Copper deficiency alters protein kinase C mediation of thrombin-induced dense granule secretion from rat platelets

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Experiments were conducted to determine if copper deficiency enhances the rate of thrombin-induced dense granule secretion by modifying the major signal transduction pathways of rat platelets. Platelets were obtained from male, weanling Sprague-Dawley rats fed diets containing either deficient (< 0.5 μg/g diet) or adequate (5.5 μg/g diet) copper for 5 weeks. Following stimulation with thrombin (0.1 U/mL), the rate of dense granule secretion as measured by ATP release was 160% higher in platelets from copper-deficient than from control rats. Inhibition of the rate of thrombin-induced ATP release by (6-aminohexyl)-1-naphthalene-sulfonamide, a calmodulin antagonist was independent of copper status. However, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, a protein kinase C inhibitor, inhibited the rate of ATP release only in platelets from copper-deficient rats. Aspirin had no effect on ATP release from platelets obtained from either copper-deficient or control rats. This suggests that copper deficiency alters the role of protein kinase C in regulating dense granule secretion. Analysis of autoradiographs showing [^{32}P]-labeled platelet proteins indicated that the phosphorylation of a 40 kDa protein, a known substrate for protein kinase C in platelets, was significantly less following thrombin stimulation in platelets from copper-deficient than from control rats. When protein kinase C was activated by phorbol 12-myristate 13-acetate prior to thrombin stimulation, ATP release was attenuated regardless of copper status. These findings suggest that protein kinase C can still function as a feedback inhibitor of platelet dense granule secretion in copper deficiency, but impaired activation of this enzyme following thrombin stimulation may prevent it from achieving full regulatory capacity.

Keywords: copper deficiency; platelets; secretion; protein kinase C

Introduction

Since the discovery by Hart et al.[1] that copper is essential for normal erythropoiesis, a number of physiological systems including the cardiovascular, nervous, skeletal, integumentary, reproductive, and immune systems[2-6] have shown a dependence on adequate dietary copper for normal function. Because a number of enzymes require copper for catalytic activity, some consequences of copper deficiency are likely associated with impaired catalysis in key metabolic pathways containing cuproenzymes. Thus, in certain instances, for example, the involvement of reduced lysyl oxidase activity in producing vascular and cardiac structural defects,[7] the pathophysiological outcomes of copper deficiency can be directly related to reduced activity of a specific copper-dependent enzyme. However, immune defects, neutropenia, anemia, glucose intolerance, and nervous system defects associated with copper deficiency cannot be explained completely by present knowledge regarding the biological functions of copper.[4]

Recent studies have shown that copper deficiency can alter the lipid and protein composition of lymphocyte plasma membranes in mice[8] and the cytoskeletal protein composition of erythrocyte membranes in rats.[9] Another study has shown that copper deficiency can enhance dense granule secretion from rat platelets and increase the association of myosin with the platelet cytoskeleton following thrombin stimulation.[10] These findings indicate that copper may have a role in maintaining normal structure-function relationships in

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cell membranes. They also suggest that altered cellular responses resulting from changes in the chemical and physical properties of cell membranes may contribute to the pathophysiological consequences of copper deficiency.

Dense granule secretion in response to thrombin stimulation is initiated by the agonist acting on a specific platelet membrane receptor.[11-13] Following the action of thrombin on its receptor, at least three signal transduction pathways can contribute to the secretory response.[14,15] Two of these pathways are regulated through the phosphorylation of proteins; one by protein kinase C and one by Ca-calmodulin dependent myosin light chain kinase. The third pathway involves the release of arachidonic acid and its conversion to thromboxane A_2, which then initiates the secretory response. The objective of the present study was to investigate the possibility that the reported hypersecretory response of thrombin-activated platelets from copper-deficient rats results from an alteration in one or more of these signal transduction pathways.

Methods and materials

Diets and animals

Diets were composed of 940.0 g of casein-based, copper- and iron-free basal diet (# TD84469, Teklad Test Diets, Madison, WI, USA),* 50.0 g safflower oil (Teklad Test Diets) and 10.0 g Cu-Fe mineral mix per kg of diet. Copper-deficient and copper-adequate diets (CuD and CuA, respectively) were obtained by using Cu-Fe mineral mixes containing either no Cu or 0.5 g Cu per kg mix as previously described.[9,10] Analysis of the diets by flame atomic absorption spectroscopy indicated that CuD diets contained < 0.5 μg Cu/g diet and CuA diets contained 5.5–6.0 μg Cu/g diet. All diets contained 53–56 μg Fe/g diet.

