Dissection of the Anti-Inflammatory Effect of the Core and C-Terminal (KPV) α-Melanocyte-Stimulating Hormone Peptides

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ABSTRACT

In this study, we analyzed the anti-inflammatory effects of α-melanocyte stimulating hormone (MSH)11–13 (KPV) in comparison with other MSH peptides in a model of crystal-induced peritonitis. Systemic treatment of mice with KPV, α-MSH, the core melanocortin peptide His-Phe-Arg-Trp, and the melanocortin receptor 3/4 agonist Ac-Nle4-c[Asp5,D-Phe7,Lys10]NH2 ACTH4-10 (MTII) but not the selective MC1-R agonist H-Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH2 (MS05) resulted in a significant reduction in accumulation of polymorphonuclear leukocyte in the peritoneal cavity. The ant-inflammatory effect of KPV was not blocked by the MC3/4-R antagonist Ac-Nle4-c[Asp5,D-Phe7,Lys10]NH2 (SHU9119). In vitro, macrophage activation, determined as release of KC and interleukin (IL)-1β was inhibited by α-MSH and MTII but not by KPV. Furthermore, macrophage activation by MTII led to an increase in cAMP accumulation, which was attenuated by SHU9119, whereas KPV failed to increase cAMP. The anti-inflammatory properties of KPV were also evident in IL-1β-induced peritonitis inflammation and in mice with a nonfunctional MC1-R (recessive yellow e/e mice). In conclusion, these data highlight that the C-terminal MSH peptide KPV exhibits an anti-inflammatory effect that is clearly different from that of the core MSH peptides. KPV is unlikely to mediate its effects through melanocortin receptors but is more likely to act through inhibition of IL-1β functions.

The pro-opiomelanocortin gene product undergoes post-translational processing to form the endogenous ligands of the melanocortin receptors [α-, β-, γ-melanocyte stimulating hormone (MSH)] and adrenocorticotropin (ACTH), which all contain the common amino acid motif His-Phe-Arg-Trp (HFRW) tetrapeptide (Wikberg et al., 2000; Getting, 2002). Five melanocortin receptors (MC-Rs) have been cloned and are positively coupled to adenylate cyclase, thus receptor activation leads to increases in intracellular cAMP (Wikberg et al., 2000; Getting, 2002). These endogenous peptides are endowed with anti-inflammatory properties, including inhibition of tumor necrosis factor-α, interleukin (IL)-1, and the CXC chemokine KC release (Getting, 2002) as well as adhesion molecule expression (Kalden et al., 1999). This is possibly due to their ability to inhibit nuclear transcription factor-κB activation (Manna and Aggarwal, 1998; Kalden et al., 1999) and protection of IκBα degradation (Ichiyama et al., 1999), thus affecting the humoral and cellular phases of inflammation (Hiltz and Lipton, 1989; Lipton and Catania, 1998). These anti-inflammatory properties have been highlighted in several experimental models of acute and chronic inflammation (for a recent review, see Getting, 2002).

At present, there is a lot of confusion within the field of whether a single MC-R mediates the anti-inflammatory effects of melanocortin peptides. One of the receptors, MC1-R, has long been regarded as the receptor responsible for the anti-inflammatory effects of α-MSH and related peptides (Wikberg et al., 2000), whereas more recently we have proposed a central role for MC3-R (Getting et al., 1999, 2001). The MC1-R mRNA, but not protein, expression has been found in an array of cells, including monocytes, B-lymphocytes, NK cells, a subset of cytotoxic T cells (Neumann Andersen et al., 2001), dendritic cells (Becher et al., 1999) as well as mast cells (Adachi et al., 1999). The expression of MC3-R mRNA and protein has been detected in rodent peritoneal and knee joint macrophages (MØ). Importantly, the receptor is functional because its activation leads to cAMP accumulation. In a series of inflammatory models, the selectively agonists have been shown to down-regulate the host inflammatory response, and this inhibition was abrogated in the presence of MC3-R, but not MC4-R antagonists (Getting et al., 1999, 2001, 2002).

