Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines

AI Q. TRUONG-TRAN, RICHARD E. RUFFIN, AND PETER D. ZALEWSKI
Department of Medicine, University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia 5011, Australia

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Damaged to the airway epithelium by inhaled noxious agents (e.g., environmental pollutants) not only compromises its protective barrier function but also results in decreased production of smooth muscle relaxant factor and increased release of proinflammatory cytokines such as interleukin-6, interleukin-8, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α (22). Although the mechanism of respiratory epithelial cell death has not been extensively studied, in at least two reports (3, 33), including an in vitro model of tracheal regeneration, it was shown that the death of these cells was due to apoptosis. Another example where apoptosis of these cells may be important is in asthma, where there is an increase in the fragility and shedding of the columnar airway epithelium (19). In these cases, apoptosis may contribute to the denuding of the respiratory epithelium and increased exposure of the basement membrane to toxic mediators of inflammatory cells, thereby exacerbating inflammation while retarding airway repair (3, 13, 19, 27).

One factor that may be significant in the biology of the respiratory epithelium is the group IIb metal zinc (Zn), an important antioxidant and anti-inflammatory agent that participates in various cellular events and is a cofactor for many metalloenzymes and transcription factors (32). Intracellular Zn exists in two discreet pools, the first of which is nonexchangeable and tightly bound to metalloenzymes and the second is a more labile and dynamic pool that is rapidly exchangeable and able to be altered by Zn deprivation or supplementation (35). One important role of labile Zn is as a survival factor that suppresses apoptosis. Studies from our laboratory (35) and others (12, 15, 16) have shown that Zn deficiency in vitro and in vivo stimulates intranucleosomal DNA fragmentation and apoptosis in lymphoid and myeloid cells as well as in intestinal epithelium, neural epithelium, and endothelium. Zalewski et al. (35) have demonstrated an inverse correlation between the level of intracellular labile Zn in lymphocytes and their susceptibility to DNA fragmentation. These results imply that a reduction below a threshold concentration in this Zn pool facilitates apoptotic DNA fragmentation.

Zn is important for the integrity, growth, and repair of epithelial tissues in the skin and gastrointestinal tract (1, 12), but its role in the respiratory epithelium has not yet been studied. We propose that intracellular labile Zn is important in this tissue as a cytoprotectant against toxins and inflammatory mediators in a way similar to that reported for the endothelium (16). Therefore, the aim of this study was to investigate where Zn is localized along and within the respiratory epithelium and to further elucidate its role in regulating apoptosis of these cells. For this study, we used a variety of cells and tissues that included 1) NCI-H292 and A549 cells (malignant cell lines representative of bronchial and alveolar epithelia, respectively), 2) primary pig and sheep ciliated epithelial cells, and 3) cryostat tissues from these animals.
To assess the levels and distribution of intracellular labile Zn, we used the ultraviolet (UV)-excitable Zn-specific fluorophore Zinquin that has previously enabled us to visualize and image distinct pools of labile Zn in a range of cell types and tissues (6, 35–37). The Zn chelator N,N',N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and the Zn ionophore sodium pyrithione (35) were used to determine the effects of changes in the intracellular Zn status of these cells on their susceptibility to undergo apoptosis induced by the oxyradical hydrogen peroxide (H$_2$O$_2$) (25) or the short chain fatty acid butyrate (24).

**METHODS**

**Materials**

The major materials were EDTA, EGTA, herring sperm DNA, Hoechst 33258, Nonidet P-40 (NP-40), dithiothreitol, sucrose, HEPES, TPEN, sodium pyrithione, amphotericin B, insulin, transferrin, epidermal growth factor (EGF), protease (from Streptomyces griseus), and retinoic acid (all from Sigma, St. Louis, MO); hematoxylin staining solution, eosin staining solution, H$_2$O$_2$ (30% wt/vol), and sodium butyrate (all from BDH, Poole, UK); LHC-9 basal medium (Clonetics, Walkersville, MD), RPMI 1640 medium, penicillin-streptomycin, EDTA-trypsin, 1-glutamine, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (CHAPS; ICN, Aurora, OH); fetal bovine serum (Biosciences, Sydney, Australia); Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden); gentamicin (David Bull Laboratories, Melbourne, Australia); Asp-Glu-Val-Asp 7-amino-4-(trifluoromethyl)coumarin (DEVDF-AFC) and Tyr-Val-Ala-Asp-AFC (YVAD-ACF; both from Kamiya Biomedical, Tukwila, WA); Val-Asp-Val-Ala-Asp-AFC (VDVAD-ACF), Leu-Glu-Val-Asp-AFC (LEVD-AFC), Trp-Glu-His-Asp-AFC (VEHD-AFC), Val-Glu-Ile-Asp-AFC (VEID-AFC), and Leu-Glu-His-Asp-AFC (LEHD-AFC; all from Calbiochem); epoxypropane and Procure 812 resin (both from Electron Microscopy Sciences, Fort Washington, PA); Tissue-Tek optimum cutting temperature compound (Miles); and ethyl-2-(methyl-4-p-toluenesulfonamido-6-quinoloxyl)acetate (Zinquin [21]; Department of Chemistry, University of Adelaide, Adelaide, Australia). All other reagents not listed were of reagent grade unless otherwise indicated.

