Copper Incorporation into Superoxide Dismutase in Menkes Lymphoblasts*

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The incorporation of copper into Cu,Zn-superoxide dismutase (SOD) was examined in Menkes lymphoblasts that express a genetic defect of copper metabolism. SOD activity was ~40% higher in Menkes than normal lymphoblasts. Since Menkes lymphoblasts contain elevated copper levels, the higher SOD activity is most likely due to near copper saturation of an apoSOD pool that is in normal lymphoblasts. Cycloheximide markedly inhibited 64Cu(II) incorporation into SOD in Menkes lymphoblasts under conditions in which no de novo synthesis of SOD protein was detected with normal lymphoblasts. The maximal amount of 64Cu incorporation into newly synthesized SOD in Menkes lymphoblasts was approximately equal to the maximal amount of 64Cu that could be incorporated into the apoSOD pool in normal lymphoblasts. The increased synthesis of SOD in Menkes lymphoblasts may play a protective role against copper toxicity in Menkes lymphoblasts. The protonophore, CCCP, markedly inhibited 64Cu incorporation into SOD in both normal and Menkes lymphoblasts, which is consistent with 64Cu incorporation into SOD within a membrane-bounded compartment in both cell types. When 64Cu-incorporation into SOD was blocked with CCCP, copper accumulated in a Superose column fraction that contains S-adenosylhomocysteine hydrolase (SAHH), which has a high affinity for copper. SAHH may play a role in delivering copper to SOD.

Menkes disease is a fatal, X-linked disease of copper metabolism which affects copper delivery to several copper enzymes, the synthesis of collagen and elastin, and brain development (1–5). A candidate gene (cDNA) for the Menkes defect was recently cloned (4–6), and the deduced amino acid sequence is homologous to metal-transporting, P-type, membrane ATPases from procaryotes (7, 8). Vulpe et al. (4) postulated that the protein encoded by the Menkes gene was within a subcellular membrane. Menkes lymphoblasts are derived by Epstein-Barr virus transformation of lymphocytes from Menkes patients (9). These cells maintain their characteristic phenotype of excess net copper accumulation when incubated with copper (9) and are also sensitive to copper toxicity.

Normal lymphoblasts were recently found to contain a pool of apoSOD1 that was activated when these cells were incubated with copper (10). The results reported here suggest that the apoSOD pool is nearly copper-saturated in Menkes lymphoblasts when grown in standard culture medium without added copper. Moreover, new SOD synthesis was detected in Menkes lymphoblasts under conditions in which no de novo synthesis of SOD was detected with normal lymphoblasts. Also, CCCP markedly inhibited 64Cu incorporation into SOD in both normal and Menkes lymphoblasts. The results suggest that SOD synthesis is induced in Menkes lymphoblasts to replenish the apoSOD pool in a membrane-bounded compartment as a protective mechanism against potential copper toxicity.

EXPERIMENTAL PROCEDURES

Materials—The Superose-12 HR 10/30 column, HPLC pump (model 2150) HPLC controller, were from Pharmacia Biotech Inc. HEPES, biocinonic acid, CCCP, and cycloheximide were from Sigma.

Cell Cultures—Human lymphoblastoid cell lines developed by transformation of peripheral B lymphocytes with Epstein-Barr virus were obtained from NIGMS, Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ). Normal human lymphoblasts (repository number GM03798) were from a normal 10-year-old Caucasian male. Menkes syndrome ( kinky hair disease) human lymphoblasts (repository number GM01982) were from a 2-year-old Caucasian male.

Human lymphoblasts were grown as suspension cultures in RPMI 1640 medium (Sigma) supplemented with 5% fetal calf serum and 5% newborn calf serum (Intergen, Purchase, NY). Cell cultures were maintained in logarithmic growth phase (0.7 × 10^6 cells/ml) by replacing cell suspensions with fresh medium as described previously (10). Cells were incubated with varying concentrations of 64Cu(NO3)2, specific activity, ~14 mCi/mg of copper (Buffalo Materials Research Center of the State University of New York at Buffalo) for the indicated durations in the normal growth medium. Cells from three 100-ml culture bottles of ~0.7 × 10^6 cells/ml were washed three times with cold phosphate-buffered saline, pH 7.4, before combining the samples for homogenization.