ABBREVIATIONS: MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropin; MC-R, melanocortin receptor; IL, interleukin; MØ, macrophage; MSU, monosodium urate; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PMN, polymorphonuclear leukocyte.
Different fragments of the anti-inflammatory melanocortin peptide α-MSH have been investigated for their efficacy, including the core region of α-MSH_{1-10} (MEHFRWG) and the C-terminal peptide α-MSH_{11-13} (KPV). α-MSH_{4-10}, identical to ACTH_{4-10}, has been shown to inhibit prostaglandin E_{1} generation and edema formation in rat skin (Gecse et al., 1980) and PMN migration and IL-1β and KC release in a model of crystal-induced inflammation (Getting et al., 1999). KPV (α-MSH_{11-13}) has been reported to reduce experimental pyresis while the core region was inactive (Richards and Lipton, 1984). KPV can also inhibit carrageenan-induced edema formation in the mouse (Hiltz and Lipton, 1990). A similar observation was noted by the same group in a model of picryl chloride in the mouse (Hiltz and Lipton, 1989) and endogenous pyrogen injected into the mouse paw (Hiltz et al., 1992). A potential mechanism of action for KPV, in analogy to that reported for α-MSH, is its ability to inhibit nuclear factor-κB activation (Mandrika et al., 2001), potentially leading to inhibition of proinflammatory cytokine synthesis.

There is a great interest from pharmaceutical companies to exploit the potent anti-inflammatory effects of the melanocortins. However, there has been much confusion regarding the mechanism behind the effects of the different MSH peptides and receptors. In this study, we systematically investigated the anti-inflammatory effects of core and C-terminal MSH peptides to understand the molecular mechanisms underlying the efficacy of these peptides. We have used an integrated approach taking advantage of recent selective MC-R antagonists, as well as a strain of mice (recessive yellow e/e; Robbins et al., 1993) without a functional MC1-R.

The effects of other melanocortin peptides were also studied for comparative purposes.

### Materials and Methods

#### Animals

Male C57 Bl.6 mice (20–22 g b.wt.) were purchased from Tuck (Battlesbridge, Essex, UK) (20–22 g b.wt.), whereas the recessive yellow e/e mouse strain mice (Robbins et al., 1993) was a kind gift from Dr. Nancy Levin (Trega Bioscience, San Diego, CA). Mice were maintained on a standard chow pellet diet with tap water ad libitum.

#### Inflammation Models

Crystal peritonitis was induced by injection of 3 mg of monosodium urate (MSU) crystals in 0.5 ml of phosphate-buffered saline (PBS) as reported previously (Getting et al., 1997). At the 6-h time point, animals were killed by CO_{2} exposure and peritoneal cavities were washed with 3 ml of PBS containing 3 mM EDTA and 25 units ml^{-1} heparin. Aliquots of lavage fluid were then stained with Turk’s solution, and differential cell counts were performed using a Neubauer hemocytometer and a light microscope (B061; Olympus, Tokyo, Japan). Lavage fluids were then centrifuged at 400g × 10 min, and supernatants were stored at −20°C before several biochemical determinations. In another set of experiments, mice were treated i.p. with 10 ng of murine recombinant IL-1β (provided by Dr. R. C. Newton, DuPont, Wilmington, DE), peritoneal cavities were lavaged 4 h later and PMN accumulation quantified as described above.

#### ELISA Measurements

Murine IL-1β and KC levels in the lavage fluids were quantified with Quantikine ELISA purchased from R & D Systems (Oxfordshire, UK). The ELISAs showed negligible (<1%) cross-reactivity with several murine cytokines and chemokines (data as furnished by the manufacturer).

