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Altered nucleotide content and changes in mitochondrial energy states associated with copper deficiency in rat platelets

W. Thomas Johnson, Steven N. Dufault, and Samuel M. Newman, Jr.

United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND USA*

Dietary copper deficiency severely reduces platelet cytochrome c oxidase activity, but the effect of this perturbation on platelet energy metabolism is unknown. In this study, the effect of copper deficiency on platelet adenine nucleotide and GTP content was determined. Structural changes in platelet mitochondria during copper deficiency also were examined. Copper deficiency caused a 24% reduction in adenosine triphosphate (ATP) content, a 113% increase in adenosine monophosphate (AMP) content, and a 36% reduction in GTP content. Because the secretory pool of ATP was not affected by copper deficiency, the changes observed in platelet nucleotide content most likely reflect an effect on the metabolic nucleotide pool. Furthermore, the number of platelet mitochondria exhibiting swollen cristae and high matrical density, characteristics suggesting that mitochondria are engaged in oxidative phosphorylation, was increased by copper deficiency. These findings indicate that copper deficiency can alter energy metabolism in platelets in a manner that is consistent with partial blockage of mitochondrial electron transport and reduced ATP production. (J. Nutr. Biochem. 6:551–556, 1995.)

Keywords: copper deficiency, platelets, nucleotides, mitochondria

Introduction

Platelets are anucleate cells that change shape, secrete the contents of their storage granules, and aggregate in response to a variety of extracellular signals. Previously, it was found that copper deficiency can affect cytoskeletal remodeling and dense granule secretion in thrombin-stimulated rat platelets by a mechanism that may involve altered signaling through a protein kinase C–dependent pathway.[1,2] Because there are no known cuproenzymes present in platelet signal transduction pathways, it is most likely that the effect of copper deficiency on thrombin-induced secretion and related signaling mechanisms is a consequence of impaired cuproenzyme activity in a nonsignaling pathway.

Platelet responses occur when stimulation triggers a series of concerted signal-transducing processes that increase the intracellular concentrations of second messengers and change the phosphorylation, enzymatic activities, and structure of proteins.[3] Because several of these processes utilize adenosine triphosphate (ATP), platelet responses require a readily available intracellular pool of ATP.[4] Thus, one-way copper deficiency could affect signal-response coupling in platelets by reducing the production of cellular energy.

Cytochrome c oxidase is a cuproenzyme that serves as the terminal complex of the mitochondrial electron transport chain. Because of its role in mitochondrial electron transport, cytochrome c oxidase is essential for the orderly flow of electrons from Krebs cycle intermediates to molecular oxygen that provides energy for ATP synthesis. Thus, reduction in cytochrome c oxidase activity has the potential to disrupt mitochondrial ATP production. However, even though copper deficiency reduces cytochrome c oxidase ac-

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Address reprint requests to Dr. W. Thomas Johnson, USDA, ARS, Grand Forks Human Nutrition Research Center, P.O. Box 9034, University Station, Grand Forks, ND 58202-9034, USA.

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tivity in a number of organs,[5,6] it usually has only a modest effect on ATP production. For instance, ATP concentrations in liver, brain, and heart of mice and rats are either unaffected or only slightly reduced when cytochrome c oxidase activity in these organs is reduced 70 to 80% by copper deficiency.[7–10] The lack of a significant effect of reduced cytochrome c oxidase activity on ATP production may be related to the abundance of mitochondria in liver, brain, and heart and the high respiratory capacities of these organs.

Unlike mitochondria in liver, brain or heart, mitochondria in platelets are small and few in number (6 to 8/cell).[11] As a result, a decrease in cytochrome c oxidase activity could have a major effect on platelet ATP content. Although previous work has shown that cytochrome c oxidase activity in platelets can be reduced in excess of 90% by severe copper deficiency,[12] it is not known whether copper deficiency can reduce platelet ATP content to the extent that it affects energy consuming processes such as signal-response coupling. Accordingly, the purpose of the present study was to investigate the effect of copper deficiency on platelet ATP content and to further characterize the effect of copper deficiency by examining platelet mitochondria for structural changes that could be relevant to altered energy metabolism.

