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Attenuated Pituitary β-Endorphin Release in Estrogen-Treated Rats (40936)

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Abstract. Using a newly developed radioimmunoassay for β-endorphin (β-END) the influence of gonadal steroids and sex difference on basal and stimulated β-END secretion was investigated. Administration of estradiol benzoate (EB), but not testosterone proprionate, tended to decrease resting levels of plasma β-END and significantly attenuated the stress-induced increase in circulating β-END in male rats. Consistent with these findings is the observation that female rats had somewhat lower nonstress and stress-elevated levels of plasma β-END as compared to males. Over 95% of the total pituitary content of β-END (12.7 ± 1.7 μg) was found to be present in the neurointermediate lobe (NIL) and treatment with EB for 5 days significantly decreased (by up to 56%) stores of β-END in the NIL yet had no effect on the small amount of β-END present in the pars distalis (PD). Since daily administration of EB produced dose-related increases in plasma levels of corticosterone and dexamethasone treatment completely prevented the stress-induced release of β-END, a possible mechanism by which treatment with EB results in decreased β-END secretion may be related to the ability of estrogen to increase circulating levels of adrenal glucocorticoids. Together, these findings suggest that adrenal glucocorticoids may be responsible for the diminished release of β-END observed here in female rats and those treated with EB as compared to normal male rats. In addition, the very high concentration of β-END in the NIL as compared to the PD provides indirect evidence for the pars intermedia being the primary source of circulating β-END in the rat.

β-Endorphin (β-END) has been shown to be concentrated in the corticotrophs of the adenohypophysis (1, 2) and to be released together with adrenocorticotropin (ACTH) in response to stressful stimuli (3–5). In addition, Vale and co-workers (6) have reported that a purified preparation of hypothalamic corticotropin-releasing factor (CRF) directly stimulated the *in vitro* release of β-END as well as ACTH by rat pituitary tissue. Together, these findings indicate that stimulatory control of both β-END and ACTH may be mediated by a common hypothalamic mechanism. Although no target site for circulating β-END has been clearly defined, its potent analgesic properties (7, 8) and dramatic release in response to painful stimuli (3–5) suggest that β-END may have a role(s) in the physiologic response to stress. Like ACTH, the secretion of β-END is inhibited by glucocorticoids (3, 9–11); however, beyond this, little is known about the hormonal regulation of β-END. Since gonadal steroids can profoundly influence the secretion of gonadotropins, prolactin, and growth hormone (12–14), the effects of estrogen, testosterone, and sex difference on basal and stress-stimulated secretion of β-END were investigated. It should be noted that because plasma and tissue concentrations of β-END presented here were determined by radioimmunoassay, these data refer to levels of β-END-like immunoreactivity.

Materials and methods. Animals, treatments, and sample collection. Mature male and female Sprague–Dawley rats (Taconic Farms, Inc., Germantown, N.Y.) were housed at 22° (lights on from 0600 to 1800) and received food and water *ad libitum* for at least 10 days prior to each experiment. In addition, all animals were gentled by daily handling for 5 days before testing. Estradiol benzoate (Sigma Chemical Co., St. Louis, Mo.) or testosterone proprionate (Calbiochem, San Diego, Calif.) was dissolved in corn oil and administered by daily subcutaneous injection in a volume of 0.25 ml per rat. Control animals received corn oil injections. Dexamethasone (Carter-Glogau Laboratories, Glendale, Ariz.) or saline ve-

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hicle treatments were given in a similar manner. Steroid- or vehicle-pretreated animals were sacrificed approximately 24 hr after the last injection. Physical immobilization was administered by taping the rats to test tube racks and placing them on their backs. Trunk blood samples (6 to 8 ml each) were collected after rapid decapitation in plastic tubes on ice containing 400 μl of 10% ethylenediamine tetraacetic acid (EDTA). Plasmas were quickly separated by centrifugation and stored at −70° until assayed. Other methods of preparing plasma, including collection in the presence of (i) greater amounts of EDTA (up to 800 μl of 10% EDTA collection per tube), (ii) heparin, (iii) *N*-ethylmaleimide (NEM, Sigma), (iv) heparin plus EDTA, or (v) NEM plus EDTA, all resulted in plasma samples having immunoassayable levels of β-END which were equivalent to those of plasma collected in 400 μl of 10% EDTA. The pituitary neurointermediate lobe (NIL) was dissected from the pars distalis (PD) and both tissues were homogenized in 0.05 *M* phosphate buffer containing 1% EDTA. Aliquots of the homogenates were removed for protein determination (Protein Assay Kit, BioRad Laboratories, Richmond, Calif.) and all samples were stored at −70° until assayed.

