INFLAMMATION AND HYPOXIA: NOVEL REGULATORS

OF MAMMALIAN COPPER HOMEOSTASIS IN

MACROPHAGES

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by

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presented by CARINE WHITE,

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LIST OF IMPORTANT ABBREVIATIONS

BCS: bathocuproine disulfonate

CCO: Cytochrome c oxidase

CCS: Copper chaperone for superoxide dismutase

COX1: Cytochrome c oxidase subunit 1

Cp: Ceruloplasmin

CTR1: Copper transporter 1

Cu: Copper

Cu/Zn SOD: Copper/Zinc superoxide dismutase

DAPI: 4',6-Diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle's Medium

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

ER: Endoplasmic reticulum

Fe: Iron

Fp: Ferroportin

H_2O_2: Hydrogen peroxide

HAEC: Human aortic endothelial cell

HIF-1α: Hypoxia-inducible factor alpha

IFN-γ: Interferon gamma

IMS: Intermembrane space

iNOS: Inducible nitric oxide synthase

LOX: Lysyl oxidase

LPS: Lipopolysaccharide

MBD: Metal binding domain

MNK: Menkes

MT: Metallothionein

NAC: N-acetyl cysteine

NO: Nitric oxide

O_2: Oxygen

$O^{\cdot}$: Superoxide

$OH^{\cdot}$: Hydroxyl radical

PBS: Phosphate buffered saline

ROS: Reactive oxygen species

RT-PCR: Real-time polymerase chain reaction

SF media: Serum-free media

SLC: Solute-linked carrier

SMC: Smooth muscle cell

Tf: Transferrin

TGN: Trans-Golgi network

TMD: Transmembrane domain

TPEC: Thioglycollate-elicited peritoneal exudate cell

TTM: Tetrathiomolybdate

WND: Wilson Disease

Zn: Zinc

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CHAPTER 1

INTRODUCTION

1.1 Copper: an essential yet potentially toxic nutrient

Copper (Cu) is an essential micronutrient and contributes to various enzymatic

processes involved in health, development and disease conditions. As a

transition metal, Cu can exist in two oxidation states, Cu^{1+} or Cu^{2+} and thus

serves as an important structural component and as a cofactor to numerous

enzymes that catalyze oxygen utilization, electron transfer, and hydrolytic

reactions. These copper-dependent enzymes include cytochrome c oxidase

(CCO), the last enzyme involved in electron transfer and oxygen utilization at

complex IV of the mitochondrial respiratory chain; copper/zinc superoxide

dismutase (Cu/Zn SOD), involved in antioxidant defense; lysyl oxidase, essential

for the cross-linking of elastin and collagen fibrils in connective tissue; dopamine

β hydroxylase; required for the synthesis of catecholamines; and tyrosinase

enzyme, involved in pigmentation (1). Imbalances in mammalian Cu homeostasis

can lead to defects in connective tissue formation, loss of pigmentation,

neurological disorders, toxicity and others. Excess Cu causes toxicity due, in

part, to the ability of Cu to non-specifically displace other metal ions from

enzymes, leading to loss of enzyme activity (2). Excess Cu is also toxic due to its

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ability to generate reactive oxygen species (ROS). The redox activity of the Cu

ion, although essential for its function as a cofactor for several enzymes, also

allows it to participate in the Fenton reaction and to generate the highly

damaging hydroxyl radical (OH$^{\bullet}$), thus increasing the need for a tight regulation

of copper homeostasis (2).

1.2 Dietary copper sources

The dietary reference intakes of Cu in adults are between 0.7 mg/day and 1.3

mg/day with potential toxic intakes levels set at doses exceeding 5 mg/day. The

total body Cu content for an adult human varies between 70 mg-100 mg (3-6).

The highest concentration of Cu in organs of healthy adult individuals is in the

liver, kidneys, brain, heart, and gastrointestinal tract, with the lowest

concentrations in the spleen and lungs (3-6). Most of the daily requirements for

copper in humans and animals are met by dietary intake. Foods rich in copper

include organ meat, especially liver and kidneys, shellfish, nuts, grains and

chocolate. Drinking water contaminated by copper pipes is also a significant

source of dietary copper (3-6).

1.3 Copper deficiency.

1.3.1 Acquired copper deficiency.

Copper deficiency has two possible etiologies, acquired or genetic. In humans, acquired copper deficiency is a known cause of sideroblastic anemia, and has been increasingly associated with neurological degeneration (7-14). Acquired copper deficiency in humans is rare, owing to copper's ubiquitous distribution and low daily requirements (15). Known causes of acquired copper deficiency include excess zinc ingestion, use of copper chelators, malabsorption, nephritic syndrome, copper-deficient parenteral nutrition and enteral feeding, and gastrectomy (15, 16). Copper deficiency may also occur in premature and malnourished infants (15, 16).

The anemia associated with copper deficiency can be attributed to impaired iron absorption, reduced iron transfer from the reticuloendothelial system to the circulation and decreased cytochrome c oxidase activity in the mitochondria (17). Iron homeostasis is severely affected by copper deficiency, mainly owing to the important role of the copper-dependent ferroxidases hephaestin and ceruloplasmin in iron uptake and tissue distribution. Hephaestin, which is expressed in the duodenal mucosa, oxidizes ferrous iron to the ferric form, thus facilitating its transfer across the basolateral membrane for loading onto apo-transferrin in the blood (17, 18). On the other hand, oxidation of iron by

ceruloplasmin facilitates its transfer from reticuloendothelial cells to apo-transferrin in the circulation (17, 18). The anemia associated with copper deficiency is associated with the formation of ring sideroblasts owing to the accumulation of iron in the mitochondria (19). This is due to the suppressed activity of cytochrome c oxidase, resulting in insufficient generation of reducing equivalents necessary to reduce mitochondrial iron from the ferric to ferrous state (20, 21). Since only ferrous iron can by used by ferrochelatase for heme synthesis (22, 23), the defect in cytochrome c oxidase activity ultimately results in intramitochondrial iron accumulation and consequently in increased production of reactive oxygen species (ROS) and damage to the mitochondrial DNA (24, 25). The hematological manifestations caused by copper deficiency are quickly reversed by copper supplementation (16, 26, 27).

Copper is an important cofactor in several enzymatic processes important for the function of the central nervous system. These include cytochrome c oxidase, copper-zinc superoxide dismutase (SOD1), and dopamine ß-hydroxylase (1). The association of neurological symptoms with copper deficiency has been increasingly recognized over the past decade (9, 10, 13, 14, 27, 28). The most common neurological symptom is ataxic myelopathy with or without neuropathy (7, 9-14, 16, 27-30). This clinical picture is similar to the progressive ataxic myelopathy or "swayback" in ruminant animals fed copper-deficient diets. Other neurological disorders associated with copper deficiency are central nervous system demyelination (13), peripheral neuropathy (31), and optic neuropathy

(31). Acute optic myeloneuropathy is a recognized adverse side effect of the copper-zinc chelator clioquinol (32). Correction of the hypocupremia in acquired copper deficiency usually arrests the neurological deterioration and may result in clinical improvement (11, 16, 26, 27).

1.3.2 Genetic copper deficiency – Menkes disease.

Despite the recent recognition of the neurological manifestations of acquired copper deficiency, there exists a well-documented precedent in the clinical syndrome Menkes disease. Menkes disease is an X-linked inherited disorder of copper malabsorption which develops in infancy and results in various neurological and systemic symptoms (33-42). In 1972, David Danks made the critical connection between the kinky/steely hair in Menkes patients and that of lambs grazing on grass grown in copper-depleted soil (38, 39). His research provided the first evidence that Menkes patients had abnormally low serum copper levels, while biopsies from patients' guts revealed unusually high levels of accumulated copper, establishing the fact that a defect in copper transport was the underlying cause of Menkes disease. This work finally led to the identification and cloning of the Menkes gene (43).

The Menkes gene codes for the Cu transporting P-type ATPase, ATP7A, also known as MNK protein (43-45). ATP7A is ubiquitously expressed in the human body except in the liver, where the related ATP7B or Wilson protein is expressed

(discussed later). ATP7A is essential for the intestinal uptake of copper and its delivery to the circulation (Fig. 1.1). Menkes disease is characterized by reduced transport of dietary copper across the basolateral membrane of intestinal enterocytes to the hepatic portal circulation, resulting in the hyperaccumulation of copper within the intestinal mucosa and the deficiency of copper in the circulation and peripheral organs (38). This copper deficiency is further exacerbated by reduced copper transport into the central nervous system across the blood brain barrier (38). Furthermore, copper transport across fetally derived placental cells is also impaired, resulting in defective transport of copper from the mother to the Menkes fetus during gestation (46, 47). The incidence of Menkes disease has been reported to vary between 1:50,000 to 1:100,000 (36). The copper deficiency symptoms associated with Menkes disease are exhibited starting the second or third month of life. These symptoms include skin laxity, hypopigmentation, kinky/steely hair, neurological impairment and convulsions (46). Autopsies of Menkes patients have revealed demyelination in spinal cord white matter, reminiscent of that observed in copper-deficient ruminants suffering from "swayback" (48). The copper-dependent enzyme lysyl oxidase is important in the cross-linking of collagen and elastin (49), and its deficient activity in Menkes disease results in vascular and microvascular system abnormalities (50). These include tortuous blood vessels, particularly affecting the blood flow in the brain (51).

Another significant defect of Menkes disease is immune system dysregulation.

Menkes patients are prone to infections, particularly of the lungs and urinary tract (52-55). Copper is critical for the function of the immune system (discussed later) (56) however, it is unknown whether the immune deficiencies in Menkes patients may be attributed to a direct role for ATP7A in immune defense. Most Menkes patients die during early childhood, however mild allelic forms of the disease exist, such as occipital horn syndrome (57) and mild Menkes disease, which are caused by a similar abnormality in copper metabolism but differ in clinical presentation and survival potential (34, 58-63). OHS is characterized by prominent protuberance of the occipital bone, which is absent in classical Menkes disease. Other OHS symptoms include skeletal abnormalities and connective tissue abnormalities, including skin laxity and blood vessel tortuosity (62, 63). OHS patients exhibit borderline average intelligence and often survive until adulthood. Mild Menkes disease patients typically lack the connective tissue defects of classical Menkes disease and present with neuro-developmental delays and mild ataxia (34, 58-61).

Despite studies identifying the mutations affecting the Menkes gene in each variation of the disease, the biochemical basis for this clinical diversity is still unknown. The current accepted treatment for Menkes disease is parenteral administration of copper (*i.e.* intravenous copper injections). When initiated in newborn Menkes disease babies, parenteral copper nutrition can prevent neurological degeneration in some, but not all, cases. Unfortunately, if treatment is started in babies with classical Menkes disease above the age of 2 months, it

does not improve the neurological degeneration. Moreover, early copper

parenteral administration does not improve the non-neurological defects such as

connective tissue laxity. Further studies are needed to develop alternative

therapies for Menkes disease and occipital horn syndrome (64-66).

FIGURE 1.1 **The ATP7A protein is critical for the entry and distribution of dietary copper.** Copper transport across the basolateral membrane of enterocytes occurs via the ATP7A protein. ATP7A is required to transport copper across the blood-brain barrier, and across the placenta. The locations of copper transport pathways mediated by ATP7A are based on sites of copper accumulation in patients with Menkes disease, and animal models. Copper is secreted from the liver in the bile via the Wilson disease protein (not shown).

Fig. 1.1
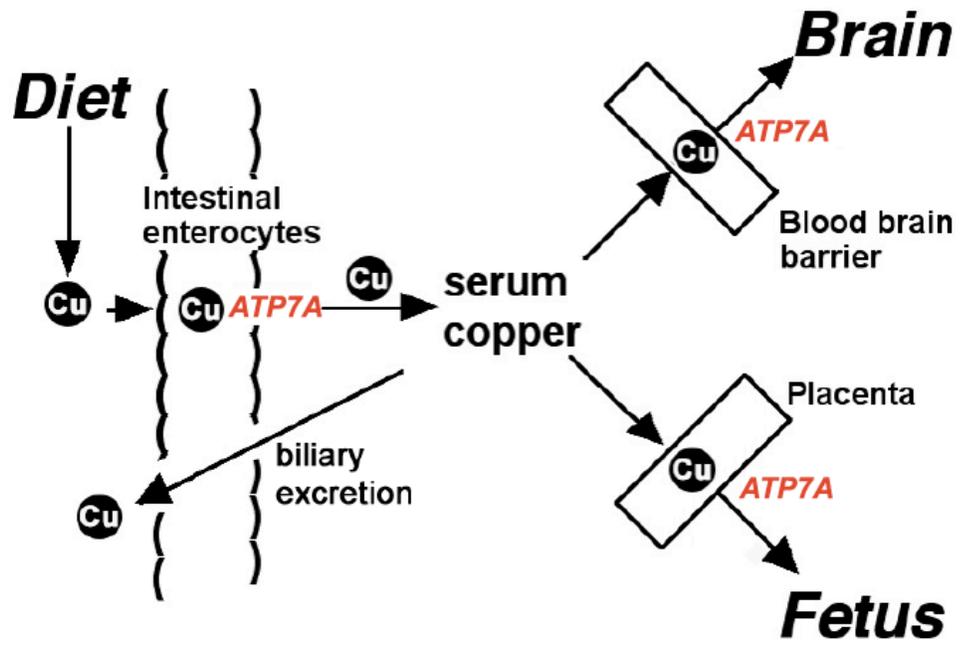
1.4 Copper toxicity

As with copper deficiency, copper toxicity can be either acquired or genetic. Acquired copper toxicity may be caused by excessive copper supplementation, food contamination, and low dietary zinc levels (67, 68). Long-term exposure to high levels of copper causes tissue damage and liver cirrhosis due to excess tissue copper accumulation. This is also true for Wilson disease, an autosomal recessive disorder of copper metabolism which results in hepatic damage presenting as progressive hepatic cirrhosis and rapidly progressive liver failure and liver fibrosis (69). Patients suffering from Wilson disease carry mutations in the gene encoding the Cu-ATPase ATP7B, or Wilson protein (69, 70). ATP7B is highly homologous to ATP7A, sharing 57% sequence identity, and is mainly expressed in the liver and brain (71). In the liver, ATP7B functions in delivering copper to the secretory pathway and also in copper export into the bile. ATP7B-dependent copper export into the bile and subsequently through the bowel is the major route of copper excretion from the body. Wilson disease greatly impairs copper transport to the secretory pathway and copper release into the bile, resulting in marked accumulation of copper in the liver, low serum levels of copper-bound ceruloplasmin and low biliary copper content (72-74). ATP7B inactivity is also associated with copper accumulation in the brain, resulting in severe neurological abnormalities such as movement disorders and psychiatric manifestations (69, 75, 76). The neurological disorders in Wilson disease patients are often accompanied with accumulation of copper deposits in the

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cornea resulting in the Kayser-Fleischer corneal pigment ring, which is a characteristic and diagnostic sign of the disease (77, 78). This is likely due to the lack of ATP7B, which is normally expressed in the retinal pigment epithelium and the ciliary body during retinal development (77, 78).

The treatment of copper toxicosis in Wilson disease involves copper chelation therapy using D-penicillamine, which binds copper and facilitates its secretion in urine (79). However, D-penicillamine administration is not always successful and may have severe side effects (80-82), which has prompted the development of alternative therapeutic treatments, such as chelation with trientine, tetrathiomolybdate (TTM) or the administration of zinc salts (83-85). Further evaluation of the efficacy and potential side effects of these alternative therapies remains to be performed.

1.5 Intestinal copper absorption

Absorption of dietary copper in mammals occurs exclusively through the digestive tract. Occasionally, copper may enter the body through the skin if applied in high concentrations in the form of ointment or if copper bracelets are worn (4, 68, 86). Copper absorption occurs primarily in the small intestine through a highly efficient mechanism. At moderate dietary intakes, 55-75% of copper is absorbed in adults, and this percentage does not drop significantly with advanced age (4, 68, 87). Interestingly, intestinal copper reabsorption from saliva

(0.33-0.45 mg/d), gastric juices (1 mg/d), pancreatic fluids (0.4-1.3 mg/d) and duodenal fluids (0.4-2.2 mg/d), contributes a total of 4-7.5 mg/d additional Cu to the digestive tract, compared to an average of 1 mg/d available from dietary sources (4, 68, 87). Copper excretion occurs mainly through the bile and sheds an average of 0.5-1.5 mg/d of body copper.

1.5.1 Absorption at the apical membrane of intestinal enterocytes.

The process of copper absorption at the apical side of intestinal epithelial cells is the subject of on-going studies. It has been suggested that intestinal Cu absorption occurs via a carrier-mediated saturable process (67, 68). Furthermore, the efficiency of Cu uptake is regulated by dietary Cu levels, *i.e.* efficiency of absorption increases when dietary Cu content is low (88). This suggests the presence of one or more specific molecules responsible for intestinal copper absorption, and the possibility that dietary copper levels regulate the expression, activity or localization of these molecules. One potential candidate is the broad spectrum metal transporter DMT1 (divalent metal transporter, also known as Nramp2/DCT1), which shows specificity for divalent metal ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} (89), and studies have shown that DMT1 transports Cu^{1+} into cultured polarized epithelial cells (90, 91). However, mammalian cell culture studies strongly support a role for the high-affinity copper transporter CTR1 in Cu uptake (92-94). CTR1 is expressed in the intestines, and is localized to the apical membrane of intestinal enterocytes (94).

CTR1 transports Cu with high specificity at a Km of $\sim$1 μM in an ATP-independent manner. There is strong accumulating evidence suggesting that CTR1 specifically transports Cu^{1+}, because yeast CTR1 requires the cell-surface Cu^{2+}/Fe^{3+} metalloreductases Fre1 and Fre2 for its optimal activity (95). Furthermore, the reducing agent ascorbate has been shown to enhance ^{64}Cu uptake in yeast and cultured mammalian cells (93). Most copper in food is present in the cupric (Cu^{2+}) form, which requires its reduction to the cuprous form (Cu^{1+}) prior to its uptake by CTR1 (68). In order to maintain copper in its reduced form in the presence of oxygen, it has been suggested that intestinal metalloreductases, possibly located in close proximity to CTR1 at the apical membrane, serve to reduce the copper and hand it off to CTR1 (96). dCytb is a duodenal metalloreductase localized at the apical membrane of intestinal enterocytes (97), and has been proposed to facilitate CTR1-mediated Cu^{1+} absorption. It is also possible that an unidentified ligand binds copper following its reduction and delivers it to CTR1 (96). Mammalian CTR1 constitutively undergoes rapid clathrin-mediated endocytosis at Cu concentrations near the Km for transport, leading to the degradation of CTR1 (98, 99). The endocytosis and degradation of CTR1 in intestinal epithelial cells may provide a mechanism to regulate Cu uptake in response to elevated dietary copper content.

The current model suggests that copper absorption at the intestinal apical membrane is primarily mediated by CTR1. A recent study using conditional intestinal knockout of CTR1 in mice ($CTR1^{int-/int-}$) demonstrates a critical role for

CTR1 in intestinal copper absorption (100). CTR1$^{int-/int-}$ mice have decreased

copper accumulation in peripheral organs and deficient activities of Cu-

dependent enzymes, leading to severe hyperaccumulation of liver Fe. The

knockout mice suffer severe post-natal growth retardation, and usually die 10 to

21 days postpartum unless rescued with an intraperitoneal (IP) injection of Cu at

days 5 or 6 after birth. The CTR1$^{int-/int-}$ mice also exhibit symptoms reminiscent of

those seen in Menkes disease, such as skin laxity, hypopigmentation, and

vascular defects, as well as cardiac hypertrophy. Surprisingly, intestinal epithelial

cells from CTR1$^{int-/int-}$ mice reveal an unexpected hyperaccumulation of

intracellular Cu (7-fold) that is not biologically available, suggesting a critical role

for CTR1 in copper distribution to intracellular chaperones (100). Decreased

activities of the Cu-dependent enzymes (such as cytochrome c oxidase), and the

accumulation of the copper chaperone CCS, which is degraded under elevated

Cu levels (discussed later), serve as indices for the low bioavailability of Cu in the

knockout mice enterocytes. These findings suggest an alternative mechanism for

Cu transport across the apical membrane. It is possible that DMT1 plays the role

of secondary intestinal copper transporter in the absence of CTR1, however,

CTR1-mediated transport appears to be critical for the bioavailability of

intracellular copper pools.

1.5.2 Transport across the basolateral membrane of intestinal enterocytes.

Copper absorption across the basolateral membrane of intestinal epithelial cells

occurs via ATP7A. ATP7A is strongly expressed in the duodenum and the upper jejunum, suggesting that these are the primary sites of intestinal Cu absorption (101). Mutations in the ATP7A gene in Menkes disease result in Cu overload in intestinal epithelial cells and copper deficiency in the circulation and peripheral organs (discussed earlier) (102, 103). Exposure of intestinal enterocytes to dietary copper stimulates the trafficking of ATP7A from its steady-state localization in the trans-Golgi network (TGN) to the basolateral membrane, where it exports copper into the circulation (101). This provides a regulated mechanism of Cu transport from the site of absorption at the apical membrane to the site of exit into the hepatic portal circulation (102).

1.6 Copper transport and distribution to tissues

On exiting the GI tract through the basolateral membrane of enterocytes into the hepatic portal blood, copper is initially bound to albumin, transcuprein and low molecular-weight amino acids such as histidine (68, 104). Albumin and transcuprein have been shown to bind Cu with high affinity and copper is readily exchanged by these 2 proteins (105). Copper-bound albumin, transcuprein and histidine are preferred substrates for the liver (68, 104), where they rapidly deliver their bound copper (68, 106, 107). A minor fraction of this copper pool is diverted to the kidneys. Copper uptake by the liver and the kidneys is thought to be mediated by CTR1 (68, 104). The liver is the major storage site of copper in mammals. In the liver, copper is either delivered to copper-dependent enzymes, incorporated into metallothionein (MT) and glutathione (108), or, when in excess,

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released into the bile via ATP7B or Wilson protein (109, 110). This mechanism is mediated by copper-dependent trafficking of ATP7B in hepatocytes. ATP7B maintains a steady-state localization in the TGN and delivers Cu to the secretory compartment to be incorporated into copper-dependent enzymes, including ceruloplasmin. In response to elevated Cu levels, ATP7B relocalizes to intracellular vesicles near canalicular membranes and pumps excess copper into the bile ducts. In the bile, copper is bound to unidentified complexes that render it unavailable for intestinal reabsorption (109, 110). ATP7B represents the major route of Cu removal in mammals. Genetic ATP7B mutations causing Wilson disease are associated with copper toxicosis, due to the inability of ATP7B to remove excess Cu from the liver and to incorporate Cu into ceruloplasmin for delivery to peripheral tissues.

1.6.1 Ceruloplasmin is the major carrier of copper in the circulation.

Ceruloplasmin-bound Cu is not part of the exchangeable plasma Cu pool, nor does ceruloplasmin readily bind or incorporate Cu when exposed to the metal. During its synthesis in hepatocytes, 6 copper atoms are incorporated into apo-ceruloplasmin via copper delivery into the secretory pathway by ATP7B to form holo-ceruloplasmin. Although Cp is primarily synthesized in the liver, other cell types express the protein, including macrophages, monocytes, astrocytes, and Sertoli cells, where ATP7A may function in delivering copper to the enzyme (111). Holo-ceruloplasmin (holoCp) is the major carrier of copper in the blood

(112-115), and serves to distribute the newly acquired/recycled copper to all peripheral organs and tissues. Cp binding has been reported in a range of tissues and cells including erythrocytes, aorta and heart, liver endothelium, leukocytes, kupffer cells and human placental cells (111). In endothelial cells, Cp binding occurs through the galactosyl recognition system (115). Also, a glycosylphosphatidylinositol-anchored form of Cp (GPI-linked Cp) has been identified in monocytes and astrocytes (116, 117). In addition to its role in copper transport, ceruloplasmin also serves to regulate cellular Fe release through its ferroxidase activity, and is thought to modulate coagulation, angiogenesis, and aid in defense against oxidative stress (111, 118-123).

1.6.2 Regulation of ceruloplasmin.

The concentration of holoCp in the plasma positively correlates with copper availability in the liver. However, Cu does not regulate the synthesis or the secretion of ceruloplasmin. Physiological conditions that increase the synthesis and secretion of ceruloplasmin include the acute-phase inflammatory response and relative oxygen depletion or hypoxia (124-132). Cp is an acute-phase reactant, and its expression is increased 2- to 3-fold in the plasma during infection, suggesting a possible antibacterial role (124-129). It has been suggested that Cp may serve as a radical scavenger, however conflicting reports have suggested a pro-oxidant role for Cp in the etiology of cardiovascular disease and diabetes (124-129). Further evidence is needed to support these

hypotheses.

On the other hand, the importance of Cp activity as a ferroxidase in hypoxia has been well described (130-132). Hypoxia is a serious condition that threatens cell survival, and can cause severe organ damage and injury in human disease (133-138). Among the physiologic responses to hypoxia is the stimulation of erythropoiesis, or the increase in the O_2-carrying capacity in the circulation via an increase in the number of circulating erythrocytes (139-142). This process stimulates hemoglobin synthesis, and thus elevates the demand for iron incorporation into heme (130-132). The increased demand for iron by erythrocytes is met by an efflux of iron from macrophages, which can store large amounts of iron due to their ability to phagocytose senescent red blood cells and to recycle the iron bound to hemoglobin (130-132). The export of iron from macrophages occurs via the ferroportin protein, and requires the oxidation of Fe^{2+} to Fe^{3+} by the ferrroxidase ceruloplasmin (130-132). The importance of the role of ceruloplasmin in iron efflux is underlined in humans and animals affected by a lack of ceruloplasmin (aceruloplasminemia) that results in the inability to export iron from tissues, and causes iron overload in parenchymal tissues, in the liver, and in the brain, often leading to severe neurological disorders (143, 144). Because ceruloplasmin is a copper-dependent protein, copper deficiency limits the incorporation of copper into the enzyme in the secretory pathway, and results in anemia due to decreased iron efflux to the circulation. Therefore, in the face of elevated demand for iron in the circulation, it is not surprising that relative oxygen

depletion or hypoxia induces ceruloplasmin expression in macrophages and other cell types (145, 146), as well as enhances its ability to oxidize Fe, thus allowing iron export via the ferroportin protein (147, 148).

Interestingly, although Cp carries ~95% of plasma Cu, and is a preferred substrate for non-hepatic tissues, the lack of ceruloplasmin protein or aceruloplasminemia in human patients does not compromise Cu-dependent enzyme activities (144, 149). This suggests that other Cu-binding proteins such as albumin can compensate for the lack of Cp and maintain copper transport and distribution to peripheral tissues. The copper taken up in peripheral tissues is mostly recycled within cells, and its turnover rate is slow. Excess Cu is released back into the circulation via ATP7A or ATP7B and recycled in the liver (150).

1.7 Distribution of copper in mammalian cells

Three regulated processes contribute to maintaining intracellular copper homeostasis, namely, Cu uptake, intracellular Cu distribution and storage, and Cu export (Fig. 1.2). The CTR1 protein is the major copper importer in mammalian cells, and serves to shuttle copper through the plasma membrane from the extracellular space into the cell cytosol (151). Following its translocation into the cytoplasm, copper is immediately sequestered and bound to small cytoplasmic proteins known as copper chaperones. It has been suggested that, in eukaryotes, cytoplasmic copper does not exist in the free unbound form (152).

The cytoplasm offers a reducing environment where Cu^{1+} can readily interact with hydrogen peroxide (H_2O_2) via the Fenton reaction to produce the highly reactive hydroxyl radical ($OH^·$) (153-155). Because of its potential to enhance the generation of oxidative stress, Cu is quickly delivered and bound to three known copper chaperones ATOX1, CCS, and COX17 which in turn deliver the Cu to the ATP7A copper transporter, to Cu/Zn superoxide dismutase (SOD1) and to mitochondrial cytochrome c oxidase, respectively (156-159) (Fig. 1.2). Excess copper is exported via copper-dependent trafficking of ATP7A to the plasma membrane (Fig. 1.2).

FIGURE 1.2. **Mammalian cellular copper homeostasis.** Copper uptake occurs via the copper importer CTR1. Copper is then bound to the copper chaperones Cox17, CCS and ATOX1, which deliver copper to cytochrome c oxidase, Cu/Zn superoxide dismutase (SOD1), and Menkes (ATP7A) protein, respectively. The ATP7A protein resides mainly in the trans-Golgi network (TGN) and delivers copper to copper-dependent enzymes like ceruloplasmin (Cp). Elevated copper levels stimulate the endocytosis of the Ctr1 and the trafficking of ATP7A to the plasma membrane. Together, Ctr1 and ATP7A function as the key regulators of mammalian cellular copper homeostasis. Both proteins are ubiquitous except in the liver where ATP7A is not expressed, and the Wilson protein (ATP7B) functions in copper export (not shown).

Fig. 1.2
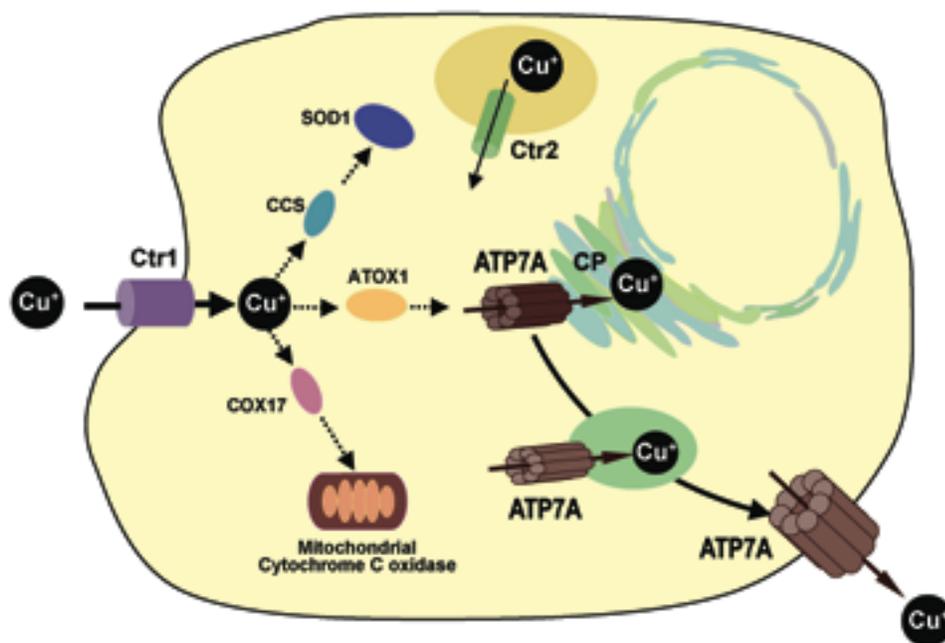
1.7.1 Copper uptake.

Copper import into eukaryotic cells occurs via the specific high-affinity Cu transporter CTR1 (151). CTR1 is an integral membrane protein that is structurally and functionally conserved from yeast to humans (94, 160, 161). In biological molecules, Cu atoms show high binding affinity to histidine nitrogen, cysteine sulfur, and methionine sulfur. The CTR1 polypeptide contains three transmembrane domains, a methionine-rich N terminus, two cysteine-histidine motifs in the C terminus and a methionine-containing MX3M motif in the second transmembrane domain (162). Mutagenesis and functional studies of yeast and human CTR1 have shown that the MX3M motif in the second transmembrane domain is essential for Cu uptake, and may serve as a Cu ligand during the transport of Cu across the plasma membrane (162). On the other hand, the N-terminal methionine-rich motifs are dispensable under Cu-replete conditions, but essential under copper-limiting conditions, suggesting that these sequences function in scavenging copper ions when extracellular copper levels are low (162).

Genetic and biochemical studies of yeast and human CTR1 suggest that this protein can form homotrimeric complexes (93, 163). Indeed human CTR1 (hCTR1) resolves as a 35-kDa and a 70-kDa band on non-reducing polyacrylamide gels, and as a 110-kDa complex when treated with crosslinking agents *in vitro*, suggesting that these bands represent the monomeric, dimeric

and trimeric forms of the protein, respectively. Recent two-dimensional crystallography and electron microscopy studies confirm that CTR1 is present as a homotrimer on the plasma membrane. This configuration of the protein is thought to form a pore or channel of nine transmembrane domains that would allow the passage of Cu ions across the lipid bilayer (100, 163-165).

Although Ctr1 is most highly expressed in the liver, its expression is ubiquitous, suggesting that it mediates copper uptake in all cells of the body (151, 166). CTR1 specifically transports Cu^{+1} at a Km of ~1-5 μM in a pH-dependent manner (2). Copper does not regulate the mRNA levels of CTR1 in cultured cells and animal models. However, studies in our lab have demonstrated that elevated Cu levels induce the trafficking of a functional epitope–tagged version of CTR1 from the plasma membrane to endosomal compartments (99). The steady-state localization of the CTR1 protein may vary depending on cell type; it may be localized to the plasma membrane or to cytoplasmic vesicles (93, 167-169). In HEK293 and CHO cells, CTR1 is mainly localized to the plasma membrane (93, 167-169). In response to elevated copper levels in these cell types, CTR1 undergoes rapid endocytosis and degradation in a time- and dose-dependent manner, suggesting that this is a mechanism by which cells regulate high-affinity Cu uptake (99, 170).

Another protein with homology to CTR1 is the Cu transporter CTR2 (151). In the yeast *saccharomyces cerevisiae*, CTR2 responds to Cu deficiency by releasing

Cu from the vacuole into the cytoplasm (171). It is possible that the mammalian CTR2 protein is similarly involved in the release of Cu from intracellular stores such as lysosomes and other compartments into the cytoplasm. Interestingly, a mammalian CTR2 homologue has been identified in mice and humans, and its function is currently not well-understood (151). Epitope-tagged mammalian CTR2 has been localized to the plasma membrane and to the late endosome and lysosomes. However, the functionality of these fusion proteins has yet to be demonstrated, as well as the localization of endogenous CTR2. Future studies and genetic animal knockout models will help elucidate the role of CTR2 in copper homeostasis.

Interestingly, recent evidence has emerged that points to a potential role for CTR1 in intracellular Cu trafficking. It has been suggested that, in addition to its role in Cu uptake at the plasma membrane, CTR1 may function in mobilizing Cu out of the endosomal compartment into the cytosol, where it would be available for binding Cu chaperones and for incorporation into Cu-dependent enzymes. This model is based on recent findings in mice bearing a specific knockout of CTR1 in the intestines (100). The knockout mice exhibited severe Cu deficiency in peripheral tissues, consistent with a critical role for CTR1 in dietary Cu absorption. Surprisingly, intestinal epithelial cells from the knockout mice exhibited 8- to 10-fold hyperaccumulation of Cu compared to wild type, suggesting alternative pathways for Cu uptake. However, despite the elevated levels of intracellular Cu in these cells, the activity of Cu-dependent enzymes

such as mitochondrial cytochrome c oxidase was depressed. It is possible that the accumulated copper may be trapped in the endosomal compartment owing to the lack of CTR1 in these cells, rendering it biologically unavailable.

It is worth noting that no known genetic diseases associated with CTR1 mutations in humans have been identified to date. This may be attributed to the essential function of CTR1 in mammalian embryonic development. Indeed, this is underscored in studies of mice carrying null mutations of CTR1, which die *in utero* due to severe copper deficiency (172).

1.7.2 Intracellular copper pathways.

1.7.2.1 Delivery of copper to Cu/Zn superoxide dismutase (SOD1).

Superoxide dismutases (SODs) are a family of metalloenzymes widely distributed in prokaryotic and eukaryotic cells that includes the Cu/Zn SOD (or SOD1) (173). These enzymes catalyze the conversion of superoxide anion $O^{\cdot}$ to hydrogen peroxide (H_2O_2) in a two-step reaction of $O^{\cdot}$ with SOD. The first step begins with the binding of the oxidized form of the enzyme (Cu^{2+}-bound) to $O^{\cdot}$, acquiring a proton, and releasing molecular oxygen. The reduced form of the enzyme (Cu^{+1}-bound) then binds a second superoxide anion, and liberates H_2O_2, returning to its oxidized state. H_2O_2 immediately becomes a substrate for the enzymes catalase and glutathione peroxidase (174). Thus, SOD enzymes play

an important role in cellular defense against superoxide-mediated oxidative stress (175).

SOD1 is a homodimeric protein located mainly in the cytosol, with a fraction located in the mitochondrial intermembrane space (176, 177, 178). Incorporation of Cu into SOD1 requires the copper chaperone for SOD1, called CCS (179, 180). The transfer of Cu from CCS to SOD1 occurs either in the cytosol, or the mitochondrial IMS since SOD1 can only cross the mitochondrial membrane as an apo-enzyme (181). The CCS protein comprises 3 domains (domains I, II and III) that carry out distinct functions during the transfer of Cu to SOD1. The N-terminal domain I is important for the capture of Cu ions through its MXCXXC Cu-binding motif. Docking of CCS to SOD1 and formation of a heterodimeric complex occurs via binding of domain II to a homologous highly conserved region in SOD1 (182, 183). Following the formation of this heterodimeric intermediate, Cu is transferred within the CCS protein from domain I to the C-terminal domain III that translocates the Cu ion to SOD1 (182-184). Domain III also functions in the Cu-dependent regulation of CCS protein ubiquitination and degradation (185). The Cu-dependent degradation of CCS is dose-dependent and serves as an indicator of intracellular copper levels. Studies in the CCS knockout mouse model (CCS^{-/-}) underscore the importance of CCS for SOD1 function. CCS^{-/-} mice exhibit normal levels of SOD1 protein in various tissues (brain, spinal cord, muscle, liver, lung, heart and kidney). However, SOD1 activity is significantly lower in these animals compared to wild-type littermates (181).

Dietary copper deficiency in animals has been associated with selective and organ-specific regulation of SOD1 transcript, protein, and activity levels. In rats fed a copper-deficient diet for 4 weeks, SOD1 activity was diminished in the heart, despite a high expression of both mRNA and apo-protein. On the other hand, a decrease in SOD1 activity, as well as a decrease in transcript and protein levels was observed in the livers of the Cu-deficient animals. However, no effect of dietary copper restriction was observed on SOD1 activity or expression levels in the brain of the same animals (186). The outcome of this study suggests that dietary copper deficiency results in tissue- and organ-specific regulation of SOD1 expression and activity levels.

Dysregulation of SOD activity has been observed in human pathologies. Deficiency in SOD1 activity plays a central role in the etiology of hepatocellular carcinoma (187). Mice deficient in SOD1 (SOD1$^{-/-}$) do not show any abnormalities during development, however, they exhibit significantly lower life spans compared to wild-type mice, as well increased neoplastic changes in the liver (188). Additionally, cancer cells generally have diminished SOD1 activity (189, 190). At the other end of the spectrum of SOD1 deficiency-associated pathologies is amyotrophic lateral sclerosis (ALS), where mutations within the SOD1 gene result in "gain of function" toxicity (191-193). ALS, or "Lou Gehrig's disease", is a selective neurodegenerative disease of motor neurons in the brain and spinal cord in which approximately 5% of cases are caused by SOD1 mutations. Symptoms include generalized weakness, muscle atrophy, and

progressive paralysis. Over 90 distinct mutations have been identified, further complicating the understanding of the underlying causes of this disease (194, 195). One potential associated mechanism is H_2O_2-mediated cell damage and toxicity caused by excess mutant SOD activity (191, 196).

