Smirnov, Igor M., Kirstin Bailey, Carol H. Flowers, Ned W. Garrigues, and Lewis J. Wesselius. Effects of TNF-α and IL-1β on iron metabolism by A549 cells, and influence on cytotoxicity. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L257–L263, 1999.—Extracellular iron, which is predominantly bound by transferrin, is present in low concentrations within alveolar structures, and concentrations are increased in various pulmonary disorders. Iron accumulation by cells can promote oxidative injury. However, the synthesis of ferritin stimulated by metal exposure for intracellular iron storage is normally protective. The cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β may alter iron metabolism by alveolar cells. In this study, we assessed the effects of TNF-α and IL-1β on iron metabolism by A549 cells and influence on cytotoxicity. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L257–L263, 1999.—Extracellular iron, which is predominantly bound by transferrin, is present in low concentrations within alveolar structures, and concentrations are increased in various pulmonary disorders. Iron accumulation by cells can promote oxidative injury. However, the synthesis of ferritin stimulated by metal exposure for intracellular iron storage is normally protective. The cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β may alter iron metabolism by alveolar cells. In this study, we assessed the effects of TNF-α and IL-1β on iron metabolism by A549 cells and influence on cytotoxicity. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L257–L263, 1999.—Extracellular iron, which is predominantly bound by transferrin, is present in low concentrations within alveolar structures, and concentrations are increased in various pulmonary disorders. Iron accumulation by cells can promote oxidative injury. However, the synthesis of ferritin stimulated by metal exposure for intracellular iron storage is normally protective. The cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β may alter iron metabolism by alveolar cells. In this study, we assessed the effects of TNF-α and IL-1β on iron metabolism by A549 cells and influence on cytotoxicity.
tion; RPMI 1640 medium and fetal calf serum (FCS) were purchased from J RH Biosciences (Lenexa, KS). Human transferrin, ferrous sulfate, penicillin, streptomycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Recombinant TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN).

Cell cultures. A549 cells (106) were allowed to adhere to 35-mm petri dishes for 2 h in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and antibiotics. Nonadherent cells were then removed, and different amounts of ferrous sulfate in 1 ml of growth medium were added. Non-transferrin-bound iron (FeSO4) or iron-loaded human transferrin was added to the medium in final concentrations up to 40 µM with and without TNF-α (20 ng) or IL-1β (20 ng). Iron, provided by ferrous sulfate must be oxidized to a ferric state before storage as a hydrous ferric oxide within ferritin.

Prior studies have utilized both ferrous and ferric iron for assessing the uptake of non-transferrin-bound iron in cell systems; however, there is evidence that ferric iron is reduced to ferrous iron before non-transferrin-bound iron uptake in some cell systems; therefore, we utilized ferrous sulfate for these in vitro studies (15). Iron concentrations up to 40 µM were utilized in studies based on initial studies demonstrating no accumulation of malondialdehyde (MDA) or cytotoxicity [MTT assay and lactate dehydrogenase (LDH) release] to A549 cells incubated in cultures containing up to this concentration of non-transferrin-bound iron or transferrin-bound iron. After incubation of cells in medium for 24 h at 37°C, the medium was removed, the cultures were washed three times with saline to remove detached cells and serum protein, and then the cell layers were seeded with silver polycrystalline in 1 ml of saline and sonicated. Butylated hydroxytoluene was added to the cells to prevent oxidative injury after collection.

L- and H-type ferritin and iron assays. The concentrations of L-ferritin were determined by Tandem-R ferritin immunoradiometric assay (Hybritech, San Diego, CA). The ELISA assay for H-ferritin was developed with the use of recrystallized human heart ferritin to develop monoclonal antibodies as previously described (33). The concentration of intracellular iron was determined with a controlled coulometric method (Ferrochem II, Environmental Science Associates, Bedford, MA) as previously described (25). This coulometric procedure measures the total electron transfer induced at two electrode surfaces by different currents applied simultaneously at each electrode. The methodology requires instilling a small (25-µl) aliquot into the reaction chamber of the coulometric equipment. Working standards for iron were prepared from certified ferric chloride suitable for standardization with atomic absorption spectrometry (Fisher Scientific, Fair Lawn, NJ). In some studies, we also measured iron concentrations by a standard colorimetric method (ferrozine) as described by Fish (9) and found these methods to yield similar results.