Methods and materials

Male Sprague–Dawley rats, 21 days old (SASCO, Inc., Omaha, NE USA),* were housed in individual stainless steel cages in a room with a 12-hr light/dark cycle and regulated temperature (22°C) and humidity (50%). The rats were fed a purified diet containing adequate copper (5.5 μg of Cu/g) and iron (45 μg Fe/g) for 5 days after their arrival at our facility. They were then divided into two weight-matched groups. One group of 24 rats was a fed copper-deficient diet and the other group of 32 rats was fed a copper-adequate diet. The initial mean weights ± SD of rats in these groups were 77 ± 4 and 77 ± 5 g, respectively. Each group was fed their respective diets and deionized water ad libitum for 5 weeks.

The diets were composed of 940 g of casein-based copper- and iron-free basal diet (No. TD84469; Teklad Test Diets, Madison, WI USA), 50 g of safflower oil (Teklad Test Diets), and 10 g of Cu-Fe mineral mix per kilogram of diet. Copper-deficient and copper-adequate diets were obtained by using Cu-Fe mineral mixes containing either no added Cu or 0.5 g of Cu/kg of mix and 3.6 g of Fe/kg of mix as previously described.[1,13] Analysis of the diets by flame atomic absorption spectrophotometry indicated that the copper-deficient diet contained 0.5 μg of Cu/g and 42.2 μg of Fe/g. The copper-adequate diet contained 5.8 μg of Cu/g and 43.5 μg of Fe/g. Copper status of the rats was determined by measuring hematocrits and hemoglobin concentrations (Coulter Counter model S-Plus IV; Coulter Electronics, Hialeah, FL USA), liver copper concentrations,[13] and plasma ceruloplasmin oxidase activity.[14,15]

Blood was withdrawn from the vena cava of anesthetized rats into 0.16 volumes of anticoagulant solution containing glucose (0.11 mol/L), sodium citrate (0.085 mol/L), and citric acid (0.071 mol/L). The blood was centrifuged at 160*g* for 20 min at 25°C (GPR Centrifuge; Beckman Instruments, Inc., Palo Alto, CA USA) to obtain platelet-rich plasma. Platelets in a 0.25 mL aliquot of platelet-rich plasma were stabilized by adding 0.25 mL of 0.1% glutaraldehyde in sodium cacodylate (0.2 mol/L, pH 7.4). After 15 min, the stabilized platelets were centrifuged at 900*g* for 10 min at 25°C. The resulting platelet pellet then was fixed overnight in 3% glutaraldehyde in sodium cacodylate (0.2 mol/L, pH 7.4), rinsed with cacodylate buffer, and treated for 1 hr at room temperature with 0.75% osmium tetroxide in cacodylate buffer. Following fixation, the platelets were dehydrated with ethanol and embedded in EMbed-812 (Electron Microscopy Sciences, Ft. Washington, PA USA). Thin sections for transmission electron microscopy were made with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined with a Phillips 300 transmission electron microscope (Phillips Electronic Co., Mahwah, NJ USA) at 60 kV.

Platelets obtained by centrifuging the remaining platelet-rich plasma at 730*g* for 10 min were washed as previously described.[16] During washing, platelet samples from 4 to 6 rats of the same dietary treatment group were combined. This step was necessary to provide enough platelets for accurate analysis of nucleotide content. The final suspensions contained 1.0×10^9 platelets/mL in a buffer, pH 7.4, containing NaCl (138 mmol/L), KCl (2.9 mmol/L), $NaHCO_3$ (12 mmol/L), NaH_2PO_4 (0.36 mmol/L), glucose (5.5 mmol/L), EDTA (1.0 mmol/L), and HEPES (5.0 mmol/L).