Preparation of Cytosols—Cells were homogenized with 150 strokes of a motor-driven (1000 rpm) homogenizer (Thomas Teflon pestle) in 0.4 ml of isotonic HEPES sucrose buffer (0.25 M sucrose, 5 mM HEPES pH 7.4). Phenylmethylsulfonyl fluoride (40 μg/ml) and leupeptin (0.5 μg/ml) were added to the homogenization buffer to inhibit proteolysis. Homogenates were centrifuged for 2 min at 1930 × g at 4 °C, and the supernatants were centrifuged for 60 min at 100,000 × g. The supernatants were filtered through a 0.22-μm Millex GV syringe filter (Milipore, Bedford, MA) before applying to the Superose column. Typical protein concentrations of lymphoblast cytosols were 10–15 mg/ml as determined by the biocinonic acid assay method using bovine serum albumin as a standard (11).

Superose 12 HPLC—The Superose 12 column was equilibrated with 0.05 mM HEPES, 0.1 mM NaCl, pH 7.4. Samples (200 μl) were injected, and the columns were eluted at a flow rate of 0.4 ml/min, as described previously (10). The amount of 64Cu in each tube (220 μl) was determined with a LKB gamma counter (model 1282), correcting for decay by a program within the counter. The radioactivity in each tube was expressed as picograms of 64Cu/mg of total cytosolic protein, and the total amount of 64Cu in a column fraction was determined by adding the 64Cu in each tube comprising the fraction.

SOD Activity—SOD assays were performed by a xanthine oxidase-

1 Abbreviations used are: SOD, superoxide dismutase; MT, metallothionein; HPLC, high performance liquid chromatography; SAHH, S-adenosyl-

homocysteine hydrolase; CCCP, carbonylcyanide m-chlorophenylhydrazone.
SOD in Menkes Lymphoblasts

RESULTS

The Distribution of $^{64}$Cu in Menkes Lymphoblast Cytosols after Incubating with $^{64}$Cu(II)—Menkes and normal lymphoblasts were incubated for 2 h with 7 $\mu$M $^{64}$Cu(II). The cell cytosols were isolated and fractionated by Superose chromatography. No significant differences were detected in the amount of $^{64}$Cu bound to proteins in the void volume fractions from normal and Menkes lymphoblasts (Fig. 1). Three additional $^{64}$Cu-binding fractions were reproducibly detected with both normal and Menkes lymphoblast cytosols. Fraction I (Fig. 1) contains SAHH which was recently proposed to play a role in copper metabolism (16, 17). The $^{64}$Cu-protein fraction peaking at tube 37 is SOD (10), and the fraction labeled MT contains metallothionein(s) as indicated below. Menkes cytosols showed much higher $^{64}$Cu binding in the MT-containing fraction than normal cytosols, which is consistent with previous studies (9). Menkes lymphoblast cytosols also showed less incorporation of $^{64}$Cu into SOD, and significantly more $^{64}$Cu-binding in fraction I than normal lymphoblasts (Fig. 1). However, by 15 h, the amounts of incorporation of copper into SOD and copper binding to a fraction I protein(s) in Menkes lymphoblasts were similar to what was detected with normal lymphoblasts (see below). As with normal lymphoblasts (10), no $^{64}$Cu incorporation or isotopic exchange into SOD was detected when $^{64}$Cu was added directly to cytosols rather than to whole lymphoblasts (data not shown).

$^{64}$Cu Concentration Dependence of $^{64}$Cu Incorporation into SOD in Menkes Lymphoblasts—Normal and Menkes lymphoblasts were incubated at varying times (1–15 h) and $^{64}$Cu concentrations (1–15 $\mu$M) to obtain varying amounts of total cellular and cytosolic $^{64}$Cu. The cytosols were isolated and chromatographed on Superose. Incorporation of $^{64}$Cu into SOD in both normal and Menkes lymphoblasts showed a hyperbolic dependence on total cytosolic $^{64}$Cu concentration (Fig. 2). The estimated maximum amounts of $^{64}$Cu incorporated into SOD were similar in normal and Menkes lymphoblasts (4.6 and 4.9 ng $^{64}$Cu per mg of protein, respectively). However, the total cytosolic $^{64}$Cu concentration at half-maximal levels of copper incorporation into SOD in Menkes lymphoblasts (206.5 ng/mg of protein) was significantly higher than with normal lymphoblasts (63.3 ng/mg of protein) (Fig. 2). Since the higher total cytosolic copper of Menkes cytosols at any extracellular copper concentration is, for the most part, due to higher levels of copper bound to elevated MT (9), these results imply that either MT-Cu is unavailable to SOD and/or that higher concentrations of copper are required for copper incorporation into SOD in Menkes lymphoblasts.

The cytosolic copper concentration dependence for binding of $^{64}$Cu to a protein(s) in fraction I, which contains SAHH, was also determined. The results are plotted in Fig. 3 as percent of maximal $^{64}$Cu bound versus cytosolic copper. Interestingly, in both normal and Menkes lymphoblasts, $^{64}$Cu-binding in fraction I showed the same relative concentration dependence (percent of maximal) as $^{64}$Cu incorporation into SOD (Fig. 3).

