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Degradation of [^{3}H]β-endorphin in rat plasma is increased with chronic stress

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With a number of acute stressors β-endorphin is released into plasma. It is unclear if β-endorphin is converted into any other biologically active products, nor is it clear if the rate or pathways of degradation are changed during chronic stress. To explore these issues, we incubated [^{3}H]β-endorphin$_h$ labeled in positions 1 and 27 with plasma from normal and chronically footshocked rats and measured the rate of conversion of the label from β-endorphin size material to smaller size material. Initial separations were done using a G-50 molecular sieving column, with subsequent characterization and identification on HPLC. By G-50 sieving, there is a time dependent formation of only one radioactive peak. HPLC identification demonstrates γ-endorphin and another unidentified peak. This enzymatic activity is increased in the plasma of chronically stressed rats.

γ-Endorphin; β-Endorphin; Stress (chronic); Opioid peptides

1. Introduction

The encoding of multiple active sequences and products within one polypeptide is a general principle in peptide biology, particularly in the opioid peptide families. Thus, most peptides are synthesized as part of a large molecular weight precursor protein which is then processed to yield smaller peptide fragments. In the case of pro-opiomelanocortin (POMC), one of the most widely studied opioid peptide precursors, there are several instances of one active sequence contained within another active sequence (Mains et al., 1977; Roberts and Herbert, 1977). In addition, there is evidence of tissue-specific processing of these sequences, so that, in general, some tissues such as the intermediate lobe process POMC to primarily small peptides such as β-endorphin and α-MSH while other tissues such as the anterior lobe process POMC to primarily ACTH and β-lipotropin (β-LPH) (Mains and Eipper, 1979; Crine et al., 1978; Eipper and Mains, 1980). In addition to these classically studied POMC products, other biologically active peptide fragments, particularly α-endorphin, γ-endorphin and γ-MSH are contained in the POMC sequence (Burbach et al., 1980a; Guillemin et al., 1976; Nakanishi et al., 1979). A number of acute stressors release β-endorphin-IR from the anterior lobe of the pituitary. Of the β-endorphin-IR released from anterior lobe, 2/3 of it is β-endorphin size by molecular sieving (Young et al., 1986; Watson et al., 1988). The rate of disappearance of β-endorphin released in plasma is rapid, with estimates of the half-life of 45 min or less (Houghten et al., 1980). It is unclear if β-endorphin released into plasma is converted into any other biologically active fragments such as β-endorphin-(1-16)(α-endorphin) or β-endorphin-(1-17) (γ-endorphin). Many peptides are

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degraded by serum (e.g. fetal calf serum) or plasma but this degrading activity for β-endorphin has not been characterized in plasma. In the case of β-endorphin degradation it is unclear if chronic drive to release or elevated glucocorticoids released subsequent to activation of the HPA axis can modulate the rate of degradation of β-endorphin in plasma.

γ-Endorphin is a naturally occurring fragment of β-endorphin (Austen et al., 1977b; Burbach et al., 1980a,b; 1981; Guillemin et al., 1976; Ling et al., 1976; Smyth and Snell, 1977). It has been demonstrated in all known POMC producing areas, both anterior and posterior pituitary, and brain (Lissitzky et al., 1978; Jegou et al., 1979; Loeber et al., 1979; Dorsa et al., 1982; Verhoef et al., 1982a,b; Vaudry et al., 1980), ileum (Opmeer et al., 1982) and testes (Tsong et al., 1982; Margioris et al., 1983; Cheng et al., 1985). The formation of γ-endorphin from β-endorphin results from proteolytic cleavage of the Leu^{17}-Phe^{18} bond. In most of these POMC producing areas γ-endorphin production is believed to result from a specific enzyme(s): γ-endorphin generating enzyme (Burbach et al., 1980b; Lebouille et al., 1984; 1987; 1985). The conversion of β-endorphin to γ-endorphin results in a substantial loss of opioid activity but produces different behavioral properties in rats including 'neuroleptic-like' activities (De Wied and Jolles, 1982; De Wied, 1978; Van Ree et al., 1978), many of which are retained by the non-opiate fragment des tyrosine γ-endorphin. Since γ-endorphin continues to demonstrate biological activities, the conversion of β-endorphin to γ-endorphin appears to be a biotransformation rather than a degradation phenomenon. Besides γ-endorphin generating enzyme, several other enzymes have been demonstrated to produce γ-endorphin: cathepsin D, rennin and endopeptidase 24.11 (Austen and Smyth, 1977a; Smyth and Snell, 1977; Graf et al., 1979a,b; 1977; 1985; Burbach et al., 1980b). Consequently, there is more than one source of γ-endorphin production in vivo.

