Agouti Antagonism of Melanocortin-4 Receptor: Greater Effect with Desacetyl-α-Melanocyte-Stimulating Hormone (MSH) than with α-MSH

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ABSTRACT
Desacetyl-α-MSH is more abundant than α-MSH in the brain, the fetus, human blood, and amniotic fluid, but there is little information on its ability to interact with melanocortin receptors. The aim of this study is to compare and contrast the ability of desacetyl-α-MSH and α-MSH to couple melanocortin receptors stably expressed in HEK293 cells, to the protein kinase A (PKA) signaling pathway. Desacetyl-α-MSH activated mouse MC1, MC3, MC4 and MC5 receptors with EC50s = 0.13, 0.98, 0.88, and 1.34 nM, respectively. Mouse agouti protein competitively antagonized α-MSH and desacetyl-α-MSH coupling to the MC1-R similarly. In contrast, mouse agouti protein antagonized desacetyl-α-MSH much more effectively and potently than α-MSH coupling the MC4-R to the PKA signaling pathway. Furthermore, mouse agouti protein (10 nM) significantly reduced (1.4-fold) the maximum response of mMC4-R to desacetyl-α-MSH and 100 nM mouse agouti significantly increased (4.8-fold) the EC50. Minimal antagonism of α-MSH coupling mMC4-R to the PKA signaling pathway was observed with 10 nM mouse agouti, whereas both 50 and 100 nM mouse agouti appeared to reduce the maximum response (1.1- and 1.3-fold, respectively) and increase the EC50 (2.5- and 3.4-fold respectively). Mouse agouti protein did not significantly antagonize either α-MSH or desacetyl-α-MSH coupling mouse MC5 and MC6 receptors. Understanding the similarities and differences in activation of melanocortin receptors by desacetyl-α-MSH and α-MSH will contribute to delineating the functional roles for these endogenous melanocortin peptides. (Endocrinology 140: 2167–2172, 1999)

IN CONTRAST TO the majority of α-MSH produced by pituitary melanotrophs, desacetyl-α-MSH is the predominant form of ACTH1–13 NH2 produced in the brain (1, 2), the fetus (3, 4), human blood (5), and amniotic fluid (6). Desacetyl-α-MSH may also be more abundant than α-MSH in the pituitary of the A<sup>vy</sup> yellow mouse (7). The biological activity of many peptide hormones and neuropeptides is altered by N-terminal acetylation; for example, β-endorphin completely loses its opiate activity, whereas N-terminal acetylation of desacetyl-α-MSH to form α-MSH enhances some activities of ACTH<sub>1–13</sub> and virtually eliminates others. α-MSH injected daily to rats is 10- to 100-fold more effective than desacetyl-α-MSH at increasing pigmentation (8), arousal, memory, attention, and excessive grooming (1). Desacetyl-α-MSH, however, is more effective than α-MSH at blocking opiate analgesia and opiate receptor binding in vivo (1). α-MSH and desacetyl-α-MSH also differentially affect feeding and weight gain. Weight gain of agouti obese mice is increased by sc administered desacetyl-α-MSH, as is food intake and fat pad weight, but α-MSH injections do not significantly increase food intake or body weight (9). Similarly, both desacetyl-α-MSH and α-MSH given sc positively stimulate neonatal rat growth during the first 2 weeks of life, but desacetyl-α-MSH is much more effective and potent than α-MSH (10). Both peptides increased muscle and brain growth, whereas desacetyl-α-MSH also induced a rise in fat deposits. Intracerebroventricular (ICV) administered α-MSH and ACTH<sub>1–24</sub> inhibit food intake in food deprived (11) and fed (12) rats, whereas ICV injections of desacetyl-α-MSH had no effect.