The Effect of Cycloheximide on $^{64}$Cu Incorporation into SOD in Menkes Lymphoblasts—Menkes lymphoblasts were preincubated for 3 h with 5 $\mu$g/ml cycloheximide and then incubated for an additional 15 h with 7 $\mu$M $^{64}$Cu(II) with cycloheximide. Cytosols were isolated and fractionated on Superose. Inhibition of protein synthesis by cycloheximide caused a significant decrease in the amount of $^{64}$Cu binding to MT (Fig. 4) in Menkes lymphoblasts as was also observed with normal lymphoblasts (10). However, in contrast to normal lymphoblasts, cycloheximide markedly inhibited incorporation of $^{64}$Cu into SOD in...
Menkes lymphoblasts (Fig. 4). The effect of cycloheximide on decreasing cytosolic $^{64}$Cu in Menkes lymphoblasts was similar in magnitude to its effect on normally lymphoblasts (10) and not large enough to account for its large inhibitory effect on $^{64}$Cu incorporation into SOD. Thus, in striking contrast to normal lymphoblasts (10), copper seems to be incorporated mainly into newly synthesized SOD in Menkes lymphoblasts rather than into a preexisting pool of apoSOD.

**The Effect of Cycloheximide and Copper on SOD Activity in Menkes and Normal Lymphoblasts**—Menkes and normal lymphoblasts were incubated with no additions (control), cycloheximide alone, 7 $\mu$M CuSO$_4$ alone, or cycloheximide plus 7 $\mu$M CuSO$_4$ for 15 h. SOD activity was $\sim$40% higher in Menkes than normal lymphoblasts when grown on standard medium without the addition of any extra copper (Table I). Surprisingly, although cycloheximide markedly inhibited $^{64}$Cu incorporation into newly synthesized SOD (Fig. 4), that had no significant effect on the total SOD activity in Menkes lymphoblasts (Table I). Also, in marked contrast to normal lymphoblasts, no significant increase in SOD activity was detected with Menkes lymphoblasts when incubated with copper (Table I), even though comparable amounts of copper were incorporated into SOD in normal and Menkes lymphoblasts.

**The Effect of High Levels of MT Bound $^{64}$Cu on Incorporation of $^{64}$Cu into SOD in Normal Lymphoblasts**—Normal lymphoblasts were preincubated for 15 h with 100 $\mu$M ZnSO$_4$ to induce MT synthesis and then incubated for 2 h with 7 $\mu$M $^{64}$Cu(II) in the same medium. Cytosols were isolated and chromatographed on Superose. Although induction of MT in normal lymphoblasts increased $^{64}$Cu binding in the MT fraction by about 30-fold that had no significant effect on the amount of $^{64}$Cu incorporated into SOD (Fig. 5), these results are consistent with a source(s) of SOD-Cu other than MT and suggest that MT cannot effectively compete with source(s) for SOD copper under these conditions. The results with zinc induction of MT also indicate that Zn(II) is not a limiting factor for $^{64}$Cu incorporation into the apoCu,Zn-SOD pool because increased Zn(II) had no effect on $^{64}$Cu incorporation.

Normal lymphoblasts were also stably transfected with the pSG-HEBO-MT-1 expression vector (see “Experimental Procedures”) to further test the effects of elevated MT bound $^{64}$Cu on the distribution of $^{64}$Cu in lymphoblast cytosols and the incorporation of $^{64}$Cu copper into apoSOD. The transfected normal
cells were preincubated for 3 h with 5 μg/ml cycloheximide and then incubated for 15 h with 7 μM 64Cu(II) in the zinc-containing medium for 2 h. ○, control; ●, zinc-treated.

Moreover, under these conditions, 64Cu incorporation into SOD was slightly less than predicted from the cytosolic 64Cu concentration dependence for lymphoblasts containing normal amounts of MT. Thus, 64Cu incorporation into SOD was clearly not increased by the large increase in 64Cu bound to MT in lymphoblasts transfected with the MT expression vector.

