Duodenal cytochrome b: a novel ferrireductase in airway epithelial cells

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Duodenal cytochrome b (Dcytb) reduces Fe3+ for cellular iron transport. A definite source of reducing equivalents for Dcytb was sought but not identified. We found no evidence for cellular iron transport. Dcytb expression was found in respiratory epithelium in vitro and in vivo and was responsive to iron concentration. Iron transport was measured in human bronchial epithelial (HBE) cells using inductively coupled plasma atomic emission spectroscopy and was demonstrated to be partially inhibited in the presence of Dcytb-blocking antibody, suggesting that Dcytb reduces Fe3+ for cellular iron transport. A definite source of reducing equivalents for Dcytb was sought but not identified. We found no evidence that ascorbate was involved but did demonstrate that O2−, possibly using ascorbate as an electron donor, which allows a divalent metal transporter, natural resistance-associated macrophage protein 2 (Nramp-2, also known as DCT1 and DMT1), to transport iron into the cell (20, 23). The expression of this ferric reductase is regulated by intracellular iron concentration and other facilitators of iron absorption, indicating that it responds to iron demand. Because many of the same proteins that regulate iron procurement and distribution in the gut also participate in iron transport in the lungs, we hypothesized that Dcytb was present in the lung to reduce Fe3+ for transport into the cell. The protein was found in airway epithelial cells, and its expression was determined to be regulated by iron concentration and thus likely contributes to the cytoprotective needs of the lungs.

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. Animal exposures. Sixty-day-old (250–300 g), male Sprague-Dawley rats were used for in vivo studies. The rats were housed in temperature- and humidity-controlled rooms. Food and water were available ad libitum. The animal protocol was approved by the Institutional Animal Care and Use Committee. We anesthetized the rats with 2–5% halothane (Aldrich Chemicals, Milwaukee, WI) and instilled the lungs with 0.5 ml of normal saline or ferric ammonium citrate (FAC, 100 μM). Twenty-four hours later, the animals were

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anesthetized and exsanguinated. The lungs were removed en bloc and inflation fixed with 10% formalin to evaluate Dcytb expression.

Measurement of Fe³⁺ reduction in vivo. Fe³⁺ reduction in the airway was measured directly in rats in vivo by ferricyanide formation. The animals were anesthetized and 10 ml of 1 mM Fe(III)NH₄(SO₄)₂ and 0.8 mM K₃Fe(CN)₆ instilled intratracheally. After 15 min, the lungs were lavaged three times with normal saline, removed, and frozen in optimum-cutting temperature compound. Sections were mounted and inspected under light microscopy. The reduction of Fe³⁺ was shown by the presence of a blue precipitate.

Immunohistochemistry. Lung sections were cut, mounted on silanetreated slides (Fisher, Raleigh, NC), and air-dried overnight. The slides were heat-fixed at 60°C in a slide dryer (Shandon Lipshaw, Pittsburgh, PA) for 10 min. Sections were deparaffinized and hydrated to 95% alcohol (xylene for 10 min, absolute alcohol for 5 min, and 95% alcohol for 5 min). Endogenous peroxidase activity was blocked with 0.6% H₂O₂ in methanol for 8 min. Slides were rinsed in 95% alcohol (xylene for 10 min, absolute alcohol for 5 min, and 100% alcohol for 5 min). Sections were deparaffinized and hydrated to 95% alcohol (xylene for 10 min, absolute alcohol for 5 min, and 95% alcohol for 5 min). Endogenous peroxidase activity was blocked with 0.6% H₂O₂ in methanol for 8 min. Slides were rinsed in 95% alcohol for 2 min, placed in deionized H₂O, and washed in PBS. After treatment with Cyto Q Background Buster (Innovex Biosciences) for 10 min, slides were incubated with Dcytb primary antibody diluted in 1% BSA (1:100; ADI, San Antonio, TX) for 45 min at 37°C in PBS. Slides were incubated with biotinylated linking antibody from Stat-Q Staining System (Innovex Biosciences) for 10 min and washed with PBS, and peroxidase enzyme label was applied from the Stat-Q Staining System (Innovex Biosciences) for 10 min at room temperature. After being washed with PBS, tissue sections were treated with 3,3'-diaminobenzidine tetrahydrochloride for 3 min. Sections were counterstained with hematoxylin, dehydrated through alcohols, cleared in xylene, and permanently cover slipped.