TfR assay. The expression of TfRs by A549 cells was measured with a previously described ELISA methodology (10). Monoclonal reagents in this method were prepared against soluble TfR rather than surface TfR so that both bound and unbound TfRs were measured. Briefly, flat-bottomed 96-well microtiter plates (NUNC-Intermed, NUNC, Naperville, IL) were coated with 2 ng/ml of monoclonal antibody diluted in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. Unreacted sites were blocked with 0.5% bovine serum albumin (BSA) in carbonate buffer for 30 min at room temperature. The plates were then washed three times with PBS-Tween (PBS-T). Purified TfR was diluted in PBS-T containing 0.5% BSA and added in a volume of 200 µl to the appropriate wells. The plates were covered, incubated for 2 h at room temperature, and washed three times with PBS-T, and then 200 µl of horseradish peroxidase-conjugated antibody diluted in PBS-T containing 1% BSA were added to all wells, and the plates were incubated an additional 2 h at room temperature. The plates were washed again three times, and 200 µl of the substrate (34 mg of o-phenylenediamine in 0.15 M citrate-phosphate buffer, pH 5.0, containing 0.01% H2O2) were added. After 30 min of incubation at room temperature in the dark, the reaction was stopped by adding 50 µl of 25% sulfuric acid. The optical density was read at 492 nm on a microplate reader (Biotek ELx808, Biotek Instruments, Winoski, VT). The sensitivity of this assay method is 0.5 µg/l.

MDA assay. MDA concentrations in the cell layers were determined with a commercially available colorimetric assay method (R&D Systems, Minneapolis, MN) and are expressed in micromoles of MDA per milligram of cell protein. This assay utilizes a chromogenic reagent that reacts with MDA at 45°C. Subsequent condensation of MDA with a second reagent yields a stable chromophore with maximal absorbance at 586 nm. The sensitivity of this assay is 0.5 µmol. Comparative studies with this method and previously described techniques with thiobarbituric acid yielded similar results (32).

MTT assay. The MTT assay assesses mitochondrial function and correlates with cell viability (20). Cells were grown in 96-well microtiter plates (Falcon, Becton Dickinson) with 5 × 104 cells plated initially in each well. The cells were cultured for 24 h in medium (RPMI 1640 medium and 10% FCS) either supplemented with iron and/or cytokines or without supplementation (control). After exposure to iron and/or cytokines, 25 µl/well of MTT stock (5 mg/ml in PBS) were added to each well. After 1 h of incubation with MTT, 100 µl of lysis buffer containing 20% (wt/vol) SDS in 50% N,N-dimethylformamide were added. The cells were incubated an additional 1 h, and then the optical density was measured at 570/630 nm with a microtiter plate reader (Dynatech). The optical density for each well was compared with lysing buffer as a blank.

LDH and protein assays. LDH was measured with a colorimetric method based on the conversion of pyruvic acid to lactic acid (procedure 500, Sigma). The LDH content of the culture supernatant is expressed as a percentage of the total LDH content of the sonicated cell layer after subtraction of the LDH content of the medium. The protein content of cell layers was measured with a protein assay reagent consisting of bicinchoninic acid (BCA Kit, Pierce, Rockford, IL), with BSA as a standard.

Statistical analysis. Data analysis was performed with ANOVA, and a P value < 0.05 was considered significant. All measurements were performed in triplicate, and the mean value from at least five separate experiments is displayed.

RESULTS

Effect of cytokines on uptake and isoferferritin synthesis in response to non-transferrin-bound iron. Treatment of A549 cells with medium supplemented with ferrous non-transferrin-bound iron (FeSO4) resulted in rapid iron uptake, with saturation by ~8 h of exposure (Fig. 1). Supplementation of the medium with FeSO4 in concentrations up to 100 µM significantly increased L-ferritin synthesis in A549 cells in a dose-dependent manner (Fig. 2). Iron-supplemented medium enhanced the synthesis of L-ferritin in preference to synthesis of H-type ferritin (Figs. 2 and 3).
Treatment of A549 cells with TNF-α or IL-1β alone was associated with accumulation of H-type ferritin and no change in L-type ferritin in the absence of any significant change in intracellular iron (Table 1). The addition of TNF-α or IL-1β to cultures of A549 cells treated with FeSO₄-supplemented medium significantly increased iron uptake (Fig. 1), suggesting cytokine-induced effects on the accumulation of iron by transferrin-independent pathways. Treatment of the cells with FeSO₄ and cytokines induced a marked increase in H-ferritin formation (Fig. 3). TNF-α and IL-1β had different effects on FeSO₄-induced L-ferritin accumulation, with TNF-α decreasing synthesis, whereas IL-1β had no effect on synthesis (Fig. 2). Both cytokines significantly increased total ferritin synthesis (H- plus L-type ferritin) compared with total ferritin induced by exposure to non-transferrin-bound iron in the absence of cytokines (Fig. 4). The increase in L-ferritin and H-ferritin associated with exposure to

