Hepcidin expression and iron transport in alveolar macrophages

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Am J Physiol Lung Cell Mol Physiol 291: L417–L425, 2006. First published April 28, 2006; doi:10.1152/ajplung.00484.2005.—Alveolar macrophages express many proteins important in iron homeostasis, including the iron importer divalent metal transport 1 (DMT1) and the iron exporter ferroportin 1 (FPN1) that likely participate in lung defense. We found the iron regulatory hormone hepcidin (HAMP) is also produced by alveolar macrophages. In mouse alveolar macrophages, HAMP mRNA was detected at a low level when not stimulated but at a high level when exposed to lipopolysaccharide (LPS). LPS also affected the mRNA levels of the iron transporters, with DMT1 being upregulated and FPN1 downregulated. However, iron had no effect on HAMP expression but was able to upregulate both DMT1 and FPN1 in alveolar macrophages. IL-1 and IL-6, which are important in HAMP augmentation in hepatocytes, also did not affect HAMP expression in alveolar macrophages. In fact, the LPS-induced alterations in the expression of HAMP as well as DMT1 and FPN1 were preserved in the alveolar macrophages isolated from IL-1 receptor or IL-6-deficient mice. When alveolar macrophages were loaded with transferrin-bound 59Fe, the subsequent release of 59Fe was inhibited significantly by LPS. In addition, treatment of these cells with either LPS or HAMP caused the diminishment of the surface FPN1. These findings are consistent with the current model that HAMP production leads to a decreased iron efflux. Our studies suggest that iron mobilization by alveolar macrophages can be affected by iron and LPS via several pathways, including HAMP-mediated degradation of FPN1, and that these cells may use unique regulatory mechanisms to cope with iron imbalance in the lung.

hepcidin; divalent metal transport 1; ferroportin 1; lipopolysaccharide; iron metabolism; inflammation

Our earlier studies revealed that most proteins involved in iron metabolism are expressed in the lung and are likely important in lung health and lung defense (16, 38, 42, 43). Among these proteins are the iron importer, divalent metal transport 1 (DMT1), and the iron exporter, ferroportin 1 (FPN1), which were recently identified in the enterocytes and appear to be responsible for nonheme iron absorption in the intestine (1, 8, 13, 18, 27). DMT1 is also involved in intracellular iron trafficking in cells, including erythrocytes (12), and FPN1 is required for iron efflux in many cells and is crucial for iron recycling in the reticuloendothelial system (reviewed in Ref. 36). Interestingly, these iron-associated proteins are synthesized in the lung mainly by two types of cells, the airway epithelial cells and alveolar macrophages (AM). The airway epithelial cells serve as the first line of defense in the upper airways against harmful environmental insults, including toxic heavy-metal-containing particles. We have found that these cells are very efficient in removing non-transferrin-bound iron and converting it to less toxic protein-bound iron (39, 43). Our studies suggest that both DMT1 and FPN1 may play important roles in this detoxification process. Indeed, rats that are deficient in DMT1 had increased lung injury compared with wild-type rats when exposed to metal-containing particles, most likely because of decreased metal transport (16).

As a unique class of macrophages, AM function primarily in lung defense against inhaled microorganisms and environmental toxins (10, 17). They appear to play a critical role in the pathogenesis of numerous pulmonary diseases, including chronic obstructive pulmonary disease (11), idiopathic pulmonary fibrosis (30, 41), asthma (5), and pulmonary sarcoidosis (6, 28). It is well established that macrophages are the major effector cells of the innate immune system. Of many types of the macrophages found in the body, AM have the most frequent contact with external stimuli. The early interaction and response to invading pathogens and other insults by the AM is essential to survival. As one of the most abundant transition metals, iron is present in all air pollution particles and can also be released in several forms from injured tissues. AM have long been recognized for their roles in iron scavenging in the lower airways and provide protection for the delicate respiratory epithelium against oxidative damage. Even more interesting, the iron-catalyzed production of reactive oxygen species in these cells is crucial for the elimination of invading microorganisms. However, the mechanisms involved in the mobilization, utilization, and detoxification of iron by AM are largely unknown; the role of these cells in iron homeostasis in the lung remains to be elucidated.

