Copper-Marginal and Copper-Deficient Diets Decrease Aortic Prostacyclin Production and Copper-Dependent Superoxide Dismutase Activity, and Increase Aortic Lipid Peroxidation in Rats[1]

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ABSTRACT Agonist challenged aortic prostacyclin production was examined in copper-adequate, -marginal and -deficient rats fed AIN-based diets providing 6.7, 1.7 and 0.8 μg Cu/g, respectively. Aortic rings were incubated in Krebs-Henseleit salts, 10 mmol/L HEPES buffer, pH 7.4, 95%:5% O_2:CO_2, 37°C, and equilibrated for 1 h. Equilibrated rings were challenged with buffer (basal), 273.0 nmol/L thrombin and angiotensin II at 84.6 pmol/L and 846.0 pmol/L. Prostacyclin production, determined at 10 minutes by RIA as 6-keto prostaglandin $F_{1\alpha}$, in basal and 84.6 pmol/L angiotensin II ring incubations was significantly reduced by 28 to 48% in copper-deficient rats. With thrombin or 846.0 pmol/L angiotensin II prostacyclin production was significantly reduced by 18 to 55% in copper-marginal and copper-deficient rats. Copper-dependent superoxide dismutase activity was significantly depressed by 30 and 57% in aortae of copper-marginal and copper-deficient rats. Lipid peroxidation, estimated by the thiobarbituric acid test, was significantly increased by 85% in copper-deficient rats, with a nonsignificant 40% increase in aortae from copper-marginal rats. The results suggest that the decreases in aortic prostacyclin production in aortea from both copper-deficient and copper-marginal rats are associated, in a dose-dependent manner, with copper-dependent superoxide dismutase depression and increases in aortic lipid peroxidation. J. Nutr. 122: 2101-2108, 1992.

INDEXING KEY WORDS:

- *dietary copper* • *superoxide dismutase*
- *aortic prostacyclin* • *rats*
- *lipid peroxidation*

Dietary copper deficiency and exogenous copper-dependent superoxide dismutase (CuSOD, EC 1.15.1.1)[3] additions have been shown to influence rat liver homogenate prostaglandin E_2 and prostaglandin $F_{2\alpha}$ production, suggesting a relationship between prostaglandin production and CuSOD (Lampi et al. 1988). Unchallenged aortic ring prostacyclin production has been shown to be depressed in severely copper-deficient rats, but not in copper-marginal animals, and SOD activity depression was associated with this reduction in prostacyclin production (Mitchell et al. 1988). However, this study (Mitchell et al. 1988) used freshly excised aortic rings incubated in a buffer system without physiologically relevant agonist stimulation and measured total superoxide dismutase activity as opposed to CuSOD activity, and no measurements of lipid peroxidation were made.

Prostacyclin, the major cyclooxygenase product of arachidonic acid metabolism by arterial endothelial and smooth muscle cells, is important in vascular homeostasis because it is a vasodilator and inhibits platelet aggregation (Dusting et al. 1982). The synthesis of prostacyclin involves prostaglandin H synthase (EC 1.14.99.1), the rate-controlling step common to all prostaglandin syntheses, and prostaglandin I synthase (EC 5.3.99.4), which converts

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[3]Abbreviations used: CuA, copper adequate; CuM, copper marginal; CuD, copper deficient; CuSOD, copper-dependent superoxide dismutase; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; SOD, superoxide dismutase.

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prostaglandin H to prostacyclin. Both of these steps have been shown to be influenced by lipid hydroperoxides (Lands et al. 1984). The cyclooxygenase activity of prostaglandin H synthase requires nanomolar quantities of activator lipid hydroperoxides (K_p = 20 nmol/L) (Lands et al. 1984, Marshall et al. 1987). Inhibition of prostaglandin H synthase occurs with lipid hydroperoxides >10 μmol/L (Markey et al. 1987, Whelan et al. 1986). Prostaglandin I synthase is especially sensitive to inactivation by lipid hydroperoxides (Moncada et al. 1976), and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) inhibits blood vessel microsome prostaglandin I synthase with a 50% inhibition constant of 1.2 μmol/L (Moncada et al. 1976). Thus, the inhibition of prostaglandin I synthase by lipid hydroperoxides seems to occur at lower concentrations than those required to cause irreversible inhibition of prostaglandin H synthase (Dusting et al. 1982).