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**Table 1. Effect of cytokines on iron uptake and ferritin synthesis in A549 cells treated with TBI**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Iron Uptake, µg/ml</th>
<th>L-Ferritin Content, ng/mg</th>
<th>H-Ferritin Content, ng/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.06 ± 0.02</td>
<td>130 ± 11.2</td>
<td>15 ± 3.1</td>
</tr>
<tr>
<td>TNF-α (20 ng)</td>
<td>0.05 ± 0.02</td>
<td>122 ± 8.1</td>
<td>283 ± 26.7†</td>
</tr>
<tr>
<td>IL-1β (20 ng)</td>
<td>0.05 ± 0.02</td>
<td>125 ± 12.4</td>
<td>208 ± 19.4*</td>
</tr>
<tr>
<td>TBI (40 ng)</td>
<td>1.55 ± 0.26</td>
<td>265 ± 14.7*</td>
<td>24 ± 6.2</td>
</tr>
<tr>
<td>TBI + TNF-α</td>
<td>0.51 ± 0.13*</td>
<td>214 ± 12.5*</td>
<td>391 ± 28.7†</td>
</tr>
<tr>
<td>TBI + IL-1β</td>
<td>0.48 ± 0.18*</td>
<td>235 ± 10.9*</td>
<td>308 ± 22.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 separate experiments. TBI, transferrin-bound iron; L, light; H, heavy; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β. All treatments were for 24 h. Significant difference compared with control group: *P < 0.01; †P < 0.0001.
FeSO₄, either with or without cytokines, began by 4 h of incubation, although ferritin accumulation did not stabilize until after 24 h of incubation (Fig. 5). The accumulation of newly synthesized H-ferritin after treatment with TNF-α or IL-1β plus FeSO₄ occurred predominantly from 8 to 24 h after exposure (Fig. 5). The relatively faster uptake of iron compared with ferritin synthesis increased the ratio of iron to ferritin within A549 cells, with greater increases in cytokine-treated cells. The ratio of iron to ferritin in A549 cells treated with FeSO₄ (40 µM) alone at 4 h was 6.7 ± 0.2 (SE), whereas in cells treated with TNF-α plus FeSO₄, the ratio was 12.2 ± 0.8 and after iron plus IL-1β, the ratio was 12.9 ± 0.9.

Effect of cytokines on iron uptake and isoferitin synthesis in response to transferrin-bound iron. Treatment with either TNF-α or IL-1β significantly decreased the accumulation of iron in cells treated with transferrin-bound iron (Table 1). There were also decreases in the synthesis of L-type ferritin after treatment with either TNF-α or IL-1β. Synthesis of H-type ferritin, however, was significantly increased by both TNF-α and IL-1β.

Effect of iron and cytokines on Tfr expression. Exposure to iron, TNF-α, or IL-1β significantly decreased A549 cell expression of Tfrs (Table 2). The effect of cytokines on Tfr expression developed rapidly and then decreased because the effects at 4 h were greater than the effects at 24 h. The effects of TNF-α and IL-1β on Tfr expression were similar to the effect of iron exposure (40 µM) at 4 h; however, the decrease induced by iron persisted through 24 h, whereas the effects of cytokine treatment had decreased substantially by 24 h.

