Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 505-511

COPPER AND THE ESTRADIOL RECEPTOR

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Received March 5, 1987

SUMMARY. Copper is an essential element in living organisms and it appears to be involved in estrogen action. This study bears on the manner in which the metal may be linked to the mechanism of this action. Divalent copper was found to induce at 37°C a several fold increase in estradiol binding to the receptors in rat uterine cytosols. An endogenous substance present in the uterine cytosol and separated from it by fractionation on a hydroxylapatite column was found to function as a potent inhibitor of the copper effect. This substance has been found so far also in human breast tissue and in some human breast tumors.

Copper is an essential element in living organisms (1, 2) and it appears to be involved in estrogen action. Administration of estrogen to the rat or the natural increases in circulating estrogens in the rat during the estrous cycle, result in increased plasma concentration of copper (3-6). Deficiency in Cu leads to infertility in the rat (7). On the other hand, the contraceptive property of the copper intrauterine device (IUD) is believed to be due to tissue absorption of copper (8). In women, increases in the levels of estrogens during pregnancy are associated with increased levels of circulating Cu (5, 9, 10). In rabbits, copper injections induce ovulation and the effect is increased by pretreatment with estrogens (11).

The studies reported here introduce information that bears directly on the manner in which copper may be linked to the mechanism of estradiol action.

MATERIALS AND METHODS

Stock Solutions: Solutions of diethylstilbestrol (Sigma) and of ^{3}H-(6,7)estradiol (New England Nuclear) were prepared in pH 7.2-7.4 phosphate buffer (PBS) containing potassium phosphate 0.01 M, NaCl 0.15 M, Na_3N 0.01 w/v and gelatin 0.1 w/v.

0006-291X/87 $1.50

Cytosol Preparation: Uteri excised from Sprague Dawley rats were homogenized in ice cold PBS, 2 ml/uterus, using Ultra-Turrax. The homogenate was centrifuged at 105000 x g for 60 min to provide the cytosol.
Fractionation of cytosol on hydroxylapatite: Hydroxylapatite (HAP) powder (Bio-Gel HTP, Bio-Rad) swollen with water was loaded to the 2.6 cc mark into a 5cc glass syringe. Cytosol, 2.5 ml, containing 13-15 mg protein, was applied to the column and eluted successively at 4°C, with: (a) 1 ml 0.1 M KCl, (b) 3 ml 0.4 M KCl in 0.001 M K_xHPO_4, (c) 2 ml 0.4 M KCl in 0.001 M K_xHPO_4, (d) 3 ml 0.1 M KCl (e) 1 ml 0.6 M K_xHPO_4, (f) 4 ml 0.6 M K_xHPO_4. The K_xHPO_4 designates an equimolar K_2HPO_4 + KH_2PO_4 mixture. The effluents from steps (b) and (f) were of consequence in this study. They are designated as solution F and as HT (hydroxylapatite treated) cytosol respectively. The latter contained more than 98% of the original protein (Bio-Rad Protein Assay).

Incubations and Binding Assay: Incubation mixtures contained 0.1 ml of each of the following: cytosol or HT cytosol, [^{3}H]-estradiol, diethylstilbestrol (DES) or PBS, KCl or solution F. The final concentration of [^{3}H]-estradiol, DES and KCl was: 5 nM, 1 µM and 0.1 M respectively. The hormone concentration was sufficient to saturate the receptor sites in the incubation mixtures and the DES, when included, was in sufficient excess to compete with the hormone for those sites. Incubations were conducted at 4°C only or followed additionally by 8 hr at 37°C and a final 2 hr at 4°C (12, 13). Hormone binding in the incubated mixtures was assayed by the dextran coated charcoal (DCC) method. Specific binding is defined as the difference in [^{3}H]-estradiol binding with DES present and absent. The results reported are mean values of multiple determinations ± standard deviation.

RESULTS

In earlier studies (12, 13), we used dextran coated charcoal to remove from target tissue cytosols low molecular weight components thought to be mediators of estradiol action and we have shown that the estradiol-receptor complexes formed in the treated cytosols are stable at 37°C. Our attempts to subsequently extract the components from the DCC for further study proved unsuccessful. We have now accomplished the isolation of such components by fractionating the cytosols on HAP columns.

The approach was to adsorb the cytosol proteins on the HAP, elute the column with non-phosphate salt solutions to separate out low molecular weight components or basic proteins (14) and finally to elute the receptor proteins with high concentration phosphate. The estrogen receptor adsorbed on HAP columns appears to suffer little loss with repeated washes with buffer (15).