1.7.2.2 Copper delivery to the mitochondria.

The mitochondrial respiratory complex IV enzyme cytochrome c oxidase (CCO) is a Cu-dependent integral membrane protein complex of the mitochondria involved in respiration and ATP synthesis. CCO is the only respiratory complex that requires copper for its function (197). The CCO complex is comprised of 13 subunits, with 3 of them (COX1-COX3) forming the core of the enzyme being encoded by the mitochondrial genome (197). COX1 and COX2 also constitute the copper-binding subunits of CCO, with 1 Cu atom incorporated into the Cu_B site of COX1, and 2 Cu atoms incorporated into the Cu_A site of COX2 (197). Since COX1 and COX2 are encoded by the mitochondrial genome, copper metallation must occur within the organelle. The only other known copper-dependent protein within the mitochondrion is SOD1, localized in the IMS. As previously mentioned, SOD1 enters the mitochondrion in its apo-form, and receives its copper within the IMS (178). The presence of 2 metalloenzymes that are metallated within the mitochondrion requires copper transport into this organelle. While CCS chaperones Cu into the mitochondrial IMS for incorporation into SOD1 (182), the Cu chaperone COX17 is required for the delivery of Cu to

CCO through a complex series of events (198). Although COX17 is present in both the cytosol and the mitochondrial IMS, it is not an obligate mitochondrial copper chaperone (197, 199). This was demonstrated in experiments tethering COX17 to the IMS by a heterologous IM-binding domain, which resulted in the exclusive localization of COX17 to the mitochondria. In these experiments, tethered COX17 reversed the respiratory defect of cox17Δ yeast cells and restored CCO activity (200). Additionally, cox17Δ cells contained mitochondrial Cu levels similar to wild type. Exactly how Cu makes its way from CTR1 at the plasma membrane into the mitochondrion is still unclear. The inability of CCS to overcome COX17 deficiency even when overexpressed at supraphysiological levels suggests the presence of one or more alternative molecules that shuttle Cu from the cytosol into the mitochondrion (96). Recently, a small Cu ligand has been identified in the cytosol and the mitochondrial matrix of yeast and mouse liver extracts (199, 201). Future studies will aim at understanding the potential role of this ligand in intracellular copper homeostasis.

The incorporation of Cu into CCO is a multi-step event that is not well understood and is the subject of ongoing studies. Evidence suggests that insertion of Cu into the Cu_B site of COX1 occurs via a transfer of Cu from COX17 to the integral membrane COX11 protein, which facilitates the insertion of Cu into COX1 (199, 201). Two additional metallochaperones have been identified, SCO1 and SCO2 that assist in the incorporation of Cu into the Cu_A site of COX2 (202-205). Another Cu-binding protein, COX19, localized to the IMS and structurally similar

to COX17, has been suggested as an additional player in the incorporation of Cu into CCO (206).

Although the pathways of Cu delivery into the mitochondrion and CCO are not well understood, the significance of this topic is underscored by inherited deficiencies in CCO in humans with mutations in copper assembly proteins. In yeast, mutations in the *cox17* gene result in a respiratory defect owing to a lack of CCO activity, since CCO depends on Cu for its activity (156, 198, 207). Additionally, COX17 knockout mice die *in utero*, revealing an essential role for COX17 in embryonic development (208). Recent studies in patients carrying mutations in either SCO1 or SCO2 revealed that liver, heart and skeletal muscle samples contained significantly lower amounts of copper compared to tissues from control subjects (209). Significantly, this effect was associated with increased copper efflux via the ATP7A protein. The authors also showed that this effect was independent of defects in CCO assembly, suggesting a novel role for the SCO1 and SCO2 proteins in the regulation of copper homeostasis via a mitochondrial signaling pathway.

1.7.2.3 Delivery of copper to the secretory pathway.

The transport of Cu from the site of CTR1-mediated uptake at the plasma membrane to the secretory pathway occurs via the Cu chaperone ATOX1. In the cytosol, ATOXI delivers Cu to one of two Cu-ATPases, ATP7A known as Menkes

protein, or ATP7B or Wilson protein, which in turn actively transport Cu into the TGN. Delivery of Cu to the secretory pathway allows its incorporation into cuproenzymes, as well as regulates its trafficking-mediated export when intracellular Cu levels are in excess.

1.7.2.3.1 The copper chaperone ATOX1.

ATOX1, also known as HAH1, is a cytosolic protein that plays a key role in delivering Cu to ATP7A and ATP7B (70, 110). Deletion of the *ATOX1* gene in mice results in intracellular copper accumulation and a decrease in the activity of Cu-dependent enzymes (210), suggesting deficient Cu-ATPase transport activity. Furthermore, the offspring of ATOX1 knockout mice suffer growth retardation, hypopigmentation and skin laxity phenotypes and increased perinatal mortality similar to Menkes patients (210), owing to impaired transfer of Cu from the placenta to the embryo.

Yeast Atx1 was originally identified as a small antioxidant protein that suppressed oxygen toxicity in yeast mutants lacking SOD1. Atx1 was subsequently shown to be a copper chaperone (211). Shortly following this discovery, a homologue in humans and other mammals was identified and named ATOX1 (212). The yeast Atx1 has been shown to facilitate the function of the P-Type Cu-ATPase Ccc2, which transports copper into the late Golgi compartment (213). Mammalian ATOX1 is a small cytosolic protein with striking

structural homology to the metal-binding domains (MBD) of the Cu ATPases

(214). ATOX1 contains a single repeat of the MXCXXC Cu-binding domain, and

this MBD has been shown to bind one Cu^{1+} molecule (215). Both ATP7A and

ATP7B carry 6 repeats of the MBD in their N-terminal regions, and, as in ATOX1,

each of these MBDs binds a single Cu^{1+} molecule. The MBDs are thought to play

a critical role in the direct interaction between ATOX1 and the ATPases during

the Cu transfer process. In vitro, Cu-ATOX1 has been demonstrated to transfer

Cu directly to the MBDs of ATP7B in a dose-dependent manner (215-219). All 6

sites on the ATP7B protein can be filled via this interaction; however, a significant

excess (5- to 50-fold) of ATOX1 over ATP7B is needed to achieve complete

saturation of the N-terminal MBDs. In vitro studies in embryonic fibroblasts from

ATOX1 knockout mice show that ATOX1 plays an important role in the Cu-

induced trafficking of ATP7A (158).

1.7.2.3.2 The Cu-ATPase ATP7A or Menkes protein.

The Menkes protein is encoded by the *ATP7A* gene and is expressed in most

mammalian tissues expect the liver (43-45, 220). This copper transporter

contains eight membrane-spanning domains and six cysteine-rich copper binding

sites at the amino terminal region that are required for high affinity copper

transport. ATP7A belongs to the P-type ATPase family whose members utilize

the energy derived from ATP hydrolysis to transport cations (221). During each

catalytic cycle, which results in the transport of Cu, the ATP7A protein undergoes

rapid auto-phosphorylation at a conserved aspartic acid residue, followed by de-phosphorylation to complete the cycle (222, 223). As discussed above, Menkes disease is a lethal genetic disease caused by mutations in the ATP7A protein, and is characterized by overall copper deficiency and severe pathophysiological consequences such as neurological impairment, convulsions, connective tissue abnormalities, skin laxity and hypopigmentation (46). These mutations in the ATP7A protein result in the reduced efflux of copper from cells (224), and the inability of the ATP7A MNK protein to transport Cu across the enterocytic basolateral membrane into the bloodstream. Mutations in the ATP7A protein may affect its copper transport activity and/or its ability to traffic to the plasma membrane (169, 225-228). Menkes disease is primarily a defect in copper efflux. Skin fibroblasts, lymphoblasts and amniocytes isolated from human Menkes patients and cultured *in vitro* accumulate copper in essential medium without additional copper. Thus, mutations in the ATP7A protein disrupt normal cellular copper homeostasis (221, 229, 230).

FIGURE 1.3. **Menkes protein traffics in response to copper in cultured**

human fibroblasts. A, Topology of the ATP7A protein. B, Copper treatment of

cultured fibroblasts induces the relocalization of ATP7A from the trans-Golgi

network (TGN) to post-Golgi vesicles and the plasma membrane. This process

results in the net export of copper and prevents the accumulation of potentially

toxic excess copper. Immunofluorescence labeling of the ATP7A protein is

shown in green. The nucleus is stained red with propidium iodide.

Fig. 1.3

A

Cu-binding sites
CxxC

Phosphorylation

N

Cytoplasm

C

Phosphatase

Lumen / Extracellular

Cu^{1+}

B

Basal medium (~1µM Cu)

+ 100 µM Copper

[Cu]

Cu+

ATP7A

Cu+
Cu+

TGN
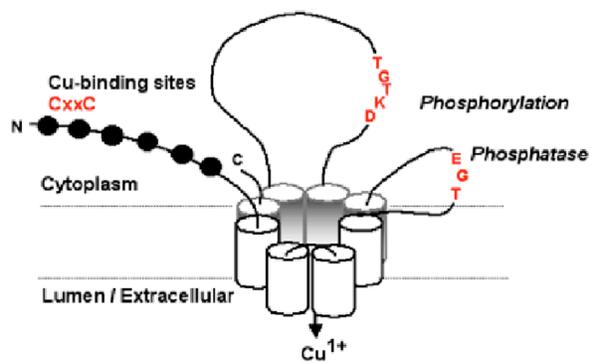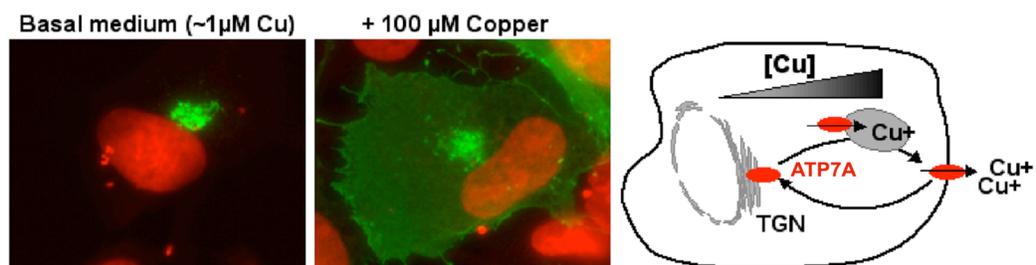
The steady-state subcellular localization of the ATP7A protein is within the final

compartment of the Golgi apparatus, also known as the trans-Golgi network

(TGN) (1, 231). ATP7A transports copper from the cytoplasm across the Golgi

membrane and delivers it to copper dependent enzymes within the TGN (232-

237). The subcellular localization of ATP7A is regulated by copper (Fig. 1.3). In

response to elevated copper levels in the cytoplasm, the ATP7A protein traffics

within post-Golgi vesicles to the plasma membrane (1, 169, 238, 239). The

copper-stimulated trafficking of ATP7A is a defense mechanism that protects the

cell from excess copper toxicity. However, ATP7A mRNA and protein levels in

cultured cells and mouse tissues do not change in response to copper availability

(1, 240). Consistent with its role in transporting copper into the bloodstream from

enterocytes and kidney tubules, and into the central nervous system across the

blood brain barrier, the ATP7A protein traffics to the basolateral membrane in

response to elevated copper concentrations in cultured polarized epithelial cells

(241).

1.7.2.3.3 Mutational analyses of copper-stimulated ATP7A protein trafficking.

Previous studies have demonstrated that the trafficking of ATP7A is dependent

on copper-binding sites closest to the first membrane-spanning domain (228).

Studies in our laboratory have demonstrated that mutations that prevent the

formation of the phosphorylated catalytic intermediate of the ATP7A protein also

block the ability of the protein to traffic in response to copper (169). Moreover, certain Menkes disease mutations, including the mutation in the Brindled mouse, totally abolish the trafficking of ATP7A in response to elevated copper (225, 226, 242). Based on these findings, it is suggested that the catalytic activity of ATP7A is essential for its trafficking function (169). However, catalysis is not a prerequisite for trafficking since our laboratory has identified a mutation that blocks the trafficking response but does not inhibit normal copper transport activity into the Golgi (226).

1.7.2.3.4 Mouse models of Menkes disease.

The murine model of Menkes disease is a collection of mice called the "mottled" mice. Each mottled mouse variant is caused by a mutation in the X-linked mottled gene ($Atp7a^{Mo}$ or Mnk), which is the orthologue of the human $ATP7A$ gene (243-250). These mutations block copper transport activity of the ATP7A protein, as evidenced by the accumulation of copper in cultured fibroblasts from mottled mice (242, 251). The mottled mice have considerable phenotypic variability, however, each mutant exhibits symptoms of copper deficiency similar to the human disease. For example, the Brindled mouse, a model of the classical disease in humans (243), carries an in-frame deletion of two codons resulting in the deletion of two amino acids in a cytoplasmic loop of the ATP7A protein (243). This mutation inhibits copper transport to cuproenzymes and copper-stimulated trafficking, despite the normal localization of the ATP7A protein in the TGN (227,

252). Brindled mice usually die within three weeks of birth, but this can be prevented by a single subcutaneous copper injection (50 µg) administered within 10 days of birth (234, 253-256). The timing of this injection is critical, since treatments that are delayed beyond the 10th day do not extend life expectancy. The copper injections have been shown to correct the neurological symptoms, such as tremors and spasms seen in untreated mutants (234), and to rescue some of the copper deficiency in peripheral tissues, although liver copper levels remain below normal (254, 256). In contrast to wild type mice in which copper injections have no lasting effect on organ copper levels (234, 253, 254), the copper-treated brindled mice exhibit patterns of Cu maldistribution similar to those seen in Menkes patients, including above-normal copper levels in enterocytes and kidneys, and reduced levels in serum and the liver. Interestingly, macrophages from the copper-treated Brindled mouse also accumulate copper (254). Although the growth of copper-rescued Brindled mice is significantly retarded in the first weeks of life, these animals reach normal weight by 60 days of age (234).

1.8 Copper homeostasis during the inflammatory response.

Infection or tissue injury promotes a variety of inflammatory responses. Monocytes accumulate at the site of inflammation where they differentiate into macrophages. Macrophages and other phagocytes, such as neutrophils, ingest microbes into the phagosome, which is accompanied by a toxic "respiratory burst" of superoxide (257). Other products of the respiratory burst include hydrogen peroxide and the hydroxyl radical (258-260). These processes are part of the innate immune response. Interestingly, the acute phase response to infections and inflammation in humans and animals is marked by changes in circulating metal levels. Both zinc and iron levels are significantly decreased, resulting the classical hypozincemia and hypoferremia, two hallmark events of the acute-phase response (261, 262). In contrast, inflammation results in an increase in copper levels in the serum, or hypercupremia (263-274). Ceruloplasmin is an acute phase protein, and the rise in serum copper levels during inflammation may be partially attributed to increased circulating holo-Cp levels (120). Indeed, it has been shown that inflammation also promotes the accumulation of the non-protein bound fraction of copper in the serum (263). Radiotracer studies with ^{64}Cu have demonstrated that copper accumulates at sites of inflammation (275), and within the exudates of wounds and burns relative to serum (276, 277).

Unlike copper, iron and zinc levels in the serum are reduced during inflammation, presumably to starve microbes of these essential nutrients. The expression of the zinc importer ZIP14 is elevated in the liver during inflammation. This is thought to increase zinc uptake into the liver and lowers its concentration in the serum (278). In the case of iron, the decrease in serum iron levels may be attributed to regulation by the peptide hormone hepcidin, which is secreted from the liver during inflammation. Hepcidin has been shown to trigger the endocytosis and reduced expression of the iron exporter ferroportin-1 in enterocytes and macrophages (116, 279), thus reducing the transport of dietary iron into the blood and the export of iron from macrophages of the reticuloendothelial system. These responses are among several others that function to limit iron as a nutrient for microbial growth (280).

1.8.1 Copper is a bactericidal agent.

Mild copper deficiency has been associated with impaired function of neutrophils and macrophages (281-284). Moreover, in cultured peritoneal macrophages, phagosomal copper levels are increased ten-fold when cells are infected with *Mycobacterium avium* in the presence of the cytokine IFN-gamma (285). Thus, copper levels may become elevated in the serum during inflammation to promote anti-microbial functions of these phagocytic cells. Consistent with this hypothesis, evidence suggests that the ability of pathogenic bacteria to export copper influences their virulence in the host. The virulence of *Pseudomonas aeruginosa*

is severely decreased (20-fold) by mutations in the copper exporter, CueA (286), and a similar attenuation of pathogenicity occurs in *Listeria monocytogenes* carrying a mutation in the copper exporter, CtpA (287). Also, 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 (288).

Copper owes its bactericidal activity to its ability to transition between Cu^{1+} and Cu^{2+}. This property of the copper ion allows it to participate in the Fenton reaction, whereby hydrogen peroxide H_2O_2 is quickly converted to the highly reactive hydroxyl radical $OH^{\bullet}$ (153-155). Reactive oxygen species (ROS), and more specifically $OH^{\bullet}$ greatly contribute to the killing of bacteria within the phagosomal compartment of immune cells. $OH^{\bullet}$ is highly toxic to cells due its ability to react with and oxidize organic molecules, such as membrane lipids, proteins and DNA, thereby compromising membrane fluidity and other essential cellular functions (289). It is not surprising, therefore, that phagocytic cells of the immune system have harnessed this property of ROS, and use it as a first line of attack against pathogens within the phagosomal compartment. This is known as the respiratory or oxidative burst, and it occurs in phagocytic cells as they initiate the degradation of internalized pathogens (257).

1.9 A role for copper in the cellular response to hypoxia.

1.9.1 Physiological and cellular responses to hypoxia.

Oxygen (O_2) utilization by cells of the body normally serves to regenerate energy in the form of ATP. Hydrolysis of ATP provides the necessary energy for maintenance, self-repair and for sustenance of tissue- and organ-specific functions. Although cells have a limited ability to generate ATP in the absence of O_2 via anaerobic respiration, the loss of O_2 supply, even for a short period of time, can threaten cell survival. Therefore, organisms have developed a number of adaptive responses to defend the O_2 supply in response to environmental changes, or to diseases that decrease the delivery of O_2 to tissues. Specialized O_2-sensing systems have evolved to detect subtle changes in O_2 tension. Among these are the arterial chemoreceptors that monitor O_2 levels in the blood and signal to the respiratory system to increase the levels of alveolar ventilation when arterial O_2 tension is too low. The liver and kidneys secrete erythropoietin, a hormone that signals to increase the number of circulating erythrocytes and the O_2–carrying capacity in the circulation (139). In addition, cells protect themselves during limitations in O_2 supply by downregulating O_2 utilization (140-142).

Rapid responses are required on the cellular level in order to protect the cell from prolonged O_2 depletion. These responses are mediated by the hypoxia-inducible factor transcription factor (discussed below), and lead to the upregulation of

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genes involved in glucose uptake, cell survival, cytoskeletal organization, extracellular matrix remodeling, cell adhesion, vascular tone, iron metabolism, and apoptosis (290-294). On the physiological level, responses to limitations in O_2 supply ultimately result in vascular remodeling, formation of new blood vessels or angiogenesis, and cell migration and proliferation.

1.9.2 Transcriptional responses to hypoxia.

Hypoxia, or low oxygen tension, is a common feature of many solid tumors (295), and has also been implicated in the etiology and the progression of Alzheimer's disease (296). In cancer, hypoxia has been linked to malignant transformation, metastasis, and treatment resistance (295). The cellular adaptation responses to hypoxia are mediated via the hypoxia-inducible factor 1 (HIF-1), a transcription factor often named "the master regulator of hypoxic responses", that upregulates hypoxia response element- (HRE-) dependent gene expression. Among HRE-dependent genes are several genes involved in glycolytic energy metabolism, angiogenesis, cell survival and erythropoiesis. Some of these are glucose transporters (GLUT), vascular endothelial growth factor (VEGF), erythropoietin, and other glycolytic genes (297, 298).

HIF-1 is a heterodimer composed of two subunits, an oxygen-regulated α subunit (HIF-1α) and a constitutively expressed β subunit (HIF-1β), both of which are ubiquitously expressed in mammalian cells (299). The HIF-1 complex regulation

is dependent on the stability of the HIF-1α subunit. HIF-1α is constitutively expressed; however, under normoxic conditions, the protein undergoes ubiquitination and is targeted for proteosomal degradation (300, 301). This involves the binding of the von Hippel-Lindau tumor suppressor protein (VHL) to an oxygen-dependent degradation domain on the HIF-1 protein. The binding of VHL to HIF-1α is regulated via hydroxylation of key proline residues on the HIF-1 protein, which is carried out by a family of HIF prolyl hydroxylases (HIF-PHs) (108, 302-304). HIF-PHs require oxygen and iron binding for their activity. Thus, during hypoxia, or in the presence of iron chelators such as desferrioxamine (DFO) or iron-displacing metals such as cobalt, HIF-PHs are inactivated, and this leads to a stabilization and accumulation of the HIF-1α protein. Thus, HIF-1 acts as a sensor of cellular oxygen tension and iron homeostasis in all cell types.

HIF-1 activation in cancer cells is highly associated with cell growth and survival, tumor development, tumor angiogenesis and poor clinical prognosis (305-308). Histopathological studies in primary tumors of the breast, colon, brain, lung, ovary and prostate have detected HIF-1 expression that could not be seen in corresponding normal tissues (308). Although hypoxia is the ubiquitous inducer of HIF-1 expression, constitutive HIF-1 expression has been detected in several nonhypoxic cancer cell lines (305) and normal tissues (309). In fact, in several cell types, other stimuli have been reported to stabilize and activate HIF-1, including epidermal growth factor (EGF), insulin, insulin-like growth factor 1 (IGF-1), angiotensin II and others (310-312). Altogether, these observations suggest a

role for distinct signaling mechanisms in the regulation of HIF-1 activity. Of relevance here are the effects of copper and mitochondrially-generated reactive oxygen species on HIF-1 expression.

1.9.3 Cu- and ROS-mediated activation of HIF-1 and HRE-dependent genes.

Transition metals have long been used as molecular mimics of hypoxia. Co^{2+}, Ni^{2+} and Mn^{2+} salts induce the expression of erythropoietin in the hepatoma cell lines HepG2 and Hep3B under normoxic conditions (313). $CuCl_2$ also stabilizes HIF-1 under normoxic conditions and stimulates the expression of HRE-dependent genes. Martin *et al.* (314) have suggested that copper activates HIF-1 by inhibiting prolyl hydroxylase activity independent of iron availability. Their experiments in hepatoma cells suggest that both copper and hypoxia increase the ceruloplasmin, VEGF and GLUT-1 mRNA levels. The authors propose that HIF-1 activation and HRE-dependent gene regulation not only serves as a sensing system for oxygen tension and iron availability, but also regulates copper metabolism.

The mechanism by which copper and other transition metals regulate HIF-1 is unknown. Several studies have implicated reactive oxygen species (ROS) in transition-metal dependent HIF-1 activation (315-319). As a transition redox metal, copper can participate in Fenton reactions, thereby catalyzing the conversion of molecular oxygen (O_2) into the powerful oxidant superoxide ($O^{•}$). In

the cellular environment, superoxide is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Copper then catalyzes the conversion of hydrogen peroxide into the harmful hydroxyl radical (OH$^\bullet$) (154, 155).

Increasing evidence suggests that it is the accumulation of ROS produced mainly by the mitochondria during low oxygen tension that mediates the activation of HIF-1. In particular, H_2O_2 has been linked to the stabilization of HIF-1 and the induction of HRE-dependent genes during hypoxia (139, 320-323). Grzenkowicz-Wydra *et al.* (323) have shown that overexpression of the human Cu/Zn superoxide dismutase SOD1 in NIH3T3 fibroblasts leads to enhanced intracellular production of H_2O_2 and stimulation of VEGF synthesis. In their study, H_2O_2 increased VEGF promoter activity, VEGF mRNA expression and VEGF protein synthesis. This effect was blocked by the simultaneous overexpression of catalase, an enzyme involved in antioxidant defense that scavenges H_2O_2 and converts it to H_2O. This is supported by further evidence that the overexpression of the antioxidants catalase and glutathione peroxidase during hypoxia prevents the stabilization of HIF-1α (324, 325).

It has been suggested that ROS stabilize HIF-1 indirectly via activation of kinase signaling cascades. Three pathways have been identified that can be activated by ROS, and in turn activate HIF-1. These are small GTPases (such as Rac or Rho), c-Src kinase, and p38a MAPK (326-328). However, the direct mechanisms by which ROS activate these signaling pathways that ultimately stabilize HIF-1α

during hypoxia have not been determined yet. In summary, it is possible that copper activates HIF-1 directly by binding and inhibiting the HIF-prolyl hydroxylases, and indirectly via its redox potential to generate ROS.

1.9.4 Generation of ROS during hypoxia.

Relative O_2 depletion can cause severe organ damage in human disease. Hypoxic tissue injury occurs during respiratory failure, systemic hypotension, and regional hypoperfusion of organs. Reactive oxygen species have been implicated in the lethal cell injury resulting from relative hypoxia and following reperfusion of ischemic heart, brain and organs (329-332). Studies have also shown that severe high-altitude hypoxia can increase the generation of cellular oxidative stress, thus causing damage to lipids, proteins and DNA (133-138).

Several cellular mechanisms for O_2-sensing and generation of ROS during hypoxia have been investigated, including NADPH oxidase (333) and cytochrome p450 (334). Diphenyleneiodonium (DPI) is a non-specific inhibitor of flavoprotein oxidases. DPI interferes with HIF1-mediated responses to hypoxia (335). Thus, it has been concluded that NADPH oxidase and/or cytochrome p450 regulate O_2 sensing and participate in HIF-1 activation during hypoxia (336). However, DPI also inhibits mitochondrial complex I (337). Inhibition of complex I suppresses ROS production by complex III (see below for further discussion; reviewed in (139)). Moreover, in B cell lines deficient in either the p22[phox] or the

gp91^{phox} subunits of the NADPH oxidase, the VEGF and aldolase mRNA responses to hypoxia are intact, suggesting that O_2-sensing mechanisms independent of NADPH oxidase exist (321, 338). The roles of NADPH oxidase and cytochrome p450 certainly require further investigation.

1.9.5 Mitochondrial ROS production regulates the responses to hypoxia.

Mitochondria are classically considered the subcellular organelles in eukaryotes that are responsible for energy production. Mitochondria consume oxygen and generate the ATP required for energy utilization, thus O_2 deprivation threatens cell survival. Recent emerging evidence now implicates mitochondria as a major source of free radicals, and as the source of signaling that regulates cell cycle, proliferation, and apoptosis. In 1973, Boveris and Chance described the production of H_2O_2 by mitochondria, and characterized H_2O_2 as a by-product of the auto-oxidation of components of the respiratory electron transport chain (339). This process, the "electron leak", occurs during respiration under conditions of sufficient O_2 supply.

New evidence now points to the mitochondria as the site of O_2 sensing during hypoxia. It has been proposed that the electron transport chain senses decreases in O_2 supply and produces ROS in response to hypoxia. The ROS released from the mitochondria act as signaling molecules and activate HIF-1 (139, 321, 324, 325, 340-344). The importance of mitochondrial signaling in the

cellular responses to hypoxia is emphasized by studies using ρ^0 cells which have been depleted of their mitochondrial DNA. ρ^0 cells lack mitochondrial DNA-derived proteins and are therefore deficient in key components of the electron transport chain, which renders them incapable of mitochondrial respiration (321). Contrary to wild-type cells, ρ^0 cells fail to stabilize HIF-1-dependent gene expression as well as fail to produce ROS during hypoxia (321, 340).

It is now widely accepted that ROS production during hypoxia primarily originates at complex III of the respiratory chain. Eukaryotic complex III is an assembly of 11 proteins encoded by nuclear and mitochondrial genes (345). Complex III accepts electrons from ubiquinol, and transfers these to cytochrome c. The Q (Ubiquinone) cycle is a major component of complex III. A pair of electrons is transferred to ubiquinone from complexes I and II. This yields ubiquinol. As cytochrome c and cytochrome c oxidase (CCO) accept single electrons sequentially, the Q cycle within complex III converts the paired transfers of complexes I and II into the sequential transfers needed for complex IV (346). This sequential transfer of electrons at complex III temporarily yields the univalently reduced ubisemiquinone. The relatively unstable ubisemiquinone radical is repeatedly generated at complex III during the electron transport process. Molecular O_2 within the mitochondrial membrane can potentially capture the electron from ubisemiquinone, yielding superoxide. The probability of this event occurring increases as the lifetime of the ubisemiquinone radical increases (342-344) . Mitochondrial inhibitors, such as Antimysin A, which increase the

half-life of ubisemiquinone, increase superoxide production at complex III (339). Genetic evidence for the requirement of complex III in the cellular responses to hypoxia is provided by studies using RNA interference against the Rieske protein. The Rieske protein is a subunit of complex III that plays a central role in generating ubisemiquinone during the electron transfer process. siRNA knockout of the Rieske protein prevents the formation of ubisemiquinone, and significantly attenuates ROS production compared to controls. Significantly, siRNA against the Rieske protein prevents the stabilization of HIF-1α during hypoxia (324, 325).

1.10 Potential role for copper in human disease.

The importance of copper as a nutrient has been underlined in this review. In addition to illnesses directly related to defects in the various copper homeostatic pathways, new connections have been drawn between copper and disease conditions such as inflammation, angiogenesis, and Alzheimer's disease. Inflammation is accompanied by an increase in serum copper levels (263-267), and copper has been shown to play a role in the proper functioning of the immune system (281-284).

A hallmark of many chronic inflammatory illnesses such as cancer, Alzheimer's disease, atherosclerosis and others is a decrease in the availability of oxygen at the site of inflammation. This lower-than-normal oxygen tension induces a wide array of cellular and physiological responses, including an increase in oxidative

stress in the inflammatory milieu, and the formation of new blood vessels along with the restructuring of the surrounding extracellular matrix. Copper availability during low oxygen tension may influence the outcome of such cellular and physiological processes and may tip the balance towards either the resolution or the exacerbation of the disease condition.

Angiogenesis, or the process of new blood vessel formation, serves to provide nutrients and oxygen to tissues where blood flow has been obstructed, and is an important component in the mechanism of tumor growth and metastasis. Copper is essential for the angiogenic process, not only in the cross-linking of newly formed extracellular matrix, but also in the transcriptional regulation of certain angiogenic responses. Copper chelation therapy has been successfully used in animal trials in the inhibition of angiogenesis in cancer (347-352). Copper is also an important component of Aβ plaques, as it serves to cross-link and aggregate the Aβ protein in the extracellular milieu, thus rendering it toxic to surrounding neurons (353-357).

Despite the emerging knowledge of a role for copper in these disease conditions, very little is understood about the role and contribution of the major regulators of copper homeostasis, CTR1 and ATP7A, in these pathophysiological events. The goal of my studies is two-fold: 1) To investigate the changes in copper homeostasis in macrophages during inflammation and gain an understanding of the contribution of these changes to the bactericidal activity of this cell type. 2) To

investigate the effect of hypoxia on the copper homeostatic pathways in

macrophage cells and gain some insight into the potential regulatory role of

oxidative stress in governing these cellular adaptations.

CHAPTER 2

A ROLE FOR THE ATP7A COPPER TRANSPORTING ATP7ASE IN
MACROPHAGE BACTERICIDAL ACTIVITY

2.1 Abstract

Copper is an essential micronutrient required for healthy immune function. This
requirement is underscored by increased susceptibility to bacterial infection in
copper deficient animals, however, a molecular understanding of copper's
importance in immune defense is unknown. To elucidate the role of copper in
innate immune function, we investigated the effect of pro-inflammatory agents on
copper homeostasis in RAW264.7 macrophages. Interferon-gamma 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 with a defect in copper export was hypersensitive to killing by
RAW264.7 macrophages, and this phenotype was dependent on ATP7A
expression. Together, these data reveal a critical role for copper transport via the

ATP7A protein in macrophage-dependent bactericidal activity and demonstrate a unique role for copper in host-pathogen interactions.

2.2 Introduction

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 (96). However, these same redox properties also confer copper with toxic properties when it is present in the free ionic form. Free copper can participate in Haber-Weiss 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.

The importance of micronutrient intake for optimal immune function is an area of intense research, and several studies have demonstrated the importance of copper. Studies in animals fed copper-deficient diets have shown impaired animal resistance to a number of pathogens including *Candida albicans* (5, 6), *Pasteurella haemolytica* (358), *Trypanosoma lewisi* (359) and *Salmonella typhimurium* (9). Other studies have shown that copper supplementation is protective against *Escherichia coli* induced mastitis in dairy cattle (10). *In vitro* studies have shown that copper deficiency impairs the bactericidal activity of neutrophils and macrophages (11-13). Moreover, a recent study demonstrated

that copper concentrations are markedly increased within the phagosomal compartment of macrophages activated by interferon-gamma (IFN-γ) and *Mycobacterium avium* (14).

The potential toxicity of copper can be attributed to its ability to transition between the cuprous (Cu^{1+}) and the cupric (Cu^{2+}) states. This property of the copper ion allows it to participate in the Fenton reaction, whereby hydrogen peroxide (H_2O_2) is quickly converted to the highly reactive hydroxyl radical $OH^{\bullet}$ (153-155). Reactive oxygen species (ROS), and more specifically $OH^{\bullet}$ greatly contribute to the killing of bacteria within the phagosomal compartment of immune cells (257). $OH^{\bullet}$ is highly toxic to cells due its ability to react with and oxidize organic molecules, such as membrane lipids, proteins and DNA, thereby compromising membrane fluidity and other essential cellular functions (289). It is not surprising, therefore, that phagocytic cells of the immune system have harnessed this property of ROS as a first line of attack against pathogens within the phagosomal compartment. This is known as the respiratory or oxidative burst, and it occurs in phagocytic cells as they initiate the degradation of internalized pathogens (257). Interestingly, the acute-phase response to infections and inflammation in humans and animals is marked by changes in circulating metal levels. Both zinc and iron levels are significantly decreased, resulting in the classical hypozincemia and hypoferremia, two hallmark events of the acute-phase response to inflammation (261, 262). In contrast, inflammation results in an increase in copper levels in the serum, or hypercupremia (263-274,

360-364). Collectively, these studies provide compelling evidence of a role for copper in innate immune defense, however, an understanding of the underlying processes at a molecular level is lacking.

On the cellular level, mammalian copper homeostasis is maintained by the concerted regulation of copper uptake and copper export. The copper importer Ctr1 is ubiquitously expressed. It is localized to the plasma membrane and transports copper across the membrane into the intracellular milieu (151, 166). Copper export from the cytoplasm is mediated by ATP7A, also known as Menkes protein. In steady-state conditions, ATP7A resides in the Trans-Golgi network (TGN) (1, 365), and delivers copper to copper-containing proteins and enzymes (232-237). Under excess copper conditions, ATP7A relocates from the TGN to the plasma membrane where it exports copper, thus protecting the cell from potential copper-mediated toxicity (1). ATP7A cycles between the TGN and the plasma membrane as it exports copper, and returns to its TGN localization (169, 238, 366) once steady-state copper homeostasis is re-established. CTR1 and ATP7A protein and mRNA levels do not respond to changes in copper concentrations. Overall, mammalian copper homeostasis is maintained via post-translational regulation of the localization of the copper importer CTR1 and the copper exporter ATP7A. However, little is known about how these major players in cellular copper homeostasis are regulated during inflammation.

The fact that inflammation is accompanied by hypercupremia, and that copper exhibits bactericidal properties suggests that immune cells of the phagocytic type may benefit from this increased availability of copper and use it as an advantage to speed up the killing of pathogens. Here, we report dramatic changes in copper homeostasis triggered by the inflammatory response in macrophages. These include elevated expression of the CTR1 copper importer, increased copper uptake, and copper-stimulated trafficking of the ATP7A copper exporter from the Golgi to vesicles that overlap with the phagosomal compartment. Significantly, RNAi-mediated depletion of ATP7A in RAW264.7 cells reduced bactericidal activity, suggesting that this process was dependent on copper transport via ATP7A. 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, and this sensitivity was dependent on ATP7A expression. These findings reveal ATP7A-mediated copper transport as a novel determinant of macrophage bactericidal activity and identify a unique interplay between copper transport activities of host and pathogen.

2.3 Materials and Methods

Preparation of stock solutions— The stock solutions of LPS, IFN-γ and Ebselen were prepared as follows:

LPS (Sigma) stock was 1000 μg/ml in 10 mg/ml bovine serum albumin solution (BSA, prepared in PBS). It is not recommended to store LPS in stock solutions

with concentrations lower than 1000 μg/ml. Stock solutions were stored at -80°C

and each tube went through the thaw-refreeze cycle no more than twice. Stock

solution was spun at high speed ($\geq$10,000 x g) for 2 minutes following thawing.

Mouse IFN-γ (BD Biosciences) stock was 25 μg/ml in 10 mg/ml BSA solution.

Storage and centrifugation conditions are the same as for LPS stock.

Ebselen (Sigma) solution was 50 mM in 1:1 DMSO:ethanol. The stock was

stored at 4°C. It is worth noting that the Ebselen solution can undergo light- and

air-induced oxidation, which reduces the shelf life of the stock solution.

Therefore, it may be necessary to monitor the decline of the antioxdiant property

of Ebselen and use prepare fresh stocks periodically.

Cell Culture— RAW264.7 cells were obtained from the American Type Culture

Collection and were maintained in Dulbecco's modified Eagle's medium

(Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum and 100

units/ml penicillin and streptomycin (Invitrogen) in 5% CO_2 at 37°C. Primary

macrophages from C57BL/6J mice were isolated by peritoneal lavage 72 h

following intraperitoneal injection with 2 ml of thioglycolate medium. ATP7A-

depleted RAW264.7 cells (ATP7A-RNAi) 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 (Invitrogen).

Control cells were transfected with the same vector expressing shRNA against

GFP (Origene). Lipofectamine 2000 (Invitrogen) was used for all transfections.

Copper Uptake Studies— Radioactive copper (^{64}Cu) was purchased from the Mallinckrodt Institute of Radiology, Washington University (Saint Louis, MO). ^{64}Cu uptake was assayed as described previously (93). RAW264.7 macrophage cells were grown in triplicate 6 well trays and cultured overnight in serum-free medium in the presence or absence of 25 ng/ml IFN-γ (BD Biosciences, Franklin Lakes, NJ). Cells were washed with serum-free DMEM medium and then exposed to 1 μM ^{64}Cu for 5 min, washed extensively in ice-cold PBS and radioactivity was measured using a gamma counter. Counts were normalized against total protein.

Bacterial Survival Assay— RAW264.7, control-RNAi or ATP7A-RNAi macrophage cells were activated by overnight treatment in serum-free DMEM medium with or without 25 ng/ml IFN-γ. Where indicated, either 20 μM CuCl$_2$ or 50 μM Ebselen was also added to the cells. Macrophages were then detached by scraping into ice-cold serum-free media, washed twice, and resuspended in triplicate in serum-free medium at a concentration of 4 x 10^6 cells/ml. *E. coli* wild-type strain W3110 and the *copA* knockout strain DW3110 (367, 368) were grown to stationary phase and mixed with 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 extracellular bacteria removed by two washes with phosphate buffered saline containing 12.5 μg/ml Gentamicin (Gibco). One set of samples (uptake group) was lysed in 0.1% (v/v) Triton X-100 solution and plated onto LB-agar

plates for counting to provide bacterial uptake values. The remaining set (kill group) was incubated for 1-2 hours as indicated at 37°C in serum-free media 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 bacterial ROS survival assay—This protocol is based on previously published methods (369). Wild-type *E. coli* W3110 was grown overnight to stationary phase. The next day, 1 ml of bacterial solution was diluted into 4 ml LB and grown for 2-3 hours to exponential phase (OD600 = 0.2 - 0.3). The bacterial solution was then pelleted and the LB discarded.

The bacterial pellet was washed twice with PBS + 67 μM EDTA followed by one wash with PBS only. The bacterial pellet was then re-suspended in buffer containing 0.1M sodium phosphate pH 7.4, and 0.15 M sodium chloride in water. The bacterial suspension was then aliquoted into 1 ml samples. Samples were run in triplicates. The sample treatments were as follows:

- Buffer only (designated as untreated group in results section)

- Buffer + 500 μM ascorbic acid

- Buffer + 500 μM ascorbic acid + 10 μM CuCl$_2$ (copper group)

- Buffer + 500 μM ascorbic acid + 500 μM H$_2$O$_2$ (H$_2$O$_2$ group)

- Buffer + 500 μM ascorbic acid + 10 μM CuCl$_2$ + 500 μM H$_2$O$_2$ (H$_2$O$_2$ + copper group)

- Buffer + 10 μM $CuCl_2$

- Buffer + 500 μM H_2O_2

The treatments were added to the bacterial solutions in the following order: 1) ascorbic acid, 2) copper, 3) H_2O_2. The samples were incubated at 37°C for 20 min. A 1/1000 dilution of each sample was plated on LB + ampicillin and bacterial survival rates were calculated and expressed as percentage survival compared to the untreated group.

