PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 306–310 (1981)

In Vivo and *ex Vivo* Effects of Copper on Rat Liver Metallothionein (41277)

FRANCES A. DAY, MYNA PANEMANGALORE, AND FRANK O. BRADY

Division of Biochemistry, Physiology and Pharmacology, School of Medicine, The University of South Dakota, Vermillion, South Dakota 57069

Abstract. In order to test the hypothesis that exogenous copper could displace endogenous zinc from metallothionein under *in vivo* and *ex vivo* conditions, a series of zinc–copper competition experiments were conducted with rat liver. Intraperitoneal injection of zinc alone resulted in a large increase in zinc and a small increase in copper associated with hepatic metallothionein 24 hr later, as compared to controls. Intraperitoneal injection with copper alone resulted in increases in both zinc and copper bound to metallothionein 6 hr later. Intraperitoneal injection of zinc at time zero and copper at 18 hr, followed by killing at 24 hr, resulted in no increase in zinc, but a significant increase in copper, bound to metallothionein.

Exposure of rat livers to copper *ex vivo* in an isolated perfusion apparatus gave different results from the *in vivo* experiments in regards to the metal composition of metallothionein. Animals were injected ip with zinc at time zero, and their livers were removed at 18 hr for a 2-hr perfusion. The zinc and copper contents of metallothionein in the groups receiving zinc *in vivo* and then either killed or perfused without additional metal were the same. The group which received zinc *in vivo* and copper *ex vivo* showed a large decrease in its metallothionein zinc content, a decrease which was matched almost exactly by a large increase in its metallothionein copper content. These results are consistent with the above-mentioned hypothesis and with recent *in vitro* results demonstrating the ability of copper to displace zinc from metallothionein [D. Holt, L. Magos, and M. Webb, Chem.–Biol. Interact. 32, 125 (1980)].

Metallothioneins are low-molecular-weight (mol wt = 6100), cysteine-rich, metal-binding proteins, found in eucaryotes. They have been implicated as functioning in zinc and copper metabolism and in cadmium and mercury detoxification. Several excellent reviews are available (1–3).

In recent years a controversy has developed over the identity of the protein which binds copper. Is it a metallothionein or is it a different low-molecular-weight protein? Evidence has been accumulating that copper is indeed a normal constituent of metallothionein, while confirmatory evidence that it is associated with another protein has not been forthcoming. Hepatic metallothioneins from many different animals are zinc- and copper-containing proteins with zinc in excess (4–13). Renal metallothioneins are also zinc- and copper-containing proteins, but with copper usually in excess (14–17). The original description of another copper-binding protein, called chelatin, synthesized in the liver in response to exogenous copper (18, 19), has not been confirmed by others. The known aerobic instability of copper-rich thioneins (15) may have been responsible for the observation of multiple chelatin-like proteins, e.g., low-molecular-weight, cysteine-rich proteins with low stoichiometry for copper (<4 per mole), as reported by several laboratories (20–23). Copper thionein, prepared carefully under *in vitro* conditions, has 10 g-atoms of copper bound per mole of protein (16).

We were of the opinion that copper was a normal constituent of metallothionein, the amount of total copper bound to thionein being dependent on the zinc status of the animal. Thus, animals receiving copper challenges would accumulate variable amounts of copper in thionein, depending on the levels of zinc and zinc thionein in their livers. Such a prediction would be consistent with the known *in vitro* affinities of thionein for divalent cations: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$ (16). In pursuit of *in vivo* and *ex vivo* data in support of this *in vitro* phenomenon we decided to study the effects of copper challenges on rat liver metallothionein, using isolated liver perfusion, with rats which had been manipulated

0037-9727/81/110306-05$01.00/0

as to their hepatic zinc status. The results of these experiments, reported herein, are consistent with the hypothesis that exogenously administered copper can displace endogenous zinc from metallothionein in rat liver under *in vivo* and *ex vivo* conditions, as predicted by the *in vitro* behavior of copper and metallothionein.

Materials and Methods. Male, Sprague–Dawley rats, weighing 200–250 g, were obtained from Sasco, Inc. (Omaha, Nebr.). Upon arrival they were housed in wire-bottomed cages in a light- and temperature-controlled room and were maintained on rat chow (Purina) and tap water *ad libitum* for several days before use.