Human lung tissue was procured during autopsies in the Durham Veterans Administration Medical Center and Duke University Medical Center under a discard protocol approved by the Duke University Institutional Review Board. Sections were prepared and stained as above for Dcytb.

Cell harvest and culture. Normal human bronchial epithelial (HBE) cells were obtained from healthy, nonsmoking adult volunteers. The protocol and consent form was approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Cells were obtained by cytologic brushing at bronchoscopy and were seeded (passage 2–4) on tissue culture-treated plates in bronchial epithelial growth medium (BEGM) supplemented with 0.5 ng/ml EGF, 5 mg/ml insulin, 0.5 ng/ml triiodothyronine, and 0.1 ng/ml retinoic acid. The purity of the airway epithelial cells was ensured by the use of specific media and by inspection of cell morphology.

Analysis of Dcytb mRNA expression by real-time quantitative PCR. Confluent HBE cells were exposed to BEGM media or to a single application of FAC (500 μM) and harvested after 3, 6, or 24 h. A second set of cells was exposed to variable concentrations of FAC (0, 125, 250, 500 μM) and harvested after 6 h to obtain a dose-response curve. RNA was prepared by lysing cells in buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 10 mM DTT. After being sheared through a 22-gauge needle, the lysate was layered over an equal volume of 5.7 M CsCl, and total RNA was pelleted by centrifugation for 2 h at 80,000 revolutions/min (rpm). Reverse transcription and DNA amplification were performed as described (8). Dcytb primer pair and fluorescent probe were designed and produced by Applied Biosystems. They contain a partial sequence of 5'-TTAACTGCACCCAGTGCATGGT-3' (accession no. NM_024843) with the amplified products crossing exons 1–2. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) primer pair and fluorescent probe were designed using a primer design program (accession no. M33197) and were obtained from Integrated DNA Technologies (Coralville, IA). The following sequences were used: GAPDH sense 5'-GAAGGTGAAGGTCGACT-3' and GAPDH antisense 5'-GAAGATGGTGATGGCAG-3'; GAPDH probe 5'-CAAGCTCTCCCGTTCTCAGGCC-3' exon 3; and GAPDH probe 5'-CAAGCTCTCCCGTTCTCAGGCC-3' exon 3.

Relative quantification of Dcytb gene expression by real-time PCR was performed by fluorogenic amplification of cDNA using the ABI Fig. 1. Ferrireductase activity is present and duodenal cytochrome b (Dcytb) is expressed in airway epithelial cells. Adult Sprague-Dawley rats were instilled intratracheally with 1 mM Fe(III)NH₄(SO₄)₂ and 0.8 mM K₃Fe(CN)₆ to measure Fe³⁺ reduction directly. After 15 min, the lungs were lavaged, removed, and frozen in optimum-cutting temperature compound. Sections were mounted and examined under light microscopy. A blue precipitate was formed by the reduction of Fe³⁺ in the presence of cyanide (A). Rat lung tissue was examined by immunohistochemistry after intratracheal instillation with normal saline (B; 0.5 ml) or ferric ammonium citrate (FAC; 100 μM in 0.5 ml; C). Slides were labeled with a primary antibody to Dcytb (1:100), developed with 3,3'-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin. B and C are shown at magnification of approximately ×100.
Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA), TaqMan Universal PCR Master Mix (Applied Biosystems), and the indicated primers and probes. Relative quantification of Dcytb and GAPDH mRNA was based on standard curves prepared from serially diluted cDNA from HBE cells. The abundance of Dcytb mRNA in each sample was standardized against that of GAPDH.