Aliquots (0.5 mL) of platelet suspension were centrifuged at 8,370*g* for 3 min at 25°C (Biofuge 15; Baxter Diagnostics, Inc., Scientific Products Division, McGaw Park, IL USA). Following removal of the supernatant, the cell pellets were resuspended in 100 μL of ice-cold 6% perchloric acid and vortexed for 5 min. The perchlorate extracts were allowed to stand for 5 min at 4°C and then centrifuged at 1,300*g* for 5 min at 4°C. The supernatants then were neutralized by adding 20 μL of cold KOH (8.6 mol/L). Following neutralization, the samples were vortexed, allowed to stand for 5 min at 4°C, and then centrifuged at 1,300*g* for 5 min at 4°C. The supernatants were removed and stored at −20°C until they were analyzed for nucleotide content. Extracts were not left in storage for longer than 72 hr.

Nucleotides were separated by using ion-pair reversed-phase high powered liquid chromatography (HPLC) (model 2350 programable pump, model 2360 gradient former, V^4 absorbance detector, Isco, Inc., Lincoln, NE USA). Aliquots (50 μL) of platelet extract were applied to a 7 μM adsorbosphere nucleotide-nucleoside column (250 × 4.6 mm; Alltech Associates, Inc., Deerfield, IL USA) and the nucleotides were eluted at 1.5 mL/min with a step gradient generated from $NH_4H_2PO_4$ (60 mmol/L), tetrabutylammonium phosphate (5 mmol/L), pH 5.0 (solvent A), and 100% methanol, tetrabutylammonium phosphate (5 mmol/L) (solvent B). The gradient conditions used were 1.0 min at 95% solvent A, 5% solvent B; 5 min at 93% solvent A, 7% solvent B; 10 min at 88% solvent A, 12% solvent B; 27 min at 85% solvent A, 15% solvent B. Nucleotide peaks were identified by coelution of standards containing 50 nmol/L of each nucleotide. The nucleotides were quantified by comparing the integrated areas of their elution peaks to those of standards containing 15 nmol/L of each nucleotide.

An aliquot of the original platelet suspension was diluted to 2.5×10^8 platelets/mL suspension buffer. The total dense granule ATP pool was then estimated by using firefly luciferase[1] to measure the amount of ATP released from the platelets following maximal stimulation with rat thrombin (Sigma Chemical Co., St. Louis, MO USA). It was found that 1.0 U/mL of thrombin was sufficient to stimulate the complete release of secretable ATP.

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Table 1 Copper concentration, plasma ceruloplasmin activity (CPLSM), hematocrit, and hemoglobin concentration in rats fed either copper-deficient (CuD) or copper-adequate (CuA) diet

Diet	Liver Cu* (μmol/kg)	CPLSM† (U/L)	Hemoglobin (g/L)	Hematocrit
CuD (n = 24)	17.9 ± 6.3‡	0.02 ± 0.024‡	100 ± 10‡	0.32 ± 0.03‡
CuA (n = 32)	199.9 ± 17.3	90.6 ± 14.4	151 ± 4	0.44 ± 0.01

*Liver Cu concentration is based on dry liver weight. Values are means ±SD.
†A unit of ceruloplasmin activity is defined as that amount which catalyzes the oxidation of 1 μmol o-dianisidine/min.
‡Means are significantly different ($P < 0.05$, Student's t-test).

Data obtained from the analysis of platelet nucleotides, expressed as means ± SD, were evaluated for statistical significance by Student's t-test for unequal variances. The frequency of changes in mitochondrial structure was evaluated by Chi-square test (SAS/STAT Version 6; SAS Institute, Inc., Cary, NC USA).

