A Role for the ATP7A Copper-transporting ATPase in Macrophage Bactericidal Activity*

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Copper is an essential micronutrient that is necessary for healthy immune function. This requirement is underscored by an increased susceptibility to bacterial infection in copper-deficient animals; however, a molecular understanding of its importance in immune defense is unknown. In this study, we investigated the role of proinflammatory agents on copper homeostasis in RAW264.7 macrophages. Interferon-γ was found to increase expression of the high affinity copper importer, CTR1, and stimulate copper uptake. This was accompanied by copper-stimulated trafficking of the ATP7A copper exporter from the Golgi to vesicles that partially overlapped with phagosomal compartments. Silencing of ATP7A expression attenuated bacterial killing, suggesting a role for ATP7A-dependent copper transport in the bactericidal activity of macrophages. Significantly, a copper-sensitive mutant of Escherichia coli lacking the CopA copper-transporting ATPase was hypersensitive to killing by RAW264.7 macrophages, and this phenotype was dependent on ATP7A expression. Collectively, these data suggest that copper-transporting ATPases, CopA and ATP7A, in both bacteria and macrophage are unique determinants of bacterial survival and identify an unexpected role for copper at the host-pathogen interface.

Copper is an essential nutrient for aerobic organisms. Its ability to exchange electrons as it cycles between cuprous and cupric states has been harnessed by enzymes that catalyze a wide variety of biochemical processes (1). However, this same redox activity also confers copper with toxic properties when it is present in the free ionic form. Free copper can participate in Fenton-like chemistry to produce the highly toxic hydroxyl radical from hydrogen peroxide and superoxide (2–4). It is therefore not surprising that organisms have evolved tightly regulated mechanisms for copper transport and intracellular distribution.

Copper is among several micronutrients found to be important for both innate and adaptive immunity (5, 6). Copper deficiency decreases interleukin-2 secretion in T cells (7), and recent studies have demonstrated a direct role for copper in the secretion of interleukin 1-α and fibroblast growth factor-1 (8). Various studies have shown that copper-deficient animals are susceptible to several pathogens, including Candida albicans (9, 10), Pasteurella hemolytica (11), Trypanosoma lewisi (12), and Salmonella typhimurium (13). Other studies have shown that copper supplementation is protective against Escherichia coli-induced mastitis in dairy cattle (14). In vitro studies have shown that copper deficiency impairs the bactericidal activity of neutrophils and macrophages (15, 16). Moreover, a recent study demonstrated that copper concentrations are markedly increased within the phagosomal compartment of macrophages activated by interferon-γ (IFN-γ)3 and Mycobacterium avium (18). Although these studies provide indirect evidence of a role for copper in immune defense, an understanding of the underlying processes at a molecular level is lacking.

Here we report dramatic changes in copper homeostasis triggered by proinflammatory agents in macrophages, a phagocytic cell type of the innate immune system. These changes include elevated expression of the CTR1 copper importer, increased copper uptake, and copper-stimulated trafficking of the ATP7A copper transporter from the Golgi to cytoplasmic vesicles that overlap with the phagosomal compartment. Significantly, silencing of ATP7A impaired bactericidal activity, suggesting that copper transport via ATP7A was required for bacterial killing. Consistent with this role, a copper-sensitive mutant of E. coli with a defect in copper export was significantly more sensitive to macrophage-mediated killing than wild type cells, and this sensitivity was dependent on ATP7A expression. These findings suggest that copper-transporting ATPases present within both macrophage and bacteria are novel determinants of this host-pathogen interaction.

EXPERIMENTAL PROCEDURES

Cell Culture—RAW264.7 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and 100 units/ml penicillin and streptomycin (Invitrogen) in 5% CO2 at 37 °C. ATP7A-silenced RAW264.7 cells were generated by stable transfection of a pRS vector harboring a 29-nucleotide short hairpin RNA against ATP7A (OriGene, Rockville, MD) and selected in 25 μg/ml puromycin.