Male weanling Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA). Upon arrival, the rats were housed in individual stainless steel cages in a temperature- and humidity-controlled room with a 12-hour light:dark cycle. All rats were fed CuA diet for 5 days after their arrival. The rats were then divided into two groups with one group remaining on CuA diet and the other starting on CuD diet. At this point, the rats weighed 65–80 g. The rats were maintained on their diets and deionized water for 35 days.

Copper status of individual rats was determined by measuring hemoglobin concentration and hematocrit (Coulter Counter model S-plus IV, Coulter Electronics, Hialeah, FL, USA), liver and plasma copper concentrations,[9] and plasma ceruloplasmin.[16,17]

*Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Measurement of platelet secretion

Blood was withdrawn from the vena cava of anesthetized rats into 0.16 volumes of anticoagulant solution containing 0.11 M glucose, 0.085 M sodium citrate and 0.071 M citric acid. Platelets were obtained from freshly drawn blood and washed as previously described.[18] The final platelet suspension contained 5×10^8 platelet/mL in a buffer containing 0.138 M NaCl, 0.0029 M KCl, 0.012 M $NaHCO_3$, 0.00036 M NaH_2PO_4, 0.0055 M glucose and 0.001 M EDTA, pH 7.4 (buffer A).

The effects of the calmodulin antagonist, N-(6-aminohexyl)-1-naphthalene-sulfonamide (W7), the protein kinase inhibitors, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H7), and N-(2-quanidinoethyl)-5-isoquinolinesulfonamide (HA1004) (50 μmol/L final concentration, Calbiochem Corp., San Diego, CA, USA), and the cyclooxygenase inhibitor, aspirin (100 μmol/L final concentration, Sigma Chemical Co., St. Louis, MO, USA), on thrombin-induced ATP secretion were assessed by incubating a mixture containing 0.2 mL of platelet suspension, 0.05 mL of inhibitor solution (0.05 mL of deionized water in controls), and 0.69 mL of buffer A at 37° C for 10 min. After incubation, 0.05 mL of luciferase:luciferin reagent (Chrono-Lume, Chrono-Log Corp., Havertown, PA, USA) were added to the mixture and the platelets activated with 0.1 U (in 10 μL) of rat thrombin (Sigma Chemical Company). Following the addition of thrombin, the rate and extent of ATP release at 37° C were measured from the slope of the linear portion and amplitude, respectively, of the curve representing the increase in luminescence with time (Lumi-Aggregonometer model 500, Chrono-Log Corp.).[10] Neither H7, W7, HA1004, nor aspirin at the concentrations used in the experiments had an effect on luciferase activity. The luminescence of 0.15 nmol of ATP was used to quantitate the amount of ATP released from the platelets.

Measurement of protein phosphorylation

Platelets were suspended at a concentration of 10^9 platelets/mL in a buffer containing 0.15 M NaCl, 0.001 M EDTA, and 0.01 M HEPES, pH 7.4. One mL aliquots of platelet suspension were incubated with 0.5 mCi [^{32}P]orthophosphoric acid (Dupont New England Nuclear Research Products, Boston, MA, USA) at 37° C for 30 min. The final concentration of orthophosphoric acid in the incubation mixture was approximately 0.06 μmol/L based on a specific activity of approximately 8800 Ci/mmol. Following incubation, the platelet suspensions were centrifuged at 730*g* for 10 min. The platelet pellets were resuspended in buffer A to a concentration of 5×10^8 platelet/mL. Platelets in a 1 mL aliquot of suspension were activated by adding 0.1 U of rat thrombin. Forty-five seconds following activation, 150 μL of platelet suspension were added to 75 μL of 6% sodium dodecyl sulfate (SDS) containing 0.188 M Tris-HCl (pH 6.8), 30% glycerol, 0.12 M dithiothreitol, and 0.003% bromophenol blue and incubated for 45 min at 37° C. Unactivated con-

