Copper Deprivation Potentiates Oxidative Stress in HL-60 Cell Mitochondria (44397)

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Abstract. **Cytochrome-*c* oxidase is the copper-dependent terminal respiratory complex (complex IV) of the mitochondrial electron transport chain whose activity in a variety of tissues is lowered by copper deficiency. Because inhibition of respiratory complexes increases the production of reactive oxygen species by mitochondria, it is possible that copper deficiency increases oxidative stress in mitochondria as a consequence of suppressed cytochrome-*c* oxidase activity. In this study, the activities of respiratory complex I + III, assayed as NADH:cytochrome-*c* reductase, complex II + III, assayed as succinate:cytochrome-*c* reductase, complex IV, assayed as cytochrome-*c* oxidase, and fumarase were measured in mitochondria from HL-60 cells that were grown for seven passages in serum-free medium that was either unsupplemented or supplemented with 50 n*M* $CuSO_4$. Fumarase activity was not affected by copper supplementation, but the complex I + III:fumarase and complex IV:fumarase ratios were reduced 30% and 50%, respectively, in mitochondria from cells grown in the absence of supplemental copper. This indicates that copper deprivation suppressed the electron transfer activity of copper-independent complex I + III as well as copper-dependent complex IV. Manganese superoxide dismutase (MnSOD) content was also increased 49% overall in the cells grown in the absence of supplemental copper. Furthermore, protein carbonyl groups, indicative of oxidative modification, were present in 100-kDa and 90-kDa proteins of mitochondria from copper-deprived cells. These findings indicate that in cells grown under conditions of copper deprivation that suppress cytochrome-*c* oxidase activity, oxidative stress in mitochondria is increased sufficiently to induce MnSOD, potentiate protein oxidation, and possibly cause the oxidative inactivation of complex I.** [P.S.E.B.M. 1999, Vol 221]

The mitochondrial electron transport chain is composed of four oligomeric enzymes: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome-*c* oxidoreductase (complex III), and ferrocytochrome-*c*:oxygen oxidoreductase (complex IV) (1). Electrochemical energy from electron transfer between these complexes to molecular oxygen drives the vectorial translocation of protons across the inner mitochondrial membrane that ultimately provides the energy for ATP synthesis. Complex IV, commonly named cytochrome-*c* oxidase, is a cuproenzyme whose catalytic activity in a variety of tissues is reduced by copper deficiency (2–4). However, a relationship between reduced cytochrome-*c* oxidase activity and defective energy metabolism in causing the pathophysiological consequences associated with copper deficiency has not been clearly established. For instance, a 70%–90% reduction in cytochrome-*c* oxidase activity during copper deficiency either has no effect or only modestly lowers brain, liver, and platelet ATP content (5–9). Thus, if reduced cytochrome-*c* oxidase activity contributes to the biological consequences of copper deficiency, then an alternative to impaired energy production as a mechanism needs to be found.

Although electron transport consumes about 85%–90% of the oxygen utilized by cells, not all of the oxygen consumed is converted to water; about 1%–2% is converted to

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superoxide (10–12). Much of the superoxide formed is converted into hydrogen peroxide by manganese-superoxide dismutase (MnSOD), but enough superoxide remains unscavanged to react further with hydrogen peroxide to form hydroxyl radical by the Haber-Weiss reaction catalyzed by mitochondrial iron (10). Thus mitochondria are a primary source for potentially damaging reactive oxygen species (ROS) in aerobic cells.

Mitochondrial ROS production is largely determined by the redox state of the electron transport chain, and ROS generation is highest when the respiratory complexes are highly reduced (10). If electron flow is blocked near the terminal end of the electron transport chain, then the reducing potential of respiratory complexes upstream from the blockage increases and ROS production increases. This principle was demonstrated in mitochondria from the flight muscles of houseflies (13). When these mitochondria were treated with the respiratory inhibitors, roteneone and antimycin A, their rate of hydrogen peroxide production was increased. The rate of hydrogen peroxide production also was increased by partial inhibition of cytochrome-*c* oxidase by cyanide or myxothiazole. Thus, even though inhibition of cytochrome-*c* oxidase by copper deficiency has little effect on mitochondrial ATP production, the inhibition could increase mitochondrial hydrogen peroxide production. It is possible, therefore, that some pathological consequences of copper deficiency occur because partial inhibition of cytochrome-*c* oxidase leads to increased oxidative stress to mitochondria that cannot be overcome by normal mitochondrial antioxidant defenses. This study examined protein oxidation and MnSOD content as signs of oxidant stress in mitochondria from a human cell line, HL-60, cultured under conditions of copper deprivation sufficient to lower cytochrome-*c* oxidase activity.