The Effects of CCCP on 64Cu Incorporation into SOD, and the Distribution of 64Cu in Menkes Lymphoblast Cytosols—Increased net copper accumulation due to decreased copper efflux is a phenotypic characteristic of Menkes lymphoblasts. In the presence of CCCP, net copper uptake by normal lymphoblasts is elevated to approximately the same abnormally high level as observed with Menkes lymphoblasts (18). Since dicyclohexylcarbodiimide, which is a specific inhibitor of ATP-synthase proton pumps had no effect on copper accumulation, the effect of CCCP was most likely due to its action as a general protonophore. Although CCCP increased net copper uptake, it markedly inhibited 64Cu incorporation into SOD in both normal and Menkes lymphoblasts (Fig. 7). Concomitant large increases in 64Cu in fraction I were detected (Fig. 7). This is an unusual result because impaired copper utilization in cells usually leads to MT induction and increased copper binding to MT rather than increased copper binding to other copper binding proteins. CCCP had only small inhibitory effects on copper binding to MT in normal and Menkes lymphoblasts, which is consistent with minimal effects of CCCP on protein synthesis under the

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**TABLE I**

SOD in Menkes Lymphoblasts

Normal and Menkes lymphoblasts were incubated with copper, cycloheximide, and cycloheximide plus copper as indicated. SOD activity was measured as described under "Experimental Procedures."

| Conditions                      | SOD activity* units/mg cytosolic protein |
|---------------------------------|-----------------------------------------|
| No additions                    | Normal: 48.2 ± 0.5 Menkes: 67.0 ± 2.7 |
| Cycloheximide, 5 μg/ml (15 h)   | Normal: 47.5 ± 1.0 Menkes: 70.5 ± 2.5 |
| CuSO4, 7 μM (15 h)              | Normal: 64.7 ± 3.8 Menkes: 66.5 ± 1.7 |
| Cycloheximide, 5 μg/ml + CuSO4, 7 μM (15 h) | Normal: 59.2 ± 0.9 Menkes: 74.0 ± 0.1 |

* Values are means ± SD for quadruplicates.

† Significantly different from control without copper p ≤ 0.0001.

‡ Significantly different from control with copper, without cycloheximide p ≤ 0.03.

§ Significantly different from Menkes lymphoblasts with copper, without cycloheximide p ≤ 0.03.

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**TABLE II**

Copper incorporation into SOD and copper binding by MT in normal lymphoblasts transfected with a MT expression vector.

Control or transfected normal lymphoblasts were incubated with 7 μM 64Cu(II) for 15 h with or without 5 μg/ml cycloheximide, and the 64Cu-labeled cytosols were fractionated on Superose.

| Cells                       | Cycloheximide | SOD † | MT ‡ | Total † |
|-----------------------------|---------------|-------|------|---------|
| Control                     | 1.3           | 2.8   | 3.1  | 7.2     |
| Control +                   | 1.7           | 2.0   | 1.7  | 5.4     |
| Transfected                 | 1.3           | 2.3   | 18.6 | 22.2    |
| Transfected +               | 1.2           | 1.6   | 13.8 | 16.6    |

* Total 64Cu in tubes under Superose fraction I.
† Total 64Cu in tubes under the SOD fraction from Superose.
‡ Total 64Cu in the MT fraction from Superose.
§ Total cytosolic 64Cu was taken as the sum of 64Cu in the SOD, MT, I fractions.
The data reported here indicate that the activity of SOD in Menkes lymphoblasts grown under standard conditions without added copper was about the same (≈40% higher) as the maximal possible SOD activity (≈35% higher) in normal lymphoblasts after copper was added (10). Since Menkes lymphoblasts accumulate and retain higher levels of copper than normal lymphoblasts, the higher SOD activity in Menkes lymphoblasts probably reflects near saturation of the apoSOD pool in Menkes lymphoblasts.

A striking abnormal characteristic of Menkes lymphoblasts is significant induction of new synthesis of SOD protein over a ~15-h incubation period. No significant SOD synthesis was detected under equivalent conditions with normal lymphoblasts (10), and the increased time dependence for copper incorporation into SOD in Menkes lymphoblasts was probably due to the requirement for new SOD synthesis. Curiously, the new holo-SOD synthesis did not appear to significantly contribute to the total SOD activity in Menkes lymphoblasts, because cycloheximide had no significant effect on SOD activity. This is despite the fact that the maximal amount of copper incorporation into newly synthesized SOD in Menkes lymphoblasts was of similar magnitude to the maximal level of copper incorporation into apoSOD in normal lymphoblasts. The lack of a significant effect of cycloheximide on SOD activity in Menkes lymphoblasts suggests that the induction of SOD synthesis and the rate of degradation or removal of SOD may both be regulated to maintain the level of holo-SOD in Menkes lymphoblasts near a maximal possible level. Equal rates of SOD synthesis and removal would also account for the fact that, unlike with normal lymphoblasts (10), $^{64}$Cu(II) incorporation into SOD was not associated with any significant increase in total SOD activity in Menkes lymphoblasts.

