Thromboxane Production in Copper-Deficient and Marginal Platelets: Influence of Superoxide Dismutase and Lipid Hydroperoxides (43523)

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Abstract. **Platelet thromboxane (TX) production was examined in response to dietary copper. Groups of eight rats were fed copper-deficient, -marginal, and -adequate diets providing 0.5, 1.7, and 7.5 μg Cu/g, respectively, with controlled dietary Se and vitamin E. Platelets were purified and washed by centrifugation. Separate platelet samples from each rat were challenged with 10 μg/ml of collagen and 1 unit/ml (27.3 n*M*) of thrombin in Tyrode's buffer, 2.0 m*M* Ca^{2+}. Platelet copper-dependent superoxide dismutase (CuSOD) activity showed a significant depression with reduced diet copper, but platelet glutathione peroxidase activity was unaffected. Challenged platelet TX production showed a significant 1.5- to 2.5-fold increase in response to both dietary copper deficiency and marginality, with highly significant negative correlations between challenged platelet TX production and platelet CuSOD activity and between TX production and copper status (liver copper). Endogenous (unchallenged) platelet lipid hydroperoxide concentrations, measured as free fatty acid hydroperoxides by a glutathione-disulfide-specific glutathione reductase recycling assay, showed a nonsignificant 47–67% increase in copper deficiency. Pooled data showed a significant 71% increase in platelet lipid hydroperoxides in copper deficiency. Platelet TX production showed a significant correlation with endogenous lipid hydroperoxides. The results suggest that dietary copper insufficiency increases platelet TX synthesis through changes in CuSOD in a dose-responsive (diet copper and platelet CuSOD activity) manner, and that platelet TX synthesis is influenced by lipid hydroperoxides (peroxide tone).**

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Regulation of thromboxane (TX) production by platelets has not been fully elucidated. TX is produced by platelets in response to agonists such as collagen and thrombin through receptor interaction and the increase in activity of several phospholipases (1). Phospholipase stimulation results in arachidonic acid release which is metabolized by prostaglandin H synthase (PGHS) to form prostaglandin (PG) H_2, the precursor common to all PG synthesis, with subsequent production of TXA_2 by platelet thromboxane synthase (2). Controlling steps in TX synthesis include phospholipase-mediated substrate arachidonate release and the regulation of cyclooxygenase activity of PGHS by cellular lipid hydroperoxides, known as peroxide tone (2). The concept of peroxide tone denotes the concentration of cellular peroxides, such as hydrogen peroxide and free fatty acid hydroperoxides (loosely referred to as lipid hydroperoxides), that influence the activity of enzymes in PG biosynthesis (2). Since hydrogen peroxide is effective in influencing PG biosynthesis at concentrations approximately 100-fold larger than those of lipid hydroperoxides, the term peroxide tone effectively denotes lipid hydroperoxide concentrations (2). Lipid hydroperoxides have been shown to be necessary for both activation and inactivation of cyclooxygenase activity (2, 3). Activation of PGHS occurs at n*M* lipid hydroperoxide concentrations, K_p = 20 n*M* for the cyclooxygenase activity of PGHS (2), but inhi-

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bition of PGHS occurs with lipid hydroperoxides at greater than 10-μM concentrations (4, 5).

PG metabolism has been shown to be influenced by nutrition. Diet may influence the cellular peroxide concentration either through changes in phospholipid arachidonate substrate (dietary linoleate) availability (6) or changes in cellular antioxidant status (2). For example, vitamin E deficiency (7–9) and selenium deficiency (10) have been shown to increase platelet TX synthesis, and copper deficiency (11) has been shown to increase challenged, recalcified, whole blood TX synthesis. Superoxide plays a role in the formation of lipid hydroperoxides (12). A decrease in cytosolic copper-dependent superoxide dismutase (CuSOD), the main cellular defense against superoxide, could lead to increased cellular peroxides. Dietary copper deficiency has been shown to decrease CuSOD in several tissues, such as the liver and aorta (13, 14), but it is not known whether platelet CuSOD responds to diet copper. Dietary copper deficiency also decreases selenium-dependent glutathione peroxidase (GSPX) in liver (13), but the effect on platelet GSPX is unknown.