**Cell Lines**

A549 and NCI-H292 cells were obtained from Dr. D. Knight (Queen Elizabeth II Medical Center, University of Western Australia, Nedlands, Australia) and cultured in RPMI 1640 medium (24). A549 cells were derived from a human alveolar cell carcinoma; they are epithelial in morphology and hyperdiploid and represent type II alveolar cells (20). NCI-H292 cells were derived from a cervical node metastasis of a pulmonary mucoepidermoid carcinoma; they are representative of the upper bronchial respiratory airways and are epithelial in morphology, containing numerous mucin-secreting granules (5).

**Respiratory Epithelial Tissues and Tracheobronchial Cell Isolation**

Pig and sheep respiratory tracts were removed from freshly killed animals and rinsed in chilled phosphate-buffered saline solution (pH 7.4). Samples were frozen in optimum cutting temperature compound and liquid nitrogen for cryostat sections or fixed in Formalin and embedded in paraffin for histological assessment. Cryostat sections were stained with hematoxylin and eosin for morphological assessment. Collagen was identified with Weigert's hematoxylin and van Gieson's stain (a gift from the Histopathology Department, University of South Australia, Adelaide, Australia). These experiments conformed to the National Health and Medical Research Council of Australia animal ethics guidelines.

To prepare isolated epithelial cells, excised segments were incubated at 4°C overnight in RPMI 1640 medium containing 0.05% protease. Dissociated cells were removed by vigorous agitation for 5 min, and enzymatic digestion was terminated with fetal bovine serum (10% final concentration). Cells were centrifuged at 150 g for 5 min to produce a cellular pellet containing isolated mucus cells, ciliated cells, and nonciliated basal cells that were used for Zinquin fluorescence studies. To prepare a highly enriched population of ciliated cells for apoptosis assays, the Percoll separation technique of Takizawa et al. (31) was used. Cell viability as determined by trypan blue exclusion was ～90%. The upper band contained a highly enriched population of ciliated cells that were used for Zn and apoptosis studies. Epithelial cells were cultured in a modified serum-free LHC-9 medium supplemented with 2 mM l-glutamine, 25 μM amphotericin B, 1 μg/ml of insulin, 1 μg/ml of transferrin, 10 ng/ml of EGF, and 10 ng/ml of retinoic acid. When extensive clumping had occurred, cell suspensions were passed two times through a 20-gauge needle before being seeded into wells.

**Depletion and Augmentation of Intracellular Labile Zn**

To deplete intracellular Zn, the cells were incubated with varying concentrations (up to 25 μM) of TPEN for 1 h at 37°C in complete culture medium. TPEN was stored as a 5 mM stock solution in DMSO at −20°C and added to the cultures at a dilution of at least 1:200. To increase intracellular Zn, the cells were incubated with 25 μM ZnSO$_4$ (a typical plasma concentration) in the presence of 1 μM sodium pyrithione. ZnSO$_4$ was added just before the addition of pyrithione.

**Quantification of Zinquin Fluorescence by Image Analysis**

Cryostat sections 3 μm thick from animal airways and alveolar tissue were fixed in acetone for 5 min at room temperature (RT) and then rinsed in PBS. Zinquin was freshly diluted in PBS to a final concentration of 25 μM and immediately pipetted onto these sections. After incubation for 30 min at RT in dark and humidified conditions, the sections were mounted with a fluorescent mounting medium (DAKO). An autofluorescence control (PBS alone) was set up for each section to ensure the specificity of Zinquin.

A549 and NCI-H292 cells were grown to semiconfluence on sterile glass coverslips in six-well plates for 48 h before the addition of Zinquin. The coverslips were then washed in PBS and incubated with 25 μM Zinquin in PBS for 30 min at 37°C. Primary epithelial cells were incubated with Zinquin (25 μM) as cell suspensions immediately after isolation. In some experiments, intracellular Zn was either depleted or supplemented as in Depletion and Augmentation of Intracellular Labile Zn before the addition of Zinquin.

Fluorescence was examined as previously described (37) with modifications. For cryostat sections, both fluorescence images and corresponding phase-contrast microscope images were captured with a ×20 objective lens and stored. For Zinquin fluorescence in cryostat sections, the outline of the airway epithelium was traced, stored, and copied onto the corresponding fluorescence image. A profile line was then drawn at 90° to the epithelial surface so that it spanned the
entire epithelium. The Video Pro image analysis system (Leading Edge) was used to quantify the fluorescence intensities at 1-pixel intervals along the profile line. For each image, seven randomly positioned profiles were obtained, and the mean fluorescence intensity was calculated for each 2.5% distance across the epithelium. As a result, 40 intervals were measured, the first beginning at the luminal surface and the last terminating at the basement membrane. For the alveolar cryostat tissue sections, 20 squares were drawn as overlays within the boundaries of the epithelium on the light images. These overlays were transferred to the corresponding fluorescence image, and the intensities were measured. The mean fluorescence intensity was collated from seven images. For transformed cells, lines were drawn around their borders, and the intensity was quantified. For all images, background fluorescence was determined and subtracted. At least 150 cells or 6–10 tissue sections in each group were analyzed.