3 The abbreviations used are: IFN-γ, interferon γ; LPS, lipopolysaccharide; RNAi, RNA interference; TTM, tetrathiomolybdate.
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(Invitrogen). Control cells were transfected with the same vector expressing short hairpin RNA against green fluorescent protein (Origene). The Rab7-Q67L plasmid was transiently transfected into RAW264.7 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Copper Uptake Studies—$^{64}$Cu uptake was assayed as described previously with minor modifications (19). RAW264.7 macrophage cells in triplicate 6-well trays were cultured overnight in 1 ml of serum-free medium with or without the indicated concentrations of IFN-γ (BD Biosciences) or LPS (Sigma). Cells were then incubated with serum-free medium containing 1 μM $^{64}$Cu for 5 min, washed extensively in ice-cold phosphate-buffered saline, and scraped into phosphate-buffered saline. Radioactivity was measured using a γ counter, and counts were determined per total protein concentration.

Bacterial Survival Assays—RAW264.7 cells alone or transfected with control RNAi or ATP7A RNAi plasmids were activated by overnight treatment in serum-free medium containing 25 ng/ml IFN-γ. Where indicated, either 20 μM CuCl₂ or 50 μM ebselen was also added to the cells. Cells were then detached by scraping into ice-cold serum-free medium, washed twice, and resuspended in triplicate in serum-free medium. Wild type E. coli (strain W3110) or the copA deletion strain (DW3110) (20, 21) were grown to stationary phase and added to the RAW264.7 macrophages at a macrophage/bacteria ratio of 1:10 or 1:1. Bacterial phagocytosis was allowed to proceed for 30 min at 37 °C, and the extracellular bacteria were removed by two washes with phosphate-buffered saline containing 12.5 μg/ml gentamicin (Invitrogen). One set of samples (uptake group) was lysed in 0.1% Triton X-100 solution and plated onto LB-agar plates for colony counting to provide bacterial uptake values. The remaining set (kill group) was incubated for 1 or 2 h (as indicated) at 37 °C in serum-free medium to allow bacterial killing to occur, lysed, and then plated onto LB-agar. Colony numbers were normalized against the total protein content of each sample, and bacterial survival was determined by dividing the number of colonies in the killing group by those in the uptake group.

In Vitro Bactericidal Assay—The in vitro survival assay was performed as previously described (22). Briefly, exponential phase cultures of E. coli were pelleted and resuspended in 1 ml of assay buffer (0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.5 mM ascorbic acid to act as a reducing agent) in the presence or absence of 20 μM copper or 50 μM copper prior to exposure to E. coli for 30 min to allow phagocytosis to occur. After removal of extracellular bacteria, bacterial survival was measured following incubations for 1 or 2 h at 37 °C and expressed as a percentage of initial internalized E. coli (mean ± S.D.; n = 3; p < 0.05, Student’s t test). Bacterial survival rates were calculated and expressed as percentage survival compared with the untreated group (mean ± S.D.; n = 3; p < 0.05). Different letters signify values of significant difference (Tukey’s post hoc analysis). A bacterial survival assay was assayed after 2 h in IFN-γ-treated RAW264.7 macrophages in the presence or absence of 20 μM copper and a 50 μM concentration of the antioxidant ebselen (mean ± S.D.; n = 3; p < 0.05, one- and two-way analysis of variance). Different letters signify values of significant difference (Tukey’s post hoc analysis).

Ceruloplasmin Activity—Conditioned medium was concentrated using Amicon Ultra-4 filter tubes (Millipore), and ceruloplasmin activity was assayed as described previously (25).