Previous work has shown that rat liver homogenate PGE_2 and $PGF_{2\alpha}$ synthesis is influenced by CuSOD (15). Copper deficiency in a collagen-challenged, recalcified, whole blood model significantly increased TX production by 35% (11). In contrast Cunnane *et al.* (16) have shown a 16% reduction in TX synthesis in the serum of moderately copper-deficient rats. Methodological differences between these two studies include several variables: challenged recalcified whole blood (11) versus unchallenged clotted blood (16), different degrees of copper deficiency, and dissimilar ages of rats and feeding protocols. The objective of this study was to examine washed platelet TX synthesis in response to dietary copper-mediated changes in platelet CuSOD using three dietary copper concentrations: deficient, marginal, and adequate. Washed platelets provide a more defined and controlled system to examine the influence of dietary copper on TX synthesis. The washed platelet system allows for adjustment of platelet number, which has been shown to be increased in copper deficiency (17), and also allows for the measurement of platelet lipid hydroperoxides and oxyradical metabolizing enzymes, such as CuSOD and GSPX, which is not possible with whole blood models. The washed platelet system also permits a short time course for challenge, since aspirin addition to platelet suspensions immediately abolishes TX synthesis. We reasoned that short time courses for challenged platelets would be important.

Materials and Methods

Materials. Diet components were obtained from United States Biochemical Corp. (Cleveland, OH). Collagen was obtained from Helena Laboratories (Beaumont, TX). *N,N'*-Bis(2-aminoethyl)-1,3-propanediamine was from Eastman Kodak (Rochester, NY) and the tetrahydrochloride salt was prepared as described previously (18). Human thrombin, EDTA, deferoxamine, HEPES, bovine serum albumin (fatty acid free, fraction V), pyrogallol, *N*-tris(hydroxymethyl)methy-3-aminopropanesulfonic acid, diethylenetriamine pentaacetic acid, and reagents for lipid hydroperoxide (19) and glutathione peroxidase assays were from Sigma Chemical Co. (St. Louis, MO). TXB_2 standard was from Cayman Chemical Co. (Ann Arbor, MI) and ^3H-TXB_2 was from NEN-Du Pont (Boston, MA). Sodium pentobarbital was from Fort Dodge Laboratories (Fort Dodge, IA). All other reagents were of analytical grade.

Animals and Diets. Weanling male Sprague-Dawley rats (Charles River, Wilmington, MA), viral-antibody free, weighing approximately 42 g, were housed individually in stainless steel cages, at 20°C, with 45% relative humidity and a 12:12-hr light:dark cycle.

The diet was a modification of the American Institute of Nutrition (AIN) recommendations (20, 21) and comprised 20% vitamin-free casein, 66.3% sucrose, 3.5% Cu- and Se-free AIN 76 mineral mix, 1% vitamin E-free AIN 76 A vitamin mix, 3% cellulose, 5% vitamin E-stripped corn oil, 0.2% choline bitartrate, and 1% powdered dextrose containing supplemental Cu. The copper concentration of diets was achieved by adding appropriate amounts of finely ground $CuSO_4 \cdot 5H_2O$, dispersed in powdered dextrose, during diet preparation and was determined by atomic absorption spectrophotometry of ashed samples to be: copper deficient (CuD), 0.5 μg Cu/g; copper marginal (CuM), 1.7 μg Cu/g; and copper adequate (CuA), 7.5 μg Cu/g. Vitamin E was added to all diets at a final concentration of 55 IU/kg (50 mg all rac-α-tocopherol/kg) by mixing the vitamin with the vitamin E-stripped corn oil prior to adding the mixing diet components. $NaSeO_3 \cdot 2H_2O$ dissolved in 10 ml of distilled water was added to mixing diet components to provide 100 μg Se/kg in all diets. We chose controlled additions of both vitamin E and Se since TX synthesis is influenced by both vitamin E and Se (7–10). At entry all rats were *ad libitum* fed CuD diet containing 0.1% *N,N'*-bis(2-aminoethyl)-1,3-propanediamine·4HCl (2,3,2-tetramine·4HCl) for 1 week (11, 14, 15). 2,3,2-Tetramine·4HCl is a specific copper chelator that does not change zinc or iron stores or excretion (18, 22). Animals were then randomly assigned to one of the three treatment groups, eight animals in each group, and *ad libitum* fed their respective diets, without the copper chelator, for 10 days. Animals were individually pair meal fed (8-hr meals offered at the beginning of the dark cycle) to CuD rats for the last 16 days. We chose pair meal feeding in order to equalize both food intake (copper and linoleate precursor of arachidonic acid) and the pattern of food consumption. Simply pair feeding CuM and CuA to CuD rats would result in CuD rats consuming diet throughout the day, whereas the pair-fed animals con-

