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The differential influences of melanocortins on nociception in the formalin and tail flick tests

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Abstract

Melanocortins exert multiple physiological effects that include the modulation of immune responses, inflammation processes, and pain transmission. In the present study we investigated the peripheral activity of natural melanocortins – α-, β-, γ1- and γ2-melanocyte stimulating hormone (MSH) – and melanocortin receptor subtypes 3 and 4 (MC3/4 receptor) antagonist HS014 in pain (formalin and tail flick) tests after peptide subcutaneous administration in mice. In the formalin test, among all substances tested only α-MSH (1 μmol/kg) statistically significantly inhibited the formalin-induced first phase pain response, however, all tested peptides (except γ1-MSH) at the dose of 1 μmol/kg produced a pronounced inhibitory effect on nociceptive behavior in the second phase and this activity was comparable with that of indomethacin (reference drug, 5 mg/kg intraperitoneally); β-MSH was also active at a dose 0.1 μmol/kg. In the tail flick test, α-MSH (1 μmol/kg) showed algesic, whereas HS014 (0.5 μmol/kg) and indomethacin (10 mg/kg) exerted analgesic activity. Other peptides did not exert any activity in the tail flick test.

These data indicate that peripherally administered melanocortin receptor agonists α-MSH, β-MSH and γ2-MSH, as well as MC3/4 receptor antagonist HS014 induced antinociception on pain/inflammatory events caused by formalin suggesting a predominant anti-inflammatory role of these peptides.

Keywords: MSH peptides; Melanocortins; Nociception; HS014; Formalin test; Tail flick test; Pain; Inflammation

1. Introduction

Melanocortins (melanocyte stimulating hormones) – α-MSH, β-MSH, γ1-MSH, γ2-MSH – and adrenocorticotropin are released in the CNS and periphery where they exert multiple physiological effects that include modulation of immune and behavioral responses, inflammation processes, and pain transmission (for reviews see: Wikberg et al., 2000; Catania et al., 2004). The effects of α-MSH, adrenocorticotropin and their fragments have been characterized in various pain models a long time before the melanocortin receptors were cloned and characterized, and the overall conclusion from these studies was that melanocortin receptor antagonists cause analgesia, whereas agonists, such as α-MSH and adrenocorticotropin, induce hyperalgesia in rats after their intracerebroventricular (i.c.v.) administration (Bertolini et al., 1979; Sandman and Kastin, 1981; Poole et al., 1992).

Although all melanocortins act as melanocortin receptor agonists, more detailed investigations have revealed some differences in their nociceptive or antinociceptive action depending on the route of the peptide administration, dosage interval, and pain inducer applied. For example, in the tail flick test, the intracisternal (i.c.) administration of α-MSH in mice induced a short hyperalgesia; and contrary, γ-MSHs acted as analgesic peptides: γ1-MSH caused a short-acting analgesia, whereas γ2-MSH caused a stable and long-term analgesia (Klusa et al., 2001). Unexpectedly, γ2-MSH analgesia was shown to be provided *via* non-melanocortin – GABA-A – receptor-mediated pathways (Klusa et al., 2001). Non-melanocortinergic mechanism of the action of the γ2-MSH was also demonstrated by other authors (Grazzini et al., 2004), showing that γ2-MSH binds to the rat sensory neuron-specific receptor type 1 and evokes

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spontaneous nociceptive behavior in paw thermal sensitivity assay after intradermal administration. In the same assay, γ1-MSH was less potent, whereas α-MSH, β-MSH and ACTH were inactive (Grazzini et al., 2004).

Several research groups have reported contradictory data on both analgesic and algesic properties of α-MSH in different pain tests. Thus, α-MSH caused analgesia in hot-plate test after i.c.v. administration in mice, on the one hand (Ohkubo et al., 1985), and hyperalgesia and reversion of the analgesic effects of morphine and β-endorphin, on the other hand (Gispen et al., 1976; Contreras and Takemori, 1984). After intraperitoneal (i.p.) administration in rats, α-MSH inhibited hyperalgesic responses caused by intraplantar injection of the interleukin-1β or prostaglandin E2 (Poole et al., 1992), however, its intravenous administration did not inhibit the prostaglandin E2-evoked hyperalgesia in the hot plate test (Follenfant et al., 1989).