Liver perfusion was performed as previously described (24); the donor animals were not starved before use. A recirculating perfusion medium, containing outdated human erythrocytes, was utilized. All livers were perfused for 30 min before additions to the perfusate were made. Perfusions were then continued for various times up to a maximal time of 120 min. At the end of a perfusion the liver was quick frozen on dry ice and stored at −20°C until further analysis was undertaken. All livers were processed individually, as previously described (14, 24–26). Metallothionein metals were determined after Sephadex G-75 column chromatography by direct analysis of the column fractions, using flame atomic absorption spectroscopy (Perkin–Elmer 303). Metallothionein elutes in the region, V_e/V_0 = 2.0 to 2.5. Quantitative data are best obtained from G-75 columns, but we also checked metallothionein metals qualitatively using DEAE-cellulose column chromatography and disc polyacrylamide gel electrophoresis. In all groups studied quantitative increases in G-75 metallothionein metals were qualitatively reflected in DEAE and PAGE metallothionein metals. Data for each liver were calculated as nanogram-atoms of metal per gram of liver wet weight. These data were then expressed as the mean ± standard error for at least four livers. Statistical comparisons between different experimental groups were performed using Fisher's t distribution for the estimation of significant differences between means (27).

In vivo competition of copper with zinc. Four groups of animals were used. One group of animals was injected ip with Zn^{2+} (10 mg/kg body wt) and was killed 24 hr later. A second group of animals was injected ip with Zn^{2+} (10 mg/kg body wt); 18 hr later Cu^{2+} (1.0 mg/kg body wt) was injected ip and the animals were killed after an additional 6 hr. A third group of animals was injected ip with Cu^{2+} (1.0 mg/kg body wt) and killed 6 hr later. A fourth group of animals was not injected with either zinc or copper.

In vivo/ex vivo competition of copper with zinc. Four groups of animals were used. One group of animals was injected ip with Zn^{2+} (10 mg/kg body wt); 18 hr later the livers were removed and perfused with Cu^{2+} added to the medium. A second group of animals was injected ip with Zn^{2+} (10 mg/kg body wt); 18 hr later the livers were removed and perfused without the addition of extra copper to the medium. A third group of animals, which had not received extra zinc *in vivo*, had their livers removed for perfusion with Cu^{2+} added to the medium. A fourth group of animals, which had not received extra zinc *in vivo*, had their livers removed for perfusion without the addition of extra Cu^{2+} to the medium. Additional details of these experiments are in the appropriate figure legends.

Results. The results of the copper competition with zinc study *in vivo* are presented in Fig. 1. As compared to controls, ip injection with zinc alone results in a large increase in zinc ($P < 0.01$) and a small increase in copper ($P < 0.01$) associated with hepatic metallothionein 24 hr later, as has been seen by many others (1–3). Zinc is greatly in excess over copper in metallothionein in this group. Intraperitoneal injection with copper alone results in statistically significant increases in both zinc ($P < 0.01$) and copper ($P < 0.01$) associated with metallothionein 6 hr later, as compared to controls, with copper slightly in excess of zinc. This was also as expected (26). The combined ip injection of zinc at zero time and copper at 18 hr with killing at 24 hr resulted in an insignificant increase ($P < 0.2$) in the amount of zinc associated with metallothionein, as compared with the

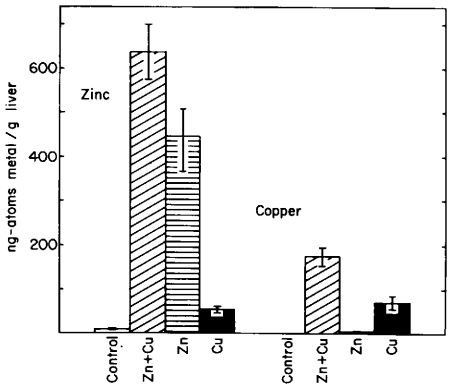

FIG. 1. *In vivo* zinc–copper competition experiment. Metal concentration (ng-atoms/g liver) of zinc (left) and copper (right) in metallothionein after *in vivo* injection of zinc and/or copper. The four experimental groups are: Control, animals receiving no treatment; Zn + Cu, animals receiving zinc (10 mg/kg) at time zero and copper (1 mg/kg) at 18 hr, and being killed at 24 hr; Zn, animals receiving zinc (10 mg/kg) at time zero, and being killed at 24 hr; Cu, animals receiving copper (1 mg/kg) at time zero, and being killed at 6 hr. The data are presented as the mean ± SE for at least four animals.

