

Bcl-2 suppresses SERCA2 expression in cystic fibrosis airways: Role in oxidant-mediated cell death

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Online Data Supplement

Online Methods Supplement

Determination of cell survival and protein concentration

Cell death was assessed by using Vybrant apoptosis assay kit (Molecular Probes, Eugene, OR), as described before (1). In this method, apoptotic cells bearing phosphatidylserine in the plasma membrane outer leaflet were identified as those binding Alexa Fluor 488-labeled annexin V and necrotic cells as those binding fluorescent DNA-binding dye SYTOX Green. Protein concentration in cell lysates was determined using the BioRad DC protein assay kit (Bio-Rad, Hercules, Ca) in a 96-well plate with bovine serum albumin as a standard.

Determination of chloride conductance

16HBE-S and 16HBE-AS were seeded on snapwell permeable supports (Corning Costar) at a density of 5×10^5 cells/ cm². At confluence (~14 day of ALI culture), the inserts were mounted in Ussing chambers (WPI, Sarasota, Florida) filled on the basolateral side with 10 ml of Krebs bicarbonate solution containing (in mM): 120 NaCl, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, 25 NaHCO₃, 10 glucose. On the apical side, 10 mM mannitol was added instead of glucose to avoid activation of the apical electrogenic Na⁺-glucose cotransporter. During the experiment, this solution was gassed with 95%O₂/5%CO₂. Experiments were conducted at 37°C. The short-circuit current (I_{sc}) was monitored continuously using a DVC1000 voltage clamp (WPI, Sarasota, Florida) and the PD was measured every 5–10 min. Cell preparations were allowed to equilibrate until stabilization of bioelectric variables took place, which required ~ 20–30 min. Basal bioelectric activity was monitored for 10 min before addition of drugs. Pharmacologic

agents were added to the apical bathing solutions and bioelectric activity was monitored for 5–15 min thereafter. Amiloride (10 μ M) and forskolin (10 μ M), were added sequentially.

Western blot

Western blots were performed as previously described in detail (2) and the membranes were probed with rabbit polyclonal antibodies against SERCA2 and SERCA3 (Affinity Bioreagents, Golden, CO) or mouse monoclonal antibodies against Bcl-2 (BD Biosciences, San Jose, CA) at 1:1,000 dilution for each, overnight at 4°C. Blots were then washed again with TBS-T and incubated with mouse anti-rabbit peroxidase-conjugated IgG (Bio-Rad, Hercules, CA) at 1:10,000 dilution, for 1 h at room temperature. Immunoreactive bands were detected using an ECL detection kit (Pierce, Rockford, IL) followed by exposure to Hyperfilm (Amersham Pharmacia Biotech Inc. UK). Total Bcl-2 content was also analysed by ELISA performed at IHCtech (Aurora, CO). Bcl-2 immunoprecipitation experiments were carried out as described previously (3). Cytochrome c oxidase, Lamin C and p65 antibodies (Affinity Bioreagents, Golden, CO) were used at a dilution of 1:1000. Anti-CFTR antibody 570 (dilution 1:500) was obtained from University of North Carolina (UNC) CFTR Antibody Distribution Program sponsored by Cystic Fibrosis Foundation Therapeutics (CFFT).

Isolation of RNA and Northern blot analysis.

The cells were washed twice with Hank's balanced salt solution (HBSS) and harvested in guanidine isothiocyanate solution (4). Total cell RNA was then purified with

CsCl centrifugation. Equal amounts of RNA (15 µg) were resolved on a 1% agarose-2.5 M formamide gel in a 20 mM MOPS buffer, pH 7.4, containing 1 mM EDTA. A standard Northern blot procedure (4) was used to transfer the RNA to a nylon membrane (Micro Separations, Westborough, MA). The SERCA2 and SERCA3 cDNAs were obtained from Origene, Rockville, MD. The cDNAs were labeled with a randomly primed labeling kit (Invitrogen, Carlsbad, Ca) and [³²P] dCTP (ICN, Irvine, CA). Blots were hybridized with the probe and autoradiographed. Quantitative analysis of Northern blots was performed with ImageQuant 1.11 (Molecular Dynamics, Sunnyvale, CA) after the blots were exposed to a phosphor screen. Northern blot data were normalized for loading efficiency with a random prime-labeled 28S rRNA probe (Ambion, Austin, TX).