We have previously shown increased releasability of anterior lobe β-endorphin to oCRF stimulation in vitro in chronically stressed rats which were re-stressed before decapitation (Young and Akil, 1985) and a potentiation of glucocorticoids on oCRF stimulated release in vitro in anterior pituitaries from chronically stressed rats (Young and Akil, 1988). Yet, in vivo both naive rats and chronically stressed rats, when challenged with an acute stress, show identical release of corticosterone, ACTH and β-endorphin. One possible explanation would be a more rapid breakdown of β-endorphin following release. To test this hypothesis, we examined the rate of degradation of [^{3}H]β-endorphin in rat plasma from control and chronically stressed rats. We now report an increased rate of degradation of β-endorphin in chronically stressed rats and that γ-endorphin is produced during the course of this degradation.

2. Materials and methods

Rats received 14 days of chronic intermittent footshock as previously described (Young and Akil, 1985). Briefly, this stress paradigm consists of daily 30 min stress sessions of intermittent footshock (1.5 mA, 12 shocks/min and 1 s duration). Blood was collected following decaptitation in either EDTA or heparin tubes from control and chronic footshock stress rats. Following centrifugation, the plasma was pooled from eight rats/group and equilibrated at 37°C in a Dubnoff Shaking metabolic incubator. Double labeled (positions 1 and 27) [^{3}H]β-endorphin (human) synthesized by one of us (RH), and was repurified on HPLC within 2 weeks of the experiments. It was mixed with unlabeled β-endorphin in a 1:10 ratio and added in a small volume Krebs Ringer bicarbonate buffer (pH 7.4) to the plasma, producing a final concentration of β-endorphin of 25 nM (approximately 25 000 c.p.m./ml). At time 0, 10, 20, 40 and 60 min, aliquots were taken, acidified to pH 2.0 and frozen on dry ice and stored at −80°C. The samples were later extracted with Sep Pak C_{18} cartridges as previously described for β-endorphin extraction (Cahill et al., 1983). To determine recovery, both the effluent and the eluate were counted for radioactivity in a Beckman LS 9000 Liquid Scintillation Counter. After lyophilization, the samples were resuspended in 1% formic acid and applied to a 1% formic acid G-50 molecular sieving column for separation by

molecular size. Half of each fraction was counted for radioactivity. The remainder of the radioactive fractions for each peak were pooled for subsequent identification by HPLC.

Two experiments were done using plasma collected in sodium EDTA. A repeat experiment used plasma from these same two groups (control and chronic footshock) collected in both sodium EDTA and sodium heparin in order to evaluate whether the method of anticoagulation affected the rate of breakdown. In addition, since corticosteroids are known to affect a number of enzymes, and daily stress sessions produced daily elevation in corticosterone, plasma from rats which received daily injection of corticosterone (30 μg/100 g body weight), or saline were compared to an unhandled non-injected control group for the rate of [^{3}H]β-endorphin conversion to products.

Final identification of the products was obtained using reverse phase HPLC. Buffer A was 50 mM potassium phosphate pH 2.7 containing 0.05% triethylamine. Buffer B was 100% acetonitrile. The gradient began at 5% B, increased to 20% B over 2 min, was left constant for 2 min then raised to 45% B over 25 min. α- and γ-endorphin were used as HPLC standards as well as β-endorphin-(18-31). The α- and γ-endorphin were obtained from Organon (Netherlands), the β-endorphin-(18-31) from Peninsula (Belmont, CA).