Cell analysis of Dcytb protein expression by Western blot analysis. HBE cells were exposed to FAC (0, 125, 250, or 500 μM) in BEGM media for 24 h. After exposure, the cells were washed with ice-cold PBS and lysed with 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS-containing protease inhibitors [Cocktail Set III: 100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin A; Calbiochem, La Jolla, CA]. Cells were harvested and sheared through a 22-gauge needle, and cell debris was pelleted by centrifugation at 500 g for 5 min. The supernatant was removed. Protein content of the lysate was determined using the Bradford assay (Bio-Rad, Hercules, CA). The sample was mixed with 4X sample loading buffer (0.5 M Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, and 0.05% bromophenol blue).

Equal quantities of cell lysate protein (100 μg) were separated by SDS-PAGE (12%) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 3% milk in PBS-Tween 20 for 1 h followed by immunoblotting overnight at 4°C using Dcytb antibody (1:100, ADI). The sample loading in Western blots. Bands were quantified using GeneTools (Syngene, Frederick, MD).

Peptide inhibition was performed to verify the specificity of the Dcytb antibody. Briefly, the antibody was incubated overnight in the presence or absence of the Dcytb peptide (ADI). The samples were then centrifuged at 15,000 rpm, and the supernatant was diluted in 5% milk and used to immunoblot as above.

Measurement of total cellular nonheme iron concentrations. HBE cells were grown to 90–100% confluence on plastic 12-well plates in 1.0 ml BEGM media. Cells were treated with a Dcytb blocking antibody or a nonspecific IgG1 antibody as negative control (1:50) for 15 min, exposed to FAC (100 μM) in HBSS, and incubated for 1 or 4 h. HBSS containing the metal was removed, the cells were washed with 1.0 ml of HBSS, and 1.0 ml of 3 N HCl and 10% TCA were added. The cells were scraped in the acid, hydrolyzed at 70°C for 18 h, and centrifuged at 600 g for 10 min, and the iron concentration of the supernatant was determined using inductively coupled plasma atomic emission spectroscopy (ICP AES) at a wavelength of 238.204 (model P30; Perkin-Elmer, Norwalk, CT). Single element standards were used to calibrate the instrument (Fisher, Pittsburgh, PA). The limit of detection was ~10 parts/billion.

To evaluate the role of O2•− in the reduction of Fe3+, HBE cells were pretreated with HBSS or HBSS and one of the following: superoxide dismutase (SOD, 0–200 U/ml), capsaicin (0–100 μM), cimetidine (0–100 μM), diphenylene iodonium (DPI, 0–100 μM), or allopurinol (0–100 μM). The cells were then exposed to FAC (100 μM) for 1 h, and iron concentration was measured by inductively coupled plasma atomic emission spectroscopy (ICP AES). Additional sets of cells were pretreated with KCN (10 μM), rotenone (10 μM), oligomycin (10 μM), antimycin (1 μM), bafilomycin (0.1 μM), or carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1–10 μM). Cells were exposed to FAC (100 μM) for 4 h, and iron concentration was measured by ICP AES.

Ascorbate measurements. To investigate the role of ascorbate in Fe3+ reduction, HBE cells were pretreated with ascorbate or 4-hydroxy-2,2,6,6-piperidinolxy, free radical (TEMPOL) to deplete cellular ascorbate content (22, 31). Cells were incubated with either ascorbate (0, 125, 250, or 500 μM) in media for 1 h or with TEMPOL (1 mM, dissolved in 0.4% DMSO) in PBS for 5 min at 37°C. The cells

