o-, relative to that seen in the non-CF cell line 16HBEo- (Figure 5A). Steady-state SERCA3 mRNA expression was not consistently elevated in CF cell lines, being elevated in CF45o- but not in CF41o- relative to 16HBEo- (Figure 5B). However, SERCA3 protein expression was increased in primary polarized airway epithelial cells from CF versus non-CF donors (Figure 5C). These findings suggest a compensatory response of SERCA3 expression to the down-regulation of SERCA2 in CF.
not cause a further decrease in its expression of SERCA2 (Figure 6C). Exposure of these cells to the inhibitor for this duration did not cause cytotoxicity.

**SERCA2 Protein Expression Is Decreased by Genetic Inhibition of CFTR Function**

The effect of interference with CFTR function was evaluated by an additional specific genetic approach. SERCA2 expression was measured in 16HBEo- cells with stable expression of sense (16HBE-S) and antisense CFTR (16HBE-AS) oligonucleotides and grown at ALI. cAMP-dependent chloride conductance was absent in antisense CFTR oligonucleotides (16HBE-AS)-containing cells (Figure E1A). SERCA2 protein, corrected for β-actin expression, was diminished in CFTR antisense-expressing cells by about two thirds relative to sense-expressing cells (Figure 7A), comparable to the extent of reduction of SERCA2 expression in primary CF relative to non-CF airway cells.

**SERCA2 Protein Expression Is Decreased by Expression of Mutant ΔF508 CFTR**

The ΔF508 CFTR mutation causes diminished channel activity, impaired processing, and reduced CFTR protein stability at the cell surface. In addition, this mutation inhibits gating of CFTR channels, resulting in a diminished rate of opening (31). Because this is the most common CFTR mutation, we sought to determine the effect of its overexpression in airway epithelial cells on SERCA2 expression. We conducted studies in minimally transformed primary human bronchial epithelial cells (UNCN3T) transiently transduced using adenoviral vectors. Compared with nontransduced, LacZ-overexpressing, and wt-CFTR–overexpressing cells (increased CFTR expression by Ad.wt-CFTR is shown by Western blot in Figure E1B), ΔF508 CFTR-overexpressing cells had decreased SERCA2 protein expression (Figure 7B). Taken together, the results from use of these three strategies for CFTR functional inhibition demonstrate a link between diminished CFTR function and SERCA2 expression.

Bcl-2–induced displacement of SERCA2 from CRDs in CF cells. Interaction of Bcl-2 with SERCA2 has been described previously (20, 32). Bcl-2 binds to and inactivates SERCA and causes SERCA displacement from the CRDs of the ER membrane (19, 20). Sucrose density gradient fractionation of purified microsomes from non-CF 16HBEo- cells and CF45o- cells was performed to localize SERCA2 in the CRDs. For the detection of...
SERCA2 distribution in the sucrose density fractions, each fraction was loaded onto the gel for Western blot analysis (Figure 8A). Fractions were collected from the top of the gradient, where fraction 1 represents the initial CRD fraction. SERCA2 of 16HBEo- cells primarily localized in the CRD fraction, whereas the major SERCA2 band of CF45o- cells migrated deeper into the gradient. Thus, sucrose density gradient centrifugation of microsomes from CF preparations showed that SERCA2 was predominantly recovered in fraction 2, whereas non-CF samples showed SERCA2 predominantly in fraction 1. SERCA2 coimmunoprecipitated with Bcl-2 in 16HBEo- and CF45o- cells (Figure 8B). However, an increased amount of Bcl-2 was detected in CRDs and the immunoprecipitates from CF cell microsomes. These figures indicate the presence of increased Bcl-2 expression and Bcl-2–SERCA2 complexes in CF. The increase in Bcl-2–SERCA2 complexes in CF cells occurred despite the fact that SERCA2 protein was decreased by about 50% in the CF cell line. These findings indicate a mechanism for decline in ER-associated SERCA2 expression and total SERCA activity due to Bcl-2 expression by Western blot. The experiments were repeated four times. (B) Results of SERCA2 expression in lysates obtained from control and adenosively transduced minimally transformed primary human bronchial epithelial cells (UNCN3T) grown on collagen-coated culture dishes. The bars represent means of data. *Significant difference (P < 0.05) from control (16HBE-S) cells. The experiment was repeated three times (n = 3 per condition).

