Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 127-134

THE EFFECT OF COPPER CHELATING DRUGS ON LIVER IRON MOBILIZATION IN THE ADULT RAT

Nancy L. Cohen, Carl L. Keen, Bo Lönnerdal and Lucille S. Hurley

Department of Nutrition, University of California, Davis, CA 95616

Received April 11, 1983

SUMMARY : To test the hypothesis that ferroxidase I (ceruloplasmin) activity is essential for iron mobilization, adult rats were fed a copper sufficient diet with or without the chelating drugs D-penicillamine and triethylenetetramine for 120 days. By day 6 of treatment and for the remainder of the experiment the drug-fed rats showed low plasma copper concentration and low ferroxidase I activity. Plasma ferroxidase II activity in the DPA and TETA groups tended to be slightly lower than that of controls. No animals became anemic. Therefore, persistent low plasma ferroxidase I does not necessarily cause anemia in the adult rat.

INTRODUCTION : A relationship between copper and anemia has been recognized since 1928 when Hart and co-workers (1) reported that rats fed a milk diet for a prolonged period of time developed an anemia that was more responsive to dietary copper supplementation than to iron supplementation. The effect of copper deficiency on hemoglobin synthesis was elucidated by Gubler et al. (2) who found that pigs fed a copper deficient diet had elevated liver iron concentration, suggesting that mobilization of liver iron was impaired. More recent studies have shown that the copper metalloenzyme, ferroxidase I (Fox I) (E.C.1.12.3.1; also called ceruloplasmin), oxidizes iron and promotes its incorporation into the plasma iron transport protein, transferrin (3,4). One mechanism proposed for copper deficiency anemia is that low dietary copper causes low plasma Fox I, leading to decreased liver iron mobilization and subsequent anemia (4). However, patients with Wilson's disease or Menkes' disease, genetic disorders of copper metabolism, often have very low plasma Fox I levels, but are not anemic. Therefore, it has been suggested that there are other compounds in plasma with ferroxidase activity. A second plasma ferroxidase (Fox II), recently isolated by Topham and Frieden (5), may be responsible for liver iron mobilization in these patients. In addition, the rate limiting enzyme in heme degradation, heme

0006-291X/83 $1.50

oxygenase, may also be involved in the accumulation of iron in the liver. Williams and co-workers (6) have shown that heme oxygenase activity may be increased in copper deficiency; increased heme breakdown could explain the increased liver iron deposition and decreased blood hemoglobin seen in copper deficiency anemia.

The present study was undertaken in order to evaluate the effects of copper deficiency in the adult rat on plasma Fox I and Fox II and liver heme oxygenase activities, and to relate these changes to the presence or absence of anemia. Copper deficiency was induced by feeding a copper sufficient diet supplemented with the drugs D-penicillamine (DPA) or triethylenetetramine (TETA), which can chelate copper and rapidly induce severe copper deficiency in the adult rat (7,8). We have made use of the chelating drugs to induce copper deficiency as we have observed that rats fed a diet deficient in copper (0.6 μg/g) for a prolonged period of time can adapt (at least partially) to the deficiency with respect to Fox I activity (unpublished observations). Adaptation to a copper deficient diet (as indicated by anemia) has also been noted by others (9), and is probably due to increased absorption and/or retention of the small amounts of copper in the diet. It is our hypothesis that rats fed the copper chelating drugs do not adapt as well to the copper deficiency, and thus can be useful probes for the study of severe copper deficiency.

MATERIALS AND METHODS : Adult 190-200g female Sprague-Dawley rats were purchased from a commercial vendor (Simonsen Labs, Gilroy, CA) and housed in stainless steel cages in a temperature and light controlled room (22-23°C, 12 hr light/dark cycle). All animals were acclimated for 7 days during which time they were fed a complete purified casein-based diet containing 10 μg Cu/g diet and 120 μg Fe/g diet. The detailed composition of the diet has been described previously (7). The rats were then fed for up to 120 days one of three diets: 1) complete purified control diet; 2) control diet supplemented with 0.83% DPA (Merck, Sharp, and Dohme, 99% purity); 3) control diet supplemented with 0.83% TETA (Aldrich Chemical Co., 99% purity). The experiment was terminated at 120 days, three times the life span of the rat red blood cell (10). Feces were collected and tail vein hematocrits were obtained throughout the experimental period. At various times after beginning the experimental diets, animals were anesthetized with ether, venous blood was collected in heparinized syringes, and livers were removed. All rats were killed between 0900 and 1100 to minimize potential diurnal variation. For each time point, 4-10 animals were used.