For UV laser confocal microscopy, a Bio-Rad MRC-1000 UV laser scanning confocal microscope system equipped with an UV argon laser was used in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode, with excitation at 363/368 nm and emission at 460 nm with a long-path filter. In the case of ciliated cells, those that were stationary were chosen for z-series imaging of 0.5-μm slices that were 2 μm apart. Images were collected with a ×40 objective water-immersion lens with a numerical aperture of 1.15. Each image was averaged over three scans by Kalman filtering.

**Transmission Electron Microscopy of Sheep Tracheal Ciliated Epithelial Cells**

Cells (10^6 cells/ml) obtained by protease treatment and Percoll separation were centrifuged at 2,000 rpm, and the pellet was resuspended in 1 ml of an electron microscopy fixative mixture (1.5% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer) overnight at 4°C. The cell suspension was centrifuged between each of the following processing steps for 1 min at 2,000 rpm. The cell pellet was postfixed in 2% osmium tetroxide and stained en bloc in 2% uranyl acetate. The cells were then dehydrated through a graded series of alcohols (70, 90, and 100%) and further processed through 100% epoxypropane, a 50:50 mixture of epoxypropane and Procure 812 resin (Electron Microscopy Sciences), and two times through 100% epoxypropane, a 50:50 mixture of epoxypropane and Procure 812 resin. The cell pellets were recombined with corresponding lysates of adherent cells to yield lysates of total cell population from each well. For all of the cell lysates, insoluble material was pelleted at 12,000 g, and the supernatants were collected and stored at −20°C until assayed.

**Measurement of Caspase Activity**

Caspase-3-like activity was assayed by cleavage of the fluorogenic substrate Z-DEVD-AFC (as described in Ref. 17). Other substrates used for the detection of various caspase activities in these experiments were YVAD-AFC (caspase-1), VDVED-AFC (caspase-2), LEVD-AFC (caspase-4), WEHD-AFC (caspase-5), VEID-AFC (caspase-6), and LEHD-AFC (caspase-8). It should be mentioned that although these substrates are preferentially cleaved by the indicated caspases, there may be some overlap. The caspase activities are expressed in fluorescence units per microgram of protein per hour.

**Experimental Design**

All experiments were repeated a minimum of three times. Typical experiments are described or data were collated as indicated. Significance was determined by Student’s t-test.

**RESULTS**

**Zinquin Fluorescence Studies**

NCI-H292 and A549 cells. Figure 1 shows corresponding UV laser confocal light transmission and Zinquin fluorescence images for NCI-H292 (A and B) and A549 (C and D) cells, representative of upper airway epithelial cells and type II alveolar epithelial cells, respectively. NCI-H292 cells had significantly higher basal levels of Zn as determined by the twofold increase in Zinquin fluorescence (mean 23.1 ± 2.2 pixels; n = 29 cells) compared with the A549 cells (mean 11.8 ± 0.3 pixels; n = 192 cells), typical of other labile Zn-poor cells (35). Zinquin fluorescence was sig-
nificantly \( (P \leq 0.05) \) quenched by 25 \( \mu M \) TPEN, which has a higher affinity for Zn than for Zinquin \((35)\). TPEN treatment reduced fluorescence to 9.7 \( \pm 0.7 \) pixels in NCI-H292 cells \((n = 28)\) and 7.2 \( \pm 0.3 \) pixels in A549 cells \((n = 187)\). Zn supplementation with exogenous \( \text{ZnSO}_4 \) and 1 \( \mu M \) sodium pyrithione increased fluorescence of NCI-H292 cells to 95.1 \( \pm 3.3 \) pixels \((4.1\text{-fold}; n = 58\) cells\) and to 59.9 \( \pm 1.6 \) pixels in A549 cells \((5.5\text{-fold}; n = 307\) cells\).

Occasional cells in nonsupplemented cultures also had an intense Zinquin fluorescence \((\text{Fig. 1D}, \text{arrow})\); these cells were larger, had nuclear changes, and may have been cycling. Figure 1E shows enrichment of these cells in cultures treated for 24 h with a mitogen, EGF \((10 \text{ ng/ml})\), in the presence of 25 \( \mu M \) \( \text{ZnSO}_4 \). The pattern of fluorescence was essentially cytoplasmic. Zn was often seen to be concentrated in the ends of pseudopodia \((\text{Fig. 1F})\) and in a perinuclear pattern \((\text{Fig. 1, F and G})\). At higher magnification \( (\times 2,500)\), some Zn-loaded cells showed a granular pattern \((\text{Fig. 1G})\), indicative of a vesicular localization.

Isolated tracheal ciliated cells. Isolated ciliated epithelial cells from the tracheobronchial region of sheep and pigs were released by proteolytic digestion and purified by Percoll separation. Figure 2 shows a transmission electron micrograph of a typical ciliated sheep tracheal epithelial cell that was used for the following Zinquin fluorescence and apoptosis studies. These cells, which were viable and had rapidly beating cilia, were incubated with Zinquin in wet suspensions, and fluorescence was analyzed by UV laser confocal microscopy. Only those ciliated cells that were not moving across the microscope field were chosen for \( z \)-series confocal images. A bright-field image and 11 fluorescence images \((0-255\text{ pixels} (A-G))\). H: high-magnification epifluorescence image \((\times 100\text{ oil})\) of Zn-supplemented A549 cell showing punctate pattern suggestive of a vesicular distribution of supplemented Zn. Bar, 2 \( \mu m \).

