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ENDOTHELIN-3 INHIBITS PROLACTIN AND STIMULATES LH, FSH AND TSH SECRETION FROM PITUITARY CELL CULTURE

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SUMMARY: The influence of endothelin-3 (ET-3) on anterior pituitary hormone secretion was investigated over a wide range of concentrations (from 10^{-14} to 10^{-6} M) and incubation times (from 4 to 48 hours). ET-3 elicited a concentration-dependent inhibition of prolactin (PRL) secretion and stimulated the release of luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) from primary monolayer cultures of anterior pituitary cells derived from female rats. The responsiveness of different pituitary cells to ET-3 differs markedly in terms of onset and duration: the maximal inhibition of PRL secretion occurred after 12 hours and the stimulation of LH, FSH and TSH reached the maximum after 4, 48 and 48 hours of incubation, respectively. These data corroborate the concept that ET-3 has an important role as a neuroendocrine modulator. Moreover, the data presented suggest different intracellular mechanisms underlying ET-3 actions.

The endothelins, a new and distinct family of regulatory peptides (1,2), were originally isolated from tissue culture media of vascular endothelial cells based on their vasoconstrictor activity (3). However, their distribution (4-6) and multiple biological activities (7,8) are not restricted to the cardiovascular system . High concentrations of ET-like immunoreactivity were found by immunocytochemical methods in the central nervous system, especially in the magnocellular hypothalamic neurons (5). Using a specific sandwich immunoassay, relatively high concentrations of endothelin-3 (ET-3) were detected in the pituitary gland (9) . Taken together, these observations led to the question whether the endothelins (ET-3, in particular) are directly involved in the regulation of pituitary hormone secretion. Recently, Samson et al. demonstrated that ET-3 has the capacity to inhibit PRL secretion *in vitro* (10). Here we describe that ET-3 is not only a potent inhibitor of PRL release but also influences other pituitary hormones such as LH, FSH and TSH as well.

MATERIALS AND METHODS: ET-3 was purchased from Peninsula Laboratories (Belmont, CA). **Pituitary cell dispersion and culture** are based on Vale's technique (11), with some modifications. Pituitary glands were collected after rapid decapitation of random cycling female rats (220-260 g body weight). Posterior lobes were removed and the anterior lobes diced into approximately 1 mm cubes. After being rinsed with sterile HEPES-buffered saline (HBS), tissue

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slices were placed in a water-jacketed spinner flask maintained at 37 C containing 10 ml of freshly prepared solution composed of collagenase (700 U/ml, Worthington, Freehold, NJ), hyaluronidase (765 U/ml, type III, Sigma Chemicals, St.Louis, MO), and bovine serum albumin (BSA, 1%, Sigma), in HBS. The cells were incubated with a constant gentle stirring for approximately 65 minutes and the dispersion was completed by gentle trituration using a siliconized glass pasteur pipette. The cells were washed three times with sterile HBS and finally resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, from Gibco, Gaithersburg, MA). The cells were plated as $6X10^4$ cells/200µl/well, in 96 well micro-titer plates (Corning Glass Work, Corning, NY). The **experiments with ET-3** were started on the fourth day of culture. The cells were washed three times with 200µl DMEM, and finally 100 µl/well of 2%FCS-DMEM were added followed by 100 µl/well test material dissolved in 0.1 % BSA-DMEM. Control wells received 0.1 % BSA-DMEM only. Each concentration of ET-3 and corresponding controls were incubated in sextuple. After different times of incubation, media were gently aspirated and stored at -25 C. The PRL, LH, FSH, TSH and GH concentration were determined by **radioimmunoassay** using NIDDK materials provided via National Pituitary Hormone Distribution Program by Dr. A.F.Parlow. The hormone concentrations were expressed as ng/ml of NIDDK rat PRL RP-3, LH RP-2, FSH RP-2, TSH RP-2 and GH RP-2, respectively. The **statistical evaluation** was performed using one way ANOVA followed by Scheffé post hoc test where appropriate (ABSTAT, Anderson Bell). A p value less than 0.05 was considered statistically significant. The concentration of ET-3 necessary for the half maximal response (EC_{50} values) were calculated using the theoretical equations (four parametric logistic function) obtained from computerized non-linear regression analysis of concentration-response curves (MINSQ. Micromath).