Hematocrits were determined by centrifugation, and blood hemoglobin values were obtained by use of a commercial cyanomethemoglobin kit (Hycel, Houston, TX). Plasma Fox I was measured by both p-phenylenediamine (PPD) oxidase and ferroxidase activities. The PPD oxidase assay of Sunderman and Nomoto was used (11). Ferroxidase activity (Fox I and Fox II) was measured by the method of Topham and Frieden (5).

Heme oxygenase activity was determined in the microsomal fraction of liver in the rats fed the diets for 120 days. These rats were fasted overnight, anesthetized with ether, and opened along the ventral midline. The superior vena cava was severed, and the organs were perfused with cold saline through the right ventricle until the liver was pale in color. Microsomes were isolated by the method of Schacter (12). Heme oxygenase activity was determined by the method of Correia and Schmid (13). The samples were measured spectrophotometrically using an Aminco DW2a spectrophotometer with 464 nm as the sample wavelength and 530 nm as the reference wavelength; E = 40 mM^{-1} cm^{-1} (14).

Plasma and liver copper and iron concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer model 370, Norwalk, CT) following wet ashing with nitric acid. Using this method, the recovery of added metals is 98-100% (15). Data were analyzed using the Student's "t" test. Within each group the mean of the values for each time point from day 1-21 was compared to the mean of the pooled day 1-21 controls. The day 120 values were compared to the day 120 controls.

RESULTS : Plasma copper concentration is summarized in Figure 1. By day 5 it was 57 and 47% of controls in DPA and TETA-fed rats, respectively, and remained at 15-57% of control values for the remainder of the experiment ($p < 0.001$).

Liver copper and iron concentrations are summarized in Figure 2. By day 5 of treatment, liver copper was 71% and 57% of controls in the DPA and TETA-fed rats, respectively, and remained at approximately 70% of controls for the remainder of the experiment ($p < 0.001$). Liver iron values were similar to those of controls in both groups at all times tested. However, at day 120, the liver iron

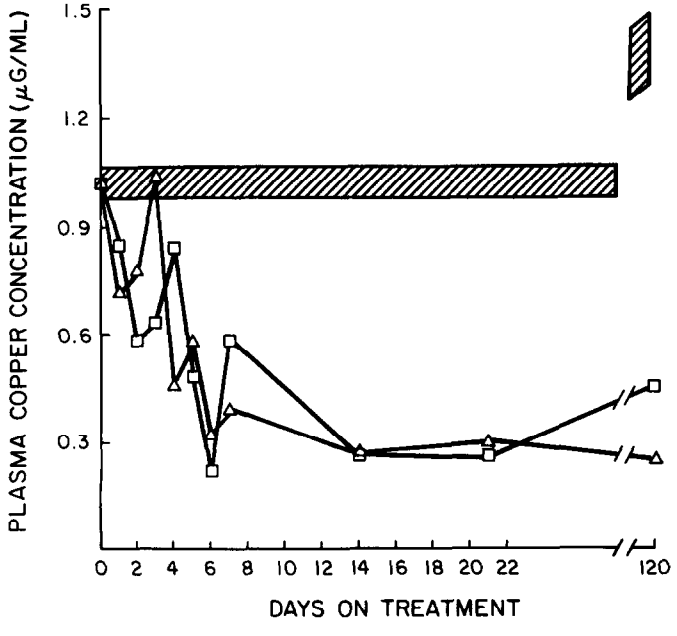

Figure 1. Plasma copper concentration of DPA (Δ) and TETA (□) fed rats. The shaded area represents the pooled means ± SEM of the day 1-21 and day 120 control values.

