GAMMA-MELANOTROPIN RESPONSE TO OVINE CORTICOTROPIN RELEASING FACTOR IN NORMAL HUMANS

James H. Meador-Woodruff, Stanley J. Watson, Virginia Murphy-Weinberg, Sylvie Jegou, Hubert Vaudry, Nabil G. Seidah, Jean Rivier, Wylie Vale, Huda Akil

Mental Health Research Institute and Department of Psychiatry, University of Michigan, Ann Arbor, Michigan 48109-0720; Laboratoire d'Endocrinologie, Faculte des Sciences et Techniques, France; Institut De Recherches Cliniques De Montreal, Clinical Research Institute of Montreal, Montreal, Canada; Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA 92037. (Reprint Requests to JHM-W)

ABSTRACT

Plasma Y-melanotropin was measured by a Y3MSH-specific radioimmunoassay before and after a single bolus intravenous infusion of ovine corticotropin releasing factor (oCRF; 0.1 ug/kg) in seven normal men. A significant increase of Y3MSH was observed 15 minutes post-oCRF infusion, which paralleled a similar increase in plasma cortisol. Gel filtration chromatography revealed that the observed increase was attributable to elevations of 9K and 4K forms of Y3MSH immunoreactivity. Affinity chromatography demonstrated that the majority of Y3MSH immunoreactivity in human plasma is glycosylated. As the smaller forms of Y3MSH are felt to have endocrine activity at the adrenal cortex, these changes may be physiologically relevant.

INTRODUCTION

Y-melanotropin (Y3MSH) is a 27-residue peptide found within the N-terminal domain of proopiomelanocortin (POMC) containing the sequence His-Phe-Arg-Trp in common with α- and β-MSH (21). There are two major dibasic cleavage sites in the 16K N-terminal region of POMC, potentially generating the extreme N-terminal peptide, Y3MSH, and joining peptide, that segment between Y3MSH and adrenocorticotropic (ACTH). In theory, Y3MSH could thus be associated with joining peptide (POMC65-103, estimated molecular weight 8-10K, depending on the extent of glycosylation), the extreme N-terminal fragment of POMC (POMC1-77, estimated molecular weight 9-11K), or alone (POMC51-77, molecular weight 3-4K). In humans, an additional cleavage site is located within Y3MSH, potentially yielding a 12-residue peptide (Y2MSH), or an 11-residue, C-amidated peptide, Y1MSH (18). These theoretical products, however, have not been demonstrated in human anterior pituitary (33,35), and a larger peptide is assumed to be the circulating form in plasma (32,33).

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The extent of processing of Y-melanotropin in the plasma of physiologically normal humans has not been elucidated. Metapyrone has been shown to facilitate an elevation of Y-melanotropin in normal humans (15) which is attributable to an elevation of 16K-sized material (33), presumably the intact N-terminal domain of POMC. The existence of more processed, smaller forms of circulating Y-melanotropin, however, may be physiologically relevant. Y3MSH, for example, is able to bind to the adrenal cortex (25), and can potentiate ACTH-induced adrenal steroidogenesis (1,2,13,24). Additionally, Y2MSH has been demonstrated to have potent cardiovascular (8,9,17) and renal (19) effects, as well as the ability to induce behavioral changes in rats (23). No potential physiological effects, however, have been demonstrated of any of the possible larger forms of Y-melanotropin.

The release of Y-MSH from the anterior pituitary into plasma is probably mediated by corticotropin releasing factor (CRF). Using an antibody specific for the N-terminal domain of POMC (POMC1-76), Chan et al. (10) demonstrated that this larger form of Y-melanotropin, ACTH, and B-endorphin were released in equimolar amounts following ovine CRF (oCRF) stimulation of cultured human anterior pituitary cells. Several reports (14,20) have since indicated that total plasma Y3MSH immunoreactivity (IR) increases in normal humans following oCRF infusion, but the specific forms released (i.e., Y3MSH, the entire 16K N-terminal domain of POMC, or intermediate-sized products) were not determined. As CRF is likely a physiological mediator of Y3MSH release, and the extent of processing of circulating Y3MSH-IR may reflect relevant endocrine activity, we undertook the present investigation to explore the effect of oCRF infusion on the pituitary release into plasma of the various possible forms of Y3MSH-IR in normal human males.

