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The Preferential Release of Beta-Endorphin From the Anterior Pituitary Lobe by Corticotropin Releasing Factor (CRF)

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YOUNG, E. A., J. LEWIS AND H. AKIL. *The preferential release of beta-endorphin from the anterior pituitary lobe by Corticotropin Releasing Factor (CRF).* PEPTIDES 7(4) 603–607, 1986.—Although a number of investigators have shown that release of ACTH is accompanied by the release of Beta-endorphin (β-End) and Beta-lipotropin (β-LPH), the proportion of the latter two peptides released with stress or by CRF is unclear. To evaluate directly the release of β-End versus β-LPH from the anterior lobe, we used molecular sieving of plasma and subsequent radioimmunoassay to measure release of both β-End and β-LPH into plasma after thirty minutes of inescapable intermittent footshock. We found a substantial increase in circulating β-End which appears to be of anterior lobe origin. The β-End does not appear to represent peripheral conversion of β-LPH to β-End since the ratio of β-LPH:β-End released remained constant between five and thirty minutes of stress, and the rate of disappearance of β-LPH is slower than the rate of disappearance of β-End following the termination of stress. Further confirmation of these findings was obtained by examining the POMC derived peptides released by pituitary cell suspensions in the presence and absence of oCRF. While unstimulated release consisted of equal proportions of β-End and β-LPH, stimulation of the anterior lobe cell suspensions with oCRF resulted in the release of two-fold more β-End than β-LPH.

Beta-endorphin Anterior pituitary lobe Corticotropin releasing factor Radioimmunoassay Beta-lipotropin

THE post-translational processing of proopiomelanocortin (POMC) to Beta-endorphin (β-End) and Beta-lipotropin (β-LPH) differs in the anterior and intermediate lobes of rat pituitary. In the anterior lobe (AL), the molar ratio of β-End to β-LPH is approximately 1:2, while in the intermediate lobe (IL), virtually all of the precursor is processed to β-End sized or smaller material [2, 5, 12]. One might expect, therefore, that the products released during stimulation of each lobe would reflect these molar ratios; the anterior lobe should release primarily β-LPH, while the IL would release β-End 1–31 or modified forms of β-End (e.g., alpha N-acetylated β-End 1–27). Consequently, the circulating levels in plasma should reflect both anterior and intermediate lobe contributions.

At rest, the majority of β-End immunoreactivity circulating in rat plasma is β-End 1–31 size [1]. The source of this plasma β-End is presumed to be intermediate lobe, since a sizeable proportion is alpha N-acetylated β-End (NAc β-End 1–31) [1]. In contrast, stress activates primarily the anterior lobe of the pituitary to release ACTH accompanied by both β-LPH or β-End [6]. Thus, if the released material truly reflects the molar ratios of stored β-LPH and β-End in the anterior lobe, after stress, approximately $^2/_3$ of the newly released β-End like immunoreactivity in plasma should be β-LPH size.

However, the existence of subpopulations of releasable pools has been shown for POMC derived peptides in the IL of the frog [8]. Similarly, we have recently shown that after repeated phasic stress the IL releases primarily NAc β-End 1–31. In the IL, however, this peptide is not the most abundantly stored [1], suggesting the existence of specifically releasable pools. Whether such pools exist for anterior lobe POMC derived peptides is not known. Using mouse anterior lobe primary cell cultures and a double labelling technique, Allen *et al.* [3] showed that the contents of older ACTH containing granules were preferentially released over newly synthesized material, when the culture was stimulated by partially purified rabbit corticotropin releasing factor. As expected, these mature granules contained both β-LPH and β-End. However, this study did not address the issue of differential release of β-LPH versus β-End. Consequently, it is not clear if the release of ACTH from the anterior lobe is accompanied primarily by the release of β-LPH, as would be expected from the molar ratio, or, if significant amounts of β-End can be released. Since older granules would have more time to continue processing, even the slowest processing reactions may have time to proceed to completion. Thus the preferential release of the more mature ACTH granules suggests that these granules could contain the most processed form, that is, β-End.

To address this issue we used a dual approach: (1) measurement of plasma levels of β-End and β-LPH before and after exposure to stress and (2) measurement of the release of β-End and β-LPH from anterior lobe pituitary cell preparations upon stimulation with ovine corticotropin releasing factor (oCRF). In addition, it is possible that sustained demand may cause some pools to be more or less releasable. Therefore, we compared the ratio of β-End and β-LPH released into plasma following exposure to 5 or 30 min footshock stress.

