Identification of an Apo-Superoxide Dismutase (Cu,Zn) Pool in Human Lymphoblasts

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Copper incorporation (\(^{64}\text{Cu}(\text{II})\)) into Cu,Zn-superoxide dismutase (SOD) was studied in human lymphoblasts. Rapid incorporation of copper with a proportionate increase in SOD activity was detected. No copper incorporation or SOD activation was detected when \(^{64}\text{Cu}(\text{II})\) was added to cell cytosols rather than to intact cells. Thus, incorporation of \(^{64}\text{Cu}\) was not due to isotopic exchange. Cycloheximide had no significant effect on copper incorporation and activation of SOD when the data were corrected for total cell copper. Thus, the data were consistent with copper incorporation into a preexisting apoSOD pool rather than newly synthesized SOD, and no new SOD synthesis was detected over a 15-h incubation period. The size of the apoSOD pool was estimated to be \(\sim 35\%\) of the total SOD in lymphoblasts. When cells were preincubated for 15 h with excess copper (15 \(\mu\text{M}\) Cu(II)), the size of the apo pool markedly decreased but was not eliminated, suggesting that the apoSOD was not due to copper deficiency. These experiments also indicated that newly arrived copper was preferentially incorporated into the apoSOD pool, while the function(s) of an apoSOD pool remains unknown. Copper binding to apoSOD may provide a rapid protective response against copper toxicity.

\(\text{Cu,Zn-superoxide dismutase (SOD)}\) is found in the cytosolic fraction of all cells (1, 2) and has also been detected in nuclei and peroxisomes (2–5). However, the cellular site(s) and stage of protein synthesis of copper incorporation into SOD remain unknown. Moreover, an apo form of SOD was detected in a variety of copper-deficient cells or in cells in which the amount of the apoSOD pool appeared to vary with the stage of differentiation (6–15). The total amount of SOD mRNA and protein may also vary with the state of differentiation of some cells types (16). The cellular location of the apo form of SOD is unknown. While some copper incorporation into apoSOD was reported when high (2 \(\text{mM}\)) concentrations of copper were added to whole cell homogenates (10), no copper incorporation has been reported for copper addition to the cytosolic fractions from cells containing an apoSOD pool. Since copper is incorporated into apoSOD in neutral buffer, this suggests that either there are factors in cell cytosols which inhibit copper incorporation or that the apoSOD pool is not cytosolic.

The time and copper concentration dependencies of copper binding to cytosolic copper binding proteins and copper incorporation into SOD were determined during incubations of lymphoblasts with \(^{64}\text{Cu}(\text{II})\). Since SOD copper does not readily exchange isotopic copper, \(^{64}\text{Cu}\) incorporation exclusively into newly synthesized SOD was anticipated. However, little or no de novo synthesis of SOD protein was detected in lymphoblasts over the time course of these experiments. Instead, an apoSOD pool was detected in lymphoblasts, which were neither copper-deficient nor differentiating. The results in this and a companion study on copper incorporation into SOD in Menkes (17) lymphoblasts (18) suggest a possible role of apoSOD pools in initial cellular defense mechanisms against copper toxicity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Superose-12 HR 10/30 column, HPLC pump (model 2150) HPLC controller, were from Pharmacia Biotech Inc. HEPES, biocinchonic acid, cycloheximide, procainamide, and all other chemicals were from Sigma. \(^{64}\text{Cu(NO}_3\text{)}_2\) was from the Buffalo Materials Research Center of the State University of New York at Buffalo. The specific activity of the isotope at the time of shipment was \(\sim 14 \text{mCi/mg of copper}\).

**Cell Cultures**—Human lymphoblasts developed by transformation of peripheral B lymphocytes with Epstein-Barr virus were obtained from NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The cells used (repository no. GM03798) were from a normal, 10-year-old, male Caucasian. Lymphoblasts were grown in 175-cm\(^2\) plastic tissue culture bottles (Falcon) as suspension cultures in 100 ml of RPMI 1640 medium (Sigma) supplemented with 5% fetal calf serum and 5% newborn calf serum (Intergen, Purchase, NY). Cells cultures were maintained at a concentration of \(\sim 0.7 \times 10^6/\text{ml}\) in logarithmic growth phase by replacing one-third to one-half of cell suspensions with fresh medium every 2nd day. \(^{64}\text{Cu(NO}_3\text{)}_2\) was added to 100 ml of cell cultures at the indicated concentrations for the indicated incubation periods. At the end of the incubation with \(^{64}\text{Cu}(\text{II})\), the cells were washed three times with isotonic phosphate-buffered saline at 4 °C before homogenization.