sume most of the diet when presented, that is, as a meal. Because the pattern of food consumption would have been different between CuD animals (consumption throughout the 24-hr period) and CuM and CuA animals (meal consumption), we trained animals to the meal-feeding protocol. Because the pattern of food consumption may influence the fatty acid composition of phospholipids, we chose the pair meal feeding protocol to avoid this potential confounding variable.

Tissue Sampling. Beginning on Day 33, six to nine rats per day, blood was collected from 15-hr fasted, sodium-pentobarbital-anesthetized rats, using 18-gauge siliconized needles, from the abdominal vena cava and allowed to flow into acid-citrate-dextrose (23), at 1/6 anticoagulant/blood. Platelet count and size (mean platelet volume) were obtained using a Coulter Counter (model S-plus IV; Coulter Electronics, Hialeah, FL). Platelets were purified by centrifugation (24) and washed twice in Ca^{2+}-free Tyrode's buffer containing 0.35% bovine serum albumin (24). Washed platelets were suspended in modified Tyrode's (10 m*M* HEPES [pH 7.4]) containing 2 m*M* Ca^{2+} and 0.35% bovine serum albumin (24), and an aliquot of this suspension was also counted for platelets. A 200-μl sample of suspended platelets (200×10^3 to 400×10^3 platelets/μl) was then aliquoted into siliconized cylindrical (7 × 45 mm) glass tubes containing siliconized magnetic stirrers (1.7 × 5 mm). Samples were stirred at 700 rpm in a multicavity heated stirrer at 37°C. Tubes magnetic stirrers and the multicavity heated stirrer were constructed specifically for platelet studies by Sienco (Morrison, CO). Platelet suspensions were challenged with 1 units/ml of thrombin (27.3 n*M*) or 10 μg/ml of collagen (final concentrations) by adding 20-μl aliquots of 10 units/ml of thrombin or 100 μg/ml of collagen prepared in modified Tyrode's buffer (10 m*M* HEPES [pH 7.4], 2 m*M* Ca^{2+}, and 0.35% bovine serum albumin). Separate samples of platelet suspensions from each rat, in duplicate, received both challenges. TXB_2 production was stopped with aspirin, 0.42 m*M* final. In preliminary studies we determined that TXB_2 production in response to 1 units/ml of thrombin or 10 μg/ml of collagen was linear up to 2 min but plateaued at 4 min. Hence, we chose 30 sec and 2 min for the collagen challenge and 15 sec and 2 min for the thrombin-challenged platelets. Also, in preliminary studies, we determined that platelets stirred at 700 rpm without a challenge of either thrombin or collagen did not produce detectable TXB_2, and that 0.42 m*M* (final) aspirin abolished platelet TX production. After aspirin addition, samples were centrifuged at 1000*g*, at 4°C for 10 min and supernatants were stored at −70°C until assayed for TXB_2. TXB_2, the spontaneous nonenzymatic degradation product of TXA_2, is stable for several months when stored at −70°C. The remaining unchallenged platelet samples were stored at −70°C for CuSOD and GSPX analysis.

In two separate experiments with the same design, washed platelet suspension aliquots were challenged with 10 μg/ml of collagen or 1 unit/ml of thrombin as described above, and the remaining unchallenged platelets were brought to 2.0 m*M* deferoxamine and stored at −70°C for lipid hydroperoxide analysis. Separate experiments were necessary since there was insufficient platelet sample to measure CuSOD, GSPX, and lipid peroxides in a single experiment.

Thromboxane Analysis. Platelet TXB_2, the spontaneous (nonenzymatic) degradation product of TXA_2, was determined by a previously validated, specific, double-antibody radioimmunoassay using ^{3}H-TXB_2 and a standard curve constructed using TXB_2 standard (25, 26).

