Copper transport into the secretory pathway is regulated by oxygen in macrophages

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Summary
Copper is an essential nutrient for a variety of biochemical processes; however, the redox properties of copper also make it potentially toxic in the free form. Consequently, the uptake and intracellular distribution of this metal is strictly regulated. This raises the issue of whether specific pathophysiological conditions can promote adaptive changes in intracellular copper distribution. In this study, we demonstrate that oxygen limitation promotes a series of striking alterations in copper homeostasis in RAW264.7 macrophage cells. Hypoxia was found to stimulate copper uptake and to increase the expression of the copper importer, CTR1. This resulted in increased copper delivery to the ATP7A copper transporter and copper-dependent trafficking of ATP7A to cytoplasmic vesicles.

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 (La Fontaine and Mercer, 2007; Madsen and Gitlin, 2007; Prohaska and Gybina, 2004). 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 proteins 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 (SLC31A1), a ubiquitously expressed homotrimeric transporter (Zhou and Gitschier, 1997). Once in the cytoplasm, small cytoplasmic proteins known as copper chaperones deliver copper to distinct target enzymes by direct protein-protein interactions. The copper chaperones, CCS and COX17 are involved in copper delivery to Cu/Zn superoxide dismutase (SOD1) in the cytoplasm, and to cytochrome c oxidase (CCO) in the mitochondria (Amaravadi et al., 1997; Casareno et al., 1998). SCO1 and SCO2 are copper chaperones that are also involved in copper delivery to CCO via a poorly understood process (Leary et al., 2004). 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 (Larin et al., 1999; Petris et al., 1996; Yamaguchi et al., 1996). 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 (Barnes et al., 2005; El Meskini et al., 2003; Petris et al., 2000; Qin et al., 2006). An additional function of ATP7A is the export of excess copper from cells. This export activity is associated with copper-stimulated trafficking of ATP7A to post-Golgi compartments, including cytoplasmic vesicles and the plasma membrane (Petris et al., 1996). The trafficking of ATP7A is triggered when cytoplasmic copper levels are elevated (Hamza et al., 2003), and this process requires both copper binding to cytoplasmic regions of the ATPase as well as its catalytic turnover (Petris et al., 2002; Strausak et al., 1999). The biological importance of the ATP7A protein is illustrated by Menkes disease, a lethal disorder of copper deficiency caused by ATP7A mutations (Kaler, 1998).

An area of copper metabolism of which we have little understanding is whether specific pathophysiological conditions lead to adaptive changes in intracellular copper homeostasis. Hypoxia is a stress that is inherent within the microenvironment of injured tissues, including dermal wounds and burns (Arnold, 1987), atherosclerotic plaques (Moreno et al., 2006) and avascular regions of solid tumors (Bristow and Hill, 2008). Cells of the myeloid lineage such a macrophages are specifically recruited to these hypoxic sites and are metabolically adapted to function within this hostile milieu. For example, it has been known for many years that neutrophils and macrophages are highly dependent on anaerobic glycolysis for ATP production, and suppress oxidative phosphorylation in the presence of hypoxia (Simon et al., 1977). In this study we investigated the impact of hypoxia on copper transport involving ATP7A.
homeostasis in the murine macrophage cell line, RAW264.7. Hypoxia resulted in increased copper uptake and enhanced the expression of the CTR1 transporter. Copper delivery to the ATP7A protein was also enhanced as evidenced by trafficking from the Golgi and enhanced copper transport into the secretory pathway to ceruloplasmin. By contrast, hypoxia triggered a decrease in the levels of other intracellular copper targets including, CCS, SOD1, and the copper-binding subunit of CCO, COX1. 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.

Results

Hypoxia stimulates trafficking of the ATP7A protein in RAW264.7 macrophages

We began this study by investigating 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 (Cobbold et al., 2002; La Fontaine et al., 1998; Petris et al., 1996). Using immunofluorescence microscopy, the ATP7A protein was found within the perinuclear region of RAW264.7 cells exposed to normoxic conditions (21% O₂), consistent with its location in the trans-Golgi network (Fig. 1A). As expected, treatment of these cells with copper resulted in the trafficking of ATP7A from the perinuclear region to cytoplasmic vesicles (Fig. 1A). Significantly, when these cells were exposed to chronic hypoxia (4% O₂ for 96 hours), the ATP7A protein was also dispersed to post-Golgi vesicles (Fig. 1A). This redistribution of the ATP7A protein required at least 48 hours 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. 1A). The intracellular location of the trans-Golgi marker protein, syntaxin 6 (STX6) and the Golgi matrix protein, GM130, were not altered by hypoxia in RAW264.7 cells (Fig. 1B,C), suggesting that the effects on ATP7A were not the result of a general effect on Golgi structure. The viability of RAW264.7 cells was not altered by these conditions and cells could be passaged continuously in 4% O₂ (data not shown).