Materials and Methods

Cell Culture and Mitochondrial Isolation. HL-60 cells, a human promyelocytic cell line, were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in static suspension in serum-free defined medium (DFM) consisting of Iscove's Modified Dulbecco's Medium (Sigma Chemical Co., St. Louis, MO) supplemented with iron, insulin, and apo-transferrin as described previously (14). Cells were subcultured in DFM at 5–6 day intervals. On the third passage in DFM, two groups of cells were established by subculturing into DFM that was either unsupplemented or supplemented with 50 n*M* $CuSO_4$ (DFM + Cu). The two groups of cells were maintained for the duration of the experiment in either DFM or DFM + Cu.

At the end of each passage in either DFM or DFM + Cu, 25×10^6 cells were reserved for mitochondrial isolation. The cells were centrifuged (200 g for 10 min, 4°C), washed in buffer containing 136 m*M* NaCl, 5 m*M* KCl, and 10 m*M* $NaPO_4$ (pH 7.4), and resuspended in 2 ml of cold buffer containing 0.25 *M* sucrose, 0.01 *M* Tris-HCl, 0.001 *M* EDTA (pH 7.6) (15). The cell membranes were ruptured by using nitrogen decompression (16). Cell suspensions were pressurized on ice in a cell disruption bomb (Parr Instrument Co., Moline, IL) for 5 min at 300 psi. Our preliminary studies using fumarase as a mitochondrial marker indicated that pressurization at 300 psi for 5 min allowed maximal recovery of mitochondria from the disrupted cells. Following disruption, the suspensions were centrifuged (750 g for 5 min, 4°C) and the pellet containing cellular debris and nuclei was discarded. The supernatant was centrifuged (12,000*g* for 10 min, 4°C) and the resultant mitochondrial pellet was resuspended in 0.05 *M* KPO_4 (pH 7.4).

Enzyme Assays. NADH cytochrome-*c* reductase (EC 1.6.99.1), succinate cytochrome-*c* reductase (EC 1.3.99.1), and cytochrome-*c* oxidase (EC 1.9.3.1) activities were measured by using the sequential assay system described by Davies (17). NADH cytochrome-*c* reductase activity represents the combined rate of electron transport by complex I + III to cytochrome-*c*, succinate cytochrome-*c* reductase activity represents the combined rate of electron transfer by complex II + III to cytochrome-*c*, and cytochrome-*c* oxidase activity represents the rate of electron transfer from cytochrome-*c* to complex IV. The reaction mixture contained 50 μ*M* cytochrome-*c*. Fumarase activity was assayed by using L-malate as substrate and monitoring the formation of fumarate specrophotometrically at 240 nm (18). All assays were performed at 30°C with 20–40 μg of mitochondrial protein. Protein concentrations were determined with bichinchoninic acid (19).

Determination of Oxidatively Modified Proteins and Mn-Superoxide Dismutase. Oxidatively modified proteins were detected by Western blot immunoassay using a commercially available kit (Oxyblot Oxidized Protein Detection Kit, Oncor, Inc., Gaithersburg, MD) based on the methodology of Shacter (20). Briefly, the carbonyl groups of mitochondrial proteins were derivatized to 2,4-dinitrophenylhydrazone by treating mitochondria with 2,4-dinitrophenylhydrazine. The proteins were then separated by SDS polyacrylamide gel electrophoresis on 10% acrylamide gels and transferred to PVDF membrane by semidry electroblotting. The blotted proteins were then screened with an antibody specific for the dinitrophenyl moiety.

MnSOD also was detected by Western blot immunoassay. Samples were prepared by mixing 150 μl of cell suspension containing 25×10^6 cells/ml with 75 μl of 0.188 *M* Tris (pH 6.8) containing 6% sodium dodecyl sulfate, 30% glycerol, 0.12 *M* dithiothreitol, and 0.003% bromophenol blue, and incubating for 45 min at 37°C. Samples containing 30 μg of protein were subjected to electrophoresis on 10% acrylamide gels and transferred to PVDF membrane by semidry electroblotting. The blots were blocked for 2 hr at room temperature with 5% dry milk in TTBS (10 m*M* Tris, 5% dry milk, 150 m*M* NaCl, 0.1% Tween 20, pH 8.0). After blocking, the blots were incubated for 2 hr with anti-MnSOD antibody (Calbiochem, San Diego, CA) diluted 1:400 in 1% dry milk in TTBS. The blots were then incu-

bated 1 hr with horseradish peroxidase-coupled anti-sheep IgG (Amersham Life Science, Inc., Arlington Heights, IL).