Immunofluorescence staining

Cells grown on glass coverslips in 6-well plates were fixed in 4% paraformaldehyde (PFA) for 10 minutes, rinsed in TBS and permeabilized with 0.4% Triton-X-100 in 10 mM sodium citrate for 20 minutes. After blocking in 5% donkey serum for 20 minutes, cells were incubated with rabbit anti-SERCA2 (Affinity Bioreagents, Golden, CO) and mouse anti-PDI (Gene Tex Inc, San Antonio, TX) for 1 hour. Negative controls included normal rabbit or mouse IgG at the same concentration as the primary antibodies used. Secondary antibodies, Texas red-conjugated donkey anti-rabbit or FITC-conjugated donkey anti-mouse were then applied for 60 minutes. Cells were mounted on slides with Prolong Gold-DAPI and allowed to dry overnight. Slides were viewed using a Zeiss, Axiovert 200M fluorescent microscope and digital images recorded using Slidebook software (both from Intelligent Imaging Innovations, Denver, CO).

SERCA2 was detected by immunohistochemistry in non-CF and CF lung tissues obtained from National Disease Research Interchange, NDRI with approval of National Jewish Institutional Committee for the Protection of the Rights of Human Subjects (NJIRB). Paraffin-embedded sections (5 μ m) were dewaxed, rehydrated, and exposed to antigen retrieval (vegetable steamer for 25 min followed by a 20 min cool down). After quenching of endogenous peroxidase and alkaline phosphatase for 10 min. (Dual Blocker; Dako, Carpinteria, CA), the nonspecific binding was blocked (Serum Free Protein Block; Open Biosystems, Huntsville, AL). The sections were then incubated for 60 min on a Dako autostainer with anti-human antibodies against SERCA2 (mouse monoclonal; 1:400, Affinity Bioreagents, Golden, CO), with protein concentration-matched mouse IgG (BD Pharmingen, San Diego, CA) for negative controls. After incubation with labeled polymer-HRP-antimouse (horseradish peroxidase-labeled polymer conjugated to goat antimouse immunoglobulin) (EnVision +HRP, Dako) for 30 min, color was developed by 3,3-diaminobenzidine (BioCare Medical, Walnut Creek, CA) combined with H₂O₂. Counterstaining was performed with hematoxylin (Open Biosystems, Huntsville, AL). Identical staining conditions were maintained during staining of non-CF and CF sections. Similarly, for *in situ* SERCA2 staining in ALI cultures, the cells growing on inserts were fixed with 4% PFA. After paraffin embedding SERCA2 or hematoxylin and eosin staining was then performed on the deparaffinized slides with sections of each insert on edge. The methods were developed in collaboration with and stains performed by IHCtech (Aurora, CO).

Isolation of microsomes

The method used for isolation of the microsomes is one modified from that described before (5). Cells were washed with 5 ml of prewarmed (37 °C) phosphate-buffered saline solution. The cells were then scraped in a solution of cold (prechilled on ice) phosphate-buffered saline with 5 mM EDTA before being transferred and collected in a single tube on ice. The cells were pelleted by centrifugation at 4000 x g for 15 min at 4 °C. The supernatant was discarded, and the resulting pellet was resuspended gently with 10 ml of phosphate-buffered saline prior to centrifugation, as before. The supernatant was then discarded, and the cells were resuspended gently with 5 ml of prechilled (on ice) hypotonic solution (10 mM Tris, pH 7.5, 0.5 mM MgCl₂). The resuspended cells were then incubated on ice for 10 min prior to the addition of 0.1 mM phenylmethylsulfonyl fluoride and 4 µg/ml leupeptin. The lysed cells were homogenized using a glass Dounce homogenizer for 30 strokes. The homogenate was then diluted with an equal volume of buffer (0.5 M sucrose, 6 mM 2-mercaptoethanol, 40 µM CaCl₂, 300 mM KCl, and 20 mM Tris, pH 7.5) before being centrifuged at 1000 x g for 10 min at 4°C. The supernatant was removed and made up to 0.6 M with KCl by the addition of an appropriate volume of a 2.5 M solution, prior to centrifugation at 100,000 x g for 60 min at 4°C to obtain the microsomal membrane fraction. The microsomal pellet was then resuspended in buffer (0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol, 20 µM CaCl₂, and 10 mM Tris, pH 7.5). The microsomal membranes were rehomogenized before being aliquoted and snap-frozen with liquid nitrogen, prior to storage at -80°C.

