0026-895X/06/7006-1850–1855$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:1850–1855, 2006

Vol. 70, No. 6
28878/3153772
Printed in U.S.A.

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ACCELERATED COMMUNICATION

[D-Trp^8]-γ-Melanocyte-Stimulating Hormone Exhibits Anti-Inflammatory Efficacy in Mice Bearing a Nonfunctional MC1R (Recessive Yellow e/e Mouse)

Stephen J. Getting, Connie W. Lam, Giovanna Leoni, Felicity N. E. Gavins, Paolo Grieco, and Mauro Perretti

The William Harvey Research Institute, Charterhouse Square, London, United Kingdom (S.J.G., C.W.L., F.N.E.G., G.L., M.P.); and Department of Pharmaceutical Chemistry and Toxicology, Universitá di Napoli, Naples, Italy (P.G.)

Received July 14, 2006; accepted September 7, 2006

ABSTRACT

Two melanocortin receptors (MC1 and MC3R) have been identified as main transducers of the anti-inflammatory effects of natural and synthetic melanocortins. In this study, we have taken advantage of the recent description of the selective MC3R agonist [D-Trp^8]-γ-melanocyte-stimulating hormone (MSH) and of the recessive yellow (e/e) mouse, bearing a nonfunctional MC1R, thereby incrementing our knowledge on this topic. Culturing peritoneal macrophages of recessive yellow (e/e) mice with [D-Trp^8]-γ-MSH led to accumulation of cAMP, indicating MC3R receptor functionality: this effect was blocked by a neutralizing antibody against MC3R. Likewise, release of the chemokine KC by urate crystals was attenuated by [D-Trp^8]-γ-MSH, and this effect was prevented by synthetic [Ac-Nle^4-c[Asp^5-2′-Nal^7,Lys^{10}]α-MSH(4–10)-NH$_2$ (SHU9119)] and natural [agouti-related protein (AGRP)] MC3R antagonists but not by the MC4R antagonist Ac-Cys-Nle-Arg-His-D-2-Nal-Arg-Trp-Cys-NH$_2$ (HS024). Systemic treatment of mice with [D-Trp^8]-γ-MSH inhibited KC release and polymorphonuclear cell accumulation elicited by urate crystals in the murine peritoneal cavity. SHU9119 and AGRP prevented the inhibitory actions of [D-Trp^8]-γ-MSH, whereas HS024 was inactive. We also demonstrate here that [D-Trp^8]-γ-MSH displays a dual mechanism of action by inducing the anti-inflammatory protein heme-oxygenase 1 (HO-1). Treatment with the HO-1 inhibitor zinc protoporphyrin IX exacerbated the inflammatory response elicited by urate crystals and abrogated the anti-inflammatory effects of [D-Trp^8]-γ-MSH. In conclusion, these data support the development of the selective MC3R agonist [D-Trp^8]-γ-MSH for the treatment of inflammatory pathologies, based on a dual mechanism of cytokine/chemokine inhibition and induction of the anti-inflammatory protein HO-1.

Melanocortins [α, β, and γ-melanocyte-stimulating hormone (MSH)] are peptides derived from a larger precursor molecule known as the pro-opiomelanocortin gene product and are little changed throughout evolution; they are traceable back to the appearance of the first vertebrates (Lipton and Catania, 1997). They exert their numerous biological effects by activating seven-transmembrane G-protein-coupled receptors, leading to adenyl cyclase activation and subsequent increases in intracellular cAMP accumulation (Wikberg et al., 2000). The *era* of molecular biology has enabled identification and subsequent cloning of five melanocortin receptors. On the one hand, this has greatly improved the understanding of the potential biological roles for melanocortins; on the other hand, it has stimulated the classification for activity of compounds associated with each receptor.

Melanocortin receptors have been shown to have a wide

Research was funded by Research Advisory Board, St. Bartholomew's and the Royal London School of Medicine and Dentistry (grant RAB04/PJ/04) and by the Arthritis Research Campaign UK (grant 17299).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.028878.

ABBREVIATIONS: MSH, melanocyte-stimulating hormone; MØ, macrophage; HO-1, heme-oxygenase 1; MSU, monosodium urate; PBS, phosphate-buffered saline; ZnPPIX, zinc protoporphyrin IX; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; SHU9119, Ac-Nle^4-c[Asp^5-2′-Nal^7,Lys^{10}]α-MSH(4–10)-NH$_2$; AGRP, agouti-related peptide; MTII, melanotan II; HS024, Ac-Cys-Nle-Arg-His-D-2-Nal-Arg-Trp-Cys-NH$_2$.