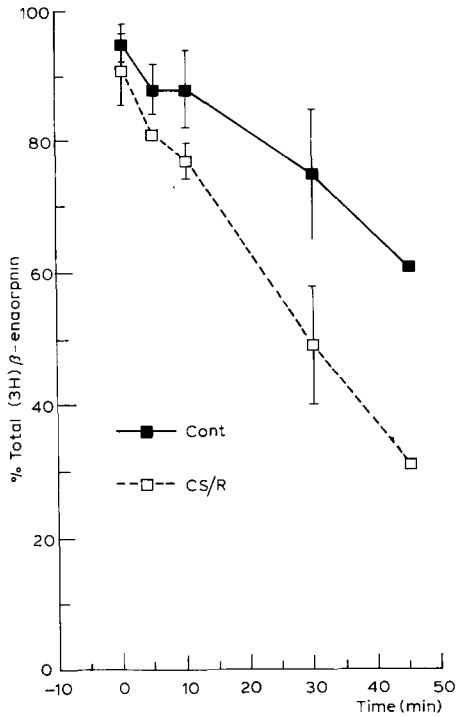

Fig. 1. Disappearance of [^{3}H]β-endorphin from rat plasma with time. Each point represents the mean of 3 experiments using plasma pooled from 10 animals/experiments. The 45 min time point was present in only 1 experiment. The percent [^{3}H]β-endorphine was estimated by molecular sieving. Note the faster disappearance of [^{3}H]β-endorphin in the chronically stressed rat plasma (CS/R, dotted line).

3. Results

Because the [^{3}H]β-endorphin was labelled in two positions (positions No. 1 and No. 27), two radioactive fragments would be expected from most enzymatic cleavages. When we applied the radioactive extracted plasma to G-50 column, only two peaks were seen, one eluting with β-endorphin (peak No. 1), the parent compound and another smaller fragment of approximately 14-17 amino acid size (peak No. 2). A time dependent decrease in [^{3}H]β-endorphin was observed (fig. 1). Along with the time dependent decrease in [^{3}H]β-endorphin, there was a time dependent increase in the breakdown peak (fig. 2). This conversion was accelerated in plasma from chronically stressed rats (figs. 1 and 2). If amino terminal cleavage of the initial tyrosine occurred, then [^{3}H]tyrosine would result and this product would not bind to the Sep Pak. Analysis of the radioactivity in the Sep Pak effluent found only small amounts (less than 5% of the total radioactivity of the sample) and no time dependent increase, suggesting [^{3}H]tyrosine formation was not occurring during the course of the incubation. We did not subject the effluent to any further analysis for characterization of this radioactivity.

For further product identification, the pooled fractions from the breakdown peak were characterized on HPLC. The results are shown in fig. 3. As can be seen, a proportion of the radioactivity co-migrates with γ-endorphin on HPLC. In addition, there is another unidentified peak. In the control plasma, the ratio of γ-endorphin to the unidentified peak is 1 : 2.2. In the chronic stress

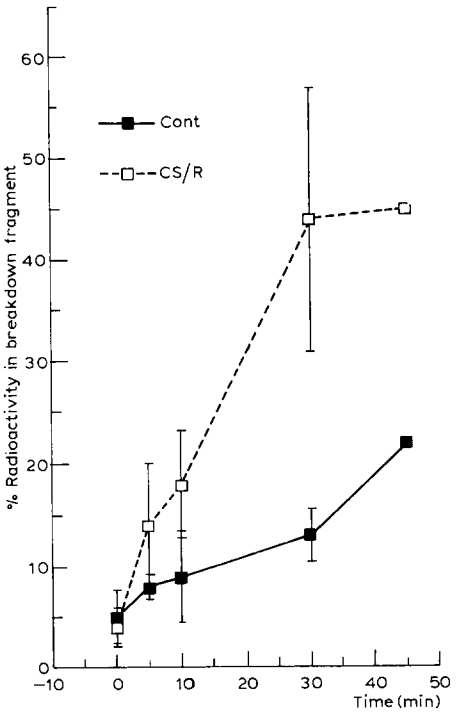

Fig. 2. Appearance of smaller size radiolabeled fragments in rat plasma collected in EDTA. Each point represents the mean of 3 experiments with pooled plasma from 10 animals/experiment. The 45 min time point is from 1 experiment. The percent smaller size ^{3}H material was determined by molecular sieving. Note faster rate of appearance of this smaller fragment in chronically stressed rats (CS/R).

plasma, the ratio is 1 : 2.1, suggesting chronic stress only accelerates the degradation, it does not produce different products. If the initial cleavage is between amino acids 17 and 18, two labeled fragments would be formed: γ-endorphin and β-endorphin-(18-31). However, this unidentified peak does not co-migrate with the β-endorphin-(18-31) standard. It is possible that the β-endorphin-(18-31) is subject to further enzymatic degradation, e.g. either aminopeptidase action or cleavage by a trypsin-like enzyme at the dibasic cleavage sites Lys-Lys in positions 28 and 29. Since the ^{3}H label is at position 27, β-endorphin-(18-27) would still retain radioactivity. Since there does not appear to be equimolar amounts of γ-endorphin and the unknown fragment, it is possible that γ-endorphin is subject to aminopeptidase action with subsequent loss of the initial tyrosine, which carries the label. Thus, we may be underestimating the amount of γ-endorphin formed.