### Drug Treatment

The melanocortin peptides Ac-Nle^{4-6}c[Asp^{5},D-Phe^{7},Lys^{10}]NH_{2} ACTH4-10 (MTI; 9.3 nmol) (Al-Obeidi et al., 1989), α-MSH (6 nmol), KPV (3–88 nmol), H-Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_{2} (MS05; 0.66–66 nmol) (Szardenings et al., 2000; Getting et al., 2003), α-MSH_{6-9} (HFRW; 104 nmol), or PBS (100 μl) was administered s.c. either alone or in combination with the MC3/4-R antagonist Ac-Nle^{4-6}c[Asp^{5},D-Phe^{7},Lys^{10}]NH_{2} ACTH4-10 (SHU9119; 9 nmol) (Hruby et al., 1995). MSU crystals were given i.p. 30 min later. In separate experiments, α-MSH (6 nmol), KPV (9 nmol), HFRW (104 nmol), MS05 (6.6 nmol), and MTII (9.3 nmol) were administered s.c. 30 min before IL-1β. Doses were selected from our previous studies and from preliminary dose-response curves (Getting et al., 1999, 2003).

Figure 1 illustrates the primary sequences of some of the peptides used. MTII, HFRW, α-MSH, and SHU9119 were purchased from Bachem (Saffron Walden, Essex, UK), whereas MS05 and KPV were kindly provided by Melacure Therapeutics AB (Uppsala, Sweden). All peptides were stored at −20°C before use and dissolved in sterile PBS (pH 7.4).

#### In Vitro MØ Activation

##### Primary MØ Culture

A rich population (>95% pure) of peritoneal MØ (5 × 10^{5}cells/well) was prepared by 2-h adherence at 37°C in 5% CO_{2}, 95% O_{2} atmosphere in RPMI 1640 medium + 10% fetal calf serum. Nonadherent cells were then washed off, and adherent cells (>95% MØ) were incubated with the reported peptides for 15 min in RPMI 1640 medium. Cells were then stimulated with 1 mg/ml MSU crystals (a concentration chosen from preliminary experiments), and the cell-free supernatants collected 2 h later (Getting et al., 1999, 2003).
Intracellular cAMP Accumulation. MØs (1 × 10⁶) were allowed to adhere for 2 h at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. MØs were then incubated with serum-free RPMI 1640 medium containing 1 mM isobutylmethylxantine and MTII (9.3 µM), KPV (3–88 µM), MS05 (6.6 µM), or the direct adenylate activator forskolin (3 µM); all were dissolved in PBS. In selected wells, MTII was incubated with the MC3/4-R antagonist SHU9119 (9 µM). In all cases after 30 min at 37°C, supernatants were removed, and cells washed and lysed. cAMP levels in cell lysates were determined with a commercially available enzyme immunoassay (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) using a standard curve constructed with 0 to 3200 fmol/ml cAMP.

Statistics

Data are reported as mean ± S.E. of n distinct observations. Statistical differences were calculated on original data by analysis of variance followed by Bonferroni’s test for intergroup comparisons (Berry and Lindgren, 1990) or by unpaired Student’s t test (two-tailed) when only two groups were compared. A threshold value of P < 0.05 was taken as significant.

Results

Evaluation of the Effect of Melanocortin Peptides in a Model of Crystal Peritonitis. The effect of KPV (3–88 nmol) and the selective MC1-R agonist MS05 (0.66–66 nmol) was evaluated in the MSU crystal peritonitis model. KPV inhibited PMN migration with a bell-shaped dose response. A maximal inhibition was seen at 9 nmol with a reduction of 42% of PMN migration (10⁶/mouse) from 7.14 ± 0.94 to 4.17 ± 0.46 × 10⁶ (n = 11, P < 0.05 versus PBS control). At the effective dose of 6 nmol (Getting et al., 1999) of α-MSH (6 nmol) caused 33% reduction in PMN migration (P < 0.05 versus PBS control; Fig. 2A).

