A role for di-acetyl α-melanocyte-stimulating hormone in the control of cortisol release in the teleost Oreochromis mossambicus

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Abstract

In stressed tilapia, Oreochromis mossambicus, total α-melanocyte-stimulating hormone (α-MSH) levels and di-acetyl α-MSH/mono-acetyl α-MSH (di:mono) ratios are elevated. We therefore investigated the role of α-MSH in the regulation of the pituitary-interrenal axis. The corticotrophic activities of des-acetyl α-MSH, mono-acetyl α-MSH and di-acetyl α-MSH were compared. These forms of α-MSH were isolated from neurointermediate lobes and tested in a superfusion experiment with homologous interrenal tissue. The corticotrophic activity of di-acetyl α-MSH was the highest, followed by that of des-acetyl α-MSH and mono-acetyl α-MSH. Apparently, acetylation of α-MSH is of functional significance for corticotrophic action. Di-acetyl α-MSH proved to be about 100 times less potent than ACTH(1–39): the half-maximal stimulating concentrations for ACTH and di-acetyl α-MSH were 0.89 nmol/l and 110 nmol/l respectively.

Surprisingly, a superfusate from neurointermediate lobes proved to be only about three times less active than a superfusate from the pituitary pars distalis, in which the corticotrophic activity is attributable to its ACTH content. When selectively stripped of all forms of α-MSH by passage through a Sepharose column coated with an antiserum against α-MSH, the neurointermediate lobe superfusate was devoid of corticotrophic activity. Thus α-MSH appears to be the corticotrophic factor in the superfusate of the neurointermediate lobe. After the same treatment, the corticotrophic activity of the pars distalis superfusate was not affected. We conclude that (in vivo) an as yet unidentified factor is co-released with α-MSH from the neurointermediate lobe, and that this potentiates its corticotrophic activity.


Introduction

Melanocyte-stimulating hormone (α-MSH) is a pro-opiomelanocortin (POMC)-derived peptide found in all vertebrates. The peptide was named MSH because of its best studied function, i.e. the regulation of melanin dispersion in melanocytes in the process of the background adaptation of amphibians (Bagnara & Hadley, 1973).

The role of α-MSH in background adaptation is equivocal in fish. In some species, melanocyte-concentrating hormone (MCH) and α-MSH in concert control the colour change of the skin (Green & Baker, 1989). However, in the molly, Poecilia latipinna, and the flounder, Pleuronectes flesus, the involvement of α-MSH in this process could not be demonstrated (Ball & Batten, 1981; Baker, Wilson & Bowley, 1984). Moreover, colour changes as an adaptation to background appear to be predominantly under neural control in fish (Green & Baker, 1989). A more common, although not consistent (Pickering, Pottinger & Sumpter, 1986), finding so far is the activation of fish α-MSH-producing cells during stress (Malo-Michele, 1980; Sumpter, Dye & Benfey, 1986). This finding is in line with recent results from higher vertebrates (Alexander, Irvine, Livesey & Donald, 1988; Carr, Saland, Samora et al. 1990; Lookingland, Gunnet & Moore, 1991).

In a previous report (Lamers, Balm, Haenen & Wendelaar Bonga, 1991) we showed that in tilapia the release of the three forms of α-MSH (des-, mono- and di-acetyl α-MSH) can be differently modulated in vitro by hypothalamic factors. When stimulated by thyrotrophin-releasing hormone (TRH), the release of di-acetyl α-MSH is relatively more enhanced than that of mono-acetyl α-MSH and this suggests a specific role for di-acetyl α-MSH. So far, to the best of our knowledge no reports have appeared on the
corticotrophic activity of di-acetyl α-MSH in lower vertebrates.
Proceeding from the observation that exposure to acid water as a stressor results in elevated plasma levels of α-MSH, and specifically of di-acetyl α-MSH (Lamers, Balm, Haenen & Wendelaar Bonga, 1990), we evaluated the potential role of the three forms of α-MSH in the pituitary-interrenal (PI) axis. To this end the corticotrophic activity of the three forms of α-MSH was determined and the potency of di-acetyl α-MSH was compared with that of adrenocorticotrophic hormone (ACTH(1-39)). In experiments in which headkidney was stimulated by products released by the neurointermediate lobe (NIL) or by the pituitary pars distalis (PD), we found an unexpectedly high stimulatory activity of the secretory products of the NIL. We conclude that the NIL co-secretes a potentiating factor for the corticotrophic action of α-MSH.