β-END conjugation and immunization procedures. Human β-END (synthesized by C. H. Li) obtained from A. Manian, National Institute of Mental Health was conjugated to bovine thyroglobulin (Sigma) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (BioRad) using weight ratios of peptide:carrier:conjugating agent of 1:10:100 as described by Goodfriend *et al.* (15). After dialyzing for 24 hr the peptide conjugate in water was emulsified with equal volumes of Freund's complete and incomplete adjuvants and approximately 100 to 200 μg of conjugate in 1 ml was administered at multiple intradermal sites in adult male New Zealand White rabbits (Dutchland Laboratory Animals, Inc., Denver, Penn.). Boosting injections were given at 5- to 8-week intervals and antisera were periodically collected by ear vein incision.

β-END radioimmunoassay (RIA) procedure. Radioiodinated β-END was prepared by the chloramine T method of Greenwood *et al.* (16) and purified chromatographically on a Sephadex G-25 (Pharmacia, Piscataway, N.J.) column. As observed with another β-END RIA (17), the presence of plasma increased specific binding of iodinated β-END to the antibody used here (A-43), resulting in increased total antibody binding and assay sensitivity. This effect was nearly maximal with the addition of 50 μl of hypophysectomized plasma to the reaction mixture (total volume, 500 μl) and greater amounts of plasma (up to 200 μl) produced only a 1 to 3% further increase in antibody binding above that observed with 50 μl. Therefore, nonplasma samples (standards and tissues) were assayed in the presence of 50 to 100 μl of plasma collected from hypophysectomized rats and experimental plasma samples were assayed in amounts ranging from 50 to 175 μl. All samples were assayed at two or three dilutions in duplicate. Separation of free from antibody-bound radioactivity was by charcoal absorption and centrifugation (18). Under these conditions, β-END antiserum A-43 used at a final dilution of 1:4000 specifically bound 35 to 40% of the iodinated β-END (10,000 cpm/tube) and was sensitive to less than 10 pg of β-END.

β-END RIA specificity. Dose−response curves of plasma and pituitary tissue extracts paralleled synthetic β-END standard (Fig. 1). Purified human β-lipotropin (obtained from L. Kapkalla and S. Reichlin) showed less than 3% cross-reactivity[1] and human α-endorphin, methionine- and leucine-enkephalin, substance P, α-MSH (Boerhringer Mannheim, Indianapolis, Ind.), human β-MSH (Peninsula Laboratories, Inc., San Carlos, Calif.), and ACTH (NIAMDD) all had less than 1% cross-reactivity in this assay. Antibody specificity was further examined by column chromatographic fractionation of the β-END immunoreactivity in NIL homogenate supernatant. When NIL supernatant was applied to Sephadex G-25 and G-75 (Pharmacia) columns (2.7 × 80 cm) and eluted with 0.1 *N* acetic acid containing 0.05% bovine serum albumin and 0.02% sodium azide (Sigma), over 95% of the

[1] See note added in proof.

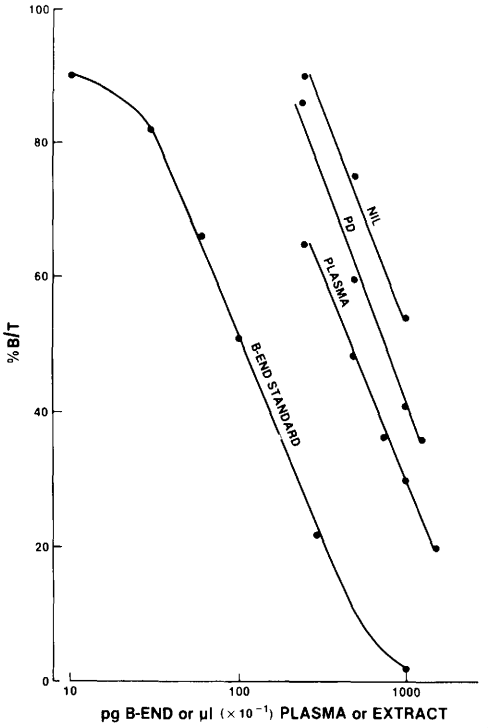

FIG. 1. Comparison of inhibition curves produced by synthetic β-END, plasma, and tissue extracts. B/T corresponds to (counts specifically bound in presence of sample/counts specifically bound in absence of sample).

eluted β-END immunoreactivity cochromatographed precisely with iodinated β-END standard. Intra- and interassay variation for plasma were 4.7 and 14.0%, respectively ($N = 10$).