Results

As shown in *Table 1*, rats fed a copper-deficient diet had significantly lower liver copper and plasma ceruloplasmin activity than the rats fed a copper-adequate diet and also became anemic. These signs are characteristic of copper deficiency [17–19] and confirm the deficient status of the rats consuming the diet containing low copper. The values for liver copper also are comparable to those observed in previous studies that utilized diets identical to those used in the present study.[1,2,12,13]

Figure 1 illustrates a typical chromatographic separation of platelet adenine nucleotides. With the chromatographic conditions employed, the adenine nucleotides were well-resolved with adenosine monophosphate (AMP) having the shortest retention time followed in order by adenosine diphosphate (ADP) and ATP. Guanosine triphosphate (GTP) was also separated and had a retention time between those of ADP and ATP. Guanosine diphosphate (GDP) and Guanosine monophosphate (GMP) were below detectable limits and could not be measured. The peak areas obtained from the chromatogram were used to evaluate the total cellular contents of ATP, ADP, AMP, and GTP shown in *Table 2*. Rat platelets have a cellular volume of about 5.5 fL. Based on the cellular contents of the adenine nucleotides given in *Table 2*, the concentrations of ATP, ADP, and AMP in platelets from rats fed adequate copper were 6.7, 2.6, and 0.4 mmol/L, respectively. These are comparable to the concentrations of 5.0, 3.5, and 0.5 mmol/L for ATP, ADP, and AMP, respectively, reported for human platelets,[4] indicating that the HPLC methodology used in the present study provided an accurate assessment of platelet nucleotide content. The data in *Table 2* also show that total platelet ATP and GTP were lower and AMP was higher in rats fed a copper-deficient diet compared with rats fed a copper-adequate diet. However, the total adenine nucleotide content, represented by the sum of the adenine nucleotides, was not affected by dietary treatment. The adenylate energy charge (EC = {[ATP] + 0.5[ADP]}/{[ATP] + [ADP] + [AMP]}), which was estimated from the total cellular ATP, ADP, and AMP contents, was also lower in platelets from rats fed a copper-deficient diet compared with rats fed a copper-adequate diet.

The secretory pool of ATP was estimated by maximally stimulating the platelets with 1 U/mL of thrombin and measuring the amount of ATP secreted. Following maximal stimulation, the total amount of ATP secreted by platelets obtained from rats fed a copper-deicient diet, 15.4 ± 3.8 nmol/10^9 cells (N = 5), was not different from the amount secreted by platelets from rats fed a copper-adequate diet, 14.0 ± 1.8 nmol/10^9 cells (N = 5).

When examined by electron microscopy, mitochondria in platelets from copper-deficient and control rats were similar in structure and existed in two untrastructural states: one with normal appearing cristae and low matrix opacity, the other with swollen cristae and high matrix opacity. *Figure 2A* shows an example of a platelet mitochondrion in which the cristae and matrix appear normal. *Figures 2B* and *2C* show mitochondria in which the matrix is dense and the cristae are swollen relative to the normal mitochondrion shown in *Figure 2A*. The mitochondria that are normal and those with dense matrix and swollen cristae appear identical to the mitochondrial conformations identified by Hackenbrock[20,21] as orthodox and condensed, respectively. Also, some mitochondria contained dense inclusions, as shown in *Figure 2D*. As shown in *Table 3*, dietary copper had a significant effect on mitochondrial conformation and dense body inclusions. Platelets from rats fed a copper-deficient diet contained more condensed mitochondria than platelets from rats fed a copper-adequate diet. The number of mito-