**Preparation of Cytosols**—The cells from three 100-ml cultures were combined and homogenized with 150 strokes of a motor driven (1000 rpm) homogenizer (Thomas Teflon pestle) in 0.4 ml of isotonic HEPES sucrase buffer (0.25 \(\text{mM}\) sucrase, 5 \(\text{mM}\) HEPES, pH 7.4) containing phenylmethylsulfonyl fluoride (40 \(\mu\text{g/ml}\)) and leupeptin (0.5 \(\mu\text{g/ml}\)) to inhibit proteolysis. Homogenates were centrifuged for 2 min at 15,000 \(\times\) g. The supernatants were centrifuged for 60 min at 100,000 \(\times\) g and then filtered through 0.22- \(\mu\text{m}\) Milex GV syringe filters (Millipore, Bedford, MA) before applying to the Superose column. Protein concentrations of cell and cytosol samples were determined by the bicinchonic acid assay (19) using bovine serum albumin as a standard. Typical protein concentrations of lymphoblast cytosols were 10–15 mg/ml.

**Superose 12 HPLC**—All solutions were filtered through 0.2- \(\mu\text{m}\) nylon filters (Nalgene, Rochester, NY) and then stored at 4 °C. Column solutions were degassed under vacuum for at least 30 min before use. The Superose 12 column was equilibrated with at least two column volumes of 0.05 M HEPES, 0.1 M NaCl, pH 7.4. The \(^{64}\text{Cu}-labeled cytosolic sample (200 \mu\text{l}) was injected through a titanium loop, and the samples were fractionated at a flow rate of 0.4 ml/min while collecting 220- \(\mu\text{l}\) fractions. (Fraction collection began 16 min after application of the sample.) Cytosol fractions were then analyzed for \(^{64}\text{Cu}(\text{II})\) in a LKB gamma counter (model 1282), correcting for decay. The amount of radioactivity

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1 The abbreviations used are: SOD, superoxide dismutase; MT, metallothionein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

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Fraction Number

Fig. 1. The distribution of $^{64}$Cu in human lymphoblasts cytosols. Lymphoblasts were incubated in RPMI 1640 medium plus 10% fetal calf serum and 7 μM $^{64}$Cu(II) for 2 h at 37 °C. Radiolabeled cytosols were fractionated by Superose 12 HPLC collecting 0.22-ml fractions. The elution positions of the molecular weight standards are indicated at the top, and the void volume ($V_v$), fractions I and II copper-binding proteins, and MT elution positions are also indicated. The $^{64}$Cu (○) and Cu,Zn-SOD activity (●) were determined in each 0.22-ml column fraction.

in each pooled $^{64}$Cu-binding protein fraction was determined by adding the radioactivities of each of the tubes constituting that fraction. Data are expressed as nanograms of $^{64}$Cu/mg of cytosolic protein.

SOD Activity—SOD activities were determined by a spectrophotometric method using xanthine oxidase (20). A unit of SOD activity represented the amount of cytosol required to decrease the absorbance of control samples (no cytosol) by 50%. SOD activities were corrected for possible mitochondrial Mn-SOD contamination by subtracting the activity remaining in 1 mM KCN. Quadruplicate assays were performed on each sample, and the data were analyzed for statistical significance by a two-tailed, Student’s t test.

Immunoprecipitation of SOD—Protein A (0.5 ml) as an insoluble cell suspension (Staphylococcus aureus) was washed once with phosphate-buffered saline (PBS) and resuspended in 0.5 ml of PBS. 50 μl of monoclonal mouse antibodies (IgG) to human recombinant Cu,Zn-SOD were added and incubated with occasional mixing for 1 h at a room temperature. Control protein A suspensions were not incubated with antibodies. The antibody-treated protein A suspensions were pelleted by 12,000 × g centrifugation for 2 min and washed once with PBS. Lymphoblast cytosols were mixed with antibody-treated (or control) protein A suspensions. After 1 h of incubation at room temperature, the protein A suspensions were pelleted by 12,000 × g centrifugation for 2 min, and the supernatants were filtered through 0.22-μm Milex GV syringe filters (Millipore, Bedford, MA) before applying the samples to the Superose 12 HPLC column.

