Research Communications

Effects of a dietary copper deficiency on plasma coagulation factor activities in male and female mice

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A previous study in this laboratory has shown that dietary copper (Cu) status mediates the development of thrombotic lesions in female mice. In the present investigation, groups (n = 16) of 6-week-old male and female Swiss-Webster mice were fed Cu-supplemented (9.0 mg Cu/kg) or Cu-deficient (0.2 mg Cu/ kg) diets and deionized water for 49–53 days. Thrombotic lesions were observed exclusively in Cu-deficient female mice. Mice fed the Cu-deficient, compared with Cu-supplemented, diet were found to have significantly increased activated partial thromboplastin time (APTT; P < 0.001) and prothrombin time (PT; P < 0.01). Significantly decreased plasma coagulation factor V (P < 0.01) and factor VIII (P < 0.0001) activities were observed in Cu-deficient mice. APTT was significantly (P < 0.01) increased while factor VIII activity was significantly (P < 0.001) decreased in female, compared with male, mice. A significant (P < 0.05) Cu × sex interaction was found for APTT. Although the mechanism involved in the development of thrombotic lesions in Cu-deficient female mice during this study is presently unclear, these results clearly demonstrate that hemostatic function may be mediated by dietary Cu status.

Keywords: copper; factor V; factor VIII; thrombosis; mice; sex characteristics

Introduction

Thrombosis has been identified as an important factor in the etiology of coronary heart disease (CHD)[1-5] and results from epidemiologic studies[6-10] suggest that elevated plasma activities of certain coagulation factors may be indicative of increased risk for CHD. Particular significance has been attached to elevated plasma factor VII activity and fibrinogen levels[11] which, it has been suggested,[12] may dispose individuals to a hypercoagulable state with attendant thrombosis.

A previous study in this laboratory[13] demonstrated that female mice fed a copper (Cu)-deficient diet exhibit extensive cardiovascular damage including large, distended atria filled with occlusive thromboses. De-

velopment of these lesions could be prevented by feeding the mice a diet containing adequate Cu; this indicates that Cu deficiency promotes thrombogenesis. Although a hemostatic role for Cu has not been demonstrated, both coagulation factors V and VIII exhibit significant structural homology with ceruloplasmin,[14] the main Cu-transport protein found in serum.[15] In addition, factor V has been shown to contain Cu.[16] These facts suggest that factors V and VIII may potentiate the effect of dietary Cu status on thrombogenesis.[13] The aim of the present investigation was to determine the effect of a dietary Cu deficiency on plasma factor V and VIII activities in male and female mice. Plasma factor VII activity and fibrinogen levels were also measured to determine if a dietary Cu deficiency could induce a hypercoagulable state similar to that previously observed in humans with increased risk for CHD.[12]

Materials and methods

Groups (n = 16) of 6-week-old male and female Taconic Swiss-Webster mice (Taconic Farms, Germantown, NY,

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J. Nutr. Biochem., 1992, vol. 3, August   **387**

Table 1 Relative heart size, hematocrit, liver copper (Cu), and plasma ceruloplasmin (Cp) activity in male (M) and female (F) mice fed copper-supplemented (S) or -deficient (D) diets[a]

	M-S	M-D	F-S	F-D	ANOVA[b] Main effects Cu	Sex	Inter[c]
Heart size	5.1	7.3	4.5	9.7	$P < 0.001$	$P < 0.01$	$P < 0.001$
(g/kg BW)[d]	(0.1)	(0.4)	(0.1)	(0.4)			
Hematocrit	0.33	0.10	0.40	0.06	$P < 0.001$	NS	$P < 0.001$
	(0.02)	(0.02)	(0.01)	(0.01)			
Cp	30.0	4.3	15.6	4.8	$P < 0.001$	$P < 0.01$	$P < 0.01$
(μ/L)	(3.4)	(0.7)	(1.8)	(0.3)			
Liver Cu	9.2	7.2	10.8	6.6	$P < 0.01$	NS[f]	NS
(μg/g)[e]	(1.5)	(0.8)	(0.6)	(0.2)			

[a]Results given as mean (standard error).
[b]ANOVA, analysis of variance.
[c]Inter, interaction.
[d]BW, body weight.
[e]Liver Cu reported on dry weight basis.
[f]NS, not significant.

USA)* were fed Cu-supplemented (9.0 mg Cu/kg diet) or -deficient (0.2 mg Cu/kg diet) diets and deionized water ad libitum for 49–53 days. Diets were identical to those of Klevay[13] but with Zn $(CH_3COO)_2 \cdot 2H_2O$ (167.82 mg/kg), $MnSO_4$ (30–76 mg/kg), and KI (0.66 mg/kg) added to diets. $CuSO_4 \cdot 5H_2O$ (39.96 mg/kg) was added only to the Cu-supplemented diet. Briefly, diets contained sucrose (544.5 g/kg), lard (280 g/kg), and casein (80 g/kg) with all known essential vitamins and minerals. Mice were exsanguinated from the inferior vena cava and blood was collected in tubes containing 3.8% (wt/vol) trisodium citrate as an anticoagulant. To compensate for the difference in hematocrit between Cu-supplemented and -deficient mice *(Table 1)*, the blood:anticoagulant ratio was changed from the standard 9:1 to 19:1 for blood samples from all animals, i.e., 26 μL trisodium citrate (3.8% (wt/vol))/0.5 mL blood.[17] Platelet-poor plasma (PPP) was prepared from whole blood by centrifugation (2000g for 20 min; 4° C) and stored at 0–4° C. All coagulation tests were performed on PPP within 4 hours of preparation.