MATERIALS AND METHODS

Experimental animals
Mature female tilapia, Oreochromis mossambicus, were bred in the laboratory. The fish were kept in 120-litre tanks with tap water at 28 °C on a grey background. The photoperiod was 12 h of direct illumination alternated with 12 h of darkness. The body weight of the animals ranged from 75 to 125 g. The fish were fed a commercial dried fish food (Trouvit, Trouw, Putten, the Netherlands). Feeding was discontinued 24 h before the experiments. Immediately after capture the fish were killed by spinal transection.

Radioimmunoassay (RIA) for α-MSH
Concentrations of α-MSH were determined in duplicate in an RIA. The antisera was raised against synthetic mono-acetyl α-MSH (Sigma, M4135, St Louis, MO, U.S.A.) and characterized in our laboratory (van Zoest, Heijmen, Cruijsen, & Jenks, 1989). Immunocytochemical experiments showed no cross-reaction of the antisem with ACTH cells in the PD of tilapia. The cross-reactivity with des-acetyl- and di-acetyl α-MSH was 100%, with ACTH(1-24) and ACTH(1-39) less than 0.5%. The antisem was used in a final dilution of 1:60 000. α-MSH was labelled with 125I (Amersham International plc, Amersham, Bucks, U.K.) by the iodogen method (Salacinsky, McLean, Sykes et al. 1981) and purified by solid phase extraction (octadecyl Bakerbond column; J. T. Baker, Phillipsburg, NJ, U.S.A.). Bound and free labelled α-MSH were separated by polyethylene glycol precipitation of the immunocomplex. The detection limit of the assay was 6 fmol. The interassay variation was 13 ± 2% and the intra-assay variation 6 ± 1%.

RIA for cortisol
Concentrations of cortisol were determined in duplicate in an RIA for cortisol as described by De Man, Hofman, Hendriks et al. (1980). The cortisol antiserum (K15) was produced in the Department of Endocrinology, Radboud Hospital, Nijmegen. The final dilution was 1:25 000. 3H-labelled cortisol was purchased from Amersham International plc. Bound and free cortisol were separated by precipitation of the immunocomplex with Sac-Cel (IDS Ltd, Boldon, Tyne & Wear, U.K.) during a 1 h contact time and 5 min centrifugation (9000 g). The interassay variation was 10 ± 3% and the intra-assay variation 6 ± 3%.

Isolation of tilapia α-MSH forms
To isolate tilapia α-MSH forms, 12 freshly dissected NILs were homogenized in 1 ml ice-cold HCl (0.1 mol/l) using a glass-to-glass Potter homogenizer device. Membranes and particulate material in the homogenate were removed by centrifugation (10 000 g, 10 min); the forms of α-MSH in the supernatant thus obtained were separated by high-performance liquid chromatography (HPLC) (Spectra Physics SP 8700, Eindhoven, The Netherlands), with a Spherisorb ODS column (Chrompack B.V., Middelburg, The Netherlands). Elution was carried out with a 1-propanol gradient (10-16%) in a buffer consisting of 0.5 mol formic acid/l plus 0.14 mol pyridine/l. The flow rate was 2 ml/min. The eluate was collected in 0-3-min fractions, dried in a Savant Speedvac concentrator and stored at -20 °C. The α-MSH content of the fractions was assessed by RIA.

Collection of blood plasma
Mixed arterial and venous blood was collected from the caudal peduncle into 50 μl glass capillaries, heparinized with ammonium heparin. The blood was diluted 1:1 in 0.9% (w/v) NaCl containing 2 mmol EDTA/l and 100 000 KIU aprotinin/ml (Sigma). After centrifugation (10 000 g, 10 min) the plasma was passed through an octadecyl Bakerbond column equilibrated with the formic acid/pyridine buffer. Total immunoreactive (ir)-α-MSH was eluted with 40% 1-propanol in formic acid/pyridine buffer. After being dried in a Savant Speedvac concentrator, α-MSH was dissolved in 0.05 mol HCl/l. The recovery of α-MSH after the Bakerbond column treatment was 35 ± 5% (n = 8) and plasma concentrations were corrected accordingly. For determination of di-acetyl α-MSH/mono-acetyl α-MSH (di:mono) ratios, samples were submitted to
HPLC, as described in detail previously (Lamers et al., 1991).