Table 1 Hemoglobin concentration, hematocrit, plasma ceruloplasmin (cplsm), and plasma and liver copper concentrations in rats fed CuD and CuA

Diet	Hemoglobin g/L	Hematocrit	Cplsm μkat/L	Plasma Cu μmol/L	Liver Cu[a] μmol/kg
CuD (N = 26)	61 ± 4[b]	0.19 ± 0.01[b]	0.02 ± 0.01[b]	0.9 ± 0.2[b]	17.3 ± 0.6[b]
CuA (N = 25)	142 ± 1	0.42 ± 0.002	0.83 ± 0.32	11.0 ± 0.5	163.7 ± 3.1

[a] Liver Cu concentration is based on dry liver weight. Means for rats fed CuD and CuA were compared by Student *t* test.
[b] $P < 0.001$.

Table 2 Effects of H7, W7, and aspirin on the rate of the ATP release from platelets stimulated by thrombin (0.1 U/mL)

	Rate of ATP Release (nmol ATP released/[min·10^9 platelets])			
Diet	Control	W7[a]	H7[b]	Aspirin
CuD (N = 22)	42.0 ± 2.7	3.7 ± 0.6	27.8 ± 1.8	46.8 ± 2.2
CuA (N = 18)	16.1 ± 3.1	1.0 ± 0.7	14.4 ± 2.0	17.7 ± 2.4

Source of variation by ANOVA: diet $P < 0.001$; inhibitor $P < 0.001$; inhibitor × diet interaction $P < 0.001$
[a] Inhibition was significant in rats fed CuD and CuA, $P < 0.0001$
[b] Inhibition was significant in rats fed CuD, $P < 0.006$

trols were prepared for electrophoresis in the same manner using 150 μL of platelet suspension that was not activated with thrombin. The samples were subjected to SDS-polyacrylamide gel electrophoresis on a 5%–16% acrylamide gradient under the conditions described by Laemmli.[19] The gels were stained with Coomassie brilliant blue R250, dried, and exposed for 96 hr at −80° C to X-ray film (X-Omat AR, Eastman Kodak Co., Rochester, NY, USA) combined with an intensifying screen (Cronex, E.I. duPont de Nemours & Co., Wilmington, DE, USA).[20] Protein phosphorylation was quantified by scanning the radiographic images with a Beckman DU-70 spectrophotometer equipped with a gel scanning accessory.

Statistical analysis

Data were evaluated by multivariate repeated measures analysis of variance (ANOVA). If interactions between main effects were significant at $P < 0.05$, comparison to the control means was by single degree of freedom contrasts (*Tables 2 and 3*) and comparison between dietary groups (*Table 4*) was by Bonferroni contrasts.[21] When appropriate, data were analyzed by Student *t* test for unequal variances.[22] All values shown are means ± SEM.

Results

In all experiments, rats consuming CuD became copper-deficient as indicated by anemia, lowered liver and plasma copper concentrations, and severely depressed plasma ceruloplasmin activity (*Table 1*). The curves for the time course of ATP secretion following activation with thrombin (*Figure 1* shows representative examples of such curves) indicated that in the absence of inhibitors the rate of ATP secretion was greater for platelets from copper-deficient rats than from copper-adequate rats. However, the extent of ATP secretion did not appear to be markedly affected by dietary Cu. It also appears from these curves that H7 inhibited ATP secretion more effectively in platelets from copper-deficient rats and that W7 may be a more effective inhibitor of ATP secretion in copper-adequate rats. When the rates of ATP secretion obtained from these curves for both dietary groups were normalized for platelet count and averaged, the rate of ATP release (*Table 2*) in the absence of inhibitors following thrombin activation was 160% higher in platelets from copper-deficient rats than in platelets from copper-adequate rats. Furthermore, incubation with 50 μmol/L H7 for 10 min prior to thrombin activation significantly inhibited the rate of ATP secretion from platelets obtained from copper-deficient rats, but did not affect the rate of ATP secretion from platelets obtained from copper-adequate rats. Regardless of which diet the rats consumed, incubation with 50 μmol/L W7 severely inhibited the rate of ATP secre-

Table 3 The effects of W7, H7, and aspirin on the extent of ATP release from platelets stimulated with thrombin (0.1 U/mL)