Platelet Superoxide Dismutase and Glutathione Peroxidase Analysis. Remaining samples of unchallenged platelets were homogenized in 73 m*M* Na phosphate (pH 7.4) and 0.1 m*M* EDTA. Platelet homogenates were centrifuged at 14,000*g* and supernatants were dialyzed for 1 hr at 4°C against 1 liter of homogenization buffer. CuSOD was assayed by the pyrogallol autoxidation inhibition assay, modified to increase sensitivity as described by Prohaska (27). Samples of dialyzed platelet homogenates were treated with 0.4 vol of 25/15 ethanol/chloroform (v/v), vortexed, and centrifuged at 14,000*g* before analysis in order to measure CuSOD. Ethanol/chloroform treatment abolishes contributions from Mn-dependent SOD activity but is without effect on CuSOD activity (27). One unit of SOD activity is defined as 50% inhibition of the initial (uninhibited) rate of pyrogallol autoxidation, which was adjusted to be 0.02 absorbance units/min (27). Hence, all CuSOD activity values are specific to this initial rate. CuSOD activity is expressed as units/10^8 platelets.

The NADPH-coupled spectrophotometric assay of Gunzler and Flohe (28) was used for GSPX analysis of dialyzed platelet homogenates. In order to measure GSPX specifically, hydrogen peroxide was used as substrate and catalase activity was inhibited with 1 m*M* sodium azide (28). GSPX activity units were expressed as μmol NADPH oxidized/min/10^9 platelets.

Lipid Hydroperoxide Analysis. Lipid hydroperoxides were measured by the glutathione-disulfide-specific glutathione reductase recycling assay described previously using *t*-butyl hydroperoxide as standard (19). Plasma samples (2.0 m*M* deferoxamine) were brought to 20% ethanol and centrifuged at 14,000*g*; platelets (2.0 m*M* deferoxamine), were homogenized in modified Tyrode's suspension buffer and centrifuged at 14,000*g*.

Copper Analysis. Diet and lyophilized liver samples were ashed at 550°C for 15 hr and analyzed by flame atomic absorption spectrophotometry (model 257; Instrumentation Laboratories, Wilmington, MA). Analyses were validated using National Institute of Standards and Technology (Gaithersburg, MD) stand-

ard bovine liver 1577a, and copper values agreed within 95–98% of the stated standard value.

Statistical Analysis. Data were analyzed by analysis of variance and differences between means were separated by Fisher's protected least significant difference test. All data analyzed for differences between means by this least significant test had overall F-values from analysis of variance that were significant ($P < 0.05$), unless otherwise stated in the legend to figures or tables. For some, data correlation analysis was performed (29).

Results

Growth of rats was normal and copper deficiency significantly depressed growth by 10–20%. In copper-marginal rats, there was no significant reduction in body weight. Liver copper concentrations, an index of copper status, were significantly different among all diet groups (Table I). Unchallenged platelet CuSOD was also significantly affected by diet copper, with a 47% and 34% decrease in CuD platelets in comparison to CuA and CuM, respectively (Table I). The approximately 20% depression in CuM platelet CuSOD was not significant. Platelet GSPX activity was low, but detectable, with no effect of copper or differences among groups (Table I). Platelet number showed a significant effect of diet copper, with a significant 18% increase in platelet number in CuD (Table I). Mean platelet volume was not affected by diet copper treatment (Table I).

TXB_2 synthesis at 2 min in response to 10 μg collagen/ml showed a significant effect of copper, with 55% and 43% elevations in CuD platelet TXB_2 production in comparison to CuA and CuM. At 30 sec, TXB_2 synthesis in response to the collagen challenge was approximately 40 pg/10^6 platelets in all groups, with no differences among groups (Fig. 1). In response to 1 unit/ml of thrombin, there was a significant effect of copper at both 15 sec and 2 min. CuD TXB_2 production was significantly increased 2.4-fold at 15 sec and 2.6-fold at 2 min in comparison to CuA platelets. CuM platelet TXB_2 production was also significantly elevated by 55% in comparison to CuA at 2 min, and the 43% elevation at 15 sec approached significance ($P < 0.08$) (Fig. 2). Platelet TXB_2 synthesis in response to the thrombin challenge showed a negative correlation with unchallenged platelet CuSOD: at 15 sec, $r = -0.50$ and $P = 0.014$, and at 2 min, $r = -0.40$ and $P = 0.079$ ($n = 19$). Thrombin-challenged platelet TXB_2 synthesis also showed a negative correlation with copper status (liver copper): at 15 sec, $r = -0.55$ and $P = 0.010$, and at 2 min, $r = -0.46$ and $P = 0.023$ ($n = 21$).