Oxygen limitation stimulates ATP7A expression and copper-dependent trafficking of ATP7A

Since the trafficking of ATP7A from the trans-Golgi network is known to be triggered by increased copper delivery to this transporter (Petris et al., 1996), we tested whether a membrane-permeable copper chelator, tetrathiomolybdate (TTM), could suppress ATP7A trafficking in response to hypoxia in RAW264.7 cells. As shown in Fig. 2A, 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. We also explored the possibility that hypoxia may also increase the expression of ATP7A in RAW264.7 macrophages. Western blot analysis demonstrated that hypoxia increased ATP7A protein levels above normoxic controls in a time-dependent manner, beginning between 24 and 48 hours (Fig. 2B). A similar increase in ATP7A levels was observed in primary peritoneal macrophages isolated from C57BL mice and cultured under hypoxic conditions (Fig. 2C). These findings suggest that hypoxia stimulates copper-dependent trafficking and expression of the ATP7A protein.

Hypoxia stimulates trafficking of the ATP7A protein in tumor-associated macrophages

We then sought to confirm whether hypoxia could elicit similar changes in ATP7A localization in hypoxic macrophages in vivo. Previous studies have demonstrated that macrophages are recruited to the hypoxic regions of solid tumors where they play important roles in promoting angiogenesis (reviewed by Mantovani et al., 2006). A prostate tumor xenograft model provided an opportunity to investigate the intracellular distribution of ATP7A in these hypoxic tumor-associated macrophages. The tumorogenic human prostate cell line, PC-3, was chosen for these studies since ATP7A expression was very low in these cells, thus allowing for easy identification of ATP7A in macrophages recruited to the tumor. PC-3 tumors were grown in immunocompromised SCID mice for 5 weeks to a size of approximately 1 cm diameter (0.5 g), and then excised and cryosectioned for immunofluorescence analysis of ATP7A expression. This revealed abundant ATP7A protein...
expression in tumor-associated macrophages that were identified using the macrophage-specific marker CD-68 (Fig. 3A). As expected, there was little if any expression of ATP7A in the PC-3 tumor cells (Fig. 3A). Consistent with previous studies, the macrophages were typically concentrated at the tumor edges, with occasional infiltration into the tumor body (Biswas et al., 2006; Lewis and Pollard, 2006; Murdoch et al., 2004). It was noted that in some macrophages ATP7A was restricted to the perinuclear Golgi complex, whereas in other macrophages ATP7A was dispersed throughout the cell in a manner reminiscent of the trafficking seen earlier in cultured hypoxic RAW264.7 cells (Fig. 3A, lower left panel). Significantly, this dispersed localization of the ATP7A protein occurred only in macrophages that coexpressed the HIF1α protein (Fig. 3B). The dispersed localization of the ATP7A protein occurred only in macrophages that coexpressed the HIF1α protein (Fig. 3B). The HIF1α transcription factor is the master regulator of gene expression responses to low oxygen, and is upregulated in macrophages within hypoxic areas of tumors (Burke et al., 2002; Talks et al., 2000). These findings, together with our earlier observations in cultured RAW264.7 cells, suggest that hypoxia triggers copper-dependent trafficking of ATP7A in macrophages.

Oxygen limitation stimulates the expression of CTR1 and copper uptake in macrophages

Based on our finding that the trafficking of ATP7A in response to hypoxia was dependent on copper, we investigated whether this might occur through increased copper uptake. Radioactive 64Cu uptake experiments were carried out using RAW264.7 macrophages that had been pre-exposed to hypoxic or normoxic conditions. A significant increase in copper uptake was found for RAW264.7 cells pre-exposed to hypoxia relative to normoxia (Fig. 4A). Interestingly, this increased copper uptake was associated with a time-dependent increase in the levels of the CTR1 copper importer (Fig. 4B). A similar increase in CTR1 expression was observed in murine primary peritoneal macrophages cultured under hypoxic conditions (Fig. 4C). Taken together with our earlier results, these findings suggest that the copper-dependent trafficking of the ATP7A protein is associated with an increase in CTR1 expression and copper uptake.