Immunofluorescence Microscopy— RAW264.7 cells or primary peritoneal macrophages were grown overnight on sterile glass coverslips in serum-free media. The next day, all treatment groups received a change of serum-free media, and where indicated, were treated with 100 ng/ml LPS, 25 ng/ml IFN-γ, or 20 μM $CuCl_2$, in the presence or absence of 50 μM Ebselen for 24 hours. Cells were then washed in ice-cold PBS, fixed in 4% (w/v) paraformaldehyde, permeabilized in 0.05% Triton X-100, and blocked overnight in 1% (w/v) casein solution. Cells were then incubated for 1 hour in the presence of primary antibodies against the C-terminal portion of ATP7A, and against mouse GM130. This was followed by three 10-minute washes in PBS, and a 1-hour incubation with the following secondary antibodies: anti-rabbit IgG conjugated to Alexa-488 (green) and anti-mouse IgG conjugated to Alexa-594 (red) (Molecular Probes). Cells received 2 washes in PBS followed by a 15-minute incubation in PBS containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI, 1:12,500 dilution), and another wash in PBS. Cells were then given a long wash in PBS

overnight at 4°C. The next day, slides were mounted on glass plates using the mounting medium Mowiol and allowed to dry before microscopy.

Immunoblot Analysis—Cells cultured in 6-well trays were scraped into ice-cold phosphate-buffered saline (PBS) and pelleted by centrifugation. After several washes in ice-cold PBS, the cells were lysed by one of two methods: 1) for ATP7A protein detection, cells were lysed by sonication in lysis buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% (w/v) SDS, 1 mM EDTA, and protease inhibitor mix (Roche Applied Science) 2) for CTR1 protein detection, cells were lysed on ice for 20 min in lysis buffer containing 62.5 mM Tris-Cl, pH 6.8, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 mM EDTA, and protease inhibitor mix. Samples were centrifuged for 10 min at 16,000 x g, and the protein concentration of the lysates was determined using a DC protein assay kit (Bio-Rad). Twenty µg of protein lysates were resuspended in loading buffer containing 100 mM dithiothreitol, separated using SDS-PAGE, transferred to nitrocellulose membranes, and detected by chemiluminescence (370). As a loading control, the same membranes were stripped and re-probed with mouse anti-tubulin antibody. For the detection of secreted ceruloplasmin, conditioned media were collected and concentrated (Amicon Ultra). The corresponding sample lysates were used to measure protein content and adjust sample loading and to probe for tubulin as a loading control.

The antibody dilutions in 1% (w/v) casein solution and incubation times were as follows: 1) for detection of ATP7A protein, an antibody against the C-terminal end

of the protein was raised by our laboratory and named MNK. Membranes were probed with a 1:5,000 dilution of this antibody for 2 hours at room temperature, 2) for detection of CTR1 protein the anti-Ctr1 antibody (a gift of Dennis Thiele, Duke University) was used at 1:500 dilution overnight at room temperature, or 3) the sheep anti-ceruloplasmin antibody (Abcam) was used at 1:250 dilution overnight at 4^oC. Western blot bands were quantified using the Quantity One software (Biorad). Each band expression was normalized against its own tubulin control, and expression is expressed as relative to the untreated control.

Latex Bead Phagocytosis and Phagosome Purification— RAW264.7 cells were activated by overnight treatment with 25 ng/ml IFN-γ and exposed to a 1/200 dilution of 3 µm latex beads (Sigma) for 90 min at 37^oC to allow phagocytosis to proceed. Cells were then washed with PBS to remove extracellular beads and fixed in paraformaldehyde and processed for immunofluorescence, as above. In other experiments, phagosomes were purified by sucrose gradient centrifugation, as previously described (371).

Ceruloplasmin Activity— Conditioned media was used to assess ceruloplasmin activity. The media was concentrated using Amicon Ultra-4 filter tubes (Millipore) and ceruloplasmin's p-phenylenediamine oxidase activity was assessed, as previously described (372). Ceruloplasmin activity values were normalized against total cell protein content. It is to be noted here that serum-containing media should be used for the successful detection of ceruloplasmin activity.

2.4 Results

Copper enhances the bactericidal activity of macrophages— We began this study by determining the impact of copper supplementation on the bactericidal activity of the murine macrophage cell line, RAW264.7. The cells were treated overnight with the pro-inflammatory cytokine interferon-gamma (IFN-γ) in the presence or absence of 20 μM copper added to the medium, and their ability to kill *E. coli* was determined the following day. Bacterial survival was significantly reduced in copper-treated RAW264.7 macrophages relative to control cells (Fig. 2.1A), suggesting that copper potentiated the bactericidal activity of these cells. One suggested mechanism for copper-mediated bacterial killing is through Fenton chemistry. The oxidative burst that follows bacterial phagocytosis by macrophages leads to the rapid production of hydrogen peroxide (H_2O_2), which can interact with intracellular copper to produce the highly reactive and toxic hydroxyl radical ($OH^{\bullet}$) (2-4). Indeed, the potentiation of bactericidal activity by copper was prevented by the antioxidant, Ebselen, (Fig. 2.1B), suggesting that it was dependent on reactive oxygen species (ROS). Copper or Ebselen treatments did not alter the phagocytosis of *E. coli* compared to control RAW264.7 cells (data not shown). Furthermore, *in vitro* exposure of *E. coli* to hydrogen peroxide (H_2O_2) in the presence of copper significantly reduced the rate of bacterial survival compared to H_2O_2 or copper alone (Fig. 2.1C). These findings suggest that copper can promote the bactericidal activity of RAW264.7 macrophages in a ROS-dependent manner.

FIGURE 2.1. **Copper enhances bacterial killing by RAW264.7 macrophage cells.** A, RAW264.7 macrophages were treated with IFN-γ in the presence of absence of 20 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 after incubations for 1 h and 2 h at 37°C and expressed as a percentage of initial internalized *E. coli* (mean ± SD; n = 3; p<0.05). B, Bacterial survival was assayed in IFN-γ-treated RAW264.7 macrophages in the presence or absence of 20 µM copper and 50 µM Ebselen (mean ± SD; n = 3; p<0.05). C, Bacterial survival was assessed following exposure of *E. coli* to 500 µM H_2O_2, 10 µM $CuCl_2$ or H_2O_2 and $CuCl_2$ combined in the presence of 500 µM ascorbic acid (mean ± SD; n = 3; p<0.05). Different letters signify values of significant difference.

Fig. 2.1
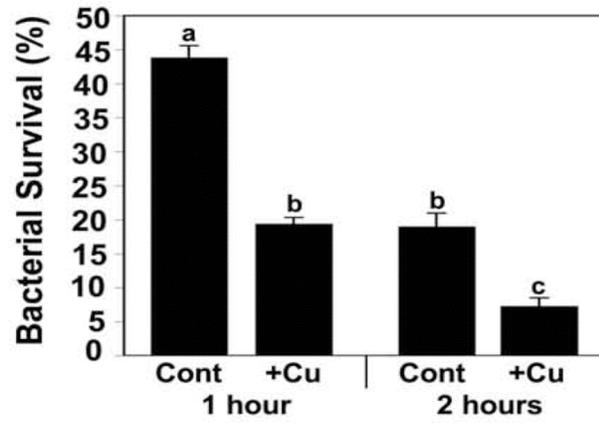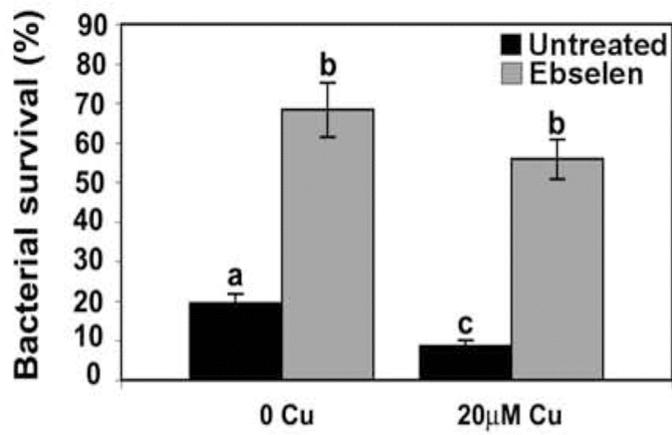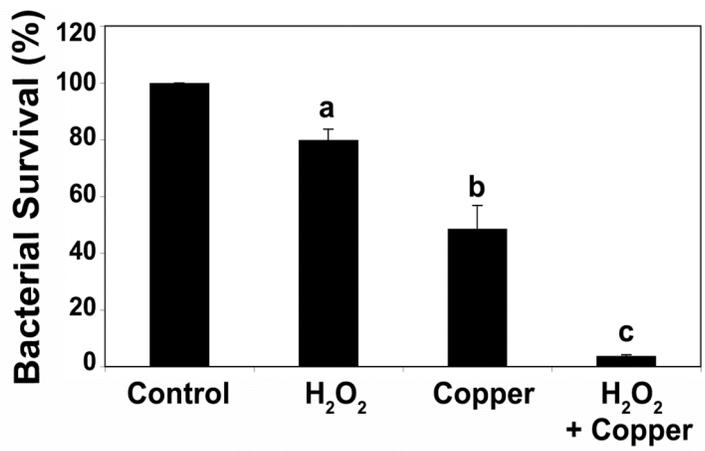
Inflammatory mediators induce the expression of CTR1 and ATP7A copper transporters— Since copper potentiated the bacteridical activity of RAW264.7 cells, we investigated whether copper homeostasis might be altered in these cells following stimulation with pro-inflammatory agents. Treatment with IFN-γ was found to stimulate copper uptake activity in RAW264.7 cells (Fig. 2.2A) and immunoblot analysis revealed an increase in the expression of the copper importer, CTR1 (Fig. 2.3A). CTR1 expression was also induced in RAW264.7 cells exposed to lipopolysaccharide (LPS), a bacterial cell wall component (Fig. 2.3B). LPS and IFN-γ also stimulated CTR1 expression in primary macrophages isolated from the peritoneum of mice (Fig. 2.3C). These findings suggest that the inflammatory response of macrophages stimulates CTR1-mediated copper uptake. Consistent with this hypothesis, the total cellular copper concentrations were elevated in LPS- and IFN-γ-treated RAW264.7 cells (Fig. 2.2B). Further evidence of elevated intracellular copper content was the finding of reduced expression of CCS in IFN-γ-treated RAW264.7 cells, a protein that is known to be proteolytically degraded in response to elevated levels of copper (185) (Fig. 2.2C). We then investigated the effect of pro-inflammatory agents on another copper transporter, ATP7A. The ATP7A protein is a copper transporting P-type ATPase responsible for delivering copper from the cytoplasm into secretory compartments and is located in the trans-Golgi network. ATP7A protein expression was increased in response to IFN-γ and LPS in RAW264.7 cells (Figs. 2.4A and 2.4B) and in primary peritoneal macrophages (Fig. 2.4C).

FIGURE 2.2. **IFN-γ alters copper homeostasis in RAW264.7 macrophages.**

A, Copper uptake activity. RAW264.7 cells were pre-treated with serum-free media ± 25 ng/ml IFN-γ for 24 hours and copper uptake in the presence of 1 μM ^{64}Cu was measured over 5 min and normalized against total protein (mean ± SD; n = 3). B, Atomic absorption spectrometry (AAS) analysis of total copper content of RAW264.7 cells treated with serum-free media, 100 ng/ml LPS or 25 ng/ml IFN-γ for 24 h. Values for copper content were normalized against total protein content of cell lysates (mean ± SD; n = 3). C, Western blot analysis of CCS protein expression in RAW264.7 macrophage cells exposed to 100 ng/ml LPS or 25 ng/ml IFN-γ. Tubulin was detected as a loading control.

Fig. 2.2
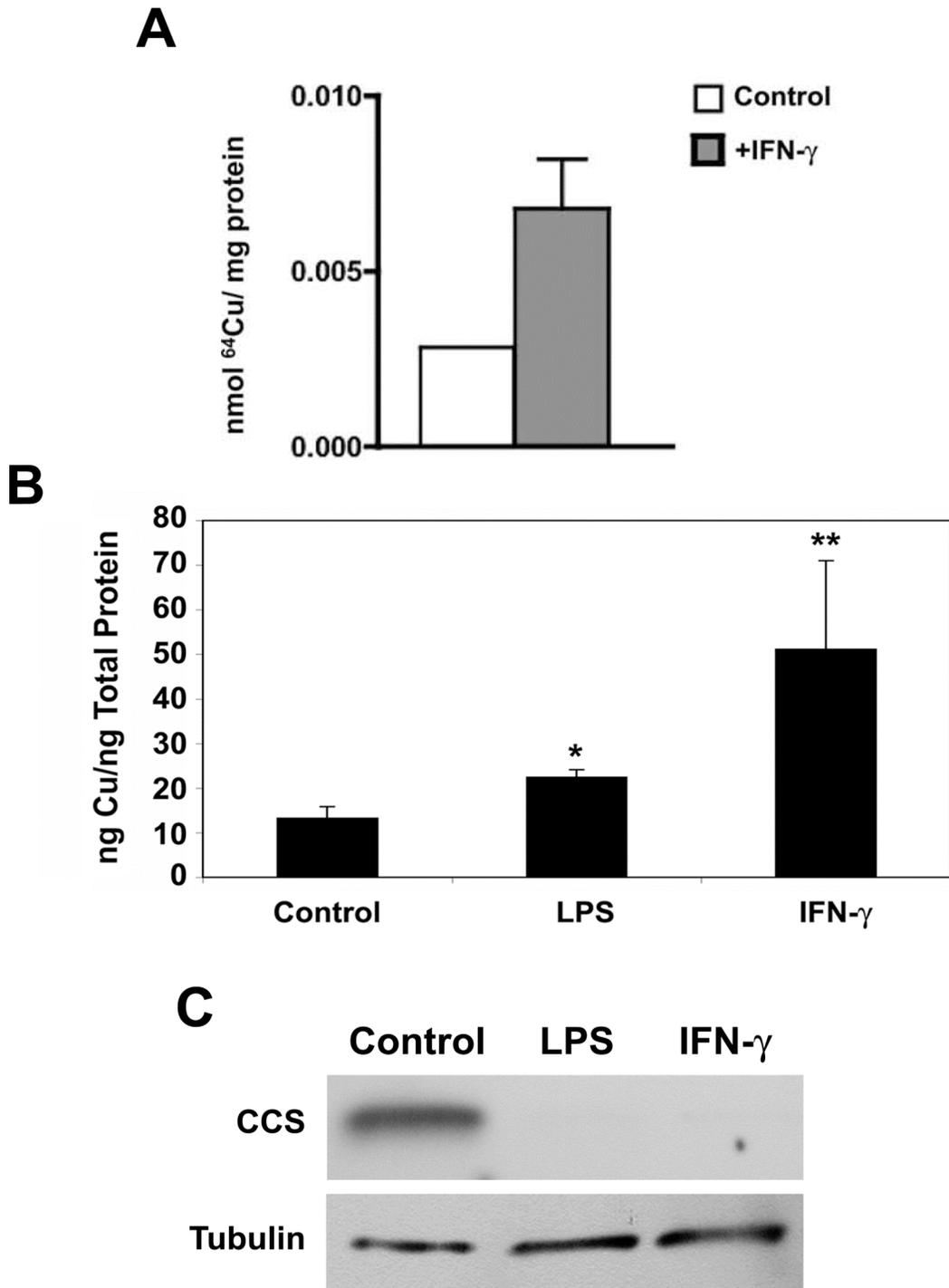
FIGURE 2.3. **Inflammatory mediators increase the expression of CTR1 in RAW264.7 and primary peritoneal macrophages**. Western blot analysis of CTR1 protein expression in: A, RAW264.7 macrophage cells exposed to a range of IFN-γ concentrations; B, RAW264.7 macrophage cells exposed to a range of LPS concentrations; C, Primary peritoneal murine macrophages cultured in serum-free media ± 100 ng/ml LPS or 25 ng/ml IFN-γ. Tubulin was detected as a loading control. The numbers shown under the immunoblot bands represent relative expression, as calculated using Quantity One software (Biorad). The expression level of each sample was first normalized against its own tubulin control. BSF (Basal serum-free media).

Fig. 2.3

A

RAW264.7

IFN-γ

ng/ml	0	5	10	25	50

CTR1   _20kDa

1   1.1   1.1   1.8   2.3

Tubulin

B

LPS

ng/ml	0	10	50	100	500	1000

CTR1   _20kDa

1   1   1.1   1.9   2.1   2.8

Tubulin

C

TPECs

BSF   LPS   IFN

30-

CTR1

20-

Tubulin

73
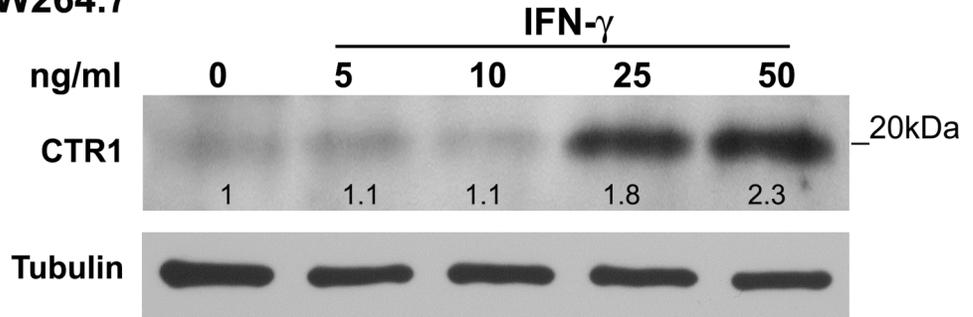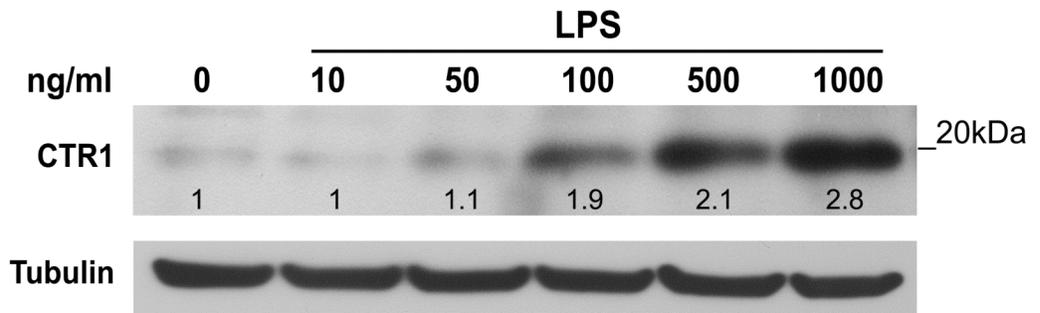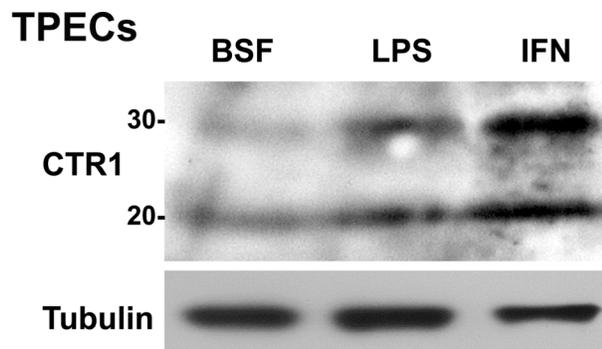
FIGURE 2.4. **inflammatory mediators increase the expression of ATP7A in RAW264.7 and primary peritoneal macrophages**. Western blot analysis of ATP7A protein expression in: A, RAW264.7 macrophage cells exposed to a range of IFN-γ concentrations; B, RAW264.7 macrophage cells exposed to a range of LPS concentrations; C, Primary peritoneal murine macrophages cultured in serum-free media ± 100 ng/ml LPS or 25 ng/ml IFN-γ. Tubulin was detected as a loading control. The numbers shown under the immunoblot bands represent relative expression, as calculated using Quantity One software (Biorad). The expression level of each sample was first normalized against its own tubulin control.

Fig. 2.4

A

RAW264.7

IFN-γ

ng/ml	0	5	10	25	50

ATP7A   _180kDa

1   2   2   2   2

Tubulin

B

LPS

ng/ml	0	10	50	100

ATP7A   _180kDa

1   1.9   3.2   4

Tubulin

C

TPECs

BSF   LPS   IFN

ATP7A   _180kDa

1   1.7   1.5

Tubulin
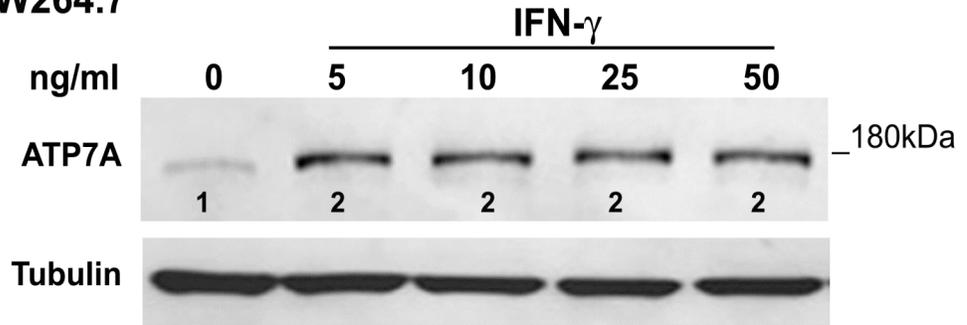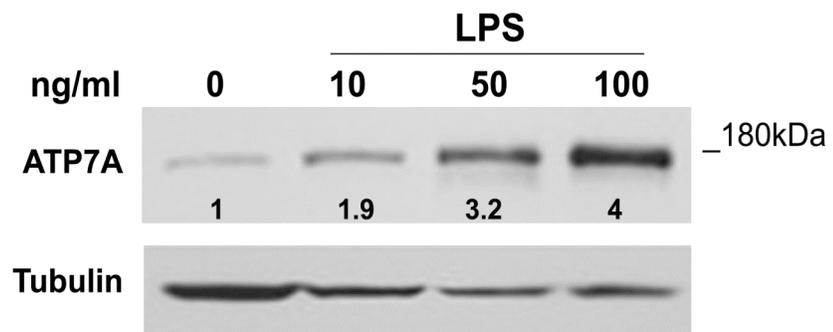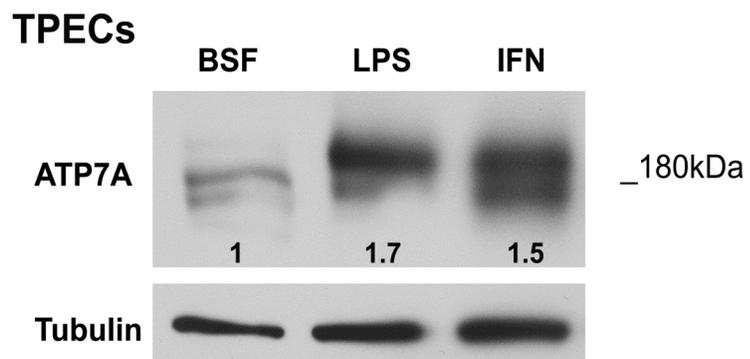
Another noteworthy finding is that both LPS and IFN-γ induced an increase in mRNA levels of the CTR1 and ATP7A transporters (Fig. 2.5). It is unknown whether this increase in mRNA levels is due to transcriptional or post-transcriptional regulation. Further studies are needed to elucidate the underlying mechanisms. Together, these results suggest that the stimulation of macrophages by inflammatory agents promotes copper uptake and increased expression of CTR1 and ATP7A proteins.

FIGURE 2.5. **Inflammatory mediators increase the expression levels of CTR1 and ATP7A mRNA.** RAW264.7 cells were grown in serum-free media ± 100 ng/ml LPS or 25 ng/ml IFN-γ for 0, 2, 6, 12 or 28 hours. RNA was purified and mRNA expression of CTR1 (A) and ATP7A (B) was quantified by real-time PCR. CTR1 and ATP7A mRNA levels were normalized against GAPDH. Fold expression is relative to a basal treatment at each time point (mean ± SD; n = 3).

Fig. 2.5
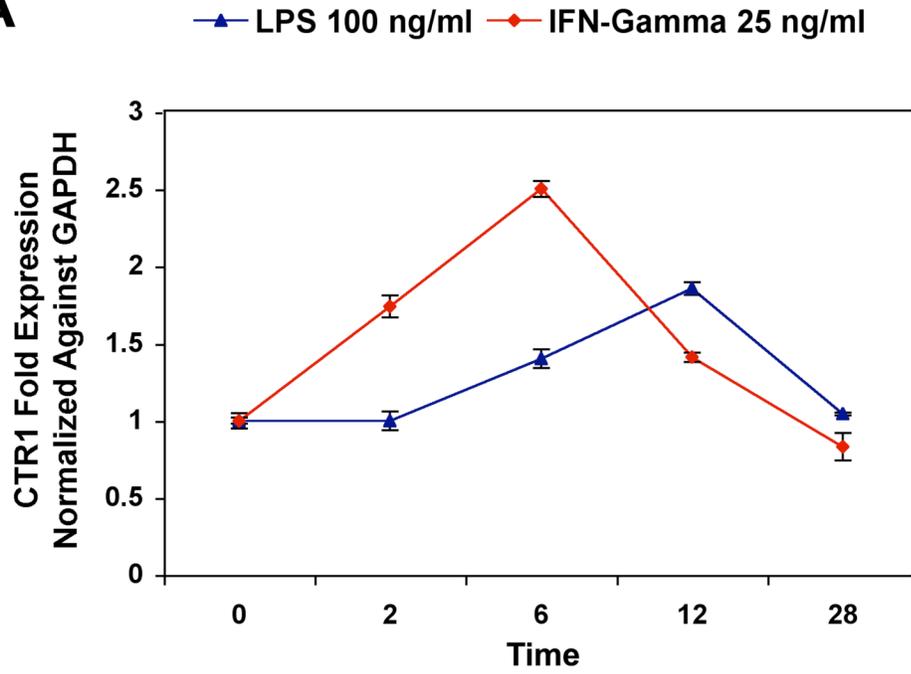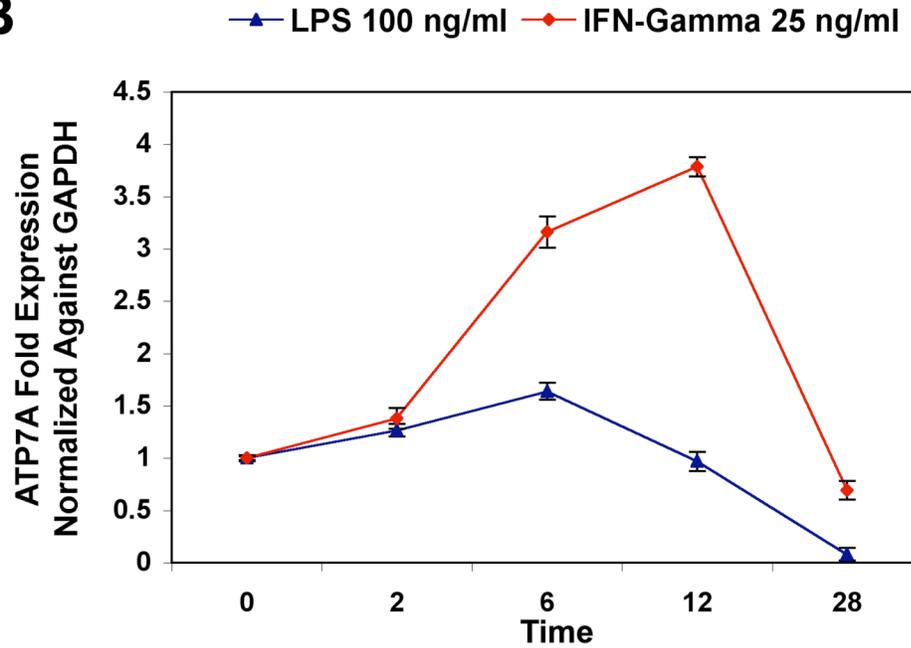
IFN-γ and LPS stimulate copper-dependent ATP7A trafficking to post-Golgi vesicles that overlap with the phagosome— Previous studies have demonstrated that elevated intracellular copper levels stimulate the trafficking of the ATP7A protein from the trans-Golgi network to post-Golgi vesicles in a variety of cell types (16-19). Based on these findings, we hypothesized that ATP7A trafficking might also be stimulated by the increased uptake and accumulation of copper in RAW264.7 cells treated with IFN-γ or LPS. Immunofluorescence microscopy was used to localize the ATP7A protein in the perinuclear region of RAW264.7 macrophages, consistent with its location in the trans-Golgi network (Fig. 2.6A, upper panel). As expected, the addition of copper to the culture medium stimulated the trafficking of ATP7A to post-Golgi vesicles (Fig. 2.6A, upper panel). Interestingly, when RAW264.7 cells were exposed to IFN-γ or LPS without copper supplementation, the ATP7A protein was also distributed to post-Golgi vesicles (Fig. 2.6A, upper panel). IFN-γ and LPS did not alter the location of the Golgi matrix marker protein GM130, suggesting that the shift in ATP7A distribution was not a result of a general Golgi disruption (Fig. 2.6A, lower panel). ATP7A trafficking was also observed in primary peritoneal macrophages in response to LPS and IFN-γ stimulation (Fig. 2.6B). Since the trafficking of ATP7A is known to be responsive to copper, we examined whether these effects of IFN-γ could be inhibited using the membrane permeable copper chelator, tetrathiomolybdate (TTM). Treatment of RAW264.7 cells with TTM suppressed the trafficking of ATP7A in response to IFN-γ consistent with a role for copper in this process (Fig. 2.7A). Interestingly, Western blot analysis indicated that the

increased levels of ATP7A in response to either IFN-γ or LPS were not blocked

by TTM (Fig 2.7B). Taken together, these findings suggest that IFN-γ stimulates

ATP7A trafficking in a copper-dependent manner that is different from the

copper-independent mechanism by which it stimulates ATP7A expression.

FIGURE 2.6. **Inflammatory mediators stimulate trafficking of the ATP7A protein in RAW264.7 and primary peritoneal macrophage cells.** A, Immunofluorescence analysis of ATP7A protein in RAW264.7 cells grown for 24 h in the presence or absence of 100 ng/ml LPS, 25 ng/ml IFN-γ, or 20 μM CuCl$_2$. Cells were fixed, permeabilized and probed with antibodies against ATP7A and anti-rabbit antibodies conjugated to Alexa-488 (green; upper panel) or antibodies against GM130 and anti-mouse IgG antibodies conjugated to Alexa-594 (red; lower panel). Nuclei were labeled with DAPI (blue). B, ATP7A traffics in response to LPS and IFN-γ in thioglycollate-elicited primary peritoneal macrophages derived from C57BL/6 mice.

Fig. 2.6

A

	Control	LPS	IFN-γ	Copper
ATP7A				
GM130				

B

	Control	LPS	IFN-γ
ATP7A			
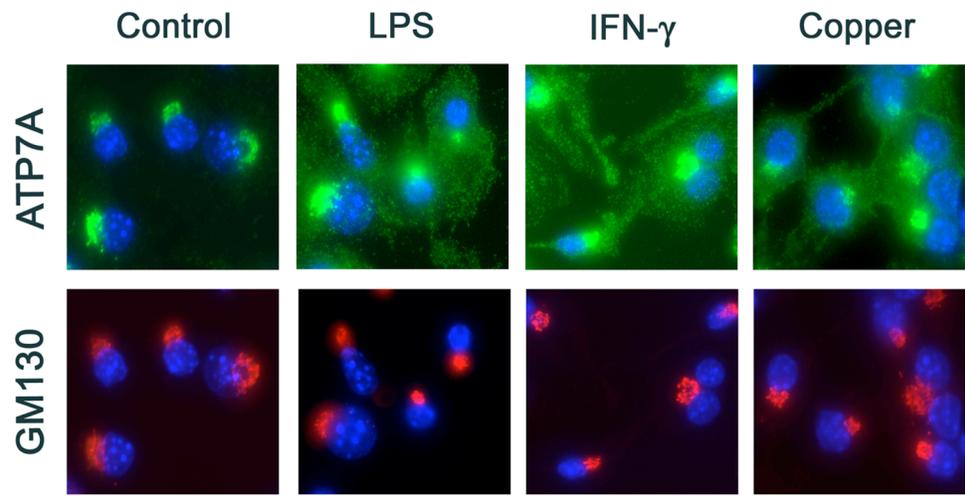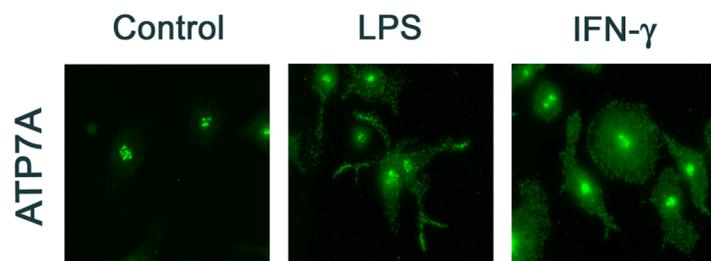
FIGURE 2.7. **The IFN-γ-stimulated trafficking of ATP7A is mediated by copper.** A, IFN-γ-induced trafficking of ATP7A is inhibited by the copper chelator tetrathiomolybdate (TTM). RAW264.7 macrophage cells were cultured in the presence of IFN-γ ± 10 nM TTM. Cells were fixed, permeabilized and probed with antibodies against for ATP7A and anti-rabbit IgG antibodies conjugated to Alexa-488 (green). Nuclei were labeled with DAPI (blue). B, the copper chelator TTM does not inhibit the increase in ATP7A protein expression in response to IFN-γ. RAW264.7 cells were treated with serum-free media or 25 ng/ml IFN-γ in the presence or absence of 10 nM TTM. ATP7A protein was detected by Western blot analysis. Tubulin was detected as a loading control. The numbers shown under the immunoblot bands represent relative expression, as calculated using Quantity One software (Biorad). The expression level of each sample was first normalized against its own tubulin control.

Fig. 2.7

A

	Control	IFN-γ	TTM	IFN- γ +TTM

ATP7A

B

	Control		IFN-γ	
[nM] TTM	0	10	0	10
ATP7A					_180kDa
	1	1.1	2.2	2.1
Tubulin				
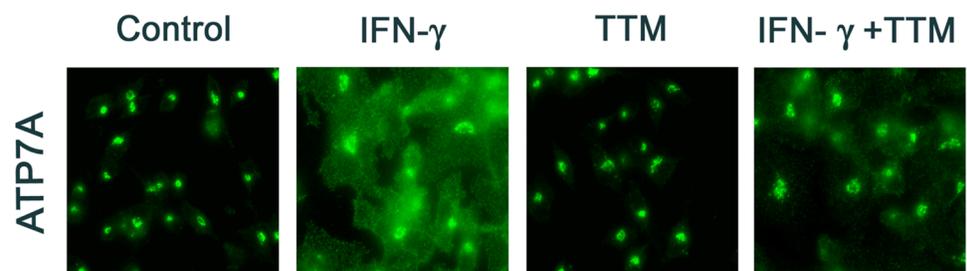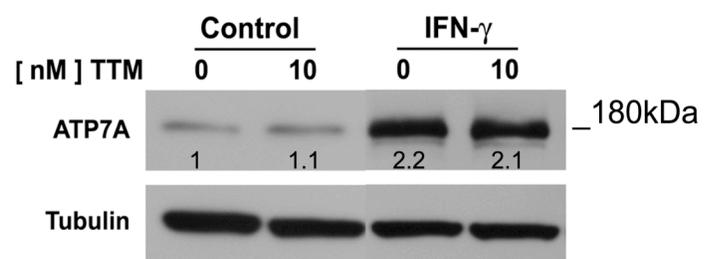
We then examined whether IFN-γ stimulates the trafficking of ATP7A to a vesicular population that overlaps with the phagosomal compartment in RAW264.7 cells. Phagocytosis of latex beads by IFN-γ-stimulated RAW264.7 cells was used to label the phagosomal compartment, and the intracellular location of ATP7A was then examined in the same cells using immunofluorescence microscopy. As shown in Figure 2.8, ATP7A was partially distributed to compartments that also phagocytosed latex beads seen in the bright field panel (Fig. 2.8A). In separate experiments, the subcellular phagosomal membrane compartment containing the internalized latex beads was isolated from RAW264.7 cells. Western blot analysis of these preparations demonstrated a marked enrichment of the LAMP-1 protein, a phagosomal marker, relative to total cell lysate (Fig. 2.8B). Significantly, the ATP7A protein was also abundant in the phagosomal fraction, which lacked a marker of the Golgi matrix, GM130. Taken together, these studies suggest that pro-inflammatory stimuli cause the increased flux of copper to the ATP7A protein and its partial redistribution to phagosomal compartments.

FIGURE 2.8. **IFN-γ induces the redistribution of ATP7A into the phagosomal compartment of RAW264.7 macrophage cells.** A, Partial co-localization of ATP7A with phagocytosed latex beads. RAW264.7 macrophages were stimulated overnight with IFN-γ and allowed to phagocytose latex beads. After fixing cells, ATP7A was detected as described above. The bright field panel shows phagocytosed beads with the adjacent panel and inset showing the localization of ATP7A (α-MNK). B, Co-fractionation of ATP7A with the phagosome. The phagosomal compartment was isolated via subcellular fractionation of membranes containing the internalized latex beads from RAW264.7 cells. This fraction (Beads) was subjected to SDS-PAGE as well as the total protein fraction (Total). Immunoblot analysis revealed abundant ATP7A in the Lamp-1 positive bead fraction, which lacked the Golgi marker protein GM130. Note that Lamp-1 was poorly detected in total lysates.

Fig. 2.8

A
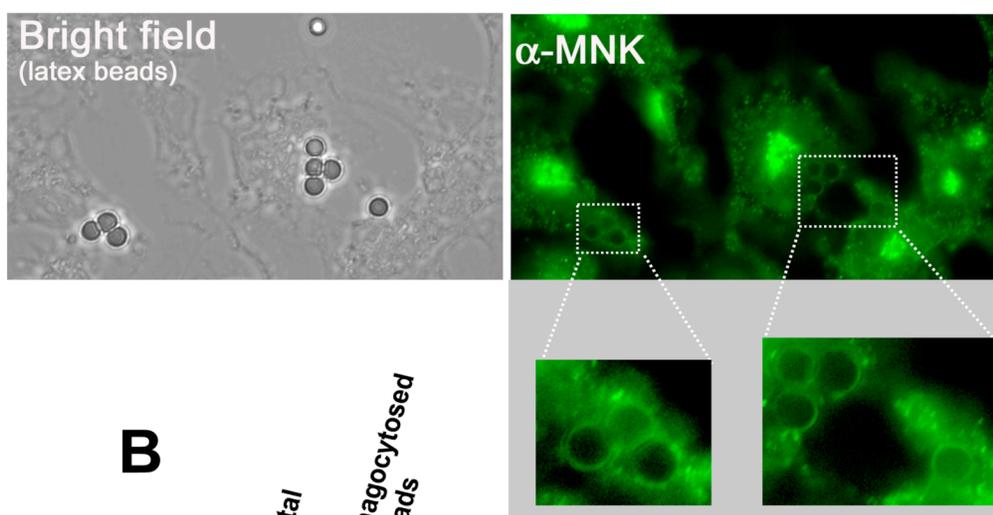
B
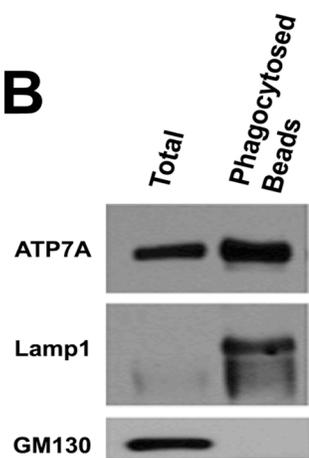
ATP7A-dependent copper transport is required for bactericidal activity of RAW264.7 macrophages— The above findings highlight the possibility that ATP7A-dependent copper transport into the phagosome is important for macrophage microbiocidal activity. To test this hypothesis, we investigated the effect of RNAi-mediated depletion of ATP7A expression on bacterial killing by RAW264.7 cells. RAW264.7 cells were stably transfected with a construct harboring a 29-nucleotide short hairpin against *ATP7A* (ATP7A-RNAi). This resulted in robust silencing of ATP7A gene expression in the ATP7A-RNAi cells, relative to control cells transfected with an irrelevant RNAi against *GFP* (Fig. 2.9A). ATP7A gene silencing did not prevent the upregulation of the inducible nitric oxide synthase (iNOS) associated with IFN-γ (373) (Fig. 2.9B). However, the activation of secreted ceruloplasmin induced by IFN-γ was suppressed in the ATP7A-RNAi cells compared to the control cells (Fig. 2.9D), whereas secreted ceruloplasmin protein levels were not affected by ATP7A gene silencing (Fig. 2.9C). This suggests that, while copper delivery to the secretory pathway is deficient in the ATP7A-RNAi cells, thereby suppressing the activity of copper-dependent enzymes such as ceruloplasmin, non copper-dependent pathways are not affected in this cell line. Significantly, ATP7A silencing reduced the bactericidal activity of RAW264.7 cells, as evidenced by the higher bacterial survival in ATP7A-RNAi cells relative to control cells (Fig. 2.10A). Moreover, the addition of copper to ATP7A-RNAi cells bypassed this reduction in bactericidal activity (Fig. 2.10A). These findings support the hypothesis that ATP7A-

dependent copper transport into the phagosome is important in bactericidal

activity of macrophages.