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and varied distribution and are found in the central nervous system, periphery, and immune cells (Catania et al., 2004). Over the last 2 decades, the pharmacological definition of each receptor has begun to be unraveled. MC1R is expressed on melanocytes and controls skin pigmentation (Abdel-Malek, 2001). MC2R is expressed on adrenocortical cells (Penhoat et al., 1989) and is involved in adrenal steroidogenesis. MC3R controls macrophage (MØ) activity (Getting, 2002), MC4R is involved in the control of obesity (Ellacott and Cone, 2004) and erectile dysfunction (Martin and MacIntyre, 2004); although MC5R has not received as much attention, it seems to be involved in the control of exocrine secretion and may also have some immunoregulatory functions (Wikberg et al., 2000).

These endogenous peptides have long been reported to be beneficial in many experimental inflammatory diseases including gouty arthritis (Getting et al., 1999, 2002), adjuvant-induced arthritis (Ceriani et al., 1994), inflammatory bowel disease (Rajora et al., 1997), and asthma (Raap et al., 2003). The beneficial effects of the melanocortins seem to be due to their ability to prevent nuclear factor κB activation (Manna and Aggarwal, 1998; Kalden et al., 1999) via the protection of IκB (Ichiyama et al., 1999), thus leading to a reduction of pro-inflammatory mediator synthesis (including cytokines) and adhesion molecule expression with the subsequent inhibition of pro-inflammatory cell migration.

Studies from our group have highlighted the role played by the MC3R in inhibiting urate crystal induced inflammation (Getting et al., 1999). The naturally occurring MC3R agonist γ_2-MSH (Roselli-Rehfuss et al., 1993) and the synthetic peptide MTII (Hruby et al., 1995) possess anti-inflammatory efficacy in a urate model of crystal-induced peritonitis (Getting et al., 2001) and rat knee joint inflammation (Getting et al., 2002). The cellular target for these actions seems to be the MØ; MC3R is the receptor predominantly responsible for mediating them (Getting et al., 1999, 2001, 2002). More recently, we have substantiated the theory that melanocortins inhibit cytokine release (<2 h) from MØ and, at later time points, (>4 h) induce the anti-inflammatory protein heme-oxygenase 1 (HO-1) (Lam et al., 2005).

The present study used both in vitro and in vivo pharmacological and genetic approaches to assess the anti-inflammatory efficacy of the selective MC3R agonist [D-Trp^8]-γ-MSH (Grieco et al., 2000). We report that [D-Trp^8]-γ-MSH displays anti-inflammatory efficacy in mice bearing a non-functional MC1R (recessive yellow e/e mouse) and that these effects are abrogated in the presence of the HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX).

Materials and Methods

Animals

Male recessive yellow (e/e) mice (20–22 g body weight) (Robbins et al., 1993; Getting et al., 2003) were a kind gift from Dr. Nancy Levin (Trega Bioscience, San Diego, USA). Mice were maintained on a standard chow pellet diet with tap water ad libitum using a 12-h light/dark cycle. Animals were used according to guidelines laid down by the ethical committee for the use of animals at St. Bartholomew's and The Royal London School of Medicine and Dentistry. Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

Models of Leukocyte Recruitment

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 (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 heparin. In different experiments, mice received, at time 0, 3 mg of monosodium urate crystals alone or together with an anti-inflammatory dose of [D-Trp^8]-γ-MSH (10 μg/mouse). Eight hours later, some animals were treated with the HO-1 inhibitor ZnPPIX given at the dose of 10 μmol/kg i.p. (Suematsu et al., 1996; Vachharajani et al., 2000). Peritoneal cavities were then washed 24 h after crystal injection. Aliquots of lavage fluid were then stained with Turk's solution, and differential cell counts 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 biochemical determinations.

ELISA Measurements

Murine KC and IL-1β 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 furnished by the manufacturer).

