Respiratory epithelial cells demonstrate lactoferrin receptors that increase after metal exposure

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Ghio, Andrew J., Jacqueline D. Carter, Lisa A. Dailey, Robert B. Devlin, and James M. Samet. Respiratory epithelial cells demonstrate lactoferrin receptors that increase after metal exposure. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L933–L940, 1999.—Human airway epithelial cells can express iron in a less reactive form than in the normal healthy state (21). However, during an inflammatory state, a recognized function of lactoferrin is the transport of iron across cell membranes to be deposited in ferritin of monocytes and macrophages (2, 29). In response to catalytically active metal, cultured respiratory epithelial cells can also increase the production of lactoferrin and ferritin to transport and store iron. Lactoferrin rarely provides metal for the nutritional requirements of the cell, except in the neonate (10), and the function of lactoferrin in iron transport is assumed to be negligible in the normal healthy state (21). However, during an inflammatory state, a recognized function of lactoferrin is the transport of iron across cell membranes to be deposited in the ferritin of monocytes and macrophages (2, 29). In response to catalytically active metal, cultured respiratory epithelial cells can also increase the production of lactoferrin and ferritin to transport and store this metal, with coordination sites fully complexed (6).

Both transferrin and lactoferrin are bound by specific receptors and internalized. The binding site for lactoferrin is different from that for the transferrin receptor, and the two ligands do not cross-react with the receptors (25). For lactoferrin to function in a manner to diminish oxidative stress, lactoferrin receptors (LfRs) on airway epithelial cells. We tested the hypotheses that 1) LfRs exist on respiratory epithelial cells and 2) exposure to both an air pollution particle, which has abundant concentrations of metals, and individual metal salts increase the expression of LfRs. Before exposure to either the particle or metals, incubation of BEAS-2B cells with varying concentrations of 125I-labeled lactoferrin demonstrated lactoferrin binding that was saturable. Measurement of 125I-lactoferrin binding after the inclusion of 100 μg/ml of oil fly ash in the incubation medium demonstrated increased binding within 5 min of exposure, which reached a maximal value at 45 min. Inclusion of 1.0 mM deferoxamine in the incubation of BEAS-2B cells with 100 μg/ml of oil fly ash decreased lactoferrin binding. Comparable to the particle, exposure of BEAS-2B cells to either 1.0 mM vanadyl sulfate or 1.0 mM iron (III) sulfate, but not to nickel sulfate, for 45 min elevated LfR activity. We conclude that LfRs on respiratory epithelial cells increased after exposure to metal. LfRs could participate in decreasing the oxidative stress presented to the lower respiratory tract by complexing catalytically active metals.

THE ATMOSPHERE CONSTITUTES a prime vehicle for the movement and redistribution of metals (27). Human activities have had a major impact on both global and regional cycles of these metals (22). As a result, there has been a significant contamination of air with their accumulation. Almost all metals in the atmosphere are associated with particles (27).

Metals are essential micronutrients utilized in almost every aspect of normal cell function. Although all living systems depend on metals to catalyze homoeostatic and synthetic functions, O2, H2O2, and -OH generated by these metals have a capacity to damage biological molecules. Consequently, metals must be transported and stored, with all coordination sites fully complexed to prevent formation of these damaging reactive oxygen species.

As a result of a potential oxidative injury, it would be of benefit to the host to sequester available metals. Such intracellular sequestration of iron by macrophages can limit its potential to generate free radicals and prevent cellular injury resulting from its exposure (23). The storage of iron in a chemically less reactive form within intracellular ferritin confers an antioxidant function to this protein and, in certain cells, provides cytoprotection in vitro against oxidants (1).

Although numerous metal chelates that present an oxidative stress to the cell are extracellular, ferritin is produced intracellularly. Therefore, sequestration and detoxification by ferritin requires that the metal be transported across the membrane. To transfer iron across a membrane, animal cells most frequently use the glycoprotein transferrin (28). However, with exposure to iron chelates, many cell types rapidly downregulate transferrin-receptor expression (11). Consequently, transferrin-dependent transport of iron across the membrane is unlikely to contribute to the sequestration of metal and the antioxidative function of ferritin.