Figure 7. Effect of inhibiting functional cystic fibrosis transmembrane regulator gene (CFTR) expression by antisense CFTR oligonucleotides (A) or by overexpressing mutated CFTR (B) on sarcocendoplasmic reticulum Ca²⁺-ATPase (SERCA2) expression. Cell lysates were prepared from polarized cultures of 16HBEo- cell line stably transfected with sense (S) and antisense (AS) CFTR oligonucleotide and analyzed for SERCA2 expression by Western blot. The bars represent means of data. *Significant difference (P < 0.05) from control (16HBE-S) cells. The experiment was repeated four times. (B) Results of SERCA2 expression analysis in lysates obtained from control and adenosively transduced minimally transformed primary human bronchial epithelial cells (UNCN3T) grown on collagen-coated culture dishes. The bars represent means of data. *Significant difference (P < 0.05) from control (LacZ) cells. The experiment was repeated three times (n = 3 per condition).

Role of diminished CFTR expression in increased Bcl-2 expression. To determine if decreased CFTR function plays a role in the enhanced expression of Bcl-2, we isolated ER microsomes from 16HBEo- cell line stably transfected with sense (S) and antisense (AS) CFTR oligonucleotide. Increased expression of Bcl-2 was observed in cells in which CFTR was decreased by expression of antisense CFTR oligonucleotides (Figure 9B). This indicates that deficient CFTR expression is sufficient to increase Bcl-2 expression. Bcl-2 analysis was also performed in lysates obtained from control and adenosively transduced primary human bronchial epithelial cells using ELISA. Cells expressing ΔF508 CFTR showed an approximately 2-fold increase in Bcl-2 expression as compared with the control cells (Figure 9C). Direct and/or indirect effects of altered CFTR function may lead to persistent endogenous activation of NF-κB in primary human bronchial epithelial cells. NF-κB regulates the transcription of Bcl-2 (33, 34) and Bcl-2 in CF airway epithelial cells (35, 36). We determined Bcl-2 content and NF-κB activation (by measuring nuclear p65) of control and TNF-treated primary HBE cells that were adenosively transduced with ΔF508 CFTR (Figure E2A). There was no change in CFTR expression with and without TNF treatment in the lacZ, wtCFTR, and ΔF508 CFTR-expressing cells. The nuclear lysates, when assessed by
Western blot showed increased p65 in the ΔF508 CFTR-expressing cells in both conditions of ΔF508 CFTR, with or without TNF treatment. Quantitative estimation of p65 in the nuclear lysates using ELISA revealed statistically significant increased nuclear p65 upon TNF treatment in the adenoviral lacZ and wtCFTR transduced cells (Figure E2B). The ΔF508 CFTR-expressing cells had increased basal nuclear p65 that was further enhanced upon TNF treatment. The cells that had increased nuclear p65 had increased Bcl-2 expression (Figure E2C).

