The melanocortin 1 receptor is the principal mediator of the effects of agouti signaling protein on mammalian melanocytes

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Accepted 2 January 2001
Journal of Cell Science 114, 1019-1024 © The Company of Biologists Ltd

SUMMARY

The agouti gene codes for agouti signaling protein (ASP), which is temporally expressed in wild-type mouse follicular melanocytes where it induces pheomelanin synthesis. Studies using purified full-length agouti signaling protein has shown that it competes with α-melanocyte stimulating hormone for binding to the melanocortin 1 receptor. We have investigated whether ASP binds exclusively to the melanocortin 1 receptor expressed on mouse melanocytes in primary culture, or additionally activates a receptor that has not been identified yet. We have compared the responses of congeneric mouse melanocytes derived from C57 Bl/6J-E+/E+, e/e, or Eso/Eso mice to α-MSH and/or ASP. E+/E+ melanocytes express the wild-type melanocortin 1 receptor, e/e melanocytes express a loss-of-function mutation in the melanocortin 1 receptor that results in a yellow coat color, and Eso/Eso is a mutation that causes constitutive activation of the melanocortin 1 receptor and renders melanocytes unresponsive to α-melanocyte stimulating hormone. Mouse E+/E+ melanocytes, but not e/e or Eso/Eso melanocytes, respond to agouti signaling protein with decreased basal tyrosinase activity, and reduction in levels of tyrosinase and tyrosinase-related proteins 1 and 2. Only in E+/E+ melanocytes does agouti signaling protein abrogate the stimulatory effects of α-melanocyte stimulating hormone on cAMP formation and tyrosinase activity. These results indicate that a functional melanocortin 1 receptor is obligatory for the response of mammalian melanocytes to agouti signaling protein.

Key words: Melanocortin 1 receptor, Agouti signaling protein, α-Melanocyte stimulating hormone, Melanocytes, Eumelanin, Pheomelanin

INTRODUCTION

In mouse follicular melanocytes, the switch between the synthesis of eumelanin, the dark brown or black pigment, and pheomelanin, the red-yellow pigment, is regulated by α-melanocyte stimulating hormone (α-MSH) and agouti signaling protein (ASP), respectively (Geschwind, 1966; Silvers, 1979; Prota, 1992). The mechanism of action of α-MSH involves binding to its specific receptor, the melanocortin 1 receptor (MC1R), which is coded for by the extension locus (Tamate and Takeuchi, 1984; Robbins et al., 1993; Suzuki et al., 1996). The MC1R belongs to a family of five different G-protein-coupled receptors with seven transmembrane domains (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992; Gantz et al., 1993a; Gantz et al., 1993b; Roselli-Rehfuss et al., 1993; Labbé et al., 1994). Binding of α-MSH to its receptor results in activation of adenylate cyclase and increased intracellular cAMP levels (Wong et al., 1974; Fuller et al., 1987; Suzuki et al., 1996). Several mutations in the mouse Mc1r gene have been identified and their consequences on the function of the receptor and the pigmentedary phenotype have been determined (Tamate and Takeuchi, 1984; Robbins et al., 1993). In the recessive yellow mouse (e/e), the yellow coat color results from a frameshift mutation in the Mc1r gene that causes deletion of a single nucleotide at position 549, resulting in premature termination of the receptor after the fourth transmembrane domain (Robbins et al., 1993). The resulting receptor is uncoupled from adenylate cyclase, thus is incapable of signaling. In the sombre (Eso/Eso) mouse, a point mutation results in the substitution of a leucine residue by a proline at codon 98 in the second transmembrane domain of the MC1R. This receptor is constitutively active and unresponsive to α-MSH (Robbins et al., 1993).

