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Possible paracrine function of α-melanocyte-stimulating hormone and inhibition of its melanin-dispersing activity by N-terminal acetylation in the skin of the barfin flounder, *Verasper moseri*

Yuki Kobayashi [a], Kanta Mizusawa [a], Takeshi Yamanome [b,1], Hiroaki Chiba [a], Akiyoshi Takahashi [a,*]

[a] School of Marine Biosciences, Kitasato University, Ofunato, Iwate 022-0101, Japan
[b] Iwate Fisheries Technology Center, Kamaishi, Iwate 026-0001, Japan

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ABSTRACT

Melanocyte-stimulating hormone (MSH) is generated from a precursor protein, proopiomelanocortin (POMC), mainly in the pituitary. The barfin flounder, *Verasper moseri*, expresses three different POMC genes (*Pomc*), among which *Pomc-c* is also expressed in the skin. Herein, we characterized the biological significance of POMC and MSH produced in barfin flounder skin. The reverse transcription polymerase chain reaction showed the expression of *Pomc-c* in isolated non-chromatophoric dermal cells. Mass spectrometry analyses of fractions of skin extract separated by high-performance liquid chromatography revealed the presence of a peptide with a molecular mass corresponding to Des-acetyl (Ac)-α-MSH-C derived from POMC-C. These results indicate that, in addition to endocrine functions, MSH in barfin flounder is associated with skin pigmentation via paracrine mechanisms. On the other hand, *in vitro* studies showed that Des-Ac-α-MSH-C dispersed pigments in both melanophores and xanthophores. These functions are similar to those of Des-Ac-α-MSH, which differs from Des-Ac-α-MSH-C only at the C-terminus, generated from POMC-A and -B. α-MSH, which has an acetyl group at the N-terminus, led to pigment dispersion in xanthophores, but showed no effect in melanophores. A series of bioassays indicated that acetylation enhances MSH activity in xanthophores, but inhibits it in melanophores, suggesting that receptors for MSHs expressed in xanthophores and melanophores are different from each other.
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1. Introduction

In teleost fish, body color changes—pigment aggregation and dispersion in chromatophores—are controlled by endocrine and nervous systems (Fujii, 2000; Fujii and Oshima, 1986). The hypothalamus-pituitary axis plays a pivotal role in the body color change employing two peptide hormones showing opposing functions. *In vitro* studies have shown that melanocyte-stimulating hormone (MSH) generated in the pituitary disperses pigment granules, while melanin-concentrating hormone (MCH) generated in the hypothalamus aggregates these granules (Baker, 1991; Castrucci et al., 1989; Fujii, 2000; Fujii and Oshima, 1986; Kawauchi et al., 1983). MSH antagonizes the activity of MCH, and *vice versa* (Burton and Vokey, 2000). Under *in vivo* conditions, MCH aggregates pigment granules (Kawauchi and Baker, 2004), but MSH does not disperse these granules (Eberle, 1988), indicating that neural control over pigment aggregation overcomes the pigment granule-dispersing function of MSH. The major function of MSH in teleosts is suggested to be participation in morphological color changes via pigment synthesis in teleost fish (Eberle, 1988; Rodrigues and Sumpter, 1984).

Barfin flounders are large flatfish that inhabit the cold sea basin around northern Japan. We showed that MSH and MCH are produced in the pituitary and hypothalamus, respectively, in barfin flounders as in other teleost fish (Amano et al., 2003, 2005; Amiya et al., 2007). Body color changes brought about by MSH and MCH in barfin flounders seem to be under similar control to those observed in other teleost fish. For instance, a bolus injection of MSH did not alter the body color, while chronic treatment with MSH turned the body color dark (Yamanome et al., 2007), and a bolus injection of MCH turned the body color pale (Takahashi et al., 2004). The function of MSH in barin flounder skin seems to be mediated through at least three melanocortin (MC) receptors (Kobayashi et al., 2007, 2008a), and that of MCH through one of the two MCH receptors (Takahashi et al., 2007).

MSH is generated from a precursor protein, proopiomelanocortin (POMC), mainly in the pituitary. The barfin flounder characteristically expresses three different POMC genes (*Pomc*, Takahashi et al., 2005, 2006). Among the three POMC transcripts, *Pomc-a* is

* Corresponding author. Fax: +81 192 44 3934.
E-mail address: akiyoshi@kitasato-u.ac.jp (A. Takahashi).
[1] Present address: Iwate Inland Fisheries Technology Center, Hachimantai, Iwate 028-7302, Japan.