Corticosterone assay and statistical analysis. Plasma levels of free corticosterone were estimated by the fluorometric method of Guillemin *et al.* (19). Data were analyzed statistically by Student's t test and $P \leq 0.05$ was chosen as the level of significance.

Results. Presented here is the development of a β-END RIA which can detect less than 10 pg of β-END without cross-reacting with related peptides including β-lipotropin (<3%).[1] As shown in Fig. 1, both rat plasma and pituitary extracts produced dose–response (inhibition) curves which were parallel to synthetic β-END standard. Confirming earlier reports (3–5), immobilization stress maximally elevated plasma β-END levels by 10 min of restraint (Fig. 2)

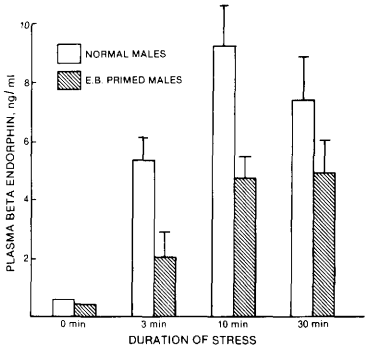

FIG. 2. Plasma levels of β-END in vehicle and EB-pretreated male rats following immobilization for either 0, 3, 10, or 30 min. Groups of six rats each received vehicle or 5 μg of EB/rat/day for 5 days before stressing. EB-treated animals had significantly lower plasma β-END levels ($P < 0.05$) by 3 and 10 min of restraint as compared to control values at the same time period.

and dexamethasone treatment (1 mg/day for 3 days, Fig. 3) completely prevented this response. Furthermore, hypophysectomy decreased circulating β-END levels to below the limit of detection (0.1 ng/ml of plasma) in both control and stressed animals.

In addition, daily injections of estradiol benzoate (EB, 5 μg per day for 5 days) tended to decrease resting levels of plasma β-END and significantly attenuated stress-induced β-END release in male rats. In

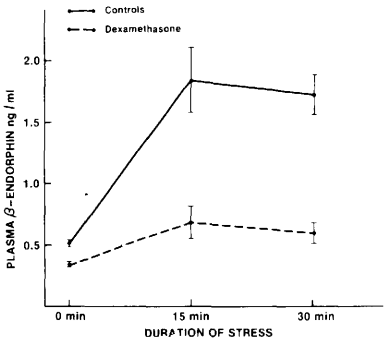

FIG. 3. Plasma β-END levels in saline- or dexamethasone-pretreated male rats following 0, 15, and 30 min of immobilization. Groups of six rats each received saline or dexamethasone (1 mg/rat/day) for 3 days before stressing. Dexamethasone significantly decreased ($P < 0.05$) plasma β-END values at all time periods as compared to the corresponding control means.

TABLE I. DOSE–RESPONSE EFFECTS OF ESTRADIOL BENZOATE TREATMENT OF PITUITARY β-ENDORPHIN

Treatment group[a] (n = 6 per group)	Neurointermediate lobe		Pars distalis	
	μg/gland	μg/mg protein	μg/gland	μg/mg/protein
Control	12.4 ± 1.7	43.4 ± 5.6	0.29 ± 0.02	0.21 ± 0.02
EB, 1 μg/day	8.7 ± 1.4*	32.0 ± 5.5*	0.30 ± 0.04	0.20 ± 0.02
EB, 3 μg/day	5.6 ± 0.8*	24.7 ± 3.6*	0.32 ± 0.05	0.19 ± 0.03
EB, 10 μg/day	7.0 ± 0.8*	24.2 ± 3.9*	0.28 ± 0.01	0.18 ± 0.01

[a] Treatments were given for 5 days and tissue samples were collected 24 hr after the last injection.
* Significantly different from control values; $P < 0.05$.

control animals, physical immobilization for 10 min increased plasma β-END levels from a resting mean of 0.57 ± 0.11 to 9.15 ± 1.36 ng/ml, whereas, in EB-treated rats, circulating β-END was elevated from a nonstress (basal) value of 0.43 ± 0.05 to 4.69 ± 0.80 ng/ml by 10 min of restraint (Fig. 2). Consistent with the diminished stress response in plasma β-END, EB treatment was also found to reduce pituitary content of β-END markedly in a dose-related fashion. As shown in Table I, total pituitary content of β-END was maximally decreased to 56% of control values by the 3 μg per day dose of EB. This reduction occurred primarily in the NIL, which was normally found to contain over 95% of pituitary β-END stores. In contrast to EB, testosterone proprionate treatment (0.5 mg/day for 5 days) did not significantly affect basal or stress-elevated levels of circulating β-END or pituitary β-END content (data not shown).

