Iron Metabolism Is Modified by the Copper Status of a Human Erythroleukemic (K562) Cell Line[1] (43465)

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Abstract. **Copper deficiency is known to result in a microcytic, hypochromic anemia. Red cells of copper-deficient animals have less hemoglobin than their copper-adequate counterparts. The objective of this work was to determine what role copper plays in maintaining hemoglobin levels. It was hypothesized that the primary defect lies in intracellular iron metabolism. The influence of copper supplementation on iron uptake and storage was examined in a cell line capable of hemoglobin synthesis. The results demonstrated that copper supplementation of human K562 cells was associated with higher cytosolic iron levels and ferritin levels. Copper supplementation of the cell culture altered the initial rate of iron uptake from transferrin and enhanced iron uptake in noninduced cells; however, in hemin-induced K562 cells, which express fewer transferrin receptors on the cell surface, copper appeared to reduce iron uptake. Subsequent studies showed that the cells were able to take up the same amount of iron from transferrin when incubated over a longer period of time (24 hr). In the noninduced (non-hemoglobin synthesizing) cells, proportionally more iron was associated with the ferritin. We concluded from these studies that copper affects both uptake and storage of iron and that copper supplementation reduces cellular iron turnover.**

[P.S.E.B.M. 1992, Vol 200]

An interaction between copper and iron has been known for some time. A lack of dietary copper impairs the utilization of iron for hemoglobin production. This results in an anemia in which the red blood cells are microcytic and hypochromic. Supplementing copper-deficient animals with iron does not alleviate the anemia. The exact biochemical mechanism is not known; this work seeks to determine at what point copper influences the utilization of iron.

Whole animal studies have shown that copper deficiency results in hepatic iron overload (1), which implies defective release of iron to the circulation. Although ceruloplasmin, through its ferroxidase activity, aids in the incorporation of iron into transferrin and, hence, its release from the liver (2), it is unlikely that the iron defect in copper deficiency is a result of depleted ceruloplasmin. Ragan *et al.* (3) showed that less than 1% of normal serum ceruloplasmin levels was required for iron incorporation into transferrin.

The hypoferremia associated with copper deficiency is transitory, but hemoglobin levels continue to decline (4), which suggests that copper may exert its influence intracellularly. Williams *et al.* (5) and Lee *et al.* (6) showed that iron uptake and heme synthesis were reduced in copper-deficient swine. Hemoglobin biosynthesis was impaired (5, 7, 8), but the heme biosynthetic enzymes were not affected by copper deficiency (9).

Copper deficiency is also known to result in lower cytochrome *c* oxidase activity (reviewed in [10, 11]). Optimal synthesis of heme from ferric iron was suggested to require an active electron transport system (5). A positive correlation between heme synthesis and cytochrome oxidase activity was found, although the correlation was weak (5). Thus, a lack of cytochrome oxidase activity does not fully explain the defect, and other cellular factors must be involved. Another difficulty in interpreting these results is that the experiments were performed with isolated mitochondria.

[1] Florida Agricultural Experiment Station Journal Series No. R-01432.

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Received January 9, 1992. [P.S.E.B.M. 1992, Vol 200]
Accepted March 3, 1992.

0037-9727/92/2004-0522$3.00/0

In order to study the intracellular utilization of iron, we chose a cell line that could be induced to use more iron, in this case, for the synthesis of hemoglobin. The K562 cell is a blast cell of the erythrocyte lineage. They can be induced to synthesize hemoglobin when incubated with 20 μmol/liter of hemin. Hemoglobin accumulation begins to occur within 24 hr and continues for up to 10 days (12). Previously, we had shown that the K562 cell expresses more binding sites for ceruloplasmin after about 48–63 hr of incubation with 20 μmol/liter of hemin (13). This suggested that the cell had a greater need for copper, which led to the working hypothesis that the increased synthesis of hemoglobin requires more copper for proper utilization of iron. Measuring changes that occur in intracellular iron due to differentiation will tell us what the normal course of events is for intracellular iron metabolism. We can then begin to discern the role of copper during the changes in iron metabolism when cells synthesize hemoglobin. Intracellular iron levels, uptake, and storage were measured in differentiating and nondifferentiating K562 cells incubated with or without a supplement of copper.