**SERCA2 is required for cell survival in oxidative stress.** CF airway epithelial cells are continuously exposed to oxidative stress presented directly or indirectly by air pollutants, bacterial endotoxins, proinflammatory cytokines, and neutrophils. For this reason, we sought to determine the potential impact of diminished SERCA2 expression on airway epithelial cell survival during challenge by three relevant prooxidant stimuli: ozone (O₃), hydrogen peroxide (H₂O₂), and TNF-α. SERCA2 siRNA was used to investigate the effect of SERCA2 expression on O₃–induced cell death in primary airway epithelial cells cultured on collagen-coated 6-well plates. First, we verified that SERCA2 protein levels were reduced in the cells expressing SERCA2 siRNA (Figure 10A). Transfection with nonspecific siRNA had no effect on SERCA2 protein expression, whereas SERCA2 siRNA–treated cells had SERCA2 protein expression decreased by 67% relative to nonspecific siRNA–treated control cells. Bcl-2 expression was not affected by SERCA2 knockdown in these cells (data not shown). Increased apoptotic and necrotic cell death was observed in the control siRNA-transfected cells upon exposure to 200 ppb O₃ for 18 hours (Figure 10B). SERCA2 siRNA increased apoptotic and necrotic cell death induced by O₃ (25.0 ± 7.0% vs. 42.0 ± 3.0% apoptotic cell death in control siRNA vs. SERCA2 siRNA–expressing cells). Besides O₃ as an environmental factor, we assessed the toxicity of TNF-α and H₂O₂ because these are abundant in CF lungs. With 10 ng/ml TNF-α, a concentration close to those found in CF airways (37), there was enhanced cell death in SERCA2 siRNA–expressing cells, but the extent of death was minimal (4.17 ± 0.30% in control vs. 7.15 ± 0.50% total cell death in SERCA2 siRNA–expressing cells). However, when TNF-α was combined with IL-1β, another cytokine abundant in CF airways, there was increased apoptotic cell death in control and 1.7-fold still greater apoptosis in SERCA2 siRNA–treated cells (Table 2). The extent of necrosis was not different in the two groups. Similarly, treatment with H₂O₂ caused enhanced apoptotic and necrotic cell death in SERCA2 siRNA–expressing cells when compared with those expressing control siRNA (Table 2).

**DISCUSSION**

Our study on the expression of SERCA2 in CF airways relative to expression in respiratory cells and tissues of non-CF individuals reveals that SERCA2 protein expression is decreased in CF airway epithelial cell lines and primary polarized airway epithelial cells grown at ALI and in vivo in distal and proximal airway epithelial tissue of subjects with CF. CFTR dysfunction-induced enhanced NF-κB activation and increased Bcl-2, which interacted with SERCA2 to translocate it from the caveolae-related domains (CRDs), were potential mechanisms causing decreased SERCA2 expression.

In the series of experiments involving primary cells grown at ALI, although there was considerable variability in expression from donor to donor, the overall extent of SERCA2 protein expression was diminished in primary CF cells. Individual variability in the primary cells could have resulted from variations in genotype, types of differentiated cells present (e.g., mucus, ciliated), the types and/or severity of infection(s) previously present, the presence of additional disease states (e.g., diabetes) in the donor (38), and/or the types of medications previously administered (39). SERCA2 protein expression was quantitatively diminished, as detected by immunohistochemistry, in epithelial cells of large and smaller airways in lungs of patients with CF. This supports the potential clinical relevance of these findings. Previous studies indicated that altered calcium homeostasis in airway epithelial cells of patients with CF is mainly a result of inflammation and infection (40, 41). The airway epithelial cell lines used in the present studies were maintained in the absence of infection and inflammation for months to years, and the primary cells examined were cultured in the absence of infection and inflammatory cells for 1 to 6 weeks. Despite the differences in cell environment,
decreased SERCA2 expression was a constant feature of CF airway epithelial cells, suggesting that it is an intrinsic feature of the disease. This is further supported by IHC studies using lung tissues of patients with CF. These tissue samples were not free of infection and chronic inflammation.