METHODS

Subjects. Seven male volunteers (age 20-30) were studied. Each subject had an unremarkable medical history as well as normal physical and psychiatric examinations. Each had normal laboratory tests including an electrocardiogram, serum electrolytes and glucose, liver, renal, and thyroid function tests, complete blood count and a urine drug screen. Written informed consent was obtained from each subject for participation in this study.

Preparation of CRF. Synthetic ovine CRF (synthesized and characterized by J.R. and W.V.) was prepared for injection by the University of Michigan Medical Center Pharmacy. Ten to 12 mg of material was dissolved in 10 ml of a vehicle solution of 5% human albumin, 0.9% benzyl alcohol, 0.001 N HCl, 0.1% lactose and 0.01% ascorbic acid, and was sterilized by filtration. This material was then diluted ten-fold in 0.9% NaCl containing 0.001 N HCl. This stock material was diluted another ten-fold with the original vehicle above, yielding a theoretical concentration of 10 ug/ml. This solution was stored in 1 ml aliquots in sterile amber glass bottles at -20°C until use. Random vials were assayed for both oCRF immunoreactivity and biological activity in rat anterior pituitary cell cultures (36). For injection, vials were thawed 10 minutes prior to infusion; all transfer syringes and needles were coated with vehicle solution, and the oCRF was diluted in this vehicle to a final concentration equivalent to 0.1 ug/kg body weight for each subject (total volume injected 0.2-0.5 ml).
Infusion paradigm. Subjects presented to the Clinical Research Center of the University of Michigan at 12:30 p.m., after having their usual lunch. Intravenous catheters were inserted in the median antecubital veins of both arms. One catheter was fitted with a heparin lock for withdrawal of blood samples; the other catheter was maintained open with a slow drip of isotonic intravenous fluid for injection purposes. At 1:30 p.m., a control injection of 0.9% saline was given; at 2:00 p.m., a vehicle-control injection was given. At 2:20 p.m., 175-200 ml of blood was withdrawn for plasma \( \gamma \text{MSH} \) determination. At 2:30 p.m., 0.1 ug/kg oCRF was injected (over 10-15 seconds), followed by a 1 ml flush with vehicle. We have previously found that the maximum response to oCRF of plasma \( \beta \)-endorphin and \( \gamma \text{MSH} \) levels occurs between 10 and 15 minutes post-oCRF infusion (unpublished observation). Accordingly, at 2:45 p.m. a second 175-200 ml blood sample was obtained for \( \gamma \text{MSH} \) quantitation. In addition, plasma was obtained for cortisol determination 5 minutes before and 15 minutes after the saline and vehicle injections as well as 5 minutes before and at 5, 10, 15, 30, 60 and 120 minutes following the oCRF infusion. Subjects were kept supine throughout the procedure, and had blood pressure and pulse monitored every 15 minutes. No subject reported any subjective experience from the infusion of oCRF; the experiment was terminated at 4:30 p.m. This paradigm is summarized in the lower portion of Figure 1.

Sample Preparation. Blood samples were obtained via the previously described intravenous cannulae. Samples for \( \gamma \text{MSH} \) determination were collected into chilled EDTA-containing vacutainers and were immediately centrifuged at 4°C; the resulting plasma was acidified to pH 2 with 1N HCl, and immediately stored at -70°C. For extraction, the plasma samples were pooled as either pre- or post-oCRF infusion for each subject. The pooled plasma samples were then extracted with Sep-Pak C\(_{18} \) cartridges as has been previously described for extracting \( \beta \)-endorphin from human plasma (7). We consistently achieve 85-90% recovery of \( \gamma \text{MSH} \) using this procedure. The extracts were then lyophilized and the dried material stored at -70°C until further processing.