Our previous studies devoted to the comparative examination of the melanocortins injected i.c. in an experimental model of brain inflammation in mice, demonstrated the anti-inflammatory action of melanocortins. Among the natural melanocortins β-MSH was the most active anti-inflammmatory agent (Muceniece et al., 2004). These data gave us enough background to focus our studies on a pain model which involves both nociceptive and inflammatory processes. Therefore, the formalin test as a suitable experimental model involving both components (Tjolsen et al., 1992; Bellasio et al., 2003) is used in the present studies. Indomethacin (i.p.), a well known non-steroidal anti-inflammatory drug, that relieves the pain, tenderness, and inflammation (swelling), was used as a reference drug. It was shown earlier that indomethacin induced a reduction of the animal paw licking pain behavior response in the second phase of the formalin test (Tjolsen et al., 1992). Besides, indomethacin was shown also to exert antinociceptive effect in the tail flick test in mice after i.p. administration (Pakulska and Czarnecka, 2002). In addition, the tail flick test was chosen as a pain model widely used in previous studies of the effects of melanocortins (Sandman and Kastin, 1981; Klusa et al., 2001; Starowicz et al., 2005). Natural melanocortin receptor agonists – α-MSH, β-MSH, γ1-MSH, γ2-MSH – were investigated after their s.c. administration in male ICR mice. For comparison, the MC3/4 receptor antagonist HS014 (s.c.) was used.

2. Materials and methods

2.1. Chemicals

Paraformaldehyde solution (37%), indomethacin, α-MSH, β-MSH, γ1-MSH, γ2-MSH and HS014 were purchased from Sigma-Aldrich, Bachem.

2.2. Animals

Male ICR mice (from the Laboratory Animals Breeding Facility, Riga Stradins University, Riga, Latvia) weighing 23–25 g were housed under standard conditions (21–23 °C, 12 h light–dark cycle) with unlimited access to standard food and water. All experimental procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

2.3. Formalin test

Formalin-induced licking paw test was adopted from the method described by Bellasio et al. 2003. All animals received the drug or saline pre-treatment 30 min prior to formalin injection. Peptides (α-MSH, β-MSH, γ1-MSH, γ2-MSH and HS014) were administered subcutaneously (s.c.) at the doses 0.1 and 1 μmol/kg and control mice received s.c. saline. Indomethacin was administered 5 mg/kg intraperitonialy (i.p.) as the reference drug. Mice were gently restrained and 30 μl of formalin solution (1.5% in saline) was injected s.c. into the plantar surface of the right hind paw, using a microsyringe with a 27-gauge needle. Each mouse was then placed in an individual clear Plexiglas observation chamber (30×20×30 cm) and the total licking time of hind paw of each mouse was registered with a stopwatch and quantified in subsequent 5-min intervals for 60 min. All assessments were carried out by blind to the treatment observers. The recording of licking time started immediately (first phase) and lasted for 5 min. The late phase (second phase) started about 15–20 min after formalin injection and lasted up to 35 min.

2.4. Tail-flick test

The tail flick test was carried out in accordance to the method described elsewhere (Dewey, 1981), with minor modification. In brief, the tail of the mouse was placed on the photo element window of a Tail flick apparatus (Model DS20, Hugo Basile, Italy) and an infrared beam was focused on the tail area, 2 cm from its base. The latency time to respond to the pain stimuli was recorded. To avoid tissue damages, the maximal exposure to the pain stimuli was restricted to 15 s. The tail flick response was tested 15, 30 and 60 min after the melanocortin administration at the doses of 1 μmol/kg and HS014 at the doses of 0.5 and 1 μmol/kg. Control mice received s.c. saline. Indomethacin was administered at doses of 5 mg/kg and 10 mg/kg i.p.

2.5. Data analysis

Data from the formalin and tail flick test are expressed as the mean nociceptive response time (s)±S.E.M, and inter-group statistics was analyzed by one-way ANOVA followed by post hoc Dunnett test using a commercial computer program GraphPad Prism. Data from the second phase of formalin test were expressed as the area under curves (AUCs). Statistical significance was set at $P<0.05$.

3. Results

3.1. Formalin test

The intraplantar injection of 1.5% formalin solution (with saline pre-treatment) into the hind paw caused an acute,