RESULTS

Copper Enhances the Bactericidal Activity of Macrophages—We began this study by testing whether copper supplementation to the growth medium of the murine macrophage cell line,
RAW264.7, might alter its bactericidal activity. Cells were stimulated overnight with the proinflammatory cytokine, IFN-γ, in the presence or absence of 20 μM copper, and their ability to kill E. coli was determined the following day. In the RAW264.7 macrophages pretreated with copper, bacterial survival was significantly reduced relative to untreated cells following either a 1- or 2-h incubation (Fig. 1A). There was no effect of copper on the uptake of bacteria by RAW264.7 cells (data not shown). These findings suggest that copper can potentiate the bactericidal activity of RAW264.7 cells. Previous in vitro studies have demonstrated that bacteria are highly sensitive to low concentrations of Cu⁺ in the presence of hydrogen peroxide (H₂O₂) (22), and we confirmed these findings in our study (Fig. 1B). These data underscored the possibility that the enhanced bactericidal activity conferred by the copper treatment of macrophages may be dependent on reactive oxygen species. This hypothesis was supported by the finding that pretreating the RAW264.7 macrophage cells with ebselen, a cell-permeable glutathione peroxidase mimetic, prevented the copper-mediated increase in bactericidal activity (Fig. 1C). Ebselen treatments did not alter the rate of phagocytosis of E. coli compared with control RAW264.7 cells (data not shown). Taken together,

**FIGURE 2. Proinflammatory agents stimulate copper uptake and CTR1 expression in RAW264.7 cells.** A, copper uptake activity. RAW264.7 cells were pretreated with serum-free medium with or without IFN-γ or LPS at the indicated concentrations for 24 h, and copper uptake in the presence of 1 μM ⁶⁴Cu was measured over 5 min and expressed per total protein concentration (mean ± S.D.; n = 3; *, p < 0.05, Student’s t test). B, Western blot analysis of CTR1 protein levels in total cell lysates of RAW264.7 macrophage cells exposed for 24 h to a range of IFN-γ and LPS concentrations. Relative band intensities against tubulin are shown in parentheses.

**FIGURE 3. Proinflammatory agents stimulate ATP7A expression in RAW264.7 cells.** Western blot analysis of ATP7A protein levels in total cell lysates of RAW264.7 macrophage cells exposed for 24 h to a range of IFN-γ (A) and LPS concentrations (B). Relative band intensities against tubulin are shown in parentheses.

**FIGURE 4. Lipopolysaccharide and IFN-γ induce trafficking of ATP7A in RAW264.7 cells.** Shown is immunofluorescence analysis of ATP7A protein in RAW264.7 cells grown for 24 h in the presence of 100 ng/ml LPS, 25 ng/ml IFN-γ, or 20 μM CuCl₂. Cells were fixed, permeabilized, and probed with antibodies against ATP7A and anti-rabbit antibodies conjugated to Alexa-488 (green) or antibodies against the Golgi marker GM130 and anti-mouse antibodies conjugated to Alexa-594 (red). Nuclei were labeled with 4’,6-diamidino-2-phenylindole (blue).
these findings suggest that copper can promote the bactericidal activity of RAW264.7 macrophages in a reactive oxygen species-dependent manner.

**Inflammatory Mediators Induce the Expression of CTR1 and ATP7A Copper Transporters**—Since copper was found to potentiate the bactericidal activity of RAW264.7 cells, we investigated whether copper homeostasis might be altered in these cells following stimulation with proinflammatory agents. Treatment of RAW264.7 macrophages with the proinflammatory agents, IFN-\(\gamma\) or LPS, stimulated copper uptake activity in a dose-dependent manner (Fig. 2A). Immunoblot analysis indicated that these effects of IFN-\(\gamma\) and LPS were associated with a dose-dependent increase in the expression of the copper importer, CTR1 (Fig. 2C). Taken together, these findings suggest that the inflammatory response of macrophages stimulates CTR1-mediated copper uptake.

We then investigated the effect of proinflammatory agents on another copper transporter, ATP7A, a copper-transporting P-type ATPase responsible for delivering copper from the cytoplasm into secretory compartments. Immunoblot analysis demonstrated that both IFN-\(\gamma\) and LPS treatments increased the expression of ATP7A protein in RAW264.7 cells (Fig. 3, A and B). A closely related copper transporter to ATP7A, known as ATP7B, was not expressed in these cells (data not shown). Taken together, these results suggest that the stimulation of macrophages by LPS or IFN-\(\gamma\) promotes copper uptake and increases expression of CTR1 and ATP7A proteins.