Visualization of protein carbonyls and MnSOD was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western blotting detection reagents and Hyperfilm-ECL, Amersham Life Science, Inc.). Immunoreactive bands representing protein carbonyls and MnSOD were analyzed by scanning densitometry (GS300 Scanning Densitometer, Hoefer Scientific Instruments, San Fransico, CA).

Statistics. Data showing the effects of copper supplementation on respiratory complex and fumarase activities and the scanned areas representing immunoreactive MnSOD on Western blots were analyzed by analysis of variance (ANOVA). Also, to minimize the effects of variations between Western blots, areas for MnSOD were ranked within individual blots and then analyzed by Freidman's nonparametric ANOVA.

Results

Passage number significantly ($P = 0.02$, ANOVA) affected the specific activity of fumarase activity in mitochondria isolated from three separate sets of cultured cells at different passages (data not shown). However, fumarase activity was not significantly affected by copper supplementation ($P = 0.32$, ANOVA). The finding that fumarase activity depended on passage number indicates that mitochondrial enrichment of the cellular fraction used to measure enzymatic activities of the respiratory complexes varied from passage to passage. To correct for the effect of variation in mitochondrial enrichment on respiratory complex activities, the measured activities were normalized to fumarase activity in the mitochondrial fraction rather than to protein concentration. The effect of copper supplementation on the ratio of respiratory complex activities to fumarase activities is shown in Table I. Copper supplementation had a significant effect on the complex I + III:fumarase and complex IV:fumarase ratios. For Passages 3 through 9, the overall means for the complex I + III:fumarase ratio were 137.8 ± 11.2 and 97.2 ± 10.2 (mean ± pooled standard error) for cells grown in copper-supplemented and unsupplemented medium, respectively. The overall means for the complex IV:fumarase ratio were 71.2 ± 6.4 and 33.6 ± 5.8 for cells grown in copper-supplemented and unsupplemented medium, respectively. Overall, the activities of complex I-III and complex IV relative to fumarase activity were about 30% and 50% lower, respectively, in mitochondria isolated from cells cultured in the absence of supplemental copper. The effects of passage and copper × passage interaction on the ratios were not statistically significant.

Preliminary examination of copper concentrations in HL-60 cells grown under conditions identical to the conditions of the present study indicated that supplementing DFM with copper increases cellular copper content. In a total of 15 samples collected from each treatment group from Passages 4 through 8, copper concentrations (mean ± SD) were 72 ± 27 pg Cu/10^6 cells and 656 ± 222 pg Cu/10^6 cells in cells grown in the absence and presence of 50 n*M* $CuSO_4$, respectively ($P < 0.05$, AVOVA). Thus, the reduction in the activity of copper-dependent complex IV in cells grown in the absence of supplemental copper in the present study is consistent with the preliminary finding that growing cells in the absence of supplemental copper lowers cellular copper concentrations.

A Western blot of HL-60 cell homogenates from copper-supplemented and unsupplemented cells at Passage 9 is shown in Figure 1. This blot is typical in that MnSOD was detected as a single immunoreactive protein band corresponding to a protein with an approximate molecular weight of 20 kDa. It also appears that MnSOD content may be higher overall at Passage 9 in the samples from unsupplemented cells. This was confirmed by scanning densitometry. Figure 2 shows the areas of the protein band representing MnSOD at Passages 3 through 9. The effect of copper supplementation was significant because the area of the MnSOD band was higher in the absence of supplemental

Table I. Ratio of Respiratory Complex Activities to Fumarase Activity in Mitochondria of HL-60 Cells Cultured in Copper Supplemented and Unsupplemented Medium