Fig. 2. Dcytb expression is altered in inflammatory lung disease. Alterations in Dcytb expression in disease were evaluated by immunohistochemistry. Human lung tissue was collected at autopsy from patients with normal lungs (A) and with chronic bronchitis (B), pulmonary fibrosis (C), and emphysema (D). Slides were labeled with a primary antibody to Dcytb (1:100), developed with 3,3′-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin. A–D shown at approximately ×400 magnification.
were assayed for ascorbate using HPLC (Waters RCM, which is visualized by light microscopy (Fig. 1)). The mucociliary epithelium over 2 wk, as previously described (29). The cells were differentiated in a plates to 80% confluence. The air-liquid interface was created by grown on collagen-coated filter supports inserted in 12-well culture plates. The cells were treated with trypsin, and a rapid acid extraction was performed using cold HClO4. The insoluble material was centrifuged, the supernatant was removed, and the cells were scraped into 35 µl 60% perchloric acid/ml and centrifuged at 20,000 g for 30 min at 4°C. The supernatant was stored at −80°C until assayed for ascorbate using HPLC (Waters RCM, Bondapak C18 column; Millipore, Marlborough, MA) with electrochemical detection (BAS model LC-4B; Bioanalytical Systems, W. Lafayette, IN; see Ref. 18).

ATP measurements. ATP content of HBE cells was measured using a commercially available kit (Molecular Probes, Carlsbad, CA). Before assay, cells were treated with trypsin, and a rapid acid extraction was performed using cold HClO4. The insoluble material was centrifuged, the supernatant was removed, and 2 M KHCO3 was added to neutralize the acid. Samples were stored at −80°C until ready to use. Measurements of extracellular O2− generation. HBE cells were grown on collagen-coated filter supports inserted in 12-well culture plates to 80% confluence. The air-liquid interface was created by removing the apical medium, and the cells were differentiated in a mucociliary epithelium over 2 wk, as previously described (29). The cells were treated at the apical surface with the Dcytb blocking antibody or a non-specific IgG1 antibody as a negative control (1:50) for 30 min. Ferricytochrome c (25 µl of 100 M solution; Sigma) was then added to the apical surface, and cells were incubated for 1 h. The absorption was measured at both 550 and 540 nm (model DU 640B spectrophotometer; Beckman).

In separate experiments, extracellular H2O2 generation was measured using Amplex Red detection as a surrogate for O2−. BEAS-2B cells, an SV40-immortalized bronchoepithelial cell line, were grown to 80% confluence as described (11). The cells were infected with AdSOD1 (34) for 4 h at multiples of 100 plaque-forming units/cell. Control cells were incubated with Ad5CMV3 (16). The cells were incubated for an additional 24 h and were then treated with the Dcytb blocking antibody or IgG1 (1:50). After 30 min, the cells were washed with HBSS and treated with Amplex Red (50 µM, Molecular Probes, Eugene, OR) and horseradish peroxidase (10 U/ml). The reaction was incubated for 60 min, and fluorescence was measured by a microplate reader at excitation 535 nm and emission 590 nm, indicating the reaction of H2O2 with Amplex Red.

Statistical analysis. Data are expressed as means ± SE. A minimum of three separate experiments was performed for each measurement. Data were compared using one-way ANOVA followed by the Fisher’s protected least square difference test. Significance was assumed at P ≤ 0.05.

RESULTS

Ferrireductase activity in the lung. Ferric reduction is required for most cellular iron uptake and has been demonstrated in vitro in airway epithelial cells. Ferrireductase activity was assessed in the lungs of rats after in vivo tracheal instillation of FAC and K3Fe(CN)6. Fe3+ reduction forms a blue precipitate, which is visualized by light microscopy (Fig. 1A). Precipitation of reduced iron was evident throughout the epithelium of the lung, with prominence along the airway epithelium.