**IFN-\(\gamma\) and LPS Stimulate Copper-dependent ATP7A Trafficking to Post-Golgi Vesicles That Overlap with the Phagosome**—Previous studies have demonstrated that elevated intracellular copper levels stimulate the trafficking of the ATP7A protein from the trans-Golgi network to post-Golgi vesicles in a variety of cell types (26–29). Given that IFN-\(\gamma\) or LPS stimulated copper uptake and increased cellular copper levels, we surmised that these proinflammatory agents might also stimulate ATP7A trafficking. Using immunofluorescence microscopy, the ATP7A protein was identified in the perinuclear region of untreated RAW264.7 macrophages, consistent with its location in the trans-Golgi network (Fig. 4, top panels). As expected, the addition of copper to the culture medium stimulated the trafficking of ATP7A to post-Golgi vesicles (Fig. 4, second from top panels). Interestingly, when RAW264.7 cells were exposed to IFN-\(\gamma\) or LPS without copper supplementation, the ATP7A protein was also distributed to post-Golgi vesicles, although these punctae were not as clearly associated with the cell periphery as in the case of copper treatment (Fig. 4, third and fourth from top panels). The location of the Golgi matrix marker protein, GM130, was not altered by IFN-\(\gamma\) or LPS, suggesting that the shift in ATP7A distribution was not the result of general Golgi disruption (Fig. 4). Because the trafficking of ATP7A is known to be responsive to copper, we examined whether these effects of IFN-\(\gamma\) could be inhibited using the membrane-permeable copper chelator tetrathiomolybdate.
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The treatment of RAW264.7 cells with TTM blocked IFN-γ-stimulated trafficking of ATP7A, consistent with a role for copper in this trafficking process (Fig. 5A). Importantly, Western blot analysis of these same cells demonstrated that the increased expression of ATP7A in response to either IFN-γ or LPS was not blocked by TTM (Fig. 5B). This finding indicated that the inhibition of trafficking by TTM was not due to suppression of ATP7A expression. In support of this conclusion, ATP7A trafficking was apparent within 3 h of IFN-γ treatment (Fig. 6A), whereas the increases in ATP7A protein expression required a longer time period (Fig. 6B).

We then examined whether IFN-γ might trigger the trafficking of ATP7A to the phagosomal compartment. The phagocytosis of latex beads was used to identify phagosomes in IFN-γ-stimulated RAW264.7 cells (Fig. 7A, Bright field), and ATP7A was then detected in the same cells using immunofluorescence microscopy (Fig. 7B). There was clear labeling of ATP7A outlining the periphery of compartments that also contained the internalized latex beads, suggesting the presence of ATP7A within phagosomes in IFN-γ-treated cells (Fig. 7B). In separate experiments, phagosomal membranes were purified from IFN-γ-treated RAW264.7 cells by subcellular fractionation of membranes containing the internalized latex beads. Immunoblot analysis of this fraction demonstrated a marked enrichment of the phagolyosomal marker LAMP-1 relative to total cell lysates, as well as abundant levels of ATP7A protein (Fig. 7C). The phagosomal fraction lacked the Golgi marker protein, GM130, indicating that the detection of ATP7A in these fractions was not an artifact of Golgi contamination (Fig. 7C). Further studies investigated whether ATP7A partially colocalized with a constitutively active mutant of Rab7, a GTPase involved in late endosome fusion and required for lysosomal biogenesis (30–32). The constitutively active mutant Rab7-Q67L is useful for co-localization studies because it results in enlarged lysosomal structures (30). RAW264.7 cells were transiently transfected with green fluorescent protein-tagged Rab7-Q67L and then exposed for 24 h to basal medium or medium containing 25 ng/ml IFN-γ. As shown in Fig. 7, in untreated cells there was little overlap between the perinuclear location of ATP7A and Rab7-Q67L-containing compartments (Fig. 7D). However, in cells stimulated with IFN-γ, the ATP7A protein was found to partially overlap with Rab7-Q67L-containing compartments (Fig. 7D). Taken together, these studies suggest that IFN-γ activation of macrophages is associated with partial redistribution of ATP7A to the phagolysosomal compartment.