	Extent of ATP Release (total nmol ATP released/10^9 platelets)			
Diet	Control	W7[a]	H7[ab]	Aspirin
CuD (N = 22)	3.44 ± 0.23	0.35 ± 0.08	2.10 ± 0.19	3.59 ± 0.21
CuA (N = 18)	2.69 ± 0.26	0.16 ± 0.09	1.99 ± 0.22	2.80 ± 0.23

Source of variation by ANOVA: diet, $P < 0.04$; inhibitor $P < 0.001$; inhibitor × diet interaction, $P < 0.004$.
[a] Inhibition by W7 and H7 was significant in rats fed CuD and CuA.
[b] Inhibition by H7 was marginally greater in rats fed CuD than in those fed CuA, $P < 0.056$.

Table 4 Inhibition by W7 and H7 of the rate and extent of ATP release from platelets stimulated by thrombin (0.1 U/mL)

Diet	Percent Inhibition			
	Rate of Release		Extent of Release	
	W7	H7	W7	H7
CuD (N = 22)	92.0 ± 3.4	25.9 ± 3.4[a]	89.8 ± 3.5[a]	34.1 ± 3.5[a]
CuA (N = 18)	93.9 ± 3.8	3.9 ± 3.8[a]	93.9 ± 3.1[a]	22.1 ± 3.1[a]

Source of variation by ANOVA: diet, $P < 0.02$ for rate of release, $P > 0.05$ for extent of release; inhibitor, $P < 0.001$ for rate and extent of release; diet × inhibitor interaction, $P < 0.05$ for rate and extent of release.
[a] Means in a column having the same superscript are significantly different ($P < 0.05$, Bonferroni contrasts).

tion. Incubation with 100 μmol/L aspirin did not affect the secretion rate.

Examination of the curves representing ATP secretion indicated that secretion was complete within 45 sec following platelet stimulation. Although not as markedly affected by copper deficiency as the rate of secretion, the total amount of ATP released for the given dose of thrombin, i.e., the extent of ATP secretion, (*Table 3*) was 28% higher for platelets obtained from copper-deficient rats than for platelets obtained from copper-adequate rats. Although the extent of ATP secretion was inhibited by W7 and H7, the inhibition was more severe with W7. Aspirin had no effect on the extent of ATP secretion.

The significant diet × inhibitor interaction terms of the ANOVA for both the rate and extent of ATP secretion indicate that dietary treatment influenced the effects of the inhibitors. By examining the percent inhibition caused by W7 and H7 on the rate and exent of ATP secretion (*Table 4*), the effect of diet on inhibition becomes apparent. The rate of ATP secretion was inhibited by W7 to the same degree regardless of dietary treatment. However, inhibition of the rate of ATP secretion by H7 was significantly greater in platelets from rats fed CuD than in those fed CuA. The extent of ATP secretion was inhibited by W7 to a slightly, but significantly, greater degree in platelets from rats fed CuA compared to those fed CuD. Inhibition of the extent of ATP secretion by H7 was greater in platelets from rats fed CuD compared with those fed CuA.

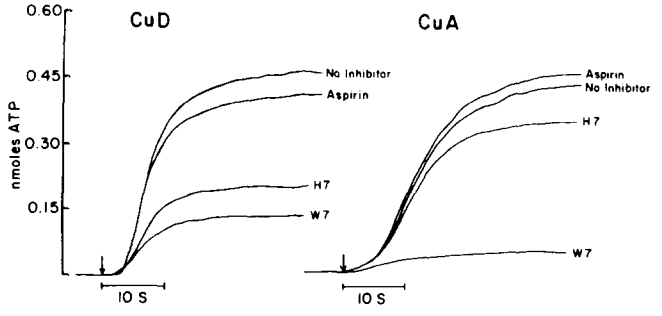

Figure 1 ATP secretion following thrombin (0.1 U/mL) activation of platelets obtained from rats fed CuD and CuA. Platelets were incubated prior to activation either in the absence of inhibitors or in the presence of 50 μmol/L H7, 50 μmol/L W7 or 100 μmol/L aspirin. The rate of ATP secretion was determined from the slope of the linear portion of the curve. The height of the curve was used to determine the extent of ATP secretion. Thrombin was added at the point indicated by the arrow.