RESULTS

(1) Time-Dependency of ET-3 Effects. First, we established the time-dependency of ET-3's effect on hormone release from anterior pituitary cells (Fig. 1). ET-3 at 100 nM concentration stimulated the release of LH, FSH and TSH and inhibited the release of PRL. ET-3 elicited rapid changes in the rate of LH and PRL secretion while the effect on FSH and TSH developed more slowly. The highest relative increase in LH, FSH and TSH secretion was observed after 4, 48 and 48 hour incubation, respectively. The inhibition of PRL secretion by ET-3 was most

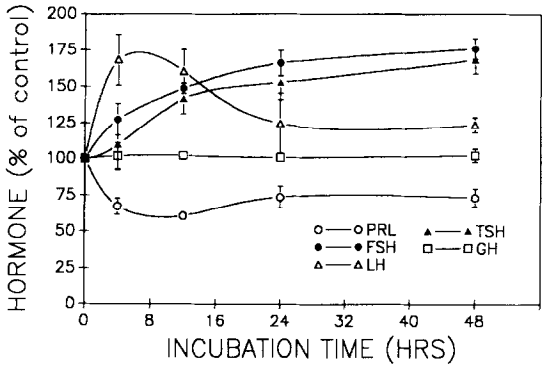

Fig. 1. Effects of 100 nM ET-3 on the secretion of PRL, GH, LH, FSH and TSH from pituitary cell cultures as a function of incubation time. The ordinate represents the changes in hormone concentration expressed as percentage of untreated control cells. Significant differences were found in hormone concentration between treated and control wells after 4, 12, 24 and 48 hours for PRL ($p<0.01$, 0.001, 0.05 and 0.01, resp.); after 4 and 12 hours for LH ($p<0.05$ and 0.01); after 4, 12, 24, and 48 hours for FSH ($p<0.05$, 0.0001, 0.0001 and 0.0001) and after 24 and 48 hours for TSH ($p<0.011$ and 0.001). The GH secretion remained unchanged by ET-3 thorough the period tested.

pronounced after 12 hour incubation period. GH secretion was not effected significantly by ET-3 during the period tested.

(2) Concentration-Dependency of ET-3 Effects. Concentration-response curves with ET-3 for each pituitary hormone were obtained using the incubation time necessary for the maximal response: 4 hours for LH, 12 hours for PRL and 48 hours for FSH and TSH (Fig. 2). ET-3