ER plays an important role in CF disease pathogenesis. The decreased SERCA2 expression was not due to a diminished ER mass in CF cells. In fact, as indicated by staining for the ER-specific protein PDI and an ER-specific fluorescent dye, CF airway epithelial cells contained similar or greater quantities of ER. These findings are generally in agreement with previous reports of expanded ER in CF (40). Although abnormal ΔF508 CFTR can be retained and degraded in ER, and transient adenoviral overexpression of ΔF508 CFTR was sufficient to cause decreased SERCA2 expression in non-CF airway epithelial cells, the findings in our study (e.g., antisense CFTR oligonucleotide expression) do not indicate that the presence of mutant CFTR(s) in the ER is the principal cause of diminished SERCA2 expression in CF airway epithelial cells.

Recent investigations on the modulation of SERCA2 activity have reported the presence of an important interaction between SERCA2 and Bcl-2, resulting in SERCA2’s translocation from CRD to membrane domains of higher density, partial unfolding, and inactivation (19, 20). Bcl-2 is capable of interaction with SERCA1 or SERCA2. In purified sarcoplasmic reticulum preparations, the addition of Bcl-2 causes significant loss of SERCA activity (20). Because of relevant reports in CF (42, 43), we determined if such an interaction occurs in CF airway epithelium. Bcl-2 protein expression and association with SERCA2 was elevated in CF relative to non-CF airway epithelial cells. Given that Bcl-2 binding to SERCA2 in other tissues can displace it from CRD and inactivate it, our findings suggest that similar mechanisms could act in lung epithelium to diminish SERCA2 protein expression and activity in the ER. We do not know whether SERCA2 is also chemically modified such that its interaction with Bcl-2 and/or its inactivation is enhanced.

A previous study in relation to CF and Bcl-2 expression indicated that Bcl-2 expression increased mucus cell metaplasia of airway epithelial cells and that insufflation of bacterial endotoxin could cause such changes in the airways of rats (42). Our studies of CF and non-CF airway epithelial cell lines and of CFTR sense and antisense oligonucleotide-expressing cells were done in the absence of bacterial infection, inflammation, and endotoxin, and these cells were maintained in the absence of these factors for extended periods of time. In addition, the cells in these experiments were not cultured at ALI. Thus, unlike our studies of primary cell cultures grown at ALI, they could not undergo differentiation to mucus-expressing cells and could not have had altered Bcl-2 expression in association with mucus cell differentiation. In this case, such culture conditions were considered advantageous because they prevented potential Bcl-2 changes due to differentiated phenotype. Some variability in SERCA2 expression in CF primary airway epithelial cells from different donors could have related to differences in mucus cell differentiation of such cultures, but this could not have occurred in the various transformed CF and non-CF cell lines we studied. Based on the absence of infection, inflammation, bacterial endotoxin, and mucus cell differentiation in our experiments pertinent to Bcl-2, our findings indicate that increased Bcl-2 expression is an inherent feature of CF airways epithelium.

CFTR dysfunction is associated with aberrations in a number of signaling pathways, including those related to inflammation and infection. The specific link(s) between CFTR dysfunction and increased NF-κB activation in CF cells remains undefined. However, there are several factors that could cause this to occur (43). Our experiment with overexpression of mutant CFTR provides further evidence that CFTR dysfunction causes increased NF-κB activation p65 mobilization to the nucleus. Increased NF-κB–mediated proinflammatory gene transcription has been reported elsewhere (24) in the cells expressing CFTR antisense oligonucleotide (16HBE-AS), which is another model we studied. NF-κB is a transcriptional regulator of Bcl-2 and other proteins of the Bcl-2 family (35, 44). Thus, increased Bcl-2 in cells with CFTR dysfunction could result, at least in part, from enhanced NF-κB activation.