Only a few suspensions of platelets from copper-deficient rats had sufficient volume to provide samples for incubation with HA1004, an inhibitor of cyclic AMP- and cyclic GMP-dependent protein kinases, once the other inhibitors had been tested. Incubation of platelets from copper-deficient rats with 50 μmol/L HA1004 had no inhibitory effect on either the rate (viz. 31.7 ± 1.7 and 32.6 ± 4.6 nmol ATP/(min·10^9 cells) in the absence and presence of HA1004, respectively, N = 5) or extent of thrombin-induced ATP secretion (viz. 5.0 ± 1.0 and 4.9 ± 1.6 nmol ATP/10^9 cells in the absence and presence of HA1004, respectively, N = 5).

Autoradiographs (*Figure 2*) showing [^{32}P]-labeled proteins indicate that phosphorylation of a 40 kDa protein occurred within 45 sec following thrombin activation of platelets from copper-deficient and copper-adequate rats. Although it appears in this representative example that phosphorylation of the 40 kDa protein was higher in platelets from copper-deficient than copper-adequate rats prior to thrombin activation, peak heights determined from densitometry scans of the autoradiographs showed that this was not generally true. Mean peak heights representing phosphorylation of the 40 kDa protein (*Figure 3*) were identical prior to platelet stimulation in copper-deficient and copper-adequate rats. However, the peak height for the 40 kDa protein was significantly less 45 sec following activation of platelets from copper-deficient compared to copper-adequate rats.

Incubation of platelets with 20 nmol/L PMA for 1 min prior to thrombin activation inhibited the rate and extent of ATP release from platelets obtained from both copper-deficient and copper-adequate rats (*Table 5*). Furthermore, ANOVA indicated that the inhibitory effect of PMA on thrombin-induced ATP secretion was independent of dietary treatment. Although PMA inhibition of the rate of ATP secretion was somewhat less in rats fed CuD (60 ± 9%) than in rats fed CuA (76 ± 2%), the difference was not statistically significant ($P = 0.2$, Student t test). PMA inhibition of the extent of ATP release was somewhat less in rats fed CuD (45 ± 8%) than in rats fed CuA (50 ± 7%), but this difference also was not significant ($P = 0.6$, Student t test). During the incubation period before thrombin stimulation, PMA induced ATP release at