This study was designed to examine prostacyclin synthesis in aortic ring incubations in response to physiologically relevant agonist stimulation in response to copper-deficient, -marginal and -adequate diets. Copper-dependent superoxide dismutase activity is dependent on dietary copper provision, and the role of CuSOD in oxyradical metabolism and lipid peroxidation is well recognized (Halliwell and Gutteridge 1989). The study was also designed to measure CuSOD activity on the same aortae samples and to examine an index of lipid peroxidation in these aortae. The hypothesis to be tested was that changes in CuSOD activity would depress aortic ring prostacyclin synthesis and that this depression would be associated with an increase in lipid peroxidation in a dose (CuSOD activity)-dependent manner. There is evidence that many commonly consumed U.S. diets provide insufficient copper, although frank copper deficiency is rare in humans (NRC 1989).

MATERIALS AND METHODS

Materials. Diet components were obtained from U.S. Biochemical (Cleveland, OH). *N,N'*-bis(2-aminoethyl)-1,3-propanediamine was from Eastman Kodak (Rochester, NY) and the tetrahydrochloride salt was prepared as described previously (Allen et al. 1987). Human thrombin (EC 3.4.21.5), human angiotensin II (acetate salt), reagents for the thiobarbituric acid estimation of lipid peroxidation, and other biochemicals were obtained from Sigma Chemical (St. Louis, MO). Reagents for the Bradford (Bradford 1976) Coomassie Blue dye binding protein assay were obtained premixed from Bio-Rad (Richmond, CA). ^{3}H-6-keto-prostaglandin $F_{1\alpha}$ was obtained from Du Pont NEN (Boston, MA) and 6-keto-prostaglandin $F_{1\alpha}$ standard from Cayman Chemical (Ann Arbor, MI).

TABLE 1

Diet composition

Component	Amount
	g/100 g
Sucrose	66.3
Casein, vitamin-free	20.0
AIN-76 mineral mix, Cu- and Se-free[1]	3.5
AIN-76A vitamin mix, vitamin E–free[2]	1.0
Cellulose[3]	3.0
Choline bitartrate	0.2
Dextrose[4]	1.0
Corn oil, vitamin E–stripped[2]	5.0

[1]Se, 100 μg/kg diet, added as $NaSeO_3 \cdot 2H_2O$ during diet preparation.
[2]Vitamin E added at a concentration of 50 mg all-*rac*-α-tocopherol/kg diet by mixing the vitamin with the vitamin E–stripped corn oil.
[3]Cellufil.
[4]Containing supplementary copper.

Sodium pentabarbital was from Fort Dodge Laboratories (Fort Dodge, IA). All other reagents were analytical grade.

Animals and diets. Viral antibody-free male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed individually in stainless steel cages at 20°C, 45% relative humidity, with a 12-h light: dark cycle. For preliminary experiments, rats weighing 250 to 300 g were allowed ad libitum access to a nonpurified diet (Prolab, Agway, Syracuse, NY). For the major study, 33 weanling rats weighing ~42 g were randomly assigned to one of three diet groups: copper-deficient (CuD), 13 rats; copper-marginal (CuM), 10 rats and copper adequate (CuA), 10 rats. Three extra rats were placed in the CuD diet group because cardiac rupture is often a consequence of extreme dietary copper deficiency. Group weights at entry were 41.7 ± 0.8 g; 42.7 ± 0.6 g and 42.4 ± 0.8 g (mean ± SEM) for the copper-deficient, -marginal and -adequate diet groups, respectively. The composition of the experiment diet (**Table 1**) met the recommendations of the AIN (1977 and 1980). During diet preparation $NaSeO_3 \cdot 2H_2O$ dissolved in 10 mL of distilled water was added to provide 100 μg Se/kg in all diets. The copper concentration of the diets was achieved by adding appropriate amounts of finely ground $CuSO_4 \cdot 5H_2O$, dispersed in powdered dextrose, during mixing of the diet components. The copper concentration of the diets was determined by atomic absorption spectrophotometry of ashed diet samples and was (μg Cu/g diet): CuD diet, 0.8 ± 0.1; CuM diet, 1.7 ± 0.1; CuA diet, 6.7 ± 0.1. Vitamin E was added to all diets at a final concentration of 50.0 mg all-*rac*-α-tocopherol/kg diet by mixing the vitamin with the vitamin E–stripped corn oil before adding it to the