Dcytb is expressed in the lung. To investigate whether Dcytb is expressed in the airway, immunohistochemistry was performed on lung tissue from adult Sprague-Dawley rats using an antibody against Dcytb. Control lungs demonstrated Dcytb labeling predominantly in the airway epithelium, with minimal expression along alveoli and vessels (Fig. 1B), reflecting the distribution of ferric reduction. Lung sections from rats instilled with FAC showed markedly increased staining for Dcytb along the airway, with enhanced staining of the alveolar epithelium and the vasculature (Fig. 1C). To determine whether Dcytb is expressed in human lung, immunohistochemistry was performed on normal lung and on lung sections obtained at autopsy from patients with inflammatory lung disease. Labeling by Dcytb antibody was seen primarily along the airway epithelium and in macrophages in normal lung tissue (Fig. 2A), with a marked extension of this distribution in the alveoli and vasculature in disease (Fig. 2B-D) similar to the pattern of distribution observed in rat lungs exposed to iron.

Regulation of Dcytb expression in the lung by iron concentration. Because certain lung diseases are associated with reactive iron in the lung, we assessed the effect of iron concentration on Dcytb expression in airway epithelial cells. Changes in Dcytb mRNA levels were measured in HBE cells stimulated with FAC using Quantitative real-time PCR, normalizing the Dcytb fluorescence to that of GAPDH. Constitutive expression of Dcytb mRNA increased modestly 3 and 6 h after exposure to FAC (500 µM) and returned toward baseline after 24 h (Fig. 3).

Fig. 3. Increased Dcytb gene expression after exposure to iron. Human bronchial epithelial (HBE) cells were exposed to FAC (500 µM) in bronchial epithelial growth medium (BEGM) for 0, 3, 6, or 24 h (A) or to FAC (0, 125, 250, or 500 µM) for 6 h (B). Total RNA was isolated and reversed transcribed. Quantitative PCR was performed using TaqMan polymerase. Fluorescence was detected on an ABI Prism 7700 sequence detector (Applied Biosystems). Relative abundance of Dcytb mRNA was normalized to that of gyceraldehyde-3-phosphate dehydrogenase (GAPDH; n = 3). Ctrl, control. *P < 0.05 compared with control.
Dcytb antibody significantly inhibited iron transport in HBE cells (Fig. 4C), whereas an irrelevant antibody (IgG1) at the same concentration produced no effect (data not shown), indicating that Dcytb participates in iron uptake in the lungs.

Dcytb is involved in iron transport. To assess whether Dcytb plays a role in iron transport in the lung, its function was inhibited using a Dcytb blocking antibody (23). The specific

FAC for 24 h. Western blot analysis demonstrated a dominant band of a molecular mass of ~33 kDa, consistent with the size of Dcytb (Fig 4A and Ref. 17) and inhibited in the presence of a Dcytb peptide (data not shown). Dcytb protein expression increased significantly in HBE cells after exposure to FAC (Fig. 4B).

**Fig. 4.** Dcytb protein expression is regulated by iron concentration and is involved in iron transport. HBE cells were exposed to FAC (0, 125, 250, or 500 μM) in BEGM media for 24 h. Whole cell lysates (100 μM) were separated by SDS-PAGE (12%) followed by immunoblotting using Dcytb antibody (1:500; ADI). Representative Western blot from at least 3 separate experiments is shown (A). The bands reflect a 33-kDa protein, consistent with the size of Dcytb (17). Densitometric measurements are graphed in relation to bands exposed to media alone (B). To evaluate whether Dcytb is involved in iron transport in the airway, HBE cells were treated with Dcytb blocking antibody (1:50) and compared with a nonspecific IgG1 antibody as a negative control (C). FAC (100 μM) was added to the media, and the cells were incubated for 1 (open bars) or 4 (filled bars) h. The cells were hydrolyzed in 3 N HCl and 10% TCA for 18 h and centrifuged. The total cellular iron content was measured using inductively coupled plasma atomic emission spectroscopy (ICPAES; λ 238.204). *P < 0.05 when compared with control.