Materials and Methods

Cell Culture and Experimental Treatments. K562 cells were purchased from the American Type Culture Collection (Rockville, MD). They were cultured in RPMI 1640 medium with 10% fetal calf serum, 2 mmol/liter of glutamine, and 50 mg/liter of gentamicin. Cultures were kept at 37°C in an atmosphere of 95% air and 5% CO_2 in a humidified chamber. Cells were maintained at a density between 2.5×10^7 and 8×10^8 cells/liter and all experiments were initiated with cells at a concentration of 5×10^7/liter. Hemin (Eastman Kodak Co., Rochester, NY) was dissolved in 7.5 g/liter of sodium bicarbonate and added to cultures at a final concentration of 25 μmol/liter. This has been well established by others to induce the synthesis of hemoglobin and cause a decrease in the surface expression of transferrin receptors (12, 14, 15) and has been shown to increase the number of ceruloplasmin binding sites (13). After 66 hr, the cells were harvested and washed three times in Dulbecco's phosphate-buffered saline (DPBS). Cell number was determined by hemocytometer counting and viability, by exclusion of trypan blue. A standard 1 g/liter of solution of copper (from metal in 0.2 ml/liter of nitric acid; GFS Chemicals, Columbus, OH) was sterile filtered and added directly to cultures for a final concentration of 0.5 mg/liter.

Cytosol Preparation. Washed cells were suspended in ultrapure, distilled, deionized water (Millipore Mill-Q water system) containing 0.5 g/liter of EDTA, 0.1 mmol/liter of phenylmethylsulfonyl fluoride, and 0.5 mg/liter of pepstatin A. They were then frozen and thawed twice to ensure disruption, and then centrifuged at 100,000*g* for 45 min at 4°C. This cytosolic preparation was used for determining Cu_2Zn_2 superoxide dismutase (Cu-ZnSOD) activity, ferritin protein, immunoprecipitation of ferritin, and copper and iron concentrations. Unless otherwise noted, the cell concentration was 10^{10}/liter.

Cu-ZnSOD Activity. Cu-ZnSOD activity was examined in cytosolic preparations by measuring the inhibition of the autoxidation of pyrogallol (Sigma Chemical Co., St. Louis, MO) spectrophotometrically at 340 nm (16). The assay was performed in 96-well microtiter plates at 25°C. Pyrogallol reagent (1 mmol/liter) was prepared in 10 mmol/liter of HCl and contained 1 mmol/liter of diethylenetriaminepentaacetic acid (DPTA). Tris buffer (50 mmol/liter; pH 8.2) also contained 1 mmol/liter of DPTA. Each reaction well contained 10 μl of cell supernatant, 50 μl of pyrogallol reagent, and 100 μl of 50 mmol/liter of Tris-HCl buffer reagent. The reaction was monitored for 3 min. The activity of Cu-ZnSOD was determined by subtracting the activity due to the mangano form of superoxide dismutase found in cell supernatants treated with 2.0 mmol/liter of KCN from the total superoxide dismutase activity. Linearity of the assay was established with known amounts of Cu-ZnSOD (Sigma) and was found to range between 10% and 65% inhibition of the autoxidation of pyrogallol. The volume of cell sample used was within this range of linearity. Enzyme activity is expressed in units/10^7 cells, with 1 unit equal to a 50% inhibition of pyrogallol's autoxidation.

Copper Analysis. Copper was analyzed by flameless atomic absorption spectrophotometry (Perkin Elmer model 460 atomic absorption spectrophotometer equipped with a graphite furnace with HGA 2200 controller and HGA ramp accessory). Samples (5 μl) of the cell cytosolic preparations were injected into graphite tubes, dried at 130°C for 40 sec (30-sec ramp time), charred at 700°C for 30 sec (15-sec ramp time), and atomized for 10 sec at 2600°C without the ramp accessory.