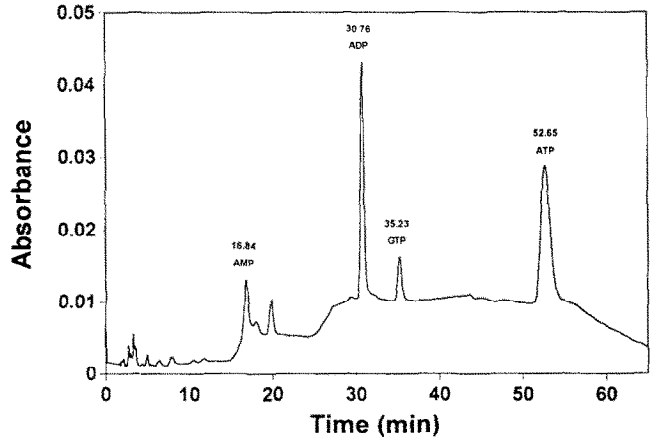

Figure 1 Chromatographic profile of AMP, ADP, GTP, and ATP obtained by reverse-phase ion-paired HPLC of neutralized perchlorate extract from platelets. Retention times for the adenine nucleotides and GTP are shown above their respective peaks.

Table 2 Total ATP, ADP, AMP, and GTP content, sum of adenine nucleotides, and adenylate energy charge in platelets from rats fed either copper-deficient (CuD) or copper-adequate (CuA) diet

Diet	ATP	ADP	AMP	GTP	Σ adenine nucleotides	Energy charge
CuD (n = 5)	28.2 ± 2.8*	14.8 ± 1.2	4.7 ± 0.9*	4.7 ± 0.9*	47.7 ± 4.3	0.74 ± 0.02*
CuA (n = 5)	37.0 ± 6.8	14.2 ± 1.0	2.2 ± 1.3	7.4 ± 1.2	53.4 ± 7.3	0.82 ± 0.04

Nucleotide content is expressed as nmol/10^9 platelets. Values are means ± SD.
*Means are significantly different ($P < 0.05$, Student's t-test).

chondria containing dense bodies also was higher in platelets from rats fed a copper-deficient diet. Although the number of mitochondria containing dense bodies was higher in platelets from rats consuming a copper-deficient diet, dense body distribution was not influenced by mitochondrial conformation. Thus, in platelets from rats fed a copper-deficient diet, 54% of condensed mitochondria and 53% of orthodox mitochondria contained dense bodies, and in platelets from rats fed a copper-adequate diet, 31% of condensed mitochondria and 32% of orthodox mitochondria contained dense bodies (conformation effect; Chi-square = 0.012, degree of freedom = 1, $P > 0.05$).

Discussion

Although copper deficiency can severely reduce cytochrome c oxidase activity in rat platelets, its effect on the adenine nucleotide content of platelets is unclear. In this study, an ion-pair reverse-phase HPLC protocol was used to separate and quantitate the adenine nucleotide content of platelets from rats that were severely copper deficient, a condition that has been shown to cause greater than 90% reduction in platelet cytochrome c oxidase activity.[12] Results from the analysis of platelet nucleotides indicated that copper deficiency did not affect the total adenine nucleotide content, represented by the sum of ATP, ADP, and AMP, but did alter their proportion, causing a 24% reduction in total ATP content and a 113% increase in total AMP content. The relative changes in ATP and AMP content are similar to those observed in platelets that were either starved in glucose-free medium containing cyanide[22] or incubated in the presence of antimycin A and 2-deoxyglucose.[23] Because cyanide inhibits cytochrome c oxidase and antimycin A inhibits the respiratory chain between cytochrome b and cytochrome c, the decrease in platelet ATP and concomitant increase in AMP when platelets are treated with these metabolic inhibitors can be attributed, at least partially, to blockage of electron transport and decreased ATP synthesis. Thus the changes observed in platelet ATP and AMP contents during copper deficiency are consistent with decreased electron transport most likely caused by reduced cytochrome c oxidase activity.