diet. Vitamin E–stripped corn oil with controlled addition of both vitamin E and selenium was used because deficiencies of either have been shown to influence aortic prostacyclin synthesis (Falanga et al. 1983, Schoene et al. 1986). At entry all rats in the three groups were allowed ad libitum access to the CuD diet containing 0.1% (by weight) specific copper chelator *N,N'*-bis (2-aminoethyl)-1,3-propanediamine·4HCl (2,3,2-tetramine·4HCl) for 1 wk to deplete body copper stores. 2,3,2-Tetramine·4HCl is a specific copper chelator that does not change iron or zinc stores or excretion (Allen et al. 1987, Twedt et al. 1988). Animals were then allowed ad libitum access to CuD, CuM or CuA diets, containing no 2,3,2-tetramine·4HCl, for 2 wk, followed by a 12 h/d access to diets for the next week. For the last 2 wk of the feeding protocol, rats in both the CuM and CuA diet groups were individually paired with rats in the CuD diet group and 8-h pair-fed the appropriate diets. Rats in the CuD diet group were allowed free access to food for 8 h and the amount each rat consumed was weighed. On the following day rats in the other groups were then fed identical amounts of their diets to the nearest 0.1 g. Meals were presented at the beginning of the dark cycle. This feeding protocol standardized and equalized food intake. Distilled deionized water, containing <63.0 nmol Cu/L, was freely available. All cages, glass food cups and water bottles were rinsed with 2 mmol/L EDTA and distilled water before use. All animal procedures conformed with U.S. NIH, Public Health Service and Animal Welfare Act guidelines and received prior approval from the Colorado State University Animal Care and Use Committee.

Tissue sampling. Rats were killed by exsanguination under sodium pentobarbital anesthesia beginning on d 42 following 12 h without food. Six to nine rats were killed per day from d 42 to 47, with equal numbers from each group. Livers were removed, frozen and lyophilized. The thoracic aorta from each rat was excised from the heart to the diaphragm, adventitia removed while chilled over ice and rinsed clean in ice-cold 0.15 mol/L saline. In preliminary experiments, rats were killed after 3 to 5 d of consuming the nonpurified diet and the aortae excised and cleaned in an identical manner. Aortic rings, 2 mm in length, were cut (surgical scalpel) from the cleaned aorta distal to the point of exit of the subclavian artery. The first two 2-mm rings were discarded and the next eight 2-mm rings were used for all incubation studies. Remaining thoracic aorta was frozen at −70°C for subsequent CuSOD activity and lipid peroxidation determinations.

Aortic ring incubations. We have previously shown that there is no difference in prostacyclin production by aortic ring incubation between Krebs Henseleit bicarbonate buffer, a physiological buffer, and Krebs Henseleit salts in 10 mmol/L HEPES, pH 7.4 buffer (Mitchell et al. 1988). Hence, for expediency, all aortic ring incubations were conducted with 2-mm aortic rings in 2.0 mL of Krebs Henseleit salts (providing (mmol/L): NaCl, 118; KCl, 4.74; $CaCl_2$, 2.56; $MgSO_4$, 1.18; KH_2PO_4, 1.18) 10 mmol/L HEPES, pH 7.4. Single aortic ring incubations were performed, in duplicate, in capped tubes with the head space flushed with 95%:5% O_2:CO_2 placed in a 37°C water bath. In preliminary experiments we found it necessary to stabilize the aortic ring incubations by equilibrating them for 1 h with incubation buffer changes at 20, 40 and 60 min in order to reduce prostacyclin production to a basal rate (see Results). This was necessary because unstabilized rings did not respond consistently to agonist challenge. Furthermore, equilibrated rings responded to a shaking (100 rpm) water bath by increasing prostacyclin production. Hence, in the main experiment equilibrated aortic rings were incubated without shaking. In additional preliminary experiments, equilibrated aortic rings from four rats fed the nonpurified diet were prepared as described. Prostacyclin production in response to 8.5, 84.6 and 846.0 pmol/L angiotensin II and in response to 27.3 and 273.0 nmol/L thrombin was examined over 10 min and compared with basal (unchallenged) aortic ring prostacyclin production. Both 8.5 pmol/L angiotensin II and 27.3 nmol/L thrombin showed no increase in prostacyclin production over basal values. With 846.0 pmol/L angiotensin II treatment there was a significant 85% increase in prostacyclin production. With 84.6 pmol/L angiotensin II there was a 26% increase in prostacyclin production over the basal value that approached statistical significance ($P < 0.09$). Thrombin treatment at 273.0 nmol/L showed a significant 50% increase over the basal value. Based on these results we selected 84.6 and 846.0 pmol/L angiotensin II and 273.0 nmol/L thrombin challenges for the main study. This was based both on the preliminary experiment described and our aim in the main experiment to measure basal and stimulated aortic ring prostacyclin production and, in addition, to measure CuSOD activity, glutathione peroxidase activity and lipid peroxidation in each of the aortae samples. Limited aortic sample, ~2.5 cm in length and weighing ~150 mg (wet weight) dictated basal and three challenges for prostacyclin production measurements in order to leave sufficient sample for CuSOD and glutathione peroxidase activity measurements and for lipid peroxidation analysis. In the main study, duplicate aortic rings from each of the 33 rats were incubated in 2 mL of incubation buffer and assigned as follows: segments 1 and 5 basal (unchallenged, buffer only), segments 2 and 6 273.0 nmol/L thrombin in buffer, segments 3 and 7 84.6 pmol/L angiotensin II in buffer and segments 4 and 8 846.0 pmol/L angiotensin II in buffer. Samples of incubation medium were removed at 10 min for analyses of prostacyclin.