Fig. 1. Labile Zn distribution in human NCI-H292 bronchial epithelial and human A549 alveolar type II epithelial cells. A and B: confocal bright-field transmission micrograph and corresponding Zinquin fluorescence, respectively, of adherent NCI-H292 cells. Note the intense cytoplasmic distribution. Bar, 10 \( \mu m \). C and D: confocal bright-field transmission micrograph and corresponding Zinquin fluorescence, respectively, of adherent A549 cells. Mean intensity of fluorescence was approximately half that of NCI-H292 cells. However, occasional cells \((D, \text{arrow})\) had intense cytoplasmic fluorescence; these were larger in size and had nuclear morphologies consistent with cycling cells. Bar, 10 \( \mu m \). E: confocal Zinquin fluorescence of A549 cells after exposure to a mitogen, EGF, in the presence of a physiological concentration \((25 \mu M)\) of extracellular \( \text{ZnSO}_4 \). Most of these cells had intense cytoplasmic fluorescence and increased size. Scale is same as in C and D. F: confocal image showing 2 major patterns of Zinquin fluorescence in unsupplemented A549 cells, with concentration of fluorescence in the pseudopodia and around the rim of the nucleus (perinuclear). Bar, 10 \( \mu m \). G: confocal image of perinuclear fluorescence in an A549 cell after Zn supplementation by Zn ionophore sodium pyrithione and 25 \( \mu M \) \( \text{ZnSO}_4 \). Horizontal bar, 10 \( \mu m \). Vertical bar, linear scale of 0–255 pixels \((A–G)\).
tent, followed by magenta and then black. These images were captured about halfway through the entire thickness of the cells. The cilia of the cells are depicted by arrows. Typical images were obtained from different pigs and sheep, and representative cells are displayed. The image reveals that Zn was concentrated in two regions: 1) in a perinuclear manner (similar to that seen in the malignant cell lines) and 2) in the cytoplasm immediately beneath the cilia. This distribution of Zinquin fluorescence was a consistent feature of all the ciliated cells examined in sheep (Fig. 3, C and D) and pigs (data not shown). In addition to Zinquin fluorescence in the apical cytoplasm, some fluorescence was also evident within the cilia themselves (e.g., Fig. 3D). Figure 3, E and F, shows a typical Zn-supplemented sheep ciliated cell as seen by epifluorescence microscopy. At the level of sensitivity of the epifluorescence microscope used, un-supplemented cells only had moderate fluorescence in the apical cytoplasm (data not shown). This was substantially increased by pre-loading of the cells with Zn with exogenous ZnSO$_4$ and the Zn ionophore sodium pyrithione (Fig. 3F). It is interesting that the supplemented Zn also localizes in the same apical region of these cells, suggesting that the Zn may be sequestered into Zn-laden vesicles.

To confirm that the Zinquin fluorescence of the ciliated cells was Zn dependent, the effect of the Zn chelator TPEN on Zinquin fluorescence was determined. Primary sheep tracheal epithelial cells purified by Percoll density centrifugation were incubated with Zinquin in the absence and presence of TPEN (final concentration of 25, 50, or 100 μM). Fluorescence was quantified by image analysis of 40–71 cells/group. In the absence of TPEN, the mean fluorescence intensity was 46.5 ± 1.9 (SE) pixels. This decreased to 16.1 ± 0.6 pixels with 25 μM TPEN (P ≤ 0.005), 9.4 ± 0.6 pixels with 50 μM TPEN (P ≤ 0.005), and 11.0 ± 0.9 pixels with 100 μM TPEN (P ≤ 0.005). Therefore, fluorescence was strongly quenched by TPEN.

Cryostat sections of tracheobronchial and alveolar epithelia. Next, we examined Zinquin fluorescence in frozen sections of sheep and pig tracheobronchial and alveolar tissues. Cryostat sections 3 μm thick were acetone fixed, incubated with and without Zinquin in PBS for 30 min, washed, and mounted for epifluorescence microscopy.

The sections incubated with PBS alone served as controls for UV autofluorescence. Autofluorescence was largely confined to the walls of blood vessels and to the lamina propria where it was colocalized with collagen as detected by staining with Weigert’s hematoxylin and van Gieson stain. Collagen fibers were stained bright red, whereas smooth muscle and other cytoplasmic proteins were stained yellow. The stained collagen and the autofluorescence were more structurally organized in the lamina propria sections, particularly in the bronchi and bronchiolar regions of pig, compared with the tracheae or sheep tissues. Importantly, there was no autofluorescence in the epithelia of any of the sections examined.

