Endorphin and MSH in Concert Form the Corticotropic Principle Released by Tilapia
(Oreochromis mossambicus; Teleostei) Melanotropes

P. H. M. BALM,1 M. L. M. HOVENS AND S. E. WENDELAAR BONGA

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

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BALM, P. H. M., M. L. M. HOVENS AND S. E. WENDELAAR BONGA. Endorphin and MSH in concert form the corticotropic principle released by tilapia (Oreochromis mossambicus; Teleostei) melanotropes. PEPTIDES 16(3) 463-469, 1995. — HPLC characterization of tilapia pituitary endorphins using an antibody specific for N-terminally acetylated endorphins yielded three major peaks in the neurointermediate lobe, but none in the pars distalis. The melanotropes secreted two of the immunoreactive products in vitro, one of which coeluted with Xenopus laevis N-ac-β-END(1-8). This immunoreactive fraction also coeluted with diacetyl-α-MSH. Evidence is presented that the noteworthy corticotropic potency of this HPLC fraction, previously attributed to diacetyl-α-MSH, results from END and MSH acting in a coordinated fashion. Confinement stress had no effect on plasma N-ac-β-END immunoreactivity, but led to a decrease in plasma α-MSH levels. Therefore, it seems unlikely that the corticotropic action of the peptides regulates the elevation of cortisol production that takes place during confinement, but it may play a role during other forms of stress that are known to activate the melanotropes.

β-Endorphin   α-MSH   Melanotropes   Tilapia (Oreochromis mossambicus)   Pituitary–interrenal axis   Stress

VARIOUS peptides derived from the multifunctional precursor protein proopiomelanocortin (POMC) are involved in the adaptation to potentially stressful conditions (6,24). Adrenal corticosteroidogenesis is one of the key targets in this respect. ACTH secreted by the pars distalis corticotropes has by far been the most studied corticotropic factor in all vertebrate classes (6,18). However, evidence is accumulating that other POMC peptides may be important for tuning corticosteroid production (18). Among these, N-terminal peptide (21,30), β-LPH (20), various forms of MSH (9,16,34), and β-endorphin (8) have been demonstrated to have direct corticotropic actions, or to modulate the action of ACTH.

The euryhaline teleost Oreochromis mossambicus (tilapia) readily acclimates to environmental changes, a capacity that is accompanied by an efficient regulation of the production of cortisol, the major corticosteroid in this (3) and other teleost species. We recently described the role of ACTH in the regulation of the cortisol-producing interrenal cells, located in the headkidney (6). In this species also, evidence for additional regulatory factors derived from POMC has been presented (2,16). In the latter study, the corticotropic function of the pars intermedia melanotropes was attributed to diacetylated α-MSH, because the HPLC fraction of NIL tissue containing this peptide potently stimulated in vitro cortisol production. Because the potency of this preparation could not completely account for the corticotropic action of the complete NIL secretory signal, the presence of additional corticosteroid secretagogues was postulated. In vitro synergistic actions of MSH and endorphin in the stimulation of adrenal corticosteroidogenesis have been documented in mammals (28). Furthermore, END was also demonstrated to potentiate the ACTH response of interrenal cells in Xenopus laevis (13). We were therefore prompted to investigate the role of endorphin in the regulation of the pituitary–interrenal axis in tilapia. To this end, tilapia N-ac-β-endorphin-like immunoreactivity was characterized using HPLC and RIA. Products purified by HPLC were subsequently tested in an in vitro bioassay. Finally, the effects of confinement stress on plasma α-MSH and N-ac-β-ENDOR were measured to assess whether these peptides could be implicated in the activation of the interrenal cortisol output under these conditions (22).

METHOD

Animals

Female tilapia (Oreochromis mossambicus), weighing 27 ± 5 g (mean ± SEM; n = 103) were obtained from our laboratory.