Aortic rings were dissolved in 0.75 mol/L aqueous NaOH and assayed for protein.

Prostacyclin analysis. Prostacyclin in incubation medium was determined as 6-keto-prostaglandin $F_{1\alpha}$, the spontaneous nonenzymatic degradation product, by a previously described and validated double-antibody RIA (Steinberg et al. 1982, van Grondelle et al. 1984). All assays were performed in duplicate under equilibrium conditions in 50 mmol/L Na phosphate buffered saline, pH 7.4, containing 0.5 g/L gelatin, at 4°C. 6-keto-Prostaglandin $F_{1\alpha}$ data were expressed as picomoles per milligram of aortic ring protein.

Superoxide dismutase activity determination. Samples of aorta from each rat were homogenized in 73 mmol/L Na phosphate, pH 7.4, 0.1 mmol/L EDTA using 200-μL glass Micro Tissue Grinders (Kontes, Vineland, NJ). Aortic homogenates (~1 mL) were centrifuged at 14,000 × *g* two times and the supernatant dialyzed against 1 L of homogenization buffer for 1 h at 4°C. For the determination of CuSOD, dialyzed homogenate samples were treated with 0.4 volumes of 25:15 (v/v) ethanol:chloroform, vortexed, centrifuged at 14,000 × *g* and assayed by the pyrogallol autoxidation inhibition assay, modified to increase sensitivity as described by Prohaska (1983). One unit of superoxide dismutase (SOD) activity is defined as 50% inhibition of the uninhibited rate of pyrogallol autoxidation. We chose an uninhibited rate of 0.02 absorbance units/min, and hence all CuSOD activity values are specific to this initial rate. We validated the assay using purified bovine erythrocyte CuSOD and *Escherichia coli* manganese-dependent SOD and established that the ethanol-chloroform treatment abolished manganese-dependent SOD activity but was without effect on CuSOD activity and on the uninhibited rate of pyrogallol autoxidation. Activity data were expressed as units per milligram of cytosolic protein.

Glutathione peroxidase activity measurement. Dialyzed aortic homogenates were analyzed for glutathione peroxidase (EC 1.11.1.9) activity by the NADPH coupled spectrophotometric procedure of Gunzler and Flohe (1985). In order to measure glutathione peroxidase specifically, hydrogen peroxide was used as a substrate and catalase (EC 1.11.1.6) activity was inhibited by 1 mmol/L sodium azide (Gunzler and Flohe 1985). Activity was expressed as units per milligram of cytosolic protein. One unit of glutathione peroxidase activity is equal to 1.0 μmol NADPH oxidized/min.

Lipid peroxidation assessment. Samples of aorta from each rat were homogenized in 73 mmol/L Na phosphate, pH 7.4, 0.1 mmol/L EDTA, 2.0 μmol/L butylated hydroxytoluene as described above, but were not dialyzed. The butylated hydroxytoluene was added to the homogenization buffer by adding 1.0 mL of a 200.0 μmol/L stock solution of butylated hydroxytoluene in 95% ethanol to 100.0 mL of homogenization buffer. Homogenates (~500 μL) were clarified by centrifugation at 14,000 × *g*, and aliquots used to assess lipid peroxidation by the thiobarbituric acid test. The thiobarbituric acid test employed was based on the Tatum et al. (1990) modification of the fluorometric assay originally described by Yagi (1976). Standard malondialdehyde solution, 1.0 μmol/L in 0.01 mol/L HCl, was freshly prepared from 1,1,3,3-tetramethoxypropane. Aliquots of malondialdehyde standard and 300-μL samples of aortic homogenate supernatant were mixed with 1.0 mL of thiobarbituric acid reagent (two parts 27.8 mmol/L thiobarbituric acid in 0.2 mol/L HCl and one part water) and 0.1 mL of 22.7 mmol/L butylated hydroxytoluene in 95% ethanol. Samples were heated in a 90°C water bath for 45 min and extracted with 2.0 mL of 2-butanol. Butanol extracts were assayed by fluorimetry (Farrand Optical, Valhalla, NY), excitation at 510 nm and emission at 560 nm. Peroxidation values were expressed as pmol malondialdehyde equivalents/μg cytosolic protein.