Exudate levels of the CXC chemokine KC were measured to ascertain whether the antimigratory effect was coupled to attenuation of the release of this mediator. KPV (9 nmol) caused a significant reduction in KC levels from 161 ± 31 to 95 ± 13 pg/ml (−41%, n = 11, *P < 0.05 versus PBS control). A comparable degree of inhibition was observed after α-MSH treatment, whereas higher doses of KPV did not modify KC levels (Fig. 2B). The selective MC1-R agonist MS05 (0.66–66 nmol), which also contains a KPV region, failed to inhibit PMN migration at any of the doses tested. However, MTII (9.3 nmol), which is a substituted cyclic peptide of the core region α-MSH₄₋₁₀, reduced PMN migration by 35% (*P < 0.05, n = 6 versus PBS control) (Fig. 2C). The peptide HFRW corresponding to α-MSH₄₋₁₀ was also found to inhibit PMN migration by ~50% (*P < 0.05, n = 6 versus PBS control), and this effect was blocked in the presence of the MC3/4-R antagonist SHU9119 (Fig. 2D). At variance from several other melanocortin peptides (Getting et al., 1999, 2001), KPV retained anti-inflammatory activity when co-injected with an equimolar dose of the MC3/4-R antagonist SHU9119 (Fig. 2D).

In Vitro Effects of Melanocortin Peptides on Chemokine and Cytokine Release from Cultured Macrophages. We have previously proposed the resident MØ as the cellular target for the action of melanocortin peptides.

Fig. 2. Anti-inflammatory effects of melanocortin peptides in urate induced inflammation. Mice were treated s.c. with KPV (3–88 nmol), α-MSH (6 nmol), or PBS (100 µl) 30 min before i.p. injections of MSU crystals (3 mg in 0.5 ml of sterile PBS) on PMN migration (A) and KC release (B) as assessed at 6-h time point. C, mice were treated s.c. with MS05 (0.66–66 nmol), MTII (9.3 nmol), or PBS (100 µl), 30 min before i.p. injections of MSU crystals, and PMN migration was assessed at 6-h time point. Data are mean ± S.E. of n = 6 mice/group. *, P < 0.05 versus control group. D, lack of effect of SHU9119 on the antimigratory actions of KPV. Mice were treated s.c. with KPV (9 nmol), HFRW (104 nmol), or PBS (100 µl) alone or in combination with 9 nmol of i.p. SHU9119, 30 min before i.p. injections of MSU crystals (3 mg), and PMN migration was assessed at the 6-h time point. Data are mean ± S.E. of n = 8 mice/group. *, P < 0.05 versus control group (no antagonist).
Thus, the in vivo experiments were complemented with the analysis of KPV effects in assays of MØ activation in vitro. Adherent cells were incubated with KPV (3–88 μM), MS05 (0.66–66 μM), MTII (9.3 μM), and α-MSH (6 μM). KPV and MS05 failed to inhibit crystal induced release of KC or IL-1β at any concentration tested. In contrast, MTII reduced KC release (−46%, *P < 0.05; Fig. 3A) and IL-1β (51%, *P < 0.05; Fig. 3B). A similar degree of inhibition was measured after cell incubation with α-MSH (Fig. 3, A and B).

**Receptor Functionality.** Determination of receptor functionality was quantified by measuring cAMP accumulation in peritoneal MØ. Forskolin (3 μM) and MTII (9.3 μM) caused a significant increase in cAMP accumulation with MTII causing a 450% increase above basal values (146 ± 22 fmol/well) (Fig. 4). This increase in cAMP was blocked in the presence of the MC3/4-R antagonist SHU9119 (9 μM) (Fig. 4). MS05 (6.6 μM) and KPV (3–88 μM) failed to elicit any detectable increase in cAMP accumulation in MØ (Fig. 4).

**Effect of KPV on MSU Crystal-Induced Inflammation in Recessive Yellow (e/e) Mice.** KPV anti-inflammatory effects were then investigated in mice with a nonfunctional MC1-R (recessive yellow e/e mice). KPV inhibited PMN migration by 32 and 35% at the dose of 3 and 9 nmol, respectively (Fig. 5A). This inhibition was not associated with a reduction in exudates levels of KC (Fig. 5B) or IL-1β (Fig. 5C).

**Effect of Melanocortin Peptides in IL-1β-Mediated Inflammation.** Some reports have linked KPV anti-inflammatory actions to blockade of IL-1β effects (Uehara et al., 2002).