1 Requests for reprints should be addressed to P. H. M. Balm.
stock. They were kept in artificial freshwater under a 12D/12L regime. To assess the effects of stress, animals were confined in groups of four in keepnets for 3 h. Three conditions were tested: four groups were confined for 3 h in their home aquarium (A; n = 16); four groups consisting of animals from the same aquarium were transported to, and confined (3 h) in, novel aquaria (B; n = 16); and four groups, each consisting of two pairs from different aquaria, were transported to, and confined (3 h) in, novel aquaria (C; n = 16). Transport between the aquaria took 5 min. There were no significant differences between groups within one treatment; therefore, values were pooled to yield n = 16 for A, B, and C. Controls were captured first from the aquaria, and were not confined (n = 8). All animals in this experiment were of the same age; there were no significant differences in body weight between the experimental groups. Blood was collected as described previously (5). Plasma was aliquotted and stored at −20°C until further analysis. Neurointermediate lobes and headkidneys were superfused in vitro as described by (4). Homogenates from freshly dissected pituitaries were prepared and subjected to HPLC as we have reported previously (6). Human acetylated endorphins (1–31), (1–27), (1–16) (Sigma, St. Louis, MO), and synthetic Xenopus laevis N-acetylated endorphin (1–8) (31) were used as markers.

**Assays**

N-ac-β-Endorphin and α-MSH were measured in HPLC fractions and plasma samples using an RIA system previously employed to measure tilapia ACTH (6). The endorphin antiserum is specific for the acetylated N-terminal of chum salmon β-endorphin (29) and has full cross-reactivity with acetylated forms of mammalian β-endorphin (10). The antiserum was used at a final dilution of 1:300,000. Sample volume was 25 μl. As standard, synthetic Xenopus laevis N-acetylated β-endorphin (1–8) was used. Dilutions of rainbow trout (Oncorhynchus mykiss) plasma were compared with the N-ac-β-END (1–8) standard curve. The α-MSH antibody used has been described previously (33). It was raised against α-MSH, but fully recognizes des-acetylated- and diacetylated α-MSH. The detection limit in our hands was 0.63 pg/tube. Plasma cortisol and ACTH were assayed following Balm et al. (6). Plasma glucose was measured with a commercial kit (Boehringer, Mannheim, Germany).

**Immunoprecipitation**

A freeze-dried NIL homogenate, dissolved in 200 μl RIA buffer (6) from which Triton X-100 was omitted, was divided in two equal aliquots. To one aliquot, 100 μl END antiserum (1:60 dilution in RIA buffer without Triton X-100) was added, and after incubation for 24 h at 4°C, immunoprecipitation was performed using a second antibody as described previously (6); the conditions for optimal immunoprecipitation — first and second antiserum concentrations — were previously determined by RIA. The remaining aliquot was incubated in the same volume without antibody (control). The two samples were subjected to HPLC, and in small portions of the fractions N-ac-β-END immunoreactivities was measured to confirm immunoprecipitation. In addition, α-MSH immunoreactivity was determined in these fractions. Subsequently, the peak HPLC fractions containing both diacetyl-α-MSH and N-ac-β-END (control) and diacetyl-α-MSH only (immunoprecipitated NIL aliquot) were dried (Speed Vac) and dissolved in incubation medium to yield 1 nM diacetyl-α-MSH in both cases. The control preparation in addition contained 750 pg/ml N-ac-β-END immunoreactivity. These solutions were administered to headkidneys from control animals incubated in vitro (n = 8 for both treatments).

**Statistics**

Results are presented as means ± SEM (n = 1). The Mann–Whitney U-test was used to assess whether results were significantly different; p < 0.05 was accepted as a statistically significant level of difference. Parameters were correlated using the method for least-squares linear regression, and the significance of the correlation coefficient (r) was assessed.
RESULTS

The radioimmunoassay appeared sensitive enough to detect endorphin-like immunoreactivity in tilapia plasma (Fig. 1). The detection limit of the assay was 2.5 pg/tube. Dilutions of pooled tilapia plasma and the standard displaced the label from the antibody in a parallel fashion. The same held for dilutions of a tilapia pituitary extract and a plasma pool from rainbow trout. On the basis of these curves, tilapia plasma appeared to contain much lower levels of endorphin than trout. No cross-reactivity with α-MSH was detected. Reversed-phase HPLC profiles of N-ac-β-END immunoreactivity displayed no peaks in the tilapia pars distalis homogenate, but multiple products in the NIL extract (Fig. 2). Two products coeluted with N-ac-β-END standards [(1–31), (1–27)], whereas the product eluting first from the column coeluted with the N-ac-β-END(1–8) standard. α-MSH immunograms from the same samples demonstrated three main peaks in the NIL, designated des-acetylated-, monoacetylated-, and diacetylated α-MSH, respectively (2, 16). Diacetylated α-MSH coeluted with the most apolar endorphin. The HPLC profile of N-ac-β-END immunoreactivity in a NIL superfusate (Fig. 3) demonstrated that the melanotropes secreted two major endorphin-immunoreactive products. Both peaks were also present in the NIL homogenate: the more apolar one coeluted with the N-ac-β-END(1–8) standard and with diacetyl-α-MSH (15.7 min).