Protein and copper analyses. Protein was analyzed by the Bradford (1976) Coomassie Blue dye binding procedure using bovine serum albumin as the standard. Aortic rings were dissolved in 0.75 mol/L NaOH before analyses; aliquots of aortic homogenate supernatants were assayed directly.

The copper content of diet and lyophilized liver samples was determined by flame atomic absorption spectrophotometry (Instrumentation Laboratories, Model 257, Wilmington, MA) on samples ashed for 15 h at 550°C. Analyses were validated using National Institutes of Standards and Technology (Gaithersburg, MD) standard bovine liver 1577a, and copper values agreed within 95 to 98% of the stated standard value.

Statistical analysis. For prostacyclin, data were analyzed by 3 × 4 factorial ANOVA, split plot design. All other data were analyzed by one-way ANOVA. The significance of differences between means were separated by Fisher's protected least significant difference test. All data analyzed for differences between means by least significant test had overall *F* tests from ANOVA that were significant ($P < 0.05$). For some data correlation analyses was performed (Steel and Torrie 1980). Values in the text are means ± SEM.

RESULTS

Rat weights at the end of the study were 247.9 ± 3.2, 234.7 ± 3.2, and 197.8 ± 6.7 g for the CuA, CuM and CuD diet groups, respectively. The CuD rats showed a significant 20% depression in weight, but there was no significant difference between the CuM and CuA diet groups.

Aortic CuSOD activity was significantly depressed 30 and 57%, in CuM and CuD diet-fed rats, respectively (**Table 2**). Aortic Se-dependent glutathione

TABLE 2

Aortic Cu-superoxide dismutase activity, glutathione peroxidase activity, lipid peroxidation and liver copper concentration of copper-deficient, -marginal and -adequate rats[1]

Group	CuSOD[2]	GSHPx[3]	MDA equivalents[4]	Liver Cu
	U/mg protein	*U/mg protein*	*pmol/μg protein*	*μmol/g dry wt*
CuA	29.6 ± 2.3[a] (10)	0.019 ± 0.001 (10)	0.39 ± 0.12[b] (7)	0.26 ± 0.01[a] (10)
CuM	20.6 ± 2.2[b] (10)	0.023 ± 0.001 (10)	0.56 ± 0.07[ab] (7)	0.18 ± 0.01[b] (10)
CuD	12.8 ± 2.3[c] (13)	0.020 ± 0.001 (13)	0.74 ± 0.15[a] (10)	0.06 ± 0.01[c] (13)

[1]Numbers in parentheses are numbers of observations (samples). CuA, CuM, CuD; copper-adequate, -marginal and -deficient diet groups. Values (means ± SEM) not sharing common letter superscripts are significantly different, $P < 0.05$.
[2]CuSOD = copper-dependent superoxide dismutase, 1 unit (U) = 50% inhibition of uninhibited rate of pyrogallol autoxidation.
[3]GSHPx = glutathione peroxidase, 1 unit (U) = 1 μmol NADPH oxidized/min.
[4]MDA, malondialdehyde, statistics on $\log_{10}$ transformed data; untransformed data shown.

peroxidase activity was unchanged by dietary copper treatment (Table 2). The estimation of lipid peroxidation, expressed as malondialdehyde equivalents (thiobarbituric acid test), is shown in Table 2. Owing to unequal variance in the three groups, $\log_{10}$ transformed data were used for statistical analyses and showed a significant effect of diet (ANOVA, $F = 3.7$, $P = 0.043$), with a significant 85% increase in aortae from CuD diet–fed rats and a nonsignificant 40% increase in aortae from CuM diet–fed rats. As an index of copper status, liver copper concentrations are shown in Table 2. There was a significant 76 and 31% decrease in liver copper in CuD and CuM diet–fed animals, respectively. There was no difference in cytosolic protein concentration of aortae from the three dietary copper groups (data not shown).