Much interest has focused on the dominant agouti locus mutations, lethal yellow (A<sup>y</sup>) and viable yellow (A<sup>y</sup>v), which give rise to mice with yellow coat color as well as obesity and noninsulin dependent diabetes (13). This phenotype is now known to be a result of ectopic expression of agouti protein (14). However, the precise mechanism by which ectopic agouti expression causes obesity and noninsulin-dependent diabetes mellitus is yet to be determined. The mouse agouti gene encodes a secreted protein primarily produced in the hair follicle (14, 15). Mouse agouti protein is normally involved in regulating the production of two pigments, phaeomelanin and eumelanin, which produce red/yellow and brown/black coloration, respectively. Agouti is expressed during the mid-portion of hair growth, and this results in hairs with a subapical yellow band on an otherwise black or brown background, commonly known as agouti banding. Another genetic locus, extension, that controls the relative amounts of phaeomelanin or eumelanin, encodes the MSH (MSH) receptor (16). α-MSH binding to the MSH receptor, now known as the melanocortin-1 receptor (MC1-R), leads to increases in cAMP and dark pigmentation. Recently the
mechanism of action of agouti on pigmentation was delineated when mouse agouti protein was discovered to be a competitive antagonist of α-MSH coupling the mouse MC1-R to the protein kinase A (PKA) intracellular signaling pathway (17, 18).

Following the cloning of the MC1-R, a family of five melanocortin receptors have been identified. Two of these, MC3-R (19, 20) and MC4-R (21, 22), are expressed in the ventromedial nucleus of the hypothalamus (VMH). This region of the brain is considered important in the regulation of feeding behavior and lesions to the VMH are associated with an increase in body weight (23). It was therefore of considerable interest when agouti protein was discovered to be an antagonist of α-MSH at the MC4-R in addition to the MC1-R (17).

One hypothesis to explain the A+/A− obesity phenotypes is that agouti protein is antagonizing the MC4-R in the VMH. Targeted deletion of the MC4-R gene recently identified this receptor to be involved in appetite control and weight regulation, as the animals lacking MC4-R are obese and hyperphagic (24). Further evidence supporting this hypothesis is in vivo antagonism of hypothalamic MC3 and MC4 receptors by 1) a synthetic peptide results in attenuation of the cyclic melanocortin analog, MTIII, induced decrease in feeding (25) and 2) transgenic mice ubiquitously expressing agouti-related protein develop obesity (26).

Despite the recent surge of interest in exploring the roles of melanocortin peptides and their receptors in feeding and weight homeostasis, there is still very little known about the functions of desacetyl-α-MSH and nothing is known about agouti protein antagonism of this peptide. In this study, we have extensively characterized α-MSH and desacetyl-α-MSH coupling mouse MC1, MC3, MC4, and MC5 receptors to the PKA intracellular signaling pathway and compared mouse agouti protein antagonism of these melanocortin peptides and their receptors.

Materials and Methods

Cells

Stably transfected HEK293 cells expressing the mouse MC1, MC3, MC4 and MC5 receptors were used. The mMC1-R expressing cell line has been described previously (27). The coding sequences of mMC3-R and mMC4-R were obtained by probing a genomic DNA library, 129 strain (Stratagene, La Jolla, CA), with the corresponding rat receptor DNA sequences. The coding region for the mMC5-R was obtained following PCR of the full-length coding region using primers designed to the published mMC5-R DNA sequence (28). Cells were grown in DMEM containing 10% newborn calf serum (NCS) and 500 μg/ml G418.

Preparation of mouse and human agouti protein

The production of recombinant human and mouse agouti has been previously described (29). A 614bp XbaI/PstI fragment of the full-length human agouti complementary DNA (cDNA) (30) or mouse agouti cDNA (14, 15) was subcloned into the baculovirus expression vector pAcMP3 (PharMingen, San Diego, CA). Virus incorporating this vector were produced by standard methods (17). Spodoptera frugiperda cells (SF-9) were propagated in Grace’s supplemented medium containing 10% CS and 0.1% pluronic F-68 and used to produce high titer viral stocks. Fifteen-liter scale production runs of human and mouse agouti were produced using Trichoplusia ni (T. ni) cells adapted to suspension (IRH Biosciences, Woodland, CA). T. ni. cells were infected 24 h postseeding at a density of 1 × 10⁶cells/ml and conditioned media from the infected cells harvested 48 h later and purified using cation exchange columns (approximately 60% and 90% purity for mouse and human respectively) or reverse phase HPLC (≥99% purity for both mouse and human). The purity was estimated from 4–20% Tris/Glycine SDS-PAGE, ISS Pro Blue staining.