In the mouse, dominant mutations in the agouti locus that result in ectopic and overexpression of ASP result in a yellow coat color, in addition to other abnormalities, the most obvious of which are obesity and increased longitudinal growth (Yen et al., 1994; Jones et al., 1996). Agouti signaling protein is known to antagonize the effects of α-MSH by competing for binding to the MC1R, and/or by inhibiting the constitutive activity of the unbound MC1R, i.e. acting as an inverse agonist (Lu et al., 1994; Blanchard et al., 1995; Siegrist et al., 1997; Suzuki et al., 1997). With the cloning of the mouse and human agouti genes and the purification of their respective products, it became feasible to elucidate the mechanism of action of ASP (Bultman et al., 1992;
Melanocytes to ASP. MC1R is absolutely required for the response of mammalian malignant melanocytes (i.e. melanoma cells), provides to ASP. The use of these melanocytes, rather than transformed shown that in melan-a mouse melanocytes, ASP blocked the with additionally activating another receptor that has not been identified yet. In the current study, we attempt to address this A pending question is whether ASP elicits its effects on mammalian melanocytes only by binding to the MC1R or by increase in cAMP levels. So far, the physiological role of ASP in the regulation of human pigmentation remains speculative.

Materials and methods

MATERIALS AND METHODS

Melanocyte cultures

Primary mouse melanocyte cultures were established from 1-day-old neonatal mouse skins. Skins were incubated in 0.25% Trypsin solution at 37°C for 90-120 minutes to allow for the separation of the epidermis from the dermis. The dermal layers of two to three skins were placed in a 15 ml centrifuge tube containing 5 ml growth medium, and vortexed vigorously for 3-5 minutes to obtain a cell suspension containing dermal hair follicles. The mouse melanocyte growth medium consisted of MCDB 153 containing 4% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 5 µg/ml insulin, 1 µg/ml transferrin, 1 µg/ml vitamin E, 0.6 ng/ml recombinant basic fibroblast growth factor, 32 nM TPA, and 5 µM dibutyryl cAMP (all obtained from Sigma, St Louis, MO). The resulting dermal cell suspension was transferred to a T-25 cm² flask, and incubated in a humidified incubator containing 5% CO₂, at 37°C. For all experiments, melanocyte cultures in passage two or three were used.

Determination of the effects of ASP and α-MSH on tyrosinase activity

Mouse melanocytes were deprived of dibutyryl cAMP for 2-3 days prior to, and for the entire duration of, the experiment. Melanocytes were plated at a density of 1.3 × 10⁵ cells per 12.5 cm² flask and allowed to attach for 48 hours. Subsequently, and every other day thereafter, the medium in each flask was removed and replaced with fresh medium, and melanocytes were treated with nothing (as control), with 1 or 10 nM α-MSH (obtained from Sigma), with 10 nM ASP, or concomitantly with α-MSH and ASP. Recombinant ASP was purified from conditioned media of insect cells infected with agouti-containing baculovirus as described previously in detail (Ollmann et al., 1998). 5 days after treatment, 2.1 µCi ³H-tyrosine (specific activity 52 mCi/mmol, Du Pont, NEN, Boston, MA; 0.7 µCi/ml medium) was added to each flask for a total of 20 hours. The medium from each flask was then transferred into a culture tube, and duplicate 1 ml samples from each tube were assayed for tyrosinase activity in situ, as described previously in detail (Abdel-Malek et al., 1989). This assay is based on determining the amount of ³H₂O released by the melanocytes to the medium as ³H tyrosine is converted to L-DOPA, a reaction catalyzed by tyrosinase, the rate-limiting enzyme in the melanin synthetic pathway. The cell number in each flask was counted with the aid of a Coulter Counter. Tyrosinase activity was expressed as dpm/10⁶ cells, and then as % of control.

cAMP determination

The effects of ASP and/or α-MSH on cAMP formation was determining using a radioimmunoassay as previously described (Suzuki et al., 1996). Mouse melanocytes were plated onto 24-well plates in the absence of dibutyryl cAMP at a density of 3 × 10⁵ cells/well. 72 hours later, the medium in each well was replaced with 0.5 ml medium containing 0.1 mM isobutyl methylxanthine, a phosphodiesterase inhibitor, and the appropriate treatment (1 nM α-MSH and/or 10 nM ASP), and the cultures were incubated for 40 minutes at 37°C. An untreated control group was included in each experiment. The reaction was stopped by the addition of 50 μl 1N HCl, and the assay was performed as described previously in detail (Suzuki et al., 1996).