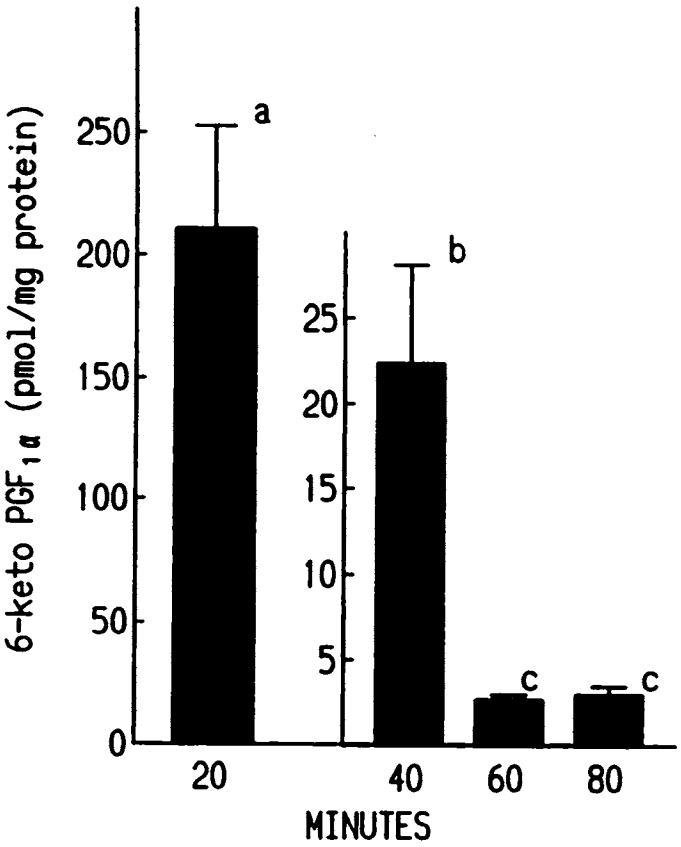

FIGURE 1 6-keto-Prostaglandin (PG)$F_{1\alpha}$ production in 20-min intervals in basal (buffer only) freshly prepared aortic ring incubations. Values are means ± SEM, $n = 6$. Values not sharing common letter superscripts are significantly different, $P < 0.05$.

Figure 1 shows the effect of equilibrating the freshly excised and cleaned aortic rings on prostacyclin production. With incubation buffer (unchallenged, buffer only) changes at 20, 40, 60 and 80 min prostacyclin production decreased by the third buffer change to ~3% of the initial rate, and remained unchanged for the next 20-min period. Based on these data all subsequent aortic ring incubations were equilibrated for 60 min with three discarded buffer changes at 20-min intervals. Equilibrated aortic ring prostacyclin production was linear over 10 min, and shaking (oscillating water bath, 100 rpm) increased prostacyclin production ~fourfold at 10 min. Shaking of equilibrated basal aortic ring incubations provided a challenge approximately equal to that of 273.0 nmol/L thrombin without shaking. There was no difference in basal prostacyclin production by equilibrated aortic rings between the eight segments used in incubation studies.

Based on the initial studies we chose 60-min equilibrated incubation conditions, without shaking, for the dietary copper study. When considering the response to challenge by control aortic rings, both 273.0 nmol/L thrombin and 846.0 pmol/L angiotensin II significantly increased prostacyclin synthesis by 80

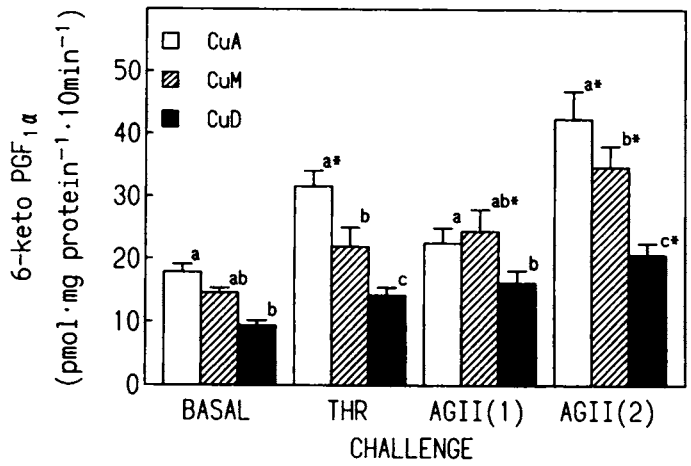

FIGURE 2 Production of 6-keto-prostaglandin (PG)$F_{1\alpha}$ in aortic ring incubations from rats fed copper-adequate (CuA), copper-marginal (CuM), and copper-deficient (CuD) diets. Challenge conditions were BASAL, buffer only; THR, 273.0 nmol/L thrombin; AGII(1), 84.6 pmol/L angiotensin II; AGII(2), 846.0 pmol/L angiotensin II. Values are means ± SEM; for copper-deficient, -marginal and -adequate groups, n = 13, 10 and 10, respectively. Within a given challenge, values not sharing common letter superscripts are significantly different, $P < 0.05$. Within a given dietary copper group, an asterisk indicates significant difference between basal and challenged conditions, $P < 0.05$.