FIGURE 2.9. **ATP7A gene silencing by siRNA suppresses copper delivery to ceruloplasmin but does not alter iNOS expression during inflammation.**

A, RNAi-mediated silencing of the ATP7A protein. Western blot analysis of ATP7A protein levels in RAW264.7 cells stably transfected with either ATP7A-RNAi or control-RNAi against *GFP*. B, ATP7A-RNAi does not suppress the IFN-γ-induced upregulation of iNOS protein expression. Western blot analysis of iNOS protein expression in control-RNAi and ATP7A-RNAi cells treated with 25 ng/ml IFN-γ for 24 hours. C, ATP7A-RNAi does not suppress the increase in secreted ceruloplasmin protein associated with IFN-γ treatment of RAW264.7 macrophages. Western blot analysis of ceruloplasmin protein in conditioned media from control-RNAi and ATP7A-RNAi cells treated with 25 ng/ml IFN-γ for 24 h. D, ATP7A-RNAi suppresses the increase in secreted ceruloplasmin activity associated with IFN-γ treatment of macrophages. Ceruloplasmin activity from concentrated conditioned media from control-RNAi and ATP7A-RNAi macrophages exposed to IFN-γ ± 2 μM $CuCl_2$ was assessed. Values for ceruloplasmin activity were normalized against total protein content of each sample (mean ± SD; n = 3; p<0.05). The numbers shown under the immunoblot bands represent relative expression, as calculated using Quantity One software (Biorad). The expression level of each sample was first normalized against its own tubulin control.

Fig. 2.9
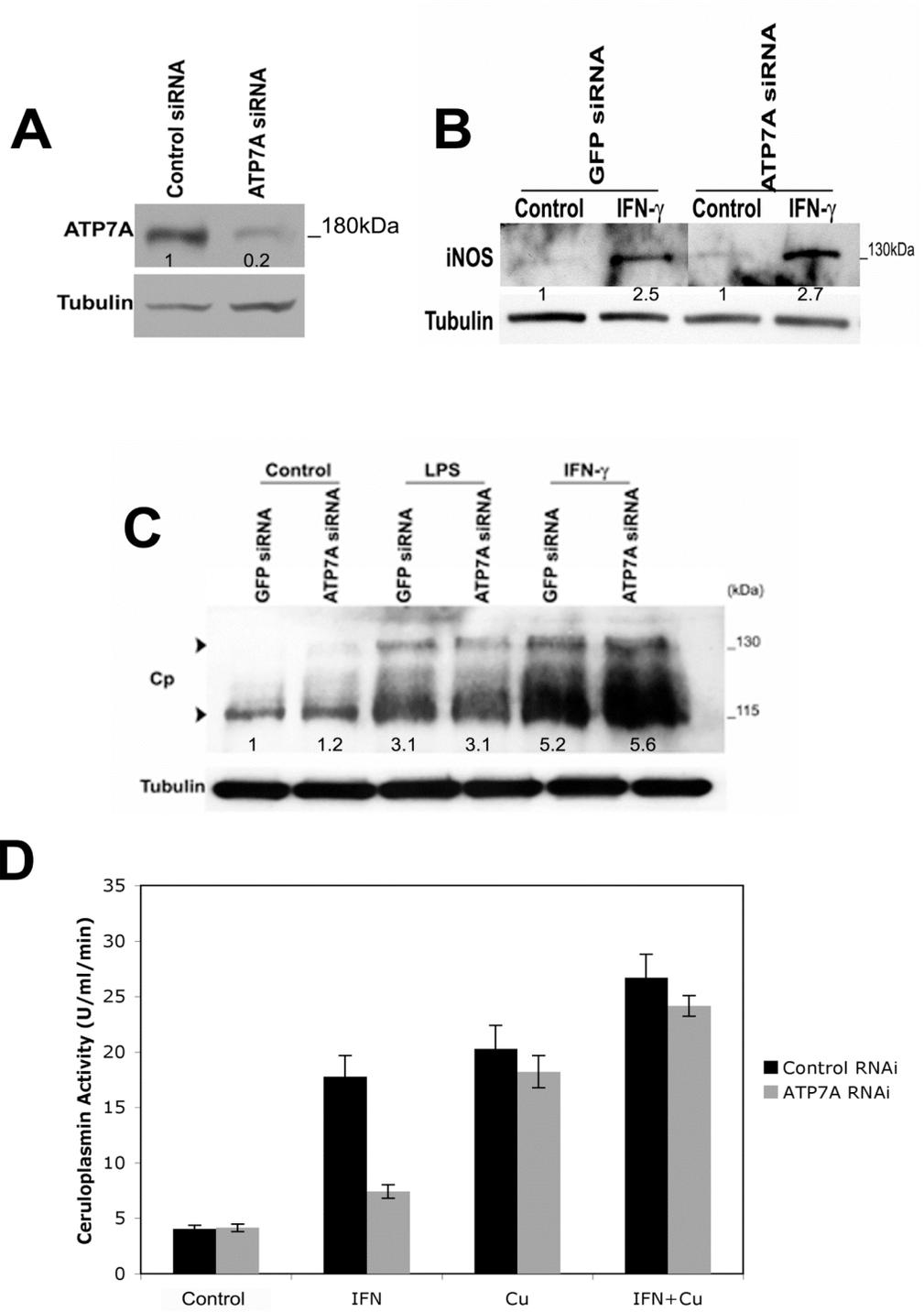
We further explored this hypothesis by testing whether the ability of *E. coli* to export copper would influence its susceptibility to killing by RAW264.7 macrophages. The CopA protein is a copper exporting P-type ATPase in *E. coli* similar to the ATP7A protein of mammals, and the ΔcopA mutant is hypersensitive to elevated copper concentrations (20). Interestingly, the ΔcopA mutant was more susceptible to killing by RAW264.7 macrophages compared to its parental wild type *E. coli* strain (Fig. 2.10B). Moreover, the survival of the ΔcopA strain was increased within the ATP7A-RNAi cells relative to control cells (Fig. 2.10C), indicating that the increased susceptibility of the ΔcopA mutant was dependent on the level of ATP7A expression within its macrophage host. In addition, *in vitro* exposure to a H_2O_2 and copper cocktail significantly reduced the survival of the ΔcopA mutant bacteria compared to wild type, suggesting that the increased copper sensitivity of the ΔcopA mutant may be ROS-mediated (Fig. 2.10D). These data suggest that copper homeostasis within both bacteria and macrophages is a determinant of bacterial survival, and identify copper as an unexpected nexus of host pathogen interactions.

FIGURE 2.10. **Bactericidal activity of RAW264.7 macrophages is dependent on ATP7A-mediated copper transport.** Control-RNAi and ATP7A-RNAi macrophage cells stimulated with IFN-γ were compared in their bactericidal activity against W3110 *E. coli* or WD3110 *E. coli* harboring a deletion of the *copA* gene. A, Percent survival of W3110 *E. coli* in control-RNAi or ATP7A-RNAi macrophages stimulated with IFN-γ $\pm$ 20 μM CuCl$_2$. The rate of bacterial survival was significantly increased in ATP7A-RNAi compared to control-RNAi macrophages. The presence of copper significantly reduced bacterial survival in both RNAi cell lines (mean $\pm$ SD; n = 3; p<0.05). B, The survival of W3110 *E. coli* in RAW264.7 macrophages was compared to that of the *copA* knockout WD3110 *E. coli.* Percent survival data is shown for 1 and 2 h kill times (post-uptake) (mean + SD; n = 3; p<0.05). C, The susceptibility of WD3110 *E. coli* is attenuated in the absence of ATP7A. Percent survival of WD3110 *E. coli* was significantly increased in ATP7A-RNAi macrophages compared to control-RNAi macrophages (mean $\pm$ SD; n = 3; p<0.05). D) Bacterial survival of W3110 *E. coli* and WD3110 (ΔCopA) *E. coli* was assessed following exposure of *E. coli* to 500 μM H$_2$O$_2$ and 10 μM CuCl$_2$ combined in the presence of 500 μM ascorbic acid. Bacterial survival rates were calculated and expressed as percentage survival compared to the untreated group (mean $\pm$ SD; n = 3; p<0.05).

Fig. 2.10

94
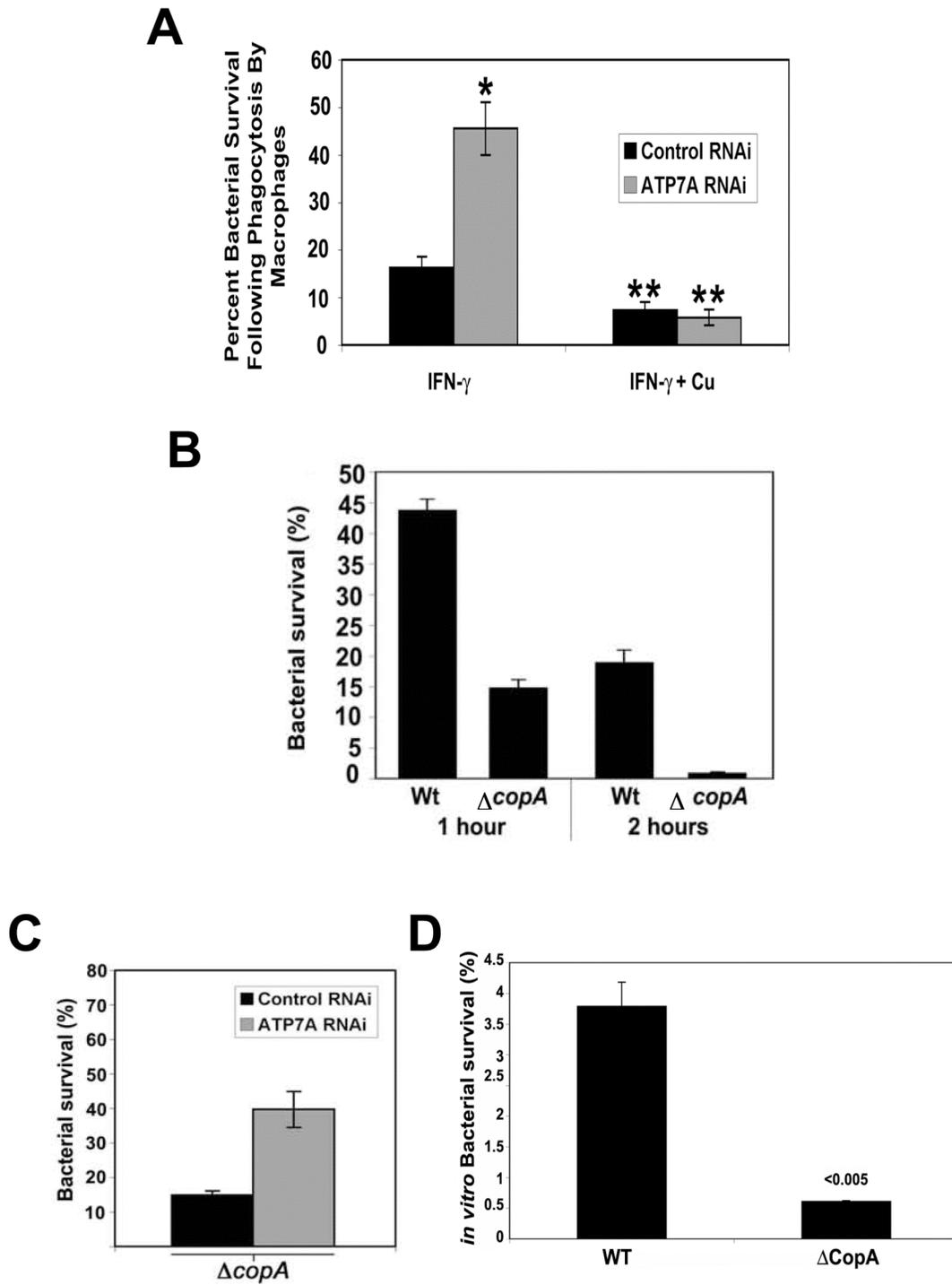
2.5 Discussion

Copper is an essential nutrient. Its deficiency has been linked to disorders such as anemia, neurodegenerative and cardiovascular defects and other illnesses (1). Inadequate copper nutrition in human patients is often associated with a decrease in the number of circulating white blood cells or neutropenia (281-284). Copper deficiency in macrophage cells results in their inability to adequately fight pathogens. Not only is copper essential for the function of the immune system, but inflammatory conditions and infections raise the body's physiological need for copper, as illustrated by the associated rise in serum copper levels in human patients (265-272, 360, 362). Furthermore, copper has long been recognized as a biocidal agent (374).

In this study, we find evidence that the biocidal property of copper has been harnessed by cells of the immune system to kill bacteria. We demonstrate for the first time that specific changes in macrophage copper homeostasis occur in response to inflammatory stimuli, and promote copper-dependent bacterial killing. Under inflammatory conditions, macrophage cells increase both uptake and retention of copper. We observe an increase in CTR1 protein levels in both the RAW264.7 murine macrophage cell line and primary peritoneal murine macrophages in response to the pro-inflammatory agents IFN-γ and LPS. This increase in CTR1 protein expression is associated with increased ^{64}Cu uptake by macrophage cells, as well as increased total copper content during inflammation.

Interestingly, inflammation also results in an increased expression of ATP7A protein, along with a copper-dependent relocalization of ATP7A from the TGN into a vesicular compartment. This trafficking process is dependent on increased flux of copper to the ATP7A protein, since it is blocked by copper chelation by TTM. Our finding of elevated expression of the CTR1 copper importer and increased copper uptake activity in response to IFN-γ, suggests that this pathway is a likely source of copper for ATP7A. Additionally, the accumulation of copper within activated macrophages suggests that the trafficking of ATP7A is associated with redistribution of copper into intracellular compartments rather than copper export.

We hypothesized that one such compartment could be the phagosome. Previous studies using x-ray microprobe analysis have demonstrated that copper levels within the phagosome increase 10-fold to approximately 180 µM in IFN-γ-stimulated macrophages exposed to *Mycobacterium avium* (285). However, no known copper transporter associated with the phagosome has been identified. Exactly how copper finds its way into the phagosomal compartment during inflammatory conditions was unknown. We hypothesized that the trafficking of ATP7A associated with inflammation would serve to partially redistribute intracellular copper into the phagosome, where there is an increased need for copper. Indeed, our studies show that ATP7A-rich vesicles partially overlap with the phagosomal compartment, as revealed by immunofluorescence microscopy and subcellular fractionation.

Our most significant finding sheds light on the role of ATP7A in bacterial killing by macrophages. Consistent with the observed pattern of ATP7A trafficking, we show that macrophage-mediated bacterial killing is dependent on the expression of the ATP7A copper transporter, as evidenced by the attenuated bactericidal activity when *ATP7A* is silenced. These findings suggest that copper transport into the phagosome via the ATP7A protein is a novel determinant of bacterial killing by macrophages.

The role of copper in bacterial killing has been attributed to its property as a transition metal and its ability to participate in Fenton reactions. During the oxidative burst associated with bacterial phagocytosis, superoxide production increases dramatically within the phagosomal compartment. Hydrogen peroxide is generated spontaneously from superoxide created by the respiratory burst; however, hydrogen peroxide is only lethal at supraphysiological millimolar concentrations that are several orders of magnitude greater than those found within activated leukocytes (375). On the other hand, studies have shown that physiological concentrations of hydrogen peroxide plus cuprous ions are lethal to *E. coli* (369), presumably owing to the formation of the OH$^\bullet$ radical, the most toxic of all reactive oxygen species (257, 289). Copper enrichment of the phagosome is thus important in providing the transition metal catalyst required for OH$^\bullet$ radical production during the oxidative burst. We propose that a lethal cocktail of copper and hydrogen peroxide may be the underlying mechanism by which ATP7A-dependent copper transport into the phagosome promotes bacterial killing. Such

a model is consistent with our finding that the combination of H_2O_2 and copper is significantly more effective at killing *E. coli in vitro* compared to H_2O_2 or copper alone. Furthermore, the enhanced bactericidal activity in copper-treated RAW264.7 macrophages is attenuated by the antioxidant Ebselen, a mimic of glutathione peroxidase that reduces the levels of intracellular H_2O_2 (376-379), suggesting a requirement for reactive oxygen species. The increased acquisition of copper by the phagocytic compartment of the macrophage host cells is accompanied by a compensatory increase in copper efflux in the ingested bacteria. Indeed, a notable finding of our study is that the copper-sensitive Δ*copA* mutant of *E. coli* is more susceptible to macrophage-mediated killing than the wild type strain. Since the CopA protein functions in the export of cytoplasmic copper across the plasma membrane (380), these findings suggest that copper export is a bacterial defense mechanism against macrophage-mediated killing. Consistent with this hypothesis, the susceptibility of the Δ*copA* mutant to killing is reduced by depletion of ATP7A in the macrophage host. These intriguing findings argue 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 is countered by copper export by the bacterium (Fig. 2.11). This concept of copper export as a defense strategy is not unique to *E. coli.* Indeed, the virulence of *Pseudomonas aeruginosa* in mice is severely decreased (20-fold) by mutations in the bacterial copper exporter, CueA (286). Additionally, the expression of several metal ion exporters including two putative copper efflux transporters, CopA1 and CopA2, are induced in *Legionella*

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pneumophila upon phagocytosis by macrophages (288). Moreover, plasmids that increase the virulence of *Klebsiella pneumoniae* and *Shigella sonnei* harbor putative copper resistance genes (381, 382).

Changes in the serum concentrations 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 (262). The proposed physiological rationale for these changes is nutrient deprivation that would serve to limit proliferation of the invading microbe (262). 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 (261, 262). In contrast to the above micronutrients, systemic copper concentrations are widely reported to increase in response to acute and chronic inflammation (263-274, 360-364). While the secreted copper-containing protein, ceruloplasmin, is partially responsible for this rise in serum copper (120), the non-protein bound fraction of copper in the serum is also increased during inflammation (263). Moreover, radiotracer studies with ^{64}Cu have demonstrated that copper accumulates at sites of inflammation (275), and within the exudates of wounds and burns relative to serum (276, 277). Although the physiological rationale of these systemic increases in copper concentration are unclear, our findings point to the possibility that such changes may provide localized reserves of copper for macrophage-mediated bactericidal activity.

FIGURE 2.11. **Model of copper-mediated bacterial killing by macrophages.**

Stimulation of macrophage cells by inflammatory signals or bacterial particles induces copper uptake via increased expression of the copper importer CTR1. The increase in intracellular copper content induces the trafficking of the copper exporter ATP7A into post-Golgi vesicles that partially overlap with the phagosomal compartment. Together with the increased expression of ATP7A protein, the trafficking of ATP7A to the phagosome drives copper transport into this compartment, where copper contributes to bacterial killing via Fenton chemistry and production of the hydroxyl radical ($OH^•$). The attack on the bacteria in the phagosomal compartment induces copper export as a pathogen defense mechanism.

Fig. 2.11

Stimulation with
Inflammatory Signals

Bacterial Phagocytosis
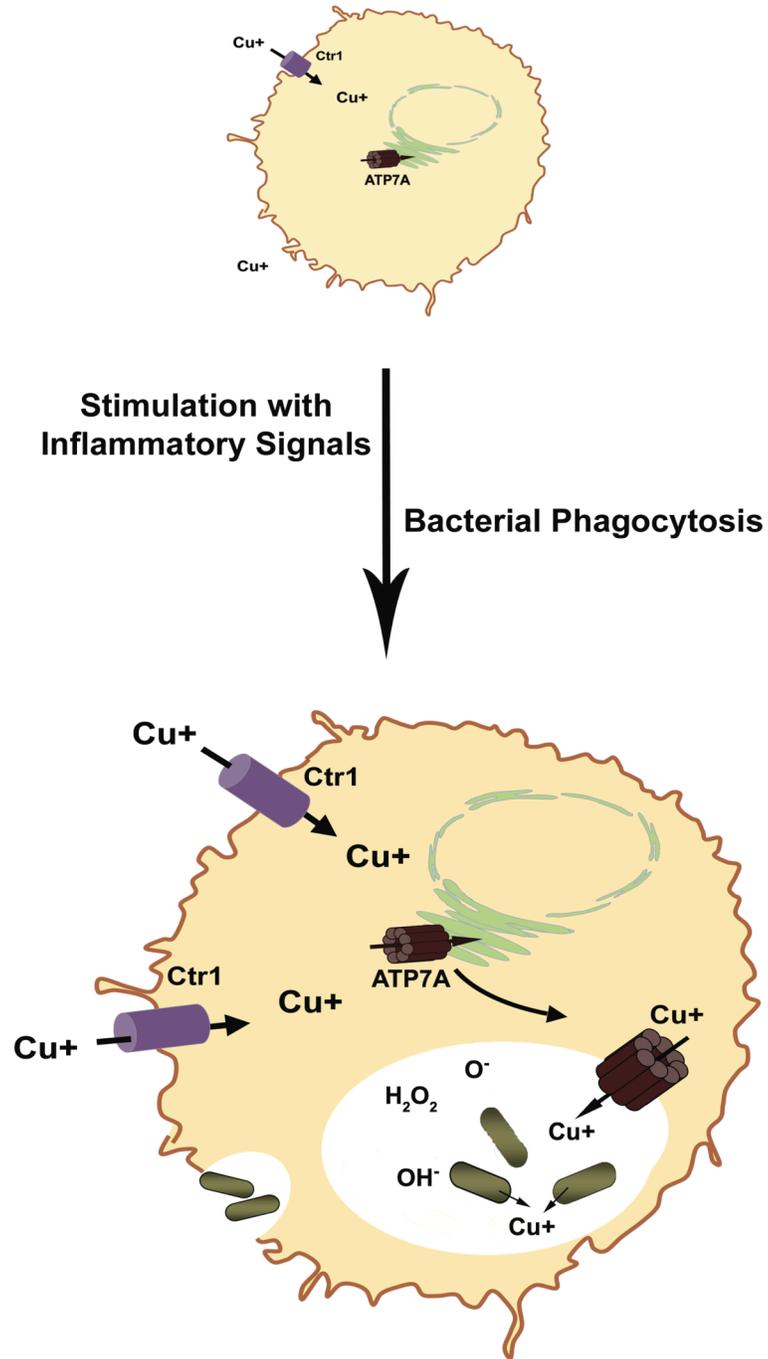
CHAPTER 3

OXYGEN IS A NOVEL REGULATOR OF COPPER METABOLISM IN MACROPHAGES

3.1 Summary

Copper is an essential cofactor of enzymes involved in a variety of important metabolic processes including ATP production, iron transport, and antioxidant defense. The maintenance of copper homeostasis requires a balance of copper uptake and export, as well as the appropriate partitioning of copper between the cytoplasm, mitochondria and secretory compartments. Although many of the proteins involved in copper homeostasis have been identified, it is unknown whether specific pathophysiological conditions lead to compensatory changes in the intracellular copper distribution. In this study, we identify striking alterations in copper homeostasis in response to hypoxia in RAW264.7 macrophage cells. Hypoxia induced the expression of the copper importer, CTR1, resulting in increased copper uptake. However, the activities of cuproenzymes superoxide dismutase and cytochrome c oxidase were inhibited by hypoxia. Significantly, copper delivery into secretory compartments via the ATP7A copper transporter was increased as evidenced by the enhanced activity of the ferroxidase ceruloplasmin, and trafficking of ATP7A to post-Golgi compartments in hypoxic macrophages *in vitro* and *in vivo*. The trafficking of ATP7A was dependent on

mitochondria-generated reactive oxygen species in hypoxic macrophages, suggesting that this process is mediated by oxidative stress. Collectively, these findings demonstrate that hypoxia alters the intracellular copper hierarchy in macrophages to favor copper delivery to the secretory pathway, and underscores the potential for other pathophysiological conditions to regulate adaptive responses involving altered copper distribution to cuproenzymes.

3.2 Introduction

Copper is a trace element that is critical for aerobic life. Its ability to accept and donate electrons has been harnessed by a select group of enzymes that function in mitochondrial respiration, connective tissue formation, pigmentation, iron oxidation, neurotransmitter processing, and antioxidant defense (110, 114, 383). However, this same redox property of copper and its ability to generate reactive oxygen species, also underscores its potential toxicity. For this reason, copper-handling pathways have evolved to deliver copper to specific sites of utilization, thereby preventing the formation of potentially damaging free ionic copper in the cytoplasm. Copper uptake in mammalian cells is mediated by CTR1, a ubiquitously expressed homotrimeric transporter (151). Once in the cytoplasm, small cytoplasmic proteins known as copper chaperones deliver copper linearly to distinct target enzymes via direct protein-protein interactions. The copper chaperones, CCS and COX17 are involved in copper delivery to Cu/Zn superoxide dismutase in the cytoplasm (SOD1) and to cytochrome c oxidase (CCO) in the mitochondria, respectively (156, 179). SCO1 and SCO2 are also

involved in copper delivery to cytochrome c oxidase via a process that is poorly understood (205). The third target for copper delivery is the ATP7A protein (or closely related ATP7B protein), a copper transporter located in the Golgi complex that receives copper from the ATOX1 copper chaperone in the cytoplasm (1, 159, 365). ATP7A transports copper into the Golgi lumen to supply copper to a select group of copper-dependent enzymes, which are either secreted from cells, or reside within vesicular compartments (232, 233, 235, 384). In addition to providing copper to secreted cuproenzymes, ATP7A is also responsible for copper export from cells. This export activity is associated with copper-stimulated trafficking of ATP7A to post-Golgi compartments, which include cytoplasmic vesicles and the plasma membrane (1). The trafficking of ATP7A is triggered when cytoplasmic copper levels are elevated (158), and this process requires both copper binding to cytoplasmic regions of the ATPase as well as its catalytic turnover (169, 228). These essential functions of the ATP7A protein are illustrated by Menkes disease, a lethal disorder of copper deficiency caused by ATP7A mutations (145).

Despite advances in our understanding of the intracellular routes of copper transport, it is unknown whether copper is differentially allocated along the three chaperone-mediated pathways to its respective targets, or whether such a hierarchy is altered by certain physiological conditions. In this study, we demonstrate using cultured RAW264.7 macrophage cells that hypoxia results in profound changes in copper homeostasis including enhanced expression of CTR1 and ATP7A proteins, copper-stimulated trafficking of ATP7A to post-Golgi

compartments, increased copper delivery to ceruloplasmin, and depletion of alternative copper targets, CCS, SOD1, and COX1, the copper-binding subunit of cytochrome c oxidase. Both the trafficking of ATP7A and the activation of ceruloplasmin were dependent on the production of mitochondrial reactive oxygen species. These findings suggest that oxygen status can regulate copper allocation to the secretory pathway for hypoxia-induced cuproenzymes, and reveal hypoxia as a unique pathophysiological regulator of intracellular copper hierarchy.

3.3 Materials and methods

Reagents and Antibodies— All reagents were from Sigma, unless otherwise indicated. The rabbit polyclonal Ctr-1 antibody (100) was a kind gift of Dennis Thiele (Duke University, Durham NC). The rabbit polyclonal ATP7A antibody raised against the C-terminal portion of the protein and was a generous gift of Dr. Elizabeth Eipper (252). Additional affinity purified anti-ATP7A antibodies were raised in rabbits against the synthetic peptide NH2-CDKHSLLVGDFREDDDTTL-COOH (Bethyl Laboratories, Mongomery TX). Mouse anti-tubulin antibody, and secondary HRP-conjugated IgG antibodies were purchased from Roche Molecular Biochemicals. Monoclonal antibody against COX I, and rabbit and mouse IgG antibodies conjugated with fluoresecent Alexa-488 and Alexa-594 were from Invitrogen (Carlsbad, CA). Antibodies against mouse GM130 and syntaxin-6 were purchased from BD Transduction Laboratories (San Jose, CA).

Antibodies against CD68 and HIF-1α were purchased from Serotec (Raleigh, NC) and Novus Biologicals (Littleton, CO) respectively. Antibodies against Cu/Zn SOD, CCS, and ceruloplasmin were purchased from Stressgen (383, 385), Santa Cruz Biotechnology (Santa Cruz, CA), and Abcam (Cambridge, MA), respectively.

Cell Lines— All cell lines were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal bovine serum and 100 units/ml penicillin and streptomycin (Invitrogen) in 5% CO_2 at 37°C. Primary macrophages were isolated by peritoneal lavage. C57BL/6J mice were injected with 0.5 ml of thioglycolate medium into the peritoneum to elicit macrophage infiltration. After 72 h, macrophages were isolated by peritoneal lavage using ice-cold PBS. Cells were seeded in six-well trays for each experiment, as described below. Hypoxia experiments were conducted at 37°C using a hypoxic incubator in which oxygen levels were lowered by air displacement with N_2 and CO_2 gas. Mitochondrial DNA-depleted RAW/Rho$^-$ cells were generated by culturing RAW264.7 cells for 26 days in media supplemented with 1 mM sodium pyruvate in the presence of 50 ng/ml ethidium bromide and confirmed by the loss of mitochondria-encoded cytochrome c oxidase subunit I (COX-1) protein from mitochondrial extracts. RNAi-mediated silencing of *ATP7A* in RAW264.7 cells was performed as described in Chapter 2. Lipofectamine 2000 (Invitrogen) was used in all transfections.

Copper uptake— Radioactive copper (^{64}Cu) was purchased from the Mallinckrodt Institute of Radiology, Washington University (Saint Louis, MO). Cells were pre-cultured in triplicate for 72 h in 6-well trays under either normoxic (21% O_2) or hypoxic (4% O_2) conditions, exposed to 1 µM ^{64}Cu for 5 min, washed extensively in ice-cold PBS and radioactivity was measured using a gamma counter. Counts were normalized against total protein.

Immunological techniques and PC-3 tumor growth— Immunofluorescence microscopy and Western blot analysis were performed as described in Chapter 2. PC-3 prostate carcinoma cells (5 x 10^6) were injected subcutaneously in one flank of each anesthetized four-week-old ICRSC-M SCID outbred mice obtained from Taconic (Germantown, NY). Mice were maintained in an approved pathogen-free institutional housing. Animal studies were conducted as outlined in the NIH Guidelines for the Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research of the Harry S. Truman Veterans Memorial Hospital. Over a period of 4 weeks solid tumors of approximately 1-cm diameter were excised from anesthetized SCID mice and flash frozen in fixative. Frozen tumors were cryosectioned, fixed in acetone for 10 minutes, washed in phosphate buffered saline (PBS) and blocked overnight in 1% (w/v) casein buffer. Immunostaining was performed using antibodies against ATP7A, CD68 and HIF-1α overnight, followed by staining with Alexa 488-conjugated anti-rabbit and Alexa 594-conjugated anti-mouse IgG antibodies for one hour. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Enzyme Assays— Cytochrome c oxidase activity assays were performed using mitochondrial preparations from RAW264.7 cells. Cells were grown in 75-cm^2 flasks and scraped using ice-cold PBS. Mitochondrial fractions were collected using the Cell Mitochondria Isolation Kit from Sigma. Cytochrome c oxidase activity was assessed using the Cytochrome c Oxidase Assay Kit from Sigma. Cytochromes c oxidase activity was normalized against mitochondrial protein content. For the cytochrome c oxidase subunit I immunoblot, mitochondrial extracts were fractionated on 4-20% (w/v) SDS-PAGE gels and probed with anti-COX-1. Superoxide dismutase assays were performed as described previously (386, 387). RAW264.7 cell lysates were fractionated using nondenaturing 12% (w/v) polyacrylamide gel electrophoresis and superoxide dismutase activity was detected by incubation of gels in nitro blue tetrazolium at room temperature. Ceruloplasmin activity in concentrated conditioned media was determined by its p-phenylenediamine oxidase activity, as previously described (179). RAW264.7 cells were grown in 6-well plates and conditioned media were collected and concentrated using Amicon Ultra-4 filter tubes (Millipore). Ceruloplasmin activity was normalized against total protein content in the cell pellets. Ceruloplasmin protein levels were detected in concentrated media using immunoblot analysis.

3.4 Results

Oxygen limitation stimulates the expression of CTR1 and ATP7A copper transporters in macrophages—We began this study by investigating the relationship between oxygen availability and copper homeostasis in the macrophage cell line, RAW264.7. Macrophages are phagocytotic cells of the adaptive immune system that are commonly recruited to hypoxic tissues. Exposure of RAW264.7 macrophages to mild hypoxia (4% O_2) increased copper uptake relative to normoxia (21% O_2) (Fig. 3.1A). Consistent with this increase in copper uptake, hypoxia stimulated the expression of the CTR1 protein within 24 h relative to normoxia (21% O_2) (Figs. 3.1B, and 3.1C). Interestingly, the expression of the ATP7A copper transporter in RAW264.7 cells and primary peritoneal macrophages was also stimulated by hypoxia, although the rate of induction was slower than for CTR1 (Fig. 3.2A, and 3.2B).

FIGURE 3.1. **Hypoxia alters copper homeostasis in RAW264.7**

macrophages. A, Copper uptake activity. RAW264.7 cells were pre-exposed to

normoxia (21% O_2) or hypoxia (4% O_2) for 72 h and copper uptake in the

presence of 1 µM ^{64}Cu was measured over 5 min and normalized against total

protein (mean + SD; n = 3). B-C, The effect of hypoxia on CTR1 protein levels in

RAW264.7 cells and thioglycollate-elicited primary peritoneal macrophages

(TPECs) under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions. B,

Immunoblot analysis of Ctr1 protein in lysates from normoxic and hypoxic

RAW264.7 cells, and C, cultured primary peritoneal macrophages. Tubulin was

detected as a loading control.

Fig. 3.1

A

^{64}Cu nmol / mg protein

Normoxia   Hypoxia

C

TPECs

72h
N   H

CTR1   _30kDa

1   2.6

Tubulin

B

RAW264.7

16h   24h   72h   96h
N   H   N   H   N   H   N   H

CTR1   _30kDa

1   1   1   1.6   1   2.5   1   2.7

Tubulin
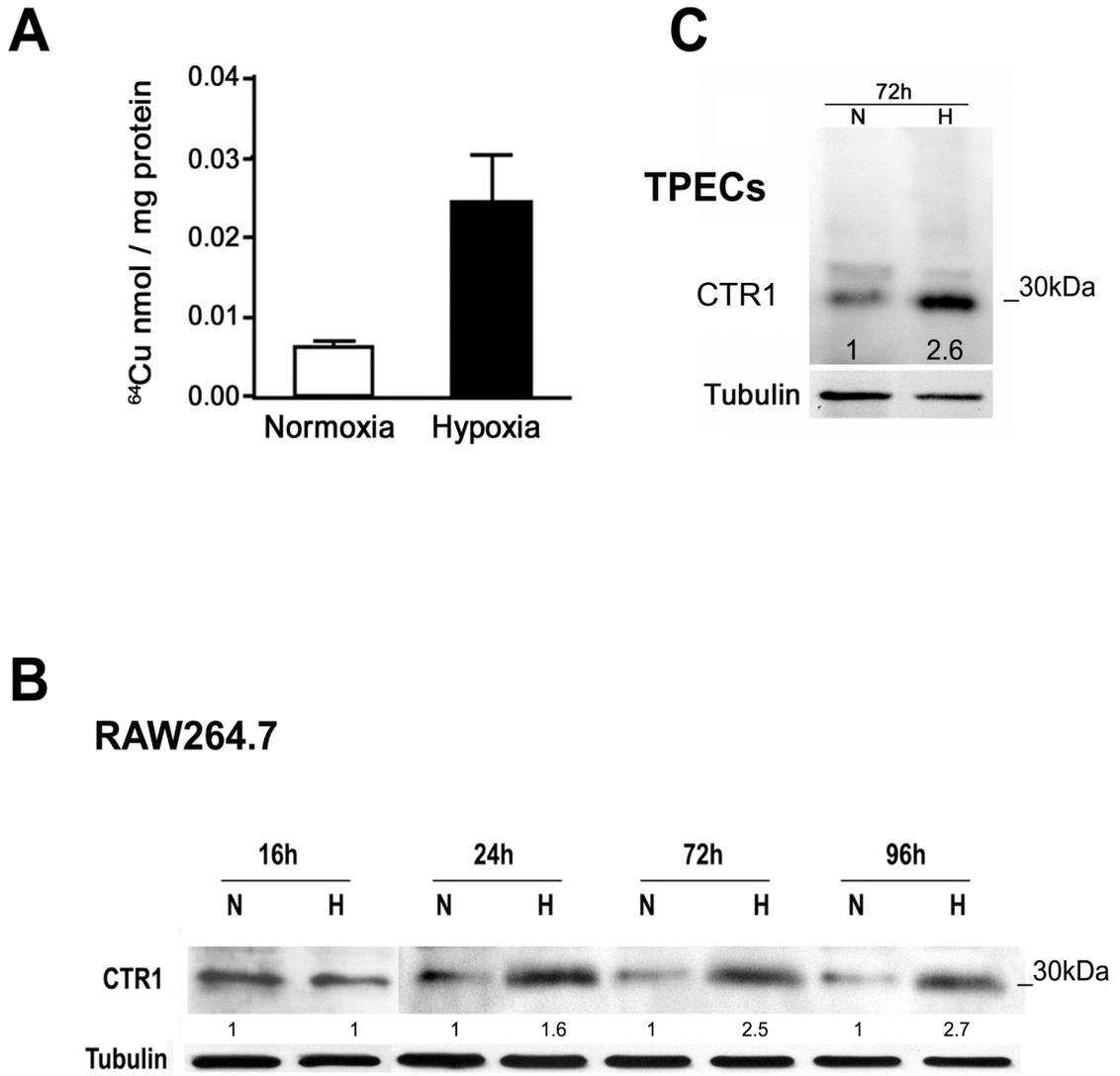
FIGURE 3.2. **Hypoxia increases the expression of ATP7A in macrophages.**

Effect of hypoxia on ATP7A protein levels in RAW264.7 cells and primary

peritoneal macrophages under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2)

conditions. A, Immunoblot analysis of ATP7A protein in lysates from normoxic

and hypoxic RAW264.7 cells, and B, cultured primary peritoneal macrophages.

Tubulin was detected as a loading control.

Fig. 3.2

A

RAW264.7

	24h		48h		72h		96h	
	N	H	N	H	N	H	N	H

ATP7A

_180kDa

| 1 | 1 | 1 | 2 | 1 | 2.5 | 1 | 1.9 |

Tubulin

B

TPECs

72h	
N	H

ATP7A

_180kDa

| 1 | 3.7 |

Tubulin
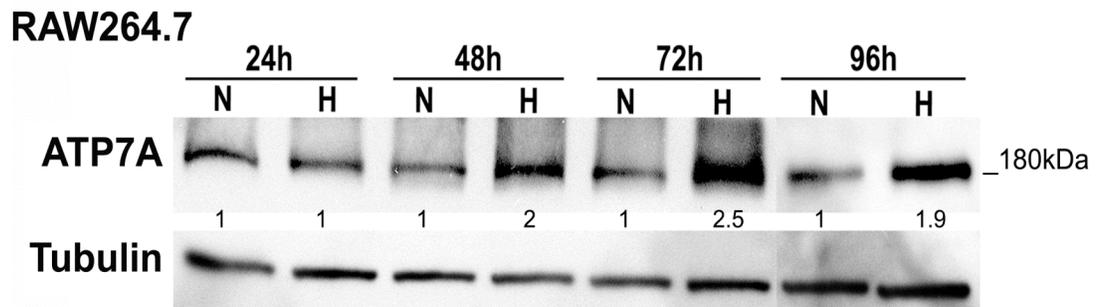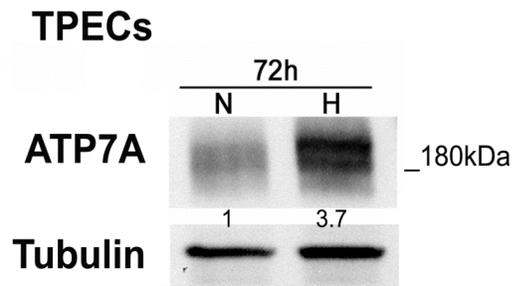
Hypoxia stimulates copper-dependent trafficking of the ATP7A protein in RAW264.7 macrophages—Since the trafficking of ATP7A from the trans-Golgi network is known to be triggered by increased copper delivery to this transporter (1), we investigated whether reduced oxygen tension might alter the localization of the ATP7A copper transporter in the murine macrophage cell line, RAW264.7. The relocalization of ATP7A from the trans-Golgi network is a key biological indicator of increased cytoplasmic copper availability and has been documented in several different cell types (1, 71, 388). Using immunofluorescence microscopy, the ATP7A protein was localized within the perinuclear region of RAW264.7 cells exposed to normoxic conditions (21% O_2), consistent with its location in the trans-Golgi network (Fig. 3.3A). As expected, treatment of these cells with copper resulted in the trafficking of ATP7A from the perinuclear region to cytoplasmic vesicles (Fig. 3.3A). Significantly, when these cells were exposed to chronic hypoxia (4% O_2 for 96 h), the ATP7A protein was also dispersed to post-Golgi vesicles (Fig. 3.3A). This redistribution of the ATP7A protein required at least 48 h of hypoxia and was not accelerated by lower levels of oxygen (data not shown). The return of hypoxic cells to normoxic conditions restored the location of the ATP7A protein to the perinuclear region, indicating that the effect of hypoxia on ATP7A was reversible (Fig. 3A). The intracellular location of the trans-Golgi marker protein, syntaxin 6, and the Golgi matrix protein, GM130, were not altered by hypoxia in RAW264.7 cells (Fig. 3.3B, and 3.3C), suggesting that the effects of hypoxia on ATP7A were not the result of a general alteration of Golgi structure. These hypoxic conditions did not alter the viability of RAW264.7

cells, which could be passaged continuously at 4% oxygen (data not shown).