Adenylyl cyclase assays

Adenylyl cyclase activity was determined directly by measuring the ability of cells to convert [3H]adenine to [3H]cAMP (31) following exposure of the cells to increasing doses of either α-MSH or desacetyl-α-MSH in the presence and absence of different doses of human or mouse agouti protein. Duplicate wells containing approximately 1 × 10⁶ cells were incubated for 2 h with 2.5 μCi of [3H]adenine in DMEM containing 10% NCS. After this the medium was aspirated and the cells washed once with PBS warmed to 37°C. The cells were then exposed for 1 h to increasing concentrations of melanocortin peptides in the presence of DMEM containing 0.1% BSA and 0.5 mM IBMX with or without mouse agouti protein. The medium with peptides plus or minus agouti protein was aspirated and the cells solubilized with 1 mL of 2.5% perchloric acid, 0.1 mM cAMP. Lysate (0.8 ml) was removed, neutralized with 80 μl of 4.2 N KOH, and 0.42 ml of H₂O. The samples were mixed and then the sediment was allowed to settle. A total of 0.1 ml of each lysate was counted in a β counter to determine the total amount of [3H]adenine incorporated into cells. cAMP was separated from the lysate following sequential chromatography over Dowex and alumina columns. cAMP was eluted from the alumina columns in 4 ml of Tris (pH 7.4) and counted in a β counter. Relative cyclase activity was calculated by determining the percentage of [3H]adenine converted into [3H]cAMP. The Kaleidagraph software package (Synergy Software, Reading, PA) was used for fitting curves to the data and calculating EC₅₀ and maximum response values.

Statistics

Comparisons between groups were made using Sigmasstat (Jandel Scientific) and one- or two-way ANOVA.

Results

α-MSH and desacetyl-α-MSH increase adenylyl cyclase activity via mouse MC1, MC3, MC4, and MC5 receptors

The melanocortin peptides, α-MSH and desacetyl-α-MSH, stimulated dose dependent increases in adenylyl cyclase activity with similar potencies on mouse MC1, MC3, MC4, and MC5 receptors. The EC₅₀ values for these peptides are shown in Table 1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MC1-R EC₅₀ (nM)</th>
<th>MC3-R EC₅₀ (nM)</th>
<th>MC4-R EC₅₀ (nM)</th>
<th>MC5-R EC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MSH</td>
<td>0.27 ± 0.06</td>
<td>0.88 ± 0.40</td>
<td>1.05 ± 0.49</td>
<td>1.34 ± 0.47</td>
</tr>
<tr>
<td>Desacetyl-α-MSH</td>
<td>0.13 ± 0.03</td>
<td>0.96 ± 0.45</td>
<td>0.53 ± 0.01</td>
<td>0.84 ± 0.19</td>
</tr>
</tbody>
</table>

Table 1. EC₅₀ values for melanocortin peptides coupling mouse melanocortin receptors stably expressed in HEK293 cells to adenyl cyclase. (Mean ± SD, n = number of dose response curves)
Mouse agouti protein is more effective and potent at antagonizing desacetyl-α-MSH than α-MSH coupling the mouse MC4-R (mMC4-R) to the PKA signaling pathway.

Mouse agouti protein potently antagonized desacetyl-α-MSH coupling of the mMC4-R to the PKA intracellular signaling pathway (Fig. 1). As shown in Fig. 1, mouse agouti protein shifted the EC50 from $2.22 \times 10^{-10}$ M to $3.51 \times 10^{-9}$ M in an apparent dose dependent manner. However, this shift was less pronounced when α-MSH was used as a ligand (Fig. 2), shifting from $1.61 \times 10^{-9}$ M to $6.12 \times 10^{-9}$ M. Mouse agouti protein (100 nm) significantly increased the EC50 (4.8-fold) for desacetyl-α-MSH (Fig. 3), whereas as little as 10 nm protein was sufficient to significantly reduce the maximum response (1.4-fold) of mMC4-R to desacetyl-α-MSH (Fig. 4). Mouse agouti protein (50 and 100 nm) appeared to increase the EC50 (2.5- and 3.4-fold, respectively) and decrease maximum response (1.1- and 1.3-fold) of α-MSH coupling mMC4-R to the PKA pathway, but these changes were not significant. Agouti protein antagonism of the maximum response was significantly greater for desacetyl-α-MSH than with α-MSH. The pA2 values are not provided since agouti antagonism of the MC4-R is not competitive.