We studied the regulation of DMT1 and FPN1 by endotoxin and iron in freshly isolated mouse AM. We found that the iron regulatory protein hepcidin (HAMP) was expressed at a high level in AM treated with lipopolysaccharide (LPS). This short peptide hormone, which is synthesized mainly by hepatocytes and secreted in the circulation, appears to play a central role in the body’s iron homeostasis, including iron absorption by enterocytes and iron recycling by tissue macrophages (review in Refs. 14, 34, and 35). It appears to modulate iron efflux by targeted degradation of FPN1 on the cell surface (32). The regulation of HAMP by LPS, inflammatory cytokines, and iron in AM was investigated. Our results suggest that inflammation can affect iron efflux from these cells via several mechanisms, including downregulation of FPN1 at the mRNA and protein levels.

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EXPERIMENTAL PROCEDURES

Collection of mouse AM. Mouse strains C57BL/6J, C3HeB/FeJ, B6.129S1-IL-1r1tm1Roml (003018; IL-1 receptor knockout), and B6.129S2-IL-6tm1Roml (002650; IL-6 knockout) were acquired from Jackson Laboratories (Bar Harbor, ME). ICR was acquired from Harlan (Indianapolis, IN). AM were collected from adult mice of the above strains. After the lung was removed from the mice postmortem, lavage was performed five times using 0.7–1 ml of sterile saline solution each time. The pooled lavage fluid was centrifuged at 600 g for 5 min, and the cell pellet was resuspended in a complete medium containing RPMI plus 10% FBS and 0.02 mg/ml gentamicin (Invitrogen, Carlsbad, CA). The resulting cell suspension contains >95% of AM.

Treatment of the cells with LPS, cytokines, and iron. A 300-μl aliquot containing 0.3 × 10^6 of AM was used for each sample. Alternatively, the same number of cells was seeded per well in a 12-well tissue culture plate. In both instances, cells were allowed to recover in an incubator at 37°C with 5% CO2 for 4 h before treatment. LPS (Sigma, St. Louis, MO) treatment was carried out at 1–100 ng/ml, and 50 ng/ml were used for individual inflammatory cytokines, including tumor necrosis factor (TNF)-α, interferon-γ, IL-1, and IL-6. Two commercial sources of all the above cytokines were used: one from Peprotech (Rocky Hill, NJ) and the other from Chemicon (Temecula, CA). Exposure of cells to iron was performed using 100–500 μM of ferric ammonium citrate (FAC, Sigma). Each treatment was conducted in triplicate or more, and the experiments were repeated several times.

Quantitative analysis of mRNA levels by real-time RT-PCR. Total RNA from AM was prepared by using Trizol reagent (Invitrogen) according to the procedure recommended by the manufacturer. Quantitative RT-PCR was performed in duplicate by using the TaqMan One-step RT-PCR Master Reagents Kits (Applied Biosystems, Foster City, CA) with detection of TaqMan fluorescence on a sequence detection system (ABI Prism 7700; Applied Biosystems). The mRNA levels of each target gene were normalized by using the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The following primer sequences were used: mouse DMT1 (common for /H11032-CAG CCT GAA CTC TAT CTT CTG AAC ACC GTG G-3; mouse DMT1 (common for +IRE and -IRE isoforms): sense 5′-AAC CAA CAA GCA GGT GGT TGA-3′, antisense 5′-CCT TGT AGA TGT CCA CAG CCA GAG T-T3′, TaqMan probe 5′-CTC CTT CTC CAG TGA CAA C-3′; mouse FPN1: sense 5′-GTG CTT GAC GAC AGT CAT TGC TGC TA-3′, antisense 5′-TTC TTT CTT GCA GCA ACT GTG T-3′, TaqMan probe 5′-AAAT CGG TCT GTG CTT GTC TTA GCT GCA GG-3′; mouse HAMP (detect both HAMP1 and HAMP2 mRNAs): sense 5′-CAT GAC CAC CAT GTA TCT-3′, antisense 5′-GGT TGC TTT CCC CTC GTG CAA CAA GGC-3′, TaqMan probe 5′-GAT AAC AGA TGA GAC AGA CTA CAG AGC TGC-3′. The GAPDH primers and probe were from TaqMan Rodent GAPDH Control Reagents (Applied Biosystems).