**Fig. 5.** Iron transport is unchanged by alterations in cellular ascorbate levels. HBE cells were incubated with varying concentrations of ascorbate for 1 h. After 4 (open bars) or 24 (filled bars) h, the medium was removed, and the cells were scraped into 35 μl 60% perchloric acid/ml and centrifuged. The supernatant was assayed for ascorbate using HPLC with electrochemical detection (A). A second set of cells were pretreated with exogenous ascorbate as above and then exposed to FAC (100 μM) for 4 h. Total cellular iron content was measured using inductively coupled plasma atomic emission spectroscopy (ICPAES, λ 238.204; B). A third set of cells was incubated with 4-hydroxy-2,2,6,6-piperidinyloxy, free radical (TEMPOL; 1 mM dissolved in 0.4% DMSO) for 5 min at 37°C. The TEMPOL was removed, and FAC (100 μM) was added. Total cellular iron concentration was measured after 1 (open bars) and 4 (filled bars) h (C). *P < 0.05 compared with control.
Sources of reducing equivalents for reduction of Fe$^{3+}$ by Dcytb. Dcytb is a member of the cytochrome $b_{561}$ family and, as such, has putative ascorbate and dehydroascorbate binding sites. Furthermore, ascorbate-dependent iron reductase activity has been demonstrated by a homolog of Dcytb in brush-border membranes (17). To investigate whether ascorbate mediates Fe$^{3+}$ reduction in the airway, cellular ascorbate levels were measured by HPLC and found to be undetectable in unstimulated control HBE cells (Fig. 5A). When these cells were supplemented with ascorbate, intracellular ascorbate concentration increased significantly after both 4 and 24 h, although levels were lower after 24 h presumably because of loss of ascorbate through utilization or oxidation. Although we elevated cellular ascorbate concentration with supplementation, the rate of iron transport did not change (Fig. 5B). HBE cells were then treated with TEMPOL to deplete intracellular ascorbate completely. Ascorbate levels remained undetectable after TEMPOL (data not shown), and iron transport was not affected (Fig. 5C). Inability to modulate iron transport by alterations in ascorbate concentration implies that Fe$^{3+}$ in airway epithelium is not reduced by ascorbate.

O$_2^*$ reduces Fe$^{3+}$ and contributes to iron transport (7, 10); therefore, to investigate the role of O$_2^*$ in iron reduction by Dcytb, we measured changes in cellular iron in the presence of inhibitors of several sources of O$_2^*$. Extracellular SOD and inhibitors of O$_2^*$ generation, cimetidine (19) and capsaicin (9), significantly decreased transcellular iron transport (Fig. 6, A and B) but did not alter Dcytb protein expression after a 24-h exposure (data not shown). Iron transport was not affected, however, in cells treated with DPI or allopurinol, inhibitors of NAD(P)H oxidoreductase and xanthine oxidase, respectively (Fig. 6C).

The mitochondrial electron transport chain can be a source of O$_2^*$ in the cell; therefore, the effect of inhibitors of respiration and oxidative phosphorylation was investigated on iron transport in HBE cells. Inhibitors of electron transport, as well as FCCP and oligomycin, decreased iron transport (Fig. 6D). Treatment of HBE cells with FCCP and oligomycin decreased
ATP production (Fig. 6E) but did not interfere directly with electron transport. This suggested that electron transport does not promote iron transport by producing \( \text{O}_2^- \) but by providing energy for cellular iron uptake.