Blood samples for corticosteroid determination were collected into chilled heparin-containing vacutainers. These samples were immediately centrifuged at 4°C, the plasma removed and stored at -70°C until assayed.

Gel chromatography. Extracted plasma samples prepared for \( \gamma \text{MSH} \) determination (equivalent to 80-100 ml of plasma) were resuspended in 400 ul 0.1% formic acid and applied to a Sephadex G-50 column (0.9 X 90 cm). The column was developed in 0.1% formic acid, and 2 ml fractions collected. Each fraction was dried by lyophilization, and resuspended in methanol/0.1 N HCl (50/50) for \( \gamma \text{MSH} \) quantitation by radioimmunoassay. Recovery of \( \gamma \text{MSH} \)-IR from this column was 90%.

Assays. \( \gamma \text{MSH} \) concentrations were determined by a specific radioimmunoassay that we have developed. The antibody is directed against a midportion region of \( \gamma \text{MSH} \) \((\text{His}^5-\text{Arg}^{14})\), and was raised in rabbits against synthetic bovine \( \gamma \text{MSH} \) conjugated to bovine serum albumin (34). The assay was performed in a 50 mM phosphate buffer, pH 7.6, containing 0.12M NaCl, 0.004M EDTA, 0.016% NaN\(_3\), 1.6% rabbit serum, and 0.1% polylysine. The standards (synthetic human Lys-\( \gamma \text{MSH} \)) and samples were added in 50 ul methanol/0.1 N HCl (50/50). The antiserum was added in 100 ul of buffer, at a final dilution of 1:25,000. The radioligand was human \([^{125}\text{I}]\)-Lys-\( \gamma \text{MSH} \), which was iodinated by the
chloramine-T method [16]; 20,000 counts in 100 ul of buffer were added per tube. The assay was run in a final volume of 250 ul under disequilibrium conditions for 48-72 hrs at 4°C. Separation was by immunoprecipitation by addition of 15 ul of sheep-anti-rabbit IgG per tube. This assay has a sensitivity of < 1 fmoles/tube, an IC20 of 2 fmoles/tube, and an IC50 of 32 fmoles/tube under disequilibrium conditions. Samples containing γ3MSH-IR in this study typically were read in the portion of the standard curve resulting in 20-30% inhibition. The antiserum has less than 1% cross-reactivity with γ1MSH, Lys-γ1MSH, γ2MSH, B-endorphin, ACTH, α-MSH, β-MSH, β-endorphin, and met-enkephalin. The intrassay coefficient of variation is 4.9%, and the interassay coefficient of variation is 10.8%.

Total plasma corticosteroids were quantitated by the competitive protein binding technique of Pegg and Keane (26) as modified by de la Peña and Goldzieher (11). The sensitivity of this assay is 0.3 ug/dl. The intra- and inter-assay coefficients of variation are 2.0% and 7.1%, respectively.

Concanavalin A Affinity Chromatography. The pre- and post-oCRF infusion plasma samples from subjects 6 and 7 were subjected to gel filtration chromatography as described above, and those fractions corresponding to 4 + 1K and 9 + 1K, as well as the fractions constituting the void volume of the column (> 13K) for each subject and for each condition (pre- and post-oCRF) were pooled. These samples were dried by lyophilization. To determine if the γ3MSH IR in these peaks existed in a glycosylated form, each peak was subjected to Concanavalin A (Con A) - Sepharose 4B chromatography, as described by Shibasaki et al. (30). Briefly, samples were resuspended in 500 ul of buffer and applied to a 500 ul Con A column that had been equilibrated with buffer. Glycosylated peptides were eluted by increasing concentrations of o-methyl-D-mannopyranoside and finally 0.1M acetic acid. 1 ml fractions from these columns were collected, dried, and assayed for γ3MSH by the previously described RIA.

Statistics. Comparisons between pre- and post-oCRF infusion levels of IR-γ3MSH and cortisol were by paired t-tests. Results are means ± S.E.M. unless stated otherwise.