Ca²⁺ ATPase (SERCA) activity

The Ca²⁺-dependent ATPase activity of microsomal membranes was measured using

the phosphate liberation assay (6). Briefly, microsomal extracts (typically 20 μg) were resuspended in 200 μl of buffer (45 mM Hepes/KOH (pH 7.0), 6 mM MgCl_2 , 2 mM NaN_3 , 0.25 mM sucrose), supplemented with 5 $\mu\text{g/ml}$ A23187 ionophore and EGTA and CaCl_2 to give a free $[\text{Ca}^{2+}]$ of 2 μM . Assays were preincubated at 37°C for 10 min prior to the addition of ATP with a final concentration of 6 mM to initiate activity. The reactions were then incubated at 37°C for 40 min, before the addition of 50 μl of 6.5% trichloroacetic acid, and the reactions were then stored on ice for 10 min before centrifugation for 5 min at 20,000 x g. Supernatant (100 μl) was added to 150 μl of buffer (11.25% (v/v) acetic acid, 0.25% (w/v) copper sulfate, and 0.2 M sodium acetate, pH 4.0). Ammonium molybdate solution (5% w/v, 25 μl) was then added, followed by the addition of 25 μl of ELAN reagent (2% w/v *p*-methyl-aminophenol sulfate and 5% w/v sodium sulfite). The samples were mixed, and the blue color was allowed to develop for 10 min prior to measuring the absorption at 870 nm using a Dynatech Laboratories enzyme-linked immunosorbent assay (ELISA) plate reader. The amount of inorganic phosphate liberated was determined by comparison with known phosphate standards. The activities were also determined in the absence of the addition of Ca^{2+} to determine non- Ca^{2+} dependent ATPase activity. All activities were calculated as pmol/min/mg of microsomal protein. Stock solutions of thapsigargin, were prepared in dimethyl sulphoxide (DMSO), when added to the assays, the amount of DMSO never exceeded 1% v/v, which was shown not to have any effect on the Ca^{2+} -dependent ATPase activity of the microsomal membranes.

Preparation of caveolae-related domains (CRDs)

For the isolation of CRDs, the microsomal membranes were lysed in 2.0 ml of ice-

cold 0.5 M sodium carbonate buffer (pH 11.0) using 20 strokes of a Dounce glass homogenizer followed by sonication and CRDs were prepared as described before (7, 8). To determine the distribution of CRD-associated proteins within the gradient, each fraction was analyzed by SDS-PAGE on 4-20% gradient gel, followed by Western blot analysis with appropriate antibodies.

Isolation of mitochondria from Optiprep density gradients

Cells were washed with 5 ml of prewarmed (37°C) phosphate-buffered saline solution. The cells were then scraped in a solution of cold phosphate-buffered saline with 5 mM EDTA (prechilled on ice) before being transferred to a universal tube on ice. The cells were pelleted by centrifugation at 1,000 x g for 15 min at 4°C. The supernatant was discarded, and the resulting pellet was resuspended gently with 10 ml of phosphate-buffered saline prior to centrifugation, as before. The pellet was suspended in 4.0 ml of isotonic buffer containing 10 mM Tris-HCl, pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.025% fatty acid-free bovine serum albumin (BSA), 10 µg/ml each of aprotinin and leupeptin and 2 mM phenylmethylsulfonyl fluoride (MSE) and homogenized using a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 600 x g for 10 min. The supernatant was then subjected to 12,000 x g centrifugation to obtain a crude mitochondrial fraction pellet. This pellet was resuspended in 2.2 ml MSE buffer and layered over a combination of Percoll/Optiprep (Accurate Chemical and Scientific, Westbury, NY) gradient prepared same day as follows (9). Each gradient was prepared in Beckman-Ultraclear 14 x 89 mm centrifuge tubes. The first step in gradient formation involved overlaying 1.74 ml of a 17% Optiprep solution on 1.74 ml of 35% Optiprep solution. Next,

4.35 ml of 6% Percoll solution were layered on top of the 17% Optiprep solution. The Optiprep and Percoll solutions were prepared using MSE buffer as diluent. Next, 2.2 ml of the resolubilized mitochondria were gently layered on top of the 6% Percoll solution. All tubes were centrifuged in a Beckman SW.41 swinging bucket rotor at 50,000 x g for 30 min. Mitochondria were harvested from the 17%/35% Optiprep interface of each gradient and placed on ice until use in Western blot analyses.