Drug Treatment

[D-Trp^8]-γ-MSH (Grieco et al., 2000) or PBS (100 μl) was administered s.c. alone or in combination with the MC3/4R antagonist SHU9119 (9 nmol) (Hruby et al., 1995), the naturally occurring MC3/4R antagonist AGRP (0.1–1 μg/mouse), and the MC4R antagonist HS024 (9 nmol). MSU crystals were given i.p. 30 min later. Doses were selected from our previous studies and from preliminary dose-response curves (Getting et al., 1999, 2003). SHU9119 was purchased from Bachem Ltd. (Saffron Walden, Essex, UK), and AGRP was purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany). 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 × 10^6/well) were prepared by 2-h adherence at 37°C in a 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 either SHU9119 (9 μM), AGRP (0.1–1 μg/ml), or HS024 (9 μM) 30 min before incubation with either PBS or the reported peptides for 30 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 were collected 2 h later (Getting et al., 1999, 2001). KC and IL-1β levels were measured by ELISA, as described above.

Intracellular cAMP Accumulation. MØs (10^5) were allowed to adhere for 2 h at 37°C in RPMI-1640 medium supplemented with 10% fetal calf serum. MØ were then incubated with serum-free RPMI-1640 media containing 1 mM 3-isobutyl-1-methylxanthine and 0.3 to 30 μg/ml [D-Trp^8]-γ-MSH or the direct adenyl activator forskolin (3 μM); all were dissolved in PBS. In separate experiment, 10 μg/ml [D-Trp^8]-γ-MSH was incubated in the presence of a recently generated polyclonal antibody toward the mouse MC3R (1:50–1:200) (Lam et al., 2005). In all cases, after 30 min at 37°C, supernatants were removed, and cells were washed and lysed. cAMP levels in cell lysates were determined with a commercially available enzyme immunoassay (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a standard curve constructed with 0 to 3200 fmol/ml cAMP.

Western Blotting Analysis

Western blotting was performed as described recently (Lam et al., 2005). In brief, samples were collected and lysed via sonication, with

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protein levels determined by Bradford assay (Bradford, 1976). Samples were electrophoresed in a 10% polyacrylamide gel in running buffer followed by transfer of proteins onto Hybond-C extra nitrocellulose membranes (GE Healthcare) in transfer buffer. Membranes were blocked overnight in blocking solution containing 5% nonfat milk solution in Tris-buffered saline containing 0.1% Tween 20 followed by HO-1 (1:2000; Nventa Pharmaceuticals, San Diego, CA) rabbit serum incubation in blocking solution. Nonspecific antibody binding was removed by washing before the addition of goat anti-rabbit antibody (1:2000). The membranes were further washed and specific antibody binding was detected by enhanced chemiluminescence system. After detection, bound antibodies were removed by incubating the membranes in acidic glycine solution (100 mM glycine, pH 2.5). The membranes were subsequently reprobed for the detection of α-tubulin (1:5000; Sigma-Aldrich Co. Ltd., Gillingham, Kent, UK) and MC3R (1:500). Images were acquired and densitometry performed using Scion Image software (ver. 1.63; Scion Corporation, Frederick, MD).

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 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

Effect of [D-Trp^8]-γ-MSH on cAMP Accumulation in Recessive Yellow e/e Peritoneal MØ. [D-Trp^8]-γ-MSH (0.3–30 μg/ml) caused an accumulation of cAMP (bell-shaped curve on graph) in peritoneal MØ from recessive yellow e/e mice with a maximal increase at 10 μg/ml [D-Trp^8]-γ-MSH (74.5 ± 31%) of the forskolin response (1000 ± 210) and a slightly lower response at 30 μg/ml. The positive controls MTII and α-MSH caused significant increases in cAMP accumulation (data not shown) (Fig. 1A).

We next evaluated whether this increase was occurring though activation of the MC3R expressed on recessive yellow e/e MØ (Getting et al., 2003) by incubating [D-Trp^8]-γ-MSH (10 μg/ml) alone or in the presence of an anti-MC3R. [D-Trp^8]-γ-MSH caused a cAMP release of 306 ± 33 fmol/well that was significantly reduced by 45% ($*$, $p < 0.05$, $n = 4$) in the presence of a polyclonal antibody directed at the MC3R at 1:50 dilution. Lower concentrations (1:100 and 1:200) of the antibody did not modulate cAMP accumulation (Fig. 1B).