Figure 3, G and H, shows typical hematoxylin and eosin-stained sections and Zinquin fluorescence images, respectively, of frozen sections of pig tracheae. The pseudostratified, ciliated epithelium was lined continuously at the luminal surface (arrow) by a region of intense Zinquin fluorescence. In the other two pigs and in the sheep, there was much less collagen. Importantly, there was no autofluorescence in the epithelium in any of the sections examined. Figure 3I shows another representative section from pig bronchi at a higher magnification and demonstrates the striking demarcation between the labile Zn-rich outer area and the relatively Zn-poor middle to inner layers of the epithelium. Similar data were observed with sheep bronchi and tracheae where there was intense Zinquin fluorescence at the luminal surface (data not shown).

The alveolar epithelium in both pig (Fig. 3J) and sheep (data not shown) had a typically dull fluorescence. No distinction in fluorescence was evident between the type I and II epithelial cells.

Figure 4 shows a compilation of data from all of the cryostat sections analyzed. The width of the Zinquin fluorescence band varied between different sections from different regions of the track and between sheep and pig specimens. To determine the mean proportion of the epithelium that was labile Zn rich, the epithelium was subdivided into 40 evenly distributed intervals across its entire width, and the mean fluorescence...
within each interval was determined. For each image, seven randomly placed profile lines (as in Fig. 3H) measuring fluorescence intensity across the epithelium were obtained, and a total of six to eight images from sections of pig and sheep tracheae and bronchioles were quantified. There were insufficient sections of sheep tracheae to do multiple analyses. Similar autofluorescence determinations were made on a separate set of images derived from sections incubated without Zinquin.

Figure 4A shows the pooled mean data for the two pig tracheae (n = 56 profile measurements). It shows the specific Zinquin fluorescence after subtraction of the background and autofluorescence readings that
were negligible (Fig. 4A, bottom). The highest Zinquin fluorescence intensities were recorded in the second to fifth intervals (from 5 to 12.5% of the epithelial width inward from the surface). Most of the Zinquin fluorescence was contained within the first seven intervals (corresponding to the outer 17.5% of the epithelium). Figure 4B shows similar data for the pig bronchioles (n = 35 profile measurements). The autofluorescence was once again negligible, and the labile Zn-rich region was largely contained within the first 12 intervals (outer 30% of the epithelium). Similar results were observed in Fig. 4C for sheep bronchioles (n = 35 profile measurements). The distribution of intense Zinquin fluorescence along the lumen of these airways confirms our previous observation in the isolated cells showing that Zn is localized in an apical manner directly beneath the cilia (Fig. 3, A–D). Because these cells are the first site of contact between the inhaled air and the respiratory tissue, they may require higher concentrations of Zn for survival.

For comparison, the corresponding intensities for alveolar epithelium of pig and sheep are shown in Fig. 4, B, right, and C, right, respectively. The mean pooled intensities for autofluorescence (Fig. 4, B, bottom, and C, bottom) and for specific Zinquin fluorescence (Fig. 4, B, top, and C, top) for pig were derived from 43 and 77 intensity measurements, respectively; for sheep alveolar epithelium, there were 39 and 45 intensity measurements, respectively. Autofluorescence intensities for these alveolar tissues were also insignificant, whereas the Zinquin fluorescence intensities were similar to those in the labile Zn-poor regions of the bronchial epithelium.

**Caspase Activation and Apoptosis Studies in Zn-Manipulated Cells**

The strong staining for Zn in the apical region of airway epithelium coupled with the relatively weak fluorescence in the alveolar epithelium is similar to that reported for procaspase-3 in human tissues by Krajewska et al. (18). These similarities, albeit in different species, in addition to the known suppressive effects of labile Zn on apoptosis, prompted us to investigate whether the labile Zn in respiratory epithelial cells might influence caspase-3 activation and apoptosis.

**Induction of caspase-3 activation and apoptosis by the Zn chelator TPEN in primary sheep ciliated epithelial cells and malignant cell lines.** To determine the effects of lowering intracellular labile Zn on caspase activation and apoptosis, primary and transformed epithelial cells were rendered Zn deficient by TPEN. Figure 5, A and B, shows the morphological changes associated with apoptosis in a typical Zn-depleted primary sheep ciliated epithelial cell. Fragmentation of the cells into apoptotic bodies (Fig. 5A), some containing remnants of the nucleus as shown by the corresponding fluorescence micrographs of chromatin stained by Hoechst dye 33258 (Fig. 5B), is a common feature of apoptotic cell death. TPEN also induced DNA fragmentation in these cells (data not shown).

In addition, apoptosis was confirmed by staining for chromatin condensation with hematoxylin and eosin. For example, A549 cells had a moderate increase in apoptotic cells from 10 ± 1.5% in control cells to 18.5 ± 1.5% when treated with 2 mM butyrate. Severe Zn deficiency created with 50 μM TPEN and coupled with 2 mM butyrate resulted in a substantial increase in apoptotic cells to 97 ± 0.71% (P ≤ 0.005). This was
constructed to determine the linearity of the rate of substrate cleavage over the 18-h period. For the conditions used in these experiments, the time course was linear (data not shown). Eighteen hours was chosen to give an adequate fluorescence signal for the limited number of cells available, especially for the primary ciliated cells.