Passage	Complex I + III/Fumarase		Complex II + III/Fumarase		Complex IV/Fumarase	
	+Cu	−Cu	+Cu	−Cu	+Cu	−Cu
3	76.4 ± 40.4 (3)	96.0 ± 13.0 (3)	22.6 ± 6.6 (3)	23.4 ± 5.6 (3)	56.9 ± 14.5 (3)	25.4 ± 4.8 (3)
4	119.2 ± 86.7 (3)	65.1 ± 22.6 (3)	20.2 ± 11.2 (3)	18.6 ± 5.2 (3)	49.2 ± 28.5 (3)	26.2 ± 2.7 (3)
5	137.1 ± 43.6 (3)	72.4 ± 4.4 (3)	35.6 ± 12.8 (3)	27.4 ± 5.8 (3)	68.2 ± 15.2 (3)	27.9 ± 3.6 (3)
6	130.5 ± 46.6 (3)	94.4 ± 41.9 (3)	21.8 ± 12.8 (3)	21.3 ± 10.5 (3)	66.7 ± 26.4 (3)	27.8 ± 9.8 (3)
7	208.2 ± 139.7 (2)	105.3 ± 31.4 (3)	44.2 ± 36.1 (2)	23.5 ± 0.8 (3)	115.2 ± 109.0 (2)	24.2 ± 3.6 (3)
8	140.0 ± 14.5 (2)	113.7 ± 22.5 (3)	18.0 ± 15.0 (2)	31.8 ± 6.7 (3)	60.8 ± 4.4 (2)	55.9 ± 27.8 (3)
9	152.9 ± 9.4 (2)	133.8 ± 7.7 (3)	33.2 ± 3.2 (2)	30.8 ± 5.2 (3)	81.9 ± 9.6 (2)	47.8 ± 8.5 (3)
ANOVA						
Cu	P = 0.01		P = 0.46		P = 0.0002	
Passage	P = 0.17		P = 0.23		P = 0.40	
Cu × Passage	P = 0.49		P = 0.41		P = 0.34	

Note. Values are means ± SD for the number of independent determinations shown in parentheses.

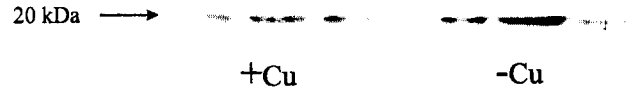

Figure 1. A Western blot showing the presence of immunoreactive MnSOD in homogenates of HL-60 cells. Each lane contains 30 μg of protein from six separate cultures of cells grown in either (Lanes 1–3) copper supplemented (+Cu) or (Lanes 4–6) unsupplemented (–Cu) medium.

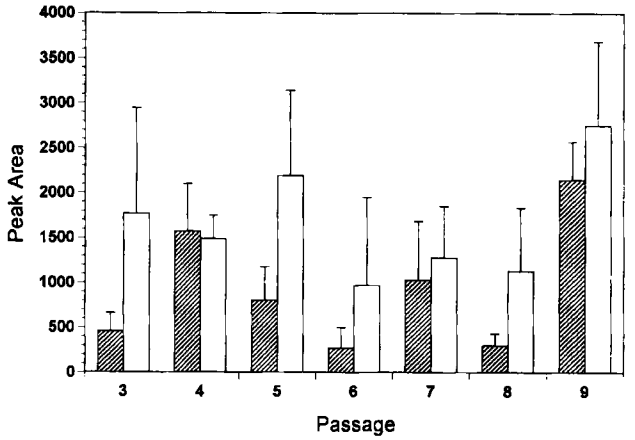

Figure 2. Areas of peaks representing immunoreactive MnSOD on Western blots of mitochondrial proteins from HL-60 cells grown in the presence (hatched bars) and absence (white bars) of supplemental copper. Values are means ± SD obtained from cells in three separate cultures at the indicated passages. All cell homogenate samples from a given passage were separated on a single electrophoresis gel (30 μg protein/lane) and blotted to produce a single immunoblot for each passage. Effect of Cu, $P = 0.001$; effect of passage, $P = 0.0007$; effect of Cu × passage, NS as determined by ANOVA.

copper at all passages except for Passage 4. The effect of passage on the area of the MnSOD band was also significant. However, the change in area across passages showed no systematic pattern. Considering that all samples collected from a single passage were blotted onto a single membrane, the effect of passage may be an artifact caused by the variations in protein transfer that normally occur between blots. To correct for this variation, the areas in each blot (i.e., each passage) were ranked and analyzed by nonparametric ANOVA. For all passages, the ranked areas (means ± SD) for the MnSOD band were 2.81 ± 1.57 and 4.19 ± 1.63 for cells grown in the presence and absence of supplemental copper, respectively ($P = 0.008$, Freidman's ANOVA). Thus, copper deprivation caused an overall increase of 49% in cellular MnSOD content.