[D-Trp^8]-γ-MSH Inhibits KC and IL-1β Release from Cultured MØ in Vitro. [D-Trp^8]-γ-MSH significantly reduced urate crystal-elicited KC release (Fig. 2A), with a calculated inhibition of 31 ± 6% ($n = 4$; $*$, $p < 0.05$ versus PBS). The antichemokine properties of [D-Trp^8]-γ-MSH were abolished by coincubation with 9 μM SHU9119 but not HS024; both antagonists were essentially inactive when administered in the presence of PBS (Fig. 2A). To determine whether this effect could occur in the presence of naturally occurring antagonist of the MC3R, we evaluated [D-Trp^8]-γ-MSH in the presence of AGRP (0.1–1.0 μg/ml). [D-Trp^8]-γ-MSH reduced KC levels by 54% from 419 ± 29 to 192 ± 10 pg/ml. Only AGRP at the highest concentration was able to abrogate the antichemokine properties of [D-Trp^8]-γ-MSH (Fig. 2B). A similar profile was observed for the cytokine IL-1β (Table 1).

Evaluation of the Anti-Inflammatory Effect of the Selective MC3R Agonist [D-Trp^8]-γ-MSH in Vivo. The effect of [D-Trp^8]-γ-MSH (10 μg/mouse) was evaluated in the MSU crystal peritonitis model. [D-Trp^8]-γ-MSH inhibited PMN migration by 48% ($n = 6$; $*$, $p < 0.05$ versus PBS control). This effect was then evaluated in the presence of 9 nmol of the MC3/4R antagonist SHU9119 or selective MC4R antagonist HS024. The attenuation of MSU crystal-induced inflammation by [D-Trp^8]-γ-MSH was prevented by the MC3/4R antagonist SHU9119 but not by the selective MC4R antagonist HS024; both antagonists were essentially inactive when administered in the presence of PBS (Fig. 3A). [D-Trp^8]-γ-MSH inhibition of PMN migration was associated with lower KC levels (Fig. 3B), and the anti-inflammatory effect of the peptide was again blocked in the presence of SHU9119 but not HS024 (Fig. 3B).

Next, we evaluated the effect of the naturally occurring MC3/4R antagonist AGRP on [D-Trp^8]-γ-MSH anti-inflammatory effects in this model of peritonitis. The synthetic peptide [D-Trp^8]-γ-MSH (10 μg/mouse) inhibited both the cellular and soluble responses produced by urate crystals. Figure 4A shows that [D-Trp^8]-γ-MSH significantly ($*$, $p < 0.05$; $n = 6$) inhibited MSU crystal-induced PMN recruitment by 47% and KC (Fig. 4B) and IL-1β (Fig. 4C) recruitment by 66% and 57%, respectively ($*$, $p < 0.05$; $n = 6$). The inhibitory effect of [D-Trp^8]-γ-MSH was abrogated by the naturally occurring antagonist AGRP on all parameters in a dose-dependent fashion; the 1-μg dose was the most effective (Fig. 4, A–C).

MC3-R and HO-1 Induction. In the final section of this study, we tested whether the selective MC3R agonist could promote HO-1 expression in MØ, an effect reported with nonselective compounds (Lam et al., 2005). To this end, we