Differentialiation of HAMP1 and HAMP2 mRNAs by restriction enzyme analysis of their cDNAs. Total RNA was reverse transcribed by using the Ambion Reverse Transcription kit (Ambion, Austin, TX) and random priming protocol. The resulting cDNA library was amplified by PCR using a primer set with sequences shared by HAMP1 and HAMP2 mRNAs. The forward primer (5′-AGG CAT GAT GCC ACT CAG CAC TCG-3′) was from nucleotide number 11 to 34, and the reverse primer (5′-TGT AGA GAG GTC AGG ATG TCG TTC TA-3′) was from nucleotide number 297 to 272 (BC021587). The resulting PCR products were then digested with Pst I or Hinf I, which produces different restriction fragments for HAMP1 and HAMP2 cDNAs.

Cell surface protein biotinylation. Freshly isolated AM resuspended in complete medium were plated in six-well plates (2 × 10^6 cells/well) and incubated at 37°C in 5% CO2 for 2 h. Cells were then incubated with a fresh aliquot of complete medium in the presence or absence of 100 ng/ml LPS or 700 nM synthetic human HAMP (Peptides International, Louisville, KY) for 16 h. The cells were then washed with PBS and exposed to cell-impermeant biotinylation reagent EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 30 min at room temperature using concentrations recommended by the manufacturer. Subsequently, cells were washed free of biotinylation reagent in PBS supplemented with 10 mM Tris and then lysed with buffer containing 1× PBS, pH 7.4, 0.05% Triton X, 1 mM vanadium sulfate oxide, and 1× protease inhibitor cocktail (Sigma). The lysate was precipitated with immobilized avidin (SoftLink Avidin; Promega, Madison, WI), eluted with 5 mM biotin in gel running dye buffer, subjected to SDS-PAGE, and subsequently blotted with the anti-FPN1 antibody as described above.

Western blot analysis. The sample was mixed with running dye buffer, separated in 7.5% ReadyGel (Bio-Rad, Hercules, CA) by Electrophoresis, and transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) as recommended by the manufacturer. The membrane was blocked with 5% nonfat milk in 1× Tris-buffered saline and 0.05% Tween 20, incubated with purified antibody against FPN1 (1), washed, and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase. The signal was developed by using Super Signal West Pico Chemiluminescent substrate (Pierce). The nitrocellulose membrane was stained with the MemCode Reversible Stain Kit (Pierce) to visualize total protein loading in each lane.

Assay for iron uptake and release. For iron uptake, AM (0.5 × 10^6) in complete medium were allowed to adhere to each well of a 12-well culture plate for 2–4 h before they were exposed to 55Fe-transferrin (2.6 μM) for 16 h. The 55FeCl3 used for the preparation of 55Fe-transferrin was from Perkin-Elmer (Wellesley, MA) with a specific activity of >3 Ci (111 GBq)/g. After removal of the iron-containing medium, the cells were rinsed with RPMI three times and then treated with 100 ng/ml of LPS for 4 h. LPS was removed, and the cells were replenished with complete medium and allowed to release iron for 4 h. The amount of 55Fe in the release medium and in the cell lysate was then determined by scintillation counting.

Statistics. Experiments are conducted in triplicate. Data are expressed as means ± SE. Differences between control and treated groups were analyzed by one-way ANOVA. Significance was measured at P < 0.05.