Figure 5 shows the large increase in DEVD-caspase activity in TPEN-treated primary (C) and NCI-H292 (D) cells. This increase was concentration dependent and plateaued, with maximal activity induced with 12.5 μM TPEN for the primary cells (2.87 ± 0.13 units·μg protein⁻¹·h⁻¹) as seen in Fig. 5C. For the NCI-H292 cells (Fig. 5D) and A549 (data not shown) cells, DEVD-caspase activity was increased in a linear manner without plateauing at any concentration of TPEN (up to 25 μM highest concentration tested). The caspase-3 activities at this concentration were 0.35 ± 0.16 units·μg protein⁻¹·h⁻¹ for NCI-H292 cells and 0.55 ± 0.03 units·μg protein⁻¹·h⁻¹ for A549 cells. Significant increases were seen at ≥6.25 μM for primary cells (1.89 ± 0.46 units·μg protein⁻¹·h⁻¹) and ≥12.5 μM for NCI-H292 cells (0.13 ± 0.09 units·μg protein⁻¹·h⁻¹) and A549 cells (0.39 ± 0.01 units·μg protein⁻¹·h⁻¹, P ≤ 0.05). These data show that the primary cells had approximately a five- to eightfold higher level of caspase-3 activity compared with the two transformed cell lines.

A range of other caspase activities were assayed to determine whether Zn chelation also affects these caspases. Caspase-6 (VEID-caspase) was moderately increased in both cell types; at 25 μM TPEN, the caspase-6 activities were 1.81 ± 0.18 units·μg protein⁻¹·h⁻¹ for primary cells and 0.16 ± 0.001 units·μg protein⁻¹·h⁻¹ for NCI-H292 cells. Caspase-2 (VDVAD-caspase) was weakly induced; at 25 μM TPEN, the caspase-2 activities were 0.33 ± 0.04 units·μg protein⁻¹·h⁻¹ for primary cells and 0.03 ± 0.01 units·μg protein⁻¹·h⁻¹ for NCI-H292 cells, although the increases after TPEN addition were still significant (P ≤ 0.05 and P ≤ 0.005, respectively). Little or no increases were seen for caspase-4 (LEVD-caspase), caspase-5 (WEHD-caspase), and caspase-9 (LEHD-caspase).

Time course of caspase activation with TPEN. The aim of the following experiment was to determine the optimal time of induction of caspase-3 activity after the decline in intracellular labile Zn induced by 25 μM TPEN. Due to limitations in the number of highly purified primary cells, this time course was performed only in NCI-H292 cells. Experiments were performed in triplicate. To monitor Zinquin fluorescence in the cells, NCI-H292 cells were cultured onto sterile glass coverslips while the caspase-3 activity was determined on the same cells that had grown on the bottom of the six-well culture plates. TPEN was added at time 0 to both populations at varying intervals up to and including 4 h.

Zinquin fluorescence was determined in 17–18 image fields (total of 140–260 cells) for each time point. Mean fluorescence intensity decreased by 38% in the first 30 min after the addition of 25 μM TPEN [from
DEVD-caspase activation was induced by treatment of the cells overnight with the apoptosis-inducer \( \text{H}_2\text{O}_2 \) (0.125 mM). Intracellular labile Zn was raised by treating the cells with 1 \( \mu \text{M} \) sodium pyrithione plus 25 \( \mu \text{M} \) ZnSO4. In cells receiving supplemented Zn, there was a 59.3% inhibition of \( \text{H}_2\text{O}_2 \)-induced DEVD-caspase activation (data pooled from 3 experiments, each in triplicate; \( P \leq 0.005 \)).

These studies show that levels of intracellular Zn can be modulated in respiratory epithelial cells in vitro to strongly influence caspase activation, DNA fragmentation, and morphological changes of apoptosis.

Synergistic interactions between TPEN and other inducers of apoptosis in DEVD-caspase activation. To determine whether Zn depletion was able to influence toxin-induced caspase-3 activation, two different classes of apoptosis-inducing agents, \( \text{H}_2\text{O}_2 \) and butyrate, were used. The results were collated from at least two experiments each performed in triplicate for the three different types of cells. Figure 7A shows the data for primary cells obtained for suboptimal concentrations of TPEN (6.25 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (125 \( \mu \text{M} \)) either alone or in combination. \( \text{H}_2\text{O}_2 \) alone gave an increase over the control value for DEVD-caspase activity of 1.24 \( \pm \) 0.12 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \); TPEN alone gave an increase of 0.52 \( \pm \) 0.14 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \), whereas the two in combination gave an increase of 2.58 \( \pm \) 0.53 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \). This increase was significantly greater than the increase that would have occurred if TPEN and \( \text{H}_2\text{O}_2 \) were acting additively (\( P \leq 0.05 \); Fig. 7A, dashed line). Similar results were found with NCI-H292 (Fig. 7B) and A549 (Fig. 7C) cells.

Synergy was also evident between 6.25 \( \mu \text{M} \) TPEN and 2 mM butyrate in both primary sheep tracheal ciliated cells and NCI-H292 cells. A549 cells were not tested. Thus in primary cells, a combination of TPEN and butyrate gave an increase in DEVD-caspase activity of 2.85 \( \pm \) 0.58 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \). This increase was significantly greater than the increase that would have occurred if TPEN and \( \text{H}_2\text{O}_2 \) were acting additively (\( P \leq 0.05 \)).