Western blot analysis for tyrosinase, tyrosinase-related proteins 1 and 2

Mouse melanocytes were plated on 60 mm dishes at a density of 5-7.5 × 10⁵ cells/dish, maintained in medium devoid of dibutyryl cAMP, and treated 72 hours later with nothing (as a control), or with 1 nM α-MSH and/or 10 nM ASP for a total of 6 days, as described above. Cell extracts were then prepared using RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0), containing the phosphatase inhibitor NaVO₄ (10 μM), and the protease inhibitors phenethylsulfonyl fluoride (200 μM), aprotinin (10 μg/ml) and leupeptin (10 μg/ml). Proteins (5 μg/lane) were loaded on 8% polyacrylamide gels and separated by electrophoresis. The separated proteins were then transblotted onto Immobilon-P membranes (Millipore, Bedford, MA), and the membranes were blocked by incubation in 5% nonfat dry milk in TBS/Tween (0.1% Tween-20 in Tris-buffered saline). The membranes were then probed with primary antibodies αPEP7, αPEP1 and αPEP8, which are raised against synthetic peptides that correspond to the unique C terminus of mouse tyrosinase, tyrosinase related protein (TRP) 1 and TRP2, respectively, at 1:1000 dilution in TBS/Tween overnight at 4°C. The membranes were then washed with TBS/Tween three times, for ten minutes each time, and incubated with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:1000, for 2 hours. Visualization of the respective bands was carried out using Enhanced ChemiLuminescence (Amersham, Arlington Heights, IL), according to the manufacturer’s instructions.

Results

Effects of ASP and/or α-MSH on tyrosinase activity and cAMP formation in primary mouse melanocyte cultures

In this study, we compared the responses to ASP and α-MSH of primary cultures of mouse follicular melanocytes that were derived from the skins of congenic 1-day-old C57 BL 6J-E⁺/E⁺, C57BL6J-e/e or C57BL6J-E⁺/E⁻ mice. E⁺/E⁺ mouse melanocytes express a normal MC1R, while e/e mouse melanocytes harbor a point mutation in the Mc1r gene that results in loss of function of the MC1R due to its uncoupling from adenylate cyclase (Tamate and Takeuchi, 1984; Robbins et al., 1993). The E⁺/E⁻ mice express a frameshift mutation that renders the MC1R constitutively active and blocks its activation by α-MSH (Robbins et al., 1993). Treatment of E⁺/E⁺ melanocytes with either 1 or 10 nM α-MSH increased tyrosinase activity by about 60%, while treatment with 10 nM
Agouti signaling protein binds the MC1 receptor

Agouti signaling protein binds the MC1 receptor and inhibited basal tyrosinase activity by about 70% (Fig. 1A). Concomitant treatment of these melanocytes with either 10 nM ASP and 10 nM α-MSH or 10 nM ASP alone reduced tyrosinase activity by 60% or 30% below basal level, respectively. In comparison, similar treatment of either e/e or Eso/Eso melanocytes with α-MSH and ASP had no significant effect on tyrosinase activity (Fig. 1B,C). Our findings that ASP inhibited basal tyrosinase activity and blocked the stimulatory effect of α-MSH in E+/E+ melanocytes led us to investigate whether or not α-MSH inhibited the α-MSH-induced increase in cAMP levels in E+/E+ melanocytes. For that, we measured cAMP formation in E+/E+ melanocytes after treatment with 10 nM α-MSH and 10 nM ASP for 40 minutes. We found that α-MSH increased intracellular cAMP levels almost fourfold; ASP alone had no significant effect, but reduced the α-MSH-induced increase in cAMP levels by 56% (Table 1). However, in e/e or Eso/Eso melanocytes, neither α-MSH nor ASP had any significant effect on control cAMP levels (data not shown).