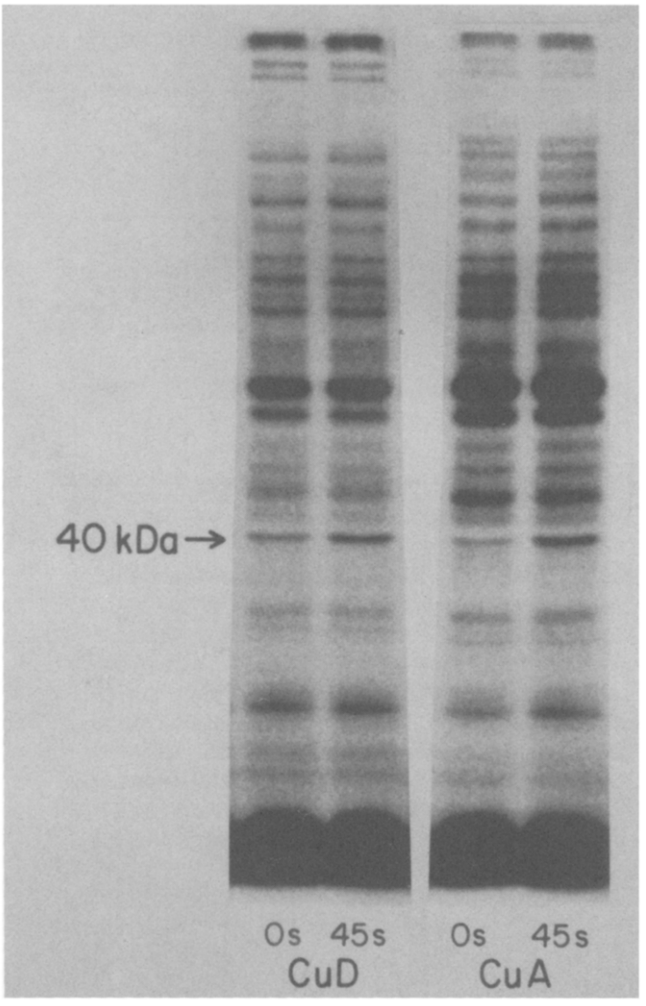

Figure 2 A representative autoradiograph showing thrombin-induced protein phosphorylation in platelets obtained from rats fed CuD and CuA. Platelets that had been labeled with ^{32}P were treated with thrombin (0.1 U/mL) for 0 sec and 45 sec. The [^{32}P]-proteins were separated by SDS polyacrylamide electrophoresis and detected by autoradiography as described in the text. The electrophoresis gels were calibrated by using myosis (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (27 kDa), and trypsin inhibitor (20 kDa).

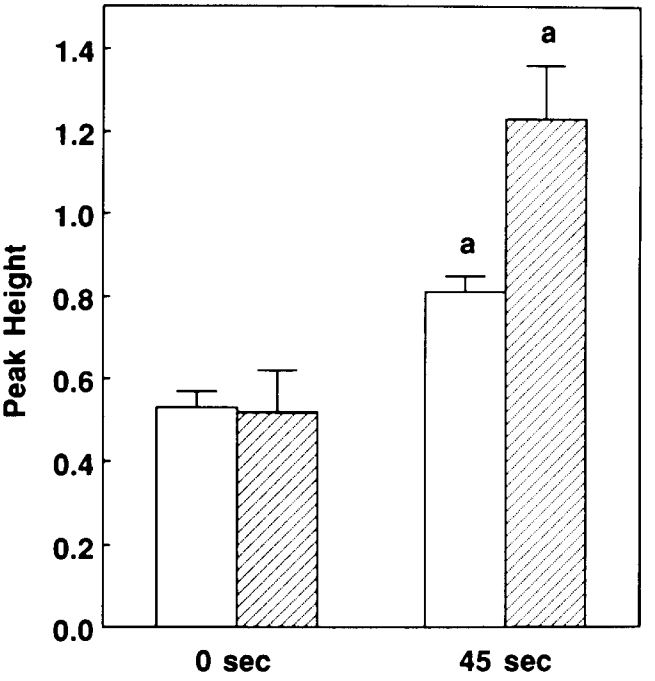

Figure 3 Phosphorylation of the 40 kDa protein following treatment of platelets obtained from rats fed CuD (open bars, N = 6), and CuA (cross-hatched bars, N = 5) with thrombin (0.1 U/mL) for 0 sec and 45 sec. Peak heights of the band representing the 40 kd protein were determined from densitometry scans of autoradiographs showing [^{32}P]-labeled proteins. The value shown for peak heights are means ± SEM for the absorbance at 580 nm. [a]CuD and CuA are significantly different ($P < 0.05$, Student t test).

rates of 0.45 ± 0.09 and 0.46 ± 0.03 nmol/(min·10^9 cells) from platelets obtained from copper-deficient and copper-adequate rats, respectively, indicating that copper status did not influence ATP release induced by PMA. Also, in this experiment copper deficiency significantly enhanced the rate of ATP secretion from thrombin-stimulated platelets but had no overall effect on the extent of ATP secretion.

Discussion

In the present study, as in a previous study,[10] the rate of thrombin-induced ATP secretion was greatly en-

Table 5 Effect of phorbol 12-myristate 13-acetate (PMA) on the rate and extent of ATP release from platelets stimulated by thrombin (0.1 U/mL)

	Rate of Release (nmol ATP/(min·10^9 cells))		Extent of Release (total nmol ATP/10^9 cells)	
Diet	Control	+PMA (20 nM)	Control	+PMA (20 nM)
CuD (N = 5)	50.4 ± 6.2	21.1 ± 2.9	7.67 ± 0.80	4.56 ± 0.74
CuA (N = 6)	19.9 ± 5.6	4.9 ± 2.7	6.16 ± 0.73	3.19 ± 0.67

Source of variation by ANOVA: diet, $P < 0.001$ for rate of release, $P > 0.05$ for extent of release; PMA, $P < 0.001$ for rate and extent of release; diet × PMA interaction, $P > 0.05$ for rate and extent of release.

hanced in platelets obtained from copper-deficient rats. However, copper deficiency had little effect on the extent of ATP release. These findings indicate that only the rate of ATP release is consistently altered by copper deficiency. Because ATP is a dense granule constituent,[23] enhancement in the rate of ATP release suggests that the mechanism for dense granule secretion is modified in platelets from copper-deficient rats.