Table 2. Effect of iron and cytokines on transferrin receptor expression by A549 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,501 ± 48</td>
<td>1,535 ± 53</td>
</tr>
<tr>
<td>TNF-α (20 ng)</td>
<td>540 ± 43*</td>
<td>850 ± 15*</td>
</tr>
<tr>
<td>IL-1β (20 ng)</td>
<td>491 ± 21*</td>
<td>861 ± 18*</td>
</tr>
<tr>
<td>Iron (40 µM)</td>
<td>499 ± 41*</td>
<td>431 ± 18*</td>
</tr>
<tr>
<td>Iron (40 µM) + TNF-α</td>
<td>521 ± 20*</td>
<td>423 ± 31*</td>
</tr>
<tr>
<td>Iron (40 µM) + IL-1β</td>
<td>498 ± 22*</td>
<td>456 ± 24*</td>
</tr>
</tbody>
</table>

Values are means ± SE in µg/ml; n = 4 separate experiments.
*Significant difference compared with control group, P < 0.05.
Cell MDA content. The addition of TNF-α or IL-1β to cell cultures or the addition of FeSO₄ (40 µM) did not significantly increase the cell layer content of MDA (Fig. 6). In contrast, the addition of both iron and TNF-α was associated with significant increases in cell MDA content. If exposure to TNF-α was delayed 24 h after FeSO₄ treatment, increases in cell MDA content were inhibited (data not shown), suggesting that the storage of iron within newly synthesized ferritin is protective. Exposure of A549 cells to transferrin-bound iron (40 µM) or the combination of transferrin-bound iron and cytokines (TNF-α or IL-1β, 20 ng) did not increase cell MDA content (data not shown).

MTT assay. Exposure of A549 cells to non-transferrin-bound or transferrin-bound iron in concentrations up to 40 µM did not significantly increase cytotoxicity as determined by the MTT assay (Fig. 7). Exposure of A549 cells to TNF-α (20 ng) or IL-1β (20 ng) induced cytotoxicity, with greater cytotoxicity associated with TNF-α compared with IL-1β. Exposure to IL-1β and either transferrin-bound or non-transferrin-bound iron did not increase cytotoxicity above levels induced by each agent alone (Fig. 7). Exposure to TNF-α was associated with a greater amount of cytotoxicity than exposure to IL-1β, and cytotoxicity was substantially enhanced by coexposure to non-transferrin-bound iron but not to transferrin-bound iron.

Release of LDH. To confirm enhancement of cytotoxicity with exposure of A549 cells to TNF-α and non-transferrin-bound iron, we measured the supernatant content of LDH to assess for induced cytolysis. A549 cells exposed to TNF-α (20 ng) and increasing concentrations of FeSO₄ up to 40 µM demonstrated a dose-dependent increase in supernatant LDH (Fig. 8).

**DISCUSSION**

In this study, we determined that TNF-α and IL-1β significantly alter iron metabolism by A549 cells, including effects on iron uptake and ferritin synthesis. Both cytokines decreased cell expression of TfRs as well as accumulation of transferrin-bound iron. In contrast, both cytokines enhanced the accumulation of extracellular non-transferrin-bound iron. We also extended prior studies (19, 30) that indicated that these cytokines induce expression of H-type ferritin. The enhanced synthesis of H-type ferritin was not fully protective against the toxicity associated with TNF-α because the enhanced uptake of non-transferrin-bound iron was associated with peroxidation of cell lipids and increased cell death.

There is normally a small amount of non-transferrin-bound iron in alveolar structures of healthy subjects, although alveolar iron is predominantly bound by transferrin (14). Iron bound by transferrin is generally prevented from catalyzing extracellular hydroxyl radical formation; however, cellular accumulation of transferrin-delivered iron can transiently increase the intracellular content of unbound iron before sequestration within ferritin, leading to enhanced oxidant stress (5). Concentrations of transferrin-bound iron in alveolar structures are increased in patients with acute respiratory distress syndrome, and our findings suggest that alveolar TNF-α and IL-1β present in this disorder may...
reduce iron uptake by alveolar epithelial cells and promote H-type ferritin synthesis (14, 27). Reduced iron uptake and enhanced ferritin synthesis induced by TNF-α and IL-1β would have potential protective effects, limiting availability of intracellular iron and decreasing the potential for iron-catalyzed oxidant injury to alveolar cells (14, 29).

Although increased alveolar concentrations of iron are present in a variety of pulmonary disorders, it is unclear how effectively iron is bound by alveolar transferrin. In some disorders, the capacity of alveolar transferrin to bind iron may be limited. In patients with cystic fibrosis, for example, intrapulmonary iron concentrations are increased and neutrophil-derived proteases in alveolar structures of these patients can cleave transferrin, impairing its capacity to effectively sequester iron (4, 28). In addition, alveolar concentrations of transferrin appear to be reduced in some types of respiratory failure, which may limit the capacity of alveolar transferrin to completely bind alveolar iron (27). The findings of this study suggest that TNF-α and IL-1β can promote the uptake of non-transferrin-bound iron by alveolar epithelial cells. In addition, the increased iron uptake may enhance TNF-α-mediated cytotoxicity to epithelial cells.