Comparison of the morphology of cultured E+/E+, e/e and Eso/Eso melanocytes

The morphology of E+/E+ melanocytes was very different from that of e/e melanocytes. The former had larger cell bodies and were multi dendritic, while the latter were thin and bipolar (Fig. 2A,B). Melanocytes from Eso/Eso mouse skins were similar to e/e melanocytes in that they were also bipolar. E+/E+ melanocytes treated for 4 or 6 days with 1 nM α-MSH exhibited extensive dendrites (Fig. 2C). On the other hand, treatment with ASP caused these melanocytes to attain a smaller cell body and to appear darker than control melanocytes due to the aggregation of melanin granules in the cytoplasm (Fig. 2D). Concomitant treatment with ASP and α-MSH resulted in inhibition of dendrite extension and in a morphology that was intermediate between that of control and ASP-treated melanocytes (Fig. 2E). Such morphological changes in response to α-MSH or ASP were not evident in either e/e or Eso/Eso melanocytes (data not shown).

Effects of ASP and α-MSH on the protein levels of tyrosinase, TRP1 and TRP2

Our previous studies on mouse melan-a cells and normal human melanocytes revealed that the inhibitory effects of ASP were associated with a decrease in the amount of tyrosinase, and a profound reduction in the expression of TRP1 and TRP2, as determined by western blot analysis (Sakai et al., 1997; Suzuki et al., 1997). Here, we found that treatment of E+/E+ melanocytes with 1 nM α-MSH for 6 days increased the amount of tyrosinase, TRP1 and TRP2, while treatment with 10 nM ASP almost abolished the expression of these three melanogenic enzymes (Fig. 3 A-C). Concomitant treatment of those melanocytes with ASP and α-MSH resulted in a profound reduction of the levels of tyrosinase and TRP-1 below

Table 1. cAMP levels of C57BL/6J-E+/E+ mouse melanocytes after treatment with ASP and/or α-MSH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP/ml (pmoles±s.e.m.)</th>
<th>% of control ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.16±0.14</td>
<td>100±6</td>
</tr>
<tr>
<td>α-MSH (1 nM)</td>
<td>8.40±0.34</td>
<td>389±16</td>
</tr>
<tr>
<td>ASP (10 nM)</td>
<td>2.79±0.28</td>
<td>129±13</td>
</tr>
<tr>
<td>α-MSH + ASP</td>
<td>3.69±0.11</td>
<td>171±5</td>
</tr>
</tbody>
</table>

Melanocytes were plated at a density of 3 x 10^5 cells/well in 24-well plates. 48 hours later, melanocytes were treated for 40 minutes with 0 (control), 1 nM α-MSH, 10 nM ASP, or both α-MSH and ASP. cAMP levels were determined by radioimmunoassay, as described in Materials and Methods.
the levels of control untreated melanocytes (Fig. 3A,B). In contrast, e/e melanocytes responded to α-MSH with a slight increase, and to ASP with minimal reduction, in the levels of tyrosinase, TRP1 and TRP2. E/o/E/o melanocytes did not respond to α-MSH and/or ASP with any alteration of the levels of tyrosinase, TRP1 or TRP2.