Fine arrows indicate the elution position of acetylated human endorphin peptides [N-acetylated Xenopus laevis endorphin(1–8) and monoacetylated α-MSH, respectively].

FIG. 2. HPLC profiles of α-MSH and N-ac-β-END immunoreactivities (IR) in tilapia pars distalis (PD) and neurointermediate lobe (NIL) homogenates; 0.5-min fractions were collected between 10 and 45 min. Fine arrows indicate the elution position of the RIA standards [N-acetylated Xenopus laevis endorphin(1–8) and monoacetylated α-MSH, respectively].

DISCUSSION

The evidence presented supports the idea that products from the melanotropes are potent regulators of corticosteroidogenesis in fish, although this principle may not be operative under all conditions requiring increased cortisol production. The data for the first time implicate a NIL N-terminally acetylated β-endorphin-like immunoreactivity in tilapia plasma (Fig. 1). The radioimmunoassay appeared sensitive enough to detect endorphin-like immunoreactivity in tilapia plasma (Fig. 1). The detection limit of the assay was 2.5 pg/tube. Dilutions of pooled tilapia plasma and the standard displaced the label from the antibody in a parallel fashion. The same held for dilutions of a tilapia pituitary extract and a plasma pool from rainbow trout. On the basis of these curves, tilapia plasma appeared to contain much lower levels of endorphin than trout. No cross-reactivity with α-MSH was detected. Reversed-phase HPLC profiles of N-ac-β-END immunoreactivity displayed no peaks in the tilapia pars distalis homogenate, but multiple products in the NIL extract (Fig. 2). Two products coeluted with N-ac-β-END standards [(1–31), (1–27)], whereas the product eluting first from the column coeluted with the N-ac-β-END(1–8) standard. α-MSH immunograms from the same samples demonstrated three main peaks in the NIL, designated des-acetylated-, monoacetylated-, and diacetylated α-MSH, respectively (2,16). Diacetylated α-MSH coeluted with the most apolar endorphin. The HPLC profile of N-ac-β-END immunoreactivity in a NIL superfusate (Fig. 3) demonstrated that the melanotropes secreted two major endorphin-immunoreactive products. Both peaks were also present in the NIL homogenate: the more apolar one coeluted with the N-ac-β-END(1–8) standard and with diacetyl-α-MSH (15.7 min). In contrast, the prominent endorphin-like product in the melano­

notrope coeluting with the N-ac-β-END(1–31) standard was a minor feature in the profile of the superfusate.

Three groups of headkidneys were challenged in vitro with either diacetylated α-MSH standard (1 nM), a pulse with the tilapia NIL HPLC fraction containing diacetyl-α-MSH (1 nM) and N-ac-β-END, or 1 nM hACTH(1–39) standard (Fig. 4). The headkidneys barely reacted to the diacetylated α-MSH standard. However, the combined tilapia diacetyl-α-MSH/N-ac-β-END pulse gave a prominent reaction, which was 38% of the response to ACTH (areas under the curve; p = 0.021). The presence of β-END appeared to be essential for the notable corticopic potency of the tilapia preparation, as demonstrated by immunoprecipitation (Fig. 5). The difference between the reactions to 1 nM diacetyl-α-MSH in the presence [Fig. 5(a)] and absence [Fig. 5(b)] of 750 pg/ml β-END immunoreactivity was highly significant (p = 0.005; areas under the curve). The effect of the pulse without β-END [Fig. 5(b)] was furthermore comparable with the response to an identical pulse with the diacetyl-α-MSH standard (Fig. 4, left panel).