METHOD

Footshock Stress

Sprague Dawley male rats (Charles River) are stressed with intermittent footshock as previously described [1,11]. The footshock stress session lasts either 5 or 30 minutes. Controls are naive, unhandled rats.

Plasma Radioimmunoassays and Chromatography

After decapitation, trunk blood is collected into tubes containing liquid NaEDTA on ice, and centrifuged to obtain plasma. The plasma is acidified with 1 N HCl to pH 3.0, and frozen immediately on dry ice and stored at −70°C until extraction. Plasma β-End immunoreactivity (β-End-IR) is assayed as described previously [4]. Briefly, peptides are extracted with Sep Pak C_{18} cartridges (Waters) using the manufacturer's recommended procedure for arginine vasopressin. β-Endorphin immunoreactivity is assayed using a β-End radioimmunoassay (RIA) with an antibody (Brenda) to the midportion of β-End 1–31. This antibody cross reacts equally well with β-LPH and β-End. Using ^{125}I β-End 1–31 as a tracer, it shows only partial cross reactivity with N-acetylated and COOH-terminal shortened forms of β-End that is dependent upon the actual form. Thus, it shows 50% cross reactivity with NaAc β-End 1–27, 80% cross reactivity with NAc β-End 1–31 and 80% cross reactivity with β-End 1–27. ACTH is measured as previously described [11] using an antibody raised to ACTH 11–24. ^{125}I ACTH 1–39 is used as a radiolabelled tracer. This antibody shows only partial (10%) reactivity with ACTH 18–39 (CLIP) and none with alpha-MSH (N-acetyl ACTH 1–13 amide). N-Acetyl β-endorphin is assayed using an N-terminus specific antibody that required acetylation for recognition by the antibody [1]. This antibody does not recognize non-acetylated β-endorphin. ^{125}I N-acetyl β-endorphin 1–27 is used as a radiolabelled tracer. To separate β-End and β-LPH sized material, molecular sieving with a G-50 Superfine, 1% formic acid column is used. For these experiments, the plasma from 10 animals, both stressed and unstressed are pooled, and applied to G-50 column for sieving (see [4] for more details). Individual fractions are dried down, resuspended in assay buffer and individually quantitated.

Pituitary Dispersed Cells Preparation

For pituitary dispersed cell preparations, the glands are removed, and separated into anterior and neuro-intermediate lobe using a conservative dissection that avoids possible contamination of the anterior lobe with the POMC rich intermediate lobe. Pituitaries are placed in oxygenated, calcium-free Krebs-Ringer bicarbonate buffer with 0.2% glucose and 0.5% bovine serum albumin (Fraction V, Sigma), 0.2% sodium bicarbonate , 0.01% lima bean trypsin inhibitor,

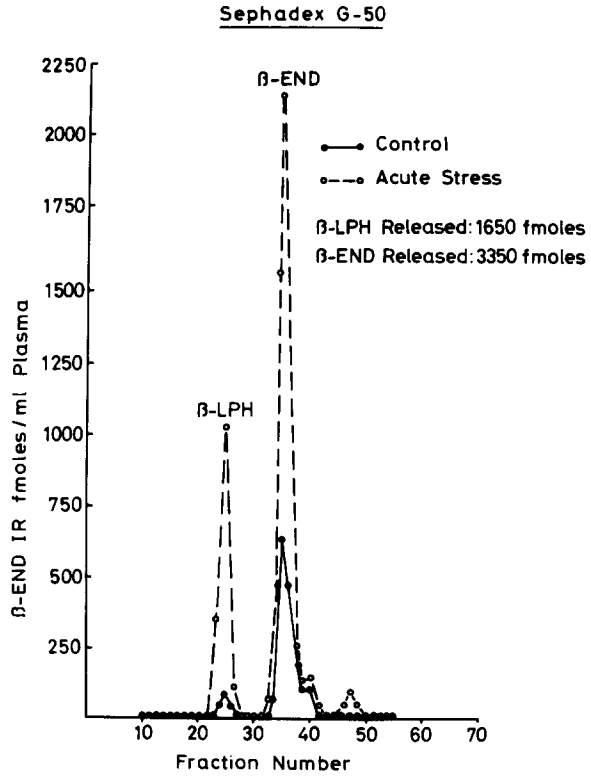

FIG. 1. G-50 column sieving of plasma from control and stressed rats. Plasma from the stressed rats shows a greater increase in β-End than β-LPH, suggesting that β-End is preferentially released during stress. The femtomoles released are calculated by subtracting control "baseline" values from "acute stress" values. The control rats show predominantly β-End-IR in plasma, which may be of IL origin.