To determine whether \( \text{O}_2^- \) involved in \( \text{Fe}^{3+} \) reduction is generated by Dcytb, cellular oxidant release was measured in the presence and absence of the Dcytb blocking antibody using a ferricytochrome \( c \) assay for \( \text{O}_2^- \) and Amplex Red for \( \text{H}_2\text{O}_2 \) (33). The reduction of ferricytochrome \( c \) was evaluated in HBE cells grown and differentiated at the air-liquid interface to model the native epithelium. When Dcytb function was inhibited in these cells, the reduction of ferricytochrome \( c \) decreased significantly, consistent with decreased production of \( \text{O}_2^- \) (Fig 7A). Because \( \text{O}_2^- \) undergoes such rapid dismutation to \( \text{H}_2\text{O}_2 \), extracellular \( \text{H}_2\text{O}_2 \) release was evaluated using Amplex Red in BEAS-2B cells infected with an AdSOD1 vector to overexpress Cu,Zn-SOD. When Dcytb function was inhibited, extracellular \( \text{H}_2\text{O}_2 \) production increased both in control cells infected with Ad5CMV3 vector and in cells overexpressing Cu,Zn-SOD (Fig. 7B). This increased oxidant leakage likely represents increased generation of both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), rather than just \( \text{O}_2^- \) detection (as by ferricytochrome \( c \)), presumably to compensate for decreased \( \text{O}_2^- \) production by Dcytb. Conversely, this may indicate decreased production of other reductants. As with cimetidine and capsaicin (inhibitors of \( \text{O}_2^- \) generation), Dcytb protein expression did not change in cells overexpressing Cu,Zn-SOD (data not shown).

**DISCUSSION**

Iron is removed from the respiratory epithelial surface by specialized systems to reduce and transport iron intracellularly (30). NTBI transport, one such system, requires \( \text{Fe}^{3+} \) reduction before its uptake across the cell membrane by the metal transporter Nramp-2. This report newly demonstrates the lung’s strong ferrireductase activity in association with Dcytb, a ferrireductase normally found in the intestine. In brief, Dcytb is also present in respiratory epithelium, responds to changes in local iron concentration, and is shown to be involved in iron uptake in airway epithelial cells. The \( \text{O}_2^- \) anion is one of the reductants, but other source(s) of reducing equivalents have not been excluded.

The importance of ferric reduction in iron transport is well described, but, in the lung, specific reducing agents for \( \text{Fe}^{3+} \) have been elusive. The airway ferrireductase activity demonstrated here indicates that the lower respiratory tract reduces \( \text{Fe}^{3+} \) before the metal is taken up by airway epithelial cells. Dcytb, an important intestinal ferrireductase, is also present in airway epithelial cells, and the inhibition of intracellular iron transport by Dcytb blocking antibody implicates it in iron transport in the lung.

Dcytb gene and protein in airway epithelial cells respond to exposure to high iron concentrations in vitro and in vivo. Here we used iron concentrations comparable to the highest levels measured in bronchoalveolar lavage fluid in patients with inflammatory lung disease (25), and the Dcytb ferrireductase response suggests that it serves to prevent iron accumulation in the airways by allowing it to be sequestered in cells. The change in gene expression is statistically significant, but it is not robust, suggesting that transcriptional regulation of Dcytb does not fully explain the change in Dcytb protein expression. Dcytb lacks a definable iron-responsive element (IRE) common in the 5’- or 3’-untranslated regions of iron-regulated genes. Therefore, further study will be required to assess the presence of binding sites for iron-responsive transcription factors in the promoter region or posttranscriptional modification of gene expression, including RNA stability.

It now seems apparent that iron concentration regulates Dcytb expression in both the gut and the lungs, but excess iron upregulates Dcytb protein expression in the lung and downregulates it in the gut (12). This suggests that different aspects of iron transport regulate Dcytb in different tissues. A similar situation is found in the regulation of the metal transporter Nramp-2. In the gut, the predominant Nramp-2 isoform contains a 5’-IRE, and its expression decreases in the presence of excess iron, whereas the lung Nramp-2 isoform lacks the IRE(−). Its expression increases in the presence of elevated iron, allowing increased iron transport in the cell for protection in ferritin. The regulation of Dcytb by iron parallels that of the IRE(−) Nramp-2 and is consistent with the proposal that Dcytb reduces \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \) for transport through Nramp-2 in airway epithelial cells.
Dcytb is an integral membrane protein of the b561 family of cytochromes that contain putative ascorbate and dehydroascorbate binding sites (23). However, we could neither demonstrate ascorbate dependence of iron uptake in HBE cells nor detect ascorbate in unstimulated HBE cells at a detection limit of 0.15–0.3 μM. We also found no increase in iron transport with ascorbate supplementation, which implies that lung Dcytb does not use ascorbate to reduce Fe3+. Few measurements of ascorbate concentrations are available in airway epithelial cells, although ascorbate is present in substantial amounts in alveolar lining fluid (3) and in alveolar type II cells in guinea pigs (6). These findings do not necessarily conflict with our HBE cell data, since ascorbate’s behavior may differ in vivo and in vitro among cell types. Brown et al. (6) commented on the relatively high concentration of ascorbate in type II cells despite limited availability of exogenous ascorbate, suggesting that type II cells may concentrate ascorbate to enhance their resistance to oxidative stress. We used primary HBE cells and did not supplement with ascorbate, but the lack of effect of ascorbate addition on iron transport suggests that it does not modulate iron reduction in these cells. This may reflect different iron transport needs in different systems, e.g., iron transport in the lung is protective, whereas, in the gut, it is nutritional.