In the subsequent experiments, liver copper values were similar: 16.8 ± 0.5, 11.6 ± 0.8, and 4.0 ± 0.4 (mean ± SE) for CuA, CuM, and CuD, respectively, with significant differences ($P < 0.05$) among all groups for the collagen experiment; 16.7 ± 0.5 and 1.4 ± 0.2 (mean ± SE) for CuA and CuD, respectively, $P < 0.001$, for the thrombin experiment. An aliquot of 10-μg/ml collagen-challenged platelets showed nonsignificant increases in TXB_2 production at 2 min similar in magnitude to that described above (Table II). Platelet lipid hydroperoxide values (unstimulated platelets) showed a nonsignificant increase of approximately 50% in CuD platelets in comparison to CuA or CuM platelets (Table II). When collagen-stimulated TXB_2 production was compared with endogenous lipid hydroperoxide concentrations, a highly significant correlation was obtained: $r = 0.60$, $P < 0.013$, $n = 16$. Plasma lipid hydroperoxide values are included (Table II) as an indicator of the validity of the assay in relation to other plasma lipid hydroperoxide values reported. In the experiment with 1 unit/ml of thrombin-stimulated platelets (no CuM group was included in this study), there was a significant 63% ($P < 0.02$) increase at 15 sec in TXB_2 production by CuD platelets (Table II). Platelet lipid hydroperoxides again showed a nonsignificant 67% increase in CuD platelets. Since analysis of variance showed no significant interexperiment difference, platelet lipid hydroperoxide values for CuD in both the collagen and thrombin experiment (Table II) were pooled, and, similarly, CuA platelet lipid hydroperoxide values in theses two experiments were also pooled. Mean ± SE for the pooled data for CuA was 8.8 ± 1.0 pmol LOOH/10^6 platelets and for CuD was 15.1 ± 2.9 pmol LOOH/10^6 platelets, with the 71% increase in LOOH of CuD platelets significant, $P < 0.05$.

Table I. Platelet Number and Volume, Platelet CuSOD, and GSPX Activity and Liver Copper of Copper-Deficient, -Marginal, and -Adequate Rats[a]

	CuA	CuM	CuD
Platelets/μl[b]	912.3 ± 37.5*	1001.6 ± 50.2*,†	1075.8 ± 41.9†
Mean platelet volume (fl)	4.8 ± 0.1*	5.0 ± 0.1*	5.1 ± 0.1*
CuSOD (units/10^8 platelets)	1.29 ± 0.11*	1.05 ± 0.13*	0.69 ± 0.04†
GSPX (units/10^9 platelets)	0.23 ± 0.02*	0.26 ± 0.06*	0.30 ± 0.07*
Liver Cu (μg/g dry)	13.6 ± 0.8*	9.1 ± 1.1†	4.8 ± 0.8‡

[a] Values are mean ± SE, $n = 6$–8. Values in rows not sharing common symbols (*, †, ‡) are significantly different, $P < 0.05$. Analysis of variance f-test was significant, $P < 0.05$, for all except mean platelet volume and GSPX.
[b] Number × 10^{-3}.