Although the function of the apoSOD pool is unknown, the similar possible maximal levels of copper incorporation into apoSOD in normal lymphoblasts and newly synthesized SOD in Menkes lymphoblasts suggests a compensatory response to depletion of the apoSOD pool in Menkes lymphoblasts. Induction of SOD synthesis and removal of SOD to maintain a maximal possible steady-state level of holo-SOD may function as a protective response against potential copper toxicity. Menkes fibroblasts are sensitive to copper toxicity (19), and Menkes lymphoblasts also show increased sensitivity to copper toxicity (unpublished results). Thus, increased SOD synthesis in Menkes lymphoblasts may provide a pool of SOD which can sequester excess copper, which is then rapidly removed to minimize copper toxicity.

The fraction of total cellular copper that is in the cytosolic fraction is much higher in Menkes than normal lymphoblasts, and this copper is mostly bound to MT, which is elevated in Menkes lymphoblasts (9). Elevated copper in the cytosolic fraction was also reported for animal models of Menkes disease (20, 21). This may reflect impairment of copper delivery to, and entry into, various organelles due to impaired function of the Menkes protein and induction of MT in response to the accumulation of nonutilizable copper. The results reported here with normal cells containing elevated levels of MT are consistent with elevated MT being a response to elevated copper rather than elevated MT being a significant contributing factor to impaired utilization of cellular copper in Menkes cells, as elevated MT levels had little or no effect on copper delivery to apoSOD in normal lymphoblasts.

The large inhibitory effect of the protonophore, CCCP on copper incorporation into SOD in normal lymphoblasts suggests that the apoSOD pool is within an intracellular, mem-

\[ N. \text{ Petrovic, A. Comi, and M. J. Ettinger, unpublished data.} \]
brane-bounded compartment rather than in the cytoplasm. The fact that copper does not activate the apoSOD when added directly to lymphoblast cytosols rather than to whole cells is also consistent with compartmentalization of the apoSOD pool. Since CCCP had similar effects on copper incorporation into SOD in normal and Menkes lymphoblasts, copper incorporation into SOD in Menkes lymphoblasts may occur in the same cell compartment as in normal lymphoblasts. That is consistent with the hypothesis that new SOD synthesis in Menkes lymphoblasts may represent a cellular response to replenish the apoSOD pool which is apparently depleted in Menkes lymphoblasts, and is also consistent with the similar maximal \( ^64 \text{Cu} \) apoSOD pool which is apparently depleted in Menkes lymphoblasts. That is consistent with the hypothesis that new SOD synthesis in Menkes lymphoblasts may represent a cellular response to replenish the apoSOD pool which is apparently depleted in Menkes lymphoblasts.

As a general protonophore, CCCP may inhibit copper delivery to SOD by dissipating a proton gradient that is required for co-transport of copper into the cellular compartment where copper incorporation into SOD is suggested to occur. Interestingly, the procaryote homologues of the putative Menkes ATPase, copper transporter may be \( \text{H}^+ \)-antiport systems (26). The defect in the Menkes gene in the patient whose lymphoblasts were used for our studies was recently reported. A five-base deletion resulting in a frameshift and presumably inactive protein was detected by reverse transcription-polymerase chain reaction (27). Thus, the similar results with CCCP with normal and Menkes lymphoblasts and the high activity of SOD in Menkes lymphoblasts suggests that a subcellular membrane copper transporter other than the Menkes protein is involved in delivery of copper to this SOD pool in lymphoblasts. This putative copper transporter protein may be the homologue of the Menkes protein in humans that was recently identified as a candidate for the defect in Wilson’s disease (28–31), or alternatively, another copper transporter that requires proton co-transport. Alternatively, the effect of CCCP may be due to sulfhydryl modification of a specific protein, as previously suggested (18).

The molecular species, pool, and mechanisms involved in copper delivery to SOD remain unknown. The results reported here suggest that MT-Cu is not a major source of SOD copper, and that MT-Cu apparently is not in rapid equilibrium with the cellular copper pool(s) that is a source(s) for SOD-Cu because increased copper on MT had no significant effect on the delivery of copper to apoSOD in normal lymphoblasts. However, increased copper in the Superose copper-binding fraction I was detected in each case in which incorporation of copper into SOD was blocked by CCCP. All of these results are consistent with copper bound to a fraction I protein(s), either residing within a copper pool that supplies copper to SOD or being in equilibrium with a pool or species that delivers copper to SOD. A protein that has been identified in the Superose fraction I that has a high affinity for copper is SAHH, and this protein has been proposed to have a role in intracellular copper trafficking (16, 17).

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