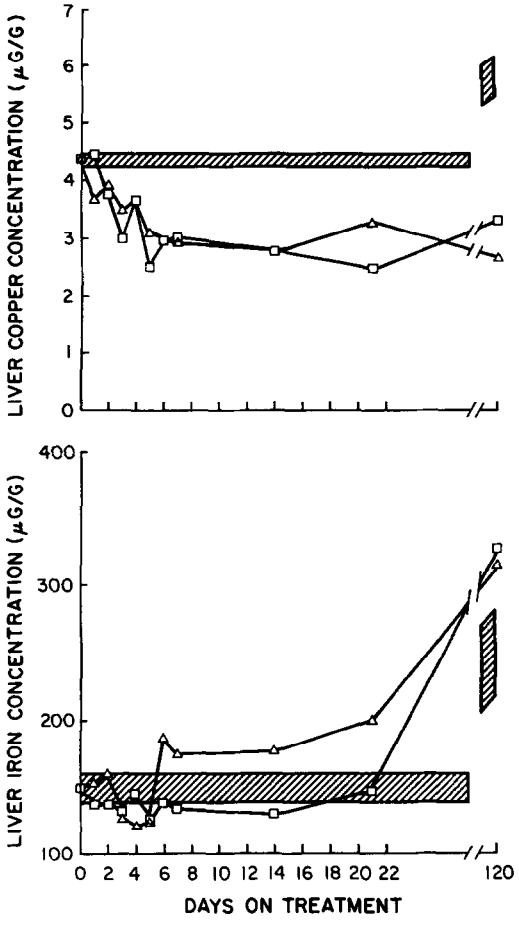

Figure 2. Liver copper and iron concentrations of DPA (Δ) and TETA (□) fed rats. The shaded area represents the pooled means ± SEM of the day 1-21 and day 120 control values.

concentration of the DPA and TETA-fed rats tended to be higher than that of controls.

Hematocrit values and hemoglobin concentrations were similar in all groups at all times. All means ranged from 90-108% of controls and 88-110% of controls for hematocrits and hemoglobin concentrations, respectively.

By day 5 of treatment plasma Fox I activity (Figure 3) was 45% and 31% of control values in DPA and TETA-fed rats, and varied between 5-42% of controls for the rest of the 120 days. The PPD oxidase activity of ceruloplasmin was consistent with its ferroxidase activity; and changes in activity were similar in both groups throughout the experiment. Fox II activity (Figure 4) in both DPA and TETA-fed rats was slightly lower than controls at some time points ($p < .05$);

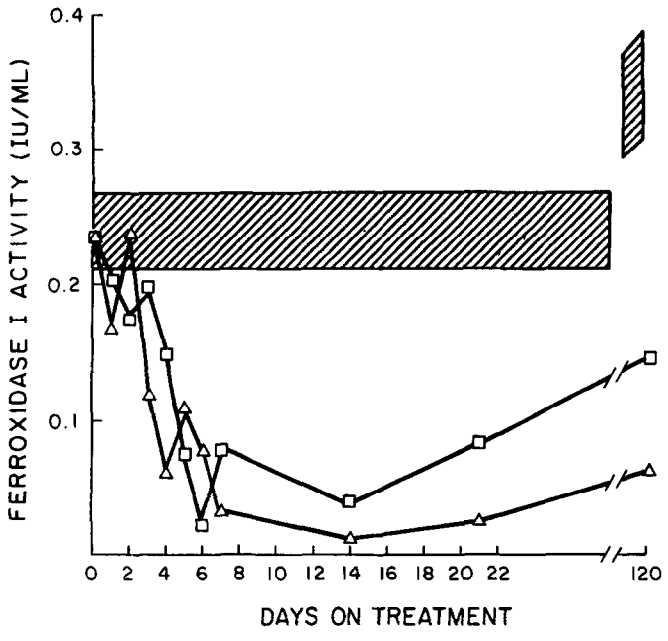

Figure 3. Plasma ferroxidase I activity of DPA (Δ) and TETA (□) fed rats. The shaded area represents the pooled means ± SEM of the day 1-21 and day 120 control values.

however there was no consistent trend. As with liver iron concentration, heme oxygenase activity of liver tended to be higher in the drug-fed rats than in controls, 0.212, 0.257, and 0.273 nmol/mg protein/hr in the control, TETA and DPA-fed groups respectively, but this difference was not statistically significant.