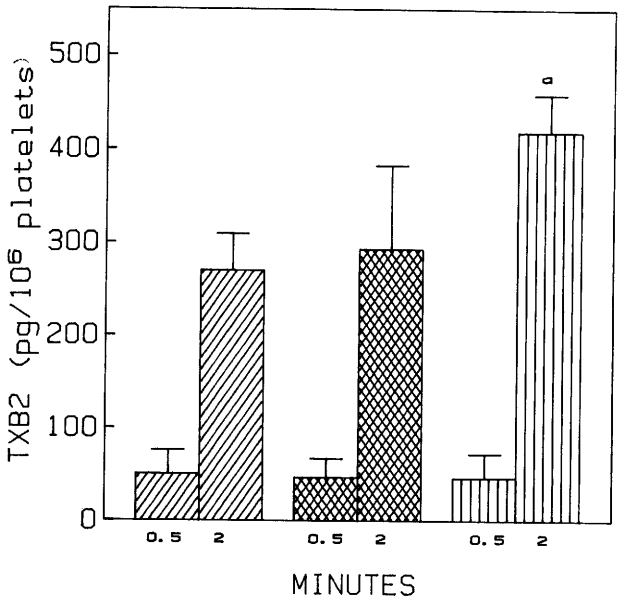

Figure 1. Washed platelet TXB_2 production in response to 10 μg collagen/ml. Copper deficient, ▥; copper marginal, ▩; copper adequate, ▨. a, At 2 min, the copper-deficient group was significantly different from the copper marginal and adequate groups, $P < 0.05$.

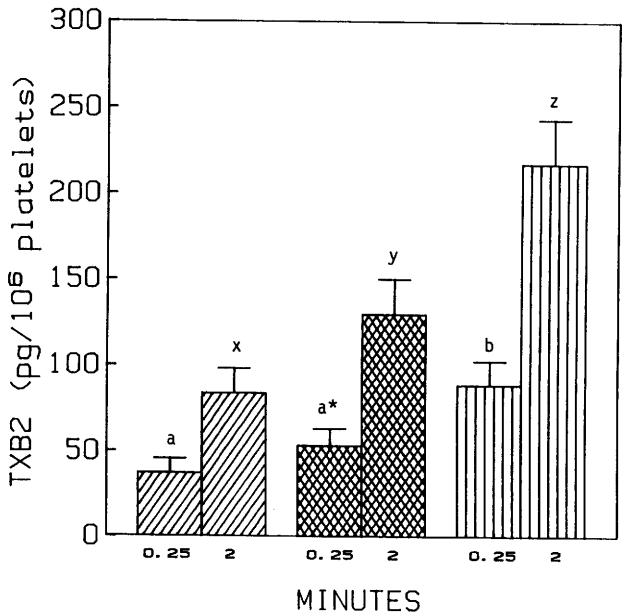

Figure 2. Washed platelet TXB_2 production in response to 1 unit/ml of thrombin. Copper deficient, ▥; copper marginal, ▩; copper adequate ▨. Values not sharing common letter superscripts are significantly different: at 15 sec, a and b, $P < 0.05$, $^*P < 0.08$ between copper adequate and copper marginal; at 2 min, x, y, and z, $P < 0.05$.

Discussion

Our objectives in this study were to resolve the differences reported in TX production in response to dietary copper reported with two whole blood models (11, 16) using a defined system (washed platelets) that allowed for the measurement of platelet CuSOD, GSPX, and lipid hydroperoxides, and to examine changes in platelet lipid hydroperoxides and their association with platelet TX production. In both of the previous whole blood studies, either challenged recalcified (11) or unchallenged clotted (16) TX production was measured with no assessment of functional blood constituent copper pools. We have now shown that in a defined system, purified washed platelets, copper deficiency significantly increases challenged platelet TX production. Using three levels of dietary copper, the degree of deficiency, indicated by liver copper and platelet CuSOD activity, resulted in an analogous increase in TX production. With thrombin-challenged platelets, both at 15 sec and 2 min, there were significant differences in TX production among all three diet copper groups. This suggests a dose-responsive (diet copper or platelet CuSOD activity) increase in platelet thromboxane production over the range of diet copper employed in this study.

Several reports have suggested that copper deficiency reduces GSPX activity by an unknown mechanism (13). However, platelet GSPX was unaffected by copper deficiency and hence cannot account for these results. Furthermore, the dose-response (diet copper) decrease observed in platelet CuSOD in copper-deficient, -marginal, and -adequate platelets has not been reported previously.

The amount of TX produced in the study of Cunnane *et al.* (16), 1.0–1.5 ng TXB_2/ml serum, is quite low for clotted blood. In the challenged, recalcified, whole blood model (11), TXB_2 production was much larger, 300–800 ng/ml, perhaps reflecting the challenge employed. In the present study, the 10-μg/ml collagen challenge appeared to proceed 2- to 3-fold more rapidly over the 2-min time course and to have more variation, in terms of TX production, than the 1 unit/ml of thrombin challenge. This may be due to the distinct platelet receptors involved in collagen and thrombin stimulation (30, 31), with a more rapid response to the collagen challenge.