Lactoferrin is a monomeric, cationic glycoprotein (molecular mass of 76.4 kDa) synthesized by myeloid cells and secretory epithelia. This single-chain, bilobed protein reversibly binds two ferric ions concomitantly with two carbonic ions. It is found in all human mucosal secretions (e.g., milk, tears, semen, and plasma) and in the specific granules of polymorphonuclear leukocytes. Lactoferrin rarely provides metal for the nutritional requirements of the cell, except in the neonate (10), and the function of lactoferrin in iron transport is assumed to be negligible in the normal healthy state (21). However, during an inflammatory state, a recognized function of lactoferrin is the transport of iron across cell membranes to be deposited in the ferritin of monocytes and macrophages (2, 29). In response to catalytically active metal, cultured respiratory epithelial cells can also increase the production of lactoferrin after exposure to catalytically active metal. LfRs exist on respiratory epithelial cells and internalize lactoferrin, providing cytoprotection in vitro against oxidants (1).
age in ferritin. We tested the hypothesis that LFRs exist on respiratory epithelial cells and increase with exposure to both an air pollution particle, which has abundant concentrations of metals, and individual metal salts. In this manner, LFRs could participate in decreasing the oxidative stress presented to the lower respiratory tract by catalytically active metal.

METHODS

Materials. The respiratory epithelial cells used in all studies were BEAS-2B cells (S6 subclone). These were obtained from Drs. Curtis Harris and John Lechner (National Institutes of Health, Bethesda, MD). This is an immortalized line of normal human bronchial epithelium derived by transfection of primary cells with SV40 early-region genes. This particular subclone undergoes squamous differentiation in response to serum. BEAS-2B cells increase lactoferrin expression after exposure to both a metal-rich particle and metal compounds comparable to primary respiratory epithelial cells (6).

BEAS-2B cells were exposed to an emission source air pollution particle. The particle employed in all studies was an oil fly ash with an average mass median aerodynamic diameter from four replicate samples of 3.60 ± 0.80 µm. This particle was collected by Southern Research Institute (Birmingham, AL) with a Teflon-coated fiberglass filter downstream from the cyclone of a power plant in Florida that was burning a low-sulfur no. 6 residual oil (collection temperature of 250–300°C). It has been chemically characterized previously and demonstrated to have high concentrations of iron, vanadium, and nickel (9). An association between exposure to a pollution particle and an oxidative stress has also been confirmed both previously and demonstrated to have high concentrations of iron, vanadium, and nickel (9).

Lactoferrin binding experiments. BEAS-2B cells were grown to 90–100% confluence in six-well tissue culture plates (Costar). The cultures were exposed to either medium or 100 µg/ml of particle for 45 min. The plates were then placed on ice, and the wells were scraped with cell scrapers (Costar) in the presence of 100 µl of lysis buffer containing 0.1% SDS, 1.0% Nonidet P-40, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml of apronin, 5 µg/ml of leupeptin, 2 mM EGTA, 1 mM vanadyl sulfate, and 1 mM sodium fluoride. The cell lysates were washed two times between a 22-gauge syringe and spun softly at 800 g for 5 min at 4°C. Supernatants (cytosolic fraction) were kept on ice and diluted 1:40 in phosphate-buffered-saline (PBS) for subsequent protein determination (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). Protein at concentrations of 10, 2.5, 0.5, and 0.1 µg was vacuum slot-blotted onto 0.45-µm nitrocellulose (Schleicher & Schuell, Keene, NH) in PBS. The blot was briefly air-dried and blocked with 10 mM Tris-0.15 M sodium chloride, pH 7.4, 10 mM calcium chloride, and 5% serotransferrin (Boehringer Mannheim) for 3 h at 4°C. The blot was then transferred to fresh blocking solution containing 100 µM 125I-lactoferrin and incubated overnight at 4°C on a rotating platform. The next day, the blot was washed two times with 10 mM Tris-0.15 M sodium chloride, pH 7.4 (washing buffer) for 10 min each, two times with washing buffer containing 0.05% Nonidet P-40 for 15 min each, and one time briefly in plain washing buffer. Radioactivity bound to each slot was determined by a Molecular Dynamics phosphorimager.