**Exposure of fish to water of low pH**

Fish were kept in 100 litre tanks of tap water. The pH of the water was gradually lowered from 7.4 to 4.0 over a 24-h period by adding H$_2$SO$_4$. The pH was controlled using pH-stat equipment (Radiometer PHM 83 + TTT 80 + ABU 80, Copenhagen, Denmark). The fish were kept at pH 4.0 for 7 days before blood was collected.

**In-vitro superfusions**

Headkidney tissue (in which the interrenal cells reside) and the pituitary gland were dissected immediately after the fish had been killed. The pituitary was bisected into NIL and PD (so that no PD tissue was left on the NIL), using a binocular microscope. Tissue thus obtained was placed on a cheese-cloth filter in a superfusion chamber and superfused with a Heps-buffered (15 mmol/1; pH 7.38) Ringer’s solution containing NaCl (132 mmol/1), KCl (2 mmol/1), CaCl$_2$.2H$_2$O (2 mmol/1), 0.25% (w/v) glucose and 0.03% (w/v) bovine serum albumin. Medium was pumped through the chambers at a rate of 30 μl/min by a Watson-Marlow 503U multichannel peristaltic pump (Smith and Nephew Watson-Marlow, Falmouth, Cornwall, U.K.). After 3 h superfusion, when the release of α-MSH and cortisol had reached an apparent steady state (as determined by RIA in pilot experiments), secretagogues were tested. In a dose–response experiment, medium supplemented with human ACTH(1–39) (Peninsula, 10 fmol/1–100 nmol/l) or di-acetyl α-MSH (1 nmol/l–5 μmol/l) was administered to the headkidneys for 10 min. When testing the products released by the NIL or PD, the outflow of the NIL or PD superfusion chambers was switched to the chambers containing the headkidney tissue for 30 min. In this set-up one fish equivalent pituitary tissue was used per fish equivalent interrenal tissue (two headkidneys). Ten-min fractions were collected with an Isco fraction collector; fractions were frozen immediately after collection and stored at −20°C until further assay. Hormone release was

![Figure 1](image-url) **Figure 1.** Effects of di-acetyl α-MSH (di-ac α-MSH, n = 9), mono-acetyl α-MSH (mono-ac α-MSH, n = 5) and des-acetyl α-MSH (des-ac α-MSH, n = 7) (each at 50 nmol/l) on release of cortisol during superfusion of headkidneys as a percentage of basal release. Shaded bars show the periods of administration of the secretagogues. Values are means ± S.E.M.
expressed in fmol/min per lobe and fmol/min per g body weight for α-MSH and cortisol respectively.

**Immunoabsorption of α-MSH**

Cyanogen bromide (CNBr)-activated Sephadex (Pharmacia, Woerden, The Netherlands) was coated with the above-mentioned anti-α-MSH serum (50 μl/ml gel) and subsequently blocked with 0.2 mol glycine/l and 3% (w/v) Polypep (Sigma). It was ascertained by RIA that the columns thus obtained extracted all ir-α-MSH from the superfusate during the length of our experiment. In control experiments, glycine/Polypep-blocked CNBr-activated columns were installed between the outlet of the NIL or PD chambers and the inlet of the headkidney chamber.

**Analysis and statistics**

Results are presented as means±s.e.m. The dose–response curves were fitted using a non-linear regression data analysis program (Leatherborrow, 1987). Statistical significance of differences was assessed using the Mann–Whitney U test.

**RESULTS**

In stressed fish the total ir-α-MSH and the di-acetyl α-MSH/mono-acetylated α-MSH peak area (di:mono) ratio in blood plasma were enhanced significantly (Table 1).

Figure 1 shows the effect of the three forms of α-MSH isolated from tilapia NILs on the release of cortisol during superfusion of homologous head-kidneys. Di-acetyl α-MSH, mono-acetyl α-MSH and des-acetyl α-MSH administered for 30 min at 50 nmol/l enhanced the release of cortisol to 1309±350% (n=8), 221±20% (n=5) and 345±68% respectively.