![Fig. 3. Effect of melanocortin peptides on KC and IL-1β release in primary cultured MØ. KPV (3–88 μM, open circles), MS05 (0.66–66 μM, filled squares), α-MSH (6 μM), MTII (9.3 μM), or PBS (dotted line) were added to adherent peritoneal MØ (5 × 10⁵) prepared from C57 Bl.6 mice, 30 min before stimulation with 1 mg/ml MSU crystals. Supernatants were removed 2 h later and cell-free aliquots analyzed for chemokine KC (A) and cytokine IL-1β (B) content using specific ELISA. Data are mean ± S.E. of n = 4 determinations. *, P < 0.05 versus relevant PBS control.](https://www.aspetjournals.org/journals/jpet/2017/636/12/)

![Fig. 4. MC-R activation in peritoneal MØ collected from C57 Bl.6 mice. Adherent peritoneal MØ (1 × 10⁵) were incubated with KPV (3–88 μM, closed square), MTII (9.3 μM), alone or in the presence of the MC3/4-R antagonist SHU9119 (9 μM). Data are mean ± S.E. of n = 4 determinations. *, P < 0.05 versus vehicle control.](https://www.aspetjournals.org/journals/jpet/2017/636/12/)

![Fig. 5. Effect of KPV on MSU crystal-induced PMN migration, KC and IL-1β release in recessive yellow (e/e) mice. Mice were treated s.c. with KPV (3–88 nmol) or PBS (100 μl), 30 min before i.p. injections of MSU crystals (3 mg). PMN migration (A) was assessed at the 6-h time point, and lavage fluids were analyzed for KC (B) and IL-1β (C) content by commercially available ELISA. Data are mean ± S.E. of n = 6 mice/group. *, P < 0.05 versus control group.](https://www.aspetjournals.org/journals/jpet/2017/636/12/)
Anti-inflammatory effects of KPV and MS05 in IL-1β-induced inflammation in recessive yellow e/e mice

Mice were treated s.c. with KPV (9 nmol), MS05 (6.6 nmol), or PBS (100 μl) 30 min before i.p. injections of IL-1β (10 ng in 0.5 ml of sterile PBS); PMN migration and KC release were assessed at the 4 h timepoint. Data are mean ± S.E. of n = 5 mice/group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PMN (10^6 per mouse)</th>
<th>KC (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4.2 ± 0.4</td>
<td>239.2 ± 37.7</td>
</tr>
<tr>
<td>KPV</td>
<td>3.2 ± 0.3*</td>
<td>251.2 ± 54.8</td>
</tr>
<tr>
<td>MS05</td>
<td>2.7 ± 0.3*</td>
<td>255.0 ± 26.8</td>
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</tbody>
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* P < 0.05 versus PBS group.

Discussion

There is a long-standing interest in understanding the molecular mechanisms responsible for the melanocortin peptide anti-inflammatory actions, which will potentially lead to the development of new therapeutics (Getting, 2002). Therefore, in this study, we have sought to determine whether the anti-inflammatory effects of this tripeptide are mediated via MC1-R or MC3-R, in analogy to the actions of other melanocortin peptides previously investigated.

In the crystal peritonitis model, α-MSH and MTII caused a significant reduction in PMN migration and associated chemokine release, confirming previous findings (Getting et al., 1999, 2001, 2002). KPV treatment caused a bell-shaped dose-response curve with maximal inhibition of PMN migration and KC release occurring at 9 nmol. These data are in agreement with previous findings because both α-MSH and β-MSH produced a bell-shaped inhibitory effect (Getting et al., 1999). Also, KPV inhibition of urate inflammation augments the list of models in which this tripeptide has been shown to inhibit inflammation elicited by irritants such as carrageenan (Hiltz and Lipton, 1990), picryl chloride (Hiltz and Lipton, 1989) as well as by endogenous pyrogen (Hiltz et al., 1992).

To gain some information on the MC-R potentially involved in these actions, the effect of the selective MC1-R agonist MS05 (Szardenings et al., 2000) was evaluated, finding that it was inactive in this model. Interestingly, MS05 has been found to be inactive in models of white blood cell recruitment and consequent tissue injury (Guarini et al., 2002; Getting et al., 2003).