0.1% soybean trypsin inhibitor and 0.1% kanamycin (KRGBAlb). Pituitaries from all rats (n=10/group) from the same group are pooled, and then treated with collagenase (Sigma, type 1) at 3 mg/ml in calcium free KRGBAlb for 60 minutes at 37°C under 95% O_2 + 5% CO_2. Every 15 minutes the fragments are dispersed by repeatedly drawing them up and down into a plastic Pasteur pipette. The cells are then filtered through a 30 micron nylon mesh and washed three times with calcium free KRGBAlb by low speed centrifugation (100×g). The cells are then resuspended in calcium containing KRGBAlb and incubated for 90 minutes to stabilize the cells. All incubations are carried out at 37°C under 95% O_2 + 5% CO_2. Aliquots of the cell preparations from each group, control and stress, are counted using a hemocytometer and the trypan blue dye exclusion method. Viability is greater than 95%. Following the stabilization period the cells are centrifuged and resuspended in fresh calcium containing KRGBAlb to a final concentration of 1×10^6 cells/ml buffer for all cultures. Aliquots of cell suspensions (0.5 ml) are incubated with either medium only (unstimulated) or medium containing 1 nM synthetic oCRF (Bachem) for 60 minutes. Both unstimulated and CRF stimulated release are set up in triplicate. Following the incubation period, the cells are centrifuged, and the medium saved and frozen at −70° centigrade until extraction. The medium is extracted using the same Sep Pak C_{18} extraction procedure used for plasma

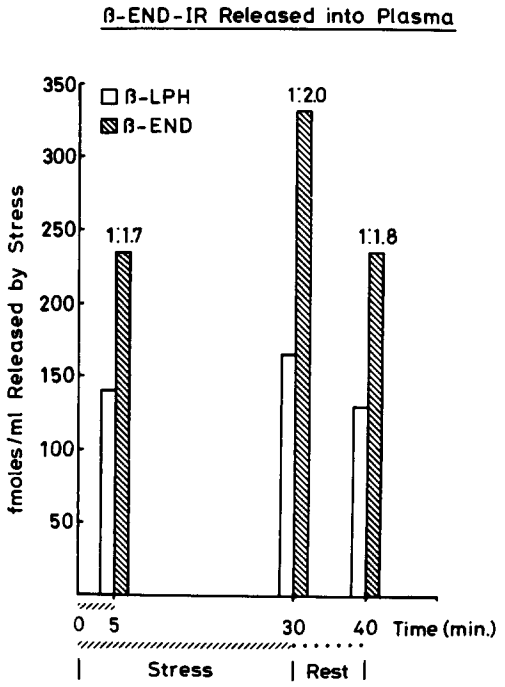

FIG. 2. Molar ratio of β-LPH to β-End in plasma after either 5 minutes or 30 minutes of stress, and 10 minutes after the end of a 30 minute footshock stress. The ratio of β-LPH to β-End released with stress remains relatively constant over this time frame. During the first 5 minutes of stress, the ratio of β-End to β-LPH released is 1.7:1. The 10 minute post stress data shows that the rate of disappearance of β-LPH is slower than β-End, suggesting peripheral conversion of β-LPH to β-End is not the source of circulating β-End.

β-End. At the end of the experiment, the AL cells are extracted by freezing and thawing in 5 N acetic acid with 0.2% bovine serum albumin. Total β-End plus β-LPH is measured using the same β-End RIA procedure as plasma. The extracted material is then sieved on a G-50 Superfine 1% formic acid column to separate β-End from β-LPH as described above. The column fractions are assayed for β-End-IR.