Interestingly, ATP7A trafficking to post-Golgi compartments in response to

hypoxia was not observed in a range of other cells types, including N2a, NIH3T3,

DLD1, HT1080 (Fig. 3.13), HEK293, HeLa, NRK, human primary aorta

endothelial cells (HAEC), and rat primary smooth muscle cells (SMC) (not

shown).

We further tested whether the membrane-permeable copper chelator,

tetrathiomolybdate, could suppress ATP7A trafficking to post-Golgi in response

to hypoxia in RAW264.7 cells. As shown in Figure 3.3D, TTM inhibited ATP7A

relocalization in response to hypoxia. These findings support the hypothesis that

oxygen limitation increases copper binding to the ATP7A protein resulting in its

trafficking from the Golgi.

FIGURE 3.3. **Hypoxia stimulates trafficking of the ATP7A protein.**

A, Immunofluorescence analysis of ATP7A protein in RAW264.7 cells grown

under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions for 96 h.

Relocalization of the ATP7A protein from the perinuclear region is shown in

hypoxic cells and normoxic cells exposed to copper (second panel). Restoration

of perinuclear labeling of ATP7A occurred upon the transfer of hypoxic cells to

normoxic media for 30 min (fourth panel). Cells were fixed, permeabilized and

probed with antibodies against for ATP7A and anti-rabbit IgG antibodies

conjugated to Alexa-488 (green). Nuclei were labeled with DAPI (blue). B-C,

Analysis of Golgi marker proteins in hypoxic RAW264.7 cells. RAW264.7 cells

were cultured under hypoxic or normoxic conditions as described in (A) and

probed using antibodies against the trans-Golgi network marker protein Syntaxin

6 (B), and the Golgi matrix protein, GM130 (C). Nuclei were labeled with DAPI

(blue). D, Hypoxia-induced trafficking of ATP7A to post-Golgi compartments is

inhibited by the copper chelator, tetrathiomolybdate. RAW264.7 cells were

cultured under hypoxic or normoxic conditions as described in (A), in the

presence or absence of tetrathiomolybdate (TTM, 5 nM). ATP7A protein was

detected as described in (A).

Fig. 3.3

A

| | Normoxia | Normoxia + Cu | Hypoxia | Hypoxia → Normoxia |

ATP7A

B

| | Normoxia | Hypoxia |

Syntaxin 6

C

| | Normoxia | Hypoxia |

GM130

D

| | Normoxia | Hypoxia | Normoxia +TTM | Hypoxia + TTM |
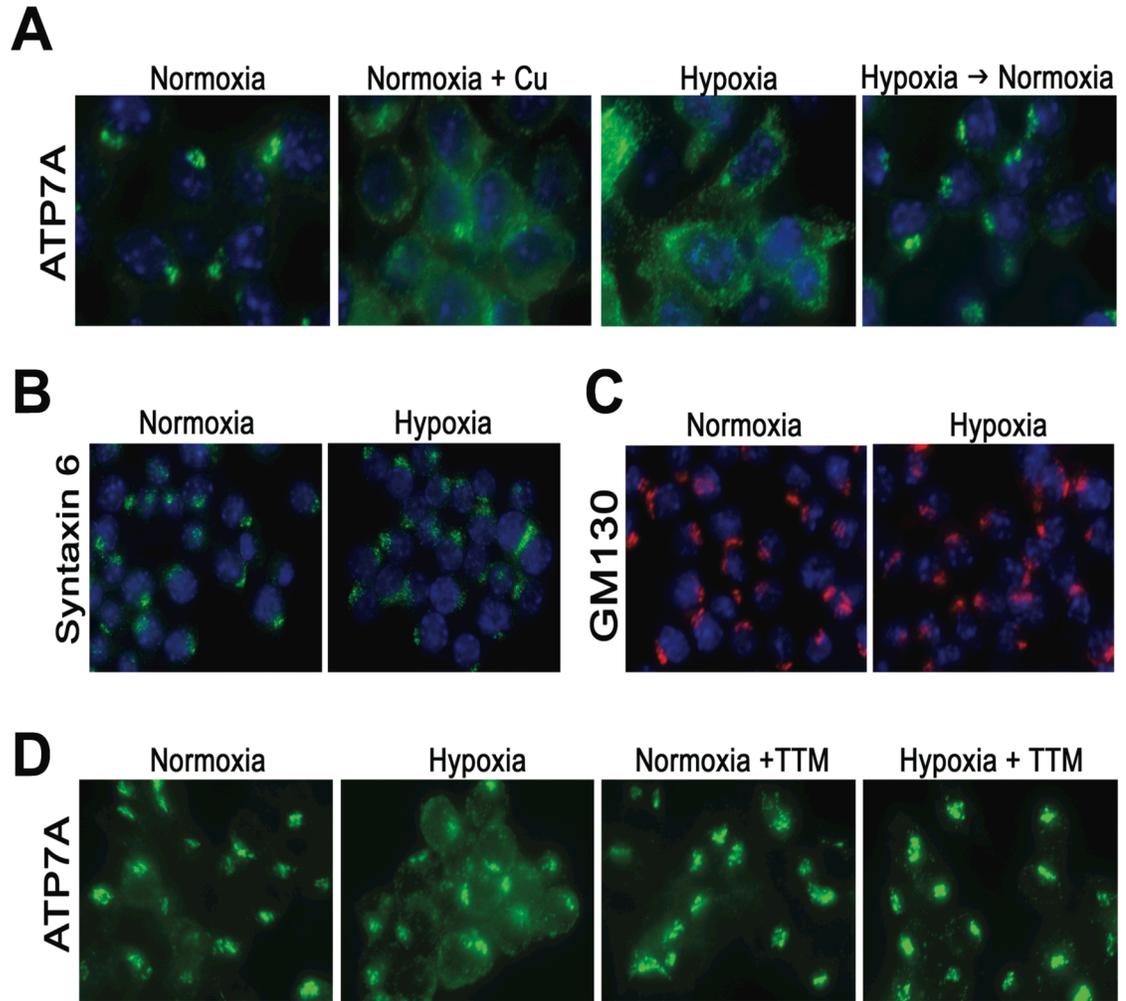
ATP7A

Hypoxia stimulates trafficking of the ATP7A protein in tumor-associated macrophages— Since previous studies have demonstrated that macrophages are recruited to the hypoxic regions of solid tumors (reviewed in (389)), the use of a human prostate tumor xenograft provided an opportunity to investigate the intracellular distribution of ATP7A in hypoxic macrophages *in vivo*. The tumorigenic human prostate cell line, PC-3, was chosen for these studies since ATP7A expression is very low in these cells, thus allowing for easy identification of ATP7A in tumor-associated macrophages. PC-3 tumors were grown in nude mice to approximately 2-cm diameter, excised and cryosectioned. Immunofluorescence analysis of ATP7A expression in PC-3 tumors demonstrated that there was abundant expression in macrophages identified using the macrophage-specific marker CD-68 (Figure 3.4A). As expected, there was little if any ATP7A expression in PC-3 tumor cells. Consistent with previous studies, these tumor-associated macrophages were concentrated at the tumor edges, with occasional infiltration into the tumor body (390-392). Significantly, we noticed that in some macrophages the location of ATP7A was restricted to the Golgi complex, whereas in other macrophages it appeared to be relocalized in a manner reminiscent of the trafficking seen in cultured RAW264.7 cells (Figure 3.4A, lower panel; arrows). Further analysis demonstrated that ATP7A was dispersed only in macrophages that co-expressed the HIF-1α protein (Figure 3.4B), a key transcriptional regulator of hypoxic gene responses whose abundance is increased in tumor-associated macrophages (393, 394). These

findings provide evidence that the hypoxia-stimulated trafficking of ATP7A occurs in macrophages *in vivo*.

FIGURE 3.4. **ATP7A trafficking in HIF-1α-positive macrophages in tumor**

xenografts. A, ATP7A is strongly expressed in tumor-associated macrophages.

Human prostate cell PC-3 tumors from SCID mice were cryosectioned and

probed with antibodies against ATP7A (green) or antibodies against the

macrophage marker CD68 (red). Upper panels are a low power magnification

showing both the tumor mass and tumor edge (x200). Note the propensity of

ATP7A and CD68 staining in cells associated with the tumor edge. A higher

magnification of the tumor edge region reveals extensive co-expression of

ATP7A in CD68-positive macrophages, as indicated by yellow signal in the

merged image. Nuclei were stained using DAPI (blue). Arrow and arrowhead

(lower left panel) reveals heterogeneous localization of the ATP7A in

macrophages, in either a perinuclear or dispersed distribution. B, The dispersed

distribution of the ATP7A occurs in HIF-1α-positive macrophages. PC-3 cell

tumors were immunostained for ATP7A (green) and HIF-1α (red). ATP7A was

restricted to the perinuclear region of cells negative for HIF-1α expression

(arrows), whereas a dispersed distribution of ATP7A was detected in cells that

were positive for HIF-1α (arrow heads) (x600).

Fig. 3.4

A

ATP7A CD68 Merge

Tumor body Tumor edge

B

ATP7A

HIF-1α
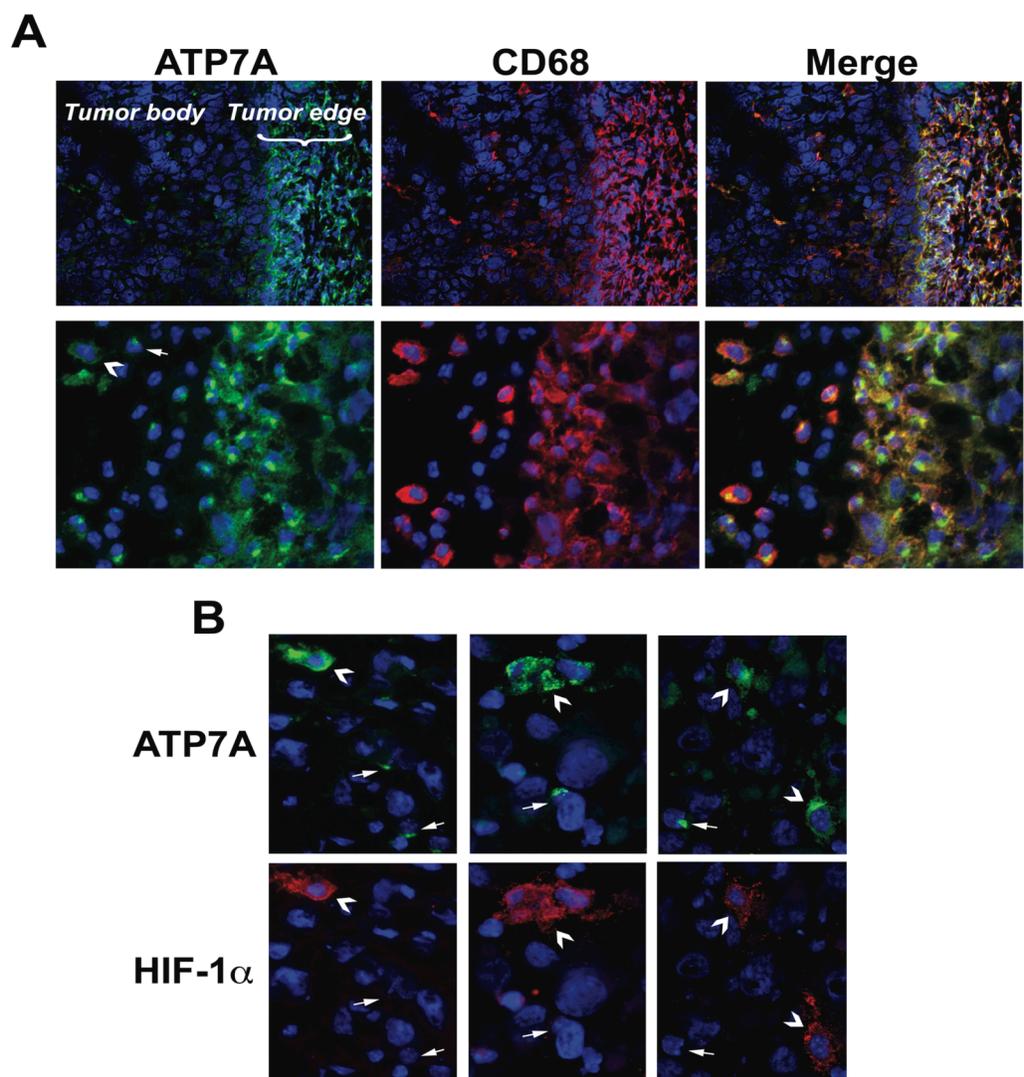
Hypoxia stimulates copper transport to ceruloplasmin via ATP7A— The major

function of ATP7A is to pump copper into the secretory pathway and supply

copper to secreted cuproenzymes. We hypothesized that a potential target for

this copper delivery in response to hypoxia might be ceruloplasmin, a

cuproenzyme that depends on copper delivery for its activity. Ceruloplasmin is a

ferroxidase secreted from macrophages and hepatocytes whose expression and

activity are stimulated by hypoxia (146, 314, 395). Hypoxia was found to increase

both the abundance and activity of ceruloplasmin secreted into the culture

medium of RAW264.7 cells relative to normoxia (Figs. 3.5A, and 3.5B). To

examine whether the increase in ceruloplasmin activity was dependent on

ATP7A copper transport activity, we measured ceruloplasmin activity in the

ATP7A-depleted cell line (described in Chapter 2). Compared to control cells,

ceruloplasmin activity in ATP7A-RNAi cells was markedly reduced under hypoxic

conditions suggesting that ATP7A copper transport activity was required for

copper delivery to ceruloplasmin (Fig. 3.5D). Consistent with this postulate, the

addition of copper to the media of these cells bypassed the requirement for

ATP7A and restored ceruloplasmin activity, indicating that the effect of *ATP7A*

gene silencing was due to a blockage of copper delivery to ceruloplasmin (Fig.

3.5D). Control experiments indicated that ATP7A silencing did not alter

ceruloplasmin protein levels in the medium relative to control cells in either

hypoxic or normoxic conditions (Fig. 3.5C). Taken together with our earlier

results, these findings suggest that hypoxia stimulates an increase in copper

delivery to ceruloplasmin via increases in CTR1-mediated copper uptake as well as ATP7A-dependent copper delivery into secretory compartments.

FIGURE 3.5. **The stimulation of ceruloplasmin activity by hypoxia is dependent on the ATP7A protein.** A, Ceruloplasmin (Cp) abundance in hypoxia. Conditioned media was collected from RAW264.7 cells grown under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions for the indicated times. The media was concentrated and subjected to non-denaturing SDS-PAGE and immunoblot analysis with anti-Cp antibody. Immunoblot of tubulin from corresponding cell lysates is shown. Lane 1 shows ceruloplasmin levels in concentrated growth media alone (M). B, Analysis of ceruloplasmin activity. Ceruloplasmin activity (p-phenylenediamine oxidase activity) was determined in the concentrated conditioned media from RAW264.7 cells following exposure to normoxia (N; 21% O_2) or hypoxia (H; 4% O_2) for 72 h. Activity was normalized against total protein content of the corresponding cell lysates (mean + SD; n = 3). C, Cp abundance in conditioned media from ATP7A-RNAi and control-RNAi RAW264.7 cells following exposure to normoxia (N; 21% O_2) or hypoxia (H; 4% O_2) for 72 h. The media was concentrated and subjected to non-denaturing SDS-PAGE and immunoblot analysis with anti-Cp antibody. Immunoblot of tubulin from corresponding cell lysates is shown. D, Ceruloplasmin activity was assessed in conditioned media from ATP7A-RNAi, ATP7A-RNAi + 20 μM $CuCl_2$, or control-RNAi RAW264.7 cell lines, as described in (B) (mean + SD; n = 3).

Fig. 3.5
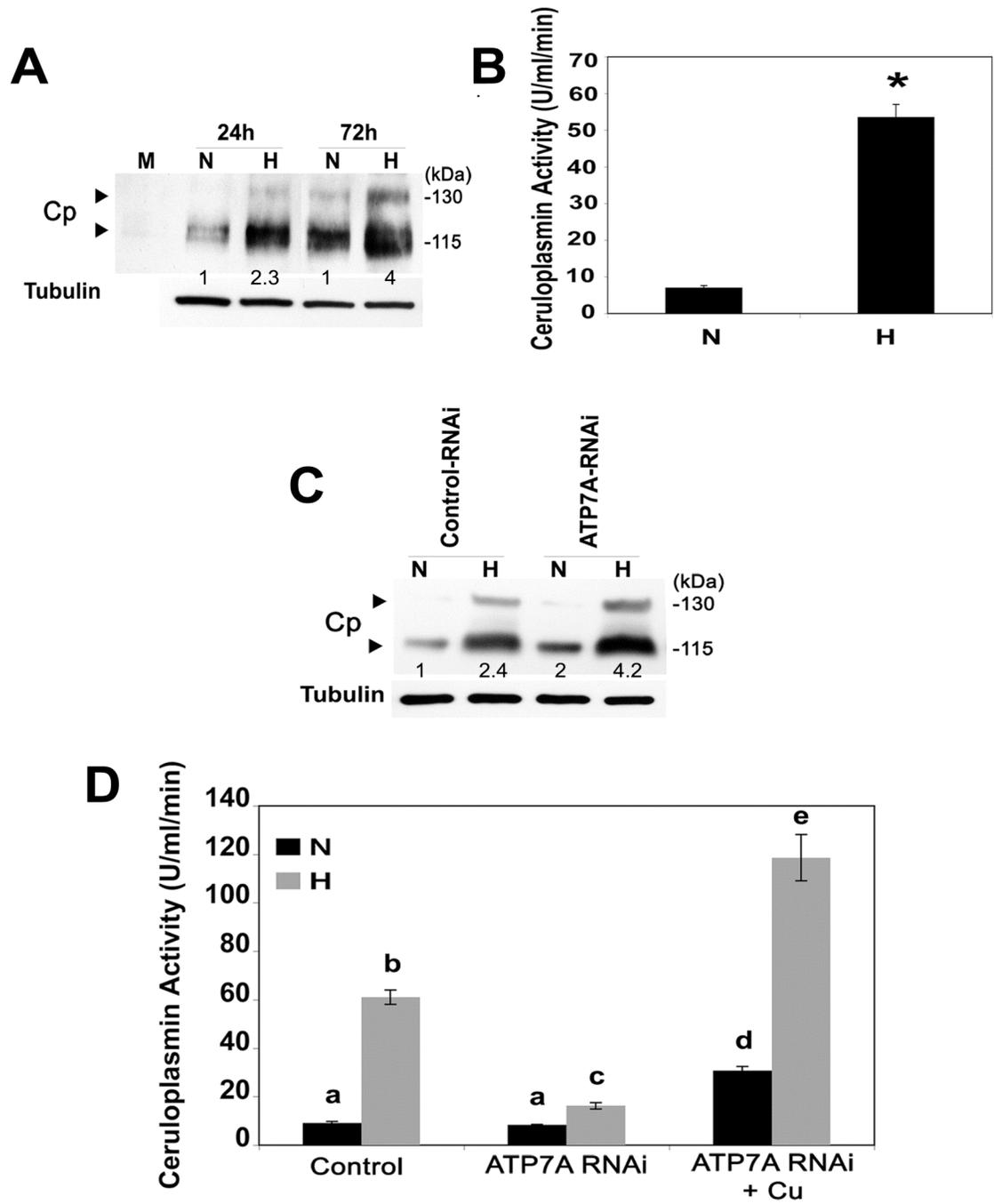
Hypoxia alters copper metabolic pathways in hypoxic macrophages—As

mentioned earlier, intracellular copper delivery to cuproenzymes occurs via three

copper known chaperone-mediated pathways, namely CCS to SOD1, COX17 to

CCO, and ATOX1 to ATP7A. To further characterize the impact of hypoxia on

copper metabolism, we tested whether the abundance and activities of SOD1

and CCO were altered in hypoxic RAW264.7 macrophages. The activity of SOD1

was reduced after 48 h exposure to 4% O_2 and completely abolished by 96 h

(Figure 3.6A, upper panel). This was also accompanied by a reduction in the

levels of both the SOD1 and CCS proteins over the same time period (Figure

3.6A lower panel and 3.6B). The activity of CCO was also markedly reduced in

mitochondrial preparations isolated from hypoxic RAW264.7 macrophages

(Figure 3.7A), and this was accompanied by the decreased expression of the

COX1 subunit of CCO which contains the CuB copper binding site (Fig. 3.7B).

Together with the above experiments, these findings suggest that hypoxia

suppresses copper delivery to SOD1 and CCO in RAW264.7 macrophages, and

enhances copper delivery to the ATP7A/ceruloplasmin pathway.

FIGURE 3.6. **Hypoxia alters the CCS/SOD pathway in RAW264.7**

macrophages. A, Effect of hypoxia on SOD1 protein expression and activity.

RAW264.7 cells were grown under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2)

conditions for the indicated times. Cell lysates were subjected to non-denaturing

SDS-PAGE for the in-gel SOD1 activity assay (top panel). Immunoblots from the

same lysates were probed with anti-SOD1 antibodies to detect SOD1 protein

(lower panel). Tubulin was detected as a loading control. B, Effect of hypoxia on

the abundance of CCS, the copper-chaperone for SOD1. The same lysates as in

(A) were subjected to SDS-PAGE and immunoblot analysis with anti-CCS

antibodies.

Fig. 3.6

A
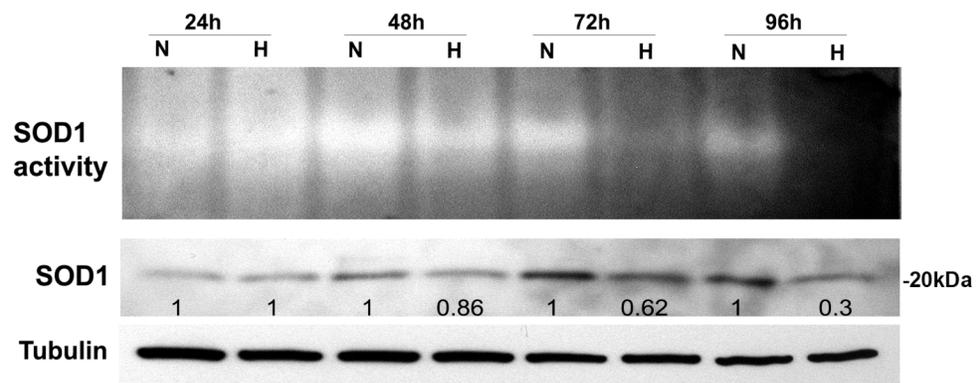
B
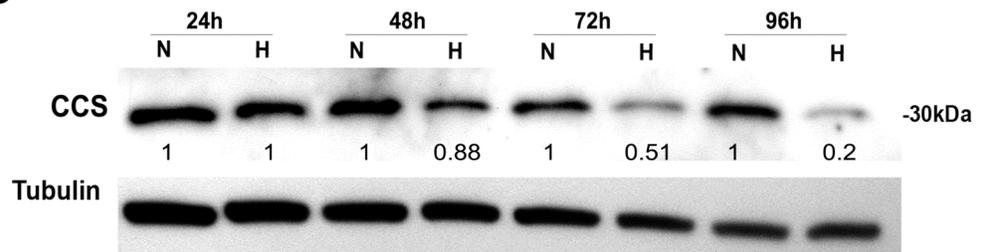
FIGURE 3.7. **Hypoxia alters cytochrome c oxidase expression and activity in RAW264.7 cells.** A, Analysis of cytochrome c oxidase activity in hypoxic (shaded bars) and normoxic (solid bars) conditions. Activity was measured in mitochondrial preparations isolated from RAW264.7 cells cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions for the indicated times. Values were normalized against total mitochondrial protein (mean + SD; n = 3). *p<0.05. B, Analysis of COX1 protein levels. Mitochondrial preparations from (A) were subjected to SDS-PAGE and probed with antibodies against COX1, the copper-binding subunit I of the cytochrome c oxidase complex. Immunoblots were probed with an antibody against mouse porin to indicate protein loading.

Fig. 3.7

A
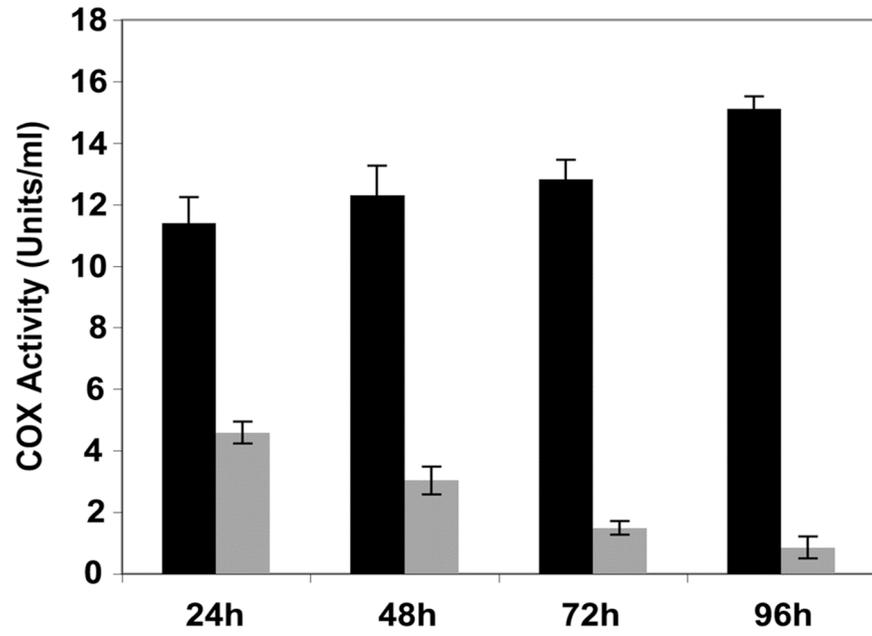
B
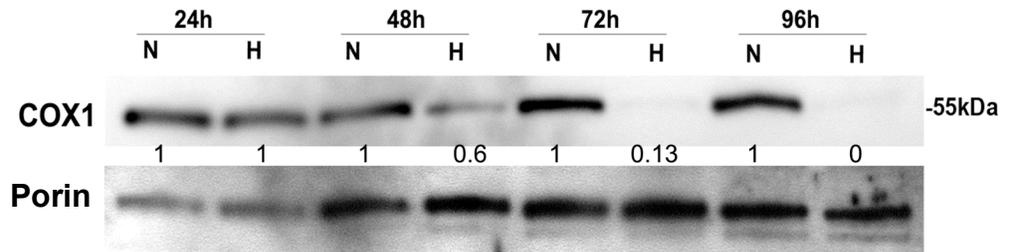
The hypoxia-induced trafficking of ATP7A and ceruloplasmin activation are dependent on mitochondria and reactive oxygen species— Recent studies have demonstrated that the mitochondria are an important sensor of oxygen limitation in mammalian cells. Mitochondrial oxygen sensing involves the generation reactive oxygen species (ROS) by the respiratory complex III of the electron transport chain which are required for the stabilization and activation of HIF-1α, the master transcriptional regulator of hypoxic responses (393, 394). To test whether ROS are required for the hypoxia-induced trafficking of ATP7A, we investigated whether a membrane-permeable antioxidant could suppress this trafficking process. Ebselen treatment of RAW264.7 cells prevented the hypoxia-induced trafficking of ATP7A (Fig. 3.8A). This suppression of ATP7A trafficking by Ebselen was not due to an inhibition of the trafficking machinery because the addition of copper elicited ATP7A trafficking in Ebselen-treated cells (Fig. 3.8A). Moreover, the addition of the oxidizing agent hydrogen peroxide to RAW264.7 cells under normoxic conditions also resulted in ATP7A trafficking (Fig. 3.8B). These findings suggest that reactive oxygen species generated by hypoxia are involved in ATP7A trafficking in RAW264.7 cells. Since the increase in ceruloplasmin activity during hypoxia requires the delivery of copper to the enzyme via ATP7A, we hypothesized that, along with inhibiting the trafficking of ATP7A, Ebselen would also inhibit the increase in ceruloplasmin activity in hypoxic macrophages. Indeed, as shown in Figure 3.8C, Ebselen suppressed the increase in ceruloplasmin activity associated with hypoxia, an effect that was overcome by the addition of copper to the Ebselen-treated cells. Interestingly,

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Ebselen treatment did not inhibit the hypoxia-induced increase in CTR1 and ATP7A expression (Fig. 3.10A, and 3.10B) nor the suppression of the CCS/SOD (Fig. 3.11) and COX (Fig. 3.12) activities associated with hypoxia.

Next, we investigated the contribution of mitochondria-generated ROS to the hypoxia-induced ATP7A trafficking. Rho-minus RAW264.7 cells (RAW/Rho⁻) lacking functional mitochondria were isolated by selection in ethidium bromide, and the depletion of the mitochondria-encoded protein, COX-1, but not the nuclear-encoded mitochondrial protein, porin was detected (Fig. 3.9A). Significantly, the trafficking of ATP7A to post-Golgi in response to hypoxia was suppressed in RAW/Rho⁻ cells suggesting that this process is dependent on mitochondria (Fig. 3.9B). The ATP7A trafficking machinery was not compromised in the RAW/Rho⁻ cells because exogenous copper was able to stimulate ATP7A trafficking. Ceruloplasmin activation under hypoxic conditions, shown earlier to be ATP7A-dependent, was also suppressed in RAW/Rho⁻ cells exposed to hypoxia, but not when copper was provided to the media (Fig. 3.9C). Taken together, these findings suggest that mitochondrially-generated ROS may facilitate the delivery of copper to ATP7A during hypoxia, thereby mediating ATP7A-dependent copper transport to ceruloplasmin.

FIGURE 3.8. **The hypoxia-induced trafficking of ATP7A and activation of ceruloplasmin are mediated by reactive oxygen species**. A, RAW264.7 cells were exposed to normoxia or hypoxia for 96 h $\pm$10 μM Ebselen and/or 20 μM CuCl$_2$. Immunofluorescence staining shows ATP7A in green. B, RAW264.7 cells were exposed for 2 h to hydrogen peroxide (H$_2$O$_2$, 500 μM). ATP7A is shown in green. C, Ceruloplasmin activity in conditioned media of RAW264.7 cells exposed to normoxia or hypoxia for 72 h $\pm$10 μM Ebselen and/or 20 μM CuCl$_2$. Ceruloplasmin activity values were normalized against total protein content of corresponding cell lysates (mean + SD; n = 3). Different letters above each value indicate significant differences $p < 0.05$.

Fig. 3.8

A

	Control	+ Ebselen	Ebselen + Cu
Normoxia			

	Control	+ Ebselen	Ebselen + Cu
Hypoxia			

B

	Control	+ H$_2$O$_2$
Normoxia		

C

Ceruloplasmin Activity (U/ml/min)

N
H

Untreated   Cu   Ebselen   Ebselen + Cu
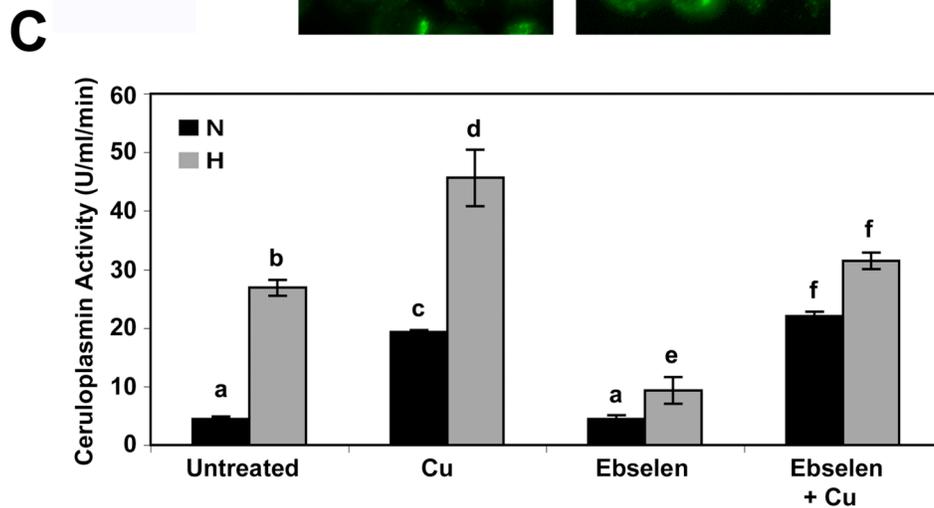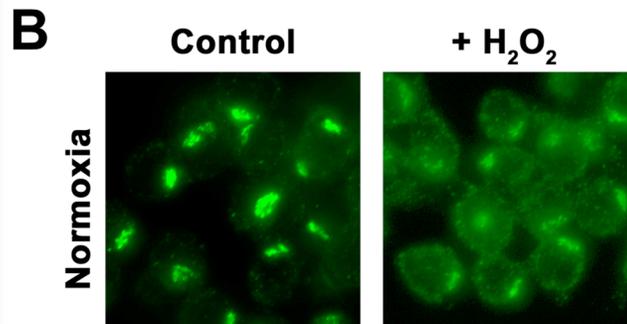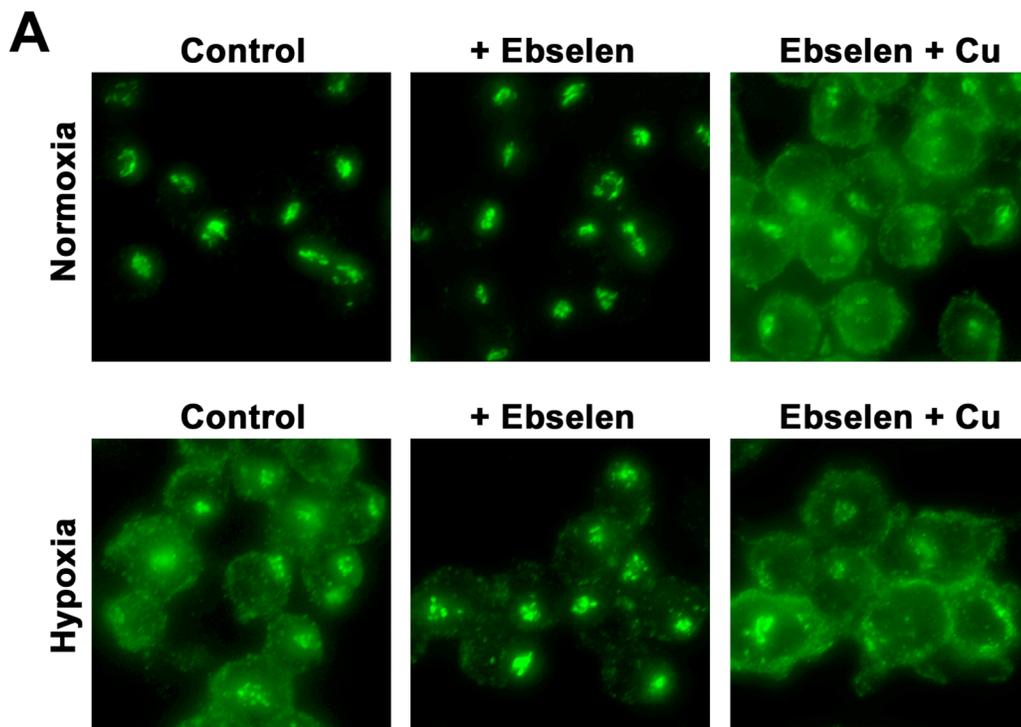
FIGURE 3.9. **The hypoxia-induced trafficking of ATP7A and activation of ceruloplasmin are mediated by reactive oxygen species generated in the mitochondria**. RAW264.7 cells were treated with ethidium bromide to deplete their mitochondrial DNA and designated RAW/Rho⁻. A, Immunoblot of COX-1 from mitochondrial extracts of RAW264.7 and RAW/Rho⁻ cells shows loss of expression of mitochondrially-encoded COX-1 in RAW/Rho⁻ cells. Porin was detected as loading control. B, Immunofluorescence imaging of ATP7A in RAW264.7 and RAW/Rho⁻ cells exposed to normoxia or hypoxia for 96 h ± 20 μM CuCl$_2$. C, Ceruloplasmin activity was measured in conditioned media of RAW264.7 and RAW/Rho⁻ cells exposed to normoxia or hypoxia for 72 h ± 20 μM CuCl$_2$. Values were normalized against total protein content of corresponding cell lysates (mean + SD; n = 3). Different letters above each value indicate significant differences $p < 0.05$.

Fig. 3.9
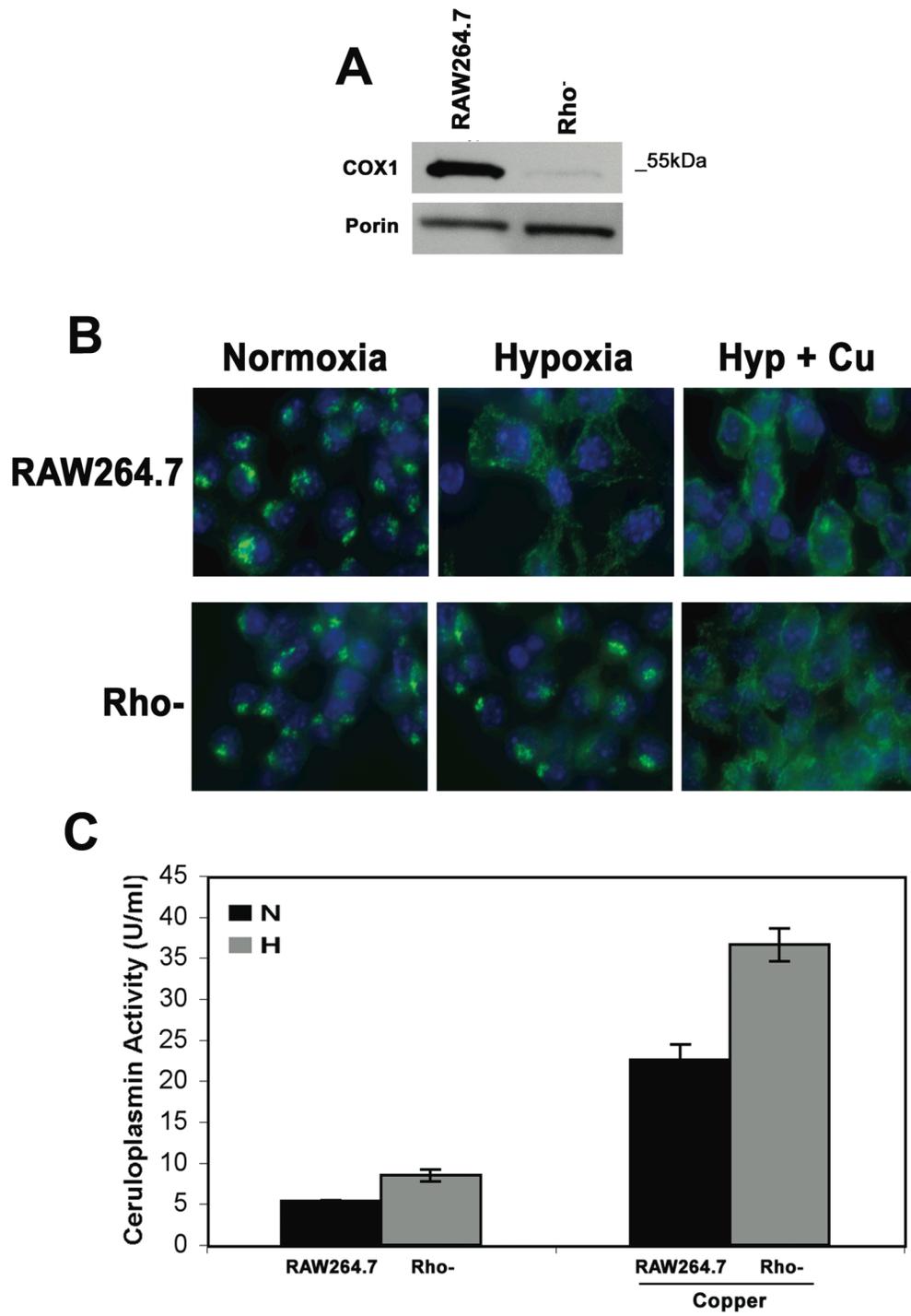
FIGURE 3.10. **Ebselen does not inhibit the increases in CTR1 and ATP7A protein expression associated with hypoxia.** RAW264.7 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions ± 50 μM Ebselen. Immunoblot analysis of A, CTR1 protein and B, ATP7A protein. Tubulin was detected as a loading control.