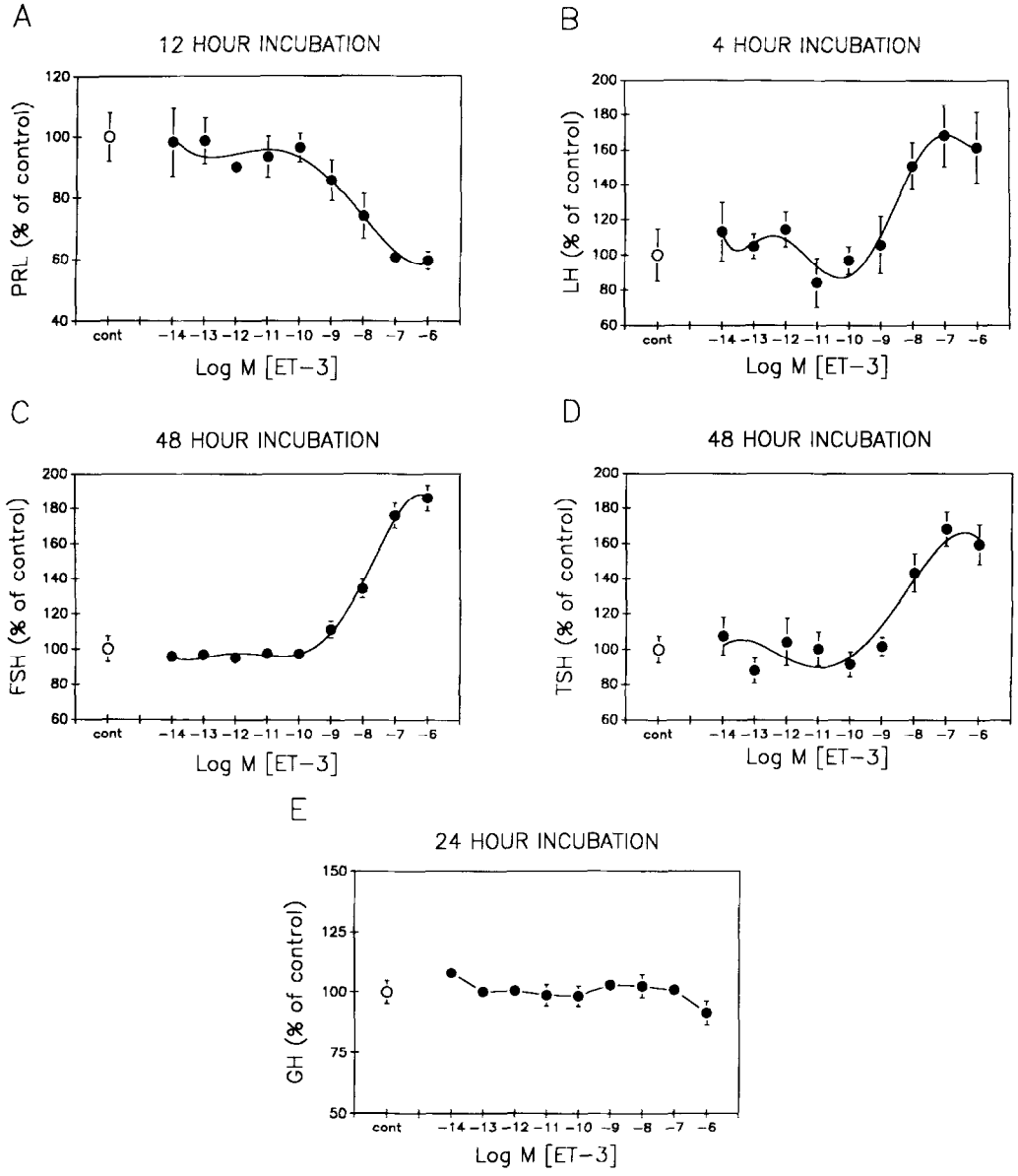

Fig. 2. Concentration-response relationships for hormone secretions of anterior pituitary cells to ET-3. The changes in hormone concentrations are expressed as the percentage of hormone concentration in the culture media of untreated cells. Panel A: PRL (hormone concentration in untreated wells was 1,538.2±121.2 ng/ml after 12 hours of incubation); panel B: LH (8.9±1.3 ng/ml, 4 hours); panel C: FSH (23.1±1.7 ng/ml, 48 hours); panel D: TSH (13.1±1.0 ng/ml, 48 hours); panel E: GH (1,715.7±80.5 ng/ml, 24 hours).

elicited a concentration-dependent decrease of PRL secretion and an increase in LH, FSH and TSH secretion. It is interesting to note, that all of the three glycoprotein hormones of the anterior pituitary which are structurally related were stimulated by ET-3. In all cases, the maximal effect was reached at 100 nM concentration of ET-3. The EC_{50} values were within a narrow concentration range: $4 < EC_{50} < 9$ nM (4.1 nM for PRL, 4.06 nM for LH, 8.7 nM for FSH and 8.5 nM for TSH).

DISCUSSION

Since the pharmacological characterization of cardiovascular and renal actions of ETs (2,7,8,12) and also ligand-binding studies (13,14) have implicated unique and specific receptors conveying ET's effects, it is very likely that the ET-responsive pituitary cells also possess ET-receptors with the same or similar specificity. Indeed, specific antagonists for dopamine (DA) or gonadotropin-releasing hormone (GnRH) receptors did not influence the PRL-lowering, or LH- and FSH-increasing effects of ETs [(10,18) and unpublished data from our laboratory] suggesting that the aforementioned effects of ET-3 do not directly involve DA or GnRH receptors.

The multiple actions of ET-3 on the pituitary cells raise the question as to whether all of these effects are initiated by the same ET-receptor. Radioligand binding studies have revealed two distinct subpopulations of endothelin binding sites in chick cardiac membrane preparations (19). However, data available to date are still insufficient to verify the existence of different ET-receptor subtypes in the pituitary gland.