The three types of stress treatment affected plasma α-MSH and N-ac-β-END levels differentially (Fig. 6). Confining fish in their home aquaria had no effect on both immunoreactivities (condition A), whereas confinement in a novel environment (conditions B and C) resulted in lower MSH levels. The specificity is also reflected in the ratio between N-ac-β-END and α-MSH immunoreactivities 1.27 ± 0.25 (controls); 1.44 ± 0.28 (A); 2.78 ± 0.48 (B, p < 0.05 from controls); 3.01 ± 0.44 (C, p < 0.01 from controls). Overall, plasma α-MSH and N-ac-β-END were positively correlated for individual fish (r = 0.70, p < 0.001; n = 50). The correlation coefficient was highest in the controls (r = 0.85, p < 0.01; n = 8), and in the animals confined in a novel environment with conspecifics not encountered previously (C; r = 0.85, p < 0.001; n = 14). There were no differences in plasma cortisol, ACTH, and glucose levels between the three confined groups; of these parameters only cortisol and glucose were elevated over controls (not shown).
phim in the regulation of the teleost interrenal. Previously, it was demonstrated that stress in rats specifically induces the release of N-ac-β-END from the intermediate lobe (1), although a function was not reported. N-terminally acetylated endorphins from the neurointermediate lobe, although nonopioid, may be more versatile messengers than previously recognized. This contention is

![Graph showing cortisol release](image)

**FIG. 4.** Stimulation of in vitro cortisol production. Di-ac-α-MSH (Sigma) was purified by HPLC prior to use; hACTH(1–39) was from Peninsula. Both standards were tested at 1 nM. The tilapia NIL HPLC fraction was dried and reconstituted in medium to also yield 1 nM diacetyl-α-MSH; the N-ac-β-endorphin concentration in this pulse was 500 pg/ml (middle panel). \( n = 6 \) for each panel.

![Graph showing cortisol release](image)

**FIG. 5.** The effect of removing N-ac-β-END on the corticotropic potency of the HPLC preparation containing di-ac-α-MSH. Two groups of headkidneys \( n = 8 \) each) were either challenged with HPLC-purified products from a complete NIL homogenate (a; control), or with a corresponding HPLC fraction from a NIL homogenate from which N-ac-β-END IR had been removed by immunoprecipitation (b). Both pulses contained 1 nM di-ac-α-MSH; the control pulse in addition contained 750 pg/ml N-ac-β-END. The small panels depict the elution positions (in min) of N-ac-β-END and α-MSH IR (in pg/fraction) in the HPLC profiles. To optimize resolution, in this experiment 0.3-min fractions were collected. Arrows indicate the fractions that were dried and administered to the headkidneys over a 30-min period. Significant differences for individual fractions between the two treatments are indicated.
supported by recent work demonstrating specific effects of N-acetylated β-END on prolactin cell recruitment in rats (12).

The data on endorphin-like material in tilapia pituitary homogenates and NIL superfusate comply with results published previously on endorphin synthesis and processing in fish (11,15). We used a heterologous RIA, but conclude that the assay detected tilapia N-acetylated endorphins on the basis of the parallism between the displacement curves obtained with the RIA standard, the tilapia pituitary homogenate, and the tilapia and trout plasma pools. This antibody has previously been used to measure salmonid N-acetylated endorphins in plasma and pituitary homogenates (24,29). Using this antibody, we observed a characteristic distribution of immunoreactivity (11,27) between PD and NIL, which demonstrates that N-acetylation of endorphins in tilapia takes place in the melanotropes exclusively. Overall, the HPLC results confirm the idea (15) that the synthesis and processing of endorphin in teleosts resemble that in mammals (35). A prominent intracellular product coeluted with N-ac-END(1–31), which probably served as a precursor for the other products. On the basis of the elution positions of the standards used, we tentatively identified one of these as the tilapia equivalent of N-ac-β-END(1–26), and a C-terminally truncated N-ac-β-END of 8–10 amino acids. Recently, splicing of endorphin at a single arginine residue, located at a position between aa 9 and 13, has been described for several vertebrate species (10,17,31), and it has been postulated that this occurs in all lower vertebrates (17). The major intracellular product may represent tilapia N-ac-β-END(1–26), a suggestion supported by the HPLC elution profile of Xenopus laevis NIL β-endorphins (31). In the rat also, N-acetylated β-endorphin(1–26), (1–27), and (1–31) are the major intracellular endorphins in the NIL (35). It is unlikely that the novel endorphin-like molecules recently postulated to occur in rainbow trout (25) would be detected by the antibody presently used. The HPLC profile obtained with the superfusate suggests that tilapia melanotropes release two major endorphins, N-ac-β-END(1–26) and a smaller C-truncated N-ac-β-endorphin.