Iron Analysis. Iron was analyzed by flameless atomic absorption spectrophotometry. Samples of the cell cytosolic preparation were first heated for 15 min at 90°C with 50 g/liter of trichloroacetic acid and cooled. After diluting 1/1 with distilled deionized water, the samples were centrifuged at 12,000*g* for 3 min at 22°C. Aliquots of the supernatant (5 μl) were injected into the HGA furnace, dried at 105°C for 20 sec, charred at 1,000°C for 25 sec, and atomized at 2,400°C for 5 sec.

Ferritin Protein. Ferritin protein was quantitated by enzyme-linked immunosorbent assay using standard methodology. Rabbit anti-human ferritin antibodies and *o*-phenylenediamine were purchased from Dako Corp., Carpinteria, CA, and human ferritin was obtained from ICN Biomedical Co., Costa Mesa, CA. Cell

cytosol preparations representing 10^{10} cells/liter were diluted 1/5 in 10 g/liter of bovine serum albumin and 10 μl were analyzed.

Radiolabeling of Transferrin. A 0.1-mmol/liter solution of apotransferrin (Sigma) was prepared in 10 mmol/liter of HEPES buffer (pH 7.4). One volume of 100-mmol/liter sodium bicarbonate was added to 9 vol of the apotransferrin. Ferrous ammonium sulfate (0.2 mmol/liter in 0.01 mol/liter of HCl) was mixed with 1×10^5 cpm of ^{59}Fe (ICN Radiochemicals Co., Costa Mesa, CA). The radioactive ferrous ammonium sulfate solution was then added in 100-μl aliquots to the apotransferrin/bicarbonate solution. One minute between additions of ferrous ammonium sulfate was allowed for equilibration. Saturation of the apotransferrin with iron was monitored in a Perkin Elmer Lambda 4B spectrophotometer at 280 nm and 470 nm. Saturated diferric transferrin has a A_{470}:A_{280} ratio of 0.0439. Transferrin that was prepared in this way was 95–97% saturated and had 2500 Bq ^{59}Fe/mg.

Iron Uptake Experiments. Two methods were used to examine iron uptake. Rate of uptake over a short time period was studied in cells that had been incubated for 66 hr with or without hemin and supplemented with copper. The cells were washed three times in DPBS to remove culture media and the copper. They were then resuspended in DPBS containing 10 g/liter of bovine serum albumin warmed to 37°C at a concentration of 10^{10} cells/liter. Radioactive ^{59}Fe-transferrin (20 μmol/liter) was added to each treatment and the cells were allowed to incubate in a 37°C water bath and aliquots were removed at times from 0 to 45 min. The aliquots were washed twice in DPBS and once in a mild acid wash (0.2 mole/liter of acetic acid, pH 2.2) to remove surface-bound transferrin (17). Radioactivity in the cell pellet was determined in a Beckman 5500 gamma counter.

Longer experiments were performed in order to measure labeling of intracellular ferritin. The treatments of hemin and/or copper were added to the cultures and the cells were allowed to incubate for 66 hr as before. Radioactive ^{59}Fe-transferrin (20 μmol/liter) was then added directly to culture flasks and the cells were incubated overnight for 20 hr. When the radioactivity was added, cells were in the log phase of growth at a concentration of $2–3 \times 10^8$ cells/liter. After the overnight incubation with the radioactivity, the cells were washed three times in DPBS and a cytosol extract was prepared as described in Materials and Methods: Cytosol Preparation (above).