Mouse agouti protein antagonized both α-MSH and desacetyl-α-MSH coupling the mMC1-R to the PKA signaling pathway.

Mouse agouti protein potently antagonized both desacetyl-α-MSH and α-MSH coupling of the mMC1-R to the PKA intracellular signaling pathway (Figs. 1 and 2). As shown in Fig. 3, mouse agouti protein shifted the EC50 in a dose-dependent manner from $1.74 \times 10^{-10}$ M to $1.02 \times 10^{-9}$ M (5.9-fold) and from $1.26 \times 10^{-10}$ M to $8.67 \times 10^{-10}$ M (5.4-fold) for α-MSH and desacetyl-α-MSH, respectively. The maximum response appeared to be decreased by 1.2- and 1.3-fold for α-MSH and desacetyl-α-MSH respectively, but these changes were not significant (Fig. 4). The pA2 values for agouti antagonism of α-MSH and desacetyl-α-MSH were 0.98 M and 0.25 M, respectively.

Mouse agouti protein has little effect on α-MSH or desacetyl-α-MSH coupling mMC3-R and mMC5-R to the PKA intracellular signaling pathway.

Mouse agouti protein (10–100 nm) had minimal effect on either α-MSH or desacetyl-α-MSH coupling the mouse MC3 and MC5 receptors to the PKA intracellular signaling pathway (Figs. 1–4).

**Discussion**

Melanocortin peptides and receptors are important in the regulation of energy balance and body weight, influencing both food intake and sympathetically mediated thermogenesis. We report that mouse agouti protein is more effective and potent at antagonizing desacetyl-α-MSH than α-MSH coupling mMC4-R to the PKA signaling pathway. Mouse agouti protein significantly decreased both the sensitivity and maximum responsiveness of the mMC4-R to desacetyl-α-MSH and appeared to decrease the sensitivity and responsiveness of the mMC4-R to α-MSH but these differences were not significant. This is the first time antagonism of desacetyl-α-MSH activating melanocortin receptors has been studied.

Desacetyl-α-MSH exists but very little is known about its function(s) or how it activates the cloned melanocortin re-
Fig. 2. Agouti protein antagonism of \( \alpha \)-MSH coupling melanocortin receptors to the PKA signaling pathway. The y-axis indicates the percent of \([3H]\)adenine converted to \([3H]\)cAMP after 1 h incubation at 37 C. Curves are representative of three separate experiments and are drawn using MACDRAW software. Points indicate the mean \( \pm \) SD of duplicate incubations.

Fig. 3. Effect of mouse agouti protein on EC\(_{50}\) for coupling of melanocortin receptors to adenylyl cyclase by \( \alpha \)-MSH and desacetyl-\( \alpha \)-MSH. Dose-response curves for adenylyl cyclase activity using either \( \alpha \)-MSH or desacetyl-\( \alpha \)-MSH were performed in the absence or presence of 10 nM, 50 nM and 100 nM mouse agouti protein and analyzed using KALEIDAGRAPH software. EC\(_{50}\) data points are means \( \pm \) SD of three separate experiments. Significant differences between the EC\(_{50}\) generated in the absence or presence of agouti protein were determined using one-way ANOVA. (*) \( P < 0.05 \).
It has previously been proposed that a defect in α-MSH acetylation could contribute to obesity and diabetic phenotypes, based on characterization of a variant "yellow" mouse. Desacetyl-α-MSH is more potent than α-MSH at increasing feeding and weight gain in yellow obese (Avy/a) mice (9) and Avy/a mice may have a higher ratio of desacetyl-α-MSH to α-MSH in the pituitary than their lean (a/a) littermates (7). The potent antagonism of desacetyl-α-MSH at the MC4-R by mouse agouti protein suggests that desacetyl-α-MSH induced increases in feeding and weight gain in obese yellow agouti mice (9), and also in neonatal rats (10), may not be through agonism of the MC4-R.