Statistics. Data are expressed as means ± SE. Measurements were made in duplicate and repeated at least once. Differences between multiple groups were compared with one-way analysis of variance (4). The post hoc test employed was Scheffé’s test. Two-tailed tests of significance were employed. Significance was assumed at P < 0.05.

RESULTS

Incubation of BEAS-2B cells with either the oil fly ash (100 µg/ml) or metal sulfates (1.0 mM) for 24 h was not associated with any significant change in cell viability as assessed by lactate dehydrogenase release relative to incubations with medium alone (18.2 ± 4.7 units). Similarly, there were no changes in the morphology of the BEAS-2B cells 24 h after exposure to either the oil fly ash or individual metal sulfates.

Incubation of BEAS-2B cells with varying concentrations of 125I-lactoferrin for 24 h demonstrated lactoferrin binding that appeared to be saturable (Fig. 1). After exposure to 100 µg/ml of oil fly ash for 24 h, binding of the lactoferrin increased approximately two- to sixfold (Fig. 1). Based on this response of the BEAS-2B cells to
these incubations with $^{125}$I-lactoferrin, a dose of 100 nM of the labeled glycoprotein was selected for further studies.

Initial time-response studies revealed a rapid increase in lactoferrin binding within 1 h after exposure to 100 µg/ml of the particle (Fig. 2A). For the 24 h of the study, there was little variance in the binding of lactoferrin by those cells incubated with medium only. Further measurements of LfR activity within the first hour after the inclusion of 100 µg/ml of oil fly ash in the incubation medium demonstrated statistically increased binding within 5 min of the exposure, which reached a maximal value at 45 min (Fig. 2B).

Previous studies (5, 16) suggested differential binding of hololactoferrin relative to that for apolactoferrin. Therefore, the specificity of LfR activity of the BEAS-2B cells was measured with inclusion of both iron-free and iron-saturated lactoferrin in the medium. Twelve-well plates of BEAS-2B cells were exposed to either the medium or the particle (100 µg/ml). Incubations with $^{125}$I-lactoferrin included wells with 10 µM either apolactoferrin or hololactoferrin. At both 45 min and 24 h, $^{125}$I-lactoferrin counts were again elevated with an increasing concentration of $^{125}$I-lactoferrin included in the medium (Fig. 3). Comparable to the previous incubations, exposure to 100 µg/ml of particle increased $^{125}$I-lactoferrin counts approximately two- to sixfold (Fig. 3). In contrast, no differences in lactoferrin binding could be demonstrated with either apolactoferrin or

Fig. 1. Dose response of $^{125}$I-labeled lactoferrin binding by BEAS-2B cells. After 24 h of exposure to either keratinocyte growth medium or 100 µg/ml of oil fly ash, cells were washed 2 times with Hanks' balanced salt solution (HBSS), and indicated doses of $^{125}$I-lactoferrin were included in incubations for 90 min. Cells were then washed 5 times with cold HBSS, disrupted into 0.5 ml of 1% Triton X-100, and counted in a gamma counter. Specific binding was quantitated by subtracting nonspecific counts from total counts. Cells exposed to medium did have lactoferrin binding that was saturable. Exposure to oil fly ash significantly increased this lactoferrin receptor (LfR) activity at all concentrations of $^{125}$I-lactoferrin. *P < 0.05 relative to cells exposed to medium at that concentration of $^{125}$I-lactoferrin.