ATP7A-dependent Copper Transport Is Required for Bactericidal Activity of RAW264.7 Macrophages—The above findings highlight the possibility that ATP7A-dependent copper transport into the phagosome might be important for macrophage microbiocidal activity. To test this hypothesis, we investigated the effect of RNAi-mediated depletion of ATP7A on bacterial killing in RAW264.7 cells. Stable transfection of a 29-nucleotide short hairpin RNAi plasmid against ATP7A resulted in robust silencing of ATP7A gene expression (ATP7A RNAi cells), relative to control cells transfected with an irrelevant RNAi against green fluorescent protein (Fig. 8A). We then confirmed that ATP7A-dependent copper transport was suppressed in the ATP7A RNAi cells by testing the activity of copper-dependent enzyme, ceruloplasmin, a ferroxidase whose expres-

FIGURE 7. IFN-γ stimulates partial trafficking of the ATP7A protein to the phagosomal compartment in RAW264.7 macrophages. A, partial co-localization of ATP7A with phagosomal membranes. RAW264.7 macrophages were stimulated overnight with IFN-γ and allowed to phagocytose latex beads to allow identification of the phagosomal compartments (Bright field). B, ATP7A was detected by immunofluorescence microscopy in the same cells as in A. C, co-fractionation of ATP7A with the phagosomal membrane marker Lamp-1. IFN-γ-stimulated RAW264.7 cells were allowed to phagocytose latex beads, and the phagosomal compartment was isolated via subcellular fractionation. The bead fraction and total protein fraction were subjected to SDS-PAGE. Immunoblot analysis revealed abundant ATP7A in Lamp-1-positive bead fraction, which lacked the Golgi marker protein GM130. Note that Lamp-1 was poorly detected in total lysates. D, partial co-localization of ATP7A with Rab7-Q67L in IFN-γ-stimulated RAW264.7 cells. Cells were transiently transfected with green fluorescent protein-tagged Rab7-Q67L plasmid and then exposed for 24 h to basal medium or medium containing 25 ng/ml IFN-γ. Cells were fixed, permeabilized, and probed with antibodies against ATP7A followed by anti-rabbit antibodies conjugated to Alexa-594.
exogenous copper, indicating that the reduced bacterial killing was attributable to a copper transport defect rather than an unrelated defect in the ATP7A-depleted cells (Fig. 8D). These findings support the hypothesis that ATP7A-dependent copper transport into the phagosome is important for the bacterial activity of macrophages.

We further explored the role of copper homeostasis in bacterial killing by testing whether the ability of _E. coli_ to export copper might influence its susceptibility to killing by RAW264.7 macrophages. The ΔcopA mutant strain of _E. coli_ is hypersensitive to elevated copper concentrations due to the role of the CopA protein in the extrusion of copper from the cytoplasm (33). The CopA protein is a copper-transporting P-type ATPase belonging to the same subfamily as the ATP7A protein. The ΔcopA mutant was markedly more susceptible to killing by RAW264.7 macrophages compared with its parental wild type _E. coli_ strain (Fig. 9A). Significantly, the susceptibility of the ΔcopA strain was dependent on ATP7A protein in the macrophage host because its survival was increased in ATP7A RNAi cells relative to control cells (Fig. 9B). Collectively, these data suggest that bacterial CopA and mammalian ATP7A are unique determinants of macrophage-dependent bacterial killing and identify an unexpected role for copper in host-pathogen interactions.

**DISCUSSION**

Copper has long been recognized as a biocidal agent. Its modern day use in algacides, fungicides, antimicrobial surfaces, and medical devices can be traced back to its early use in the treatment of wounds by ancient Egyptian and Roman civilizations (34). In this study, we identify for the first time that specific changes in macrophage copper homeostasis mediate bacterial killing. Macrophage activation by proinflammatory agents triggered increased copper uptake activity and a striking copper-dependent relocalization of the ATP7A protein to a vesicular distribution, which partially overlapped with the phagosomal compartment. In support of this trafficking event, a recent analysis of the macrophage phagosomal proteome identified ATP7A in this compartment (35). Significantly, macrophage-mediated bacterial killing was dependent on the expression of the ATP7A copper transporter, as evidenced by the
attenuated bactericidal activity when ATP7A was silenced. These findings suggest that copper transport into the phagosome via the ATP7A protein is a novel determinant of bacterial killing by macrophages. Consistent with this hypothesis, recent studies using x-ray microprobe analysis have demonstrated that copper levels within the phagosome increase 10-fold to ~180 μM in IFN-γ-stimulated macrophages exposed to M. avium (18).