An experiment comparing EDTA vs. heparin as an anticoagulant was performed. This experiment was carried out in control and chronic stress rats and alternated EDTA and heparin blood collection for each pair of rats. The data revealed that the half-life of [^{3}H]β-endorphin was shorter in plasma collected in heparin than plasma collected in EDTA (table 1B, fig. 4), but the accelerated degradation rate of β-endorphin in plasma from chronically stressed rats was still observed. These data suggest that (1) there is approximately a 3-fold acceleration in the degradation of [^{3}H]β-endorphin to its product in plasma from rats which were chronically stressed, and (2) that EDTA slows down this enzymatic degradation. A final experiment compared the effects of daily

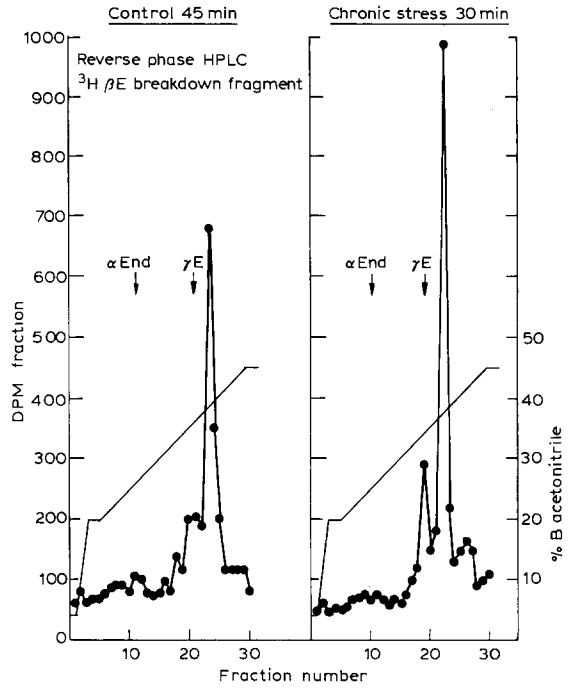

Fig. 3. HPLC profile of small size [^{3}H]β-endorphin degradation fragments using pooled G-50 sieving fractions from rat plasma collected in EDTA. In both profiles one-third of the radioactivity co-migrates with the γ-endorphin standard on HPLC. Pooled sieving fractions from other time points demonstrated the same degradation pattern. This suggests that some of the small size material formed during these studies is [^{3}H]γ-endorphin.

TABLE 1

Half-life of [^{3}H]β-endorphin in rat plasma (min), mean ± S.D.

		Control	Chronic stress	No. of experiments
(A)	EDTA	72 ± 20	26 ± 6.3 [a]	3
(B)	Heparin	29	13	
	EDTA	58	30	

[a] P = 0.026, two-tailed t-test in comparison to control

corticosterone injection on the rate of degradation of [^{3}H]β-endorphin in rat plasma. It demonstrated no difference in the half-life of [^{3}H]β-endorphin between unhandled control, daily saline injected and daily corticosterone injected rats (data not shown). The basal corticosterone values in plasma did not differ between groups since the study was conducted 24 h after the last injection.

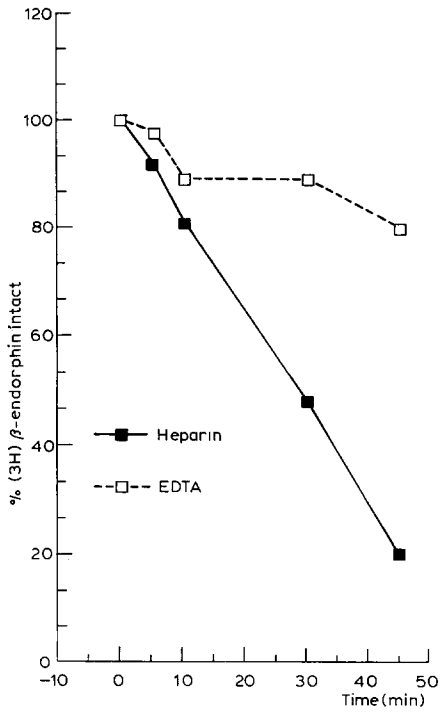

Fig. 4. Comparison of EDTA and heparin anticoagulant on the degradation of [^{3}H]β-endorphin. Although the degradation proceeds faster in heparized blood, the degradation is accelerated in chronically stressed rats to approximately the same extent (2-fold).