RESULTS

Distribution of Copper in Cytosols after Incubating Cytosols or Intact Lymphoblasts with $^{64}$Cu(II)—Lymphoblasts were incubated with 7 μM $^{64}$Cu(II) in culture medium supplemented with 10% fetal calf serum for 2 h, and the $^{64}$Cu-labeled cytosols were fractionated on Superose columns. The amount of $^{64}$Cu in the void volume was variable and most likely reflected variable particulate contamination of the cytosols. Three $^{64}$Cu-labeled protein fractions eluted after the void volume. Fraction I (Fig. 1) contains S-adenosylhomocysteine hydrolase which was recently found to have a high affinity for copper (21, 22). This was confirmed by Western blots (data not shown). Copper-binding fraction III contains metallothionein(s) (MT) as indicated by the apparent molecular weight, and the elution of MT in this fraction after transfection of lymphoblasts with a MT-expression vector (18). Since it was known from prior studies that Cu,Zn-SOD elutes near the elution volume where the $^{64}$Cu-binding fraction II was detected, the fractions were assayed for SOD activity. Maximal SOD activity occurred at the peak of $^{64}$Cu-incorporation; and the amount of SOD activity correlated well with the amount of $^{64}$Cu detected in each tube comprising fraction II (Fig. 1). Interestingly, copper incorporation into this fraction required intact cells as no significant $^{64}$Cu-binding in fraction II was detected when $^{64}$Cu(II) was added directly to lymphoblast cytosols (data not shown). $^{64}$Cu incorporation into fraction II with intact cells was unlikely due to isotopic exchange with copper in holo-SOD as none occurred when $^{64}$Cu was added directly to cell cytosols that contain active SOD.

The Effect of Immunoprecipitation of SOD on the Cytosolic Distribution of $^{64}$Cu—SOD was immunoprecipitated from lymphoblast cytosols to determine if $^{64}$Cu incorporation into SOD completely accounted for Superose $^{64}$Cu-binding fraction II. Lymphoblasts were incubated with $^{64}$Cu(II), the cytosols were isolated, and SOD was immunoprecipitated with a monoclonal antibody to human SOD bound to protein A. Control cytosols were treated with protein A without antibody. The effects of immunoprecipitation on the distribution of $^{64}$Cu and SOD ac-
tivity are shown in Fig. 2, A and B. Both SOD activity and $^{64}$Cu incorporation into copper-binding fraction II decreased proportionally, while the amount of $^{64}$Cu in copper-binding fractions I and MT were not significantly affected. Thus, copper incorporation into fraction II reflects $^{64}$Cu incorporation in SOD.

The Concentration Dependence of $^{64}$Cu Incorporation into SOD in Intact Lymphoblasts—Lymphoblasts were incubated at varying times (2–15 h) and $^{64}$Cu concentrations (2–15 $\mu$M) to obtain varying amounts of cytosolic $^{64}$Cu. The isolated cytosols were chromatographed on Superose. The amounts of $^{64}$Cu in SOD were determined by calculating the areas under the SOD plot. Since the plot of $^{64}$Cu incorporated into SOD versus total cytosolic $^{64}$Cu followed a simple saturation curve (data not shown), the maximum amount of $^{64}$Cu that was incorporated into SOD, and the cytosolic $^{64}$Cu concentration needed to reach 50% of maximal incorporation of $^{64}$Cu into SOD ($K_{0.5}$) could be estimated. Maximal incorporation was $\approx 4.6$ ng of $^{64}$Cu/mg of cytosolic protein, and $K_{0.5}$ was $\approx 6.3$ ng of cytosolic $^{64}$Cu/mg of cytosolic protein.

The Effects of Cycloheximide on the Distribution of $^{64}$Cu in Lymphoblast Cytosols and $^{64}$Cu Incorporation into SOD—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) and cycloheximide to determine whether protein synthesis was required for copper incorporation into SOD. Control cells were incubated without cycloheximide. Cytosols were isolated and fractionated on Superose columns. Increasing the incubation time from 2 h (Fig. 1) to 15 h (Fig. 3) increased the total $^{64}$Cu incorporated in all three copper-binding protein fractions in the control cytosols (Fig. 3). Cycloheximide caused a large decrease in the amount of $^{64}$Cu in the MT fraction and a small increase in $^{64}$Cu in fraction I (Fig. 3). Copper incorporation into SOD decreased significantly. However, cycloheximide also led to decreases in total cytosolic $^{64}$Cu levels. The amounts of copper incorporated into SOD with and without cycloheximide were not significantly different when corrected for the total amount of cytosolic copper under these conditions (Table I).