Fig. 3.10

A

B
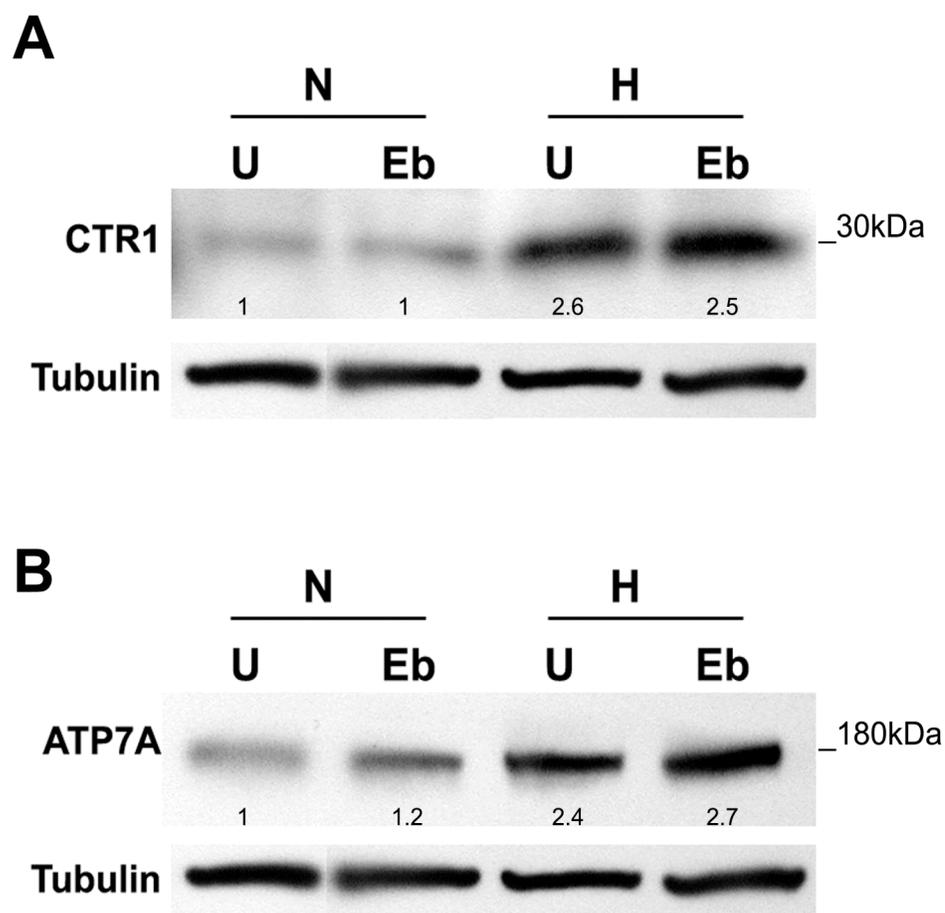
FIGURE 3.11. **Ebselen does not inhibit the hypoxia-induced changes in CCS/SOD pathway in RAW264.7 macrophages.** RAW264.7 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions ± 50 μM Ebselen. A, Cell lysates were subjected to non-denaturing SDS-PAGE for the in-gel SOD1 activity assay (top panel). Immunoblots from the same lysates were probed with anti-SOD1 antibodies to detect SOD1 protein (lower panel). B, The same lysates as in (A) were subjected to SDS-PAGE and immunoblot analysis with anti-CCS antibodies. Tubulin was detected as a loading control.

Fig. 3.11
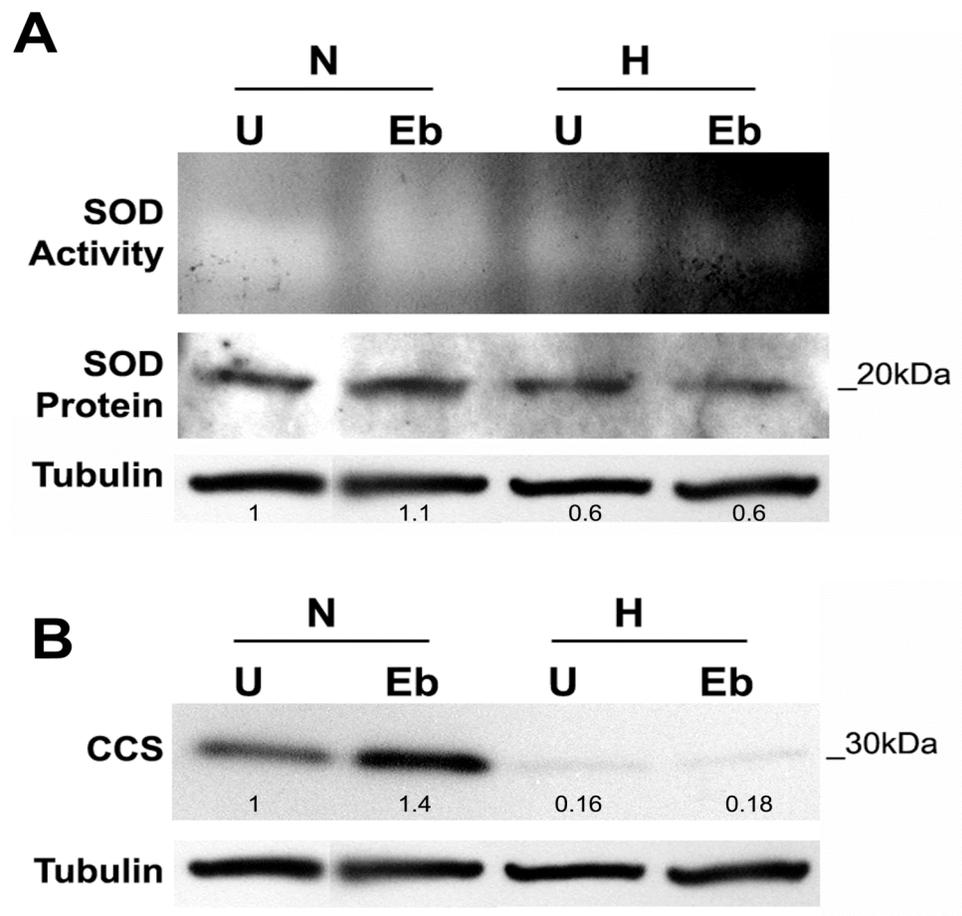
FIGURE 3.12. **Ebselen does not inhibit the hypoxia-induced changes in the cytochrome c oxidase pathway in RAW264.7 macrophages.** RAW264.7 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions ± 50 μM Ebselen. A, Analysis of COX1 protein levels. Mitochondrial preparations were isolated from RAW264.7 cells cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions for 72 h and subjected to SDS-PAGE and probed with antibodies against COX1, the copper-binding subunit I of the cytochrome c oxidase complex. Immunoblots were probed with an antibody against porin to indicate protein loading. B, Analysis of cytochrome c oxidase activity in hypoxic (shaded bars) and normoxic (solid bars) conditions. Activity was measured in mitochondrial preparations from (A). Values were normalized against total mitochondrial protein (mean + SD; n = 3). *$p < 0.05$.

Fig. 3.12
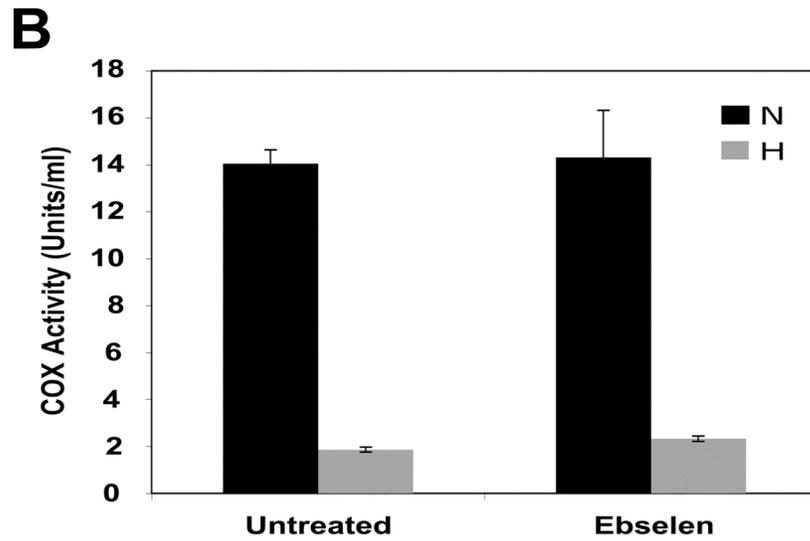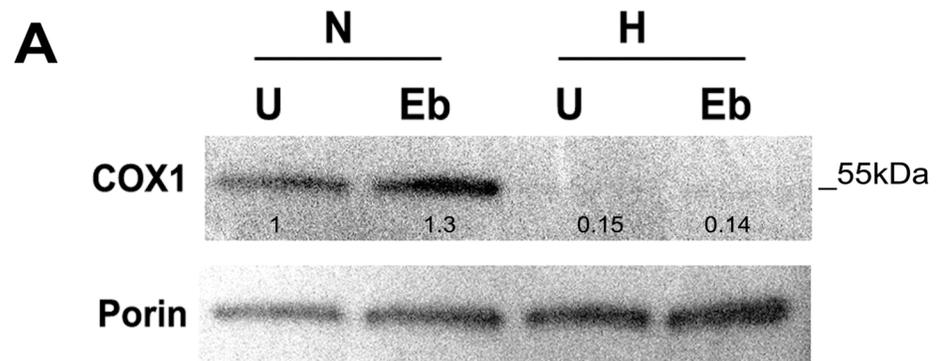
Effect of hypoxia on the copper pathways in non-macrophage cell lines— We

investigated whether the observed changes in copper pathways induced by

hypoxia in macrophage cells also occur in other cell lines. Interestingly, ATP77A

did not traffic to post-Golgi in response to hypoxia in N2a, NIH3T3, DLD1,

HT1080 cells (Fig. 3.13), HEK293, human primary aortic endothelial cells

(HAECs), and rat primary smooth muscle cells (SMCs) (not shown).

Furthermore, hypoxia did not induce the coordinated increases in CTR1 and

ATP7A protein expression, along with the suppression of the activities of the two

other copper pathways, *i.e.* the CCS/SOD and the cytochrome c oxidase

pathways in any of the cell lines tested (Fig. 3.14, 3.15, and 3.16). These

observations suggest that the observed changes in copper homeostasis in

response to hypoxia are unique to macrophages. Further studies are required to

determine whether these effects also occur in other cells of the myeloid lineage.

It is worth noting that although hypoxia did not induces changes in the copper

pathways in N2a cells that were similar to those observed in macrophage cells,

there were some interesting changes that may be of relevance to neuron biology.

In response to hypoxia, the expression of CTR1, CCS, SOD1, as well as the

activity of SOD1 were induced in N2a cells.

FIGURE 3.13. **ATP7A does not traffic in response to hypoxia in various cell lines.** Immunofluorescence analysis of ATP7A protein in in N2a, NIH3T3, DLD1, and HT1080 cells grown under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions for 96 h. Cells were fixed, permeabilized and probed with antibodies against ATP7A and anti-rabbit IgG antibodies conjugated to Alexa-488 (green).

Fig. 3.13
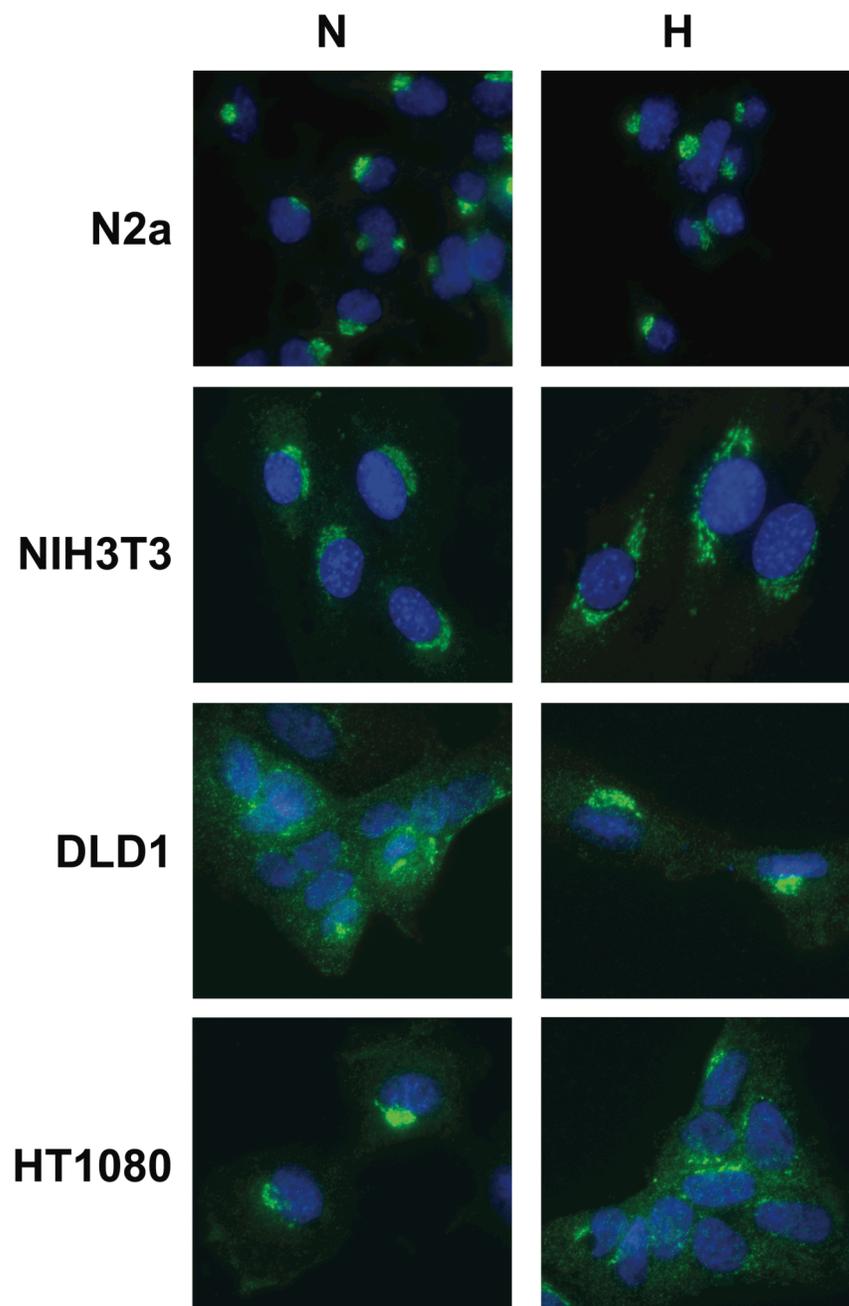
FIGURE 3.14. **Effect of hypoxia on CTR1 and ATP7A protein expression levels in various cell lines.** N2a, NIH3T3, DLD1, HT1080 and HEK293 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions. Immunoblot analysis of A, CTR1 protein and B, ATP7A protein. Tubulin was detected as a loading control. CTR1 protein levels were too low to detect in N2a, NIH3T3 and HEK293 cells.

Fig. 3.14

A

N2a   DLD1

N   H   N   H

CTR1   _30kDa

1   1.5   1   0.5

Tubulin

B

N2a   NIH3T3   DLD1   HT1080   HEK293

N   H   N   H   N   H   N   H   N   H

ATP7A   _180kDa

1   1   1   1   1   1   1   1   1   1.3

Tubulin
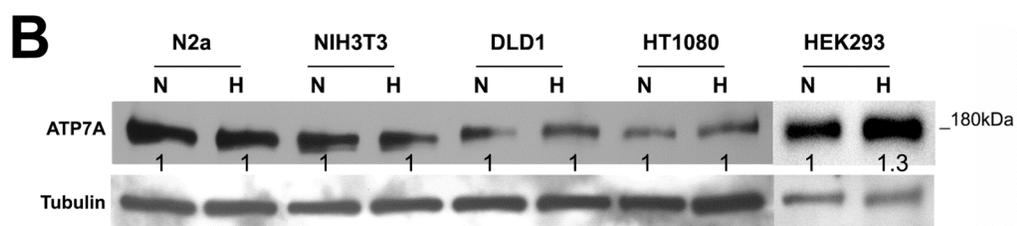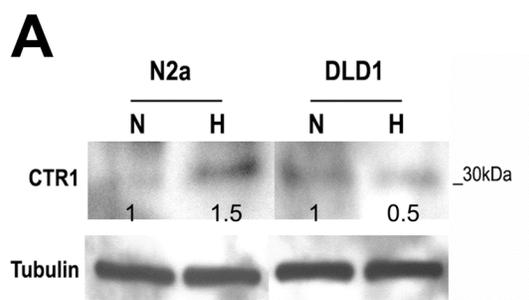
FIGURE 3.15. **Effect of hypoxia on the CCS/SOD pathway in various cell lines.** N2a, NIH3T3, DLD1, HT1080 and HEK293 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions. A, Cell lysates were subjected to non-denaturing SDS-PAGE for the in-gel SOD1 activity assay (top panel). Immunoblots from the same lysates were probed with anti-SOD1 antibodies to detect SOD1 protein (lower panel). B, The same lysates as in (A) were subjected to SDS-PAGE and immunoblot analysis with anti-CCS antibodies. Tubulin was detected as a loading control.

Fig. 3.15

	N2a		NIH3T3		DLD1		HT1080		HEK293	
	N	**H**	**N**	**H**	**N**	**H**	**N**	**H**	**N**	**H**
SOD Activity										
SOD Protein	1	1.1	1	1	1	1	1	1.2	1	0.85
Tubulin										

_20kDa

	N2a		NIH3T3		DLD1		HT1080		HEK293	
	N	**H**	**N**	**H**	**N**	**H**	**N**	**H**	**N**	**H**
CCS	1	1.3	1	1.2	1	1	1	1.2	1	0.8
Tubulin										

_30kDa

149
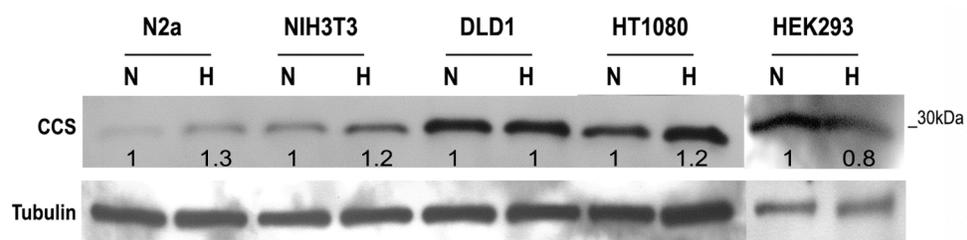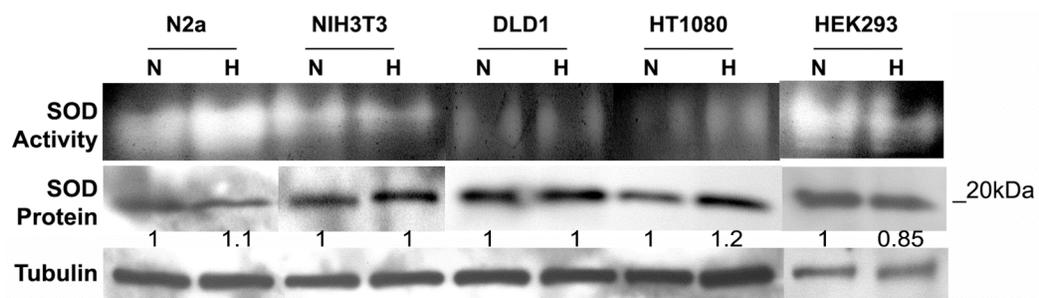
FIGURE 3.16. **Effect of hypoxia on the cytochrome c oxidase pathway in various cell lines.** N2a, NIH3T3, DLD1, HT1080 and HEK293 cells were cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions. A, Analysis of COX1 protein levels. Mitochondrial preparations isolated from cells cultured under normoxic (N; 21% O_2) or hypoxic (H; 4% O_2) conditions were subjected to SDS-PAGE and probed with antibodies against COX1, the copper-binding subunit I of the cytochrome c oxidase complex. Immunoblots were probed with an antibody against porin to indicate protein loading. B, Analysis of cytochrome C oxidase activity in hypoxic (shaded bars) and normoxic (solid bars) conditions. Activity was measured in mitochondrial preparations from (A). Values were normalized against total mitochondrial protein (mean + SD; n = 3). *$p<0.05$.

Fig. 3.16

A

B
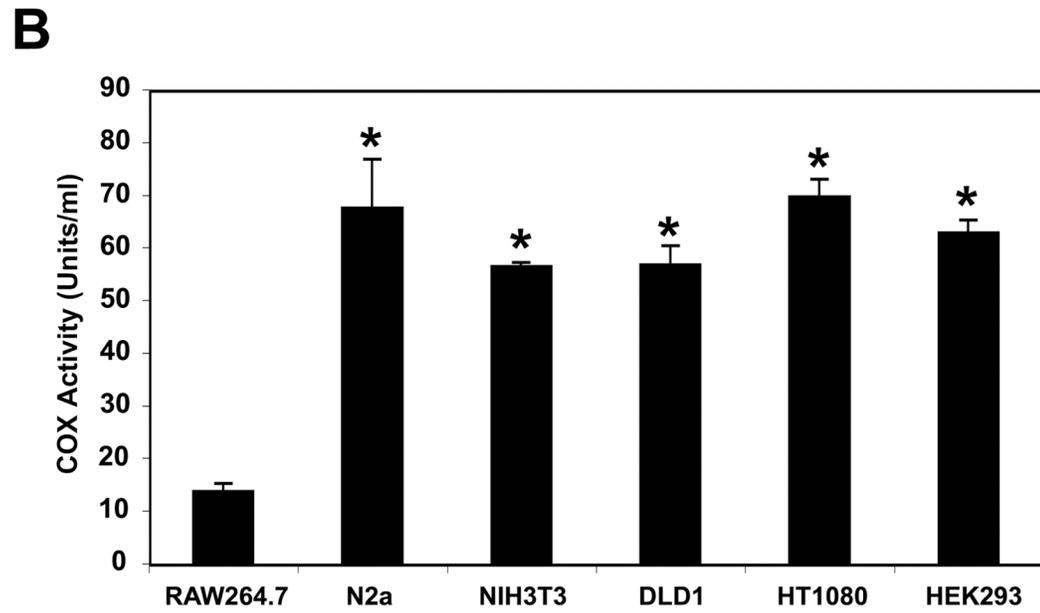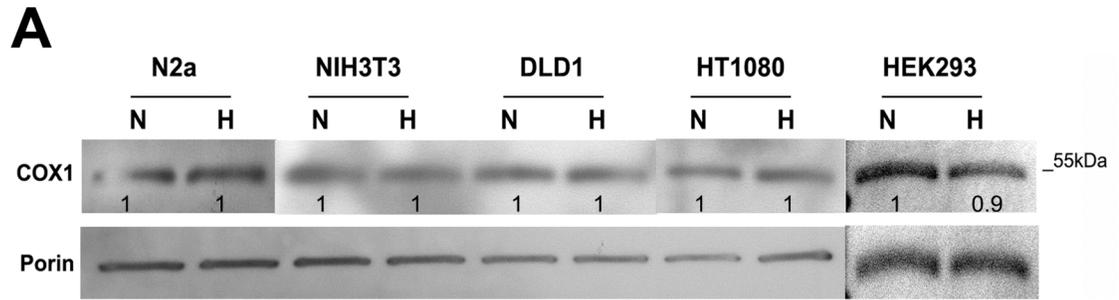
3.5 Discussion

Hypoxia, or relative O_2 depletion, is a serious condition that threatens cell survival, and can cause severe organ damage and injury in human disease. Respiratory failure, systemic hypotension and ischemia promote hypoxic tissue injury in great part mediated by oxidative stress (133-138). Therefore, physiological and cellular responses have evolved to defend the oxygen supply (139-142). Rapid responses are required on the cellular level to protect the cell from prolonged O_2 depletion. These responses are mediated by the hypoxia-inducible factor transcription factor 1 alpha (HIF-1α), and lead to the upregulation of genes involved in glucose uptake, cell survival, cytoskeletal organization, extracellular matrix remodeling, vascular tone, iron metabolism, and apoptosis (290-294). On the physiological level, responses to limitations in O_2 supply ultimately result in vascular remodeling, cell migration and proliferation, and formation of new blood vessels or angiogenesis.

Macrophage cells commonly encounter hypoxia as they migrate away from the vasculature, therefore, these cells have developed unique adaptive mechanisms allowing them to function in a hostile environment. We have identified a series of unexpected changes in macrophage intracellular copper metabolism in response to hypoxia, as illustrated by the model in Figure 3.17. These include the elevated expression of the CTR1 copper transporter and increased copper uptake; a decrease in the abundance and/or activity of CCS, SOD1 and CCO; and

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increased copper delivery to ATP7A and ceruloplasmin in the secretory pathway. Significantly, such changes provide the first evidence that the hierarchy of intracellular copper distribution is subject to modulation by environmental stimuli.

Our findings suggest that this coordinate reprogramming of copper metabolism functions to enhance copper availability to the ATP7A protein for copper delivery to the secretory pathway. This was evidenced by the requirement for ATP7A for ceruloplasmin activation in response to hypoxia as well as the copper-dependent relocalization of the ATP7A protein from the TGN to a post-Golgi compartment, a well-described indicator of increased copper flux to ATP7A. Ceruloplasmin is a ferroxidase synthesized in the liver and macrophages that plays a critical role in cellular iron export by oxidizing iron, a necessary step in the iron loading of transferrin in the blood (396). This function of ceruloplasmin is particularly critical during hypoxia where the mobilization of iron into the blood is important for hematopoeisis (146).

Further evidence for a shift in the intracellular hierarchy of copper metabolism in response to hypoxia was our finding of decreased levels of CCS protein and SOD1 activity. By diminishing the flux of copper from CCS to SOD1, this would allow more copper to be conserved for the ATP7A protein. Whether such adaptations respond directly to reduced oxygen levels is unknown, however, CCS degradation may be triggered in hypoxic macrophages in response to the higher copper uptake activity (Fig. 3.1), since elevated intracellular copper

concentrations are known to cause CCS degradation in other cells (185). Like SOD1, the activity of cytochrome c oxidase was also diminished by hypoxia in RAW264.7 cells and this was associated with a decrease in COX1 protein levels, the Cu_B-site containing subunit of cytochrome c oxidase. A decrease in cytochrome c oxidase activity has been observed in hypoxic macrophages in previous studies as part of a metabolic shift from oxidative phosphorylation to glycolysis for the bulk of ATP production (397, 398). By eliminating intracellular copper delivery to COX1, this may serve the dual purpose of preventing wasteful copper delivery to cytochrome c oxidase as the cell undergoes a metabolic shift to glycolysis, as well as diverting precious copper stores to secretory compartments via ATP7A.

A particularly notable finding of this study was the requirement for reactive oxygen species and mitochondrial function for both the activation of ceruloplasmin in hypoxic RAW264.7 macrophages, and the induction of ATP7A trafficking. Recent studies indicate that the mitochondria are a critical sensor of oxygen in mammalian cells and communicate this information to a master regulator of hypoxia-responsive gene expression, the HIF-1α protein (139, 321, 324, 325, 340-344). HIF-1α is constitutively degraded under oxygen-replete conditions, however, the stimulated production of ROS at complex III of the mitochondrial electron transport chain is essential for HIF-1α stabilization under hypoxic conditions (139, 321, 324, 325, 340-344). Based on these findings, the ability of mitochondrial ROS to stimulate ceruloplasmin activity and ATP7A

trafficking may occur via a HIF-1α-dependent mechanism. Alternatively, the ROS may function indirectly by displacing labile copper ions from intracellular ligands, thereby increasing copper availability for ATP7A. Although this latter mechanism would be the first such type of ROS-mediated regulation of copper homeostasis in mammalian cells, a precedent exists in yeast where nitric oxide alters copper homeostasis by displacing copper from the AceI transcription factor (35). Other examples of ROS-mediated regulation of iron and zinc homeostasis also exist. For example, NO and H_2O_2 can displace iron from the IRP1/2 proteins, thereby allowing these proteins to regulate a select group of mRNAs encoding proteins involved in iron homeostasis (36-39). In the case of zinc, several studies have demonstrated that NO and H_2O_2 release labile zinc from metallothionein which then stimulates the activity of the metal-responsive transcription factor, MTF-1, a major regulator of zinc homeostasis (40, 41). Interestingly, the ROS generated during hypoxia in macrophage cells did not mediate the downregulation of the CCS/SOD and COX17/CCO pathways. Further studies are required to elucidate the mechanisms by which hypoxia downregulates these copper pathways.

It appears that the above reported changes in copper homeostasis are somewhat unique to macrophages. ATP7A trafficking and/or changes in SOD1 and CCO activity were not observed in our analysis of cultured cells from a variety of sources including HeLa (cervical carcinoma), HEK293 (human embryonic kidney), N2a (neuroblastoma), primary human aortic endothelial cells, and primary rat smooth muscle cells. The reasons for this are not fully understood.

The macrophage-specific effects of hypoxia on copper homeostasis may be attributable to inflammatory responses, which in these cells overlap with responses to hypoxia (42, 43). Consistent with this postulate, our studies demonstrate that pro-inflammatory agents can stimulate copper-dependent ATP7A trafficking in macrophages under normoxic conditions.

Macrophages are highly adapted to hypoxic environments and are prominent in avascular sites of diseased tissue, and the hypoxic regions of different types of tumors (391). By releasing pro-angiogenic cytokines that promote vascularization of hypoxic tissues, macrophages promote tissue repair and tumor growth (44-47). This property is thought to underlie the positive relationship that exists between the macrophage content of tumors and a poor clinical outcome (48-53). It is, therefore, intriguing that copper also possesses pro-angiogenic and tumorigenic properties, and several studies have demonstrated that copper chelation is an effective suppressor of tumor vascularization and growth (54-57). Whether the adaptive changes in macrophage copper metabolism described in this study underlie copper's role in tumor growth is unknown, and the subject of on-going experiments.

The prioritization of copper distribution to ATP7A is a finding that is novel in mammals, but has parallels in microorganisms. For example, in the photosynthetic microbe *Chlamydomonas reinhardtii*, the copper-containing protein plastocyanin is degraded in response to copper-deficiency, which is

thought to be an adaptive process to preserve copper for more critical enzymes such as cytochrome c oxidase (399). It will be of interest to determine whether redistribution of intracellular copper is stimulated by pathophysiological conditions in other mammalian cell types. For example, copper is required for melanin production via tyrosinase, norepinephrine synthesis via dopamine b hydroxylase, and collagen cross-linking via lysyl oxidase, and the activity of each of these biochemical pathways can be initiated by several physiological or developmental cues in specific cell types (reviewed in (4)). The challenge of future studies will be to address whether this adaptive upregulation of cuproenzyme activity is achieved via adaptive changes in pathways that intracellular copper distribution.

FIGURE 3.17. **Schematic model of macrophage copper homeostasis pathways altered by hypoxia**. Effects of hypoxia (H) on specific steps in intracellular copper handing are shown based on the findings of this study. Hypoxia-stimulated expression of the CTR1 copper importer and copper uptake (**1**). Hypoxia-stimulated decrease in CCS expression (**2**) and activity of SOD1 (**3**). Mitochrondrial CCO activity was diminished by hypoxia associated with reduced expression of COX1 (**4**). The hypoxia-induced increase in ATP7A expression (**5**), copper-dependent trafficking of ATP7A (**6**), and ATP7A-dependent copper transport to ceruloplasmin (**7**), are ROS-dependent (**8,9**).

Fig. 3.17
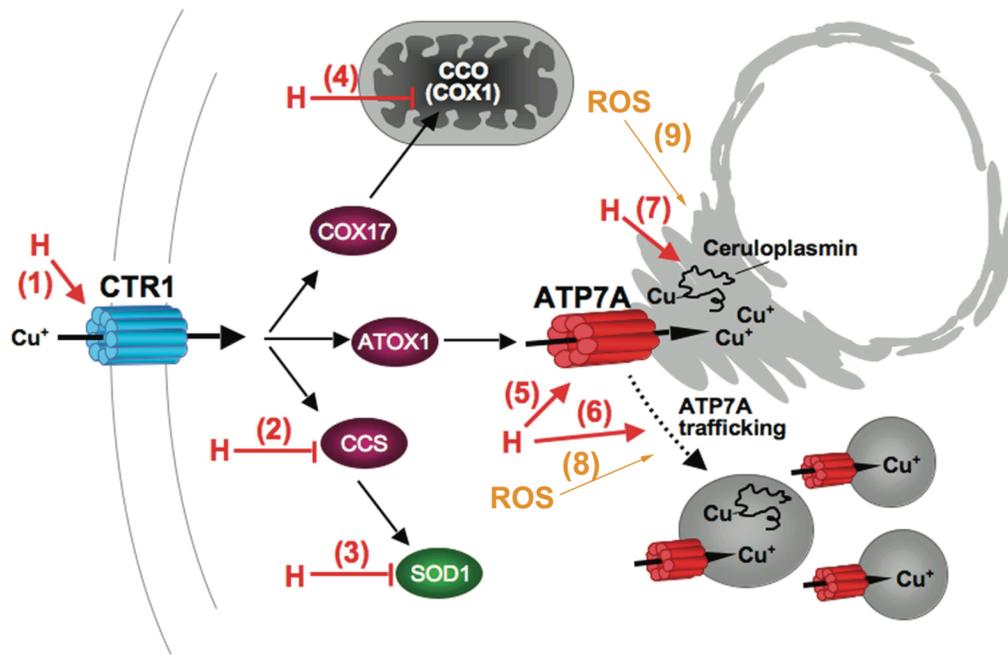
CHAPTER 4

SUMMARY AND FUTURE PERSPECTIVES

4.1 ATP7A-mediated copper transport into the phagosome enhances the bactericidal activity of macrophages.

Copper has long been recognized as a bactericidal agent. Its modern day uses in fungicides, antimicrobial surfaces and fabrics, anti-fouling marine paints and hygienic medical devices can be traced back to its early uses in ancient civilizations (374, 401). The uses of copper as a sanitizing agent in ancient Egyptian and Roman civilizations ranged from its addition to drinking water, to its topical administration to sores and skin infections (374). Today, the effectiveness of copper as a bactericidal agent has further expanded its uses in the healthcare industry (401). Brass, an alloy of 67% copper and 33% zinc, is the metal of choice for the manufacture of hospital doorknobs (402, 403), owing to its proven self-sanitizing properties. Copper is also increasingly incorporated into the fibers used in the manufacture of hospital beddings and linens, as well as the facemasks, gloves and clothing worn by healthcare professionals (404, 405). Several studies have highlighted the importance of copper for the function of the immune system (281-284). Our data provide exciting new evidence of a novel role for ATP7A in promoting bacterial killing via transport of copper into the phagosome of macrophage cells. We also show changes in copper homeostasis

that accommodate the increased need for copper in macrophage cells during bacterial killing. These changes include a higher rate of copper uptake via increased expression of the CTR1 copper importer. Another novel finding is the increases of CTR1 and ATP7A mRNA levels that occur in response to inflammation. It is unknown whether the elevated mRNA levels are due to transcriptional or post-transcriptional regulation by inflammatory mediators. Further studies are needed to elucidate the mechanisms mediating these responses in macrophages.

Our findings suggesting that transport of copper to the phagosome via ATP7A is an important player in macrophage bactericidal activity are consistent with previous reports of common bacterial infections, especially those of the respiratory tract in Menkes disease infants carrying genetic mutations in the ATP7A gene (50, 406-408). However, it is uncertain whether this susceptibility to infection arises from defects in ATP7A-dependent activities of macrophages, or impairment of other processes such as connective tissue formation (114). Clearly, a more direct test of the importance of ATP7A in innate immune function *in vivo* will be to generate a myeloid-specific ATP7A knockout mouse model. An additional model for testing the importance of copper in macrophage bactericidal activity will be a myeloid-specific CTR1 knockout mouse. If copper is of importance in bactericidal activity of macrophages *in vivo* we anticipate the following outcomes in ATP7A and CTR1 myeloid knockout mice: 1) decreased clearance of intraperitoneally-injected bacteria in the myeloid ATP7A$^{-/-}$ and

myeloid CTR1$^{-/-}$ mice as compared to wild type controls; 2) decreased viability of the myeloid ATP7A$^{-/-}$ and myeloid CTR1$^{-/-}$ mice as compared to wild type controls following bacterial infection.

Our findings also indicate interplay of opposing copper regulatory mechanisms in bacteria and host. The transport of copper into the phagosome via ATP7A is opposed by the export of copper from the cytoplasm of *E. coli* mediated by the CopA copper exporter. Deletion of the *copA* bacterial gene severely impaired the resistance of *E. coli* to killing within the macrophage phagosome. Microbial copper export in response to phagoctosis by macrophages has been previously demonstrated in several bacterial species (288, 381, 382). Understanding microbial resistance mechanisms is of great importance in designing new preventative and therapeutic avenues, particularly in the health care industry where pathogen infections severely compromise patient welfare and recovery, spurring the increased use of copper in alloys for the manufacture of hospital surfaces over the past decade (reviewed in (401)). Copper has become popular in the biomedical environment as the bactericidal and fungicidal metal of choice and copper alloys are the only solid surface currently approved by the FDA as "antimicrobial". However, intriguing new evidence for a potential role for copper in promoting fungal survival within the phagosome has emerged. In contrast to bacteria which upregulate the expression of copper export mechanisms as a protection against copper-mediated toxicity within the phagosome, the fungus *Cryptococcus neoformans* responds to phagosomal stress by increasing its

copper acquisition (409, 410). In fact, the expression of the copper importer CTR4 was increased in *Cryptococcus neoformans* following phagocytosis by J774A.1 macrophage cells (410). This was accompanied by an increase in the expression of copper-dependent cryptococcal antioxidant systems, suggesting a role for copper in promoting cryptococal resistance to killing by macrophages (410). Neurological infections with *Cryptococcus neoformans* resulting in meningitis are common in AIDS patients, who are often vulnerable to such infections due to their high levels of serum copper. This emerging new evidence suggests that although copper status of the host might be beneficial in the case of bacterial infection, this may not be the case for fungal infections. When considering the use of copper supplementation of patients or animals as a treatment strategy, it will be important to tailor such treatments to the particular infective agent.. Clearly, further work is needed to comprehensively assess of the role of copper in various bacterial and fungal species of significance to human health. Understanding the role of phagosomal copper in the killing or survival of different pathogenic species can ensure tailored treatments of human infections as well as better understanding of the impact of copper nutrition and physiological copper status on human health.

4.2 Hypoxia modulates copper homeostasis in macrophage cells.

One of the earliest physiological responses to tissue injury and infection is an increase in vascular permeability and blood flow to the affected area (411, 412).