Hyoxia stimulates copper transport to ceruloplasmin via ATP7A

The major function of ATP7A is to pump copper into the secretory pathway to supply copper to secreted cuproenzymes. We hypothesized that a potential target for this copper delivery in response to hypoxia might be ceruloplasmin, a copper enzyme that requires copper delivery into the secretory pathway. Ceruloplasmin is a ferroxidase secreted from macrophages and hepatocytes, the expression and activity of which are stimulated by hypoxia (Martin et al., 2005; Mukhopadhyay et al., 2000). Hyoxia was found to
Hypoxia differentially affects intracellular copper pathways

Having established that hypoxia increases the delivery of copper to ceruloplasmin via the ATP7A copper transporter, we explored the effect of hypoxia on two additional targets of intracellular copper pathways. These include Cu/Zn-superoxide dismutase (SOD1) in the cytoplasm and CCO in the mitochondria. Hypoxia resulted in a time-dependent decrease of SOD1 activity in RAW264.7 macrophages (Fig. 6A). Interestingly, this reduction in SOD1 activity was associated with a marked reduction in the level of CCS protein, which is the copper chaperone required for copper delivery to SOD1 (Fig. 6B). The activity of the copper enzyme, CCO, was also markedly reduced in mitochondrial preparations isolated from hypoxic RAW264.7 macrophages (Fig. 6C), and this was accompanied by reduced levels of COX1 protein, a copper containing subunit of CCO (Fig. 6D). Hypoxia did not result in detectable changes in other copper chaperones COX17, SCO1 or SCO2, which are required for copper delivery to CCO (data not shown). Taken together, these findings suggest that hypoxia differentially impacts intracellular copper handling pathways by decreasing copper delivery to SOD1 and CCO, while up-regulating copper delivery to the secretory pathway via ATP7A.

Discussion

Hypoxia is a stress that is commonly encountered by macrophages as they migrate away from the vasculature. Accordingly, this cell type is uniquely adapted to function within this hostile microenvironment. In this study we demonstrate that reduced oxygen levels promote adaptive changes in intracellular copper homeostasis in macrophages by specifically enhancing copper delivery to the biosynthetic pathway via the ATP7A protein (illustrated in Fig. 7). Copper-dependent trafficking of the ATP7A protein from the trans-Golgi network is one of the clearest biological indicators of increased cytoplasmic copper concentrations. Previous studies have shown that copper-stimulated trafficking is dependent

Fig. 4. Hypoxia stimulates copper uptake and CTR1 expression in RAW264.7 macrophages. (A) Copper uptake activity. RAW264.7 cells were pre-exposed to normoxia (21% O2) or hypoxia (4% O2) for 72 hours and 64Cu uptake was measured over 5 minutes. Values were normalized against total protein concentrations (mean + s.d.; n=3; *P<0.05). (B,C) The effect of hypoxia on CTR1 protein levels in RAW264.7 cells (B) and primary peritoneal macrophages (C) cultured under normoxia (N; 21% O2) or hypoxia (H; 4% O2) for the indicated times. Immunoblot analysis was used to detect CTR1 protein in lysates using anti-CTR1 antibodies. Tubulin was detected as a loading control. Relative CTR1 band intensities at each time point, normalized against tubulin, are shown for each normoxic and hypoxic pair.

Fig. 5. ATP7A-dependent copper transport is required for hypoxia-stimulated ceruloplasmin activity. (A) Immunoblot analysis of ceruloplasmin (Cp) secreted from RAW264.7 cells grown under normoxic (N; 21% O2) or hypoxic (H; 4% O2) conditions for the indicated times. Conditioned medium was concentrated and subjected to non-denaturing SDS-PAGE and immunoblot analysis with anti-Cp antibodies. Tubulin levels from corresponding cell lysates are also shown. Lane 1 is a negative control of concentrated growth medium alone (M). (B) Hypoxia stimulates ceruloplasmin activity. Ceruloplasmin activity (p-phenylenediamine oxidase activity) was measured in the concentrated conditioned medium from RAW264.7 cells following exposure to normoxia (N; 21% O2) or hypoxia (H; 4% O2) for 72 hours. Activity was normalized against total protein content of the corresponding cell lysates (mean + s.d.; n=3). (C) RNAi-mediated silencing of the ATP7A protein. Immunoblot analysis of ATP7A protein levels in RAW264.7 cells stably transfected with either ATP7A-RNAi or control-RNAi. (D) Ceruloplasmin activity was measured in conditioned medium from the ATP7A-RNAi or control-RNAi cells exposed to hypoxia (N; 21% O2) or hypoxia (H; 4% O2) for 72 hours (mean + s.d.; n=3). Note the failure to activate ceruloplasmin in ATP7A-depleted cells, and the restoration by addition of copper to the growth medium (+Cu).