An evaluation of the possible influence of sex difference on the β-END response to physical immobilization is presented in Fig. 4. This experiment was carried out in the morning and females from all stages of the reproductive cycle were randomly assigned to treatment groups. In male rats, physical restraint for 15 and 30 min increased plasma β-END from a control value of 0.21 ± 0.06 to 2.39 ± 0.31 and 2.91 ± 0.57 ng/ml, respectively. Under the same test conditions, blood β-END levels in female rats were increased from a control mean of less than 0.10 ng/ml (detection limit) to 1.72 ± 0.22 and 2.06 ± 0.17 ng/ml by 15 and 30 min of restraint stress. After 45 min of restraint, plasma β-END levels in male rats had fallen into the range of female values at the same time interval. Although the peak levels of plasma β-END produced by restraint stress in female rats were less than those observed in males ($P < 0.05$), it should be noted that basal levels were also lower in females as compared to males (less than 0.10 vs 0.21 ± 0.06 ng/ml). When a value of 0.01 ng/ml was assigned to those samples which contained less than detectable amounts of β-END, resting levels of plasma β-END in female rats were significantly lower ($P < 0.05$) than those observed in males.

Because EB treatment consistently resulted in adrenal hyperplasia (data not shown), the possibility that adrenal glucocorticoids might be involved in the inhibitory effect of estrogen on stimulated β-END release was investigated. Treatment with 1, 3, or 10 μg of EB per day for 5 days resulted in a dose-related increase in plasma levels of corticosterone (Fig. 5). At the highest dose of EB, corticosterone

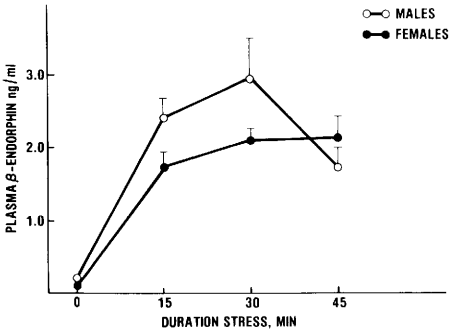

FIG. 4. Time course effects of restraint stress on plasma β-END levels in normal male and female rats (n = 6 per group). Females had significantly lower ($P < 0.05$) plasma β-END values by 0, 15, and 30 min of immobilization as compared to male values at the same time period.

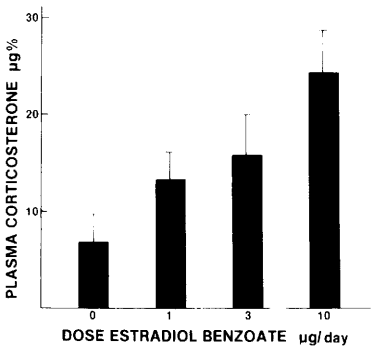

FIG. 5. Dose-related increases in plasma corticosterone levels by EB treatment. Groups of six male rats each were treated with either 0, 1, 3, or 10 μg of EB/rat/day for 5 days. Mean values for the groups receiving 3 and 10 μg of EB were significantly increased ($P < 0.05$) over the vehicle-injected control value.

levels in plasma were increased 3.6-fold above the vehicle-injected control value.

Discussion. A sensitive and reliable radioimmunoassay has been developed for measuring plasma and tissue levels of β-END-like immunoreactivity. Although human β-END was utilized for the development of the present assay, the likeness in structure between human, camel, ovine, and porcine β-END molecules (20) suggests that a human β-END RIA can be applied to the measurement of rat β-END. Using the RIA described above it was shown that in rats, physical immobilization evokes a rapid and dramatic increase in plasma β-END levels and this response is prevented by hypophysectomy or dexamethasone pretreatment. These findings, which are in agreement with previous reports (3–5), further suggest the possible role of β-END in the physiologic response to stress and confirm the potent inhibitory effect of glucocorticoids on pituitary β-END secretion.