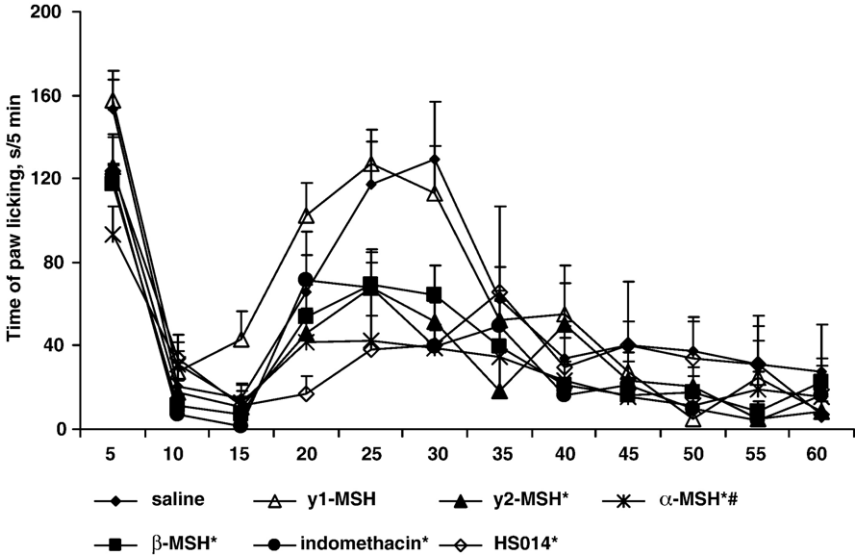

Fig. 1. Analgesic effect of the melanocortins (1 μmol/kg, s.c.) and HS014 (1 μmol/kg, s.c.) in the formalin test in mice. Melanocortins, HS014 and indomethacin (5 mg/kg, i.p.) were injected 30 min before intraplantar formalin injection. Licking of the injected paw was recorded as the total time (s) per 5 min for a total duration of 60 min. Data are calculated as means ± S.E.M. for 7–8 animals per group. #$P<0.05$ vs. control group in the first phase. *$P<0.05$ vs. control group in the second phase.

immediate nociceptive response, i.e., licking of the injected paw, which lasted for 5 min (early phase), followed by a later phase starting 15–20 min after formalin injection and lasting for an additional 25–35 min (Fig. 1). After the pre-treatment with melanocortins (s.c.) or HS014 at the dose 1 μmol/kg s.c., or indomethacin at 5 mg/kg i.p. prior to formalin injection, only α-MSH statistically significantly inhibited the formalin-induced response in the first phase (Fig. 1). As can be seen from the data expressed as AUCs, all peptides (natural and synthetic HS014) at dose 1 μmole/kg (with exception of γ1-MSH) and indomethacin caused reduction of the nociceptive behavior in the second phase (Fig. 2). β-MSH (but not other peptides) showed this activity already at the dose of 0.1 μmol/kg.

3.2. Tail flick test

In the tail flick test, the latency time of the pain response for control mice was about 5–7 s (Fig. 3). Among all tested peptides, only α-MSH at the dose 1 μmol/kg possessed algesic activity after 30 and 60 min following s.c. administration, whereas HS014 at the dose 0.5 μmol/kg exerted short-term (only after 15 min following administration) analgesic activity (Fig. 3). The reference

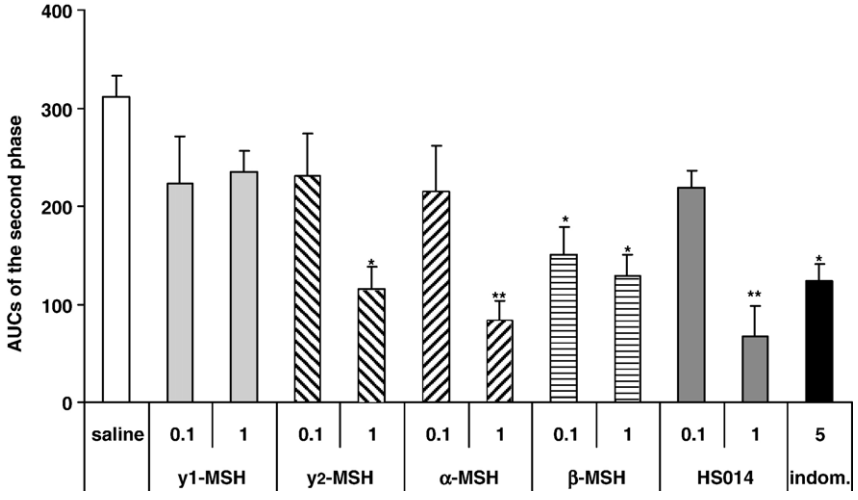

Fig. 2. Antinociceptive effect of melanocortins, HS014 and indomethacin in the formalin test in mice. Melanocortins, HS014 (both at dose 0.1 and 1 μmol/kg, s.c.) and indomethacin 5 mg/kg i.p. were injected 30 min before intraplantar formalin injection. Formalin (1.5%; 30 μl) was injected s.c. into the plantar surface of the right hind paw of the mouse. Time spent licking the paw was considered as a measure of nociceptive intensity. Areas under curves (AUCs) of the second phase were calculated from 15 to 30 min after formalin injection. Bars represent means ± S.E.M. for 7–8 animals per group. *$P<0.05$ vs. saline-treated group. **$P<0.01$ vs. saline-treated group.