Activated partial thromboplastin time (APTT; sensitive to deficiencies of coagulation factors VIII, IX, XI, and XII), prothrombin time (PT; sensitive to deficiencies of coagulation factors V, VII, X, and prothrombin), and coagulation factor activities were measured by micromodified clot-based assays.[18] APTT and PT were determined by measuring the clotting time observed following recalcification (20 μL; 25 mmol $CaCl_2$) of PPP (20 μL) in the presence of either APTT reagent (20 μL; Sigma A-7668) or thromboplastin (20 μL; Sigma T-0263) at 37° C. Coagulation factor activities were determined by measuring the PT (factor V or VII) or APTT (factor VIII) obtained following addition of 10-fold diluted PPP (20 μL) to a substrate plasma severely deficient in factor V, VII, or VIII (20 μL; Sigma F5D, F7D, or F8D, respectively). PPP was diluted with Owren's veronal buffer, pH 7.35. Pooled PPP from mice fed Purina Rodent Lab Chow 5001 (Purina Mills, Inc., St. Louis, MO, USA) was serially diluted 10-fold (Factor V, VII, or VIII activity = 1.00) to

160-fold (factor V, VII, or VIII activity = 0.0625) with Owren's veronal buffer, pH 7.35, and used to generate calibration data for the factor V, VII, and VIII assays. Plots of log_{10} (APTT or PT) versus log_{10} (factor V, VII, or VIII activity) were linear and were used to determine the percentage factor V, VII, and VIII activities in PPP from experimental mice. Plasma fibrinogen levels were determined by the method of Claus.[19] Plasma ceruloplasmin (Cp) activity was assayed by the method of Schosinsky et al.[20] Dietary and liver Cu were determined by flame atomic absorption spectrophotometry following $H_2SO_4/HNO_3/H_2O_2$ digestion[21] of the diet sample or lyophilized organ.

Data were analyzed by two-way analysis of variance (ANOVA).[22] Scheffé contrast analyses were performed on data from any ANOVA with a significant $(P < 0.05)$ Cu × sex interaction.

Results

Both male and female Cu-deficient mice exhibited significant $(P < 0.01$ and $P < 0.001$, respectively) cardiac enlargement *(Table 1)*. No significant difference in heart size was found between Cu-supplemented male and female mice, but Cu-deficient females had significantly $(P < 0.01)$ larger hearts than the corresponding males. Cu-deficient mice had significantly $(P < 0.001)$ lower hematocrit than Cu-supplemented animals; a significant $(P < 0.001)$ Cu × sex interaction was also noted *(Table 1)*. Plasma Cp activity was significantly $(P < 0.001)$ lower in Cu-deficient, compared with -supplemented, mice *(Table 1)*. Cp activity was significantly $(P < 0.01)$ lower in Cu-supplemented female than male mice. Significantly $(P < 0.01)$ lower liver Cu was found in Cu-deficient, compared with -supplemented, mice *(Table 1)*.

Both APTT and PT were significantly $(P < 0.001$ and $P < 0.01$, respectively) influenced by Cu status *(Table 2)*. While PT was significantly $(P < 0.01)$ increased in both male and female Cu-deficient mice, a significantly increased APTT was found only in female Cu-deficient, compared with Cu-supplemented, mice.

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Table 2 Plasma activated partial thromboplastin time (APTT); prothrombin time (PT); and factor V, VII, and VIII activities and plasma fibrinogen in male (M) and female (F) mice fed copper-supplemented (S) or -deficient (D) diets[a]

	M-S	M-D	F-S	F-D	ANOVA[b] Main effects Cu	Sex	Inter[c]
APTT (s)	24.7 (1.3)	26.1 (1.2)	25.1 (0.5)	31.9 (1.1)	$P < 0.001$	$P < 0.01$	$P < 0.05$
PT (s)	11.6 (0.2)	12.4 (0.3)	11.1 (0.3)	12.5 (0.6)	$P < 0.01$	NS[d]	NS
F-V[e]	1.39 (0.08)	1.01 (0.08)	1.24 (0.04)	1.12 (0.12)	$P < 0.01$	NS	NS
F-VII[e]	1.58 (0.09)	1.50 (0.12)	1.30 (0.14)	1.32 (0.11)	NS	NS	NS
F-VIII[e]	1.32 (0.06)	1.12 (0.05)	1.15 (0.04)	0.84 (0.05)	$P < 0.001$	$P < 0.001$	NS
Fibrinogen (g/L)	0.9 (0.1)	1.4 (0.4)	1.1 (0.1)	0.8 (0.1)	NS	NS	NS

[a]Results given as mean (standard error).
[b]ANOVA, analysis of variance.
[c]Inter, interaction.
[d]NS, Not significant.
[e]F-V, Factor V; F-VII, Factor VII; F-VIII, Factor VIII.
Activities reported as a fraction of the activity present in pooled normal plasma from adult mice (arbitrarily assigned a value of 100%).