On the other hand, the marked differences in the incubation times necessary to develop maximal response of lactotrophs (PRL), gonadotrophs (LH,FSH) and thyrotrophs (TSH) suggest different intracellular events underlying ET-3 effects. Moreover, ETs likely share one or more intracellular signaling systems (15,16) with well known pituitary hormone secretagogues such as, e.g., DA, TRH, or VIP (17). Therefore, the ET(s) should also be considered as potential modulators of the responsiveness of certain pituitary cells to other hormones. It has been reported that ET-1 at concentrations over 1 nM stimulate gonadotropin release in perifused pituitary cells (18). Interestingly, subthreshold concentrations of ET-1 can evoke a rapid oscillation of the intracellular Ca^{2+} concentration in gonadotroph cells indicating that the target

cells are capable of perceiving the presence of a modulator far below the "effective" concentration (18). Concerning the integrative capacity of the cells, this phenomenon could provide an important signaling mechanism for certain types of modulator molecules.

Since the actual concentrations of the different ETs around the pituitary cells *in vivo* is not known, these *in vitro* data as well as those of others'(10,18) provide only suggestive or circumstantial evidence for the regulatory role of ETs on pituitary hormone secretion *in vivo*. Moreover, primary cell cultures often posses higher sensitivity to certain agonists (probably because more efficient receptor/effector coupling occurs) than the same cells *in vivo* which makes the evaluation of the physiological relevance of these data rather uncertain. The elucidation of physiological significance of the contribution of ETs to the regulation of pituitary hormone secretion still awaits data from *in vivo* experiments with specific ET-antibodies and with synthetic receptor antagonists.

In summary, our data provided further evidence for the PRL release-inhibiting capability of ET-3 and extend the potential range of biological actions of ETs to modulate other pituitary hormone secretion such as LH, FSH and TSH.

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REFERENCES

1. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi,T., Goto, K., and Masaki, T. (1989) Proc.Natl.Acad.Sci.USA 86, 2863-2867.
2. Yanagisawa, M., and Masaki, T. (1989) TIPS 10, 374-378.
3. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y.Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki,T. (1988) Nature 332, 411-415.
4. Shinmi, O., Kimura, S., Sawamura, T., Sugita, Y., Yoshizawa, T., Uchiyama, Y., Yanagisawa, M., Goto, K., Masaki, T., and Kanazawa, I. (1989) Biochem. Biophys.Res.Commun. 164, 587-593.
5. Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson,S.J., Kimura, S., Uchiyama, Y., Polak, J.M., Masaki, T., and Kanazawa, I. (1990) Science 247, 462-464.
6. Shinmi, O., Kimura, S., Yoshizawa, T., Sawamura, T., Uchiyama, Y., Sugita, Y., Kanazawa, M., Yanagisawa, M., Goto, K., and Masaki, T. (1989) Biochem. Biophys.Res.Commun. 162, 340-346.
7. Miller, W.L., Redfield, M.M., and Burnett, J.C. (1989) J.Clin.Invest. 83, 317-320.
8. Simonson, M.S., Wann, S., Men,P., Dubyak, G.R., Kester, M., Nakazato, Y., Sedor, J.R., and Dunn, M.J. (1989) J.Clin.Invest. 83, 708-712
9. Matsumoto, H., Suzuki, N., Onda, H., and Fujino, M. (1989) Biochem.Biophys.Res.Commun. 164, 74-80.
10. Samson, W.K., Skala,K.D., Alexander,B.D., and Huang,F.-L.S. (1990) Biochem.Biophys. Res.Commun. 169, 737-743.

11. Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R. (1972) Endocrinology 91, 562-572.
12. Van Renterghem, C., Vigne, P., Barhanin, J., Schmid-Alliana,A., Frelin, C., and Lazdunski, M. (1988)Biochem.Biophys.Res.Commun. 157, 977-985.
13. Gu, X.-H., Casley, D., and Nayler, W. (1989) Eur.J.Pharmacol. 167, 281-290.
14. Neuser, D., Zaiss, S., and Stasch, J.-P. (1990) Eur.J.Pharmacol. 176, 241-243.
15. Simonson, M.S., and Dunn, M.J. (1990) Hypertension 15 (Suppl.13), I5-I12.
16. Simonson, M.S., and Dunn, M.J. (1990) FASEB J. 4, 2989-3000.
17. Lamberts, S.W.J., and MacLeod, R.M. (1990) Physiological Reviews 70, 279-318.
18. Stojilkovic, S.S., Merelli, F., Iida, T., Krsmanovic, L.Z., and Catt, K.J. (1990) Science 248, 1663-1666.
19. Watanabe, H., Miyazaki,H,, Kondoh,M., Masuda,Y., Yanagisawa,M., Masaki,T. and Murakami,K. (1989) Biochem.Biophys.Res.Commun. 161, 1252-1259.