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exclusively expressed in the pituitary, while *Pomc-b* and *Pomc-c* are expressed in extra-pituitary tissues in addition to the pituitary. The expression levels of *Pomc-c* seem to be greater than those of *Pomc-b* in extra-pituitary tissues (Takahashi et al., 2005). *Pomc* expression in extra-pituitary tissues, including the skin, has also been shown in mammals, and POMC-derived peptides such as adrenocorticotropic hormone (ACTH), MSH, β-endorphin (β-END) have been identified in cultured skin cells (the review of Slominski and Wortsman, 2000). These reports indicate the presence of autocrine and/or paracrine systems in pigment production in a wide range of vertebrates.

This study aimed to evaluate the role of *Pomc-c* expressed in barfin flounder skin. Therefore, we assessed the expression levels of *Pomc-c* in the skin with regard to the background color, cell types expressing *Pomc-c*, and presence of POMC-derived peptides; moreover, we evaluated the effect of these peptides on pigment dispersion.

2. Materials and methods

2.1. Fish

Immature barfin flounder, *Verasper moseri*, were obtained from Iwate Fisheries Technology Center, Iwate, Japan, and all experiments were conducted according to Guidelines for the Care and Use of Animals of Kitasato University. The fish were reared in indoor tanks with running seawater under a natural photoperiod. The body sizes of fish used were 24.3–30.7 cm in total length (TL) and 231–445 g in body weight (BW). Skin samples used for gene expression tests, peptide analysis, and bioassays were collected from fish anesthetized with 0.2% 2-phenoxyethanol or ice-cold water, respectively.

2.2. Peptides

α-MSH was purchased from the Peptide Institute (Osaka, Japan). Des-Ac-α-MSH-C and Des-Ac-α-MSH were synthesized and purified according to the previously described methods (Takahashi et al., 1995). The amino acid sequence of barfin flounder α-MSH derived from POMC-A is identical to those from POMC-B and many other vertebrate species (Takahashi and Kawauchi, 2006). α-MSH-C derived from POMC-C is different from those from POMC-A and -B at the C-terminus (Thr-amide or Val-amide). Here, α-MSH refers to the peptides derived from POMC-A and -B, and α-MSH-C indicates the peptide derived from POMC-C.

2.3. Reverse transcription (RT)-PCR for POMC mRNA in skin and pituitary

RNA samples were prepared from the skin of body and fin of both dorsal and ventral surface (eyed side and non-eyed side, respectively) and the pituitary using Isogen (Nippon gene, Tokyo, Japan). The amount of RNA was estimated by spectrophotometry. For each tissue, an equal amount of total RNA (50 ng/μl) from three individuals was combined and subjected to amplification using the One-Step RT-PCR kit (Qiagen, Hilden, Germany). Primers amplifying the POMC-A cDNA fragment were 5′-TGG TCA GCG GAG ACG ACA AC-3′ and 5′-GTG AAG ATG TGA ACA GAT TC-3′, those amplifying POMC-B cDNA were 5′-GGC CAG AGG AGC TGT CAG T-3′ and 5′-CTG CCT GTC GAC GAC CTG TGT A-3′, and those amplifying POMC-C cDNA were 5′-CAT CTG CAA CAA CCT GAG CA-3′ and 5′-TCC TTG TCT AGA ATG TTC CTC-3′. The amplified cDNA spans the boundary between exons 2 and 3 of *Pomc-a*, *Pomc-b*, and *Pomc-c* to distinguish products of POMC mRNAs from those of genomic DNA. Primers amplifying the β-actin cDNA fragment were 5′-TGA AGT ACC CCA TCG AGC AC-3′ and 5′-AGG ATC TTC ATG AGG TAG TC-3′, which straddled one of the introns (unpublished data). PCR products were electrophoresed using 3% agarose gel (Agarose S, Nippon Gene) and visualized with 0.025% ethidium bromide. Photographs were taken using a Densitograph (Atto, Tokyo, Japan).