to 120% in CuA rings incubations, in agreement with the preliminary data in rats fed nonpurified diet. With a challenge of 84.6 pmol/L angiotensin II there was a nonsignificant 26% increase in CuA ring incubations.

Total aortic protein, expressed per unit length or on a weight basis, was not different among the three dietary copper groups. When considering the response of aortic ring incubations from CuD, CuM and CuA diet–fed rats to a particular challenge, prostacyclin production by basal (unchallenged) incubations was depressed by 18% in CuM and significantly depressed by 48% in aortic rings from CuD diet–fed rats (**Fig. 2**). With both thrombin and 846.0 pmol/L angiotensin II there was a significant difference among all three groups in prostacyclin synthesis. In comparison with ring incubations from CuA diet-fed rats, aortic ring prostacyclin syntheses from CuD and CuM diet-fed rats were significantly depressed by 55 and 35%, respectively, in response to thrombin, and significantly depressed by 51 and 18%, respectively, in response to 846.0 pmol/L angiotensin II (Fig. 2). Dietary copper and challenge interacted in affecting prostacyclin production; from ANOVA treatment (challenge) × group (dietary copper), $P = 0.0016$. However, this interaction was due to the response to angiotensin II at the low dose, 84.6 pmol/L. With angiotensin II challenge of 84.6 pmol/L there was a significant 28% decrease in aortic ring prostacyclin synthesis in CuD diet–fed rats, but no effect on synthesis in aortae from CuM diet–fed rats. Hence, under challenged conditions, with the exception of the 84.6 pmol/L angiotensin II challenge, the response to a dietary copper deficit, both frank and marginal, was a dose-dependent decrease in prostacyclin production.

DISCUSSION

We have improved the aortic ring incubation system originally described (Mitchell et al. 1988) to include an equilibration (resting) protocol that allows for the measurement of agonist-mediated prostacyclin production. Freshly excised and cleaned aortic ring incubations had high prostacyclin production, which probably reflects the stimulus of excision, cleaning and ring preparation. Prostacyclin synthesis decreased exponentially with equilibration and reached base line value at 60 min (Fig. 1). Similar results using an equilibration protocol have been obtained with ex vivo perfused canine artery (Brunkwall et al. 1989) and rat pulmonary artery ring (Wang and Voelkel 1989) prostacyclin production. Equilibrated ring preparations were responsive to the thrombin and angiotensin II challenges. However, the increase in prostacyclin production elicited by these challenges was approximately half of that in freshly excised and prepared aortic rings. The same response was observed in ex vivo perfused canine arteries (Brunkwall et al. 1989). Hence, it is possible that either phospholipid arachidonate pools are depleted by the initial burst of prostacyclin production or that irreversible inhibition of prostaglandin H synthase or prostaglandin I synthase activity, due to this initial burst, has occurred. In prolonged perfusion studies with ex vivo canine arteries, similar results have been observed, and addition of exogenous arachidonic acid could not compensate for the diminished responsiveness observed in challenged arteries after the initial burst of prostacyclin production (Brunkwall et al. 1989). This implies that aortic ring phospholipid arachidonate pools are sufficient to respond to the challenges and produce prostacyclin, and suggests that irreversible inhibition of prostaglandin H synthase may have occurred during artery excision and ring preparation. The 1-h equilibration (resting) protocol is insufficient time for synthesis of new prostaglandin H synthase (Brotherton and Hoak 1983). Furthermore, the repetitive buffer changes during the resting protocol indicate that prostacyclin production in response to challenges is a true response and not merely a washout of accumulated, but unreleased, prostacyclin.