**DISCUSSION**

The purification of the mouse agouti gene product, ASP, has allowed the investigation of the mechanism(s) by which ASP signals and antagonizes the effects of α-MSH on target cells (Bultman et al., 1992; Miller et al., 1993). That α-MSH induces eumelanin synthesis while the expression of ASP switches to pheomelanin synthesis in mouse follicular melanocytes has
been recognized for decades (Geschwind, 1966; Silvers, 1979). Loss-of-function mutations in the mouse Mc1r gene, as in e/e mice, or gain-of-function mutations in the agouti gene result in a similar pigmentary phenotype, namely yellow coat color (Tamate and Takeuchi, 1984; Robbins et al., 1993; Yen et al., 1994). These observations led to a number of speculations about the mechanism of action of ASP, particularly about the nature of the receptor(s) it binds. Several studies have provided evidence that ASP acts as a competitive inhibitor of α-MSH binding to the MC1R (Lu et al., 1994; Blanchard et al., 1995; Siegrist et al., 1997; Suzuki et al., 1997). It has also been proposed that ASP acts as an inverse agonist of the MC1R, and results in MC1R desensitization (Siegrist et al., 1997; Suzuki et al., 1997). Recently, we obtained data that showed that ASP downregulates Mc1r mRNA levels in normal human melanocytes (unpublished observations).

It has been reported that ASP alone inhibits melanin formation in melan-a cells, B16 mouse melanoma cells and normal human epidermal melanocytes (Hunt and Thody, 1995). In those experiments when ASP and α-MSH were concomitantly used at equal concentrations, ASP failed to antagonize the melanogenic effect of α-MSH. This result is not surprising, as in competition assays, the antagonist should be used at 10-100-fold higher concentration than the agonist in order to displace it effectively from the receptor. In another study, using mouse B16 melanoma cells, it was found that ASP at relatively higher concentrations inhibited α-MSH-induced melanin production, and additionally abrogated the stimulatory effects of forskolin and cholera toxin (Siegrist et al., 1997). An important observation was reported in that study, namely that B16 G4F cells that lack detectable MC1R expression failed to respond to ASP. The sum of these results suggested that ASP acts as a competitive inhibitor of α-MSH, and that expression of the MC1R is required for melanocytes to respond to ASP. Those results were obtained using malignant melanoma cells, and thus did not resolve the issue of whether or not ASP acts on normal mammalian melanocytes exclusively by binding to the MC1R, or if ASP additionally binds to another receptor that is as yet unknown. Using normal human epidermal melanocytes, we previously found that they responded to ASP with a reduction in basal tyrosinase activity and abrogation of the mitogenic and melanogenic effects of α-MSH, forskolin and dibutyryl cAMP (Suzuki et al., 1997). We also found that ASP displaced 125I-NDP-α-MSH from the human MC1R. The ability of ASP to antagonize effectors downstream from the MC1R in mouse melanoma cells and normal human melanocytes is difficult to explain. One speculation is that binding of ASP to the MC1R may stimulate a signaling pathway that blocks the activation of cAMP-dependent targets.

In the present study, we relied on the use of melanocytes derived from three congenic mice strains that differ only in the Mc1r alleles expressed. Comparison of the responses of E+/E+ melanocytes to those of e/e and Eso/Eso revealed that only E+/E+ melanocytes expressing wild-type MC1R responded to α-MSH with a significant stimulation of cAMP formation and tyrosinase activity, and to ASP with a reduction in basal tyrosinase activity and abrogation of the effects of α-MSH on tyrosinase activity and cAMP levels (Fig. 1 A-C; Table 1). The inhibitory effect of ASP on tyrosinase activity in control melanocytes may be due to the antagonism by ASP of melanocortins that are present in fetal calf serum in the growth medium, or that are synthesized by the melanocytes themselves. Alternatively, binding of ASP to the MC1R may inhibit one of the pathways that regulate tyrosinase activity, such as the protein kinase C pathway (Park et al., 1993). This latter possibility explains the lack of reduction of cAMP levels in melanocytes treated only with ASP (Table 1). The melanogenic response of E+/E+ melanocytes to α-MSH also included an increase in the protein levels of tyrosinase, TRP1 and TRP2 (Fig. 3A-C). These effects were completely abrogated by concomitant treatment with ASP. However, neither e/e melanocytes with loss-of-function mutation in the Mc1r gene, nor Eso/Eso melanocytes with a constitutively active MC1R responded significantly to either α-MSH or ASP. The small increase in tyrosinase, TRP1 and TRP2 levels in e/e melanocytes after α-MSH treatment might be attributed to residual activity of the mutant MC1R.