2.4. Quantification of POMC mRNA by real-time RT-PCR

Barfin flounders were reared in an indoor 4 kl black or 4 kl white tank for 43 days in winter. Each tank contained 15 fish. After rearing for 43 days, 6 out of 15 fish in each tank were subjected to skin sampling. TL and BW of black-reared fish were 22.5 ± 0.5 cm and 193 ± 12 g, respectively ($n = 6$), and those of white-reared fish were 23.1 ± 0.5 cm and 201 ± 14 g, respectively ($n = 6$). Skin samples were taken from dorsal and ventral body. Pituitaries were also dissected out. Total RNA was extracted from the skin and pituitary using the RNeasy Mini Kit (Qiagen) with QIAcube (Qiagen). After estimating the amount of total RNA by spectrophotometry, samples were diluted to 3 ng/μl using yeast tRNA solution (50 ng/μl) and stored at −80 °C until analysis.

Absolute amounts of mRNA for POMC-A, -B, and -C were determined using standard sense RNAs prepared as follows. Fragments of POMC mRNAs were amplified from pituitary cDNA using the HotStarTaq Mater Mix Kit. Primer sets were 5′-GGC CGA ACG ACA AGA AGA CA-3′ (forward) and 5′-TGG AGC TTC TCC TGC TCC C-3′ (reverse) for POMC-A, 5′-AAC TCC CGC CTC CTC ATC TC-3′ (forward) and 5′-GAA GAA GAG ACA GAG CGG AG AAT G-3′ (reverse) for POMC-B, and 5′-TTT CAC CAG AGC AGC GAA GAG-3′ (forward) and 5′-TAG AGT AGG AGC GTC GCT CGT-3′ (reverse) for POMC-C. The amplified cDNAs were ligated into pT7 Blue T-Vector and then subjected to sequence analysis to determine the direction. These recombinant DNAs were cut with *Bam* HI at one site near the 3′ terminal ends of the inserts. RNA synthesis was performed according to the manufacturer's instructions using MAXIscript (Ambion, Austin, TX, USA). The synthesized cRNAs were quantified and then serially diluted in yeast tRNA solution (50 ng/μl).

Real-time quantitative PCR was carried out with an ABI Prism 7700 Sequence Detection System and TaqMan One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems, Foster City, CA, USA). TaqMan probes and primers were designed using Primer Express (Applied Biosystems). The probes span the boundary between exons 1 and 2 of *Pomc-a*, *Pomc-b*, and *Pomc-c*. For the assay of POMC-A mRNA, the sequences of primers were 5′-CGG AGA CGA CAA CCA ATG CT-3′ (forward) and 5′-AGG AGG AGG AGG AGG AGG CC-3′ (reverse), and that of the probe was 5′-(Fam)-TCA ACG ATG AGA GCA GCA TGA TGG AGT GTA-(Tamra)-3′. For the assay of POMC-B mRNA, the sequences of primers were 5′-TGG TGG CTG TGG TGG TAG TG-3′ (forward) and 5′-AGG TCA GAG TGA CAG AGG TCG ATA-3′ (reverse), and that of the probe was 5′-(Fam)-AGG AGG TGA ACG ATG AGA GAA GCA TGA ATG-(Tamra)-3′. For the assay of POMC-C mRNA, the sequences of primers were 5′-GAC GCA AGG AGA AGA GCG AG-3′ (forward) and 5′-ATC GCT ACC AAG AGC CAA CG-3′ (reverse), and that of the probe was 5′-(Fam)-ACA TTT GGA AAA GGC TGA AGA TGG TGT GTC-(Tamra)-3′. The PCR mixture (10 μl) contained Master Mix without uracil-*N*-glycosylase, MultiScribe, and RNase Inhibitor Mix, 9.0 pmol of each forward and reverse primer, and 2.5 pmol of fluorogenic probe (Applied Biosystems). The reaction was started with reverse transcription at 48 °C for 30 min, followed by amplification: 95 °C for 10 min (activation of AmpliTaq Gold), 40 cycles at 95 °C for 15 s (denaturation), and 60 °C for 60 s (annealing and extension). In the assays, several doses of standard POMC-A, B, or C sense RNA (3×10^5, 3×10^6, 3×10^7, 3×10^8, 3×10^9, and 3×10^{10} copies) and unknown total RNA samples (9 ng) were subjected to assays in triplicate. The amount of mRNAs was calculated

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