Copper deficiency also caused a 36% reduction in platelet GTP content. This reduction may be symptomatic of metabolic stress induced by reduced ATP and increased AMP contents of platelets during copper deficiency. In human platelets, either glucose starvation or hydrogen peroxide treatment caused a 40 to 50% reduction in GTP concentration as ATP concentration decreased and AMP concentration increased.[24] Under these conditions, the reduction in GTP concentration may be related to the accumulation of AMP. As AMP accumulates, the rate of inosine-5′-monophosphate (IMP) formation by AMP deaminase increases. However, some of the IMP is salvaged through its conversion to adenylsuccinate by adenylsucci-

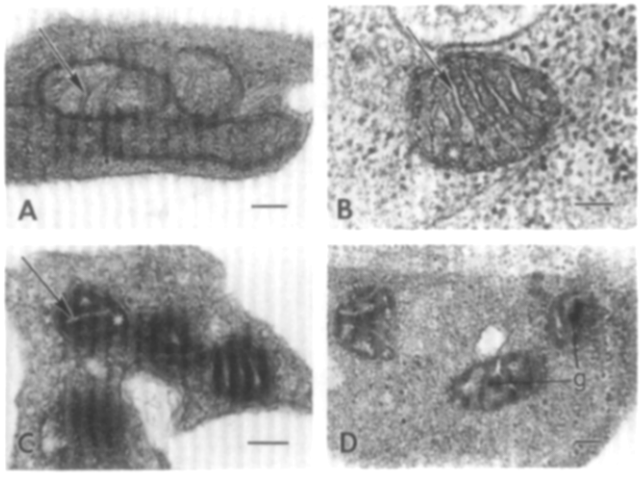

Figure 2 Electron micrographs showing the variations in mitochonrial structure encountered in rat platelets. (A) An orthodox mitochondrion showing normal cristae (arrow) and normal matrical density. (B and C) Eamples of condensed mitochondria showing swollen cristae (arrows) and high matrical density (asterisks). (D) Condensed mitochondria containing dense bodies (g). The bar represents 0.1 μm. These variations in mitochondrial structure were found in platelets regardless of the copper status of the rats from which the cells were obtained.

Table 3 Mitochondrial conformation and dense body content in platelets obtained from rats fed either copper-deficient (CuD) or copper-adequate (CuA) diet

Diet	No. of mitochondria examined	No. of condensed mitochondria	No. of mitochondria with dense bodies
CuD	109	71 (65%)*	57 (52%)†
CuA	75	16 (21%)	24 (32%)

*Significant diet effect: Chi-square = 34.0, degree freedom = 1, $P < 0.0001$.
†Significant diet effect: Chi-square = 7.4, degree freedom = 1, $P < 0.01$. The percentages given in parentheses represent the percent of the number of mitochondria examined in each dietary group.

nate synthetase. Adenylsuccinate then is converted to AMP by adenylsuccinate lyase.[11] In this salvage pathway, adenylsuccinate synthetase specifically requires GTP as a substrate. Thus, as platelet ATP falls and AMP increases during copper deficiency, GTP also may fall as it is utilized to salvage and conserve adenine nucleotides.

While the changes in platelet nucleotide content during copper deficiency indicate that ATP production is impaired, changes in mitochondrial structure also suggest that the cells are experiencing a shortage of ATP. Unlike heart mitochondria which become vacuolated and fragmented during copper deficiency,[25–27] platelet mitochondria undergo structural changes that are suggestive of a normal response to an altered metabolic state rather than of mitochondrial disruption. It has been shown by Hackenbrock et al.[20,28] that isolated mitochondria undergo reversible ultrastructural alterations as a result of transitions between respiratory states 4 and 3. In state 3, the active respiratory state, the mitochondria assume a condensed configuration in which the matrix becomes highly electron opaque. The condensed mitochondrial configuration also has been observed in intact cells in which ADP generation has been induced with 2-deoxyglucose.[21] Under these conditions it was shown that the condensed configuration was characteristic of mitochondria actively engaged in oxidative phosphorylation. In the present study, a number of platelet mitochondria had high matrical density and swollen cristae. The structural similarity of these particular mitochondria to the condensed mitochondria shown in Hackenbrock's work[20,21,28] suggests that they also are in the condensed configuration and are actively engaged in oxidative phosphorylation. It was found that copper deficiency increased the number of mitochondria in the condensed configuration. Because all platelet samples were treated identically during isolation and preparation, a likely explanation for this shift in mitochondrial configuration is that the reduction in platelet ATP content during copper deficiency caused metabolic stress that led to a compensatory increase in the number of mitochondria engaged in oxidative phosphorylation.