How might this enrichment of copper promote bacterial killing by macrophages? One possibility is the copper-catalyzed production of the hydroxyl radical from hydrogen peroxide via Fenton-like chemistry. Although hydrogen peroxide is generated spontaneously from superoxide created by the respiratory burst, it is only lethal to bacteria at supraphysiological millimolar concentrations (36). However, physiological concentrations of hydrogen peroxide and cuprous ions are lethal to E. coli (22). Iron-mediated hydroxyl radical production is also toxic to bacteria in vitro (37); however, a role for iron in macrophage bactericidal activity is unlikely because iron accumulation in the phagosome is decreased in IFN-γ-stimulated macrophages, and iron treatment of macrophages reduces bactericidal activity (38). These findings, together with those of our study, suggest that a lethal combination of copper and hydrogen peroxide may be the underlying mechanism by which ATP7A-dependent copper transport into the phagosome promotes bacterial killing.

An important finding of our study was that the copper-sensitive ΔcopA mutant of E. coli was more susceptible to macrophage-mediated killing than the wild type strain. Because the CopA protein functions in the extrusion of cytoplasmic copper across the plasma membrane (33), these findings suggest that copper export is a bacterial defense mechanism against macrophage-mediated killing. Consistent with this hypothesis, the susceptibility of the ΔcopA mutant to killing was ameliorated by depletion of ATP7A in RAW264.7 macrophage cells. These findings suggest that copper transport by both host and pathogen is a unique and mutually opposing tactic in the struggle for supremacy; i.e. ATP7A-mediated copper transport into the phagosome appears to be countered by copper extrusion by the bacterium. Indeed, there is evidence to suggest that copper export might be a defense strategy that is generally applicable to other pathogenic bacteria. For example, the virulence of Pseudomonas aeruginosa in mice is severely decreased (20-fold) by mutations in the copper exporter, CueA (39). Additionally, the expression of several metal ion exporters, including two putative copper efflux transporters, CopA1 and CopA2, are induced in Legionella pneumophila upon phagocytosis by macrophages (40). Moreover, plasmids that increase the virulence of Klebsiella pneumoniae and Shigella sonnei harbor putative copper resistance genes (41, 42).

In light of our findings, it is notable that bacterial infections, especially those of the respiratory tract, are commonly reported in infants with Menkes disease, in whom genetic mutations in the ATP7A gene give rise to copper deficiency (43–46). However, it is uncertain whether this susceptibility to infections arises from defects in ATP7A-dependent activities of macrophages or impairment of other copper-requiring processes. Deciphering the role of ATP7A in innate immunity in vivo will require myeloid-specific ATP7A knock-out mice, which we are currently generating.

It is notable that changes in the serum concentration of micronutrients following infection are characteristic of the acute phase inflammatory response. Serum levels of iron, zinc, selenium, retinol, riboflavin, and pyridoxine are all reported to decrease following inflammatory insult (47). The proposed physiological rationale for these changes is nutrient deprivation to limit proliferation of the invading microbe (47). A well documented example is the contest between host and pathogen over limiting iron stores, which plays a critical role in determining the outcome of infection (48). In contrast, however, systemic copper concentrations are widely reported to increase in response to acute and chronic inflammation (49–65). Although the secreted copper-containing protein, ceruloplasmin, is partially responsible for this rise in serum copper (66), the non-protein-bound fraction of copper in the serum is also increased during inflammation (49). Moreover, radiotracer studies with 64Cu have demonstrated that copper accumulates at sites of inflammation (67) and within the exudates of wounds and burns where there exists an abundance
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of macrophages (68, 69). Our findings underscore the possibility that these changes in systemic copper concentrations may provide localized reserves of copper for macrophage-mediated bactericidal activity.

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