PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 528–531 (1981)

Correlation of Lysyl Oxidase Activation with the *p*-Phenylenediamine Oxidase Activity (Ceruloplasmin) in Serum (41102)

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Abstract. A dietary deficiency in copper decreases the lysyl oxidase activity in aortic tissue of growing chickens. The decrease has been correlated with a lowering of serum copper and ceruloplasmin (measured as PPD oxidase activity). Injections of $CuSO_4$ restored enzyme function and brought serum copper and ceruloplasmin levels back to normal. However, when the $CuSO_4$ was given to chicks that had received two injections of estradiol-17β, a much stronger increase in lysyl oxidase in response to $CuSO_4$ was observed. The enhanced response correlated with an enhanced increase in ceruloplasmin, linking the serum copper protein with the restoration of lysyl oxidase activity.

Lysyl oxidase is a copper-dependent enzyme. It requires copper to function as a catalyst and to maintain a constant level of activity in aortic tissue. The enzyme catalyzes the formation of allysines, the active aldehyde centers which give rise to crosslinks in collagen and elastin (1). Lysyl oxidase activity fails in animals that are fed diets low in copper, but is restored by refeeding copper-supplemented diets or injecting $CuSO_4$ intraperitoneally (2). The close interplay between copper and an associated metalloenzyme is evident. Not so evident, however, is a biochemical understanding of the mechanism.

Very little is known of the process by which metal ions are transported in the blood to the tissues. Even less is known of how metal ions enter cells to engage the binding sites of enzymes. The lysyl oxidase system may provide a model for elucidating such events, at least to the extent of identifying transport components for delivering copper to the metal-free enzyme. Studies *in vitro* have suggested that lysyl oxidase activation coincides with the binding of copper ions to newly synthesized molecules of apoenzyme (3). Although copper ions may limit the activation of this enzyme *in vitro*, free metal ions are not likely to be the immediate source of the protein-bound metal in the intact animal; a serum protein or a complex of copper with specific amino acids (4) probably performs this function *in vivo*. The present study examines this question closely.

Numerous studies have provided evidence that ceruloplasmin transports copper from liver to the peripheral tissues (5, 6) and may even decide the fate of copper intracellularly (7, 8). This serum copper protein, synthesized in liver (9, 10), may be the vehicle transporting copper to aortic tissue. In earlier studies we associated the activation of lysyl oxidase with an undefined copper-rich protein fraction in serum (11, 3). The present work provides additional evidence for an activation factor and further identifies it with the *p*-phenylenediamine (PPD) oxidase activity (ceruloplasmin) in the serum.

Materials and Methods. Preparation of diets, care and feeding of animals, was as previously described (3).

PPD oxidase activity was measured in fresh serum. The method of Houchin (12) was used and activity has been expressed as international units (IU) (13). IU refer to the number of micromoles of oxidized product formed per minute per liter of serum. Because of the very low amounts of PPD oxidase activity in chick serum, 0.2 ml of serum was incubated for 1 hr at 25° in an otherwise standard assay. Controls used serum proteins dissolved in 5.0 m*M* NaN_3.

Estradiol-17β was obtained from Sigma Chemical Company and used without further purification. The crystalline hor-

0037-9727/81/040528-04$01.00/0

mone was dissolved in 1,2-propanediol at a final concentration of 0.5 mg/ml. Injections of the hormone into the animals were made subcutaneously at a dose of 1 mg/kg body wt. Chicks received two doses at 24-hr intervals. $CuSO_4$, when given, was administered intraperitoneally at a dose of 1 mg/kg body weight at the time of the second hormone injection.

Measurement of lysyl oxidase activity in aortic tissue followed a previous procedure (2). Enzyme activity has been expressed in terms of counts of ^3H_2O released into the medium when the substrate is oxidized. Controls were run to correct for the nonenzyme release of tritium.

Serum copper concentrations were determined by atomic absorption analysis after diluting the serum with an equal volume of deionized water.

Results. With very little copper in the diet, lysyl oxidase activity in aorta falls to less than 5% of the control (copper-fed) activity in 8 days (2). That fall has now been correlated with an equally severe drop in serum copper and PPD oxidase activity (Table I). Giving one injection of $CuSO_4$ (1 mg/kg body wt) restored enzyme activity and brought the serum copper and PPD oxidase activity back to near-normal levels within hours. Also seen in Table I are the effects of estrogen administration on the return of lysyl oxidase activity. The hormone, administered twice over a 48-hr period, had no effect on serum copper levels, PPD oxidase activity, or lysyl oxidase activity in these deficient animals. However, when $CuSO_4$ was given after the second injection, there was a much stronger increase in lysyl oxidase activity than that elicited by $CuSO_4$ alone. Moreover, that increase was associated with an equally strong enhancement in PPD oxidase activity (Table I). Serum copper levels did not show a heightened response to the hormone–$CuSO_4$ combination, suggesting the PPD oxidase, not the serum copper was more responsive to the hormone treatment.