RESULTS

Footshock stress releases several POMC derived peptides into plasma, as previously reported [6]. ACTH, a marker of anterior lobe release, increases 500% following footshock stress (30±10 [SD] fmoles/ml to 166±19 [SD] fmoles/ml). Likewise, β-End-IR increases 500% following this same stressor (50±14 [SD] fmoles/ml to 277±16 [SD] fmoles/ml). In contrast, following footshock stress, N-acetyl-β-End, a marker of intermediate lobe release, increases only 80% in plasma (30±6 [SD] fmoles/ml to 52±7 [SD] fmoles/ml). Taken as a set, these data indicate that acute footshock stimulates release from the anterior lobe to a much greater extent than it stimulates release from the intermediate lobe. Thus, the changes in β-End-IR seen following footshock appear to result from anterior lobe release into plasma of β-End and β-LPH.

To characterize the forms of β-End-IR in plasma, pooled plasma was applied to a G-50 column and the fractions were assayed for β-End-IR. The data from plasma sieving are seen in Fig. 1. Both β-End and β-LPH are present in rat plasma at rest and both are released with stress. Since the control "baseline" plasma sieving data may be influenced by β-End-IR of IL origin, to characterize the forms of β-End-IR released by stress, we subtracted control "baseline" values from the acute stress values before calculating the ratio. The ratio of β-End to β-LPH released by stress is approximately 2:1. Since the anterior lobe from these animals contains mostly β-LPH(β-LPH:β-End is 1.2:1 for the control group and 1.4:1 for the acute stress group), while the plasma shows mostly β-End, granules containing β-End, the most processed form, appear to be preferentially released. An ACTH-RIA of these same G-50 column sieving fractions show femtomolar totals in close agreement with the total of β-LPH plus β-End, supporting the conclusion that the non-acetylated β-End is primarily of anterior lobe origin. (1) Further assurance that the IL is not responsible for the β-End size material released by stress is provided by the data that the intermediate lobe contributes primarily N or C terminally modified β-End [2,12], which shows only partial crossreactivity with our antibody under these conditions, and (2) even if the IL contributed to the β-endorphin-IR released with stress, C-terminally modified β-End is separated from authentic β-End on the sieving column and NAc-β-End was measured in these samples. If the NAc β-End-IR released with stress showed 100% cross reactivity with our antibody (which it does not), only 709 fmoles of N-acetyl β-End is present while 2380 fmoles of β-End size material was measured. The difference is 1671 fmoles of β-End size material compared to 869 fmoles β-LPH size material, yielding a ratio of β-End to β-LPH of 1.9:1. Thus, the evidence converges that the great majority of the β-End seen in plasma following stress is of anterior lobe origin.

To examine the possibilities that either different pools may be released over time or that the peripheral conversion of β-LPH to β-End occurs during the course of our stress (30 minutes), we examined a shorter stress, 5 minutes, and compared ratios of β-End size material to β-LPH at the end of the 30 minutes of stress and 10 minutes following the 30 minute stress. The data in Fig. 2 shows that the ratio of β-End-size material to β-LPH size material released by stress remains constant across all stress conditions. During the first 5 minutes of stress, when the rate of release is far greater than the rate of degradation, the ratio of release of β-End to β-LPH is 1.7:1 suggesting that β-Endorphin is preferentially released even during the early stress period. If peripheral conversion of β-LPH to β-End were occurring, one would expect the ratio of β-End to β-LPH to increase ten minutes post stress as β-LPH was converted to β-Endorphin. Using these data, we can actually calculate the rate of disappearance of β-LPH and β-End during this post stress period. While β-End-IR disappears at a rate of 13 fmoles/min, β-LPH disappears at the rate of 5.5 fmoles/min, which does not suggest that β-LPH is being converted to β-End. In fact, it suggests that β-LPH may be more stable to breakdown in plasma than β-End. Thus, these ratios do not suggest that peripheral conversion of β-LPH to β-End is the source of the circulating β-End.