In comparison to platelets from control rats, platelets from copper-deficient rats have more mitochondria that contain dense bodies. While the composition of mitochondrial dense bodies is not known with certainty, they may be composed of phospholipoprotein and are capable in vitro of binding calcium.[29] Increases in the number, size, and density of mitochondrial dense bodies have been reported in many pathological and experimental states and may result from intramitochondrial calcification.[30] Therefore, calcium accumulation may have caused the increased frequency of mitochondrial dense bodies in platelets from copper-deficient rats. Although the changes in mitochondrial configuration during copper deficiency suggest that more mitochondria are engaged in oxidative phosphorylation, the fact that the frequency of dense body appearances in mitochondria was independent of configuration indicates that the increase in dense body numbers during copper deficiency may have resulted from a defect either in calcium uptake or in the regulation of calcium uptake.

It is evident from the findings of the present study that copper deficiency can perturb adenine nucleotide metabolism in rat platelets and cause a decrease in total ATP content. Because the ATP content of the secretory pool was not affected by copper deficiency, the decrease in total platelet ATP most likely represents a decrease in metabolically available ATP. The question is whether or not this decrease is sufficient to alter platelet responses. The pool of ATP that is metabolically available can be estimated from the difference between total ATP content and the amount of ATP in the secretory pool. Using this estimation, the metabolic pool was 12.8 nmol ATP/10^9 cells and 23.0 nmol ATP/10^9 cells in platelets from copper-deficient and control rats, respectively. This represents about a 45% decrease in the metabolic ATP pool in platelets from copper-deficient rats. A range of 0.89 to 0.94 has been reported for the energy charge of platelets from a variety of species.[31] While the estimated adenine energy charge of 0.82 in platelets from control rats fed adequate copper is low in comparison, our estimate should be interpreted with caution because it is based on total cellular content of adenine nucleotide and does not take into account the amounts of ATP and ADA that, as a result of being sequestered in secretory granules, do not contribute to the energy charge. However, our estimates indicate that the energy charge in rat platelets may be reduced about 10% by copper deficiency. In human platelets, shape change, aggregation, dense granule secretion, and α-granule secretion are unaltered by up to 60% reductions in metabolic ATP and 5% reductions in energy charge.[32] Thus platelet responses, while resilient to large decreases in metabolic ATP, are sensitive to modest reductions in energy charge. It is possible, therefore, that the estimated 10% reduction in energy charge is more important than the 45% reduction in metabolic ATP in altering platelet responses during copper deficiency.

It may be concluded that under conditions of dietary copper deficiency known to reduce platelet cytochrome c oxidase activity by at least 90%,[12] platelets exhibit some signs of metabolic stress, such as decreased ATP, increased AMP, decreased GTP, decreased energy charge, and ultrastructural changes in mitochondria associated with an increase in oxidative phosphorylation. However, even though cytochrome c oxidase activity can be drastically reduced by copper deficiency, the present findings suggest that platelets may conserve ATP by increasing the number of mitochondria engaged in oxidative phosphorylation and by using GTP to synthesize ATP. The resulting reduction in platelet GTP may help explain how copper deficiency can affect signal transduction. It has been reported that GTP, through its interaction with a G-protein, increases the sensitivity of platelet dense granule secretion to Ca^{2+} and acts synergistically with thrombin to elicit the secretory response.[33] Therefore, it is possible that the effects of copper deficiency on platelet responses cannot be attributed solely to the reduction of cellular ATP and energy charge but also to the reduction of GTP that occurs as the platelet tries to maintain ATP levels needed to ensure its responsiveness.

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