Fig. 2. Time response of lactoferrin binding by BEAS-2B cells. Cells were exposed to either medium or 100 µg/ml of ash for indicated hours (A) or minutes (B). After 2 washes with HBSS, 100 nM $^{125}$I-lactoferrin was included in cell incubations for 90 min. Cells were then washed 5 times with cold HBSS, disrupted into 0.5 ml of 1% Triton X-100, and counted in a gamma counter. Specific binding was quantitated by subtracting nonspecific counts from total counts. Binding did not vary with time in cells incubated with medium. LfR activity was immediately maximal after exposure to ash (A). After 8 h, this lactoferrin binding was diminished and continued to decrease at 16 and 24 h. When measured between 5 and 60 min, LfR activity was significantly elevated at all times after ash exposure relative to incubations with medium (B). Greatest LfR activity was at 45 min. *P < 0.05 relative to cells exposed to medium at that time point.
hololactoferrin as the cold competitor to determine nonspecific binding (Fig. 3).

The biological effects of this oil fly ash, such as oxidant generation, tyrosine phosphorylation, and cytokine release, have been associated with metals contained in the particle (3, 26). We therefore tested the association of LfR increase with the presence of metals. Addition of the metal chelator deferoxamine (1.0 mM) to BEAS-2B cells with 100 µg/ml of oil fly ash significantly decreased lactoferrin binding at 45 min (Fig. 4A). Similarly, the oxidant scavenger dimethylthiourea at a

![A](image1)  
![B](image2)

Fig. 3. Binding of apolactoferrin (APO) and hololactoferrin (HOLO) by BEAS-2B cells. Cells were exposed to either medium or 100 µg/ml of ash for 45 min (A) and 24 h (B). After 2 washes with HBSS, 125I-lactoferrin and either cold APO or cold HOLO were added to a final concentration of 10 µM, and incubations continued for 90 min. Cells were then washed 5 times with cold HBSS, disrupted into 0.5 ml of 1% Triton X-100, and counted in a gamma counter. Specific binding was quantitated by subtracting nonspecific counts from total counts. There were no differences in 125I-lactoferrin binding by BEAS-2B cells with either APO or HOLO. Disparities could not be demonstrated at either 45 min or 24 h. Exposure to ash again increased LfR activity 2- to 6-fold. *P < 0.05 relative to cells exposed to medium at that concentration of 125I-lactoferrin.

![A](image3)  
![B](image4)

Fig. 4. Deferoxamine (A) and dimethylthiourea (DMTU; B) diminished LfR activity in BEAS-2B cells after ash exposure. Cells were exposed to either medium or 100 µg/ml of ash for 45 min. Deferoxamine and DMTU were also included in one-half of the incubations at a final concentration of 1.0 mM each. After 2 washes with HBSS, 100 nM 125I-lactoferrin was included in cell incubations for 90 min. Cells were then washed 5 times with cold HBSS, disrupted into 0.5 ml of 1% Triton X-100, and counted in a gamma counter. Specific binding was quantitated by subtracting nonspecific counts from total counts. Although 45 min of exposure to ash significantly increased (*) LfR activity, both deferoxamine and DMTU significantly decreased (**) lactoferrin binding by BEAS-2B cells.
final concentration of 1.0 mM diminished 125I-lactoferrin counts at 45 min of incubation with oil fly ash (Fig. 4B). These results supported the increase in LfR activity of the BEAS-2B cells being both a metal- and oxidant-dependent response.

The biological effects of exposure to oil fly ash can be associated with its concentrations of metals (3, 6, 9, 24, 26).

Exposure of respiratory epithelial cells to either 1.0 mM vanadyl sulfate or 1.0 mM iron (III) sulfate for 45 min significantly elevated LfR activity (Fig. 5). However, incubation of BEAS-2B cells with 1.0 mM nickel sulfate for the same duration of time had no effect (Fig. 5). Inclusion of 1.0 mM deferoxamine for a 45-min incubation significantly diminished lactoferrin binding after 1.0 mM vanadyl and iron (III) sulfates (Fig. 5).