It is known that O2− reduces Fe3+ in airway epithelial cells (15), which raises the possibility that Dcytb produces the O2− for ferrireduction. Here we found that inhibitors of O2− generation, cimetidine and capsaicin, decreased iron transport in airway epithelial cells. In contrast, inhibitors of NAD(P)H oxidoreductase and xanthine oxidase had no effect on Fe3+ reduction and transport in HBE cells. This suggests that multiple sources of O2− exist in the lung. When Dcytb function was inhibited with blocking antibody, we found a 50% decrease in ferricytochrome c reduction, indicating a decrease in the production of an extracellular reducing agent, such as O2−. However, the compensatory increase in Amplex red measured in both control cells and those overexpressing Cu,Zn-SOD suggests increased production of oxidants. Amplex red measures H2O2 produced by the dismutation of O2− and H2O2 produced by peroxidases and other sources. A simple explanation is that Dcytb serves as a source of O2− or other reducing equivalents for ferric reduction in the lung, but, when inhibited, alternative sources of O2− and other oxidants will compensate. Although O2− was inhibited by cimetidine, capsaicin, and overexpression of Cu,Zn-SOD, these agents did not alter Dcytb expression, suggesting that decreased reductant availability rather than protein regulation is responsible for decreased iron transport in airway epithelial cells. Further investigation is needed to determine the sources of O2− production for Fe3+ reduction and to establish whether Dcytb does generate O2− in the lung.

The mitochondrial electron transport chain has been implicated in the reduction and intracellular transport of Fe3+ in bacteria (32). Our data demonstrate that multiple inhibitors of the electron transport chain decrease iron transport in HBE cells. In addition, FCCP and oligomycin, which block oxidative phosphorylation and ATP production, also block iron transport. Therefore, the decrease in iron transport after compromise of mitochondrial function is likely because of loss of energy production. Although the electron transport chain produces O2−, the diffusivity of O2− is limited by its reactivity; thus, mitochondria probably do not provide reducing equivalents for ferric reduction at the cell membrane.

In conclusion, we have identified a novel ferrireductase activity in the lung that depends on Dcytb. We have shown that Dcytb is expressed in airway epithelium and responds positively to iron concentration. The importance of Dcytb relative to other sources of ferrireductase activity in the lung will require further study, as will the role of O2− in Fe3+ reduction and iron transport and the exact source(s) of reducing equivalents for Dcytb. Although we blocked iron transport partially with a range of inhibitor types, no single inhibitor impeded iron transport completely. This implies redundancy in the lung’s iron transport capacity that protects it from excess iron and the generation of oxidative stress. This redundancy appears both in the sources of reducing equivalents and in the presence of multiple pathways to take up and package iron (30). The ability to regulate Dcytb expression in response to iron concentration is likely lung protective because Dcytb expression is high in inflammatory lung diseases associated with disordered iron metabolism. Whether this represents a defensive response to shield the epithelium from excess iron or whether it is part of the general defense against oxidative stress remains to be determined.

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