**TABLE 1.** Total immunoreactive α-MSH (ir-α-MSH) and peak area di-acetylated α-MSH/mono-acetylated α-MSH (di:mono) ratios in tilapia plasma from control and low-pH stressed fish. Values are means±s.e.m. with the numbers of experiments shown in parentheses

<table>
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<th>Total ir-α-MSH (pmol/l)</th>
<th>Ratio of di:mono</th>
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<tbody>
<tr>
<td>Control</td>
<td>294±2±50·9 (6)</td>
<td>0·34±0·09 (4)</td>
</tr>
<tr>
<td>Stressed</td>
<td>1012±1±44·0 (3)**</td>
<td>0·68±0·07 (3)*</td>
</tr>
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*P<0·05, **P<0·01 compared with respective controls (Mann-Whitney U test).
were 2.2 ± 0.2, 1.4 ± 0.2 and 2.0 ± 0.4 fmol cortisol/min per g respectively. We then compared the dose-response curves of ACTH(1–39) and tilapia di-acetyl α-MSH (Fig. 2). ACTH and tilapia di-acetyl α-MSH were administered for 10 min. The dose required to obtain half-maximal stimulation (ED50) calculated for ACTH(1–39) was 0.9 nmol/l and for di-acetyl α-MSH 110 nmol/l. Under these particular experimental conditions di-acetyl α-MSH gave the same maximal response as ACTH(1–39): 704 ± 160 and 726 ± 60% of basal respectively (P > 0.15).

Superfusate from tilapia NILs administered for 30 min to headkidney in superfusion caused a stimulation of the release of cortisol of 190 ± 31% (n = 9). The PD superfusate enhanced the release of cortisol to 515 ± 30% (n = 6) of basal release. Basal values were 2.6 ± 0.4 and 1.9 ± 0.2 fmol cortisol/min per g for NIL and PD pulses respectively and were designated 100% (Fig. 3).

The headkidneys cleared 65.2 ± 3.4% of the α-MSH from the medium, as calculated from the concentrations of α-MSH in the NIL superfusate entering (16 ± 1 nmol/l, n = 9) and leaving (5 ± 1 nmol/l, n = 9) the headkidney chamber. The stimulation of cortisol release as an effect of the NIL superfusate that had passed through the unloaded column was 191 ± 45% of the basal release. This was not significantly different from ‘directly’ administered NIL superfusate. The corticotrophic activity of the NIL superfusate that had passed through the anti-α-MSH-coated column had completely disappeared (97.2 ± 7.9% of basal release). The effects of PD superfusates that had passed through control and anti-α-MSH-coated columns did not differ significantly from the effects of ‘directly’ administered PD superfusate (Fig. 4). The 10–20 min shift in the response peak observed in experiments where immunoabsorption columns were used was due to an increased dead volume of the superfusion set-up under these conditions.

**DISCUSSION**

Four major conclusions can be drawn from the results presented. First, during exposure to water of low pH the total ir-α-MSH as well as the di:mono ratio in tilapia blood plasma is enhanced. Secondly, three forms of α-MSH were shown to have corticotrophic effects in a homologous assay, with di-acetyl α-MSH being about six times more active than mono-acetyl α-MSH and about four times more active than des-acetyl α-MSH at a concentration of 50 nmol/l. Thirdly, di-acetyl α-MSH proved to be more than 100 times less potent than human ACTH(1–39) in stimulating cortisol release in vitro. Fourthly, a NIL superfusate stimulated cortisol release to an extent comparable with a PD superfusate (NIL superfusate was only about three times less active than PD superfusate), although it was demonstrated that the corticotrophic effect of the NIL superfusate depended on the α-MSH in the superfusate.

We have provided evidence that during exposure to water of low pH not only the total ir-α-MSH levels but also the di:mono ratio in the blood plasma are elevated. These findings indicate that α-MSH is involved in adaptation to this kind of stressor, as previously reported for some other kinds of stressors (Malo-Michele, 1980; Sumpter et al. 1986). The increase in the di:mono ratio indicates that di-acetyl α-MSH might be of primary importance in this function. This in-vivo enhancement of the di:mono ratio indicates that TRH may be involved in the stimulation of α-MSH cells during exposure to low pH because we have previously reported elevated di:mono ratios by in-vitro stimulation of α-MSH release with TRH (Lamers et al. 1991).

We have here shown that in a homologous bioassay using tilapia interrenal tissue, all three forms of α-MSH (Lamers et al. 1991) significantly stimulated cortisol release. Corticotrophic effects of α-MSH (Kawauchi, Kawasoe, Adashi et al., 1984; Vinson, Whitehouse, Bateman et al. 1984; Delarue, Leboulenger, Netchitalo et al. 1990) and di-acetyl α-MSH (Dell, Etienne, Panico et al. 1982) have been reported before in higher vertebrates. However, di-acetyl α-MSH proved to be the most active, with des-acetyl α-MSH being more active than mono-acetyl α-MSH. For trout, Rance & Baker (1981) found that des-acetyl α-MSH was more potent than mono-acetyl α-MSH in stimulating cortisol release in vitro. The difference in these experiments was substantial. Thus, although acetylation is of functional significance, there is no correlation between the degree of acetylation and corticotrophic activity.