RESULTS

We desired to demonstrate that any effect we might find on plasma γ3MSH levels in this study was specific to the oCRF infusion, as opposed to any non-specific stress effects. The average response of plasma cortisol to oCRF is shown in Figure 1. The mean baseline cortisol level remained unperturbed by the control injections, but showed a rapid increase within 10 minutes of the oCRF injection, returning to baseline levels after several hours. These data suggest that the oCRF is physiologically active and able to reach the pituitary and facilitate the release of POMC-derived peptides (i.e., ACTH, as indirectly observed by the increase in plasma cortisol), and that the endocrine effect seen is specific to the oCRF injection, and not a stress response to injections per se (as indicated by unchanging baseline cortisol levels pre- and post-control injections).

The γ3MSH-IR from a typical subject is shown in Figure 2. Immunoreactivity both pre- and post-oCRF infusion was found in fractions of the void volume of the Sephadex G-50 column (molecular weight > 13K), in
fractions of 8-10K (fractions #31-35), and in a collection of smaller peaks of 3.1-5.0K (fractions #41-55). In several subjects (including the one shown in Figure 2), several fractions with molecular weights less than 3.1K contained Y\textsubscript{3}MSH-IR. The immunoreactivity in these fractions presumably is due to smaller forms of Y\textsubscript{3}MSH, although not to the predicted cleavage products Y\textsubscript{2}MSH or Y\textsubscript{1}MSH, as the antiserum does not recognize these specific peptides. The total plasma Y\textsubscript{3}MSH-IR before and after oCRF infusion for each subject is summarized in Figure 3. Each subject manifested an increased plasma level of total Y\textsubscript{3}MSH-IR after injection of oCRF, ranging from a 1.3 to 16.5 fold elevation. The mean group change from pre- to post- infusion was from 0.26 ± 0.09 fmoles/ml to 0.53 ± 0.11 fmoles/ml, a 2.0 fold increase (t = -3.146, p < 0.01). Similarly, the mean group plasma cortisol levels measured at the same two time points increased 2.4 fold, from 6.14 ± 1.21 ug/dl to 14.98 ± 1.83 ug/dl (t = -8.393, p < 0.0005).

To determine which forms of Y\textsubscript{3}MSH-IR contributed to the observed 2-fold increase of total Y\textsubscript{3}MSH-IR, the amount of immunoreactivity found in the 3-5K ("4K"), 8-10K ("9K"), and void volume (>13K) fractions both before and after

![Figure 1](image_url)  

**Figure 1.** Plasma cortisol levels in seven subjects at various times before and after control and oCRF injections. Data are means ± S.E.M. The lower half of the figure summarizes the times of the injections and sample collections.
Figure 2. Representative Sephadex G-50 gel filtration profiles of \( \gamma_3 \text{MSH-IR} \) in extracted plasma samples both pre- and post-oCRF infusion (Subject 3). Molecular weights of peptide standards are indicated (12.4K, cytochrome C; 4.5K, \([\text{I}^{125}\text{]}\text{ACTH} \); 3.1K, \([\text{I}^{125}\text{]}\gamma_3\text{MSH})

oCRF infusion was determined from the gel filtration elution profiles (Figure 2) for each subject. These data are shown in Figure 4. The total increase of \( \gamma_3 \)MSH-IR was attributable to elevations of both the 9K fractions, which rose from a mean level of 0.79 fmoles/ml to 2.08 fmoles/ml \((t = -1.609, p < 0.1)\), and the 4K fractions, which increased from 0.10 to 0.19 fmoles/ml \((t = -2.515, p < 0.025)\). There was no increase observed for the immunoreactivity found in the void fractions.

Because \( \gamma_3 \)MSH has been reported to exist in a glycosylated form in bovine anterior pituitary as determined by Con A affinity chromatography [30], we attempted to determine if human plasma \( \gamma_3 \)MSH-IR was glycosylated in two of these subjects. The 4K, 9K and void fractions, both pre- and post-oCRF infusion were found to be glycosylated. Between 80 and 100\% of the \( \gamma_3 \)MSH-IR in the 4K fractions was retained by Con A columns; 89-100\% of the 9K fractions and 69-100\% of the void volume fractions were similarly retained. A representative experiment is summarized in Figure 5. In a control experiment, less than 10\% of synthetic human \( \gamma_3 \)MSH was retained by a Con A column.