Immunoprecipitation of Ferritin. Ferritin was immunoprecipitated from the cytosol of cells incubated overnight with ^{59}Fe-transferrin. Rabbit anti-human ferritin antibodies (Dako) were diluted 1/20 in DPBS and 10 μl of diluted antibody were incubated with 100 μl of cytosol extract equivalent to 10^{10} cells/liter for 3 hr. Controls were cytosol extracts incubated with no primary antibody and cytosol extracts to which an excess of unlabeled ferritin protein was added. After 3 hr, 10 μl of *Staphylococcus aureus* (Cowan strain) (Sigma) were added and incubated for 20 min. The suspension was centrifuged at 10,000*g* for 3 min at 25°C. The pellet was washed twice with DPBS. Radioactivity was determined in the washed pellets using a Beckman 5500 gamma counter.

Data Treatment and Statistics. Results are expressed on a per cell basis due to the greater hemoglobin protein and total DNA levels in the hemin-treated cells. Data were analyzed by one-way analysis of variance using the InStat statistical program by GraphPad. An adjusted *t* test with *P*-values corrected by the Bonferroni method was used for multiple comparisons. A *P*-value of less than 0.05 was defined as significant.

Results

Copper supplementation of noninduced K562 cells resulted in a 2-fold greater copper concentration, but had little effect on Cu-ZnSOD activity (Table I). In hemin-induced cells, copper supplementation elevated the intracellular copper levels and doubled the activity of Cu-ZnSOD. These two indices of copper nutriture were correlated with a greater cytosolic level of iron. The copper-treated noninduced cells had nearly twice the level of iron as noninduced control cells. The copper-treated hemin-induced cells had 20% greater iron concentration compared with the hemin-induced control cells.

Uptake of ^{59}Fe from transferrin into cells was measured to determine whether alterations in the rate of uptake were responsible for alterations in intracellular iron levels. Copper-supplemented noninduced K562 cells took up more radioactivity during the first 10 min than the noninduced controls (Fig. 1). By 45 min, both copper-treated and control cells had taken up the same amount of radioactive iron. Hemin-induced cells showed the opposite effect: Copper supplementation appeared to result in a slower uptake of ^{59}Fe from transferrin. Thus, the rate of uptake, at least in the hemin-treated cells, could not account for the greater cytosolic iron levels. But again, as in the noninduced cells, there was no significant difference in the radioactivity found in the cell pellet after 30 min of incubation.

The rate of iron uptake did not explain why the cells had a greater level of cytosolic iron; therefore, the relationship between copper supplementation and long-term iron uptake and storage was investigated. The percentage of added ^{59}Fe from transferrin found in the cytosol of cells over a longer time period was not significant among groups; the cells took up about 10% of the radioactivity that was added (Table II). Copper supplementation of the culture media resulted in

Table I. Copper and Iron Levels and Cu-ZnSOD Activity Is Enhanced by Copper Supplementation[a]

	Copper (ng/10^7 cells)	Cu-ZnSOD activity (units/10^7 cells)	Iron (ng/10^7 cells)
Noninduced	2.91 ± 0.66*	1.93 ± 0.28†	31.81 ± 1.16‡
Noninduced + Cu	6.13 ± 0.60†	2.33 ± 0.17†,§	55.34 ± 2.75§
Hemin	5.23 ± 0.60†	1.40 ± 0.16*	37.09 ± 1.19*
Hemin + Cu	7.77 ± 0.65§	2.79 ± 0.45§	46.30 ± 2.38†

[a] Cells were treated with or without 25 μmol/liter of hemin and/or 0.5 mg/liter of copper for 66 hr. Cu-ZnSOD activity was determined in extracts of the cytosol using pyrogallol as described in Materials and Methods. Copper and iron were determined by atomic absorption spectrophotometry, as described. The results are reported as the mean ± SD of six replicates. Means of values in a column without a common symbol (*, †, ‡, §) are significantly different by a *t* test where the Bonferroni's correction of the *P*-value is less than 0.05.