The Effect of Preincubation with Nonradioactive Copper on the Distribution of $^{64}$Cu in the Cytosol and $^{64}$Cu Incorporation into SOD—Lymphoblasts were preincubated with 15 $\mu$M CuSO$_4$ for 15 h and then incubated with cycloheximide (5 $\mu$g/ml) for 3 h followed by 7 $\mu$M $^{64}$Cu(II) for 15 h in the same medium. Control cells were not preincubated with copper. Although the amount $^{64}$Cu incorporated into the apoSOD pool decreased, preincubation with copper did not eliminate the apoSOD pool (Fig. 4). The amount of $^{64}$Cu in fraction I increased in cells preincubated with copper by about the same amount as the decrease in $^{64}$Cu incorporation into SOD with or without cycloheximide present. This is the expected result if the apoSOD pool in lymphoblasts was estimated from the maximal copper incorporation into this pool as $\approx 35\%$ of the total SOD in lymphoblasts.

| Table I |
|---|
| The effect of cycloheximide on copper incorporation into SOD and SOD activity |
| Human lymphoblasts were incubated with 7 $\mu$M $^{64}$Cu(II) for 15 h with or without cycloheximide (5 $\mu$g/ml), and the radiolabeled cytosols were fractionated on Superose. Copper incorporation into SOD was determined by determining the total $^{64}$Cu in the SOD fraction. Total cytosolic copper represents the total $^{64}$Cu in the three major $^{64}$Cu-binding fractions, i.e. fraction I, SOD (fraction II), and MT (Fig. 1B). SOD activities (mean ± S.D. of four experiments) were determined as described under “Experimental Procedures.” |

| Incubation conditions | $-\text{Cycloheximide}$ | $+\text{Cycloheximide}$ |
|---|---|---|
| SOD activity and $^{64}$Cu | | |
| Total cytosolic $^{64}$Cu* | 7.829 ± 0.635 | 5.370 ± 0.220 |
| $^{64}$Cu in SOD* | 2.808 ± 0.270 | 2.032 ± 0.182 |
| $^{64}$Cu in SOD per total cytosolic copper | 0.357 | 0.378 |
| Increase in SOD activity (units/mg cytosolic protein)$^a$ | 16.5 ± 3.8 | 11.0 ± 1.0 |
| Increase in SOD activity per total cytosolic $^{64}$Cu | 2.107 | 2.048 |
| Increase in SOD activity per $^{64}$Cu in SOD | 5.876 | 5.413 |

* Nanogram/mg of cytosolic protein; mean ± S.D. of three experiments.

$^a$ The SOD activity in cytosols from untreated cells was 48.2 ± 0.5 units/mg of cytosolic protein.
fraction I contains a copper-binding protein(s) that either supplies copper to apoSOD or equilibrates with a copper pool that is a major source of apoSOD copper. The amount of $^{64}$Cu bound in the MT fraction increased in these experiments (Fig. 4), most likely due to isotopic exchange with copper after induction of MT by copper during the preincubation period.

The results of the preincubation with copper experiments suggested that newly arrived copper is preferentially incorporated into apoSOD without significant mixing with preexisting copper pools. When lymphoblasts were incubated with 15 $\mu$M CuSO$_4$ for 15 h at 37 °C in standard growth medium. The cells were washed with medium, and cycloheximide (5 $\mu$g/ml) was added. After pretreatment with cycloheximide for 3 h, incubation was continued in the same medium plus 7 $\mu$M $^{64}$Cu(II) for 15 h. Cytosols were fractionated on Superose collecting 0.22-ml fractions. Data are shown for control cells (C) that were not preincubated with stable Cu(II) and the cells that were preincubated (M) with Cu(II). The minimal effects of cycloheximide on copper incorporation and activity and the effects of immunoprecipitation of SOD on copper incorporation and activity. That copper incorporation was correlated with the detection of an apoSOD pool in a variety of copper-deficient systems (6–9, 13, 14). ApoSOD pools were also inferred for differentiating K562 cells (10, 11, 15) and differentiating HL-60 cells (12). However, the apoSOD pool in lymphoblasts that was detected here is unlikely to be due to copper deficiency, and no cell-differentiating factors were used in the lymphoblast studies. Thus, the results with lymphoblasts represent the first example of a copper-replete, nondifferentiating cell having an apoSOD pool. The possibility of normocupric, nondifferentiating cells having an apoSOD pool had been suggested by Harris (13).

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**FIG. 4.** The effect of preincubating with copper on subsequent $^{64}$Cu incorporation into apoSOD. Lymphoblasts were preincubated with 15 $\mu$M CuSO$_4$, 15 $\mu$M Cu(II) in standard growth medium. The cells were washed with medium, and cycloheximide (5 $\mu$g/ml) was added. After pretreatment with cycloheximide for 3 h, incubation was continued in the same medium plus 7 $\mu$M $^{64}$Cu(II) for 15 h. Cytosols were fractionated on Superose collecting 0.22-ml fractions. Data are shown for control cells (C) that were not preincubated with stable Cu(II) and the cells that were preincubated (M) with Cu(II).