To determine whether metal-induced LfR expression requires protein or mRNA synthesis, BEAS-2B cells were incubated for 1 h with either 1.0 µg/ml of cycloheximide or 2.0 µg/ml of actinomycin D before a 45-min exposure to 100 µg/ml of oil fly ash and 1.0 mM metal sulfates. Neither cycloheximide nor actinomycin D had an effect on lactoferrin binding by BEAS-2B cells incubated with medium, the particle, or metal salts (Fig. 6). Contrasting this, a 1-min preincubation of the respiratory epithelial cells with 0.25% trypsin after 45 min of exposure to either medium, the particle, or metal sulfates significantly decreased 125I-lactoferrin counts (Fig. 7).

We next studied the role of calcium in 125I-lactoferrin binding in particle-exposed BEAS-2B cells. Forty-five-minute incubations of BEAS-2B cells with the particle (100 µg/ml) were repeated with either 1.0 mM CaCl₂, 1.0 mM MgCl₂, or 1.0 mM calcium chelator EGTA included. Although neither CaCl₂ nor MgCl₂ had any effect on the LfR activity of BEAS-2B cells, EGTA significantly decreased the 125I-lactoferrin counts after incubation with both medium alone and oil fly ash (Fig. 8).

Finally, the distribution of LfR activity in respiratory epithelial cells was measured after a 45-min exposure to 100 µg/ml of oil fly ash. 125I-lactoferrin counts in the cytoplasm demonstrated no significant changes after incubation with both medium alone and oil fly ash (Fig. 9). These results imply that changes in lactoferrin binding by BEAS-2B cells after exposure to the particle may be distinct to the membrane.

DISCUSSION

LfRs were first described on the intestinal cells of rabbits (14). Similarly, the cells of the small intestine in mice, monkeys, piglets, and humans exhibit LfRs that are specific for that species (8). In addition, monocytes, macrophages, leukemic cells, activated lymphocytes, and hepatocytes display high-affinity binding sites for lactoferrin (8, 13, 20). LfRs, with a molecular mass approximating 110,000–114,000 Da, have been isolated...
and described from enterocytes and peripheral blood lymphocytes (7, 8, 10, 13).

Before any exposure, respiratory epithelial cells were demonstrated to also express high-affinity binding sites for lactoferrin that were saturable. After incubation with a particle containing significant concentrations of metals, these LfRs increased in number on the BEAS-2B cells. This response of LfRs is much quicker than that exhibited by hepatocytes after in vitro exposure to metals (17) but is similar to that after mitogen stimulation in which LfRs remained elevated for at least 2 days (14).

Binding of apolactoferrin and hololactoferrin was equivalent at both 45 min and 24 h after exposure to the particle. These results are analogous to rat hepatocytes and mouse enterocytes that bind iron-depleted and iron-loaded chelators equally (7). However, there are other cells that seem to prefer metal-saturated lactoferrin (5, 16).

LfR activity has been demonstrated to be regulated by the iron status of the cells (17). Hepatocytes from adult rats treated with ferric ammonium citrate increased both the binding and endocytosis of lactoferrin (17). Two- to sixfold increases in 125I-lactoferrin binding were observed after metal exposure. The characteristics of the response supported a requirement for the translation of existing mRNA, but de novo transcription did not appear to be required. Subsequently, it was
hypothesized that the expression of LfR activity could be regulated by an iron-responsive element similar to ferritin and the transferrin receptor. Comparable to the hepatocyte response to ferric ammonium citrate (17), the increased LfR activity on BEAS-2B cells was diminished by the metal chelator deferoxamine. The oxidant scavenger dimethylthiourea also decreased 125I-lactoferrin counts, suggesting that metal-catalyzed radicals could participate in the increased expression of LfR activity after exposure to this particle. Finally, a role for metals in lactoferrin binding by BEAS-2B cells was further supported by increases after exposure to two metals included in high concentrations in this particle (i.e., iron and vanadium).