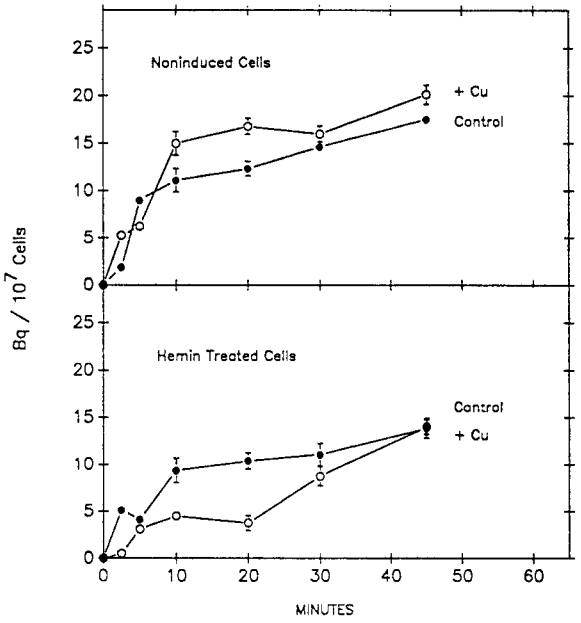

Figure 1. Short-term uptake of ^{59}Fe from transferrin in K562 cells. Cells were treated with or without 25 μmol/liter of hemin and/or 0.5 mg/liter of copper for 66 hr before the experiment. After washing and resuspending in DPBS-bovine serum albumin, 20 μmol/liter of ^{59}Fe-transferrin were added. Aliquots were removed at the times indicated and washed, and radioactivity in the cell pellet was determined. The data are the means and SD; $n = 4$.

greater ferritin levels in both the hemin-treated cells as well as the noninduced cells. The proportion of radioactivity associated with immunoprecipitable ferritin was greater in the copper-supplemented noninduced cells than in the non-copper-treated noninduced cells. This effect was not seen in the hemin-treated cells.

Discussion

Supplementing the cell culture media with copper increased both intracellular copper and the activity of a copper-requiring enzyme. The amount of copper in RPMI 1640 culture medium containing 10% fetal bovine serum is 0.5 μmol/liter. This level is adequate for growth, because copper supplementation did not stimulate the growth rate. Physiologic serum levels of copper are approximately 2 μmol/liter, but we do not know how to relate this to the requirement of the cells in culture. The cells that were not supplemented with copper had an alteration in the metabolism of iron. The magnitude of this effect was small because these cells were not overtly deficient in copper. However, it is possible that the cell culture medium is marginal in copper since supplementation increased the intracellular copper level and enhanced the activity of a copper-requiring enzyme. Copper supplementation led to changes in the intracellular metabolism of iron.

According to Dean *et al.* (12), noninduced K562 cells do not synthesize much hemoglobin; the intracellular levels are about 1 pg/cell compared with 10–15

Table II. Copper Enhances Iron Storage by K562 Cells[a]

	Cytosolic ^{59}Fe (%)	Ferritin antibody-reactive ^{59}Fe (%)	Ferritin (ng/10^5 cells)	^{59}Fe incorporation into ferritin (pg ^{59}Fe/ng ferritin)
Noninduced	10.5	38.4 ± 3.1*	11.8 ± 1.3†	219 ± 17*
Noninduced + Cu	10.9	49.2 ± 4.4‡	16.0 ± 0.6*	283 ± 25‡
Hemin	9.9	50.8 ± 5.4‡	16.7 ± 0.7*	228 ± 24*‡
Hemin + Cu	10.2	47.8 ± 0.4‡	20.5 ± 1.2‡	220 ± 2*

[a] Cells were treated with 25 μmol/liter of hemin and/or 0.5 mg/liter of copper for 66 hr. ^{59}Fe-labeled transferrin (20 μmol/liter) was added and incubated with the cells in culture for 20 hr. The cells were washed with 0.2 mmol/liter of acetic acid and radioactivity in an aliquot of the cell pellet was determined by gamma counting. Radioactive ferritin was immunoprecipitated from cytosolic extracts and ferritin protein was determined by enzyme-linked immunosorbent assay, as described in Materials and Methods. The results are reported as the mean ± SD of four replicates. Means of values in a column without a common symbol (*, †, ‡) are significantly different by a *t* test where the Bonferroni's correction of the *P*-value is less than 0.05.