Macrophage cells migrate away from the vasculature into the sites of injury or infection and commonly encounter varying degrees of oxygen depletion (391). Rapid cellular responses are initiated that allow these cells to cope with the low oxygen tension in the extravascular milieu (290-294). The HIF-1α transcription factor coordinately upregulates the expression of genes involved in metabolism and angiogenesis, among many other processes (290-294). These cellular responses ensure adaptation to the hypoxic conditions, as well as increased oxygen flow to the microenvironment via promoting neo-vascularization. Interestingly, accumulating evidence suggests that inflammatory mediators stabilize HIF-1α in the presence of oxygen (411, 412). Furthermore, inflammatory mediators and hypoxia activate common signaling pathways and often lead to converging biological outcomes (reviewed in (411, 412)). Following our observation that inflammatory mediators regulates copper homeostasis in macrophages, we investigated the effect of relative hypoxia on copper homeostatic pathways in these cells.

Our studies indicate that hypoxia induces an increased flux of copper into the cell, associated with an increase in CTR1 protein expression. The increase in copper flux resulted in copper-dependent trafficking of the ATP7A protein, as well as increased ATP7A-dependent copper delivery to the secretory pathway, as evidenced by the enhanced ceruloplasmin activity in hypoxia. Interestingly, hypoxia led to the downregulation of the two other cellular copper pathways, *i.e.* the CCS/SOD1 and the mitochondrial cytochrome c oxidase pathways. We

suggest that the downregulation of these alternative cellular copper pathways might allow for the prioritization of copper flux into the secretory pathway via ATP7A. Ceruloplasmin and lysyl oxidase are two examples of secreted copper-dependent enzymes that may benefit from this increased copper delivery, since the expression of both proteins is enhanced under hypoxic conditions (146, 314, 395, 413, 414).

Our finding that ceruloplasmin is a target of increased copper delivery to the secretory pathway during hypoxia is in agreement with the function of this protein in iron homeostasis. Ceruloplasmin is a ferroxidase required for cellular iron export, which is a critical step in the loading of iron onto transferrin in the blood (396). This process is also an adaptive response to hypoxia to meet the increased iron demand of hematopoeisis (146). Thus, the prioritization of copper delivery to ceruloplasmin via ATP7A may ultimately function to regulate iron homeostasis in response to hypoxia.

Other hypoxia-induced cuproenzymes that are also potential targets of ATP7A-dependent copper delivery into secretory compartments include the lysyl oxidase family of enzymes. These proteins function in the cross-linking of collagen and elastin within connective tissue (1) and, importantly, play a key role in hypoxia-induced tumor metastasis (31). It would be of interest to test the ATP7A dependency for lysyl oxidase activation in hypoxia, as this may provide potential future therapeutic avenues in the prevention of tumor growth and metastasis,

since lysyl oxidase expression during hypoxia is associated with tumor

metastasis. Another important copper-dependent role for the lysyl oxidase

enzymes is in embryogenesis and development. LOX$^{-/-}$ mice suffer from impaired

connective tissue formation resulting in cardiovascular and diaphragm instability,

leading to perinatal death (415, 416). Interestingly, connective tissue and

cardiovascular defects are associated with Menkes disease (417), and were

recently observed in CTR1$^{int-/Int-}$ mice which lack CTR1 in the intestine (100). It is

possible that low oxygen availability encountered by the embryo especially in the

early days of gestation may drive both lysyl oxidase expression and copper

delivery to the enzyme via the adaptive responses observed in our studies. It

would be interesting to investigate the regulation of copper homeostasis in

developing embryos undergoing adaptation to low oxygen availability *in vivo*.

4.3 ATP7A traffics in response to hypoxia in tumor-associated macrophages.

A particularly intriguing finding of our study was the strong expression of ATP7A

in tumor-associated macrophages. Several studies have shown that copper

induces proliferation of human endothelial cells in the absence of exogenous

cytokines (418, 419), and copper chelation prevents endothelial cell proliferation

in vitro (420, 421) and *in vivo* (422). Copper is also known to promote the

expression and secretion of pro-angiogenic cytokines from endothelial cells and

other cell types (423, 424). Moreover, copper has been shown to play an

important role in angiogenesis, and copper chelation via TTM has proven to be

an effective suppressor of tumor growth in animals (347-352, 421, 425, 426). It is, therefore, possible that the adaptive changes in macrophage copper homeostasis described in this study underlie copper's role in tumor growth. Additionally, our studies suggest that the changes affecting the copper homeostatic pathways in response to hypoxia are specific to macrophages.

Macrophages are highly prominent in tumors, constituting up to 80% of the cell mass in breast carcinoma (427) and their presence in high numbers is associated with poor prognosis (reviewed in (428)). Exposure of tumor-associated macrophages to the hypoxic milieu activates their pro-angiogenic properties (429). Future studies in our lab will aim at investigating the role of macrophage copper homeostasis in promoting tumor growth and angiogenesis. Does copper delivery to avascular tumor sites occur via macrophage recruitment and copper release? Studies that test this hypothesis will include measuring tumor growth in myeloid-specific ATP7A and CTR1 knockout mice. If indeed macrophage copper contributes to tumor growth, we expect tumor growth rates and tumor sizes to be significantly reduced in the knockout mice as compared to wild type. It will also be interesting to investigate whether the previously reported expression (314) and secretion (430, 431) of pro-angiogenic cytokines induced by hypoxia could be suppressed by the knockdown of ATP7A and CTR1 proteins in cultured macrophages. The effect of ATP7A and CTR1 knockdown on angiogenesis could be determined both *in vitro* and *in vivo*. The *in vitro* studies involve testing the ability of conditioned media from ATP7A-siRNA, CTR1-siRNA,

or control-siRNA RAW264.7 macrophages, to promote endothelial tube formation when co-cultured with endothelial cells (432, 433). A role of ATP7A and CTR1 in promoting tumor angiogenesis would be supported by the reduced pro-angiogenic activity of conditioned media ATP7A-RNAi, and CTR1-RNAi as compared to control-RNAi RAW264.7 cells. The extent of vascularization in tumors grown in myeloid-specific ATP7A and CTR1 knockout mice as compared to wild type mice is another means of assessing the contribution of macrophage copper to tumor angiogenesis *in vivo*.

4.4 ATP7A trafficking in response to hypoxia is stimulated by mitochondrial oxidative stress.

Another novel finding in our study is the trafficking of ATP7A in response to oxidative stress. Additionally, the ATP7A trafficking, as well as the activation of ceruloplasmin during hypoxia,were inhibited by the antioxidant Ebselen and by depletion of mitochondrial DNA in RAW267.4 cells (RAW/Rho$^-$). As discussed in chapter 1, ROS generated at complex III of the respiratory chain has recently been shown to be the major contributor in the stabilization of HIF-1α (324, 325, 342-344). Further in-depth studies of the effect of mitochondrial ROS on ATP7A trafficking and the activation of its potential target in the secretory pathway are required. For this, I propose using siRNA interference against the Rieske iron-sulfur protein. This will inhibit the formation of ubisemiquinone at complex III and thus will inhibit ROS generation by the mitochondria during hypoxia. Genetic knockdown of the Rieske protein has been successfully achieved elsewhere

(324, 325). Other chemical methods of inhibiting the mitochondrial respiratory chain at specific complexes exist, such as the use of rotenone (complex I inhibitor), myxothiazol (complex III inhibitor), Antimysin A (complex III inhibitor), potassium cyanide and sodium azide (complex IV inhibitors). However, these drugs are extremely toxic to cells when used for long durations of time. My preliminary experiments using varying dosages of these drugs suggest that the health of macrophage cells is severely compromised when exposed to such chemicals for 96 h, which is the time required to induce significant trafficking of the ATP7A protein.

4.5 Conclusion

Our studies provide exciting new evidence that macrophage copper homeostasis is regulated by pro-inflammatory agents. Importantly, we show that ATP7A-mediated copper transport serves to enhance the bactericidal activity of macrophages. We also demonstrate that hypoxia regulates intracellular copper delivery in macrophages, including a downregulation of the CCS/SOD1 and mitochondrial CCO pathways, and the upregulation of the CTR1/ATP7A axis, resulting in increased copper uptake, as well as increased activity of the copper-dependent enzyme ceruloplasmin.

Our data provide exciting new insight into the roles played by CTR1 and ATP7A proteins and copper in cancer and chronic inflammatory diseases that are often

accompanied by poor oxygen supply and an increase in cellular oxidative stress levels. Future studies will provide a better understanding of the molecular mechanisms by which copper homeostasis is regulated by inflammation and hypoxia, and may provide insight into potential future therapeutic avenues in the treatment of chronic inflammatory diseases with obstructive ischemic components and angiogenesis in cancer.

BIBLIOGRAPHY

1.   Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J. Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. Embo J 1996;15(22):6084-95.

2.   Pena MMO, Lee J, Thiele DJ. A delicate balance: Homeostatic control of copper uptake and distribution. J Nutr 1999;129:1251-60.

3.   Cousins RJ. Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol Rev 1985;65(2):238-309.

4.   Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. Am J Clin Nutr 1996;63(5):797S-811S.

5.   Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. Copper transport. Am J Clin Nutr 1998;67(5 Suppl):965S-71S.

6.   Margalioth EJ, Schenker JG, Chevion M. Copper and zinc levels in normal and malignant tissues. Cancer 1983;52(5):868-72.

7.   Greenberg SA, Briemberg HR. A neurological and hematological syndrome associated with zinc excess and copper deficiency. J Neurol 2004;251(1):111-4.

8.   Kumar N, Ahlskog JE, Gross JB, Jr. Acquired hypocupremia after gastric surgery. Clin Gastroenterol Hepatol 2004;2(12):1074-9.

9.   Kumar N, Crum B, Petersen RC, Vernino SA, Ahlskog JE. Copper deficiency myelopathy. Arch Neurol 2004;61(5):762-6.

10.   Kumar N, Gross JB, Jr., Ahlskog JE. Myelopathy due to copper deficiency. Neurology 2003;61(2):273-4.

11.   Kumar N, Gross JB, Jr., Ahlskog JE. Copper deficiency myelopathy produces a clinical picture like subacute combined degeneration. Neurology 2004;63(1):33-9.

12.   Kumar N, Low PA. Myeloneuropathy and anemia due to copper malabsorption. J Neurol 2004;251(6):747-9.

13.   Prodan CI, Holland NR, Wisdom PJ, Burstein SA, Bottomley SS. CNS demyelination associated with copper deficiency and hyperzincemia. Neurology 2002;59(9):1453-6.

14.   Schleper B, Stuerenburg HJ. Copper deficiency-associated myelopathy in a 46-year-old woman. J Neurol 2001;248(8):705-6.

15.   Williams DM. Copper deficiency in humans. Semin Hematol 1983;20(2):118-28.

16.   Bottomley SS. Sideroblastic anemias. 11 ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004.

17.   Andrews NC. Disorders of iron metabolism and heme synthesis: iron deficiency and related disorders. 11 ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004.

18. Vulpe CD, Kuo YM, Murphy TL, et al. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet 1999;21(2):195-9.

19. Matthes T, Rustin P, Trachsel H, et al. Different pathophysiological mechanisms of intramitochondrial iron accumulation in acquired and congenital sideroblastic anemia caused by mitochondrial DNA deletion. Eur J Haematol 2006;77(2):169-74.

20. Flatmark T, Romslo I. Energy-dependent accumulation of iron by isolated rat liver mitochondria. Requirement of reducing equivalents and evidence for a unidirectional flux of Fe(II) across the inner membrane. J Biol Chem 1975;250(16):6433-8.

21. Romslo I. Energy-dependent accumulation of iron by isolated rabbit reticulocyte mitochondria. Biochim Biophys Acta 1974;357(1):34-42.

22. Porra RJ, Jones OT. Studies on ferrochelatase. 1. Assay and properties of ferrochelatase from a pig-liver mitochondrial extract. Biochem J 1963;87:181-5.

23. Porra RJ, Jones OT. Studies on ferrochelatase. 2. An in vestigation of the role offerrochelatase in the biosynthesis of various haem prosthetic groups. Biochem J 1963;87:186-92.

24. Gattermann N, Retzlaff S, Wang YL, et al. Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia. Blood 1997;90(12):4961-72.

25. Reddy PL, Shetty VT, Dutt D, et al. Increased incidence of mitochondrial cytochrome c-oxidase gene mutations in patients with myelodysplastic syndromes. Br J Haematol 2002;116(3):564-75.

26. Goodman BP, Chong BW, Patel AC, Fletcher GP, Smith BE. Copper deficiency myeloneuropathy resembling B12 deficiency: partial resolution of MR imaging findings with copper supplementation. AJNR Am J Neuroradiol 2006;27(10):2112-4.

27. Kumar N, Elliott MA, Hoyer JD, Harper CM, Jr., Ahlskog JE, Phyliky RL. "Myelodysplasia," myeloneuropathy, and copper deficiency. Mayo Clin Proc 2005;80(7):943-6.

28. Hedera P, Fink JK, Bockenstedt PL, Brewer GJ. Myelopolyneuropathy and pancytopenia due to copper deficiency and high zinc levels of unknown origin: further support for existence of a new zinc overload syndrome. Arch Neurol 2003;60(9):1303-6.

29. Hayton BA, Broome HE, Lilenbaum RC. Copper deficiency-induced anemia and neutropenia secondary to intestinal malabsorption. Am J Hematol 1995;48(1):45-7.

30. Kumar N, McEvoy KM, Ahlskog JE. Myelopathy due to copper deficiency following gastrointestinal surgery. Arch Neurol 2003;60(12):1782-5.

31. Gregg XT, Reddy V, Prchal JT. Copper deficiency masquerading as myelodysplastic syndrome. Blood 2002;100(4):1493-5.

32. Konagaya M, Matsumoto A, Takase S, et al. Clinical analysis of longstanding subacute myelo-optico-neuropathy: sequelae of clioquinol at 32 years after its ban. J Neurol Sci 2004;218(1-2):85-90.

33.   Danks DM. Of mice and men, metals and mutations. J Med Genet 1986;23:99-106.

34.   Danks DM. Copper deficiency in humans. Ann Rev Nutr 1988;8:235-57.

35.   Danks DM. The mild form of Menkes disease: progress report on the original case. J Med Genet 1988;30:859-64.

36.   Danks DM. Disorders of copper transport. In: Scriver CR, Beaudet AL, Sly WM, Valle D, editors. The metabolic and molecular basis of inherited disease. 7th ed. New York: McGraw-Hill; 1995. p. 2211-35.

37.   Danks DM, Camakaris J, editors. Mutations affecting trace elements in humans and animals. New York and London: Plenum Press; 1983.

38.   Danks DM, Campbell PE, Stevens BJ, Mayne V, Cartwright E. Menkes's kinky hair syndrome:an inherited defect in copper absorption with wide-spread effects. Pediatrics 1972;50:188-201.

39.   Danks DM, Cartwright E, Stevens BJ, Townley RR. Menkes' kinky hair disease: further definition of the defect in copper transport. Science 1973;179(78):1140-2.

40.   Ma YX, Zhang DX, Kawabata T, et al. Copper and iron-induced oxidative damage in non-tumor bearing LEC rats. Path Int 1997;47:203-8.

41.   Menkes JH. Kinky hair disease. Pediatrics 1972;50(2):181-3.

42.   Menkes JH, Alter M, Stegleder GK, Weakley DR, Sung JH. A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. Pediatrics 1962;29:764-79.

43.   Mercer JF, Livingston J, Hall B, et al. Isolation of a partial candidate gene for Menkes disease by positional cloning. Nat Genet 1993;3(1):20-5.

44.   Chelly J, Tumer Z, Tonnerson T, et al. Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nature Genet 1993;3:14-9.

45.   Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nature Genet 1993;3:7-13.

46.   Kaler SG. Diagnosis and therapy of Menkes syndrome, a genetic form of copper deficiency. Am J Clin Nutr 1998;67:1029S-34S.

47.   Mann J, Camakaris J, Danks DM. Copper metabolism in mottled mouse mutants: defective placental transfer of Cu-64 to foetal brindled (Mo^{br}) mice. Biochem J 1980;186:629-31.

48.   Tan N, Urich H. Menkes' disease and swayback. A comparative study of two copper deficiency syndromes. J Neurol Sci 1983;62(1-3):95-113.

49.   Murakami H, Kodama H, Nemoto N. Abnormality of vascular elastic fibers in the macular mouse and a patient with Menkes' disease: ultrastructural and immunohistochemical study. Med Electron Microsc 2002;35(1):24-30.

50.   Uno H, Arya S. Neuronal and vascular disorders of the brain and spinal cord in Menkes kinky hair disease. Am J Med Genet Suppl 1987;3:367-77.

51.   Yoshikawa H, Fueki N, Sugai K, Araki A, Sakuragawa N, Iio M. Decreased blood flow and oxygen metabolism in the cerebellum, brain stem and thalamus in a case with Menkes kinky hair disease. Brain Dev 1989;11(6):426-9.

52.   Gunn TR, Macfarlane S, Phillips LI. Difficulties in the neonatal diagnosis of Menkes' kinky hair syndrome--trichopoliodystrophy. Clin Pediatr (Phila) 1984;23(9):514-6.

53.   Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH. A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. Pediatrics 1962;29:764-79.

54.   Danks DM, Campbell PE, Walker-Smith J, et al. Menkes' kinky-hair syndrome. Lancet 1972;1(7760):1100-2.

55.   Sullivan JL, Ochs HD. Copper deficiency and the immune system. Lancet 1978;2(8091):686.

56.   Prohaska JR, Lukasewycz OA. Effects of copper deficiency on the immune system. Adv Exp Med Biol 1990;262:123-43.

57.   Takagi S, Takahashi K, Katsura Y, Matsuoka T, Ohsaka A. Basic fibroblast growth factor modulates the surface expression of effector cell molecules and primes respiratory burst activity in human neutrophils. Acta Haematol 2000;103(2):78-83.

58.   Gerdes A-M, Tonnesen T, Pergament E, et al. Variability in clinical expression of Menkes syndrome. Eur J Pediatr 1988;148:132-5.

59.   Kaler SG, Gallo LK, Proud VK, et al. Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. Nature Genet 1994;8:195-202.

60.   Procopis P, Camakaris J, Danks DM. A mild form of Menkes' syndrome. J Pediatr 1981;98:97-100.

61.   Westman JA, Richardson DC, Rennert OM, Morrow G. Atypical Menkes steely hair disease. J Med Genet 1988;30:853-8.

62.   Byers PH, Siegel RC, Holbrook KA, Narayanan AS, Bornstein P, Hall JG. X-linked cutis laxa: defective cross-link formation in collagen due to decreased lysyl oxidase activity. New Eng J Med 1980;303:61-5.

63.   Kuivaniemi H, Peltonen L, Palotie A, Kaitila I, Kivirikko KI. Abnormal copper metabolism and deficient lysyl oxidase activity in a heritable connective tissue disorder. J Clin Invest 1982;69(3):730-3.

64.   Kaler SG, Buist NRM, Holmes CS, Goldstein DS, Miller RC, Gahl WA. Early copper therapy in classic Menkes disease patients with a novel splicing mutation. Ann Neurol 1995;38:921-8.

65.   Kaler SG, Das S, Levinson B, et al. Successful early copper therapy in Menkes disease associated with a mutant transcript containing a small in-frame deletion. Biochem Mol Med 1996;57:37-46.

66.   Kodama H, Murata Y, Kobayashi M. Clinical manifestations and treatment of Menkes disease and its variants. Pediatr Int 1999;41(4):423-9.

67.   Linder MC. Nutrition and metabolism of the trace elements. In: Linder MC, editor. Nutritional Biochemistry and Metabolism. 2nd edition ed. New York: Elsevier; 1991. p. 215-76.

68.   Linder MC. Biochemistry of Copper. New York: Plenum Press; 1991.

69.   Gitlin JD. Wilson disease. Gastroenterology 2003;125(6):1868-77.

70.   Lutsenko S, Barnes NL, Bartee MY, Dmitriev OY. Function and regulation of human copper-transporting ATPases. Physiol Rev 2007;87(3):1011-46.

71.	La Fontaine SL, Firth SD, Camakaris J, et al. Correction of the copper transport defect of Menkes patient fibroblasts by expression of the Menkes and Wilson ATPases. J Biol Chem 1998;273(47):31375-80.

72.	Langner C, Denk H. Wilson disease. Virchows Arch 2004;445(2):111-8.

73.	Schilsky ML, Irani AN, Gorla GR, Volenberg I, Gupta S. Biliary copper excretion capacity in intact animals: correlation between ATP7B function, hepatic mass, and biliary copper excretion. J Biochem Mol Toxicol 2000;14(4):210-4.

74.	Sugawara N, Sato M, Yuasa M, Sugawara C. Biliary excretion of copper, metallothionein, and glutathione into Long-Evans Cinnamon rats: a convincing animal model for Wilson disease. Biochem Mol Med 1995;55(1):38-42.

75.	Ferenci P. Review article: diagnosis and current therapy of Wilson's disease. Aliment Pharmacol Ther 2004;19(2):157-65.

76.	Harada M. Wilson disease. Med Electron Microsc 2002;35(2):61-6.

77.	Borjigin J, Payne AS, Deng J, et al. A novel pineal night-specific ATPase encoded by the Wilson disease gene. J Neuroscience 1999;19(3):1018-26.

78.	Krajacic P, Qian Y, Hahn P, Dentchev T, Lukinova N, Dunaief JL. Retinal localization and copper-dependent relocalization of the Wilson and Menkes disease proteins. Invest Ophthalmol Vis Sci 2006;47(7):3129-34.

79.	Walshe JM. The story of penicillamine: a difficult birth. Mov Disord 2003;18(8):853-9.

80.	Brewer GJ, Fink JK, Hedera P. Diagnosis and treatment of Wilson's disease. Semin Neurol 1999;19(3):261-70.

81.	Jablonska-Kaszewska I, Dabrowska E, Drobinska Jurowiecka A, Falkiewicz B. Treatment of Wilson's disease. Med Sci Monit 2003;9 Suppl 3:5-8.

82.	Pandit A, Bavdekar A, Bhave S. Wilson's disease. Indian J Pediatr 2002;69(9):785-91.

83.	Brewer GJ, Johnson V, Dick RD, Kluin KJ, Fink JK, Brunberg JA. Treatment of Wilson disease with ammonium tetrathiomolybdate. II. Initial therapy in 33 neurologically affected patients and follow-up with zinc therapy. Arch Neurol 1996;53(10):1017-25.

84.	Milanino R, Marrella M, Moretti U, et al. Oral zinc sulphate as primary therapeutic intervention in a child with Wilson disease. Eur J Pediatr 1989;148(7):654-5.

85.	Smolarek C, Stremmel W. [Therapy of Wilson disease]. Z Gastroenterol 1999;37(4):293-300.

86.	Walker WR. The results of a copper bracelet clinical trial and subsequent studies. Clifton, NJ: Humana Press; 1982.

87.	Turnlund JR, Keyes WR, Anderson HL, Acord LL. Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope 65Cu. Am J Clin Nutr 1989;49(5):870-8.

88.	Turnlund JR, Keyes WR, Peiffer GL, Scott KS. Copper absorption, excretion and retention by young men consuming low dietary copper determined by using the stable isotope 65Cu. Am J Clin Nutr 1998;67:1219-25.

89.	Gunshin H, Mackenzie B, erger UV, et al. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 1997;388:482-8.

90.   Arredondo M, Munoz P, Mura CV, Nunez MT. DMT1, a physiologically relevant apical Cu1+ transporter of intestinal cells. Am J Physiol Cell Physiol 2003;284(6):C1525-30.

91.   Ferruzza S, Sambuy Y, Ciriolo MR, et al. Copper uptake and intracellular distribution in the human intestinal Caco-2 cell line. Biometals 2000;13(2):179-85.

92.   Eisses JF, Chi Y, Kaplan JH. Stable plasma membrane levels of hCTR1 mediate cellular copper uptake. J Biol Chem 2005;280(10):9635-9.

93.   Lee J, Pena MM, Nose Y, Thiele DJ. Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 2002;277(6):4380-7.

94.   Puig S, Thiele DJ. Molecular mechanisms of copper uptake and distribution. Curr Opin Chem Biol 2002;6(2):171-80.

95.   Rees EM, Thiele DJ. Identification of a vacuole-associated metalloreductase and its role in Ctr2-mediated intracellular copper mobilization. J Biol Chem 2007;282(30):21629-38.

96.   Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol 2008;4(3):176-85.

97.   McKie AT, Barrow D, Latunde-Dada GO, et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 2001;291(5509):1755-9.

98.   Guo Y, Smith K, Lee J, Thiele DJ, Petris MJ. Identification of methionine-rich clusters that regulate copper-stimulated endocytosis of the human Ctr1 copper transporter. J Biol Chem 2004;279(17):17428-33.

99.   Petris MJ, Smith K, Lee J, Thiele DJ. Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1. J Biol Chem 2003;278(11):9639-46.

100.   Nose Y, Kim BE, Thiele DJ. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab 2006;4(3):235-44.

101.   Nyasae L, Bustos R, Braiterman L, Eipper B, Hubbard A. Dynamics of endogenous ATP7A (Menkes protein) in intestinal epithelial cells: copper-dependent redistribution between two intracellular sites. Am J Physiol Gastrointest Liver Physiol 2007;292(4):G1181-94.

102.   Llanos RM, Mercer JF. The molecular basis of copper homeostasis copper-related disorders. DNA Cell Biol 2002;21(4):259-70.

103.   Lutsenko S, Petris MJ. Function and Regulation of the Mammalian Copper-transporting ATPases: Insights from Biochemical and Cell Biological Approaches. J Membr Biol 2003;191(1):1-12.

104.   Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. Copper Transport. Am J Clin Nutr 1998;67:965S-71S.

105.   Linder MC, Lomeli NA, Donley S, et al. Copper transport in mammals. Adv Exp Biol Med 1999;448:1-16.

106.   Owen CAJ. Biochemical aspects of copper. Copper deficieny and toxicity. Park Ridge, N.J.: Noyes; 1982.

107.   Weiss KC, Linder MC. Copper transport in rats involving a new transport protein. Am J Physiol 1985;249:E77-E88.

108. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001;292(5516):468-72.

109. Harris ZL, Gitlin JD. Genetic and molecular basis for copper toxicity. Am J Clin Nutr 1996;63:836S-41S.

110. La Fontaine S, Mercer JF. Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. Arch Biochem Biophys 2007;463(2):149-67.

111. Fox PL, Mazumder B, Ehrenwald E, Mukhopadhyay CK. Ceruloplasmin and cardiovascular disease. Free Radic Biol Med 2000;28(12):1735-44.

112. Field LS, Luk E, Culotta VC. Copper chaperones: personal escorts for metal ions. J Bioenerg Biomembr 2002;34(5):373-9.

113. Petris MJ. The SLC31 (Ctr) copper transporter family. Pflugers Arch 2004;447(5):752-5.

114. Prohaska JR, Gybina AA. Intracellular copper transport in mammals. J Nutr 2004;134(5):1003-6.

115. Tavassoli M, Kishimoto T, Kataoka M. Liver endothelium mediates the hepatocyte's uptake of ceruloplasmin. J Cell Biol 1986;102(4):1298-303.

116. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306(5704):2090-3.

117. Patel BN, David S. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. J Biol Chem 1997;272(32):20185-90.

118. Floris G, Medda R, Padiglia A, Musci G. The physiopathological significance of ceruloplasmin. A possible therapeutic approach. Biochem Pharmacol 2000;60(12):1735-41.

119. Fortna RR, Watson HA, Nyquist SE. Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions. Biol Reprod 1999;61(4):1042-9.

120. Fox PL, Mukhopadhyay C, Ehrenwald E. Structure, oxidant activity, and cardiovascular mechanisms of human ceruloplasmin. Life Sci 1995;56(21):1749-58.

121. Hannan GN, McAuslan BR. Modulation of synthesis of specific proteins in endothelial cells by copper, cadmium, and disulfiram: an early response to an angiogenic inducer of cell migration. J Cell Physiol 1982;111(2):207-12.

122. Harris ED. A requirement for copper in angiogenesis. Nutr Rev 2004;62(2):60-4.

123. Mazumder B, Mukhopadhyay CK, Prok A, Cathcart MK, Fox PL. Induction of ceruloplasmin synthesis by IFN-gamma in human monocytic cells. J Immunol 1997;159(4):1938-44.

124. Engstrom G, Hedblad B, Stavenow L, Lind P, Janzon L, Lindgarde F. Inflammation-sensitive plasma proteins are associated with future weight gain. Diabetes 2003;52(8):2097-101.

125. Engstrom G, Janzon L, Berglund G, et al. Blood pressure increase and incidence of hypertension in relation to inflammation-sensitive plasma proteins. Arterioscler Thromb Vasc Biol 2002;22(12):2054-8.

126.   Engstrom G, Lind P, Hedblad B, Stavenow L, Janzon L, Lindgarde F. Effects of cholesterol and inflammation-sensitive plasma proteins on incidence of myocardial infarction and stroke in men. Circulation 2002;105(22):2632-7.

127.   Engstrom G, Lind P, Hedblad B, Stavenow L, Janzon L, Lindgarde F. Long-term effects of inflammation-sensitive plasma proteins and systolic blood pressure on incidence of stroke. Stroke 2002;33(12):2744-9.

128.   Engstrom G, Stavenow L, Hedblad B, et al. Inflammation-sensitive plasma proteins, diabetes, and mortality and incidence of myocardial infarction and stroke: a population-based study. Diabetes 2003;52(2):442-7.

129.   Lind P, Hedblad B, Stavenow L, Janzon L, Eriksson KF, Lindgarde F. Influence of plasma fibrinogen levels on the incidence of myocardial infarction and death is modified by other inflammation-sensitive proteins: a long-term cohort study. Arterioscler Thromb Vasc Biol 2001;21(3):452-8.

130.   Attieh ZK, Mukhopadhyay CK, Seshadri V, Tripoulas NA, Fox PL. Ceruloplasmin ferroxidase activity stimulates cellular iron uptake by a trivalent cation-specific transport mechanism. J Biol Chem 1999;274(2):1116-23.

131.   Kosman DJ. FET3P, ceruloplasmin, and the role of copper in iron metabolism. Adv Protein Chem 2002;60:221-69.

132.   De Domenico I, McVey Ward D, Kaplan J. Regulation of iron acquisition and storage: consequences for iron-linked disorders. Nat Rev Mol Cell Biol 2008;9(1):72-81.

133.   Bailey DM, Davies B, Young IS. Intermittent hypoxic training: implications for lipid peroxidation induced by acute normoxic exercise in active men. Clin Sci (Lond) 2001;101(5):465-75.

134.   Magalhaes J, Ascensao A, Soares JM, et al. Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. J Appl Physiol 2005;99(4):1247-53.

135.   Magalhaes J, Ascensao A, Soares JM, et al. Acute and severe hypobaric hypoxia-induced muscle oxidative stress in mice: the role of glutathione against oxidative damage. Eur J Appl Physiol 2004;91(2-3):185-91.

136.   Magalhaes J, Ascensao A, Viscor G, et al. Oxidative stress in humans during and after 4 hours of hypoxia at a simulated altitude of 5500 m. Aviat Space Environ Med 2004;75(1):16-22.

137.   Moller P, Loft S, Lundby C, Olsen NV. Acute hypoxia and hypoxic exercise induce DNA strand breaks and oxidative DNA damage in humans. Faseb J 2001;15(7):1181-6.

138.   Oishi Y, Taniguchi K, Matsumoto H, Ishihara A, Ohira Y, Roy RR. Muscle type-specific response of HSP60, HSP72, and HSC73 during recovery after elevation of muscle temperature. J Appl Physiol 2002;92(3):1097-103.

139.   Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 2006;91(5):807-19.

140.   Budinger GR, Duranteau J, Chandel NS, Schumacker PT. Hibernation during hypoxia in cardiomyocytes. Role of mitochondria as the O2 sensor. J Biol Chem 1998;273(6):3320-6.

141.   Chandel NS, Budinger GR, Choe SH, Schumacker PT. Cellular respiration during hypoxia. Role of cytochrome oxidase as the oxygen sensor in hepatocytes. J Biol Chem 1997;272(30):18808-16.

142.   Land SC, Buck LT, Hochachka PW. Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. Am J Physiol 1993;265(1 Pt 2):R41-8.

143.   Harris ZL. Aceruloplasminemia. J Neurol Sci 2003;207(1-2):108-9.

144.   Shukla N, Maher J, Masters J, Angelini GD, Jeremy JY. Does oxidative stress change ceruloplasmin from a protective to a vasculopathic factor? Atherosclerosis 2006;187(2):238-50.

145.   Kaler SG. Metabolic and molecular bases of Menkes disease and occipital horn syndrome. Pediatr Dev Pathol 1998;1(1):85-98.

146.   Sarkar J, Seshadri V, Tripoulas NA, Ketterer ME, Fox PL. Role of ceruloplasmin in macrophage iron efflux during hypoxia. J Biol Chem 2003;278(45):44018-24.

147.   Harris ED, Reddy MC, Qian Y, Tiffany-Castiglioni E, Majumdar S, Nelson J. Multiple forms of the Menkes Cu-ATPase. Adv Exp Med Biol 1999;448:39-51.

148.   McKie AT, Marciani P, Rolfs A, et al. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 2000;5(2):299-309.

149.   Meyer LA, Durley AP, Prohaska JR, Harris ZL. Copper transport and metabolism are normal in aceruloplasminemic mice. J Biol Chem 2001;276(39):36857-61.

150.   Turnland JR. Human whole-body copper metabolism. Am J Clin Nutr 1998;67:960S-4S.

151.   Zhou B, Gitschier J. hCTR1: A human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci USA 1997;94:7481-6.

152.   Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 1999;284(5415):805-8.

153.   Jimenez Del Rio M, Velez-Pardo C. Transition metal-induced apoptosis in lymphocytes via hydroxyl radical generation, mitochondria dysfunction, and caspase-3 activation: an in vitro model for neurodegeneration. Arch Med Res 2004;35(3):185-93.

154.   Kadiiska MB, Mason RP. In vivo copper-mediated free radical production: an ESR spin-trapping study. Spectrochim Acta A Mol Biomol Spectrosc 2002;58(6):1227-39.

155.   Wardman P, Candeias LP. Fenton chemistry: an introduction. Radiat Res 1996;145(5):523-31.

156.   Amaravadi R, Glerum DM, Tzagoloff A. Isolation of a cDNA encoding the human homolog of COX17, a yeast gene essential for mitochondrial copper recritment. Hum Genet 1997;99:329-33.

157.   Casareno RLB, Waggoner D, Gitlin JD. The copper chaperone CCS directly intereacts with copper/zinc superoxide dismutase. J Biol Chem 1998;273(September 11):23625-8.

158. Hamza I, Prohaska J, Gitlin JD. Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. Proc Natl Acad Sci U S A 2003;100(3):1215-20.

159. Larin D, Mekios C, Das K, Ross B, An-Suei Y, Gilliam TC. Characterization of the interaction between the Wilson and Menkes disease proteins and thke cytoplasmic copper chaperone, HAH1P. J Biol Chem 1999;274(40):28497-504.

160. Maryon EB, Molloy SA, Zimnicka AM, Kaplan JH. Copper entry into human cells: progress and unanswered questions. Biometals 2007;20(3-4):355-64.

161. Nose Y, Rees EM, Thiele DJ. Structure of the Ctr1 copper trans'PORE'ter reveals novel architecture. Trends Biochem Sci 2006;31(11):604-7.

162. Puig S, Lee J, Lau M, Thiele DJ. Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. J Biol Chem 2002;277(29):26021-30.

163. Pena MM, Puig S, Thiele DJ. Characterization of the Saccharomyces cerevisiae high affinity copper transporter Ctr3. J Biol Chem 2000;275(43):33244-51.

164. Aller SG, Unger VM. Projection structure of the human copper transporter CTR1 at 6-A resolution reveals a compact trimer with a novel channel-like architecture. Proc Natl Acad Sci U S A 2006;103(10):3627-32.

165. Dancis A, Yuan DS, Haile D, et al. Molecular characterization of a copper transport protein in S. cerevisiae: an unexpected role for copper in iron transport. Cell 1994;76:393-402.

166. Lee J, Prohaska JR, Dagenais SL, Glover TW, Thiele DJ. Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. Gene 2000;254(1-2):87-96.

167. Klomp AE, Juijn JA, Van Der Gun LT, Van Den Berg IE, Berger R, Klomp LW. The N-terminus of the Human Copper Transporter 1 (hCTR1) is localized extracellularly, and interacts with itself. Biochem J 2002;Pt.

168. Klomp AE, Tops BB, Van Denberg IE, Berger R, Klomp LW. Biochemical characterization and subcellular localization of human copper transporter 1 (hCTR1). Biochem J 2002;364(Pt 2):497-505.

169. Petris MJ, Voskoboinik I, Cater M, et al. Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate. J Biol Chem 2002;277(48):46736-42.

170. Holzer AK, Samimi G, Katano K, et al. The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. Mol Pharmacol 2004;66(4):817-23.

171. Rees EM, Lee J, Thiele DJ. Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter. J Biol Chem 2004;279(52):54221-9.

172. Lee J, Prohaska JR, Thiele DJ. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl Acad Sci U S A 2001;98(12):6842-7.

173. Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995;64:97-112.

174.   Huffman DL, O'Halloran TV. Function, structure, and mechanism of intracellular copper trafficking proteins. Annu Rev Biochem 2001;70:677-701.
175.   Johnson F, Giulivi C. Superoxide dismutases and their impact upon human health. Mol Aspects Med 2005;26(4-5):340-52.
176.   Steingrimsdottir H, Rowley G, Dorado G, Cole J, Lehmann AR. Mutations which alter splicing in the human hypoxanthine-guanine phosphoribosyltransferase gene. Nucl Acids Res 1992;20:1201-8.
177.   McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244(22):6049-55.
178.   Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC. A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. J Biol Chem 2001;276(41):38084-9.
179.   Casareno RL, Waggoner D, Gitlin JD. The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. J Biol Chem 1998;273(37):23625-8.
180.   Culotta VC, Klomp LWJ, Strain J, Casareno RLB, Krems B, Gitlin JD. The copper chaperone for superoxide dismutase. J Biol Chem 1997;272:23469-72.
181.   Wong PC, Waggoner D, Subramaniam JR, et al. Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc Natl Acad Sci U S A 2000;97(6):2886-91.
182.   Field LS, Furukawa Y, O'Halloran TV, Culotta VC. Factors controlling the uptake of yeast copper/zinc superoxide dismutase into mitochondria. J Biol Chem 2003;278(30):28052-9.
183.   Schmidt PJ, Kunst C, Culotta VC. Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1. J Biol Chem 2000;275(43):33771-6.
184.   Culotta VC, Yang M, O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. Biochim Biophys Acta 2006;1763(7):747-58.
185.   Caruano-Yzermans AL, Bartnikas TB, Gitlin JD. Mechanisms of the copper-dependent turnover of the copper chaperone for superoxide dismutase. J Biol Chem 2006;281(19):13581-7.
186.   Lai CC, Huang WH, Askari A, et al. Differential regulation of superoxide dismutase in copper-deficient rat organs. Free Radic Biol Med 1994;16(5):613-20.
187.   Liaw KY, Lee PH, Wu FC, Tsai JS, Lin-Shiau SY. Zinc, copper, and superoxide dismutase in hepatocellular carcinoma. Am J Gastroenterol 1997;92(12):2260-3.
188.   Elchuri S, Oberley TD, Qi W, et al. CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. Oncogene 2005;24(3):367-80.
189.   Oberley TD. Mitochondria, manganese superoxide dismutase, and cancer. Antioxid Redox Signal 2004;6(3):483-7.
190.   St Clair D, Zhao Y, Chaiswing L, Oberley T. Modulation of skin tumorigenesis by SOD. Biomed Pharmacother 2005;59(4):209-14.

191. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 1994;264(5166):1772-5.

192. Beckman JS, Carson M, Smith CD, Koppenol WH. ALS, SOD and peroxynitrite. Nature 1993;364(6438):584.

193. Furukawa Y, O'Halloran TV. Posttranslational modifications in Cu,Zn-superoxide dismutase and mutations associated with amyotrophic lateral sclerosis. Antioxid Redox Signal 2006;8(5-6):847-67.

194. Beckman JS, Esetvez AG, Barbeito L, Crow JP. CCS knockout mice establish an alternative source of copper for SOD in ALS. Free Radic Biol Med 2002;33(10):1433-5.