RESULTS

LPS induced HAMP synthesis and altered the expression of both FPN1 and DMT1 in AM. We have found that AM express both iron importer DMT1 and iron exporter FPN1, which can affect iron mobilization in these cells and iron homeostasis in the lung. Because HAMP has been shown to be a major iron regulatory hormone via its ability to regulate FPN1, we investigated whether AM in their unique location in the lung can produce HAMP during normalcy and/or inflammation. AM freshly isolated from mouse lung were incubated in the absence or presence of 10 or 100 ng/ml of LPS for 4 or 16 h. The level of HAMP mRNA was determined in these cells by real-time RT-PCR. In untreated cells, only a low level of HAMP mRNA was detected with a HAMP-to-GAPDH ratio ~0.012. However, an LPS dosage-dependent increase of HAMP mRNA was observed, and as much as 20- to 80-fold increase was detected after the cells were exposed to LPS. The LPS-induced HAMP expression was seen in AM from several strains of mice, including C57BL/6J, C3HeB/FeJ, and ICR. Shown in Fig. 1A is a representative result using AM derived from ICR mice.
To determine whether the expression of iron transporters in AM is also altered during inflammation, the levels of DMT1 and FPN1 mRNAs were quantified in cells treated with or without LPS for 4 or 16 h. Although DMT1 was upregulated by LPS (Fig. 1B), FPN1 was downregulated (Fig. 1C). Similar to what was observed with HAMP, there was an LPS dosage-dependent response for the expression of these two iron transporters in cells treated for 4 or 16 h with LPS.

LPS upregulated HAMP1 but not HAMP2 expression in AM. Although there is only one copy of HAMP gene in humans, two HAMP genes have been detected in mice, with HAMP1 being the human homologue that has been shown to possess iron regulatory activity. The function of HAMP2 is not clear. However, these two genes are highly similar in both cDNA sequences and regulatory region (25). To determine whether the expression of both HAMP genes is induced in AM during inflammation, RNAs isolated from LPS-treated cells were used as templates to prepare cDNAs using primers common to HAMP1 and HAMP2. The cDNA products of 287 bp in size were then subjected to restriction enzyme analysis. The resulting HAMP cDNA is predicted to have one \( \text{PstI} \) site, which gives rise to 170- and 117-bp fragments, but no \( \text{HinfI} \) site, and not HAMP2 cDNA, which has one \( \text{HinfI} \) site but no \( \text{PstI} \) site. On the other hand, HAMP2 cDNA would have one \( \text{HinfI} \) site but no \( \text{PstI} \) site. Figure 2A shows the restriction digest products from two cDNA preparations and demonstrates that only HAMP1 mRNA and not HAMP2 mRNA was detectable in AM after LPS treatment. As a control for enzyme digest, HAMP2 cDNA derived from untreated mouse spleen was digested with \( \text{PstI} \) and \( \text{HinfI} \), and the digest products with predicted restriction patterns are shown in Fig. 2B.

Time course-dependent alteration of HAMP, DMT1, and FPN1 gene expression after LPS treatment. The mRNA levels of HAMP, DMT1, and FPN1 in AM were followed at 2, 4, 8, or 24 h after LPS treatment. In cells treated with either 10 or 100 ng/ml of LPS, the HAMP mRNA level was increased significantly within 2 h and peaked around 4 h after treatment. By 24 h, the HAMP mRNA level in LPS-treated cells was approaching the same level as in untreated cells (Fig. 3A). Similar time course-dependent alteration in mRNA level was observed for DMT1 (Fig. 3B), which includes both \( \text{IRE}^+ \) and \( \text{IRE}^- \) isoforms, and for \( \text{IRE}^- \) isoform of DMT1 alone (Fig. 3C). The mRNA level for FPN1, on the other hand, appeared to decrease shortly after LPS exposure but was not much different from untreated control after 4–8 h, and decreased again after 24 h of treatment (Fig. 3D). In most of the experiments conducted, FPN1 level was decreased after the cells were exposed to LPS for a short period of time (2–4 h) as well as for overnight (16–24 h), such as that shown in Fig. 1. However, in some cases, a decrease in FPN1 was observed at 2 h but not at 4 or 8 h after LPS exposure. In fact, 8 h of LPS
treatment usually resulted in either a slight increase or no change in the level of FPN1. The reason for this variation is unclear but may reflect the unstable nature of freshly isolated AM. It is also possible that FPN1 levels could be fluctuated during 24 h of LPS treatment as a consequence of the combined effect of various regulatory pathways associated with LPS stimulation. A more detailed analysis of time course-dependent alteration of FPN1 is needed to address this question.