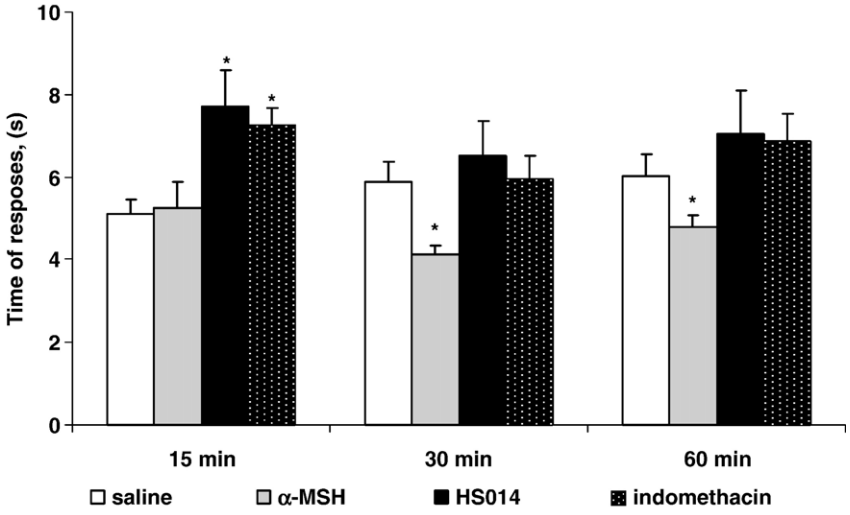

Fig. 3. Effect of the α-MSH (at dose 1 μmol/kg, s.c.), HS014 (at dose 0.5 μmol/kg, s.c.) and indomethacin (10 mg/kg) in the tail-flick test in mice. Antinociception was recorded for 15, 30 and 60 min after the s.c administration of the melanocortins and HS014 as well as i.p. indomethacin. *$P<0.05$ vs. saline-treated group.

drug indomethacin 5 mg/kg did not show analgesic activity whereas at dose 10 mg/kg had a similar antinociceptive effect to HS014. Other peptides tested were ineffective.

4. Discussion

The present study was designed to investigate the effects of melanocortins on nociception after their peripheral (s.c.) administration in mice in two different pain models. The formalin test was chosen as a simple two-phase model of tonic pain and localized inflammation (Tjolsen et al., 1992). In addition, the tail flick test was applied as a model that has been used widely to study central algesic/analgesic action of melanocortins (Sandman and Kastin, 1981; Klusa et al., 2001; Starowicz et al., 2005).

In the first phase of the formalin test, the only peptide which influenced nociceptive behavior in mice was α-MSH which exerted the antinociceptive effect; other peptides and indomethacin, a reference drug were inactive. However, in the second phase of the formalin test all melanocortin receptor agonists (except γ1-MSH) and MC3/4 receptor antagonist HS014 acted as antinociceptive agents with a potency order: β-MSH > α-MSH = HS014 > γ2-MSH. Although formalin test is not a novel pain model, up to now it was used only for testing melanocortin receptor synthetic agonist melanotan II (MTII) and several melanocortin receptor antagonists (SHU9119, HS014, AgRP, and JKC-363) after their intrathecal and i.c.v. administrations (Bellasio et al., 2003). It was found that MTII increased nociception in both phases of the formalin test, whereas antagonists reduced the pain responses only in the second phase (Bellasio et al., 2003), thus confirming the general assumption that after central (intrathecal or i.c.v.) administration melanocortin receptor agonists induce algesic effects whereas antagonists act as analgesic agents.