References:

- E1. Ahmad S, Ahmad A, McConville G, Schneider BK, Allen CB, Manzer R, Mason RJ, White CW. Lung epithelial cells release ATP during ozone exposure: Signaling for cell survival. *Free Radic Biol Med* 2005;39:213-226.
- E2. Ahmad S, Ahmad A, Ghosh M, Leslie CC, White CW. Extracellular ATP-mediated signaling for survival in hyperoxia-induced oxidative stress. *J Biol Chem* 2004;279:16317-16325.
- E3. Dremina ES, Sharov VS, Kumar K, Zaidi A, Michaelis EK, Schoneich C. Anti-apoptotic protein bcl-2 interacts with and destabilizes the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). *Biochem J* 2004;383:361-370.
- E4. Riddle SR, Ahmad A, Ahmad S, Deeb SS, Malkki M, Schneider BK, Allen CB, White CW. Hypoxia induces hexokinase ii gene expression in human lung cell line A549. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L407-416.
- E5. Wootton LL, Argent CC, Wheatley M, Michelangeli F. The expression, activity and localisation of the secretory pathway Ca^{2+} -ATPase (SPCA1) in different mammalian tissues. *Biochim Biophys Acta* 2004;1664:189-197.

- E6. Michelangeli F, Munkonge FM. Methods of reconstitution of the purified sarcoplasmic reticulum (Ca²⁺)-Mg²⁺)-ATPase using bile salt detergents to form membranes of defined lipid to protein ratios or sealed vesicles. *Anal Biochem* 1991;194:231-236.
- E7. Dremina ES, Sharov VS, Schoneich C. Displacement of SERCA from SR lipid caveolae-related domains by bcl-2: A possible mechanism for SERCA inactivation. *Biochemistry* 2006;45:175-184.
- E8. Li C, Duan W, Yang F, Zhang X. Caveolin-3-anchored microdomains at the rabbit sarcoplasmic reticulum membranes. *Biochem Biophys Res Commun* 2006;344:1135-1140.
- E9. Guo D, Nguyen T, Oghi M, Tawfik H, Ma G, Yu Q, Caldwell RW, Johnson JA. Protein kinase C-epsilon coimmunoprecipitates with cytochrome oxidase subunit iv and is associated with improved cytochrome-c oxidase activity and cardioprotection. *Am J Physiol Heart Circ Physiol* 2007;293:H2219-2230.

Table E1. Demographics of airway tissue donors for cells

Donor No.	Category	Age/Sex (yr)	COD	Genotype
1	NTD	44/M	IVH	
2	NTD	48/M	Head trauma	
3	NTD	16/F	Head trauma	
4	(Tissue from UNC)	11/F		
5	NTD	40/M	CVA	
6	(Tissue from Miami)	22/M		
7	NTD	47/M	Anoxia	
8	NTD	24/M	MVA/ head trauma	
9	NTD	68/F	Head trauma	
10	TD	14/M		
11	NTD	22/M	CVA	
12	TD	42/M	antifreeze poisoning	
13	(Tissue from UNC)	61/M		
14	CF transplant	40/M		$\Delta F508 / ?$
15	CF transplant	22/F		Not Genotyped
16	CF transplant	14/F		$\Delta F508 / \Delta F508$
17	CF transplant	35/F		$\Delta F508 / \Delta F508$
18	CF transplant	24/M		$\Delta F508 / \Delta F508$
19	CF transplant	34/M		$\Delta F508 / \Delta F508$

20	CF transplant	31/M	$\Delta F508 / 1898+IG>A$
21	CF transplant	17/M	$\Delta F508 / \Delta F508$

Definition of abbreviations: COD=cause of death, NTD = nontransplant donor, lung unsuitable because of acute injury, age, etc.; TD = excess airway from lung transplant donor; ? = mutation not identified, IVH= intraventricular hemorrhage, CVA= cardiovascular arrest, MVA= motor vehicle accident.