Figure 3. Plasma concentrations of total \( \gamma_3 \)MSH-IR and cortisol in seven subjects both pre- and 15 minutes post-oCRF infusion. The increase of mean plasma level (dotted lines) from pre- to post-oCRF infusion is significant for both \( \gamma_3 \)MSH (paired t-test, \( p < 0.01 \)) and cortisol (paired t-test, \( p < 0.0005 \)).
Figure 4. Concentration of Y3-MSH-IR in various peaks from gel filtration chromatography of plasma samples both pre- and post-oCRF infusion in seven subjects. A. 4 + 1K (fractions #41-55); B. 9 + 1K (fractions #31-35); C. fractions of the void volume of the column (fractions #22-29). The increase of mean plasma level (dotted lines) from pre- to post-oCRF infusion is significant for the 4K fractions (paired t-test, \( p < 0.025 \)), and approaches significance for the 9K fractions (paired t-test, \( p < 0.1 \)); there is no significant difference of the immunoreactivity found in the void fractions between the pre- and post-oCRF conditions.
DISCUSSION

Ovine CRF infusion in the present study resulted in a doubling of total \( \gamma_3 \text{MSH-IR} \) in normal men, in reasonable agreement with several previous studies. Motomatsu and co-workers (20) found up to a two-fold increase in circulating levels of immunoreactive N-terminal peptide (POMC1-76) after injection of 100 \( \mu \text{g} \) of oCRF in 4 subjects. Hale and co-workers (14), using a radioimmunoassay specific for \( \gamma_3 \text{MSH} \) demonstrated about a 1.5-fold increase in \( \gamma_3 \text{MSH-IR} \) following injection of 100 \( \mu \text{g} \) of oCRF in 6 subjects. Using a much smaller dose of oCRF (5-7 \( \mu \text{g} \)), we were able to observe increases of the same order as previous investigators employing much higher oCRF doses. We selected the 0.1 \( \mu \text{g/kg} \) dose of oCRF as we found in an earlier study (Watson, S.J., Lopez, J., Young, E.A., Vale, W., Rivier, J., Akil, H., submitted) that this results in plasma levels of oCRF in the normal physiological range. It may be that the pool of releasable \( \gamma_3 \text{MSH} \) is limited and that the system is maximally driven

![Diagram](image)

**Figure 5.** Representative elution profile of \( \gamma_3 \text{MSH-IR} \) in plasma from a Concanavalin A (Con A)-Sepharose 4B affinity column. Plasma samples were chromatographed on a Sephadex G-50 column as described in the text, and the 4K, 9K, and void volume peaks pooled. Each peak was then chromatographed on a Con A-Sepharose 4B affinity column. Glycosylated material was eluted with increasing concentrations of \( \alpha \)-methyl-D-mannopyranoside (\( \alpha \text{MM} \)) and 0.1M acetic acid (\( \text{AcOH} \)). This profile is for the 4K fractions of subject 6 following oCRF infusion.
after being exposed to this low-dose infusion of oCRF, so that the infusion of more oCRF, at least in the short run, is incapable of facilitating the release of more Y3MSH.