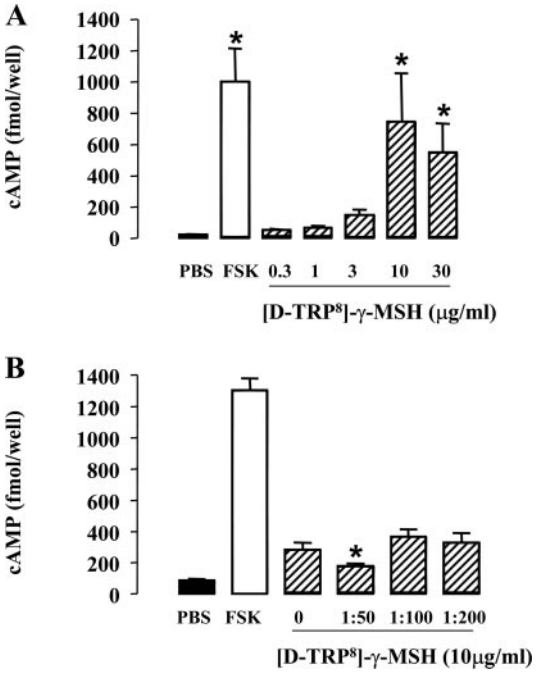

Fig. 1. Melanocortin receptor activation in peritoneal MØ collected from recessive yellow e/e mice. A, adherent peritoneal MØ (1×10^5) were incubated with [D-Trp^8]-γ-MSH (0.3–30 μg/ml), PBS, and forskolin (3 μM). Data are expressed as femtomoles per well. B, the effect of [D-Trp^8]-γ-MSH (10 μg/ml) alone or in the presence of an antibody to the MC3R (1:50–1:200 dilution) for 30 min before determination of intracellular cAMP. Data are mean ± S.E. of $n = 4$ determinations. $*$, $p < 0.05$ versus PBS.

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used e/e mice and incubated them with the anti-inflammatory dose of [D-Trp^8]-γ-MSH (10 μg/mouse). Figure 5 shows the data obtained over 24 h. Activation of MC3-R by [D-Trp^8]-γ-MSH promoted HO-1 induction with a plateau effect at 8 to 18 h (Fig. 5). We also monitored MC3R and α-tubulin expression and found no major changes over this time course (Fig. 5).

Next, we sought to determine whether this induction was pivotal to the anti-inflammatory effects elicited by [D-Trp^8]-γ-MSH in a murine model of urate peritonitis. The functional relevance of the MC3R/HO-1 axis was tested by means of the HO-1 inhibitor ZnPPIX. The inflammatory response to urate crystals at 24 h (Getting et al., 1997) was used here, injecting mice with [D-Trp^8]-γ-MSH 30 min before 3 mg of MSU crystals at time 0; the HO-1 inhibitor was administered 8 h after MSU crystal injection. Figure 6 reports these data. As expected, [D-Trp^8]-γ-MSH exerted a 47% (*, $p < 0.05$) reduction in PMN influx as measured 24 h after crystal injection. The anti-inflammatory nature of HO-1 in this model of peritonitis was confirmed by the augmented PMN influx (22% increase; *, $p < 0.05$) measured in mice treated with ZnPPIX (Fig. 6). It is noteworthy that when ZnPPIX was given to mice previously treated with [D-Trp^8]-γ-MSH, the antimigratory effect of the melanocortin derivative was no longer evident. These in vivo data indicate a major functional role for [D-Trp^8]-γ-MSH–induced HO-1, at least with respect to the delayed anti-inflammatory effect measured 24 h after crystal inflammation.

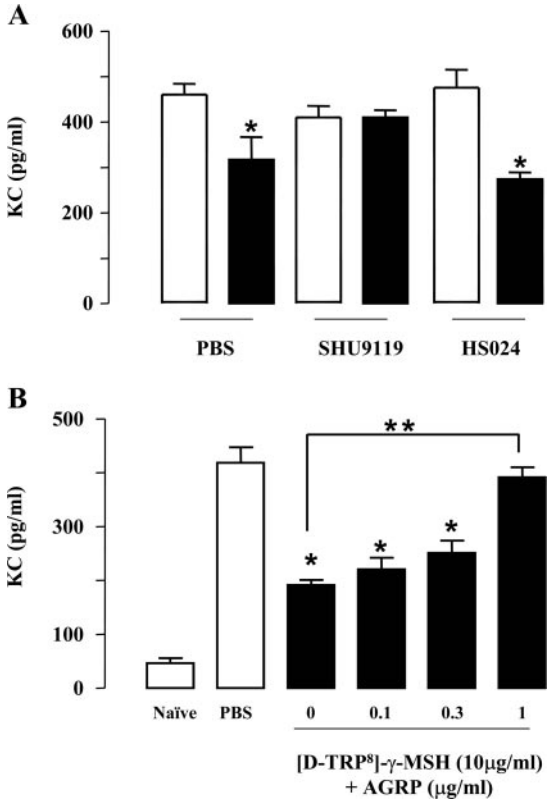

Fig. 2. Effect of [D-Trp^8]-γ-MSH on KC release from primary cultured MØ. [D-Trp^8]-γ-MSH (10 μg/ml) (black bars) or PBS (white bars) were added to adherent peritoneal MØ (5×10^6) from recessive yellow e/e mice 30 min before stimulation with 1 mg/ml MSU crystals. The antagonists SHU9119 (9 μM) and HS024 (9 μM) (A) and AGRP (0.1–1 μg/ml) (B) were added 30 min before agonist treatment. Supernatants were removed 2 h later, and cell-free aliquots were analyzed for the chemokine KC content using specific ELISA. Data are mean ± S.E. of $n = 4$ determinations. *, $p < 0.05$ versus relevant PBS control; **, $p < 0.05$ AGRP (1 μg/ml) versus AGRP (0 μg/ml) group.