**DISCUSSION**

This paper is the first to visualize and quantify labile intracellular Zn in different populations of airway epithelium. There have been no previous studies of Zn distribution in the respiratory system due to lack of sensitive techniques to visualize and quantify tissue Zn. Here, we used the Zn fluorophore Zinquin, which is specific and sensitive for labile intracellular Zn in the nanomolar range (6, 21, 36, 37). Zinquin detects the free or more loosely bound (labile) pools of intracellular Zn. The major findings of this present study are that 1) tracheobronchial epithelial cells are relatively rich in labile Zn compared with alveolar epithelial cells, 2) this Zn is especially concentrated in the mitochondria-rich, apical cytoplasmic region immediately below the cilia, and 3) chelation of Zn with TPEN results in the rapid decrease in butyrate-induced caspase activity but not in apical 

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**Fig. 6.** Time course for induction of caspases in Zn-depleted respiratory epithelial cells. Labile Zn content (A) and caspase-6 activity (B) of NCI-H292 cells treated with 25 \( \mu \text{M} \) TPEN and assayed at time 0 and at 30- to 60-min intervals. Symbols are the same as in Fig. 5, C and D. Data are means \( \pm \) SD of triplicates from a typical experiment repeated 3 times. Labile Zn content (as quantified by Zinquin fluorescence) decreased after TPEN treatment. This was followed by a rapid increase in caspase-3 activity. Caspase-6 activity increased, but other caspases did not. Significant difference from untreated cells: \( * P \leq 0.05 \).

*the control value of 23.7 \( \pm \) 1.3 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \) (DEVD-caspase activity); \( P \leq 0.005 \) and by a further 7% (not significant) in the next 30 min (Fig. 6A). DEVD-caspase activity did not increase in the first 60 min, but there was a 65% increase over the next 75 min and a further 79% increase over the next 60 min (\( P \leq 0.005 \)).

Because DEVD-caspase (caspase-3) is activated from the zymogen form by other caspases (including caspase-6 and caspase-9), we sought to determine whether any of these caspases were activated before caspase-3 in TPEN-treated cells. Figure 6B shows a large increase in VEID-caspase (caspase-6) activity from the control value of 0.086 to 0.20 units \( \cdot \mu \text{g protein}^{-1} \cdot \text{h}^{-1} \) (\( P \leq 0.05 \)). However, this occurred between 3 and 4 h after the addition of TPEN. There was no early increase in caspase activity of any of the other caspases (1, 2, 4, 5, or 9) tested.

**Suppression of DEVD-caspase activation in Zn-supplemented primary respiratory epithelial cells.** The previous studies have shown that lowering the intracellular labile Zn content by TPEN induces DEVD-caspase activation. The effect of increasing intracellular labile Zn content in primary sheep tracheal ciliated cells on DEVD-caspase activation was determined next.
activation of caspase-3-like activity and downstream events of apoptosis as well as in the markedly enhanced susceptibility of these cells to toxin-induced apoptosis.

Our first finding of labile Zn lining the apical and luminal sides of the entire length of the conducting airways may have an analogy with the concept of Zn in galvanization where Zn acts to protect the underlying steel from oxidation and corrosion. It has been proposed that Zn became particularly important in eukaryotic cell evolution at about the time the atmosphere was becoming oxygen rich and the cells were developing mitochondria (7). Therefore, Zn, as one of the major antioxidants in our body, may play an important role in the protection of the respiratory epithelium. Several properties of Zn contribute to its antioxidant functions. First, unlike Cu and Fe, Zn exists in only one oxidation state (II) and therefore cannot react with oxidants to generate potentially damaging oxyradicals (7); second, Zn may protect cellular membranes, proteins, and DNA from oxidative damage (32); and third, Zn is an important component of the antioxidant Cu/Zn superoxide dismutase (8).

The finding of a low labile Zn content in A549 cells derived from transformed alveolar type II epithelium compared with that in the transformed bronchial epithelial NCI-H292 cells is consistent with the observation of low Zinquin fluorescence in cryostat sections of alveolar epithelium of sheep and pigs. The difference in this fluorescence intensity between the two malignant cell lines cannot be attributed to environmental factors because both cell lines had been maintained for several generations in the same culture medium. Rather, variations in fluorescence may be due to intrinsic differences in the capacities of the two cell types to take Zn up from the medium, perhaps as a consequence of altered levels of Zn transporters in the cell membrane (23). We hypothesize that the higher Zn content in the upper conducting airway epithelium reflects the greater need to protect these cells from foreign inhaled particles and other noxious agents.

It is important to mention that Zinquin binds to intracellular labile Zn with a dissociation constant ($K_d$) of $10^{-27}$ M. By labile, we mean the pools normally participating in Zn fluxes and ionic exchange, most readily altered in imbalances of Zn homeostasis, and not tightly bound to metalloproteins ($K_d > 10^{-11}$ to $10^{-13}$ M) (35). The distribution of total Zn is not known but is likely to be similar to the distribution of metalloenzymes in these cells and, therefore, is uniformly distributed throughout the cellular organelles (32). The depletion of intracellular Zn was achieved with TPEN, a membrane-permeable heavy metal chelator that binds intracellular labile Zn ([log association constant ($K_a$) $15.6$] via appropriately spaced nitrogens in the ring structure, but has minimal affinity for calcium or magnesium (4). This chelator has a much higher affinity for Zn than does Zinquin (log $K_{a1}$ 6.4 and log $K_{a2}$ 7.1, where $K_{a1}$ and $K_{a2}$ are the association constants for the formation of Zinquin-Zn and (Zinquin)$_2$-Zn complexes, respectively) and quenches Zinquin fluorescence in solution and within cells (35).