Increased LfR activity in hepatocytes and respiratory epithelial cells after metal exposure contrasts with the results of similar exposures of intestinal epithelial cells to metals. This supports a differential regulation of LfR activity by metals. LfR activity in intestinal epithelial cells (e.g., Caco-2 and HT29-18-C1: colon carcinoma cells) can be increased rather than decreased by iron depletion (19). These disparities in the response of LfR activity can reflect dissimilar functions of the receptor in the intestinal and lung tissues (17). In the intestine, LfRs potentially could operate to meet nutritional requirements for metals and therefore would increase with iron depletion. However, it is improbable that the LfR could serve this same function in respiratory epithelial cells. It is more feasible that they scavenge excess concentrations of metals to prevent an oxidative injury. Therefore, the receptors increase in the respiratory epithelial cells, with elevations in metal concentrations in an attempt to lessen the catalysis of free radicals and the subsequent tissue injury.

Characterization of LfR activity in respiratory epithelial cells revealed some similarities with LfRs on other cells. Analogous to other cells, LfR expression was not blocked by the inclusion of either cycloheximide or actinomycin D in the incubation (17). Cycloheximide affects cytosolic ribosomes to inhibit protein synthesis, whereas actinomycin D binds to DNA to block the movement of RNA polymerase. This lack of an effect of either cycloheximide or actinomycin D and the rapid increase in specific lactoferrin binding (within 5 min) both suggest that a posttranslational modification of the protein or a translocation could contribute to the elevated LfR activity after metal exposures. In addition, changes in the affinity constant could be an alternative explanation for increased binding of lactoferrin after exposure to the particle and metals. However, elevations in LfR activity after incubation of hepatocytes with iron were suppressed with anisomycin, a protein synthesis inhibitor, supporting a dependence of LfR expression on ongoing protein formation in this specific cell type (19).

Pretreatment with trypsin diminished LfR activity in respiratory epithelial cells comparable to that in a previous investigation (12). We assume that this decrement is the result of the proteolytic activity of the enzyme on the LfR at the surface of the cell. Similarly, the calcium chelator EGTA diminished LfR activity. Lactoferrin binding to several different cells is calcium dependent (15, 18). The diminished 125I-lactoferrin counts after exposure of the BEAS-2B cells to both EGTA and the particle or metal sulfates may reflect an analogous dependence of receptor expression in these cells on calcium availability. Alternatively, EGTA can also bind metals and could be functioning to decrease the exposure of the BEAS-2B cells.

A previous study (13) has demonstrated a ratio of 1:2.5 for the presence of lactoferrin surface and/or intracellular receptors in one specific cell. In this study, we found that exposure of respiratory epithelial cells to oil fly ash particle had no effect on the activity of LfRs in the cytoplasm. This evidence taken together with the lack of any effect of cycloheximide and actinomycin D and the very rapid increase in expression of LfR activity suggests either a conformational change that exposes a lactoferrin-binding site in preexisting LfRs or a translocation of preformed receptors to the membrane.

The potential of lactoferrin to contribute to metal sequestration denotes an importance of this induction of LfR activity in respiratory epithelial cells by metals. Numerous metals contained in the particle can be associated with an oxidative stress. After exposure to the particle, respiratory epithelial cells have demonstrated an increase in ferritin protein (6). Lactoferrin also increased after exposure to the particle (6). The lactoferrin produced by respiratory epithelial cells could function to transport metal included in chelates that present an oxidative stress to the respiratory tract. After reaction with the metal, the complex appears to undergo endocytosis by cells with LfRs, and the metal would then be transported to intracellular ferritin. In the respiratory tract, cells with LfRs include alveolar macrophages. We have demonstrated that respiratory epithelial cells also have LfRs. These results imply that lactoferrin transport by LfRs could participate in the detoxification of metal and its catalyzed oxidative stress by the respiratory epithelial cell.

This report has been reviewed by the National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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