In particular, this latter product was of interest because it coeluted with diacetyl-α-MSH. It has previously been demonstrated that this HPLC fraction possesses a remarkable corticotropic potency (2,16). The data presented here confirm these results, but suggest that this potency results from the combined action of diacetyl-α-MSH and the tilapia endorphin identified in this study. In our bioassay we were unable to stimulate cortisol production with Xenopus N-ac-β-END(1–8) (not shown). However, until the tilapia endorphin coeluting with this peptide has been sequenced and tested, we cannot exclude that the smaller tilapia endorphin has some intrinsic corticotropic potency. Research into the capacity of β-endorphin to regulate adrenal corticosteroidogenesis in vertebrates has led to conflicting results (8,23). Because removal of either β-END (this study) or α-MSH (16) from the preparations tested completely (16) or to a large extent (this study) reduces the corticotropic effects, we ascribe the notable potency of the tilapia HPLC preparation to a concerted action of the MSH and END forms. Because removal of END by immunoprecipitation reduced the corticotropic potency of the tilapia HPLC preparation to that of synthetic diacetylated-α-MSH, we furthermore exclude the possibility that additional products, potentially coeluting with diacetyl-α-MSH and the β-END, are of major importance. We recently demonstrated that the two ACTH-immunoreactive products synthesized by tilapia (6) do not coelute with the two products of interest in the present study. At the diacetyl-α-MSH concentration tested (1 nM), the preparation appears more potent than reported previously (16). This likely follows from the different pulse durations; the 10-min period employed earlier (16) might have been too short to reach the nominal concentration in the incubation chambers. The low corticotropic potency of diacetyl-α-MSH in tilapia reported here implies that in this respect tilapia can be compared with the rat, in which the corticotropic potency of diacetyl-α-MSH does not exceed that of monoaexyl-α-MSH (9). Interestingly, also in the rat, β-END and α-MSH have combined effects on in vitro corticosteroidogenesis (28). The physiological relevance of the MSH/END action was addressed by investigating the effect of stress on plasma α-MSH and N-ac-β-END levels. The correlation observed between the two immunoreactive fractions in the plasma of the controls confirms results observed on salmonids (27). In contrast with the situation in salmonids, however, tilapia plasma contained similar amounts of α-MSH and N-ac-β-END. The remarkable END/MSH molar ratio of 22 measured in rainbow trout plasma (24) therefore might be specific for this latter species. The different effects of various confinement protocols observed in this study emphasize the importance of factors such as the intensity of the stress and social interactions in the response of tilapia melanotropes to stressors, in accordance with our previous results on corticotropic function during stress in this species (6). We employed different stress treatments, because in fish the type and the intensity of the challenge is an important variable determining the influence of stress on the melanotropes (19,27). The fact that α-MSH and β-END remained strongly correlated concurrently with a selective effect on plasma α-MSH might indicate differential effects of stress on the half-lives of the hormones. Alternatively, the data could indicate the ability of tilapia melanotropes to modulate their secretory profile, thereby lending support to the concept of multiple secretory pools in melanotropes, recently developed for another lower vertebrate (32).
In mammals, stressors generally lead to an increase in α-MSH output (1,7). It could be argued that the reduction in plasma α-MSH levels by confinement in tilapia was effected by the potent feedback exerted by cortisol on the melanotropes of this species (4), a mechanism undocumented until recently in higher vertebrates (26). However, because plasma cortisol levels did not differ between the three stressed groups, this is unlikely. Taken together, the in vivo results do not point to a major role of the melanotropes in the interrenal response of the interrenal to confinement in this species, as has also been suggested for rainbow trout (22). The possibility that the melanotropes regulate the interrenal cells under other conditions should not be ruled out, because our data further identify the corticotropin identity of the melanotrope signal. Previous research on higher (1,34) and lower (16,19) vertebrates has confirmed the potential role of the melanotropes in the stress response. Perhaps the key to understanding the relevance of these regulations lies in the appreciation of the influence of the type of challenge, and of the idea that factors other than α-MSH are likely of importance.

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