Assuming the estradiol-17β augmented the amount of administered copper absorbed into the system, serum copper and PPD oxidase levels were followed closely after one injection of $CuSO_4$ into estrogen-treated and nontreated chicks. In one 4-hr study shown in Fig. 1, both groups had high values of serum copper 15 min after receiving $CuSO_4$. By 4 hr the serum copper had declined to preinjection levels, the rate of return was about the same for both groups. The PPD oxidase activity in the serum of these same chicks rose slowly over the interval. That of the treated appeared to rise at a faster rate, a difference that was noticeable for at least 10 hr (Fig. 2B). In the study shown in Fig. 1 the nontreated chicks were slower to elevate PPD oxidase activity in response to the injected $CuSO_4$.

In a second study PPD oxidase and lysyl oxidase activities were followed over a longer interval of time. After receiving $CuSO_4$ neither hormone-treated nor nontreated showed an appreciable increase in lysyl oxidase for the first 2 hr (Fig. 2A). However, after 3 hr the lysyl oxidase activity in the treated appeared to rise at a slightly faster rate than that of the nontreated. In this same study PPD oxidase activity in hormone-treated chicks also accelerated at a more rapid pace (Fig. 2B)

TABLE I. CORRELATION OF LYSYL OXIDASE ACTIVITY WITH SERUM COPPER AND PPD OXIDASE ACTIVITY: EFFECT OF $CuSO_4$ AND ESTROGEN[a]

Treatment	N	Serum copper (μg/ml)	PPD oxidase (IU)	Lysyl oxidase (^3H_2O/hr/g) $\times 10^{-3}$
Copper fed	4	0.20 ± 0.07	1.14 ± 0.18	103.6 ± 5.1
Copper deficient	4	0.09 ± 0.01	0.35 ± 0.01	2.7 ± 0.8
+ Estrogen	4	0.09 ± 0.01	0.37 ± 0.02	2.7 ± 1.2
+ $CuSO_4$	4	0.16 ± 0.01	0.92 ± 0.05	62.8 ± 7.3
+ Estrogen, $CuSO_4$	4	0.14 ± 0.03	1.35 ± 0.05	88.7 ± 3.2

[a] Serum copper and lysyl oxidase were determined 24 hr after $CuSO_4$; PPD oxidase was determined 4 hr after $CuSO_4$. Data ± S.D.

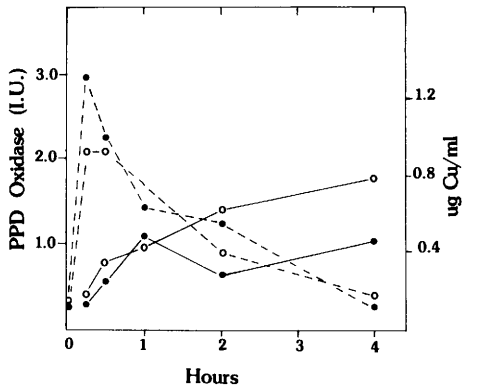

FIG. 1. Influence of estrogen on copper-induced changes in PPD oxidase and serum copper. Groups of 8-day-deficient chicks received one injection of $CuSO_4$ (1 mg/kg body wt) with (○) and without (●) prior treatment with estradiol-17β. Each point shown represents the average of three separate determinations. Solid line, PPD oxidase; broken line, serum copper.

confirming earlier data (Fig. 1) and further suggesting a positive correlation between the PPD oxidase and lysyl oxidase activities. Whereas PPD oxidase activity fell to normal levels after 10 hr (about 1.0 IU), the lysyl oxidase activity remained elevated for at least 20 hr, the margin of difference between the estrogen-treated and non-treated was sustained even at this time. These data confirm that the PPD oxidase activity and not the serum copper levels have a more direct bearing on the activation of lysyl oxidase. The heightened increase in the copper-induced lysyl oxidase activity appears to occur through an increase in PPD oxidase activity.

Discussion. Serum copper and PPD oxidase levels respond to copper depletion and repletion. As to which of these parameters regulates lysyl oxidase activity is the question investigated in the present study.

Estrogen has been shown to raise the serum copper and PPD oxidase in rats (14) and humans (15, 16). In fowl one injection of estradiol sufficed to raise the protein-bound iron in plasma several fold through a specific increase in the serum protein phosvitin (17–19) and subsequent to a more direct increase in the copper-dependent ferroxidase activity (20). Combining these observations led us to hypothesize that estrogen treatment may cause an increase in PPD oxidase, serum copper, or both leading directly to an increase in lysyl oxidase activity. No such increase in lysyl oxidase was observed. However, neither did the serum copper nor PPD oxidase activity change in response to the estradiol. Appar-