Increased expression of Bcl-2 in CF airway epithelium could have beneficial and/or detrimental effects. It could increase proliferation and diminish cell death via its antiapoptotic action. Increased proliferation in the submucosal gland and in basal cells of CF airways has been described elsewhere (45), and this could have an important role in airway epithelial regeneration and

Figure 10. Sarcoendoplasmic reticulum Ca2+-ATPase (SERCA2) is essential for cell survival during oxidative stress. (A) SERCA2 knockdown using SERCA2 siRNA. Top: representative Western blot of SERCA2 expression in primary human bronchial epithelial cells (grown on collagen-coated culture dishes) (1) mock-transfected or transfected with (2) control or (3) SERCA2 siRNA. Bottom: Quantitation of SERCA2 knockdown. The bars represent means of data. *Significant difference (P < 0.05) from control siRNA-transfected cells (n = 3; three individual experiments). For assessing ozone toxicity, primary human airway epithelial cells were transfected either with a control or SERCA2 siRNA for 24 hours and exposed to 0 ppb or 200 ppb ozone for 18 hours. After exposure, cells were analyzed for cell death using Vybrant apoptosis assay kit. The Alexa Fluor-488–labeled annexin-positive apoptotic and Sytox green-positive dead cells were quantified by flow cytometry (B). The columns represent means of data. *Significant difference (P < 0.05) from 0 ppb control cells. **Significant difference from 0 ppb control cells (n = 3). This experiment was repeated four times.
TABLE 2. SERCA2 KNOCKDOWN ENHANCES CELL DEATH

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<thead>
<tr>
<th>Treatment</th>
<th>Control siRNA</th>
<th>SERCA2 siRNA</th>
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<tr>
<td></td>
<td>Apoptosis (%)</td>
<td>Necrosis (%)</td>
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<td>Apoptosis (%)</td>
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<tr>
<td>TNF + IL-1β (10 ng/ml for 18 h)</td>
<td>17.53 ± 1.83</td>
<td>8.54 ± 0.55</td>
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<tr>
<td>H2O2 (500 μM for 18 h)</td>
<td>9.66 ± 0.33</td>
<td>14.9 ± 0.85</td>
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* Significant difference from control siRNA-treated cells (P < 0.05). n = 3, experiment repeated three times.

Definition of abbreviation: SERCA2 = sarcoendoplasmic reticulum Ca2+-ATPase; TNF = tumor necrosis factor.

Serca2 knockout (Serca2a+/−) mice showed substantially enlarged infarction after cardiac ischemia (60). In addition, they showed impaired postischemic myocardial relaxation and reduced postischemic myocardial contractile function. These animals had higher diastolic intracellular calcium without oxidative stress and higher intracellular calcium after the oxidative stress of reperfusion. The level of reactive oxygen species production was not increased in the SERCA2 knockouts, but the level of dysfunction related to oxidative stress was increased. Thus, there are precedents for substantial alterations in cell survival and function in the heart with comparable changes in SERCA2 expression to those seen in CF, relative to non-CF, lung cells.

Intracellular calcium signaling depends upon SERCA activity. [Ca2+]i can regulate a number of important functions pertinent to airway epithelial cells, including mucus secretion, ciliary contraction and motility, signal transduction, and cell survival. Diminished SERCA activity in CF could dampen such intracellular calcium signals and blunt adaptive chloride export. Novel purinergic compounds in development for treatment of CF are directed at stimulating intracellular calcium signals by acting on extracellular ATP receptors that activate this process (61). It was recently reported that ATP-induced increased short circuit currents were lower in CF than non-CF, small airway epithelial cell cultures (62). Taken together, these data suggest that SERCA inhibitor therapy would not likely be beneficial in CF and that SERCA inhibition may worsen airway epithelial cell survival under oxidative stress and decrease alternative calcium-dependent chloride transport.

Hence, investigation of an alternate approach using SERCA-stimulating interventions in clinically relevant models of CF may be warranted by the present study.

Conflict of Interest Statement: S.A. holds a provisional patent for use of SERCA activators in CF. This patent was filed on October 2, 2008, after the original submission of this manuscript. No funds have been received as a result of this patent filing. A.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.S.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.N.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.W.W. holds a provisional patent for use of SERCA activators in CF. This patent was filed on October 2, 2008, after the original submission of this manuscript. No funds have been received as a result of this patent filing.

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References