The findings presented here also show that the administration of estrogen results in an attenuation of the stress-induced release of pituitary β-END. This suppression of β-END is probably due, at least in part, to an increase in circulating levels of glucocorticoids caused by estrogen treatment. Several laboratories have reported that in humans, estrogen treatment leads to an elevation of plasma concentrations of free (biologically active) as well as protein-bound (inactive) cortisol and a reduction in pituitary ACTH secretion (21–23). Reported here in rats is a dose-related increase in blood levels of free corticosterone and a marked reduction in pituitary stores and release of β-END following EB treatment. By contrast, administration of testosterone proprionate (0.5 mg/day for 5 days) had no effect on blood levels of corticosterone or pituitary content or release of β-END. Together these findings support the view that glucocorticoids are involved in the physiologic regulation of pituitary β-END secretion and may mediate the suppression of β-END release associated with estrogen treatment. In addition, a possible direct action of estrogen on the pituitary gland also exists since Vale and co-workers have observed that estrogen inhibited the *in vitro* release of ACTH (personal communication) and presumably β-END as well. The failure of testosterone proprionate treatment to modify pituitary β-END secretion significantly *in vivo* in the present experiments is in agreement with the observation that dihydrotestosterone had no effect on the *in vitro* release of β-END by cultured rat anterior pituitary cells (24). Taken together, these observations suggest that androgens are not important regulators of pituitary β-END secretion in the adult rat.

Interestingly, the β-END content of the NIL is subject to certain endocrine manipulation (EB treatment) while that of the AP is not (Table I). Since over 95% of total pituitary β-END is found in the NIL, this provides indirect evidence that the pars intermedia is the primary source of circulating β-END.

Consistent with the suppression of β-END release by EB treatment is the observation that basal and maximal stress-induced levels of plasma β-END were somewhat lower in female rats as compared to males. These differences may be a consequence of increased concentrations of estrogen and/or plasma-free corticosterone although the exact mechanism(s) responsible cannot be deduced from the findings of the present study alone. Since basal levels of β-END in the female rats were lower

than those observed in males (below 0.10 vs 0.21 ± 0.06 ng/ml), the proportional increase in circulating β-END evoked by the restraint stress may have been similar between the two groups. It is yet to be determined whether the absolute level of blood β-END or its increase relative to basal levels is of more importance with respect to the physiologic actions of β-END.

It should also be noted that agents other than glucocorticoids, estrogen, and CRF can influence β-END secretion. It has been reported that progesterone and dopamine decreased whereas norepinephrine increased the *in vitro* release of both β-END and ACTH by rat pituitary tissue (6, 24). Since each of these compounds are thought to be normally present in hypophyseal portal blood, their possible influence on the β-END responses observed here must also be considered.

The basal levels of plasma β-END reported here are comparable to those published by Akil *et al.* (17) and Höllt *et al.* (2) but lower than those reported by Guillemin and co-workers (3). With respect to pituitary tissue, the present finding of high concentrations of β-END in the NIL as compared to the PD is consistent with the findings of immunohistochemical studies which have demonstrated a similar distribution of β-END immunoreactivity in the rat pituitary (1, 25). In addition, Liotta *et al.* (26) found very low levels of β-END in the rat PD by RIA. Similarly, others have reported (2, 27) marked differences between the concentration of β-END in the rat NIL and PD although the magnitudes of these differences were not as great as that observed here. The reasons for this variation in reported values of plasma and pituitary β-END contents noted above may reflect differences in antibody specificities and/or extraction procedures; however, there is general agreement on the ability of stresses to increase plasma β-END levels and on the relatively higher concentration of β-END in the NIL as compared to the PD.

The present findings confirm earlier reports on the effects of stress and glucocorticoids on the secretion of pituitary β-END. Furthermore, it was shown that estrogen treatment leads to a depletion of pituitary stores of β-END and diminished β-END release in response to physical immobilization. These effects may be secondary to an increase in circulating glucocorticoids caused by estrogen treatment although a possible direct inhibitory action of estrogen on the pituitary gland exists.

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Note added in proof. While this manuscript was in press preparations of purified human β-lipotropin were obtained from Drs. A. Parlow and D. Orth and both were found to crossreact on an equimolar basis with synthetic β-endorphin (β-END) in the β-END RIA described above. Subsequently, the β-END-like immunoreactivities present in rat plasma and pars distalis have been characterized by G-50 gel filtration as described for the neurointermediate lobe under Materials and Methods. It was found that over 95% of the β-END immunoreactivity present in plasma, like that in the neurointermediate lobe, coeluted with synthetic β-END. By contrast, chromatographic separation of acetic acid extracts of pars distalis revealed three peaks of β-END-like immunoreactivity; one peak eluted in the void volume, while a second and third peak coeluted with β-lipotropin (A. Parlow) and synthetic β-END, respectively. The relative amounts of the total β-END immunoreactivity eluted as peaks 1, 2, and 3 were 11, 46, and 43%, respectively. Thus, with regard to the data presented in this report it appears that almost all of β-END-like immunoreactivity present in both rat plasma and neurointermediate lobe resembles true β-END in size, whereas two or more larger molecules also contribute to the β-END-like immunoreactivity present in rat pars distalis.

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