Western blots of mitochondria treated with 2,4-dinitrophenylhydrazine contained a range of proteins that were immunoreactive to antidinitrophenyl antibody. This indicated that numerous mitochondrial proteins contain 2,4-dinitrophenylhydrozone derivatives of carbonyl groups. Densitometry scans of immunoblots containing derivatized mitochondrial proteins from HL-60 cells harvested at Passages 4 and 9 are shown in Figure 2. At Passage 4, the profiles for derivatized proteins in mitochondria from cells grown in copper-supplemented and unsupplemented medium were similar, showing the presence of carbonyl groups in proteins with molecular weights between 80 and 210 kDa. At Passage 9, derivatized carbonyl groups were still observed in proteins in the 80–210-kDa range, but derivatized carbonyls were diminished in proteins with molecular weights of 90 and 100 kDa in the mitochondria of copper-supplemented cells. Carbonyl groups were also present in the 90 and 100-kDa proteins of mitochondria isolated from cells grown in unsupplemented and copper-supplemented medium at Passage 7, but were somewhat diminished in the copper-supplemented cells (data not shown).

Discussion

In the present study, the activities of complex I + III (NADH cytochrome-*c* reductase) and complex IV (cytochrome-*c* oxidase) relative to fumarase activity were reduced in HL-60 cells cultured in the absence of supplemental copper. Furthermore, the activities and the effect of copper supplementation on the activities of complexes I + III and IV were both independent of passage number. This indicates that complex I + III and IV activities were in a steady state across the passages and that the absence of copper supplementation suppressed their steady state activities. The reduction in complex I-III activity could reflect an effect of copper deprivation on either complex I or complex III. However, copper deprivation did not affect complex II-III activity. If copper deprivation impaired complex III activity, then both complex I-III and Complex II-III would have been affected. Thus, the reduction in complex I-III activity caused by copper deprivation is a result of impaired complex I activity.

Our findings regarding MnSOD suggest that reduced complex I and IV activities in copper-deprived cells potentiated mitochondrial oxidative stress. The expression of MnSOD, a mitochondrial matrix enzyme involved in the detoxification of superoxide, can be increased by H_2O_2 and other oxidants (21, 22). Its close proximity to the electron transport chain, a major source of ROS, and its sensitivity to induction by oxidants indicates that MnSOD may be induced specifically by ROS generated by the mitochondria. That MnSOD can be induced by an increase in ROS production caused by inhibition of respiratory complexes is substantiated by the observation that the rate of superoxide production by mitochondria is inversely proportional to MnSOD content in skin fibroblasts from patients with complex I deficiency (23). Thus, the overall increase in immunoreactive MnSOD in cells grown in the absence of supplemental copper indicates that the reduction in complex I and IV activities that accompanied copper deprivation was sufficient to increase mitochondrial ROS production and create a pro-oxidant environment capable of inducing MnSOD. However, MnSOD was not affected by copper supplementation in Passage 4 even though the activities of complexes I and IV were higher in copper-supplemented cells. This discrepancy suggests that even though copper supplementation raised the activities of complexes I and IV in Passage 4, oxidative stress may not have been sufficiently amelio-

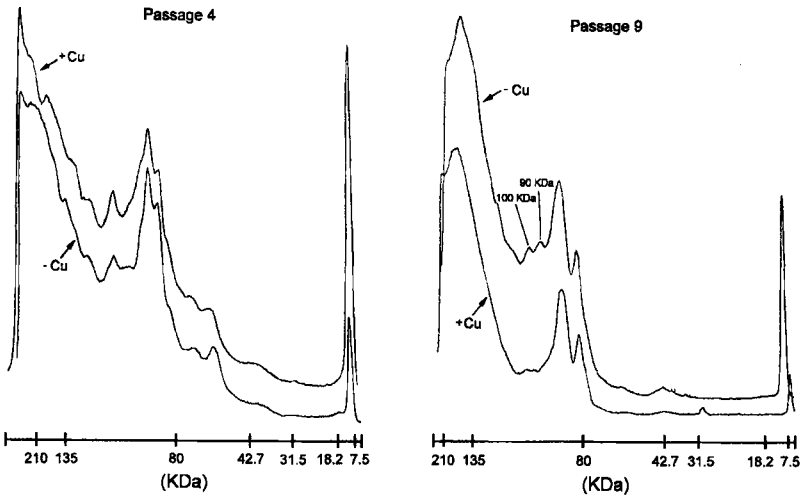

Figure 3. Densitometry scans showing the immunoreactive dinitrophenyl derivatives of carbonyl groups in mitochondrial proteins from HL-60 cells at Passages 4 and 9 in the presence (+Cu) and absence (–Cu) of supplemental copper. Isolated mitochondria were derivatized with dinitrophenylhydrazine, and the derivatized proteins were separated by electrophoresis, blotted, and detected by antidinitrophenyl antibody as described in Materials and Methods. Each profile represents 1.5 μg of mitochondrial protein. Migration distances for molecular weight standards are shown on the horizontal axis.

rated by copper supplementation to lower the induction of MnSOD.