Effects of cytokines on the expression of HAMP, DMT1, and FPN1. Induction of HAMP synthesis in hepatocytes during inflammation has been reported to depend on IL-6 and/or IL-1 signaling pathways (20, 23, 31). It is not clear whether this is also true in other cells/tissues that are capable of synthesizing HAMP. Because macrophages can produce several cytokines such as IL-1, IL-6, and TNF-α upon stimulation by endotoxin, we investigate whether the observed alteration in HAMP, DMT1, and FPN1 expression could be mediated through these inflammatory mediators. Interferon-γ, which can be produced by infiltrating T lymphocytes in the lung, was also included in this study. As shown in Fig. 4, no single cytokine alone appeared to significantly affect the expression of HAMP, DMT1, or FPN1 gene in AM after 4 h of treatment, whereas LPS altered the mRNA levels of all three genes in the same experiment. Similar results were obtained when cytokines from two different commercial sources were used, and no significant alteration in the expression of the above three genes was observed even after the cells were treated with these cytokines for 20 h (data not shown).

To determine whether IL-1 or IL-6 signaling pathway are essential for LPS-induced alteration of HAMP, DMT1, or FPN1 gene expression in AM, experiments were conducted using AM isolated from mice deficient in IL-1r1 (receptor for IL-1) or IL-6. AM derived from wild-type mice with the same genetic background (C57BL/6J) were included in the same experiments for comparison. The mRNA levels of HAMP, DMT1, and FPN1 in cells treated without or with LPS (100 ng/ml) for 4 h were quantified by real-time RT-PCR and compared. Figure 5 shows the degree of changes in the mRNA levels between untreated and LPS-treated cells for each of the three genes in three strains of mice. The response of HAMP, DMT1, or FPN1 gene on LPS stimulation was very similar among IL-1r1 knockout, IL-6 knockout, and wild-type mice. There was no significant difference in the degree of alteration in mRNA levels for any of these three genes among three groups of mice. Similar results were obtained in IL-6 plus NOS double-knockout mice compared with wild-type mice (data not shown). Although this study did not include NOS single knockout mice, our results suggest that nitric oxide is not likely involved in the regulation of these genes as well.

Iron upregulated DMT1 and FPN1 but has no effect on HAMP expression in AM. Iron overload in vivo has been shown to induce HAMP production in the liver, which is
independent of HAMP induction by inflammation (19, 37). We investigated whether HAMP expression in AM can also be upregulated by iron. After the cells were exposed to 50 μM of FAC for 4 h, no significant change in HAMP mRNA level was detected (Fig. 6A). There was also no significant alterations in mRNA levels for DMT1, although a slight increase in FPN1 mRNA was detected (Fig. 6A). The HAMP mRNA level was not significantly altered, even when the cells were treated with 500 μM of FAC for 16 h (Fig. 6B). Under this condition, the mRNA levels for both DMT1 and FPN1 were increased severalfold (Fig. 6B). Our results indicate that iron does not affect HAMP expression in AM. However, exposure of AM to iron increased the expression of both DMT1 and FPN1 genes and could consequently promote the mobilization of iron, which involves both uptake and release of the metal, in these cells.