To explain the effects of desacetyl-α-MSH on feeding and weight gain we propose that desacetyl-α-MSH activates the MC3-R, which in contrast to the MC4-R, is expressed not only in the VMH but also in the arcuate nucleus where the blood brain barrier is leaky, and is not significantly antagonized by agouti protein. We also propose that desacetyl-α-MSH activates the MC5 receptor that is expressed in adipose tissue and muscle (32). The MC5-R couples to the PKA signaling pathway in response to desacetyl-α-MSH and mouse agouti protein is not an antagonist of either α-MSH or desacetyl-α-MSH coupling this receptor to the PKA signaling pathway. Hence, increased activity of the MC3 and MC5 receptors in response to desacetyl-α-MSH in the yellow obese mouse may contribute to the mechanism for agouti-driven obesity. Although targeted deletion of the MC5-R in mice did not lead to an obese phenotype (33), the MC5-R has recently been linked with human obesity (34).

Mouse agouti protein antagonism of desacetyl-α-MSH induced cAMP increases via the mMC4-R is not consistent with competitive antagonism. This is in contrast with the competitive antagonism of α-MSH coupling the mMC1-R via the PKA signaling pathway (17, 18). Levels of mouse agouti protein (10 nM), which are without effect on α-MSH coupling the mMC4-R, significantly reduce the maximum response resulting from desacetyl-α-MSH activation of this receptor. Increasing agouti concentrations fail to further decrease the maximum response for the ligand. Recently antagonism of α-MSH coupling the hMC4-R to the PKA signaling pathway by agouti-related protein (26) was shown to be inconsistent with competitive antagonism. The role this effect plays in energy homeostasis is not clear. Interestingly, administration of 5 mg/day desacetyl-α-MSH, but not α-MSH, to obese yellow mice (Avy) increases feeding and weight gain and 15 mg/day α-MSH, but not desacetyl-α-MSH, induces eumelanin synthesis (9). Furthermore, NDP-α-MSH, a superpotent analog of α-MSH, when sc administered to obese yellow mice, reverses fur pigmentation but not obesity and insulin resistance (35). It appears therefore that α-MSH can compete with agouti at the MC1-R, but neither α-MSH nor desacetyl-α-MSH when administered sc can compete with agouti antagonism at the MC4-R. Furthermore, administration of desacetyl-α-MSH actually potentiates the agouti-driven obesity phenotype. We offer two possible explanations for the decrease in maximum response induced by mouse agouti protein. First, agouti protein may down-regulate the MC4-R independent of melanocortin peptides or potentiate melanocortin peptide induced down-regulation. This issue will be resolved when antibodies to the MC4-R become available.
and regulation of receptor protein can be studied. Second, agouti protein may bind irreversibly to the MC4-R.

In summary, mouse MC1, MC3, MC4, and MC5 receptors are similarly activated by both α-MSH and desacetyl-α-MSH. Mouse agouti protein antagonizes both α-MSH and desacetyl-α-MSH coupling MC1-R and MC4-R to the PKA signaling pathway; a greater effect is seen with desacetyl-α-MSH than α-MSH for the MC4-R only. Mouse agouti protein antagonizes desacetyl-α-MSH on the MC4-R by decreasing both the sensitivity and maximum response. Understanding the similarities and differences in activation of melanocortin receptors by desacetyl-α-MSH and α-MSH will contribute to delineating the functional roles for these endogenous melanocortin peptides.

References

25. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD 1997 Role of mela
32. Boston BA, Cone RD 1996 Characterization of melanocortin receptor subtype expression in murine adipose adipose tissues and in the 3T3-L1 cell line. Endocrinology 137:2043–2050