The oxidation of proteins converts some of their amino acid side chains to carbonyl derivatives, and the presence of carbonyl groups in proteins can be used as a marker of ROS-induced protein oxidation (20, 24, 25). Protein carbonyl groups were readily detectable in mitochondrial proteins with molecular weights between 80 and 210 kDa in cells grown in either copper-supplemented or unsupplemented medium. This finding is consistent with previous findings showing that oxidative damage to mitochondrial proteins, lipids, and DNA is a normal consequence of aerobic metabolism (11, 12). The similarity in the pattern of mitochondrial proteins containing carbonyl groups in unsupplemented and copper-supplemented cells in Passage 4 suggests that the unsupplemented and supplemented cells experienced similar levels of oxidative stress. Copper supplementation also did not change MnSOD content at Passage 4. Thus, the impact of oxidative stress on mitochondria as indicated by oxidative modifications of mitochondrial proteins and the induction of MnSOD was not reversed by copper supplementation at Passage 4. However, carbonyl groups that had been present in 90- and 100-kDa proteins in Passage 4, shortly after copper supplementation had begun, were essentially undetectable by the ninth passage in copper-supplemented medium. The additional burden of oxidized mitochondrial proteins with molecular weights of 90 and 100 kDa in cells grown continuously in the absence of supplemental copper indicates that copper deprivation potentiates normal oxidation of mitochondrial proteins. This is accordant with the hypothesis that copper deprivation increases mitochondrial oxidative stress by lowering complex I and IV activities and increasing ROS production by the electron transport chain.

Our finding that copper deprivation reduces NADH:cytochrome-*c* reductase activity of HL-60 cell mitochondria is consistent with a previous study showing that copper deficiency reduces this activity in hepatic mitochondria (17). It has also been shown that copper deficiency, possibly by reducing the capacity to oxidize NADH, impairs glutamate oxidation in brain mitochondria (6). Although these findings all suggest that copper deprivation produces a defect in complex I that inhibits the oxidation of NADH, they are difficult to interpret because complex I is not a cuproenzyme and is not directly dependent on copper for catalytic activity. Loss of mitochondrial integrity may have contributed to the reduction of complex I activity. However, if loss of mitochondrial integrity were a factor, then the succinate: cytochrome-*c* reductase activity of complex II-III would also have been affected, but copper deprivation did not affect this activity. Furthermore, losses in mitochondrial integrity that may have influenced respiratory complex activities were adjusted by normalizing the activities to fumarase activity. Thus, loss of mitochondrial integrity cannot fully account for reduced complex I activity in copper-deprived cells. Although an explanation for the loss of complex I activity is beyond the scope of the present study, mitochondrial protein oxidation was potentiated in cells grown in the absence of supplemental copper, and oxidative damage to complex I may have contributed to the loss of its activity. This explanation is supported by a previous study showing that several enzymes located in the mitochondrial inner membrane, including complex I, can be oxidatively inactivated (26). However, the effect of copper deprivation on complex I may be more specific than general oxidative inactivation of enzymes associated with the inner mitochondrial membrane. It is known that copper deficiency depresses the nuclear-encoded subunits IV and V of cytochrome-*c* oxidase in heart mitochondria (27, 28). Although reasons for the supression are not clear, one hypothesis is that copper deficiency impairs importation of the nuclear-encoded subunits into the mitochondria. A similar mechanism could affect complex I in HL-60 cells grown in the absence of supplemental copper. Complex I has 32 nuclear-encoded subunits (29), and failure to import one or more of them into the mitochondria of copper-deprived cells could specifically lower complex I activity.

In summary, our study showed that HL-60 cells grown in the absence of supplementary copper exhibit inhibited complex I and IV activities, increased MnSOD content and

oxidative modifications of some mitochondrial proteins. Together, these finding suggest that copper deprivation can increase oxidative stress in mitochondria by suppressing respiratory complex activity and increasing mitochondrial ROS production. Although suppression of cytochrome-*c* oxidase activity during copper deficiency is usually not sufficient to impair mitochondrial ATP production (5–9), it may potentiate oxidative stress in mitochondria and cause oxidative lesions whose accumulation contributes to the pathological consequences of copper deficiency.

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