Table E2. Demographics of airway tissue donors for IHC

Donor No.	Category/COD	Age/Sex (yr)	Genotype
1.	NAT/ Cancer	56/M	
2.	MVA	42/M	
3.	NAT/ Cancer	63/M	
4.	MVA	36/F	
5.	CVA	44/F	
6.	Cerebral edema	12/M	$\Delta F508 / \Delta F508$
7.	ARF	14/F	$\Delta F508 / ?$
8.	CF	19/F	$\Delta F508 / ?$
9.	CF	18/M	$\Delta F508 / \Delta F508$
10.	CF	41/M	$\Delta F508 / ?$

Definition of abbreviations: COD=cause of death, NAT=normal adjacent tissue, ? = mutation not identified, ARF= Acute Respiratory Failure, MVA= motor vehicle accident, CVA= cardio vascular arrest.

Legends to Figures:

Figure E1. CFTR function in CFTR sense and antisense oligonucleotide-expressing 16HBEo- cells (A) and CFTR expression in adenovirally transduced primary airway epithelial cells (B). A representative blot of two individual experiments.

Figure E2. Mutated CFTR-dependent increases in NF- κ B and Bcl-2 in primary human airway epithelial cells. Primary airway epithelial cells cultured on collagen coated dishes were adenovirally transduced with LacZ, wtCFTR and Δ CFTR. After 24 h cells were treated with TNF (10 ng/ml, 18 h). Cells lysates and nuclear lysates were prepared. Top panel of part A shows CFTR expression. Lower panel shows Western blot of p65 in the nuclear lysate. Panel B is quantification of nuclear translocation of NF- κ B as measured by ELISA. The bars represent mean of data and * indicates significant difference ($p < 0.05$) from untreated cells. The experiment was repeated two times with $n=3$. The lowest panel C is a Western blot for Bcl-2 in whole cell lysates. The experiment was repeated 3 times and representative blot is shown.

Figure E1

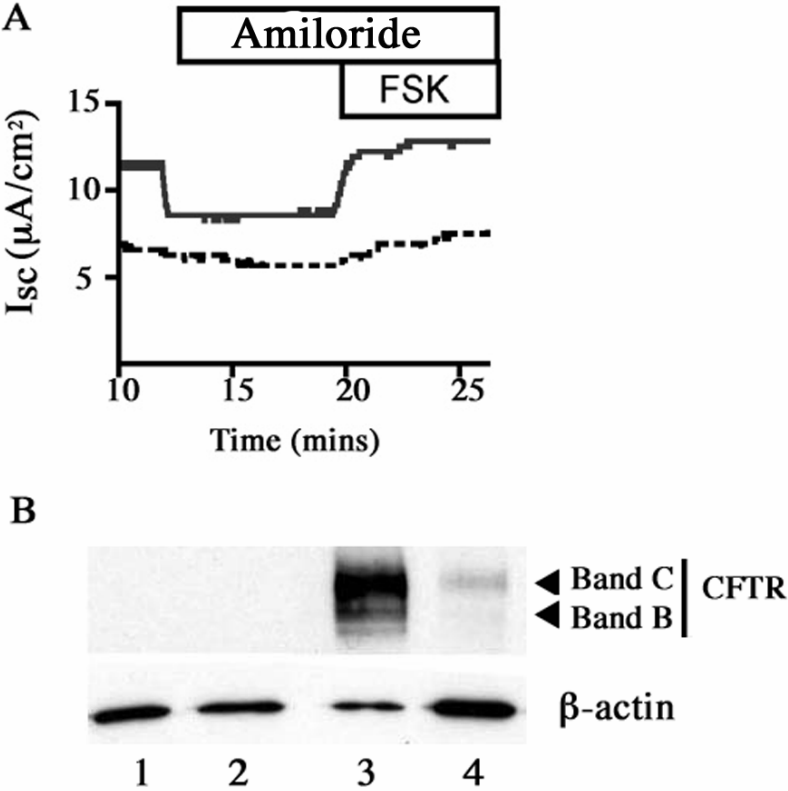


Figure E2