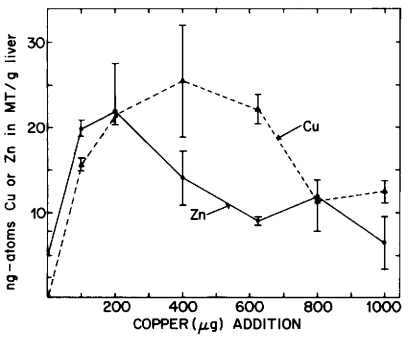

FIG. 2. Accumulation of copper and zinc in metallothionein from perfused livers as a function of the dosage of copper added to the perfusion medium. Metal concentration (ng-atoms/g liver) of copper (▲– – –▲) and zinc (●——●) in metallothionein after perfusion of the isolated livers for 30 min without added copper and then for 90 min with the indicated dosages of copper added to the perfusion medium (100 ml). The data are presented as the mean ± SE for at least four perfused livers.

combined groups receiving zinc alone and copper alone, and a significant increase in the amount of copper associated with metallothionein, whether compared to the group receiving copper alone ($P < 0.001$) or to the combined groups receiving zinc alone and copper alone ($P < 0.001$). As compared to controls, both zinc and copper were significantly ($P < 0.001$) elevated in this group.

In Fig. 2 are shown the results of *ex vivo* exposure of livers to various amounts of exogenous copper (0–1000 μg) in the perfusion medium on the zinc and copper content of metallothionein. A dosage of 625 μg added copper in 100 ml of perfusion medium was used for the *in vivo–ex vivo* competition study, the results of which are presented in Fig. 3.

As can be seen, the results of exposing rat livers to copper *ex vivo* are different from exposing them *in vivo* in regard to the metal content of their metallothionein. The zinc and copper contents of metallothionein in the groups receiving zinc *in vivo* and then either killed (Fig. 1) or perfused (Fig. 2) were the same. However, the group which received zinc *in vivo* and copper *ex vivo* showed a large decrease in its metallothionein zinc content ($P < 0.05$), a decrease which was matched almost exactly by a large increase in its metallothionein copper content ($P < 0.001$). This amount of copper was five times greater ($P < 0.001$) than the amount of copper incorporated *ex vivo* into metallothionein in the perfused livers of rats which had not been previously exposed to exogenous zinc.

Discussion. The hypothesis that exogenously administered copper can displace zinc from endogenous hepatic metallothionein under *in vivo* and *ex vivo* conditions is supported by the experiments reported above. These results are consistent with the demonstrated ability of copper to displace zinc from metallothionein under *in vitro* conditions, using the purified protein (16). Increased amounts of copper are incorporated into metallothionein *in vivo* in the livers of rats which had received exogenous zinc, as compared to animals which received only copper ($P < 0.01$). The increase in the amount of zinc in the hepatic metallothionein of the animals receiving zinc and copper, as compared to those receiving zinc alone, is probably due to a migration of zinc into the liver from extrahepatic stores in response to the stress to the animal of copper injections, since total hepatic zinc increases under these conditions. This is a reasonable

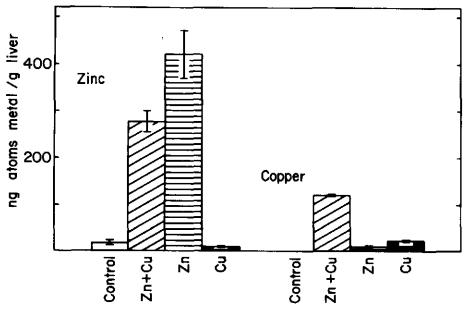

FIG. 3. *In vivo–ex vivo* zinc–copper competition experiments. Metal concentration (ng-atoms/g liver) of zinc (left) and copper (right) in metallothionein after *in vivo* induction of zinc thionein, followed by isolated liver perfusion in the presence of added copper. The four experimental groups are: Control, animals receiving injection *in vivo*, and whose livers were perfused without added copper; Zn + Cu, animals receiving zinc *in vivo* (10 mg/kg) at time zero, and whose livers were removed at 18 hr for perfusion in the presence of 625 μg copper added to the medium; Zn, animals receiving zinc *in vivo* (10 mg/kg) at time zero, and whose livers were removed at 18 hr for perfusion without additional copper in the medium; Cu, animals not receiving any *in vivo* treatment, and whose livers were removed for perfusion in the presence of 625 μg copper added to the medium. All livers were perfused for 30 min with no additions to the medium and then for an additional 90 min ± added copper. The data are presented as the mean ± SE for at least four perfused livers.