When tested for corticotrophic potency in dose-response studies, the ED50 of di-acetyl α-MSH was 123 times higher than that of ACTH(1–39). This suggests that di-acetyl α-MSH (and α-MSH in general) is of no relevance for the control of cortisol release. Indeed, Rance & Baker (1981) drew this conclusion. However, this does not correspond to our observations on the corticotrophic activity of α-MSH in a more physiological set-up. In this experiment, we serially coupled the NIL or PD superfusion chambers and headkidney superfusion chambers. Unexpectedly, the NIL superfusate was only 2.7-fold less active than the PD superfusate. Furthermore, the PD and NIL superfusate produced the same pattern of release of cortisol. The fact that α-MSH was cleared from the
medium by the headkidneys during the superfusion indicates a specific binding of the peptide. To assess to what extent the 'NIL effect' may be attributed to α-MSH, we selectively stripped the superfusate of all α-MSH by immunoabsorption; this treatment abolished the stimulatory effect of the NIL superfusate completely. In contrast, the same treatment did not influence the effect of the PD superfusate, for which the stimulatory effect is attributed to ACTH. We therefore conclude that the corticotrophic action of the NIL may be significant compared with that of the PD.

What then can explain the difference between the 123-fold higher corticotrophic potency of ACTH compared with that of purified di-acetyl α-MSH on the one hand and the only 2.7-fold difference in corticotrophic activity of a PD and a NIL superfusate on the other? One may predict a higher release of α-MSH to the blood than of ACTH. At this time we have no access to a reliable assay for tilapia ACTH, but, according to Sumpter et al. (1986), the blood plasma levels (on a molar basis) of α-MSH in trout are about tenfold higher than those of ACTH in vivo. Proceeding from this we predict that di-acetyl α-MSH, which makes up 25% of the total ir-α-MSH found in the plasma of our fish, is 2.5 times more abundant than ACTH. Taking into account the 123-fold higher potency of ACTH compared with di-acetyl α-MSH and the relative amount of the peptides in vivo we are left with a factor of 123/2.5 = 49.2. This still does not explain the only 2.7-fold lower corticotrophic activity of NIL superfusate. We therefore tentatively conclude that a factor is co-released with α-MSH from the NIL which potentiates its effect. A NIL superfusate contains several other POMC-derived peptides released by the α-MSH cells and (glyco)peptides from the pars intermedia periodic acid Schiff's positive (PIPAS) cells, the second most abundant cell type in the NIL. Moreover, arginine-vasotocin and serotonin released from the neural lobe are known to stimulate corticosteroid secretion in goldfish in vivo (Fryer & Leung, 1982) and amphibians in vitro (Kloas & Hanke, 1990; Delarue, Lefebvre, Idres et al. 1988). Furthermore, one could speculate that the different forms of α-MSH in a cocktail potentiate each other’s corticotrophic activities.
The presence and spontaneous release of ACTH from NILs in vitro have been reported earlier in goldfish (Tran, Fryer, Bennett et al. 1989). This has not been confirmed by investigations on the teleost Prochilodus platensis (Estivariz & Itturiza, 1985), the rainbow trout (Rodrigues, Jenks & Sumpter, 1983) and the Australian lungfish (Joss, Dores, Crim & Beshaw, 1990). Although only picomolar concentrations of ACTH would have been sufficient to stimulate cortisol release, we conclude that in our experiments the corticotrophic activity of the NIL superfusate was not due to ACTH, because the corticotrophic activity was removed by an α-MSH antiserum which does not cross-react with tilapia ACTH and human ACTH (van Zoest et al. 1989).

We conclude that, although a much higher dose of di-acetyl α-MSH than of ACTH(1–39) is required to obtain a similar corticotrophic effect in vitro, its role in the PI axis is of comparable importance to that of ACTH under physiological circumstances. These findings and previously published observations of stress-induced enhancement of α-MSH cell activity in mammals (Alexander et al. 1988; Carr et al. 1990; Lookingland et al. 1991) warrant further study of the corticotrophic action of di-acetyl α-MSH in the adaptation of fish and higher vertebrates to stress.

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