Basal prostacyclin production as a function of dietary copper is in agreement with our previous study and showed a significant reduction in rings from the CuD but not the CuM group (Mitchell et al. 1988). With challenges of 273.0 nmol/L thrombin and 846.0 pmol/L angiotensin II, the effect of dietary copper treatment was apparent in all three groups,

with prostacyclin production significantly depressed to the greatest extent in CuD aortae. Both thrombin and angiotensin II are physiologically relevant arterial challenges. Copper-dependent SOD activity was significantly depressed in both aortae from CuD and CuM diet–fed rats (Table 2), and the magnitude of the depression was significantly greater in CuD than in CuM diet–fed rats. Because there have been several reports that dietary copper deficiency depresses glutathione peroxidase activity by an unknown mechanism (Allen et al. 1988, Jenkinson et al. 1982, Prohaska 1991), we assayed this activity in aortae. Glutathione peroxidase levels were low, but detectible, with no difference between groups (Table 2). As an index of aortic lipid peroxidation, we chose the thiobarbituric acid test because small amounts of aortae were available, and we recognize the imprecision and limitations of this test (Janero 1990). Spurious metal-catalyzed autoxidation of sample polyunsaturated lipids was controlled by inclusion of butylated hydroxytoluene (Janero 1990). There was a significant effect of dietary copper on aortic malondialdehyde equivalents, with a significant 85% increase in this index of peroxidation in aortae from CuD diet–fed rats, and a non-significant increase in aortae from CuM diet–fed rats (Table 2).

Overall consideration of these data suggests that dietary copper insufficiency decreases aortic CuSOD activity, and that the depression in aortic prostacyclin synthesis is associated, in a dose-responsive manner, with decreased CuSOD activity. The association of CuSOD activity with prostacyclin production was examined by correlation analysis. In basal, 273.0 nmol/L thrombin, and 846.0 pmol/L angiotensin II incubations there was a significant correlation between CuSOD activity and prostacyclin production, with r values of 0.63, 0.54 and 0.55, respectively ($P < 0.01$ and $n = 32$ in each case). The association between aortic lipid peroxidation index and prostacyclin production was also explored. There was a significant negative correlation between malondialdehyde equivalents and prostacyclin production in basal incubations ($r = -0.44$, $P < 0.03$, $n = 24$), and this association approached significance in thrombin and 846.0 pmol/L angiotensin II incubations ($r = -0.38$, $P = 0.067$; $r = -0.37$, $P = 0.073$, respectively, $n = 24$). The significant negative correlation between aortic ring lipid peroxidation and prostacyclin production, and the significant positive correlation between aortic ring prostacyclin production and CuSOD activity suggests that decreased aortic CuSOD activity may result in increased aortic lipid peroxidation and hence decreased aortic prostacyclin production. Other studies have suggested that frank copper deficiency decreases hepatic CuSOD activity and increases in vivo lipid peroxidation assessed by breath ethane production in rats (Saari et al. 1990). Hence, the finding in this study of decreased aortic CuSOD and of increased lipid peroxidation in aortic tissue, although measured by the thiobarbituric acid test, is consonant with this evidence of increased lipid peroxidation in living animals (Saari et al. 1990).

As regards the mechanism of the depression in prostacyclin production, we suggest that lipid peroxidation, as a consequence of depressed CuSOD activity, is inhibiting prostaglandin I synthase, or prostaglandin H synthase. As was mentioned previously, it is possible that irreversible inhibition of prostaglandin H synthase or prostaglandin I synthase, as a consequence of the initial burst of prostacyclin production in freshly excised and prepared rings, may have occurred. Hence, we cannot exclude the possibility that this effect may have occurred during the excision or ring preparation stages rather than during the equilibration and subsequently challenged phase. The possibility of prostaglandin H synthase or prostaglandin I synthase inhibition during aortic ring preparation, as opposed to prostaglandin I synthase or prostaglandin H synthase inhibition after equilibration and subsequent challenge, still suggests that lipid peroxidation due to depressed CuSOD can account for these results. These possibilities are consonant with the known sensitivity of prostaglandin I synthase to lipid hydroperoxides (Moncada et al. 1976) and the irreversible inhibition of prostaglandin H synthase by lipid hydroperoxides (Lands et al. 1984). This explanation is also consistent with the depression in prostacyclin production observed in vitamin E (Falanga et al. 1983, Karpen et al. 1981, Okuma et al. 1980, Spitz et al. 1985) and selenium deficiency studies (Schoene et al. 1986).

These findings may be of relevance to human health because there are many reports that commonly consumed diets contain insufficient copper to replace daily losses, although frank copper deficiency is rare in humans (NRC 1989). The copper concentration of the CuM diet used in this study, 1.7 μg/g, is close to the mean of 1.86 μg Cu/g of dry human diets reported by Klevay (1977).

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