Since the *in vivo* plasma studies are complicated by the contribution of IL and the breakdown of both β-LPH and β-End, we elected to study anterior pituitary release more directly, using anterior lobe dispersed cell preparations from either naive unhandled rats (control) or rats which had been stressed immediately prior to decapitation (acute stress). Previous studies within our laboratory [9] had indicated that

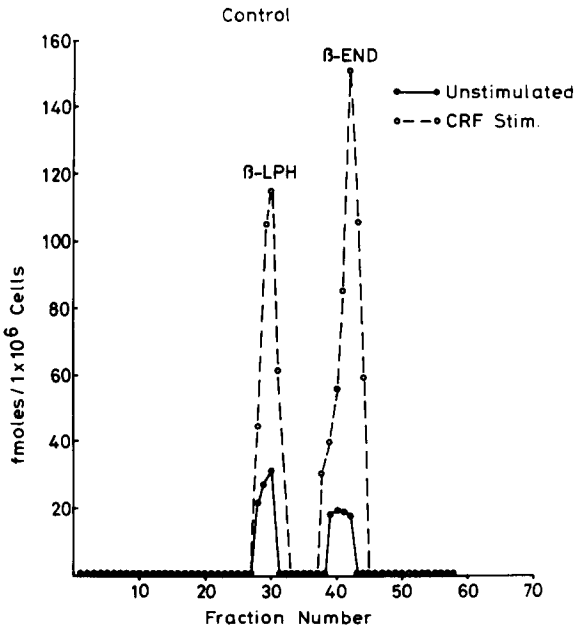

FIG. 3. G-50 column sieving of medium from control anterior lobe suspensions with and without CRF. The baseline (unstimulated) release shows a 1:1 ratio of β-End to β-LPH. This contrasts with plasma data, where *both* anterior lobe and IL are contributing to pools of circulating β-End-IR, making it difficult to determine the forms released by anterior lobe at *rest*. However, with CRF stimulation, the ratio of β-End to β-LPH of released material is 2:1, suggesting the anterior lobe releases primarily β-End.

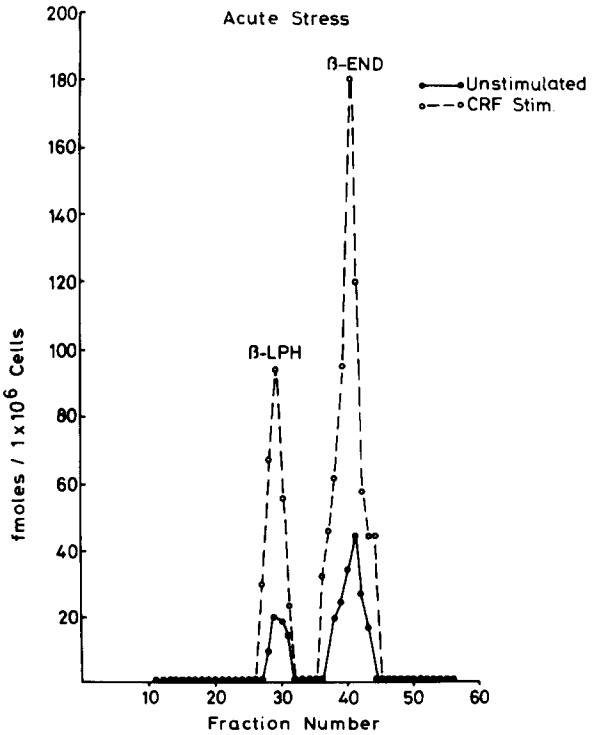

FIG. 4. G-50 column sieving of medium from anterior lobe dissociated cells from rats which were stressed immediately before sacrifice. The unstimulated release is primarily β-End, in contrast to anterior lobe suspensions from control rats that show equal proportions of β-End and β-LPH released at rest. With CRF stimulation, the anterior lobe suspensions from both control and stressed rats demonstrate the predominance of β-End release over β-LPH release (β-End:β-LPH for control=2:1; β-End:β-LPH for acute stress=2.3:1).