increase both the abundance and the activity of ceruloplasmin secreted into the culture medium of RAW264.7 cells relative to normoxia (Fig. 5A,B). To examine whether the increase in ceruloplasmin activity was dependent on ATP7A copper transport activity, we depleted ATP7A expression in RAW264.7 cells using RNAi-mediated gene silencing (ATP7A/RNAi cells; Fig. 5C). Control cells were transfected with a construct expressing an irrelevant RNAi against GFP (Fig. 5C). Compared with 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. 5D). Consistent with this postulate, the addition of copper to the medium 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. 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 (data not shown). Taken together with our earlier results, these findings suggest that hypoxia stimulates an increase in copper delivery to ceruloplasmin by increasing CTR1-mediated copper uptake as well as ATP7A-dependent copper delivery into secretory compartments.
O₂-dependent regulation of copper homeostasis

on ATP7A copper transport activity as well as copper binding to cysteines within its amino-terminal region (Strausak et al., 1999; Petris et al., 2002). Our finding that hypoxia stimulated the relocalization of ATP7A to post-Golgi vesicles in a copper-dependent manner is consistent with increased copper binding to this protein, as well as an increase in its copper transport activity into the secretory pathway. This was supported by the finding that the increased activity of ceruloplasmin, an enzyme that acquires copper within secretory compartments, was dependent on ATP7A expression in hypoxic cells.

In contrast to ceruloplasmin, the abundance and/or activity of CCS, SOD1 and CCO were reduced by hypoxia. These findings provide the first evidence that the pathways of intracellular copper distribution can be differentially regulated in response to an environmental stress. By reducing the flow of copper from CCS to SOD1, this may provide a mechanism to redirect copper to the ATP7A protein. Like SOD1, the activity of CCO was also diminished by hypoxia in RAW264.7 cells and this was associated with a decrease in levels of COX1 protein, the subunit of CCO containing the CuB site. Although a decrease in CCO activity has been previously reported in hypoxic macrophages to facilitate the metabolic shift from oxidative phosphorylation to glycolysis (Murphy et al., 1984; Simon et al., 1977), our findings highlight the possibility that COX1 depletion serves an additional purpose of diverting precious copper stores to secretory compartments via ATP7A.

The finding that ceruloplasmin was a recipient of increased copper delivery to the secretory pathway 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 (Nittis and Gitlin, 2002). This process is an adaptive response to hypoxia to meet the increased iron demand of hematopoiesis (Sarkar et al., 2003). Thus, the prioritization of copper delivery to ceruloplasmin via ATP7A may ultimately function to regulate iron homeostasis in response to hypoxia. The finding that ceruloplasmin trafficking in hypoxic cells occurred concurrently with ceruloplasmin activation raises the possibility that under hypoxic conditions, copper delivery to ceruloplasmin may occur in post-Golgi vesicles rather than in the trans-Golgi network where copper loading normally takes place. Thus, ATP7A trafficking may not simply reflect an increased flux
of copper to this protein, but facilitate copper-loading of ceruloplasmin in post-Golgi compartments. Indeed, a recent study demonstrating a subset of ATP7A protein was required in post-Golgi melanosomes for the copper loading of tyrosinase is consistent with this model (Setty et al., 2008). A particularly intriguing finding of our study was the strong expression of ATP7A in tumor-associated macrophages. Copper has been shown to play an important role in angiogenesis, and copper chelation via TTM has proved to be an effective suppressor of tumor growth in animals (Alessandri et al., 1984; Camphausen et al., 2004; Cox et al., 2003; Cox et al., 2001; Pan et al., 2003a; Pan et al., 2003b; Pan et al., 2002; Redman et al., 2003; Teknos et al., 2005). It is therefore tempting to speculate that the adaptive changes in macrophage copper homeostasis described in this study may underlie the role of copper in tumor growth.

A notable finding of our study was that the changes in copper homeostasis in response to hypoxia appear to be restricted 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 (data not shown). The macrophage-specific effects of hypoxia on copper homeostasis may reflect inflammatory responses, since hypoxia is known to specifically activate inflammatory pathways in macrophages (Rius et al., 2008). Consistent with this postulate, our unpublished studies demonstrate that pro-inflammatory agents can stimulate copper-dependent ATP7A trafficking in macrophages under normoxic conditions. It will be of interest to determine whether other physiological conditions regulate changes in the intracellular distribution of copper in other mammalian cell types. For example, copper transport via ATP7A is required for melanin production via tyrosinase, norepinephrine synthesis via dopamine β-hydroxylase, and collagen cross-linking via lysyl oxidase, and each of these cuproenzymes is stimulated by particular physiological cues in specific cell types. The challenge of future studies will be to address whether this involves adaptive changes that promote an increase in ATP7A-dependent copper transport into the secretory pathway.