interpretation in light of the stress responses manifested by increases in zinc thionein, as seen under other conditions (28).

Because of the increased zinc thionein levels seen in the *in vivo* study for the animals treated with zinc and with copper, as compared to those treated separately with the two metals, we decided to examine copper competition with zinc thionein under *in vivo/ex vivo* conditions, looking at copper displacement of zinc from metallothionein in isolated perfused rat livers. Uptake of zinc by the livers from the perfusion medium is minimal [(24, 25) and this study], so we expected to avoid the increases in zinc thionein seen in the *in vivo* study when zinc and copper were administered sequentially. This indeed proved to be the case. The incorporation of copper *ex vivo* into the metallothionein of livers from animals which had been induced *in vivo* with zinc was greatly increased over that seen with livers from animals which had not received exogenous zinc. In addition the total incorporation of copper into metallothionein in the Zn + Cu group matched the total decrease of zinc in metallothionein in this group, as compared with animals which had received zinc *in vivo* and were perfused without additional copper in the perfusion media.

The simplest interpretation of the results of these studies is that copper is able to displace zinc from preexisting rat hepatic metallothionein under *in vivo* and combined *in vivo/ex vivo* conditions. This is consistent with the recent report (16) that copper can displace cadmium and zinc from rat renal metallothionein under mild *in vitro* conditions. It remains to be proven whether copper itself is an *in vivo* inducer of thionein or whether copper administration disturbs the zinc homeostasis of the liver, resulting in increased levels of zinc thionein to which copper can then bind. We favor the latter interpretation.

Other possible explanations involve redistribution of zinc and copper among proteins in the hepatic cytosol and into and out of the liver itself. We looked at these possibilities, but nothing of consequence or unexpected was observed.

Chromatography of hepatic cytosol on Sephadex G-75 and analysis for zinc and copper usually reveal three types of metal binding peaks: peak 1 with mol wt >40,000 daltons, containing several/many metal binding proteins; peak 2 with mol wt ~30,000 daltons, containing carbonic anhydrase and superoxide dismutase; and peak 3 with mol wt <10,000 daltons, containing metallothionein. When a liver is exposed to excess metal (Zn^{2+}, Cu^{2+}, Cd^{2+}), much of this metal will be found in peak 1 until sufficient thionein is synthesized to bind this excess. If sufficient thionein is already present, as zinc thionein, then excess Cu^{2+} and Cd^{2+} can bind to it, displacing endogenously bound Zn^{2+}.

Our observations in this study are the same as mentioned above. *In vivo* study: Zn + Cu treatment, no copper in peak 1, no change in the copper in peak 2, copper increased in peak 3; Zn-only treatment, no copper in peak 1, no change in the copper in peak 2, no copper in peak 3; Cu-only treatment, copper in peak 1 increased, no

change in the copper in peak 2, copper increased in peak 3. Zinc in the three peaks changed/did not change in an expected manner. *In vivo/ex vivo* study: same results for copper as above. For zinc there was one difference, a 20–25% decrease in peak 1 zinc occurred during perfusion of the liver. We did not monitor serum zinc and copper levels in the *in vivo* study, but we did analyze the perfusion media metals during the course of the perfusions: zinc levels did not change, while copper levels reflected a rapid uptake of exogenous copper ($t_{1/2}$ ~6 min) during the first 10 min of perfusion, followed by a much slower disappearance of copper ($t_{1/2}$ ~2 hr) during the remainder of the perfusion.

Still another point of contention might be that copper stimulated degradation of zinc thionein and/or synthesis of thionein. The conditions of our experiments were such that thionein mRNA levels and protein synthesis were maximally stimulated (29–31), making the latter possibility unlikely. Other workers (15) have demonstrated that the reverse of the former is true; copper-rich thioneins are degraded more rapidly than zinc-rich thioneins.