TABLE 1

In vitro effect of [D-TRP^8]-γ-MSH on MSU crystal-stimulated mediator release

[D-TRP^8]-γ-MSH (10 μg/ml) or PBS was added to adherent peritoneal MØ (5×10^6) from recessive yellow e/e mice 30 min before stimulation with 1 mg/ml MSU crystals. The antagonist AGRP (0.1–1 μg/ml) was added 30 min before agonist treatment. Supernatants were removed 2 h later, and cell-free aliquots were analyzed for the chemokine IL-1β content using specific ELISA. Data are mean ± S.E. of $n = 4$ determinations.

Antagonist	Agonist	IL-1β
		pg/ml
PBS	PBS	243 ± 8
PBS	[D-Trp^8]-γ-MSH	151 ± 3* (−38%)
AGRP 0.1 μg/ml	[D-Trp^8]-γ-MSH	166 ± 15* (−32%)
AGRP 0.3 μg/ml	[D-Trp^8]-γ-MSH	207 ± 14
AGRP 1 μg/ml	[D-Trp^8]-γ-MSH	263 ± 6

* $P < 0.05$ vs. relevant PBS control.

Discussion

In this study, we investigated the anti-inflammatory efficacy of the selective MC3R agonist [D-Trp^8]-γ-MSH in mice bearing a nonfunctional MC1R (recessive yellow e/e mouse) in acute inflammation, and found an important role for HO-1 in mediating these anti-inflammatory effects. The selective MC3R agonist [D-Trp^8]-γ-MSH, the naturally occurring MC3R antagonist AGRP, and the recessive yellow (e/e) mouse have been used to obtain this result.

We have shown previously that MC3R mRNA is present on murine resident peritoneal MØ (Getting et al., 1999) and that

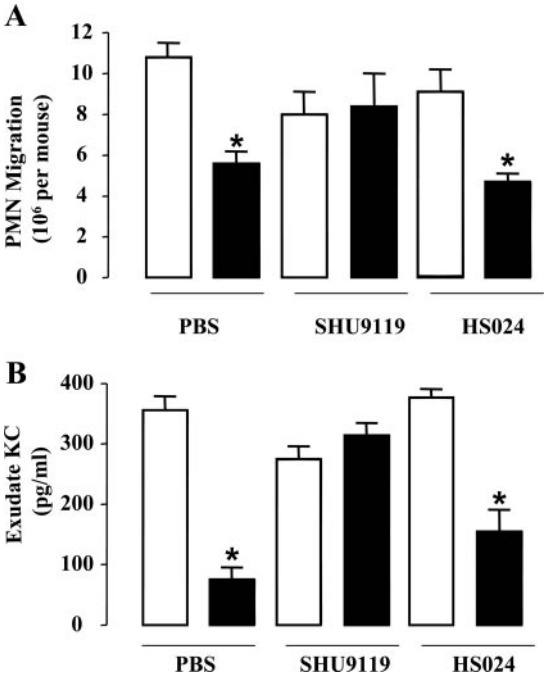

Fig. 3. Anti-inflammatory effects of [D-Trp^8]-γ-MSH in urate-induced inflammation. Recessive yellow e/e mice were treated s.c. with [D-Trp^8]-γ-MSH (10 μg; black bars) or PBS (100 μl; white bars) 30 min before i.p. injection of MSU crystals (3 mg in 0.5 ml of sterile PBS). SHU9119 (9 nmol i.p.) and HS024 (9 nmol i.p.) were administered to mice 30 min before PBS or [D-Trp^8]-γ-MSH administration, and PMN migration (A) was assessed at the 6-h time point. KC levels were assessed using a commercially available ELISA in cell-free supernatants (B). Data are mean ± S.E. of $n = 6$ mice per group. *, $p < 0.05$ versus control group.