Materials and Methods

Reagents and antibodies

All reagents were from Sigma (St Louis, MO), unless otherwise indicated. The rabbit polyclonal CTR1 antibody (Nose et al., 2006) was kindly provided by Dennis Thiele (Duke University, Durham, NC). The rabbit polyclonal antibody against ATP7A was raised against the C-terminal portion of the protein and was a generous gift from Elizabeth Epper (Stevenson et al., 2003). Additional affinity purified anti-ATP7A antibodies were raised in rabbits against the synthetic peptide NH2-CDKHSLVGGFREDDDTTL-COOH (Bethyl Laboratories, Montgomery, TX). Anti-tubulin antibody, and secondary HRP-conjugated antibodies were purchased from Roche Molecular Biochemicals. Antibodies against COX1, and fluorescent Alexa Fluor 488- and Alexa Fluor 594-conjugated antibodies were from Invitrogen (Carlsbad, CA). Antibodies against GM130 and syntaxin 6 were purchased from BD Transduction Laboratories (San Jose, CA). Antibodies against CD68 and HIF1α were purchased from Serotec (Raleigh, NC) and Novus Biologicals (Littleton, CO), respectively. Antibodies against Cu/Zn-SOD, CCS and ceruloplasmin antibodies were purchased from Stressgen (Ann Arbor, MI), Santa Cruz Biotechnology (Santa Cruz, CA), and Abcam (Cambridge, MA), respectively.

Cell culture

All cell lines were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and 100 u/ml penicillin and streptomycin (Invitrogen) in 5% CO2 at 37°C. Primary macrophages were isolated by peritoneal lavage. C57BL/6J mice were injected with 2 ml of thioglycolate medium into the peritoneum to elicit macrophage infiltration. After 72 hours, macrophages were isolated by peritoneal lavage using ice-cold PBS. Cells were seeded in six-well plates for each experiment as described. Hypoxic atmospheres were generated by displacement with N2 and CO2 gas using a trigaic hypoxic incubator. RNAi-mediated silencing of ATP7A in RAW264.7 cells was performed by stable transfection of a pRS vector expressing a 29 nucleotide short hairpin (sh) RNA against ATP7A (Origene, Rockville, MD) followed by selection in 25 μg/ml puromycin. Control cells were transfected with the same vector expressing shRNA against GFP. Lipofectamine 2000 (Invitrogen) was used in all transfections.

Copper uptake

Radioactive copper (64Cu) was purchased from the Mallinckrodt Institute of Radiology, Washington University (Saint Louis, MO). Cells were pre-cultured in triplicate for 72 hours in 6-well plates under either normoxic (21% O2) or hypoxic (4% O2) conditions, and then exposed to 1 μM 64Cu for 5 minutes, washed extensively in ice-cold PBS and radioactivity measured using a gamma counter. Counts were normalized against total protein.

Immunocytochemistry and PC-3 tumor xenografts

Immunofluorescence microscopy and western blot analysis were performed as described previously (Mao et al., 2007). PC-3 prostate carcinoma cells (5×104) were injected subcutaneously into one flank of anesthetized 4-week-old ICRSC-M SCID mice obtained from Taconic (Germantown, NY). Mice were maintained in an approved pathogen-free institutional housing and 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. After a period of 4 weeks solid tumors of appropriately 1 cm diameter were excised from anesthetized mice and flash frozen in isopentane. Frozen tumors were cryosectioned, fixed in acetone for 10 minutes, washed in phosphate-buffered saline (PBS) and blocked overnight in 1% casein in PBS. Immunostaining was performed using antibodies against ATP7A, CD68 or HIF1α, followed by staining with Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-mouse antibodies, as indicated in the figure legends. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI).

Enzyme assays

CCO activity assays were performed using mitochondrial preparations from RAW264.7 cells obtained using the Cell Mitochondria Isolation Kit from Sigma. CCO activity was measured using a CCO assay kit (Sigma) according to the manufacturer’s instructions and activity was normalized against mitochondrial protein content.

Superoxide dismutase assays were performed as described previously (Flohé and Otting, 1984). Briefly, RAW264.7 cell lysates were fractionated using nondenaturing 12% 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 (Sunderman and Nomoto, 1970). RAW264.7 cells were grown in 6-well plates and the conditioned medium was collected and concentrated using Amicon Ultra-4 filter tubes (Millipore). A low level of endogenous ceruloplasmin activity in concentrated medium alone was subtracted from that of conditioned medium for each experiment. Ceruloplasmin activity was normalized against total protein content in the cell pellets. Ceruloplasmin protein levels were detected in concentrated medium using immunoblot analysis.

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References