this same short term dispersed cell system showed differences in the rate of biosynthesis and processing of POMC induced by stress and these same changes are still present several hours later. Therefore, the CRF and arginine vasopressin (AVP) released *in vivo* during the time of the stress may continue to influence the release of β-End and β-LPH *in vitro*. Consequently, we examined both baseline (unstimulated) and CRF stimulated release to determine the ratio of β-End to β-LPH in both situations. The data are shown in Fig. 3 and 4. In preparations from control animals without CRF (Fig. 3), the baseline ratio of β-End to β-LPH released into the medium is 1:1. Addition of 1 nM CRF causes the total β-End/β-LPH-IR to increase by 3 fold, but the ratio of β-End to β-LPH is now 1.6 to 1. Since unstimulated release may represent a number of different physiological mechanisms including cell death and may continue to occur even in the presence of CRF, we subtract baseline release from total release to obtain CRF stimulated release similar to the calculations used to determine forms released into plasma. After unstimulated release is subtracted from total release, then oCRF induced secretion of β-End:β-LPH in a 2:1 ratio. This ratio is very close to the ratio of β-End to β-LPH in plasma after 30 minutes of footshock stress (β-End:β-LPH is 1.9:1). In anterior lobe cell suspensions from stressed rats (Fig. 4), that is, rats already exposed to endogenous CRF and other endogenous secretagogues *in vivo*, the baseline release is predominantly β-End (2.8:1). Addition of CRF to the medium stimulates more release but induces little change in the ratio of β-End to β-LPH released (2.4:1). If unstimulated release is subtracted from total release, the ratio of β-End to β-LPH is 2.3:1. It is unclear if this higher proportion of β-End release seen in anterior lobe suspensions following acute stress represents a real difference from the 2:1 ratio seen following CRF stimulation of anterior lobe suspensions from control rats. However, in all cases stimulated secretion is predominantly β-End. This occurs despite the fact that molecular sieving of the cells from these same suspensions show a β-End:β-LPH ratio of 0.45:1 for control cultures and 0.4:1 for acute stress cultures.

DISCUSSION

In summary, both the *in vivo* stress studies and the *in vitro* CRF and stress studies indicate that β-End is preferentially released over β-LPH from the anterior lobe by approximately 2:1. The time course study shows that this pattern is not due to conversion of β-LPH to β-endorphin, since the half-life of β-LPH in plasma appears to be longer than the half-life of β-End. The preferential release of β-End from the anterior lobe has a number of implications for our understanding of the regulation of biosynthesis, processing, and release. While we have only examined a single stressor *in vivo* and a single releaser *in vitro*, and while we have not examined time points shorter than five minutes, the con-

vergence of the evidence within the current framework is rather striking. Our data suggest that β-End is the preferred secreted end product derived from the processing of the carboxy-terminal domain of POMC. Together with the data of Allen *et al.* [3], the results indicate that the most mature ACTH granules may contain β-End. Thus, although the initial processing of POMC may stop at the conversion of POMC to ACTH and β-LPH, processing may continue within some of the granules to form β-End. These granules appear to form the most releasable pool. It also points out that what exists in the releasable pools may not match the molar ratios of peptides seen in the gland as a whole. Thus, while the usual ratio of β-LPH to β-End in the anterior pituitary is 2:1, 2-fold more β-End than β-LPH is released by stress and CRF—i.e., four times more than predicted by the ratio of materials stored in the gland.

The ability of anterior pituitary cells from stressed rats to continue to release 2-fold more β-End than β-LPH, even in the face of mild depletion to 80% control values (previously reported, [11]), suggests that the pool of β-End can be replenished very quickly during the course of the stress and shortly thereafter. This is consistent with our previous findings [9] that post-translational processing is increased following acute stress, speeding the conversion of POMC to its final products.

There exists an alternative explanation to the idea that different populations of releasable pools are related to the maturity of the granule. Our results can be equally well explained by suggesting that β-LPH is converted to β-End in a release-coupled fashion. Such a phenomenon has been proposed in frog, where the authors have suggested that the last step in the post-translational modification of alpha-MSH is coupled to secretion [10]. While no such phenomenon has been demonstrated in mammals, our current work does not allow us to distinguish between the two alternative explanations. Similarly, it is possible that rather than subpopulations of releasable pools, there are subpopulations of secreting cells, and that the actively secreting cells produce primarily β-End.

Regardless of the mechanisms, the liberation of a larger than expected molar ratio of β-End to β-LPH points to the anterior lobe as an important source of circulating plasma β-End. The dissociation between the ratio of β-End to β-LPH in the anterior lobe versus the ratio of these peptides released from the anterior lobe into plasma suggests that simply measuring and sieving the whole anterior lobe does not provide the proper information to predict the forms released. Therefore, in species such as man, in which β-LPH is even more predominant in the anterior lobe than in rat [7], the anterior lobe may still release substantial β-End in times of stress or with CRF stimulation. Consequently, the ratio of β-End to β-LPH must be evaluated in both unstimulated (basal) conditions and with stress or CRF infusion.

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