The forms of Y3MSH-IR released following oCRF stimulation in normal humans have not been elucidated (14,20). Using an antibody directed against the N-terminal peptide of POMC (POMC\(^{1-70}\)), Chan et al. (10) demonstrated the release of two forms of Y-MSH-IR, a B-lipotropin-sized (10-11K) and a larger form, from cultured human anterior pituitary cells following oCRF stimulation. The major forms of Y3MSH-IR detected in the present investigation were 4K and 9K forms, presumably representing native, glycosylated Y3MSH, and Y3MSH extended with the extreme N-terminal fragment of POMC (4,10,14,19,28), although extension of Y3MSH with joining peptide has been reported in porcine pituitary (6). One might argue that larger forms were present, but due to sample collection or processing methods, breakdown was allowed to occur and we measured these breakdown products instead of the native circulating forms. While this is a possibility it seems unlikely, as this same procedure preserves intact the multiple molecular species of ACTH and B-endorphin. Inspection of Figure 1 indicates several peaks in the 3-5K range. The column system utilized for separation in this study is one we have previously developed for resolving different forms of B-endorphin; the observed peaks actually differ from each other only by a few hundred daltons. The reason for this heterogeneity is not apparent, but probably reflects variable degrees of glycosylation of intact Y3MSH, or some other form of post-translational modification.

While smaller forms of Y3MSH-IR exist in human plasma under normal physiological conditions, it may be that the processing of the N-terminal domain of POMC is altered in various pathological states. A number of investigators have determined the sizes of Y3MSH-IR under other conditions. Nakao et al. (22) demonstrated a large form (B-lipotropin-sized, 10-11K) in plasma from patients with Addison's and Nelson's syndromes. Another report (5) showed a 16K form in Nelson's syndrome patients' plasma, and both a 16K and a smaller form in tumor extract and plasma of a patient with ectopic ACTH syndrome. Tanaka et al. (33) showed an increase in 16K-sized Y3MSH-IR following metyrapone in normal males. In general, the observed forms of Y3MSH-IR in these non-physiological states tends to be large. Of interest, however, is the report of Watsman et al. (35), which demonstrated elevations of 4K, 6K and 11K forms of Y3MSH-IR following "maximal stress" (cardiac arrest). While cardiac arrest cannot be considered a "normal" stressor, it would cause maximal driving of physiological regulatory mechanisms; it is of note that these authors found smaller forms of Y3MSH-IR under these circumstances. The release of smaller forms of Y3MSH-IR following oCRF stimulation has not been previously reported; the results of the present study suggest that this physiological perturbation results in the release of processed forms of Y3MSH.

The release of smaller, processed forms of Y3MSH-IR under physiological conditions may be endocrinologically relevant. The smallest form of Y3MSH-IR (Y3MSH, 3-4K) has been shown to have the ability to bind to the adrenal cortex (25) and enhance ACTH-induced steroidogenesis (1,2,13,24). No physiological action has been demonstrated for the larger forms of Y3MSH-IR. It is interesting to note that the vast majority of circulating Y3MSH-IR at both rest and post-CRF stimulation is of 4K and 9K sizes. On the other hand, there
have been several communications (12,27,29,31) indicating that human pituitary contains predominately a larger form (i.e., B-lipotropin-sized) of \( \gamma_3 \text{MSH-IR} \), similar to reports of bovine pituitary (3,32). Young et al. (37), however, have recently demonstrated in the rat that although B-lipotropin is the primary product stored in the anterior lobe, CRF promotes the preferential release of B-endorphin into plasma. Thus, the findings of the current study are not altogether surprising; it may be that in the human, the POMC products B-endorphin and \( \gamma_3 \text{MSH} \) are stored in the pituitary as larger peptides, but the smaller, possibly more biologically active forms may be preferentially released.

We also determined that the circulating forms of \( \gamma_3 \text{MSH-IR} \) in human plasma are to a large extent glycosylated. The determination of glycosylation of \( \gamma_3 \text{MSH} \) has not been previously reported in human plasma, but is in agreement with the finding of glycosylated forms of \( \gamma_3 \text{MSH-IR} \) in bovine anterior and intermediate pituitary (30) and in human pituitary (4). The significance of this post-translational modification is not known, but may facilitate the biochemical activity of this peptide, or may protect it from intracellular degradation (30).

The function of \( \gamma_3 \text{MSH} \) is not known. That circulating levels of processed, possibly physiologically active forms of this peptide are significantly elevated following oCRF infusion suggests that \( \gamma_3 \text{MSH} \) serves a role in modulating the neuroendocrine responses of the hypothalamic-pituitary-adrenal axis.

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