The increased TX production observed in response to copper deficiency cannot be attributed to the increased platelet number of copper deficiency, since platelet TX production was expressed on a 10^6 platelet basis. A previous report has noted a significant increase in mean platelet volume in copper deficiency, but platelet number was not measured (17). However, the present study showed no effect of copper on platelet volume. In the study of Johnson and Dufault (17), the degree of copper deficiency, as indicated by liver copper, was considerably more severe than in this study. Hence, our results cannot be attributed to increased platelet volume, and this is significant since larger platelets have been shown to produce more TX (32, 33). We do not have an explanation for the increase in platelet number observed in CuD, but an increase in platelet number has been reported previously in extreme copper deficiency (17, references therein).

In vitro studies, using purified cyclooxygenase with

Table II. Collagen- and Thrombin-Stimulated Platelet TXB_2 Production and Platelet and Plasma Lipid Hydroperoxide (LOOH) Concentration of Copper-Deficient, -Marginal, and -Adequate Rats[a]

	CuA	CuM	CuD
Collagen-stimulated platelets			
TXB_2 (pg/10^6 platelets)	401.0 ± 80.1*	426.7 ± 75.3*	675.6 ± 176.8*
Plasma LOOH (μM)	3.8 ± 0.7*	3.8 ± 0.7*	4.2 ± 0.4*
Platelet LOOH (pmol/10^6 platelets)	7.4 ± 1.2*	6.4 ± 1.3*	10.9 ± 2.3*
Thrombin-stimulated platelets			
TXB_2 (pg/10^6 platelets)	149.1 ± 23.5*		243.2 ± 26.5†
Platelet LOOH (pmol/10^6 platelets)	9.8 ± 4.0*		16.4 ± 1.3*

[a] Values (mean ± SE), n = 6–8, not sharing common symbols (*, †) are significantly different, $P < 0.02$. Collagen (10 μg/ml) and thrombin (1 unit/ml) stimulated TXB_2 at 2 min and 0.25 min, respectively. Platelets were stirred at 700 rpm (37°C).

added (exogenous) lipid hydroperoxides, have shown the importance of peroxide tone in stimulating cyclooxygenase activity (34). However, peroxide tone regulation of the cyclooxygenase activity of PGHS remains an *in vitro* concept because its relevance to tissue PG production has not been demonstrated. In the subsequent experiments, we applied a specific lipid hydroperoxide assay which measures free fatty acid hydroperoxides (19) to plasma and unchallenged platelets. Plasma lipid hydroperoxide values were approximately 2- to 3-fold higher than those reported for human plasma and rabbit plasma as measured by the cyclooxygenase activation assay of Lands (34, 35). However, the values are considerably lower than the typical overestimation of peroxide tone provided by the malondialdehyde-thiobarbituric acid reactivity test (36). Unchallenged (endogenous) platelet lipid hydroperoxide concentrations have not been measured before. We chose to measure endogenous (unchallenged) values, without extraction, because the time course of platelet tone is unknown and also because we wished to examine initial (endogenous) platelet hydroperoxide concentrations and correlate these with subsequently challenged platelet TXB_2 production. In both of the experiments in which platelet lipid hydroperoxide concentrations were measured, there was a nonsignificant increase in lipid hydroperoxides from copper-deficient platelets. However, TX production showed a highly significant correlation with endogenous lipid hydroperoxide values. This is the first direct report to show that the peroxide tone of a tissue (platelets) influences prostaglandin (platelet TX) production. Furthermore, pooling of the lipid hydroperoxide concentration data in CuD platelets from these two experiments, and similarly pooling CuA platelet lipid hydroperoxide concentration data, showed that copper deficiency significantly ($P < 0.05$) increased endogenous CuD lipid hydroperoxide concentrations by approximately 71%. The only previous model to show the importance of peroxide tone involves purified, *in vitro*, cyclooxygenase stimulation by added (exogenous) lipid hydroperoxides (34), and the indirect implication of peroxide tone in models in which antioxidant nutrients were deficient (2). Hence, these results indicate the importance of peroxide tone in the regulation of tissue TX production. Furthermore, these results also suggest that dietary copper, by influencing CuSOD activity, is also a constraint on tissue (platelet) TX synthesis independent from Se-dependent GSPX activity (3). The lipid hydroperoxide assay employed measures free fatty acid hydroperoxides and not phospholipid hydroperoxides (19). However, homogenization of platelets in the Ca^{2+}-containing Tyrode's suspension buffer may activate phospholipases and release these phospholipid hydroperoxides as free fatty acid hydroperoxides. Hence, this assay may reflect total potential endogenous lipid hydroperoxides.