DISCUSSION : At day 120, the control values for plasma copper, Fox I, Fox II, and liver copper and iron were higher than the pooled day 1-21 control values. This is probably an effect of maturation and accumulation of trace elements with age. Despite prolonged low Fox I activity, anemia was not induced in the adult rats in this study. This finding is consistent with that of Prohaska (16) who reported that low ceruloplasmin and low total ferroxidase activity also did not result in anemia in the genetically copper deficient brindled mouse.

There are several possible explanations for the absence of anemia in the presence of low ferroxidase activity. First, Fox I activity may be critical only when there is a low or marginal level of dietary iron. Weisenberg et al. (17) have recently reported that copper deficient weanling rats fed adequate iron were

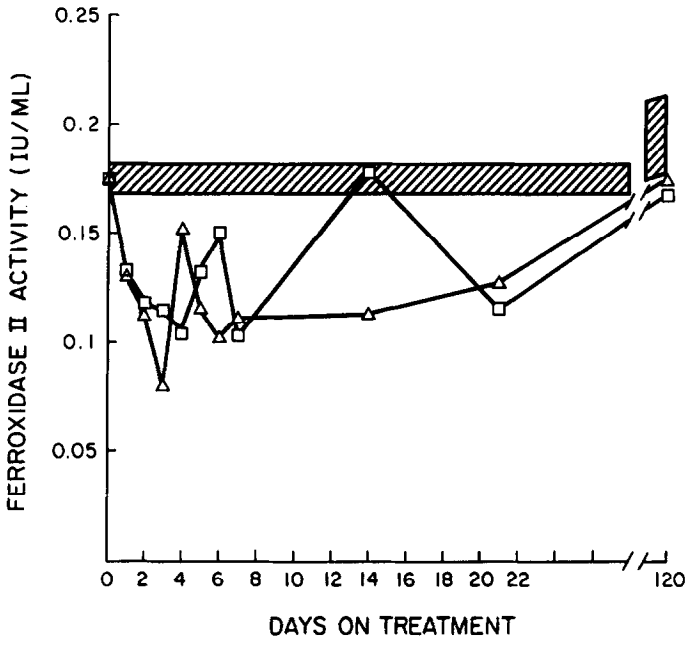

Figure 4. Plasma ferroxidase II activity of DPA (Δ) and TETA (□) fed rats. The shaded area represents the pooled means ± SEM of the day 1-21 and day 120 control values.

not anemic despite low ceruloplasmin levels, although marginally iron deficient rats fed a copper deficient diet did become anemic. As the diet used in the present study contained adequate iron (120 μg Fe/g diet), low Fox I activity may not have had functional significance with regard to iron mobilization. Alternatively, even the low activity of Fox I plus the activity of Fox II may have been sufficient to oxidize the liver iron. Roeser et al. (18) suggested that plasma ceruloplasmin levels must be less than 1% of control values in order to observe anemia, but anemia has been reported at levels of plasma ceruloplasmin higher than this (4,15). A third possibility is that the activity of Fox II alone is responsible for liver iron mobilization. The activity of this enzyme was not reduced very much by copper deficiency in the adult rat in the present study, and it may have been sufficient for iron oxidation. Topham and co-workers (19) found that rats fed a copper deficient diet did develop low levels of Fox II by day 14 of treatment, but the age of their rats was not specified.

Age may be an important consideration, as high ferroxidase activity may be necessary only under conditions of rapid hemoglobin synthesis such as growth or

pregnancy. For example, many studies cite copper deficiency anemia in weanling pigs or rats (1,4,6,17,20,21). Pregnant animals may also require high ferroxidase activity, as pregnant rats fed copper deficient diets or diets containing 0.83% DPA became copper deficient and accumulated liver iron (7,21). However, pregnant rats fed diets containing 0.83% TETA were copper deficient but did not have elevated liver iron (unpublished observations). It is possible that DPA and TETA produce different effects on iron metabolism by chelating copper at different points in its metabolism. As the specific metabolic site at which copper concentration is reduced may be critical for different functions, these chelating drugs may serve as useful tools in the study of mechanisms of copper deficiency effects (22).