Plasma factor V and VIII activities were significantly ($P < 0.01$ and $P < 0.001$, respectively) lower in Cu-deficient than in -supplemented mice *(Table 2)*. In addition, female mice were found to have significantly ($P < 0.001$) lower factor VIII activity than males irrespective of dietary Cu status *(Table 2)*. No significant changes in either plasma factor VII activity or fibrinogen concentrations were found in this experiment *(Table 2)*. Although a number of the coagulation factor activities in *Table 2* exceed those observed in pooled normal plasma from reference mice, this discrepancy may result from the high fat content of the experimental diet (280 g lard/kg diet) because it has been shown that there is a direct association between fat intake and coagulation factor VII activity in humans.[23] Thus, the high fat content of the experimental diet may have resulted in a general increase in coagulation factor activities in mice fed this diet, compared with the reference mice.

Discussion

Mice fed the Cu-deficient, in contrast to the Cu-supplemented, diet exhibited significant cardiac enlargement and lowered hematocrit, plasma Cp activity, and liver Cu *(Table 1)*. Such changes are indicative of a Cu-deficient state in experimental animals.[24–28] Copper deficiency was further confirmed by observation of decreased heart and kidney Cu, decreased erythrocyte copper-zinc superoxide dismutase (EC 1.15.1.1.) activity, and increased liver iron (Lynch and Klevay, data not shown). Between days 40–48 of this experiment six Cu-deficient female mice died. These animals exhibited cardiac enlargement with thrombotic lesions similar to those observed previously.[13] No deaths oc-

curred in any of the other experimental groups. These observations are in contrast to those from experiments using rats that found lethal effects of a dietary copper deficiency exclusively in male animals.[29] The reason for such a contrasting sex-specific effect of a dietary copper deficiency in two different animal species is presently unknown. The significant changes in APTT and PT observed in Cu-deficient, compared with Cu-supplemented, mice *(Table 2)* indicate that Cu deficiency may cause dysfunction of both the intrinsic and extrinsic coagulation pathways. The increased APTT and PT may result from the decreased factor VIII and V activities, respectively, which are also found in the Cu-deficient mice *(Table 2)*. However, although no significant changes in plasma factor VII activity or fibrinogen levels were found in this experiment *(Table 2)*, altered activities of other, unmeasured, coagulation factors may not be excluded from having influenced APTT and PT. A significant sex effect on coagulation factor activity was only found for factor VIII; females exhibited lower activity than males *(Table 2)*. Although at least two studies in humans have found a similar sex distribution of factor VIII activity,[30,31] others have found no sex differences.[32,33]

The observed changes in APTT, PT, and factor V and VIII activities in the Cu-deficient mice clearly indicate that dietary Cu status may mediate hemostatic function. However, the mechanism involved is presently unclear. Although factor V has been shown to contain Cu,[16] a functional role for this metal has not yet been defined.[34] There is presently no experimental evidence that factor VIII contains Cu.[35] Therefore it seems unlikely that the decreased activities of these coagulation factors found in Cu-deficient mice may have resulted from a loss of functional Cu from the

apoprotein molecules. Furthermore, the decreased co-agulation factor activities found in the Cu-deficient mice do not seem to correlate with the increased incidence of thrombotic events observed in these animals.[13] However, a similar pathology is observed in human disseminated intravascular coagulation[36] and in some individuals with hereditary clotting factor abnormalities including factor V deficiency.[37] Perhaps the reduced plasma factor V and VIII activities observed in this study were the result of lower circulating amounts of these proteins rather than a reduction in activity per se. However, further experiments will be necessary to determine if this hypothesis is valid.

Although a daily Cu intake of 1.5–3.0 mg has been estimated as safe and adequate for adults,[38] many diets may fail to provide this amount.[39,40] Calculations[40] based on 10 dietary surveys reveal that 35% of the daily diets in the United States contain less than 1.0 mg of Cu. Amounts below 1.0 mg/day have been shown to be insufficient for more than 31 men and women in Cu-depletion experiments.[41-44] Furthermore, because it has been proposed[13,39,40,45] that CHD may be related to a dietary Cu deficiency, the results of these surveys and this investigation may be germane to the etiology of this disease in some individuals.

In conclusion, it has been shown that dietary Cu status may mediate hemostasis by altering plasma factor V and VIII activities, however, the mechanism involved is presently unclear.

Acknowledgments

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