195. Crow JP, Sampson JB, Zhuang Y, Thompson JA, Beckman JS. Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. J Neurochem 1997;69(5):1936-44.

196. Furukawa Y, O'Halloran TV. Amyotrophic lateral sclerosis mutations have the greatest destabilizing effect on the apo- and reduced form of SOD1, leading to unfolding and oxidative aggregation. J Biol Chem 2005;280(17):17266-74.

197. Cobine PA, Pierrel F, Winge DR. Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. Biochim Biophys Acta 2006;1763(7):759-72.

198. Glerum DM, Shtanko A, Tzagoloff A. Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem 1996;271(24):14504-9.

199. Cobine PA, Pierrel F, Bestwick ML, Winge DR. Mitochondrial matrix copper complex used in metallation of cytochrome oxidase and superoxide dismutase. J Biol Chem 2006;281(48):36552-9.

200. Maxfield AB, Heaton DN, Winge DR. Cox17 is functional when tethered to the mitochondrial inner membrane. J Biol Chem 2004;279(7):5072-80.

201. Cobine PA, Ojeda LD, Rigby KM, Winge DR. Yeast contain a non-proteinaceous pool of copper in the mitochondrial matrix. J Biol Chem 2004;279(14):14447-55.

202. Dickinson EK, Adams DL, Schon EA, Glerum DM. A human SCO2 mutation helps define the role of Sco1p in the cytochrome oxidase assembly pathway. J Biol Chem 2000;275(35):26780-5.

203. Horvath R, Lochmuller H, Stucka R, et al. Characterization of human SCO1 and COX17 genes in mitochondrial cytochrome-c-oxidase deficiency. Biochem Biophys Res Commun 2000;276(2):530-3.

204. Horng YC, Cobine PA, Maxfield AB, Carr HS, Winge DR. Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome C oxidase. J Biol Chem 2004;279(34):35334-40.

205. Leary SC, Kaufman BA, Pellecchia G, et al. Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. Hum Mol Genet 2004;13(17):1839-48.

206.   Rigby K, Zhang L, Cobine PA, George GN, Winge DR. characterization of the cytochrome c oxidase assembly factor Cox19 of Saccharomyces cerevisiae. J Biol Chem 2007;282(14):10233-42.
207.   Beers J, Glerum DM, Tzagoloff A. Purification, characterization and localization of Yeast Cox17p, a mitochondrial copper shuttle. J Biol Chem 1997;272(52):33191-6.
208.   Takahashi Y, Kako K, Kashiwabara S, et al. Mammalian copper chaperone Cox17p has an essential role in activation of cytochrome C oxidase and embryonic development. Mol Cell Biol 2002;22(21):7614-21.
209.   Leary SC, Cobine PA, Kaufman BA, et al. The human cytochrome c oxidase assembly factors SCO1 and SCO2 have regulatory roles in the maintenance of cellular copper homeostasis. Cell Metab 2007;5(1):9-20.
210.   Hamza I, Faisst A, Prohaska J, Chen J, Gruss P, Gitlin JD. The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis. Proc Natl Acad Sci U S A 2001;98(12):6848-52.
211.   Lin S-J, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC. A role for the *saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. J Biolog Chem 1997;272(14):9215-20.
212.   Klomp LW, Lin S-J, Yuan DS, Klausner RD, Culotta VC, Gitlin JD. Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. J Biol Chem 1997;272(14):9221-6.
213.   Pufahl RA, Singer CP, Peariso KL, et al. Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science 1997;278(5339):853-6.
214.   Wernimont AK, Huffman DL, Lamb AL, O'Halloran TV, Rosenzweig AC. Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. Nat Struct Biol 2000;7(9):766-71.
215.   Walker JM, Tsivkovskii R, Lutsenko S. Metallochaperone Atox1 transfers copper to the N-terminal domain of the Wilson's disease protein and regulates its catalytic activity. J Biol Chem 2002.
216.   Achila D, Banci L, Bertini I, Bunce J, Ciofi-Baffoni S, Huffman DL. Structure of human Wilson protein domains 5 and 6 and their interplay with domain 4 and the copper chaperone HAH1 in copper uptake. Proc Natl Acad Sci U S A 2006;103(15):5729-34.
217.   Banci L, Bertini I, Cantini F, et al. Solution structure and intermolecular interactions of the third metal-binding domain of ATP7A, the Menkes disease protein. J Biol Chem 2006;281(39):29141-7.
218.   Banci L, Bertini I, Ciofi-Baffoni S, Chasapis CT, Hadjiliadis N, Rosato A. An NMR study of the interaction between the human copper(I) chaperone and the second and fifth metal-binding domains of the Menkes protein. Febs J 2005;272(3):865-71.
219.   Yatsunyk LA, Rosenzweig AC. Cu(I) binding and transfer by the N terminus of the Wilson disease protein. J Biol Chem 2007;282(12):8622-31.
220.   Paynter JA, Camakaris J, Mercer JFB. Analysis of hepatic copper, zinc, metallothionein and metallothionein-Ia in the developing sheep. Eur J Biochem 1990;190:149-54.

221.   Herd SM, Camakaris J, Christofferson R, Wookey P, Danks DM. Uptake and efflux of copper-64 in Menkes'-disease and normal continuous lymphoid cell lines. Biochem J 1987;247(2):341-7.

222.   Voskoboinik I, Brooks H, Smith S, Shen P, Camakaris J. ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells. FEBS Letters 1998;438:178-82.

223.   Voskoboinik I, Greenough M, La Fontaine S, Mercer JF, Camakaris J. Functional studies on the Wilson copper P-type ATPase and toxic milk mouse mutant. Biochem Biophys Res Commun 2001;281(4):966-70.

224.   Qian Y, Tiffany-Castiglioni E, Harris ED. Functional analysis of a genetic defect of copper transport (Menkes disease) in different cell lines. Am J Physiol 1996;271:C378-84.

225.   Ambrosini L, Mercer JFB. Defective copper-induced trafficking of the Menkes protein in patients with mild and copper-treated classical Menkes disease. Hum Mol Genet 1999;8:1547-55.

226.   Kim BE, Smith K, Petris MJ. A copper treatable Menkes disease mutation associated with defective trafficking of a functional Menkes copper ATPase. J Med Genet 2003;40(4):290-5.

227.   La Fontaine S, Firth SD, Lockhart PJ, Brooks H, Camakaris J, Mercer JF. Intracellular localization and loss of copper responsiveness of Mnk, the murine homologue of the Menkes protein, in cells from blotchy (Mo blo) and brindled (Mo br) mouse mutants. Hum Mol Genet 1999;8(6):1069-75.

228.   Strausak D, La Fontaine S, Hill J, Firth SD, Lockhart PJ, Mercer JF. The role of GMXCXXC metal binding sites in the copper-induced redistribution of the Menkes protein. J Biol Chem 1999;274(16):11170-7.

229.   Goka TJ, Stevenson RE, Hefferan PM, Howell RR. Menkes disease: a biochemical abnormality in cultured human fibroblasts. Proc Natl Acad Sci USA 1976;73:604-6.

230.   Horn N. Copper incorporation studies on cultured cells for prenatal diagnosis of Menkes disease. Lancet 1976;1:1156-8.

231.   Yamaguchi Y, Heiny ME, Suzuki M, Gitlin JD. Biochemical characterization and intracellular localization of the Menkes disease protein. Proc Natl Acad Sci US 1996;93:14030-5.

232.   Barnes N, Tsivkovskii R, Tsivkovskaia N, Lutsenko S. The copper-transporting ATPases, menkes and wilson disease proteins, have distinct roles in adult and developing cerebellum. J Biol Chem 2005;280(10):9640-5.

233.   El Meskini R, Culotta VC, Mains RE, Eipper BA. Supplying copper to the cuproenzyme peptidylglycine alpha-amidating monooxygenase. J Biol Chem 2003;278(14):12278-84.

234.   Mann JR, Camakaris J, Danks DM, Walliczek EG. Copper metabolism in mottled mouse mutants: copper therapy of brindled (Mobr) mice. Biochem J 1979;180(3):605-12.

235.   Petris MJ, Strausak D, Mercer JFB. The Menkes copper transporter is required for the activation of tyrosinase. Hum Mol Genet 2000;9:2845-51.

236.   Royce PM, Camakaris J, Danks DM. Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome. Biochem J 1980;192(2):579-86.

237. Superti-Furga A, Royce PM, Steinmann B. Congenital dopamine beta-hydroxylase deficiency. Lancet 1987;1(8534):693.

238. Petris MJ, Camakaris J, Greenough M, La Fontaine S, Mercer JFB. A C-terminal di-leucine is required for localization of the Menkes protein in the transGolgi network. Hum Mol Genet 1998;7:2063-71.

239. Petris MJ, Mercer JFB. The Menkes protein (ATP7A, MNK) cycles via the plasma membrane in basal and elevated extracellular copper using a C-terminal di-leucine endocytic signal. Hum Mol Genet 1999;8:2107-21175.

240. Paynter JA, Grimes A, Lockhart P, Mercer JFB. Expression of the Menkes gene homologue in mouse tissues: lack of effect of copper on the mRNA levels. FEBS lett 1994;351:186-90.

241. Greenough M, Pase L, Voskoboinik I, Petris MJ, O'Brien AW, Camakaris J. Signals regulating trafficking of Menkes (MNK; ATP7A) copper-translocating P-type ATPase in polarized MDCK cells. Am J Physiol Cell Physiol 2004;287(5):C1463-71.

242. La Fontaine S, Firth SD, Lockhart P, Brooks H, Camakaris J, Mercer JFB. Activity and intracellular localization of Mnk, the murine homologue of the Menkes protein in cells from blotchy (Moblo) and brindled (Mobr) mouse mutants. Hum Mol Genet 1999;8:1069-75.

243. Grimes A, Hearn C, Lockhart P, Newgreen D, Mercer JFB. Molecular basis of the brindled mouse mutant (*Mo^{br}*): a murine model of Menkes disease. Hum Mol Genet 1997;6:1032-42.

244. Levinson B, Vulpe C, Elder B, et al. The mottled gene is the mouse homologue of the Menkes disease gene. Nature Genet 1994;6:369-73.

245. Levinson B, Packman S, Gitschier J. Deletion of the promoter region in the Atp7a gene of the *mottled dappled* mouse. Nature Genet 1997;16:224-5.

246. Reed V, Boyd Y. Mutation analysis provides additional proof that mottled is the mouse homologue of Menkes' disease. Human Mol Genet 1997;6:417-23.

247. Das S, Levinson B, Vulpe C, Whitney S, Gitschier J, Packman S. Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse. Am J Hum Genet 1995;56:570-6.

248. Cecchi C, Biasotto M, Tosi M, Avner P. The *mottled* mouse as a model for human Menkes disease: identification of mutations in the *Atp7a* gene. Hum Molec Genet 1997;6:425-33.

249. Ohta Y, Shiraishi N, Nishikimi M. Occurrence of two missense mutations in Cu-ATPase of the macular mouse, a Menkes disease model. Biochem Mol Biol Int 1997;43(4):913-8.

250. Murata Y, Kodama H, Abe T, et al. Mutation analysis and expression of the mottled gene in the macular mouse model of Menkes disease. manuscript 1996.

251. Masson W, Hughes H, Papworth D, Boyd Y, Horn N. Abnormalities of copper accumulation in cell lines established from nine different alleles of mottled are the same as those found in Menkes disease. J Med Genet 1997;34:729-32.

252.   Steveson TC, Ciccotosto GD, Ma XM, Mueller GP, Mains RE, Eipper BA. Menkes protein contributes to the function of peptidylglycine alpha-amidating monooxygenase. Endocrinology 2003;144(1):188-200.

253.   Royce PM, Camakaris J, Mann JR, Danks DM. Copper metabolism in mottled mouse mutants. The effect of copper therapy on lysyl oxidase activity in brindled (Mobr) mice. Biochem J 1982;202(2):369-71.

254.   Yoshimura N, Kida K, Usutani S, Nishimura M. Histochemical localization of copper in various organs of brindled mice after copper therapy. Pathol Int 1995;45(1):10-8.

255.   Tanaka K, Kobayashi K, Fujita Y, Fukuhara C, Onosaka S, Min K. Effects of chelators on copper therapy of macular mouse, a model animal of Menkes' kinky disease. Res Commun Chem Pathol Pharmacol 1990;69(2):217-27.

256.   Prohaska JR. Repletion of copper-deficient mice and brindled mice with copper or iron. J Nutr 1984;114(2):422-30.

257.   Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197-223.

258.   Weiss SJ, Rustagi PK, LoBuglio AF. Human granulocyte generation of hydroxyl radical. J Exp Med 1978;147(2):316-23.

259.   Weiss SJ, King GW, LoBuglio AF. Superoxide generation by human monocytes and macrophages. Am J Hematol 1978;4(1):1-8.

260.   Weiss SJ, King GW, LoBuglio AF. Evidence for hydroxyl radical generation by human Monocytes. J Clin Invest 1977;60(2):370-3.

261.   Doherty CP. Host-pathogen interactions: the role of iron. J Nutr 2007;137(5):1341-4.

262.   Prentice AM, Ghattas H, Cox SE. Host-pathogen interactions: can micronutrients tip the balance? J Nutr 2007;137(5):1334-7.

263.   Auer DE, Ng JC, Thompson HL, Inglis S, Seawright AA. Acute phase response in horses: changes in plasma cation concentrations after localised tissue injury. Vet Rec 1989;124(10):235-9.

264.   Morimoto A, Sakata Y, Watanabe T, Murakami N. Characteristics of fever and acute-phase response induced in rabbits by IL-1 and TNF. Am J Physiol 1989;256(1 Pt 2):R35-41.

265.   Akcil E, Yavuz G, Kocak M. Effects of inflammation and antiinflammatory treatment on serum trace elements concentrations. Biol Trace Elem Res 2003;93(1-3):95-104.

266.   Milanino R, Marrella M, Gasperini R, Pasqualicchio M, Velo G. Copper and zinc body levels in inflammation: an overview of the data obtained from animal and human studies. Agents Actions 1993;39(3-4):195-209.

267.   Ward WF, Molteni A, Ts'ao C, Ischiropoulos H. Serum copper concentration as an index of experimental lung injury. Adv Exp Med Biol 1989;258:287-302.

268.   Shah I, Lewkow LM, Khilanani U. Correlation of hypercupremia with other acute phase reactants in malignant lymphoma. Cancer 1983;51(5):851-4.

269.   Yilmaz A, Sari RA, Gundogdu M, Kose N, Dag E. Trace elements and some extracellular antioxidant proteins levels in serum of patients with systemic lupus erythematosus. Clin Rheumatol 2005;24(4):331-5.

270. Koyanagi A, Kuffo D, Gresely L, Shenkin A, Cuevas LE. Relationships between serum concentrations of C-reactive protein and micronutrients, in patients with tuberculosis. Ann Trop Med Parasitol 2004;98(4):391-9.
271. Srinivas U, Braconier JH, Jeppsson B, Abdulla M, Akesson B, Ockerman PA. Trace element alterations in infectious diseases. Scand J Clin Lab Invest 1988;48(6):495-500.
272. Hallgren R, Feltelius N, Lindh U. Redistribution of minerals and trace elements in chronic inflammation--a study on isolated blood cells from patients with ankylosing spondylitis. J Rheumatol 1987;14(3):548-53.
273. Conforti A, Franco L, Milanino R, Totorizzo A, Velo GP. Copper metabolism during acute inflammation: studies on liver and serum copper concentrations in normal and inflamed rats. Br J Pharmacol 1983;79(1):45-52.
274. Seyrek A, Kocyigit A, Erel O. Essential trace elements selenium, zinc, copper, and iron concentrations and their related acute-phase proteins in patients with vivax malaria. Biol Trace Elem Res 2005;106(2):107-15.
275. Beveridge SJ, Garrett IR, Whitehouse MW, Vernon-Roberts B, Brooks PM. Biodistribution of 64Cu in inflamed rats following administration of two anti-inflammatory copper complexes. Agents Actions 1985;17(1):104-11.
276. Voruganti VS, Klein GL, Lu HX, Thomas S, Freeland-Graves JH, Herndon DN. Impaired zinc and copper status in children with burn injuries: need to reassess nutritional requirements. Burns 2005;31(6):711-6.
277. Jones PW, Taylor DM, Williams DR, et al. Using wound fluid analyses to identify trace element requirements for efficient healing. J Wound Care 2001;10(6):205-8.
278. Liuzzi JP, Lichten LA, Rivera S, et al. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci U S A 2005;102(19):6843-8.
279. Knutson MD, Oukka M, Koss LM, Aydemir F, Wessling-Resnick M. Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin. Proc Natl Acad Sci U S A 2005;102(5):1324-8.
280. Andrews NC. Anemia of inflammation: the cytokine-hepcidin link. J Clin Invest 2004;113(9):1251-3.
281. Babu U, Failla ML. Respiratory burst and candidacidal activity of peritoneal macrophages are impaired in copper-deficient rats. J Nutr 1990;120(12):1692-9.
282. Babu U, Failla ML. Copper status and function of neutrophils are reversibly depressed in marginally and severely copper-deficient rats. J Nutr 1990;120(12):1700-9.
283. Torre PM, Harmon, R. J., Sordillo, L. M., Boissonneault, G. A., Hemken, R. W., Trammell, D. S., & Clark, T. W. **Modulation of bovine mononuclear cell proliferation and cytokine production by dietary copper insufficiency.** *J Nutr Immunol* 1995;3:3-20.

284. Becton DL, Schultz WH, Kinney TR. Severe neutropenia caused by copper deficiency in a child receiving continuous ambulatory peritoneal dialysis. J Pediatr 1986;108(5 Pt 1):735-7.

285. Wagner D, Maser J, Lai B, et al. Elemental analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol 2005;174(3):1491-500.

286. Schwan WR, Warrener P, Keunz E, Stover CK, Folger KR. Mutations in the cueA gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of Pseudomonas aeruginosa in mice. Int J Med Microbiol 2005;295(4):237-42.

287. Francis MS, Thomas CJ. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of Listeria monocytogenes. Microb Pathog 1997;22(2):67-78.

288. Rankin S, Li Z, Isberg RR. Macrophage-induced genes of Legionella pneumophila: protection from reactive intermediates and solute imbalance during intracellular growth. Infect Immun 2002;70(7):3637-48.

289. Cabiscol E, Tamarit J, Ros J. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol 2000;3(1):3-8.

290. Greijer AE, van der Groep P, Kemming D, et al. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol 2005;206(3):291-304.

291. Gunton JE, Kulkarni RN, Yim S, et al. Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. Cell 2005;122(3):337-49.

292. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 2003;23(24):9361-74.

293. Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. Oncogene 2000;19(54):6297-305.

294. Wykoff CC, Sotiriou C, Cockman ME, et al. Gene array of VHL mutation and hypoxia shows novel hypoxia-induced genes and that cyclin D1 is a VHL target gene. Br J Cancer 2004;90(6):1235-43.

295. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93(4):266-76.

296. Matsuzaki S, Manabe T, Katayama T, et al. Metals accelerate production of the aberrant splicing isoform of the presenilin-2. J Neurochem 2004;88(6):1345-51.

297. Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. Curr Opin Genet Dev 2001;11(3):293-9.

298. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000;88(4):1474-80.

299. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;270(3):1230-7.

300. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 1998;95(14):7987-92.

301. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem 1999;274(10):6519-25.

302. Ivan M, Kondo K, Yang H, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001;292(5516):464-8.

303. Epstein AC, Gleadle JM, McNeill LA, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107(1):43-54.

304. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294(5545):1337-40.

305. Akakura N, Kobayashi M, Horiuchi I, et al. Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. Cancer Res 2001;61(17):6548-54.

306. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 2000;60(17):4693-6.

307. Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat Med 2000;6(12):1335-40.

308. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59(22):5830-5.

309. Stroka DM, Burkhardt T, Desbaillets I, et al. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. Faseb J 2001;15(13):2445-53.

310. Richard DE, Berra E, Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem 2000;275(35):26765-71.

311. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. Embo J 1998;17(17):5085-94.

312. Zhong H, Chiles K, Feldser D, et al. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60(6):1541-5.

313. Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science 1988;242(4884):1412-5.

314.   Martin F, Linden T, Katschinski DM, et al. Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood 2005;105(12):4613-9.

315.   Gao N, Ding M, Zheng JZ, et al. Vanadate-induced expression of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor through phosphatidylinositol 3-kinase/Akt pathway and reactive oxygen species. J Biol Chem 2002;277(35):31963-71.

316.   Gao N, Jiang BH, Leonard SS, et al. p38 Signaling-mediated hypoxia-inducible factor 1alpha and vascular endothelial growth factor induction by Cr(VI) in DU145 human prostate carcinoma cells. J Biol Chem 2002;277(47):45041-8.

317.   Gao N, Shen L, Zhang Z, et al. Arsenite induces HIF-1alpha and VEGF through PI3K, Akt and reactive oxygen species in DU145 human prostate carcinoma cells. Mol Cell Biochem 2004;255(1-2):33-45.

318.   Soucy NV, Klei LR, Mayka DD, Barchowsky A. Signaling pathways for arsenic-stimulated vascular endothelial growth factor-a expression in primary vascular smooth muscle cells. Chem Res Toxicol 2004;17(4):555-63.

319.   Skinner HD, Zhong XS, Gao N, Shi X, Jiang BH. Arsenite induces p70S6K1 activation and HIF-1alpha expression in prostate cancer cells. Mol Cell Biochem 2004;255(1-2):19-23.

320.   Bell EL, Emerling BM, Chandel NS. Mitochondrial regulation of oxygen sensing. Mitochondrion 2005;5(5):322-32.

321.   Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A 1998;95(20):11715-20.

322.   Duranteau J, Chandel NS, Kulisz A, Shao Z, Schumacker PT. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem 1998;273(19):11619-24.

323.   Grzenkowicz-Wydra J, Cisowski J, Nakonieczna J, et al. Gene transfer of CuZn superoxide dismutase enhances the synthesis of vascular endothelial growth factor. Mol Cell Biochem 2004;264(1-2):169-81.

324.   Brunelle JK, Bell EL, Quesada NM, et al. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab 2005;1(6):409-14.

325.   Guzy RD, Hoyos B, Robin E, et al. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 2005;1(6):401-8.

326.   Emerling BM, Platanias LC, Black E, Nebreda AR, Davis RJ, Chandel NS. Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. Mol Cell Biol 2005;25(12):4853-62.

327.   Hirota K, Semenza GL. Rac1 activity is required for the activation of hypoxia-inducible factor 1. J Biol Chem 2001;276(24):21166-72.

328.   Sato H, Sato M, Kanai H, et al. Mitochondrial reactive oxygen species and c-Src play a critical role in hypoxic response in vascular smooth muscle cells. Cardiovasc Res 2005;67(4):714-22.

329.   Cross CE, Halliwell B, Borish ET, et al. Oxygen radicals and human disease. Ann Intern Med 1987;107(4):526-45.

330. Kuehl FA, Jr., Humes JL, Ham EA, Egan RW, Dougherty HW. Inflammation: the role of peroxidase-derived products. Adv Prostaglandin Thromboxane Res 1980;6:77-86.

331. Lemasters JJ, Caldwell-Kenkel, J. C., Gao, W., Nieminen, A.-L., Herman, B., and Thurman, R. G. Hypoxic, ischemic and reperfusion injury in the liver. In: Das DK, editor. Pathophysiology of Reperfusion Injury. Boca Raton, FL: CRC; 1992. p. 101 - 35.

332. Marotto ME, Thurman RG, Lemasters JJ. Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: protection by allopurinol. Hepatology 1988;8(3):585-90.

333. Acker H. Cellular oxygen sensors. Ann N Y Acad Sci 1994;718:3-10; discussion 1-2.

334. Fandrey J, Seydel FP, Siegers CP, Jelkmann W. Role of cytochrome P450 in the control of the production of erythropoietin. Life Sci 1990;47(2):127-34.

335. Gleadle JM, Ebert BL, Ratcliffe PJ. Diphenylene iodonium inhibits the induction of erythropoietin and other mammalian genes by hypoxia. Implications for the mechanism of oxygen sensing. Eur J Biochem 1995;234(1):92-9.

336. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 1996;76(3):839-85.

337. Holland PC, Clark MG, Bloxham DP, Lardy HA. Mechanism of action of the hypoglycemic agent diphenyleneiodonium. J Biol Chem 1973;248(17):6050-6.

338. Wenger RH, Marti HH, Schuerer-Maly CC, et al. Hypoxic induction of gene expression in chronic granulomatous disease-derived B-cell lines: oxygen sensing is independent of the cytochrome b558-containing nicotinamide adenine dinucleotide phosphate oxidase. Blood 1996;87(2):756-61.

339. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 1973;134(3):707-16.

340. Chandel NS, Schumacker PT. Cells depleted of mitochondrial DNA (rho0) yield insight into physiological mechanisms. FEBS Lett 1999;454(3):173-6.

341. Dawson TL, Gores GJ, Nieminen AL, Herman B, Lemasters JJ. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. Am J Physiol 1993;264(4 Pt 1):C961-7.

342. Rana M, de Coo I, Diaz F, Smeets H, Moraes CT. An out-of-frame cytochrome b gene deletion from a patient with parkinsonism is associated with impaired complex III assembly and an increase in free radical production. Ann Neurol 2000;48(5):774-81.

343. Sun J, Trumpower BL. Superoxide anion generation by the cytochrome bc1 complex. Arch Biochem Biophys 2003;419(2):198-206.

344. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 1985;237(2):408-14.

345. Iwata S, Lee JW, Okada K, et al. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science 1998;281(5373):64-71.

346.   Saraste M. Oxidative phosphorylation at the fin de siecle. Science 1999;283(5407):1488-93.

347.   Cox C, Merajver SD, Yoo S, et al. Inhibition of the growth of squamous cell carcinoma by tetrathiomolybdate-induced copper suppression in a murine model. Arch Otolaryngol Head Neck Surg 2003;129(7):781-5.

348.   Pan Q, Bao LW, Kleer CG, Brewer GJ, Merajver SD. Antiangiogenic tetrathiomolybdate enhances the efficacy of doxorubicin against breast carcinoma. Mol Cancer Ther 2003;2(7):617-22.

349.   Pan Q, Bao LW, Merajver SD. Tetrathiomolybdate inhibits angiogenesis and metastasis through suppression of the NFkappaB signaling cascade. Mol Cancer Res 2003;1(10):701-6.

350.   Pan Q, Kleer CG, van Golen KL, et al. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res 2002;62(17):4854-9.

351.   Redman BG, Esper P, Pan Q, et al. Phase II trial of tetrathiomolybdate in patients with advanced kidney cancer. Clin Cancer Res 2003;9(5):1666-72.

352.   Teknos TN, Islam M, Arenberg DA, et al. The effect of tetrathiomolybdate on cytokine expression, angiogenesis, and tumor growth in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 2005;131(3):204-11.

353.   Bush AI. The metallobiology of Alzheimer's disease. Trends Neurosci 2003;26(4):207-14.

354.   Dikalov SI, Vitek MP, Mason RP. Cupric-amyloid beta peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. Free Radic Biol Med 2004;36(3):340-7.

355.   Lee JY, Mook-Jung I, Koh JY. Histochemically reactive zinc in plaques of the Swedish mutant beta-amyloid precursor protein transgenic mice. J Neurosci 1999;19(11):RC10.

356.   Miller LM, Wang Q, Telivala TP, Smith RJ, Lanzirotti A, Miklossy J. Synchrotron-based infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with beta-amyloid deposits in Alzheimer's disease. J Struct Biol 2006;155(1):30-7.

357.   Suh SW, Jensen KB, Jensen MS, et al. Histochemically-reactive zinc in amyloid plaques, angiopathy, and degenerating neurons of Alzheimer's diseased brains. Brain Res 2000;852(2):274-8.

358.   Jones DG, Suttle NF. The effect of copper deficiency on the resistance of mice to infection with Pasteurella haemolytica. J Comp Pathol 1983;93(1):143-9.

359.   Crocker A, Lee C, Aboko-Cole G, Durham C. Interaction of nutrition and infection: effect of copper deficiency on resistance to Trypanosoma lewisi. J Natl Med Assoc 1992;84(8):697-706.

360.   Freeland JH, Cousins RJ, Schwartz R. Relationship of mineral status and intake to periodontal disease. Am J Clin Nutr 1976;29(7):745-9.

361.   Lamand M, Levieux D. Effects of infection on plasma levels of copper and zinc in ewes. Ann Rech Vet 1981;12(2):133-6.

362.	Cesur S, Kocaturk PA, Kavas GO, Aksaray S, Tezeren D, Ciftci U. Serum copper and zinc concentrations in patients with brucellosis. J Infect 2005;50(1):31-3.

363.	Deghaidy FS, Mahklady FA, Makhlouf LM, el-Ayat AA. Serum zinc and copper levels in bilharzial patients. J Egypt Soc Parasitol 1989;19(1):205-9.

364.	el Hawy AM, Abdel Rahman MM, Rozik S, Fawzy AF, el Gammal HA. Assessment of serum copper and zinc levels in chronic Schistosoma mansoni patients with and without hepatic involvement before and after praziquantel therapy. J Egypt Soc Parasitol 1993;23(1):55-62.

365.	Yamaguchi Y, Heiny ME, Suzuki M, Gitlin JD. Biochemical characterization and intracellular localization of the Menkes disease protein. Proc Natl Acad Sci U S A 1996;93(24):14030-5.

366.	Petris MJ, Mercer JF, Camakaris J. The cell biology of the Menkes disease protein. Adv Exp Med Biol 1999;448:53-66.

367.	Grass G, Rensing C. Genes involved in copper homeostasis in Escherichia coli. J Bacteriol 2001;183(6):2145-7.

368.	Stoyanov JV, Magnani D, Solioz M. Measurement of cytoplasmic copper, silver, and gold with a lux biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of Escherichia coli. FEBS Lett 2003;546(2-3):391-4.

369.	Elzanowska H, Wolcott RG, Hannum DM, Hurst JK. Bactericidal properties of hydrogen peroxide and copper or iron-containing complex ions in relation to leukocyte function. Free Radic Biol Med 1995;18(3):437-49.

370.	Helman RG, Adams LG, Pierce KR, Bridges CH, Bailey EM. The role of lysosomes in the pathogenesis of copper-induced hepatotoxicity: morphological studies. J Comp Pathol 1985;95(1):25-35.

371.	Desjardins M, Huber LA, Parton RG, Griffiths G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol 1994;124(5):677-88.

372.	Sunderman FW, Jr., Nomoto S. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. Clin Chem 1970;16(11):903-10.

373.	Xie QW, Cho HJ, Calaycay J, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256(5054):225-8.

374.	Dollwet HHAaS, J.R.J. Historic uses of copper compounds in medicine. Trace Elem Medic 1985;2(2):80-7.

375.	Liu X, Zweier JL. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. Free Radic Biol Med 2001;31(7):894-901.

376.	Schewe T. Molecular actions of Ebselen--an antiinflammatory antioxidant. Gen Pharmacol 1995;26(6):1153-69.

377.	Schewe C, Schewe T, Wendel A. Strong inhibition of mammalian lipoxygenases by the antiinflammatory seleno-organic compound Ebselen in the absence of glutathione. Biochem Pharmacol 1994;48(1):65-74.

378.   Nikawa T, Schuch G, Wagner G, Sies H. Interaction of Ebselen with glutathione S-transferase and papain in vitro. Biochem Pharmacol 1994;47(6):1007-12.

379.   Noguchi N, Gotoh N, Niki E. Effects of Ebselen and probucol on oxidative modifications of lipid and protein of low density lipoprotein induced by free radicals. Biochim Biophys Acta 1994;1213(2):176-82.

380.   Rensing C, Fan B, Sharma R, Mitra B, Rosen BP. CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A 2000;97(2):652-6.

381.   Chen YT, Chang HY, Lai YC, Pan CC, Tsai SF, Peng HL. Sequencing and analysis of the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43. Gene 2004;337:189-98.

382.   Jiang Y, Yang F, Zhang X, et al. The complete sequence and analysis of the large virulence plasmid pSS of Shigella sonnei. Plasmid 2005;54(2):149-59.

383.   Madsen E, Gitlin JD. Copper deficiency. Curr Opin Gastroenterol 2007;23(2):187-92.

384.   Qin Z, Itoh S, Jeney V, Ushio-Fukai M, Fukai T. Essential role for the Menkes ATPase in activation of extracellular superoxide dismutase: implication for vascular oxidative stress. Faseb J 2006;20(2):334-6.

385.   Brewer GJ, Askari F, Lorincz MT, et al. Treatment of Wilson disease with ammonium tetrathiomolybdate: IV. Comparison of tetrathiomolybdate and trientine in a double-blind study of treatment of the neurologic presentation of Wilson disease. Arch Neurol 2006;63(4):521-7.

386.   Flohe L, Otting F. Superoxide dismutase assays. Methods Enzymol 1984;105:93-104.

387.   Zhou B, Gitschier J. hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci U S A 1997;94(14):7481-6.

388.   Cobbold C, Ponnambalam S, Francis MJ, Monaco AP. Novel membrane traffic steps regulate the exocytosis of the Menkes disease ATPase. Hum Mol Genet 2002;11(23):2855-66.

389.   Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev 2006;25(3):315-22.

390.   Biswas SK, Gangi L, Paul S, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). Blood 2006;107(5):2112-22.

391.   Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006;66(2):605-12.

392.   Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood 2004;104(8):2224-34.

393.   Burke B, Tang N, Corke KP, et al. Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. J Pathol 2002;196(2):204-12.

394.	Talks KL, Turley H, Gatter KC, et al. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 2000;157(2):411-21.
395.	Mukhopadhyay CK, Mazumder B, Fox PL. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. J Biol Chem 2000;275(28):21048-54.
396.	Nittis T, Gitlin JD. The copper-iron connection: hereditary aceruloplasminemia. Semin Hematol 2002;39(4):282-9.
397.	Murphy BJ, Robin ED, Tapper DP, Wong RJ, Clayton DA. Hypoxic coordinate regulation of mitochondrial enzymes in mammalian cells. Science 1984;223(4637):707-9.
398.	Simon LM, Robin ED, Phillips JR, Acevedo J, Axline SG, Theodore J. Enzymatic basis for bioenergetic differences of alveolar versus peritoneal macrophages and enzyme regulation by molecular O2. J Clin Invest 1977;59(3):443-8.
399.	Merchant S, Bogorad L. Rapid degradation of apoplastocyanin in Cu(II)-deficient cells of Chlamydomonas reinhardtii. J Biol Chem 1986;261(34):15850-3.
400.	van de Sluis BJA, Breen M, M. N, et al. Genetic mapping of the copper toxicosis locus in Bedlington terriers to dog chromosome 10, in a region syntenic to human chromosome region 2p13-p16. Hum Mol Genet 1999;8(3):501-7.
401.	Borkow G, Gabbay J. Copper as a biocidal tool. Curr Med Chem 2005;12(18):2163-75.
402.	Chohan ZH, Pervez H, Rauf A, Scozzafava A, Supuran CT. Antibacterial Co(II), Cu(II), Ni(II) and Zn(II) complexes of thiadiazole derived furanyl, thiophenyl and pyrrolyl Schiff bases. J Enzyme Inhib Med Chem 2002;17(2):117-22.
403.	Zhao ZH, Sakagami Y, Osaka T. Toxicity of hydrogen peroxide produced by electroplated coatings to pathogenic bacteria. Can J Microbiol 1998;44(5):441-7.
404.	Borkow G, Gabbay J. Putting copper into action: copper-impregnated products with potent biocidal activities. Faseb J 2004;18(14):1728-30.
405.	Spencer RC. Epidemiology of infection in ICUs. Intensive Care Med 1994;20 Suppl 4:S2-6.
406.	**Gunn TR** MS, and **Phillips LI. Difficulties in the neonatal diagnosis of Menkes' kinky hair syndrome--trichopoliodystrophy.** Clin Pediatr (Phila) 1984;23(9):514-6.
407.	Agertt F, Crippa AC, Lorenzoni PJ, et al. Menkes' disease: case report. Arq Neuropsiquiatr 2007;65(1):157-60.
408.	Kreuder J, Otten A, Fuder H, et al. Clinical and biochemical consequences of copper-histidine therapy in Menkes disease. Eur J Pediatr 1993;152(10):828-32.
409.	Hu G, Cheng PY, Sham A, Perfect JR, Kronstad JW. Metabolic adaptation in Cryptococcus neoformans during early murine pulmonary infection. Mol Microbiol 2008.

410.   Waterman SR, Hacham M, Hu G, et al. Role of a CUF1/CTR4 copper regulatory axis in the virulence of Cryptococcus neoformans. J Clin Invest 2007;117(3):794-802.

411.   Frantz S, Vincent KA, Feron O, Kelly RA. Innate immunity and angiogenesis. Circ Res 2005;96(1):15-26.

412.   Zagorska A, Dulak J. HIF-1: the knowns and unknowns of hypoxia sensing. Acta Biochim Pol 2004;51(3):563-85.

413.   Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006;440(7088):1222-6.

414.   Csiszar K. Lysyl oxidases: a novel multifunctional amine oxidase family. Prog Nucleic Acid Res Mol Biol 2001;70:1-32.

415.   Hornstra IK, Birge S, Starcher B, Bailey AJ, Mecham RP, Shapiro SD. Lysyl oxidase is required for vascular and diaphragmatic development in mice. J Biol Chem 2003;278(16):14387-93.

416.   Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol 2005;167(4):927-36.

417.   Kuivaniemi H, Peltonen L, Kivirikko KI. Type IX Ehlers-Danlos syndrome and Menkes syndrome: the decrease in lysyl oxidase activity is associated with a corresponding deficiency in the enzyme protein. Am J Hum Genet 1985;37(4):798-808.

418.   Hu GF. Copper stimulates proliferation of human endothelial cells under culture. J Cell Biochem 1998;69(3):326-35.

419.   Alessandri G, Raju K, Gullino PM. Mobilization of capillary endothelium in vitro induced by effectors of angiogenesis in vivo. Cancer Res 1983;43(4):1790-7.

420.   Camphausen K, Sproull M, Tantama S, et al. Evaluation of copper chelation agents as anti-angiogenic therapy. Bioorg Med Chem 2003;11(19):4287-93.

421.   Camphausen K, Sproull M, Tantama S, et al. Evaluation of chelating agents as anti-angiogenic therapy through copper chelation. Bioorg Med Chem 2004;12(19):5133-40.

422.   Brem SS, Zagzag D, Tsanaclis AM, Gately S, Elkouby MP, Brien SE. Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. Am J Pathol 1990;137(5):1121-42.

423.   Sen CK, Khanna S, Venojarvi M, et al. Copper-induced vascular endothelial growth factor expression and wound healing. Am J Physiol Heart Circ Physiol 2002;282(5):H1821-7.

424.   Bar-Or D, Thomas GW, Yukl RL, et al. Copper stimulates the synthesis and release of interleukin-8 in human endothelial cells: a possible early role in systemic inflammatory responses. Shock 2003;20(2):154-8.

425.   Alessandri G, Raju K, Gullino PM. Angiogenesis in vivo and selective mobilization of capillary endothelium in vitro by heparin-copper complex. Microcirc Endothelium Lymphatics 1984;1(3):329-46.

426.   Cox C, Teknos TN, Barrios M, Brewer GJ, Dick RD, Merajver SD. The role of copper suppression as an antiangiogenic strategy in head and neck squamous cell carcinoma. Laryngoscope 2001;111(4 Pt 1):696-701.

427.   Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 2002;196(3):254-65.

428.   Lewis C, Murdoch C. Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 2005;167(3):627-35.

429.   Knighton DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ. Oxygen tension regulates the expression of angiogenesis factor by macrophages. Science 1983;221(4617):1283-5.

430.   Mandinova A, Soldi R, Graziani I, et al. S100A13 mediates the copper-dependent stress-induced release of IL-1alpha from both human U937 and murine NIH 3T3 cells. J Cell Sci 2003;116(Pt 13):2687-96.

431.   Prudovsky I, Mandinova A, Soldi R, et al. The non-classical export routes: FGF1 and IL-1alpha point the way. J Cell Sci 2003;116(Pt 24):4871-81.

432.   Davis GE, Senger DR. Extracellular matrix mediates a molecular balance between vascular morphogenesis and regression. Curr Opin Hematol 2008;15(3):197-203.

433.   Koh W, Stratman AN, Sacharidou A, Davis GE. In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. Methods Enzymol 2008;443:83-101.

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