Protein phosphorylation by Ca^{2+}-calmodulin-dependent myosin light chain kinase[24,25] and protein kinase C[26,27] had been implicated in platelet dense granule secretion. The rate and extent of ATP secretion from thrombin-activated platelets were severely inhibited by W7, a calmodulin antagonist.[28] Although inhibition of the rate of ATP secretion by W7 was not influenced by copper status, W7 was slightly less effective as an inhibitor of the extent of ATP release in platelets from copper-deficient rats. However, because W7 was a potent inhibitor of ATP secretion in platelets obtained from rats fed copper-deficient as well as copper-adequate diets, it cannot be concluded that the slight influence copper status had on W7 inhibition of the extent of ATP secretion represents a biological effect of copper deficiency on the role of calmodulin in dense granule secretion. The severity of the W7 inhibition of ATP release in thrombin-activated platelets from copper-deficient and copper-adequate rats indicates that a Ca^{2+}-calmodulin-dependent process, possibly catalyzed by myosin light chain kinase, is obligatory for dense granule secretion to occur regardless of copper status.

The primary and most consistent effect of copper deficiency on thrombin-induced ATP secretion from platelets is enhancement of the rate at which ATP is secreted. Furthermore, the rate of ATP secretion was inhibited by H7, a protein kinase C inhibitor,[29,30] only in platelets from copper-deficient rats. Cyclic AMP- and cyclic GMP-dependent protein kinases are also inhibited by H7.[29] However, HA1004, which more effectively inhibits cyclic AMP- and cyclic GMP-dependent protein kinases than protein kinase C,[29] can be used as a control for the effects of H7 on these cyclic nucleotide-dependent kinases. In the present study, HA1004 did not inhibit either the rate or extent of ATP secretion in platelets from copper-deficient rats. Therefore, the enhanced inhibitory effect of H7 on the rate of thrombin-induced ATP secretion in platelets from copper-deficient rats is most likely caused by inhibition of protein kinase C. It may be concluded from these findings that copper deficiency enhances the rate of thrombin-induced ATP release by modifying the contribution of protein kinase C to the mechanism of dense granule secretion.

Following platelet activation with thrombin, protein kinase C is translocated to the plasma membrane where it is fully activated by the synergistic actions of diacylglycerol, phosphatidyl serine, and Ca^{2+}.[27,31] One of the proteins phosphorylated by protein kinase C subsequent to platelet stimulation has a molecular weight of 40 kDa.[27] Although the function of the 40 kDa protein in platelet responses is not known, its phosphorylation is useful as an intracellular indicator of protein kinase C activity. In the present study, phosphorylation of the 40 kDa protein following thrombin stimulation was suppressed by copper deficiency. This finding suggests that copper deficiency can alter participation of protein kinase C in platelet dense granule secretion by reducing the activity of protein kinase C following thrombin activation.

Although protein kinase C had been implicated in platelet dense granule secretion,[26,27] its exact role is not completely understood. It has been proposed that protein kinase C can operate a feedback mechanism that serves to limit thrombin-induced secretion.[32,33] One way in which protein kinase C may serve as a feedback inhibitor is by phosphorylating platelet myosin. Phosphorylation by protein kinase C can inhibit the actin-activated ATPase activity of platelet myosin that has been prephosphorylated by myosin light chain kinase. This inhibition occurs because phosphorylation of myosin by protein kinase C decreases myosin's affinity for actin.[34] Therefore, the activation of protein kinase C that occurs following platelet stimulation by thrombin may decrease association of actin with myosin and inhibit the contractile activity of the cytoskeleton. Because platelet dense granule secretion involves granule centralization prior to fusion with the surface connected canalicular system (SCCS),[35] impairment of cytoskeletal contractile activity can slow the rate of granule centralization and inhibit dense granule secretion. Thus, suppression of protein kinase C activity by copper deficiency may enhance actin association with myosin and reduce feedback inhibition of dense granule secretion. Such a reduction in feedback inhibition may explain the enhanced rate of ATP secretion observed in the present study. Because the affinity of myosin for actin would be increased, suppression of protein kinase C activity following activation may also explain the previously reported increase in actin-myosin association in thrombin-activated platelets from copper-deficient rats.[10]