Frank O. Brady is a recipient of a Research Career Development Award of the National Institute of Environmental Health Sciences (ES 00022). This research was supported by a grant from NIEHS (ES 01288).

Note added in proof. A recent report (32) has demonstrated that copper chelatin is indeed a metallothionein.

1. Kojima, Y., and Kägi, J. H. R., Trends Biochem. Sci. **3**, 90 (1978).
2. Kägi, J. H. R., and Nordberg, M. (eds.), "Metallothionein." Birkhäuser Verlag, Basel/Boston/Stuttgart (1979).
3. Webb, M. (ed), "The Chemistry, Biochemistry, and Biology of Cadmium." Elsevier/North-Holland, New York (1979).
4. Mason, R., Bakka, A., Samawickrama, G. P., and Webb, M., Brit. J. Nutr. **45**, 375 (1981).
5. Bremner, I., and Marshall, R. B., Brit. J. Nutr. **32**, 283 (1974).
6. Bremner, I., and Marshall, R. B., Brit. J. Nutr. **32**, 293 (1974).
7. Bremner, I., Brit. J. Nutr. **35**, 245 (1976).
8. Bremner, I., and Davies, N. T., Brit. J. Nutr. **36**, 101 (1976).
9. Bremner, I., and Young, B. W., Biochem. J. **155**, 631 (1976).
10. Bremner, I., Hoekstra, W. G., Davies, N. T., and Young, B. W., Biochem. J. **174**, 883 (1978).
11. Bremner, I., Hoekstra, W. G., Davies, N. T., and Young, B. W., Chem.–Biol. Interact. **23**, 355 (1978).
12. Webb, M., Plastow, S. R., and Magos, L., Life Sci. **23**, 1901 (1979).
13. Riordan, J. R., and Richards, V., J. Biol. Chem. **255**, 5380 (1980).
14. Brady, F. O., Mason, R., and Webb, M., J. Biol. Chem. **256**, 3931 (1981).
15. Cain, K., and Holt, D. E., Chem.–Biol. Interact. **28**, 91 (1979).
16. Holt, D., Magos, L., and Webb, M., Chem.–Biol. Interact. **32**, 125 (1980).
17. Suzuki, K. T., Arch. Environ. Contam. Toxicol. **8**, 255 (1979).
18. Premakumar, R., Winge, D. R., Wiley, R. D., and Rajogopalan, K. V., Arch. Biochem. Biophys. **170**, 267 (1975).
19. Premakumar, R., Winge, D. R., Wiley, R. D., and Rajagopalan, K. V., Arch. Biochem. Biophys. **170**, 278 (1975).
20. Irons, R. D., and Smith, J. C., Chem.–Biol. Interact. **18**, 83 (1977).
21. Riordan, J. R., and Gower, I., Biochem. Biophys. Res. Commun. **66**, 678 (1975).
22. Porter, H., Biochem. Biophys. Res. Commun. **56**, 661 (1974).
23. Rydén, L., and Deutsch, H. F., J. Biol. Chem. **253**, 519 (1978).
24. Panemangalore, M., and Brady, F. O., J. Biol. Chem. **253**, 7898 (1978).
25. Panemangalore, M., and Brady, F. O., J. Nutr. **109**, 1825 (1979).
26. Day, F. A., Coles, B. J., and Brady, F. O., Bioinorg. Chem. **8**, 93 (1978).
27. Hodgeman, C. D., "CRC Standard Mathematical Tables," pp. 250–251, Chemical Rubber Pub. Co., Cleveland (1959).
28. Brady, F. O., Life Sci. **28**, 1647 (1981).
29. Squibb, K. S., Cousins, R. J., and Feldman, S. L., Biochem. J. **164**, 223 (1977).
30. Shapiro, S. G., Squibb, K. S., Markowitz, L. A., and Cousins, R. J., Biochem. J. **175**, 833 (1978).
31. Shapiro, S. G., and Cousins, R. J., Biochem. J. **190**, 755 (1980).
32. Winge, D. R., Geller, B. L., and Garvey, J., Arch. Biochem. Biophys. **208**, 160 (1981).

Received March 6, 1981. P.S.E.B.M. 1981, Vol. 168.