To highlight a potential MC-R activation, we evaluated in vivo the effects of KPV in the presence of receptor antagonists and also in recessive yellow e/e mice, which lack a functional MC1-R (Robbins et al., 1993). KPV retained anti-inflammatory activity in mice pretreated with the MC3/4-R antagonist SHU9119. Importantly, the same occurred in recessive yellow e/e mice, together, these data would suggest that KPV exhibits an anti-inflammatory effect that does not involve either MC1, 3, or 4-R. This inability to function at these MC-Rs is in agreement with previous results showing that KPV was inactive on MC1-R expressed on a RAW264.7 MØ cell line (Mandrika et al., 2001).

Searching for MC-R-independent effects of KPV, we used IL-1β-induced peritonitis. In fact, this tripeptide shows its exclusive ability to interfere with IL-1β binding to its own receptor (type I) (Mugridge et al., 1991), which drives the neutrophil accumulation process (Perretti and Flower, 1993). A potential explanation for this is given by the fact that the tripeptide KPV is structurally similar to an antagonist of the IL-1 receptor, the peptide KPT (Ferreira et al., 1988), which inhibits the development of new therapeutics (Getting, 2002). Therefore, in this study, we have sought to determine whether the anti-inflammatory effects of this tripeptide are mediated via MC1-R or MC3-R, in analogy to the actions of other melanocortin peptides previously investigated.
the KPV sequence. Interestingly, this set of data is in agreement with previous work in which α-MSH was shown to inhibit IL-1α-induced migration of neutrophils into subcutaneous sponges (Mason and Van Epps, 1989) whereas the effect observed with MS05 is novel. It is of interest that the ability of KPV to antagonize the effects of IL-1β has also been reported in IL-1-induced anorexia (Uehara et al., 1992) and hyperalgesia (Follenfant et al., 1989). The lack of effect of HFRW on IL-1β experiments confirms the lack of MC-R activation and would suggest that peptides that do not contain KPV exert their anti-inflammatory effect by inhibiting the release of chemokines and cytokines rather than their action.

Primary culture of murine MØ (Getting et al., 1999) was used to measure release of the CXC chemokine KC and cytokine IL-1β in response to urate crystal application. This in vitro model of MØ activation is susceptible to inhibition by the melanocortin peptides, including MTII and γ2-MSH (Getting et al., 1999, 2001). As previously observed (Getting et al., 1999, 2001), MTII and α-MSH caused significant reduction in release of these proinflammatory mediators. However, KPV and the selective MC1-R agonist MS05 failed to inhibit the release of either mediator. This lack of effect of MS05 has recently been reported (Getting et al., 2003), whereas the inactivity of KPV in this experimental condition is reported here for the first time.

The notion that the anti-inflammatory effects of KPV might be independent from MC-R was further challenged using an assay of receptor functionality. We have previously shown that MC3-R is present on the MØ plasma membrane, and here we could show that MTII caused intracellular cAMP accumulation. These effects were abrogated in the presence of the MC3-R agonist SHU9119. KPV failed to evoke a cAMP response in mouse peritoneal MØ, confirming that the core region (HFRW) is required for binding and activation of the receptor (Wickberg et al., 2000). Conversely, the MC1-R agonist MS05, which has very low affinity for the MC3-R (Szaradenins et al., 2000), failed to cause cAMP accumulation. The failure of KPV to induce cAMP has previously been observed in RAW264.7 macrophages (Mandrika et al., 2001) and in cells transfected with different melanocortin receptors as reported in a recent review (Wickberg et al., 2000). It has also been suggested using molecular modeling and ligand docking experiments that the core region is the sequence required to interact with MC-Rs (Prusis et al., 1997).

In conclusion, the melanocortin peptide KPV was able to inhibit PMN migration and generation of proinflammatory mediators in a model of urate peritonitis. This inhibition did not seem to be associated with MC-R activation and could be better explained by inhibition of IL-1β effects. Our results show that at least two pharmacophores, the core region (HFRW) involved in MC-R activation and the KPV C-terminal tripeptide, which is able to counteract specific cytokines. These findings are thus of fundamental importance for the drug developmental strategies, including determination of targets, exploiting the therapeutic potential attributed to MSH peptides.

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