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it is genuinely translated to protein by Western blotting (Getting et al., 2001). More importantly, the receptor is expressed on the cell protrusions of the MØ as shown by immuno-gold labeling by electron microscopy (Getting et al., 2003); it is functionally active as determined by cAMP accumulation (Getting et al., 2003). This activation leads to a down-regulation of experimental inflammation (Getting et al., 1999, 2001, 2003); the initial phase of inflammation is inhibition of cytokine synthesis within the first 2 h and at later time-points (>4 h) induction of the anti-inflammatory protein HO-1. In the present study, we set out to examine these early and more delayed downstream events subsequent to MC3R activation using the highly selective MC3R agonist [D-Trp^8]-γ-MSH (Grieco et al., 2000). We have also used the recessive yellow (e/e) mouse to confirm and restrict the scope onto MC3R.

At first, we tested whether the selective MC3R agonist [D-Trp^8]-γ-MSH could induce cAMP accumulation in MØ from the recessive yellow e/e mouse. Indeed, a response was measured, and this was blocked in the presence of a polyclonal antibody directed against the MC3R (Lam et al., 2005) in a concentration-dependent manner. This ability to induce cAMP was associated with a reduction in the release of the pro-inflammatory chemokine KC from MØ, an effect blocked by the MC3/4R antagonist SHU9119 but not by the MC4R antagonist HS024. This highlights not only the antichemokine properties of this selective peptide, but in view of its selectivity (Grieco et al., 2000), it adds further weight to our proposition that MC3R is a main target for modulating inflammation. These data also add this peptide to the list of other melanocortin peptides (including α-MSH, ACTH 4–10, γ_2-MSH, and MTII) known to inhibit KC release from cultured MØ (Getting et al., 1999, 2001). To further support the

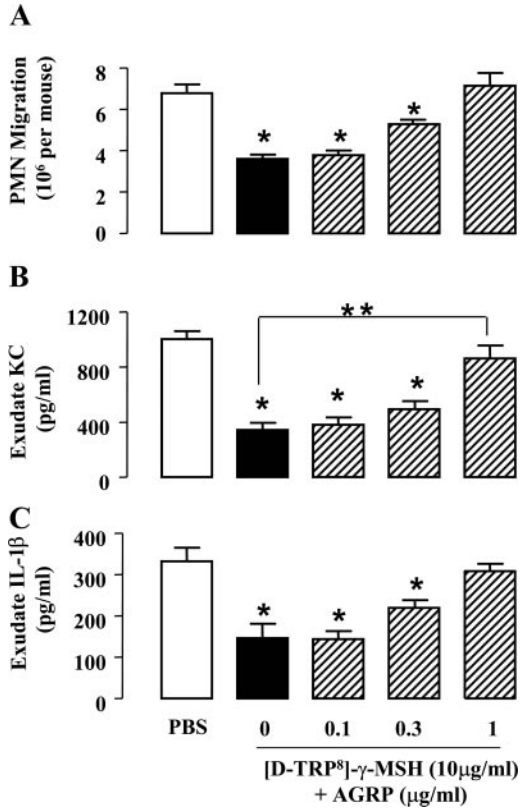
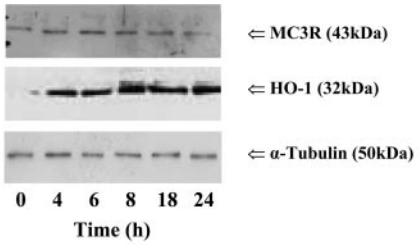


Fig. 4. Effect of [D-Trp^8]-γ-MSH on MSU crystal-stimulated PMN migration in the presence of the naturally occurring MC3/4R antagonist AGRP in vivo. Mice were treated with either PBS (100 μl i.p.) or AGRP (0.1–1.0 μg i.p.) 30 min before treatment with either [D-Trp^8]-γ-MSH (10 μg s.c.) or PBS (100 μl s.c.). Thirty minutes later, mice received MSU crystals (3 mg in 0.5 ml of sterile PBS i.p.). Mice were killed 6 h later, and PMN migration (A) was assessed by light microscopy and KC (B) and IL-1β (C) content in cell-free lavage by commercially available ELISA. Data (mean ± S.E.M. of $n = 6$ mice per group), *, $p < 0.05$ versus PBS control; **, $p < 0.05$ AGRP (1 μg/ml) versus AGRP (0 μg/ml) group.