Inhibition of platelet protein kinase C by H7 can inhibit dense granule secretion by impairing dense granule fusion with the SCCS.[36] This indicates that besides having a feedback inhibitory role, protein kinase C can also promote dense granule secretion by facilitating the labilization and fusion of granule membranes with the SCCS. Because copper deficiency reduces protein kinase C activity in thrombin-activated platelets, granule fusion with the SCCS may be inhibited, but not sufficiently to overcome the reduced capacity of protein kinase C to regulate dense granule secretion by feedback inhibition. Thus, the net effect of copper deficiency leads to an enhanced rate of dense granule secretion. However, additional inhibition by H7 may impair membrane fusion to such an extent that defective dense granule fusion becomes the limiting factor and thrombin-induced ATP secretion is inhibited even though cytoskeletal contractibility and granule centralization are enhanced. In platelets from rats fed adequate copper, protein kinase C activity is

not impaired following thrombin activation. Under this circumstance, H7 inhibition of protein kinase C may decrease feedback inhibition of dense granule secretion to a greater extent than it inhibits dense granule fusion with the SCCS. Thus, H7 is not as effective in inhibiting thrombin-induced ATP secretion in platelets obtained from rats fed adequate copper as it is in platelets from copper-deficient rats.

The phorbol ester, PMA, can activate protein kinase C by substituting for diacylglycerol.[27] This permits activation of protein kinase C to occur without the usual transmembrane signaling events that elicit diacylglycerol production. In our study, pretreatment of platelets with PMA inhibited thrombin-induced ATP secretion. Although PMA inhibition of the rate of ATP secretion was slightly less in copper-deficient compared to copper-adequate rats, the inhibitory effect of PMA was not influenced significantly by copper status. These findings suggest that copper deficiency does not directly affect the ability of protein kinase C to function as a feedback inhibitor of dense granule secretion, provided that protein kinase C is activated prior to thrombin stimulation of the platelets. Thus, if impaired feedback inhibition by protein kinase C contributes to enhanced dense granule secretion in thrombin-activated platelets from copper-deficient rats, most likely it is because copper deficiency interferes with the activation of protein kinase C and prevents it from achieving its full capacity for catalytic activity and feedback inhibition. The slight reduction in PMA inhibition of the rate of thrombin-induced ATP secretion observed in platelets from copper-deficient rats is also consistent with the possibility that copper deficiency may interfere with PMA activation of protein kinase C and diminish its capability as a feedback inhibitor.

Cyclooxygenase[14,15] also can have a role in platelet dense granule secretion.[14,15] Cyclooxygenase is a key enzyme in the formation of thromboxane A_2 from arachidonic acid that is released from membrane phospholipids during platelet activation. Thromboxane A_2 can induce dense granule secretion in platelets, but is normally not a factor in the secretory response of thrombin-activated platelets except at very low thrombin concentrations (< 0.04 U/mL).[14] At the thrombin concentration (0.1 U/mL) used in our study, aspirin, which inhibits cyclooxygenase rapidly and irreversibly,[37,38] had no effect on ATP secretion in platelets from copper-deficient or copper-adequate rats. These findings indicate that under our experimental conditions, thromboxane A_2 did not generally mediate dense granule secretion or specifically contribute to the enhanced rate of dense granule secretion in platelets from copper-deficient rats.

The results of the present study indicate that abnormally low protein kinase C activity contributes to the enhanced rate of dense granule secretion in thrombin-activated platelets from copper-deficient rats. Although the mechanism through which copper deficiency may reduce protein kinase C activity in thrombin-activated platelets is not clear, impairment of protein kinase C activation, decrease in the platelet content of protein kinase C, or impaired interactions with regulatory ligands may be involved. Because protein kinase C is involved in regulating receptor mediated responses of a variety of cell types in addition to platelets,[39] further investigations into the role of copper in maintaining the regulatory functions of protein kinase C may provide new insights into how copper deficiency produces such diverse pathophysiological consequences.

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