Iron release from AM was significantly reduced after LPS treatment. HAMP has been shown to inhibit iron efflux in several cell types, including the macrophage cell line J774 (21). Because HAMP was greatly induced in AM shortly after LPS treatment, we investigated if LPS affects iron efflux in these cells. Freshly isolated AM adhered to culture plates were allowed to take up iron for 16 h by incubation with 55Fe-transferrin. After removal of iron-containing medium, the cells were incubated in the absence or presence of 100 ng/ml LPS for 4 h and subsequently allowed to release iron in a replenished culture medium for an additional 4 h. The amounts of 55Fe released in medium from LPS-treated and untreated cells were compared. Shown in Fig. 7A is a representative result of three experiments, which demonstrated that LPS significantly reduces iron efflux from AM. Shown in Fig. 7B is the total intracellular 55Fe before release, which is the sum of 55Fe in release medium and in cell lysate after release. No significant difference in total cellular 55Fe between untreated and LPS-treated cells was detected before release. A similar result was obtained from another experiment that measured intracellular 55Fe before release (data not shown). Therefore, the observed reduction of iron efflux in LPS-treated cells is not the result of a reduction in total cellular iron.

Cell surface FPN1 in AM decreased after LPS and HAMP treatment. The HAMP-induced inhibition of iron efflux has been shown to be associated with targeted degradation of cell surface FPN1 by HAMP in several studies. However, most of these studies either investigate the FPN1 fusion protein expressed by the transfected genes (21) or the FPN1 in cells grown in culture, such as bone marrow-derived macrophages (7). It is not known if this is also true for FPN1 in primary cells of terminally differentiated macrophages such as AM. We therefore investigated the effects of LPS and HAMP on FPN1 in freshly isolated AM by incubating the cells in the absence or presence of 100 ng/ml LPS or 700 nM of chemically synthesized HAMP for 16 h. At the end of incubation, total cell lysates were prepared and subjected to Western blot analysis.
and consequently affect iron transport in these cells. FPN1 in AM, the mature functional immune cells in the lung, both LPS and HAMP can cause the reduction of cell surface described in METHODS. The amounts of $^{55}$Fe re-

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system, the contribution of AM in iron recycling is not known. The detection of a high level of FPN1 in human and rodent AM (44) indicated that these macrophages may use the same FPN1-mediated iron-exporting pathway to release iron in less reactive forms, which may eventually be expelled from the lung (2). Indeed, searches on protein sequence databases (26) and genetic evidence (9) both suggest that FPN1 may be the sole transmembrane iron exporter in mammalian cells.

The molecular weight of the FPN1 band detected in AM (90–110 kDa) appears to be higher than that observed in the intestine and spleen but is consistent with that found in both airway epithelial cells and AM in humans (44). The identity of this band is supported by many experimental data. First, the intensity of this band increased when lung cells were exposed to iron in a dosage-dependent manner, which was in agreement with our results derived from immunohistochemical studies (44). The iron-dependent upregulation of FPN1 in lung cells was further supported at the RNA level by our data from both RT-PCR and in situ hybridization experiments (44). Furthermore, we have demonstrated in this paper that this band was downregulated when cells were treated with LPS or HAMP. FPN1 with higher-than-calculated molecular weight has been reported in other tissues, including brain (3), in which 80 and 110 kDa bands were found. Experiments using various denaturing conditions to determine if the shift in molecular weight of the FPN1 in AM is attributable to glycosylation have yielded uninterpretable results because of either resistance to deglyco-
sylation or aggregation of the protein. Interestingly, a recent study (4) demonstrated that FPN1 derived from duodenum, spleen, and liver migrated differently on SDS-PAGE and could be partly explained by posttranslational glycosylation in different tissues.

Our findings that both DMT1 and FPN1 are regulated by LPS and iron in AM further support the importance of these two transporters in iron metabolism in these cells. Although the mRNA level of DMT1 was upregulated and that of FPN1 was downregulated by LPS, the mRNA levels of both genes were increased when the cells were exposed to iron. Suppression of FPN1 synthesis by LPS indicated that iron efflux from AM could be reduced during infection or inflammation. In addition to regulating FPN1 expression at the mRNA level, LPS has been shown to indirectly regulate FPN1 at the protein level by inducing the synthesis of HAMP, which interacts with and degrades FPN1. We have found that HAMP, which is believed to be synthesized mainly by hepatocytes and released in the circulation, is also produced by AM. Our finding that HAMP