In the described procedure, step b elutes an endogenous factor (F) which as described below regulates the activation of estradiol receptor by divalent copper. Elution steps c, d and e serve to remove residual F prior to the elution of F-free receptors (HT cytosol) with phosphate.

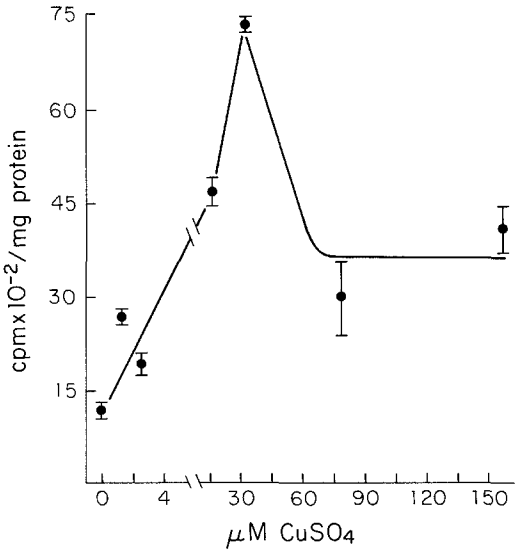

Fig. 1. Effect of Cu on Estradiol Binding in Rat Uterine Cytosol. Cytosol was prepared from a pool of six mature uteri excised at random phase of the estrous cycle. The cytosol was fractionated on HAP column to obtain the HT cytosol which was then incubated with [^{3}H]-estradiol ± DES and inclusions of $CuSO_4$ up to 157 μM. Incubation was 16 hr at 4°C followed by 8 hr at 37°C and a final 2 hr at 4°C. Binding was assayed by the DCC method. Specific binding is shown.

Fig. 1 shows the striking effect of $CuSO_4$ additions on the specific [^{3}H]-estradiol binding in rat uterine HT cytosol at 37°C. At peak effect, noted here with 32 μM $CuSO_4$, the copper ion induces a five fold increase in estradiol binding. When, however, the endogenous factor F is included in the incubation mixture, the binding increase is virtually eliminated. This is clearly illustrated in the results shown in Fig. 3 and discussed further on.

Solutions incubated at 4°C showed minor effects of copper salt addition on estradiol binding. Also, substituting for copper other biologically essential divalent metal ions in the form of $CaCl_2$, $MgCl_2$ or $ZnCl_2$ had no effect on specific binding.

Ovariectomized rats injected with estradiol undergo a biological response which can be followed by measuring the estradiol receptor in the uterine cytosol. The results of such an experiment, shown in Fig. 2 follow in pattern those reported by others (16-19), namely, hormone binding capacity is depleted to a minimum at about 4 hr and reaches a maximum in an "overshoot" at about 24 hr after the estradiol injection. We conducted a study of how Cu(II) and F,