We now need to determine and compare the Zn distribution in respiratory tissues of humans and other species. Our studies have shown that upper respiratory epithelial cells are rich in Zn, leading us to speculate that constant insults to this tissue by proteases and cytokines from inflammatory cells would cause a significant loss of Zn. This may contribute to the known hypozincemia that is a reported feature of asthma (10, 11, 17). In a similar way, Zn deficiency also arises in burn patients where there is enhanced cell turnover in the damaged skin (28).

As discussed earlier (14), Zn may have a special antioxidant role in the vicinity of mitochondria, which
release potentially damaging oxyradicals during the generation of energy. This is a particular hazard for the ciliated cells lining the airways because these cells rely heavily on this energy for rapid beating of the cilia. Zn may exert a protective, antioxidant role in these cells against oxidants both inhaled down the airways and released from mitochondria as a by-product of oxidative metabolism. It will be interesting to determine whether other antioxidants (e.g., glutathione and Cu/Zn superoxide dismutase) are similarly concentrated in this apical region.

Apical Zn may be derived from Zn reservoirs existing in a possible vesicular and perinuclear pattern. This was particularly evident in Zn-supplemented transformed cells when viewed at high magnification. In the primary epithelial cells, the perinuclear concentration of Zn was most pronounced on the apical side of the nucleus and is reminiscent of the distribution of Zn in secretory cells such as the Zn-rich pancreatic islets (37). Whether any of the apical Zn in the ciliated cells is contained within secretory granules and is destined for secretion into the pericellular fluid is not clear. Measurement of the Zn content in this fluid should be informative. The confocal studies also indicated a significant content of labile Zn within the cilia themselves. Zn is a well-known stabilizer of microtubules (26) and may have a similar role in the axonemes of the cilia.

We propose that the major function of apical labile Zn in the ciliated cells is cytoprotective and involves suppression of cell death by apoptosis and perhaps also necrosis. It is interesting to note that our observed distribution of labile Zn in these cells closely matches that of the major executioner enzyme in apoptosis, caspase-3, as detected by immunocytochemistry in human tracheobronchial epithelium (18). The close proximity of labile Zn, with procaspase-3 and the rapid activation of this enzyme after Zn depletion, raises the question of whether apical cytoplasmic Zn can act to suppress the induction of this caspase. A similar conclusion was reached by Aiuchi et al. (2) and Wolf and Eastman (34) in nonrespiratory cell lines. Zn is only a weak suppressor of active caspase-3 but a potent suppressor of caspase-6 at low micromolar concentrations (29) and possibly also of caspase-9 (34). However, both of these caspases contribute to the activation of caspase-3 (29, 30, 34). Caspase-6 was activated in our Zn-depleted respiratory epithelial cells, but there was no evidence that this event occurred before the activation of caspase-3. One implication of the relatively late (after 3 h) increase in caspase-6 activity is that this enzyme is also required during the effector phase where it cleaves lamin proteins, leading to nuclear collapse (30). Because it has been shown that caspase-3 and caspase-6 act synergistically to trigger morphological changes in apoptosis (30), the induction of both caspases in Zn-depleted cells is consistent with other models of apoptosis. Activated caspase-9, on the other hand, was undetected in our Zn-deprived cells. This could be due to an absence of this particular enzyme in primary sheep ciliated epithelial cells. These issues are currently being pursued with cell-free extracts.

Induction of high rates of apoptosis has previously been observed in the gastrointestinal epithelium of Zn-deficient animals (12). Furthermore, Zn deficiency rendered this tissue more susceptible to damage by toxins (9). Similar in vivo animal studies are required to answer the question of whether the respiratory epithelium is also affected in Zn deficiency. Our experiments in vitro with primary and malignant respiratory epithelial cells support the hypothesis that Zn deficiency renders the airway epithelium more susceptible to damage by apoptosis-inducing toxins. That this occurred with two different types of an apoptosis inducer is consistent with a generalized effect of Zn depletion on increased susceptibility to apoptosis. It is important to remember that once caspase-3 has been activated in a damaged cell, then the cell is committed to apoptosis regardless of the presence of Zn. However, Zn will suppress the death of those cells that have not yet had their caspase-3 enzymes activated.

Our findings suggest that Zn suppresses caspase activation in these cells and might have potential therapeutic implications in epithelial protection. Studies are now required to establish the intracellular labile Zn status of airway epithelial cells in vivo in healthy and asthmatic subjects and to correlate these levels with altered caspase activity and susceptibility to apoptosis. In conclusion, we have shown an unexpectedly high concentration of labile Zn lining the airway epithelium in pigs and sheep and propose that this Zn exerts a largely protective role against cell death induced by oxidants derived either from the lumen or by-products of oxidative metabolism.

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