with anti-FPN1 antibody. There was no significant difference in total FPN1 levels among untreated, LPS-treated, and HAMP-treated cells (data not shown). We have shown that most of the FPN1 protein is present in intracellular compart-
ments in AM (43). If LPS and HAMP affect only the surface FPN1 in AM, a small alternation in total FPN1 may not be detectable by this method. We then analyzed the amount of both intracellular FPN1 and surface FPN1 in AM treated with and without LPS by using the techniques of surface biotinyla-
tion and avidin precipitation. The result of Western blotting shows that, although no difference was detected in intracellular FPN1 (Fig. 8A), cell surface FPN1 levels were reduced significantly (Fig. 8B) in LPS-treated cells compared with untreated cells. Similarly, surface FPN1 in HAMP-treated cells was also reduced significantly compared with that in untreated cells (Fig. 8C). This downregulation was repeatedly observed in many experiments. As shown in Fig. 8, the molecular weight of the FPN1 band in mouse AM (~90–110 kDa) is higher than that detected in the intestine and spleen but is consistent with our previous findings in human lung cells (44). The identity of this FPN1 band in AM is further supported by several findings, as documented in the DISCUSSION. Our studies demonstrated that both LPS and HAMP can cause the reduction of cell surface FPN1 in AM, the mature functional immune cells in the lung, and consequently affect iron transport in these cells.

**DISCUSSION**

Iron can be taken up by AM in several forms via different pathways. These include transferrin receptor-mediated uptake of transferrin-bound iron, CD163 receptor-mediated uptake of Hb-hepatoglobin complexes (22), and phagocytosis-mediated uptake of erythrocytes, large iron complexes, and iron-containing pollution particles. The presence of transmembrane iron importer DMT1 in AM suggested that DMT1-mediated trans-
port could be another pathway of iron uptake by these cells. The contribution of this pathway may depend on the microen-
v
vironment in the lung and the forms of iron present in alveolar fluid. We have found that uptake of iron by mouse AM in the form of $^{55}$FeCl$_3$ is ~30% efficient compared with $^{55}$Fe-transferrin (N. Nguyen, K. Callaghan, and F. Yang, unpublished results). Release of iron from AM has been demonstrated in vitro (40). Despite recent intense investigations on iron release in reticuloendothelial (RE) macrophages, including splenic macrophages and bone marrow-derived macrophages, little is known about how AM export iron. Although FPN1 has been shown to be the key iron exporter in iron recycling of the RE

![Fig. 7. Effect of LPS on iron efflux in AM. AM were allowed to take up $^{55}$Fe-transferrin, treated with LPS, and then allowed to release iron as described in METHODS. The amounts of $^{55}$Fe re-

duced iron efflux in AM. As shown in B, there is no significant difference in total intracellular $^{55}$Fe before release between untreated and LPS-treated cells. CPM, counts/min. *P < 0.05 compared with untreated cells.](http://ajplung.physiology.org/)

![Image](http://ajplung.physiology.org/)
Expression in AM is greatly induced by LPS supports the finding by others (29) that inflammation/infection-induced HAMP production could also occur at the sites of injury/infection where the innate immune cells encounter the invading microorganisms. The need for a quick response to inflammation locally in the lung raises the possibility that HAMP may act on AM themselves to efficiently shut down iron efflux from these cells.