Finally, another possible explanation for the absence of anemia observed in the present study is that Fox I may act in some capacity other than as a ferroxidase. For example, it may serve as a copper donor to another enzyme that actually has direct ferroxidase activity. The role of heme oxygenase in heme breakdown and liver iron deposition and the hypothesis that its elevation in copper deficiency causes increases in these functions cannot be evaluated from the present experiment, as neither anemia nor elevated heme oxygenase activity occurred.

In summary, the results of this study show that persistent low plasma Fox I does not necessarily cause anemia or elevated liver iron in the adult rat. There are several possible explanations for the lack of anemia: 1) Fox I may be critical for iron mobilization only in cases of low or marginal dietary iron; 2) even a low activity of Fox I may be sufficient to oxidize liver iron; 3) the activity of Fox II may be sufficient to oxidize liver iron; 4) high ferroxidase activity may be necessary only under conditions of rapid hemoglobin synthesis; 5) Fox I may not function as a ferroxidase, but may transport copper to the compound directly responsible for iron oxidation. These possibilities are currently being investigated.

REFERENCES :

1. Hart, E.B., Steenbock, H., Waddell, J., and Elvehjem, C.A. (1928) J. Biol. Chem. 77, 797-812.

2. Gubler, C.J., Lahey, M.E., Chase, M.S., Cartwright, G.E., and Wintrobe, M.M. (1952) Blood 7, 1075-1091.
3. Osaki, S., Johnson, D.A., and Frieden, E. (1966) J. Biol. Chem. 241, 2746-2751.
4. Evans, J.C. and Abraham, P.A. (1973) J. Nutr. 103, 196-201.
5. Topham, R.W. and Frieden, E. (1970) J. Biol. Chem. 245, 6698-6705.
6. Williams, D.M., Burk, R.F., Jenkinson, S.G., and Lawrence, R.A. (1981) J. Nutr. 111, 979-983.
7. Keen, C.L., Mark-Savage, P., Lonnerdal, B., and Hurley, L.S. (1983) Drug-Nutrient Interactions 2, 17-34.
8. Keen, C.L., Cohen, N.L., Lonnerdal, B., and Hurley, L.S. (1982) Lancet 1, 1127.
9. Lukasewycz, O.A. and Prohaska, J.R. (1982) J. Nat. Cancer Inst. 69, 489-493.
10. Schalm, O.W. (1975) Veterinary Hematology, p. 362, Lea and Febiger, Philadelphia.
11. Sunderman, F.W. and Nomoto, S. (1970) Clin. Chem. 16, 903-910.
12. Schacter, B.A. (1978) Methods Enz. 52, 367-372.
13. Correia, M.A. and Schmid, R. (1975) Biochem. Biophys. Res. Comm. 65, 1378-1384.
14. Maines, M.D. and Kappas, A. (1975) J. Biol. Chem. 250, 4171-4177.
15. Clegg, M.S., Keen, C.L., Lonnerdal, B., and Hurley, L.S. (1981) Biol. Trace Element Res. 3, 107-115.
16. Prohaska, J.R. (1981) Nutr. Res. 1, 159-167.
17. Weisenberg, E., Halbreich, A., and Mager, J. (1980) Biochem. J. 188, 633-641.
18. Roeser, H.P., Lee, G.R., Nacht, S., and Cartwright, G.E. (1970) J. Clin. Invest. 49, 2408-2417.
19. Topham, R.W., Sung, C.S., Morgan, F.G., Prince, W.D., and Jones, S.H. (1975) Arch. Biochem. Biophys. 167, 129-137.
20. Lee, G.R., Cartwright, G.E., and Wintrobe, M.M. (1968) Proc. Soc. Exp. Biol. Med. 127, 977-981.
21. Williams, D.M., Lee, G.R., and Cartwright, G.E. (1974) Am. J. Physiol. 227, 1094-1097.
22. Keen, C.L., Lonnerdal, B., and Hurley, L.S. (1982) Inflammatory Diseases and Copper, (J.R.J. Sorenson, ed.), pp. 109-121, Humana Press, Clifton, NJ.