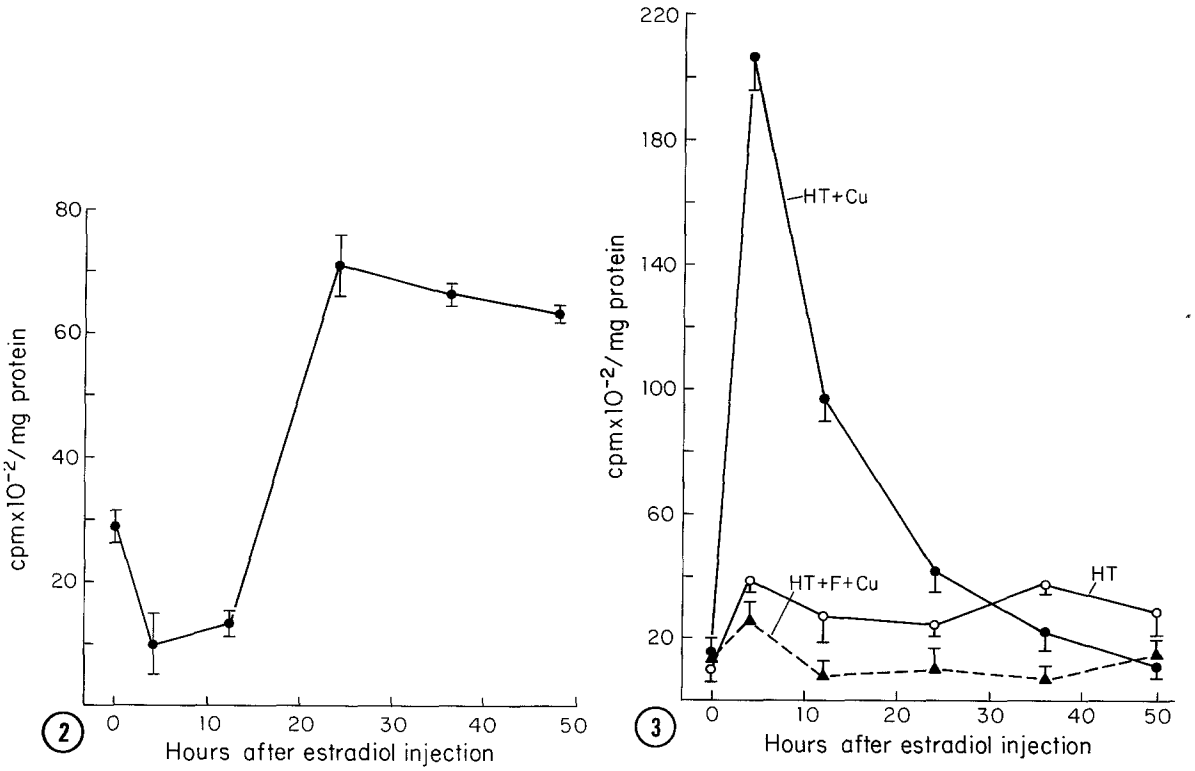

Fig. 2. Estradiol Binding in Cytosol of Uteri Excised from Ovariectomized Rats at Various Times Following Estradiol Injection. Ovariectomized mature rats were primed with a 10 µg subcutaneous injection of estradiol on day 25 and again on day 26 after the operation. Eighty hours after the last injection they were injected again with 10 µg of estradiol and groups of 5 rats were killed 4, 12, 24, 36 and 48 hr later. Cytosols prepared from the excised uteri were incubated with [^{3}H]-estradiol ± DES at 4°C. Hormone binding was assayed by the DCC method. Specific binding is shown.

Fig. 3. Effects of F and Cu on Estradiol Binding in Cytosol of Uteri Excised from Ovariectomized Rats Following Estradiol Injection. Each of the cytosols described in Fig. 2 was fractionated on a HAP column to obtain the HT cytosol and the factor F. The HT cytosols were incubated with [^{3}H]-estradiol ± DES in the presence and absence of 30 µM $CuSO_4$ or of $CuSO_4$ and F combined. Incubation was 16 hr at 4°C followed by 8 hr at 37°C and a final 2 hr at 4°C. Binding was assayed by the DCC method. Specific binding is shown.

in their effects on estradiol binding, reveal the biological responses shown in Fig. 2. Portions of the uterine cytosols from the ovariectomized rats were chromatographed on HAP to separate the F and to obtain the HT cytosols which were then incubated with [^{3}H]-estradiol ± DES in the presence and absence of $CuSO_4$ or of $CuSO_4$ + F. The results in Fig. 3 show that the addition of Cu(II) induces a several fold increase in hormone binding with a distinct peak in the very time region which in 4°C incubations with untreated cytosols (Fig. 2) is characterized by binding depletion. When the respective F solutions are in-

cluded in the incubation mixtures Cu(II) addition fails to promote hormone binding.

DISCUSSION

This study deals with two linked findings. One is that Cu(II) induces a large increase in estradiol binding to the receptor in rat uterine cytosol, and the second is that the uterine cytosol contains an endogenous component which inhibits the Cu(II) effect.

As stated earlier, copper is an essential element and there is considerable evidence that it may be involved in the actions of reproductive hormones (1-11). Therefore, the observations with exogenous Cu(II) described here, very likely reflect a natural copper based mechanism. The relevance of the Cu(II) effects to estradiol action is particularly well supported by the results obtained with ovariectomized rats in the process of responding to an estradiol injection. The striking, sharp pulse of increased hormone binding in the HT cytosols (Fig. 3), induced by the presence of Cu(II), reflects a biological response to the estradiol. The fact that the Cu(II) effect is eliminated by an endogenous factor, is further support for the biological relevance of the phenomenon.