Our results showed that activities of melanocortin receptor agonists after their peripheral administration differ from those following central injections. Thus, the agonist α-MSH had an analgesic effect in both phases of the formalin test, whereas other agonists β-MSH and γ2-MSH were active only in the second phase. At the same time γ1-MSH was inactive in both phases of formalin test. Although in the previous studies (Bellasio et al., 2003) it was found that HS014 (0.5–5 nmol/mouse i.t. and i.c.v.) showed tendency to exert antinociceptive effect, at the corresponding dosage interval it was not statistically significant. Our studies with higher HS014 doses (up to the 1 μmol/kg s.c.) demonstrated statistically significant antinociceptive effect in the second phase of the formalin test. That is in good agreement with the earlier reported central analgesic activity of the melanocortin receptor antagonists (Bellasio et al., 2003). Thus, comparison of melanocortin agonists (α-, β- and γ2-MSH) and antagonist (HS014) in our studies have revealed that there is no difference between their influence on nociceptive behavior in the second phase of the formalin test, since administered peripherally they acted as pain-alleviating substances, suggesting that a common, probably non-melanocortin-ergic mechanism underlies this activity. Since the second phase of the formalin test more closely models the events leading to chronic pain and the development of inflammation (paw edema and central sensitization) by further inducing of the overproduction of inflammatory substances, such as cytokines, prostaglandins, NO (Hassanzadeh and Ahmadiani, 2006; Chen et al., 1999; Shibata et al., 1989), pain-reducing effects obtained in our studies allow to suggest that melanocortins injected peripherally may play a role of anti-inflammatory agents. Besides, melanocortins (with exception of γ1-MSH) behaved similarly to indomethacin which has been shown to reduce the pain behavior response in the second phase of the formalin test. This activity of the indomethacin was already described earlier (Tjolsen et al., 1992) and explained by inhibition of LPS-induced NO release, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression and NF-KB translocation in nuclei in RAW264.7 macrophage cell line (Chen et al., 1999). Therefore, we suggest that pain/inflammation modulating effect of the melanocortin agonists may have common mechanisms to

those characteristic for indomethacin. One of such possible common mechanisms could be the inhibition of the NO overproduction. These suggestions are in good agreement with our recent data (Muceniece et al., 2004) demonstrating that α-, β- and γ-MSHs (injected intracisternally in mice) considerably inhibited the NO overproduction induced by LPS in brain tissue *ex vivo*.

If formalin test has revealed some common components of action of melanocortin agonists (except γ1-MSH), MCR3/4 antagonist HS014 and indomethacin, the tail flick test showed diversity of effects caused by these substances. Interestingly, α-MSH, the only natural peptide, which showed algesic activity in tail-flick test after its peripheral s.c. administration (present data), that coincided with its central (i.c.) algesic activity (Klusa et al., 2001), displays analgesic activity in both phases of the formalin test. Obviously both the route of administration and different pain stimuli — thermal (tail-flick) and chemical (formalin) play an important role in realization of α-MSH activities.

The question about participation of specific melanocortin receptors in these activities remains to be elucidated, however one may suggest that they are involved to a much greater extent in α-MSH-induced algesic effects (tail-flick) than in analgesic (formalin test), particularly in the second phase of formalin test where all peptides (with exception of γ1-MSH) showed a common-analgesic action. Earlier in the experiments with the synthetic peptide MTII it was already shown that MTII algesic effects in both phases of the formalin test where blocked by the melanocortin receptor antagonists (Bellasio et al., 2003). At the same time neither γ1- nor γ2-MSH did show peripheral analgesic effect that contrasts to their analgesic activity observed in the tail flick test after i.c. administrations (Klusa et al., 2001), when γ1-MSH caused a mild and short-term analgesia, whereas γ2-MSH demonstrated stable and prolonged analgesia. Moreover, γ2-MSH-induced analgesia was prevented by previous administration of GABA-A receptor antagonist bicuculline and potentiated by muscimol, an agonist of this receptor, suggesting that γ2-MSH analgesia is essentially mediated via GABA-A-ergic processes (Klusa et al., 2001). Therefore we suggested that non-melanocortinergic mechanisms might appear at the central level of pain perception. After peripheral administration γ2-MSH might be metabolized to a form which is inactive in the tail-flick test or here used dose of γ2-MSH might be not comparable with that following central administration. Unlike, synthetic peptide HS014 showed short-term peripheral analgesia that was comparable to that of indomethacin in the tail flick test, indicating different molecular events involved in their action in comparison to other melanocortins.

Summing up the data obtained, one may consider that peripheral (s.c.) administration of melanocortin receptor agonists α-MSH, β-MSH, and γ2-MSH are capable of producing a predominant analgesic activity in the second phase of formalin test. β-MSH was the most active among all tested melanocortins, since its activity was observed at a dose of 0.1 μmol/kg vs. 1 μmol/kg for other peptides. γ1-MSH was ineffective in all tests, and the synthetic cyclic peptide HS014, an MCR3/4 antagonist, acted similarly to natural melanocortins in suppressing pain in the second phase of formalin test, and similarly to indomethacin in tail flick test. One may suggest that the diversity of the pain modulating activities of the melanocortin receptor ligands might indicate their different pharmacokinetics and crosstalks with non-melanocortin-ergic pathways.

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