4. Discussion

In summary, we have demonstrated that the degradation of [^{3}H]β-endorphin is accelerated in rats which had received chronic footshock treatment. In addition, one-third of the radiolabeled product co-migrated with γ-endorphin on HPLC. Although only one-third of the degradation product co-migrates with γ-endorphin following footshock stress, the amount of non-acetylated β-endorphin released from anterior lobe is approximately 133 fmol/ml which could yield 45 fmol/ml of γ-endorphin. Both γ- and β-endorphin have known opiate activity, but γ-endorphin is much less potent than β-endorphin at the opiate receptor. It does, however, have a different non-opioid set of behavioral effects (De Wied and Jolles, 1982; De Wied, 1978; Van Ree et al., 1978). Thus, this conversion of β-endorphin to γ-endorphin may act to both modulate the opioid effects of β-endorphin as well a encode a different message unique to γ-endorphin. The nature of the enzyme responsible for this conversion is unclear; however, given the lysosomal nature of cathepsin D, it is likely that this enzyme may be present in plasma.

The major metabolite (2/3 of the radioactivity on HPLC purification) remains unidentified. It is a fragment that is more hydrophobic than γ-endorphin, β-endorphin-(1-18), or β-endorphin-(1-21) (Burbach et al., 1981). It also is not the C terminal (β-endorphin-(18-31) by HPLC identification. Thus, at the current time we are unable to identify this fragment, and whether it represents the N or C terminal of β-endorphin. However, the demonstration of this fragment may suggest that there is cleavage of β-endorphin at a site other than the Leu^{17}-Phe^{18} bond. Thus another enzyme may be responsible for this cleavage in plasma. Whether one enzyme is responsible for the formation of both this major metabolite and γ-endorphin in plasma is also unclear. If these two metabolites are generated by two different enzymes (e.g. cathepsin D and another enzyme) then

both enzymatic activities are induced with chronic stress, and the ratio of γ-endorphin to the major metabolite remains constant across time points, suggesting a degree of co-regulation. Alternatively, one enzyme with differential preferences for these sites could be responsible for the formation of both metabolities.

The demonstrated degradation of β-endorphin to other products in the bloodstream suggests that 'steady state' plasma measures even following stress may not give a true picture of release but only reflect a balance between release and degradation. In addition, the fact that this process is induced during chronic stress resulting in a shorter half-life of circulating materials demonstrates that there are, even at this level, regulatory elements to prevent 'over secretion'.

This level of regulation represents only one of numerous alterations in the hypothalamo-pituitary adrenal axis following repeated stress. Using this same stress model system we have previously shown (1) a 2-fold increase in ACTH and β-endorphin anterior pituitary content in chronically stressed animals (Young and Akil, 1985), (2) an increase in POMC mRNA in these rats (Shiomi et al., 1986), (3) the preferential secretion of β-endorphin from anterior lobe in vivo with stress or in vitro in anterior pituitary cell suspensions (Young et al., 1986), and (4) increased releasability of β-endorphin to oCRF in pituitary cell suspensions from chronically stressed rats which are re-stressed prior to sacrifice (Young and Akil, 1985). Despite these alterations, the chronically stressed animals show plasma β-endorphin at the end of 30 min of stress identical to those of a naive rat similarly stressed. The current data suggest that accelerated breakdown of β-endorphin in vivo may account for our failure to detect increased β-endorphin release in these chronically stressed rats. The possible increased activity of a non-specific lysosomal enzyme such as cathepsin D in chronic stress suggests that ACTH may likewise be subject to a similar increase in the degradation in vivo. If the increased enzymatic activity results from 'γ-endorphin generating enzyme', this enzyme also shows high affinity for ACTH (Lebouille et al., 1987), and has been shown to be regulated in both anterior pituitary, intermediate pituitary and hypothalamus by glucocorticoids (Lebouille et al., 1988). Thus, such an induction of γ-endorphin generating activity with chronic stress may have importance for other hypothalamic pituitary adrenal axis hormones.

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