Our finding that TNF-α and IL-1β induce synthesis of H-type ferritin in A549 cells both with and without supplemental iron is consistent with prior observations reported with different cell lines (19, 30). Although exposure to iron alone induced synthesis of almost solely L-type ferritin in A549 cells, ferritin synthesized in response to iron and cytokines was more evenly divided between L-type and H-type ferritin, with TNF-α inducing synthesis of >50% H-type ferritin. The substantial increase in synthesis of H-type ferritin in response to TNF-α and IL-1β may enhance sequestration of intracellular iron and thereby contribute to limiting iron-catalyzed oxidative injury (8).

The findings of this study suggest that release of TNF-α or IL-1β by alveolar macrophages or other alveolar cells could decrease the uptake of transferrin-bound iron and enhance the synthesis of total ferritin in alveolar cells in response to iron uptake. These effects would decrease the intracellular availability of catalytic iron. Consistent with this hypothesis, there was no evidence of increased cytotoxicity in A549 cells exposed to transferrin-bound iron and cytokines. In contrast, both cytokines increased the uptake of unbound iron, with substantial increases by 4 h after treatment. Increased synthesis of H-type ferritin in response to iron and cytokines was also induced by both cytokines; however, a substantial increase in the cell content of H-type ferritin did not develop until between 8 and 24 h after exposure, resulting in an initial phase of increased cell iron content relative to ferritin stores. The greater cytotoxicity associated with TNF-α and non-transferrin-bound iron may be due, at least in part, to the decreased synthesis of L-type ferritin, which was induced by TNF-α but not by IL-1β. TNF-α also decreases the A549 cell content of glutathione within 4 h, and glutathione is important in protecting against iron-induced tissue injury (17, 21). TNF-α-induced increases in the cell iron-to-ferritin ratio during the initial 8 h of iron exposure, together with the negative effects on intracellular glutathione, may promote lipid peroxidation and loss of cell viability.

Treatment with TNF-α or IL-1β decreased expression of TfRs in A549 cells, a finding similar to the effects previously reported in a monocyte cell line (8), although enhanced expression has been noted in other cell lines (30). Consistent with this observation, both cytokines decreased the uptake of transferrin-bound iron in vitro. In contrast, both cytokines increased the uptake of non-transferrin-bound iron, indicating enhanced transferrin-independent pathways of iron uptake. The mechanisms contributing to enhanced transferrin-independent iron uptake induced by TNF-α or IL-1β are not clear from our studies. However, a prior study (22) has described a transferrin-independent pathway for cellular iron uptake that is stimulated by hydroxyl radical generation, and TNF-α-induced intracellular generation of reactive oxygen species may promote hydroxyl radical generation (15). The mechanism by which IL-1β promotes transferrin-independent iron uptake by A549 cells, however, is not clear from these studies.

A role for intracellular oxidants in TNF-α-mediated cytotoxicity is supported by prior observations that antioxidants, including iron chelators, inhibit TNF-α-induced lysis of L929 cells (24, 26). Our finding that combined exposure to non-transferrin-bound iron and TNF-α induced lipid peroxidation and enhanced cell death compared with TNF-α alone supports the concept that intracellular iron is involved in TNF-α-mediated cell injury. Although the mechanism of enhanced cytotoxicity is uncertain, increased concentrations of unbound iron may catalyze intracellular generation of highly reactive hydroxyl radicals.

In summary, these studies demonstrate that both TNF-α and IL-1β alter iron metabolism by A549 cells, with differing effects on transferrin-bound and non-transferrin-bound iron. Both cytokines decreased cell expression of TfRs and decreased cell uptake of transferrin-bound iron. In contrast, both cytokines enhanced the uptake of non-transferrin-bound iron by A549 cells. Both cytokines also increased H-ferritin synthesis; however, H-type ferritin synthesis was not fully protective because combined exposures to non-transferrin-bound iron and TNF-α alone suggests that increased availability of non-transferrin-bound iron within the lungs could enhance TNF-α-mediated toxicity to alveolar epithelial cells.

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