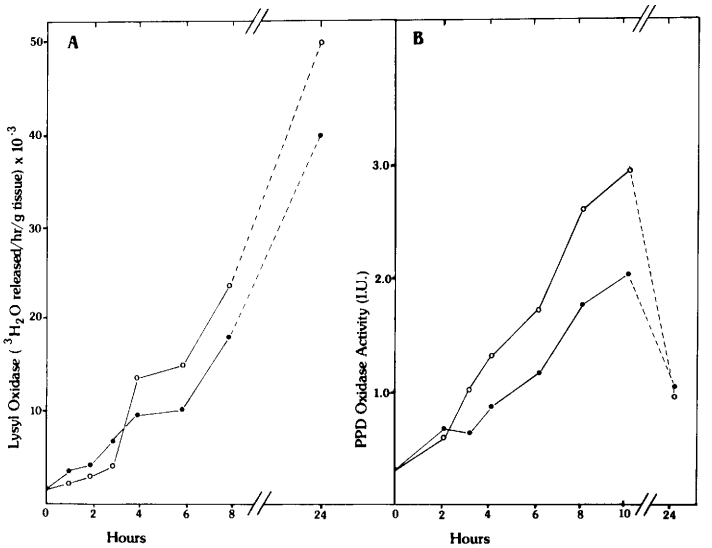

FIG. 2. (A) Time course of changes in aortic lysyl oxidase activity and (B) PPD oxidase activity following $CuSO_4$:effect of estrogen. See Fig. 1 for details. Values shown were obtained from three chicks that received $CuSO_4$ with (○) or without (●) estrogen.

ently the hormone will not mobilize copper in deficient chicks. One reason may be that 8-day-deficient chicks have about one-fourth the liver copper concentration as 8-day copper-fed chicks (Balthrop and Harris, unpublished observations).

Only when $CuSO_4$ was given with the hormone was lysyl oxidase activation observed. Lysyl oxidase activity which, in deficient animals, normally responds to very low copper showed an even greater response when estradiol preceded the copper. Significantly, that response correlated with an enhanced increase in the azide-sensitive PPD oxidase activity which measures ceruloplasmin (21). Simply raising the copper levels in the blood did not increase PPD oxidase (Fig. 1), nor did serum copper levels per se appear to respond to the hormone or correlate with the enhancement of lysyl oxidase. Thus a link between the serum protein and lysyl oxidase has been established. More direct proof that ceruloplasmin actually activates lysyl oxidase must await the isolation of the chick serum protein. Our results support the proposed transport-delivery function of ceruloplasmin in copper metabolism and extend the findings to the aortic tissue (22).

Supported in part by Grant PCM 77-25400 from the National Science Foundation. We acknowledge with gratitude the advice of Dr. Barry C. Starcher in setting up the assay procedure for PPD oxidase.

1. Pinnell, S. R., and Martin, G. R., Proc. Nat. Acad. Sci. USA **61**, 708 (1968).
2. Harris, E. D., Proc. Nat. Acad. Sci. USA **73**, 371 (1976).
3. Rayton, J. K., and Harris, E. D., J. Biol. Chem. **254**, 621 (1979).
4. Harris, D. I. M., and Sass-Kortsak, A., J. Clin. Invest. **46**, 659 (1967).
5. Owen, C. A., Jr., Amer. J. Physiol. **209**, 900 (1965).
6. Marceau, N., and Aspin, N., Amer. J. Physiol. **222**, 106 (1972).
7. Marceau, N., and Aspin, N., Biochim. Biophys. Acta **293**, 338 (1973).
8. Hsieh, H. S., and Frieden, E., Biochem. Biophys. Res. Commun. **67**, 1326 (1975).
9. Owen, C. A., Jr., and Hazelrig, J. B., Amer. J. Physiol. **210**, 1059 (1966).
10. Neifakh, S. A., Monakhov, N. K., Shaposhnikov, A. M., and Zubzhitski, Y. N., Experientia **25**, 337 (1969).
11. Rayton, J. K., and Harris, E. D., Fed. Proc. **36**, 1122 (1977).
12. Houchin, O. B., Clin. Chem. **4**, 519 (1958).
13. Rice, E. W., Anal. Biochem. **3**, 452 (1962).
14. Meyer, J., Meyer, A. C., and Horwitt, M. K., Amer. J. Physiol. **194**, 581 (1958).
15. Russ, E. M., and Raymunt, J., Proc. Soc. Exp. Biol. Med. **92**, 465 (1956).
16. Johnson, N. C., Kheim, T., and Kountz, W. B., Proc. Soc. Exp. Biol. Med. **102**, 98 (1959).
17. Greengard, O., Mendelsohn, N., and Gordon, M., Science **147**, 1571 (1965).
18. Beuving, G., and Gruber, M., Biochim. Biophys. Acta **232**, 529 (1971).
19. Ali, K. E., and Ramsay, W. N. M., Biochem. J. **110**, 36P (1968).
20. Planas, J., and Frieden, E., Amer. J. Physiol. **225**, 423 (1973).
21. Rice, E. W., Clin. Chim. Acta **5**, 632 (1960).
22. Frieden, E., *in* "Copper in the Environment" (J. O. Nriagu, ed.), p. 241. Wiley, New York (1979).

Received September 1, 1980. P.S.E.B.M. 1981, Vol. 166.