Overall consideration of these data indicates that both dietary copper deficiency and marginality significantly increase platelet TX synthesis through changes in platelet CuSOD in a dose-responsive (diet copper and platelet CuSOD activity) manner and that platelet TX synthesis is influenced by peroxide tone.

These results may have relevance to human health since there have been numerous reports that commonly consumed human diets contain insufficient copper to replace daily losses (37). However, severe copper deficiency is rare in human beings (37). The copper concentration of the CuM diet used in this study is very close to the mean value of 1.86 μg Cu/g dry human diets reported by Klevay (38).

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Multiple Pathways Lead to Activation of the Survival Mechanism in Quiescent BALB/c-3T3 Cells (43524)

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Abstract. **The survival of density-arrested quiescent murine BALB/c-3T3 cells in serum-free Dulbecco's medium requires the presence of cell growth factors or second messenger agonists. The protein synthesis inhibitor anisomycin blocks the survival-mediating action of the basic fibroblast growth factor (bFGF) and of 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but has little or no effect on the protective action of platelet-derived growth factor or 8-bromoadenosine 3′:5′-cyclic monophosphate (Br-cAMP). The effects of anisomycin are concentration dependent in the range from 2.5 to 25 μM and show that the survival-enhancing abilities of bFGF and TPA critically require protein synthesis, whereas those of platelet-derived growth factor and Br-cAMP do not. The survival-mediating action of bFGF and TPA can also be blocked with the RNA synthesis inhibitors actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), whereas the action of platelet-derived growth factor and Br-cAMP is largely resistant. Results on the time course of action of DRB, a selective inhibitor of the synthesis of mRNA precursor molecules, suggest that the RNA required for the survival-enhancing action of bFGF and TPA is present in cells at the time of serum withdrawal and addition of the survival factor and has a half-life greater than 3 h. The new evidence provides further support for the hypothesis that protection of serum-deprived, density-arrested BALB/c-3T3 fibroblasts against death can be achieved either via pathways that entail the synthesis of protein and RNA (e.g., via diacylglycerol-protein kinase C) or via pathways that do not involve *de novo* biosynthesis (e.g., via cAMP-protein kinase A).**

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The survival of density-inhibited quiescent BALB/c-3T3 fibroblasts in the absence of serum requires the presence of growth factors (1–3). In their absence, most of the cells die with approximately first-order kinetics over the course of 5 hr from serum withdrawal (2, 3). The death process is characterized by contraction of the cells and cytoplasmic blebbing at the plasma membrane. This mode of death is unusual in that it is not associated with the commonly recognized signs of either necrosis (swelling before rupture) or apoptosis (nuclear condensation and lobulation before cleavage of nuclear DNA and cell fragmentation) (3).

Of special note are the observations that second messenger agonists can replace growth factors in protecting density-inhibited cells against death (2, 3). We have reported that whereas the survival-enhancing activity of some growth factors and second messenger agonists is dependent on protein and RNA synthesis, that of others is not or is less so (1–3). Our present hypothesis is that the cellular death process in serum-deprived, density-arrested BALB/c-3T3 cells is initiated by an as yet unidentified event whose occurrence is prevented by the presence of appropriate growth factors and/or cytokines acting as survival factors (4). Different factors may use distinct and sometimes multiple pathways that presumably converge on a mechanism critical for the maintenance of cellular integrity (4).

In the present studies, we have investigated the effects of the duration of treatment of cells with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of messenger precursor RNA synthesis, on the survival of serum-deprived quiescent BALB/c-3T3 cells mediated by platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), 8-bromoadenosine 3′:5′-cyclic monophosphate (Br-cAMP), or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The results of

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