We suggest the following explanation for the observations. The administration of a single estradiol dose to an estrogen deprived rat initiates rapid nuclear processing which leads to the synthesis of a significant quantity of receptors in the uterine cytoplasm. The new receptors are not phosphorylated and therefore not able to bind estradiol (20, 21). In subsequent periods some phosphorylation by the cytoplasmic kinase takes place and this manifests itself as the estradiol binding "overshoot" invariably observed in $4^{o}C$ incubations. We think that the Cu(II) effect may rest with the ability of this ion to activate a kinase to phosphorylate the receptor, which progresses efficiently at $37^{o}C$, and that peak estradiol binding in the presence of Cu(II) occurs in the period of the highest accumulation in the cytoplasm of newly synthesized nonphosphorylated receptors. The decline of this quantity with time as seen in Fig. 3 may reflect, at least in part, the gradual translocation of

the receptors to the nucleus where they become firmly anchored and not solubilized during homogenization. Recent evidence indeed points to the predominantly nuclear localization of the estradiol receptors (22, 23).

The physiological function of the endogenous factor F may be the control of hormone action by the down regulation of receptor phosphorylation perhaps by blocking the activating capacity of Cu(II).

The hypothetical description of the functions of Cu(II) and F is supported by extensive studies (20, 21), which substantiate phosphorylation as a key process in receptor activation for estradiol binding. Auricchio et al (20) have located the kinase only in the cytoplasm of target tissues and an inactivating phosphatase, whose presumed task is to terminate hormone action by receptor dephosphorylation, only in the nucleus.

ACKNOWLEDGMENT

This study was supported by grant PDT-281 from the American Cancer Society. Gregory J. Mullen provided very competent technical assistance in all phases of this work.

REFERENCES

1. Biological Roles of Copper (1980) Ciba Foundation Symposium 79, Excerpta Medica, New York
2. Prasad, A.S. (1978) in Trace Elements and Iron in Human Metabolism, Chapter 2, Plenum, New York
3. Johnson, N.C., Kheim, T. and Kountz, W.B. (1959) Proc. Soc. Exptl. Biol. Med. 102, 98-99
4. Russ, E.M. and Raymunt, J. (1956) Proc, Soc. Exptl. Biol. Med. 92, 465-466
5. Scheinberg, I.H., Cook, C.D. and Murphy, J.A. (1954) J. Clin. Invest. 33, 963-968
6. Sato, N. and Henkin, R.I. (1973) Amer. J. Physiol. 225, 508-512
7. Hall, G.A. and Howell, J. McC. (1969) Brit. J. Nutr. 23: 41-45
8. Okereke, T., Sternlieb, I., Morell, A.G. and Scheinberg, H. (1972) Science 177, 358-360
9. Henkin, R.I., Marshall, J.R. and Meret, S. (1974) Amer. J. Obstet. Gynecol. 110, 131-134
10. Lahey, M.E., Cubler, C.J., Cartwright, G.E. and Wintrobe, M.M. (1953) J. Clin. Invest. 32, 329-339
11. Suzuki, M., Watanabe, S. and Hoshii, M. (1965) Endocrinology 76, 1205-1207
12. Fishman, J.H. (1983) Biochem. Biophys. Res. Commun. 110, 713-718
13. Fishman, J.H. (1983) Endocrinology 113, 1164-1166

14. Bernardi, G. (1971) in Methods in Enzymology (Jakoby, W.B., ed.) Vol. XXII, pp. 325-339, Academic Press, New York
15. Sakai, D. and Gorski, J. (1984) Biochemistry 23, 3541-3547
16. Mester, J. and Baulieu, E.E. (1975) Biochem. J. 146, 617-623
17. Katzenellenbogen, B.S., Bakoo, H.S., Ferguson, E.R., Lan, N.C., Tatee, T., Tsai, T.S. and Katzenellenbogen, J.A. (1979) Recent Prog. Hormon. Res. 35, 359-400
18. Diaz Chico, B.N.D., Gonzales, A.S. and Benitez, J.G. (1984) J. Steroid Biochem. 20, 1135-1139
19. Terakawa, N., Aono, T., Tsutsumi, H. and Matsumoto, K. (1985) J. Steroid Biochem. 22, 475-480
20. Auricchio, F., Migliaccio, A., Castoria, G., Lastoria, S. and Rotondi, A. (1984) in Progress in Cancer Research and Therapy, (Bresciani et al, eds.) Vol. 31, pp. 49-62, Raven Press, New York
21. Raymoure, W.J., McNaught, R.W., Greene, G.L. and Smith, R.G. (1986) J. Biol. Chem. 261, 17018-17025.
22. King, W.J. and Greene, G.L. (1984) Nature 307, 745-747
23. Welshons, W.V., Lieberman, M.E. and Gorski, J. (1984) Nature 307, 747-749