Although only one HAMP gene exists in humans, two HAMP genes, HAMP1 and HAMP2, are found in mice. Although HAMP1 is specifically expressed in the liver, HAMP2 is strongly expressed in the pancreas (25). However, the iron regulatory activity has only been identified in HAMP1, the human homologue, when HAMP1 and HAMP2 transgenic mice were studied (25). Induction of HAMP1 but not HAMP2 in AM by LPS suggested that HAMP may play a role in lung defense against infection/inflammation by creating an iron-limited environment in lower respiratory fluid. In fact, we have shown that LPS treatment significantly reduced iron release from AM. Using the techniques of surface biotinylation and avidin precipitation, we have also demonstrated that both LPS and HAMP can target and degrade the FPN1 on the surface but not the intracellular fraction of these cells. Our observations appeared to be different from what was found in other types of macrophages. First, a decrease in the total FPN1 level after LPS treatment was detected in other types of macrophages (7, 21) but not in AM even though FPN1 mRNA was downregulated in these cells. Second, downregulation of surface FPN1 in AM did not appear to be as prominent as reported in other systems (7, 21). In addition, >4–8 h exposure were needed to detect the alteration in surface FPN1 level in AM. It is not clear why FPN1 in AM behaves differently from that in other types of cells. Possible explanations may include differences in subcellular localization and/or posttranslational modification such as glycosylation, which could affect the stability of FPN1. Nevertheless, the effect of LPS and HAMP on surface FPN1 in AM has been observed consistently in all of the experiments conducted. In our previous publication (24), we reported that both splenic and peritoneal macrophages from mice also produce HAMP upon LPS stimulation and is likely to directly affect the iron pool in the body independent of hepatic HAMP. HAMP production may therefore be characteristic for all cells in the macrophage lineage. Nevertheless, we have found that HAMP mRNA was induced by LPS at a much higher level in AM than in the other two types of tissue macrophages studied. Based on HAMP-to-GAPDH mRNA ratios in LPS-treated cells, the HAMP mRNA level in AM was 100-fold higher than that in splenic macrophages and 10-fold higher than that in peritoneal macrophages (N. Nguyen, K. Callaghan, and F. Yang, unpublished results). Although the effect of HAMP on iron efflux in different lung cells other than AM awaits investigation, this finding further supports the importance of both HAMP and iron homeostasis in lung defense.

Hepatic expression of HAMP is upregulated during inflammation but downregulated during iron overload (19, 37). Contrary to in vivo observations, HAMP in primary hepatocytes and HepG2 cells appeared to be downregulated by iron (15, 33). In our experiments, exposure of freshly isolated AM to iron had no effects on HAMP expression, although iron was able to upregulate both DMT1 and FPN1. This is not surprising but consistent with the iron-detoxification function of AM, which can take up catalytically active iron and most likely release iron in less toxic protein-bound forms such as transferrin and ferritin (40). Upregulation of DMT1 and FPN1 but not HAMP in AM would be desirable under the condition of...
iron overload in alveoli. Our previous work also demonstrated that iron did not induce HAMP synthesis in mouse splenic macrophages in culture (24). Apparently, HAMP expression may be regulated differently in different cells/tissues. This is also reflected in its response to inflammatory cytokines. Although HAMP synthesis has been shown to be regulated by IL-1 and IL-6 in hepatocytes (20, 23, 31), none of the cytokines in our studies, including IL-1, IL-6, TNF-α, and interferon-γ, had a significant effect on the expression of HAMP in AM. Interestingly, DMT1 and FPN1, both regulated by LPS, were also not affected by these cytokines. Furthermore, LPS-induced alteration of HAMP, DMT1, and FPN1 gene expression was not different between AM derived from wild-type mice and mice deficient in IL-1 receptor or IL-6. Similar results were obtained in IL-6 plus NOS double-knockout mice compared with wild-type mice (data not shown). Although this study did not include NOS single-knockout mice, our results suggest that nitric oxide is not likely involved in the regulation of these genes either. The response of these genes to LPS was greatly reduced in Toll-like receptor 4 (TLR4)-deficient mice (data not shown), but the mechanism(s) involved in LPS-induced regulation of these genes is not clear. Our studies, however, suggest that DMT1, FPN1, and HAMP genes may be regulated by a similar pathway(s) in AM during an inflammatory response.

In summary, we have shown that the two key iron transporters DMT1 and FPN1 were regulated by LPS and iron in AM. Upon exposure to LPS, HAMP gene expression was greatly induced, which can potentially act on AM themselves to effectively degrade surface FPN1 and inhibit iron efflux from the cells. HAMP produced by AM can also affect iron transport in other lung cells and thus play a key role in iron homeostasis in the lung during infection/inflammation. Because iron imbalance can affect lung defense against infection and lead to iron-mediated oxidative injuries, lung cells may have their own regulatory pathways to achieve iron homeostasis.

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REFERENCES