pg/cell after 10 days of incubation with hemin. Noninduced K562 cells also express a greater number of transferrin receptors on the cell surface than hemin-treated cells (14, 15). Therefore, noninduced K562 cells take up more iron from transferrin than hemin-induced cells. This was true in our study as well. Iron uptake proceeds by two mechanisms: rapid transferrin-receptor-mediated endocytosis (17) and a slower phase in which iron is released from transferrin prior to uptake (18, 19). Our iron uptake studies showed a rapid phase (within 5 min), followed by a plateau and then a slower linear phase.

Copper supplementation appeared to enhance the rate of iron uptake from transferrin in the cells having more transferrin receptors (noninduced cells). On the other hand, copper supplementation decreased iron uptake from transferrin in the cells having fewer transferrin receptors (hemin-treated cells). Copper may be important in some step during transferrin-mediated endocytosis. Williams *et al.* (5) found that the uptake of iron from transferrin into reticulocytes was impaired in copper deficiency. Reticulocytes take up a majority of iron via transferrin-mediated endocytosis (19). Changes occur in the membranes of erythrocytes (20), platelets (21), and lymphocytes (22) when animals are made copper deficient. Copper deficiency also results in an increased expression of transferrin receptors on the surface of rat splenocytes (23). Our results show that providing copper will enhance iron uptake in those cells having a greater number of transferrin receptors. It suggests that the defect in iron metabolism that results from copper deficiency may occur at the plasma membrane and that this membrane defect may alter cellular iron turnover.

Over the long term, the cells were able to compensate for the differences in iron uptake. After 30–45 min, the cell had accumulated about the same amount of radioactivity whether or not copper was present. Yet the copper-supplemented cells had a greater level of cytosolic iron. Copper supplementation was associated with greater ferritin protein levels, regardless of whether or not they had been provided a source of iron (hemin). In addition, the noninduced cells put a greater proportion of that iron into the ferritin. Hemin-treated cells also had greater ferritin levels after treatment with copper, but did not increase the proportion of iron to ferritin, perhaps because iron was also utilized for hemoglobin synthesis.

The regulation of ferritin synthesis has been the subject of many recent reviews (24–27). The role of copper in ferritin metabolism has not been well studied. In the late 1970s, Theil and Calvert (28) observed an increase in ferritin in the spleen of sheep treated with excess dietary copper levels. Copper appeared to influence the reutilization of iron from old erythrocytes. Normally, the reticulocytes of the spleen phagocytose old erythrocytes and release the iron back into the circulation in two phases. The rapid phase of release was not affected while the slower phase was reduced by copper treatment, which resulted in retention of iron by the spleen of copper-treated sheep. After copper treatment, spleen ferritin took up iron faster and released it slower (29). The copper (II) complex of glysyl-histidyl-lysyl also inhibits the release of iron from ferritin (30). The molecular mechanism of how copper might reduce iron release from ferritin is not known.

In summary, although the rate of iron uptake from transferrin was not affected over the long term, the cell accumulated more iron that was subsequently stored as ferritin when the cell was supplemented with copper. Copper appears to enhance the storage of intracellular iron and, as a result, to reduce the rate of cellular turnover of iron. Therefore, if a lack of copper reduces the cell's ability to store iron, then a lack of copper would reduce the availability of intracellular iron for hemoglobin synthesis and the organism would become anemic. This should also be acknowledged as an important factor when iron deficiency anemia is treated. This study suggests that adequate amounts of copper should be provided when dietary iron supplements are prescribed in order to reduce iron turnover and maintain iron levels.

This project was supported in part with funds provided by the Center for Nutritional Sciences, University of Florida.

This work was presented in part at the 75th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlanta, GA, April 1991 (Percival SS, Armstrong E. Copper's influence on iron metabolism in K562 cells [Abstract 5339]. FASEB J **5**:A1291.

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