Unlike e/e or Eso/Eso melanocytes, E+/E+ melanocytes responded to α-MSH with extensive increase in dendrite formation and in the size of melanocytes (Fig. 2C). Dendrite extension in response to α-MSH has been described previously in human melanocytes and was attributed to increased actin stress fibers and adhesion to extracellular components (Abdel-Malek et al., 1995; Scott et al., 1997). Human melanocytes exhibited the same morphological changes after treatment with dibutyryl cAMP, suggesting that these alterations are mediated by activation of the cAMP pathway. Treatment of E+/E+ melanocytes with ASP reduced their size and the extent of their dendriticity, and completely reversed the morphological effect of α-MSH (Fig. 2E).

Among the above three different mouse melanocyte cultures, e/e melanocytes were the slowest in their proliferation, and became extremely pigmented, owing to accumulation of melanin in response to continuous exposure to dibutyryl cAMP in the growth medium. On several occasions, e/e melanocytes died in culture after becoming extensively pigmented, possibly owing to accumulation of cytotoxic melanin by-products that were not diluted by cell division. There is convincing evidence that α-MSH counteracts oxidative stress in epidermal cells (Haycock et al., 2000). Thus, it is plausible that e/e melanocytes have increased sensitivity to oxidative DNA damage, owing to their inability to respond to α-MSH. We speculate that the observed cytotoxicity of those melanocytes is due to the loss-of-function mutation that possibly acts in a dominant negative fashion.

The significance of our current results is that they are based on the use of primary cultures of congenic mouse melanocytes that express wild-type MC1R or naturally occurring mutations in the Mc1r. Using these melanocytes, rather than transformed melanoma cells (as used in previous studies by other investigators; Siegrist et al., 1997), we provide conclusive biochemical evidence that the MC1R is the principal mediator of the biological effects of ASP. This conclusion is corroborated by previous findings that Chinchilla mice that express the e mutation but have a normal agouti gene (A/A, MC1R+/MC1R+, cch/cch) have the same coat color as Chinchilla mice with the e mutation and the lethal yellow mutation in the agouti gene (A/-, MC1R-/MC1R-, cch/cch) that results in increased and ectopic expression of ASP (Ollmann et al., 1998). The latter observation suggests that expression of a non-functional MC1R renders melanocytes unresponsive to ASP, even when ASP is expressed at abnormally high levels. The
results obtained using mice strains that harbor mutations at the extension and/or agouti locus provide strong support for the notion that the MC1R is the key mediator of the effects of melanotropins and ASP on mammalian melanocytes.

In mice, eumelanin and pheomelanin synthesis in follicular melanocytes is primarily determined by MC1R function and proper temporal expression of ASP (Silvers, 1979; Tamate and Takeuchi, 1984). In humans, several population studies have concluded that specific mutations in MC1R are strongly associated with red hair (i.e. pheomelanogenic) phenotype (Valverde et al., 1995; Smith et al., 1998). Additionally, mutations that disrupt the normal expression of proopiomelanocortin, the precursor peptide for melanotropins and other bioactive peptides, result in red hair and significant metabolic dysfunctions (Krudel et al., 1998). So far, no mutations in the human agouti gene have been identified, and the regulation of ASP expression in humans remains elusive.

The results hereby presented take us a significant step forward in understanding the signaling pathway of ASP. Further studies will be aimed at identifying the exact binding site of ASP on the MC1R and the second messengers involved in the signaling pathway that leads to the biological effects of ASP.

Supported in part by R01 ES 06882 and R01 ES 09110 (to Z. A. M.). The authors extend their thanks to Ms Joan Griggs for her excellent secretarial assistance.

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