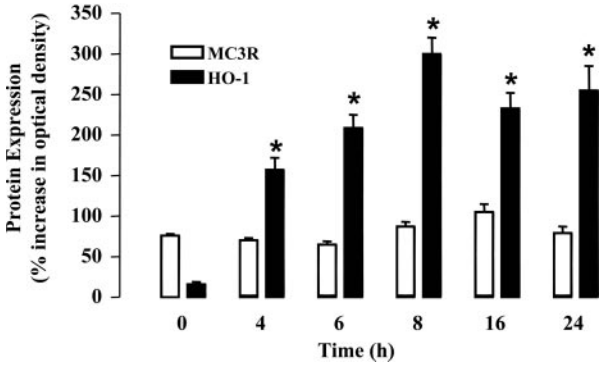


Fig. 5. Time-dependent [D-Trp^8]-γ-MSH-induced HO-1 protein expression in vivo. Recessive yellow e/e mice were treated i.p. with [D-Trp^8]-γ-MSH (10 μg/mouse) at time 0, and peritoneal cells were collected at the reported time points. Protein expression of HO-1, MC3R and α-tubulin were assessed using Western analysis. Representative blots are shown. Values in the bar graph are mean ± S.E.M. of three experiments with cells collected from three mice each time. *, $p < 0.05$ versus time 0 expression.

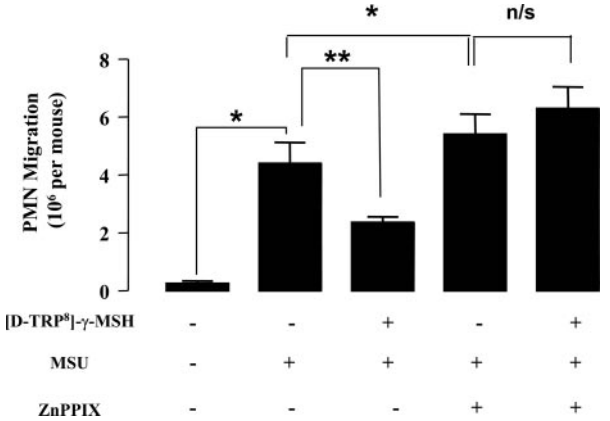


Fig. 6. Effect of [D-Trp^8]-γ-MSH ± ZnPPIX on MSU crystal-induced inflammation. Mice were pretreated with [D-Trp^8]-γ-MSH (10 μg/mouse; i.p.) for 30 min, followed by MSU crystal (3 mg/mouse; i.p.) ± ZnPPIX (10 μmol/kg i.p., 8 h after MSU crystal injection) for 24 h. Resident MØ were taken from the peritoneal cavity for analysis. Aliquots were stained with Turk solution, and PMN counts were performed using a Neubauer hematocytometer. Data are expressed as mean ± S.E.M. of six mice per group. *, $p < 0.05$ vs. control; **, $p < 0.05$ [D-Trp^8]-γ-MSH + MSU versus MSU alone.

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causal role of MC3R, natural antagonist AGRP (Wikberg et al., 2000) was tested on the anti-inflammatory efficacy of [D-Trp^8]-γ-MSH. The MC3R antagonist AGRP attenuated the ability of [D-Trp^8]-γ-MSH to switch off MØ in vitro chemokine KC and IL-1β production. Therefore, the natural occurring antagonist AGRP might have modulatory activities also on the anti-inflammatory and homeostatic actions of melanocortin peptides.

We next sought to add in vivo relevance to these in vitro findings. Systemic administration of the selective MC3R agonist [D-Trp^8]-γ-MSH (Grieco et al., 2000) inhibited MSU crystal-induced PMN migration, and this was associated with a reduction in the levels of the pro-inflammatory chemokine KC in the exudates. The MC3/4R antagonist SHU9119, but not the MC4R antagonist HS024, blocked the inhibition in PMN migration and KC release. In addition, under these in vivo experimental conditions, AGRP (given at the dose of 1 μg/mouse) antagonized the protective effects exhibited by [D-Trp^8]-γ-MSH on PMN migration and KC and IL-1β release, thus supporting the conclusion made above.

In the final experiments, we sought to determine whether the anti-inflammatory effects exhibited by [D-Trp^8]-γ-MSH could be due to increases in the anti-inflammatory protein HO-1, which is exclusive to the MØ (Willis et al., 1996, 2000). Elevation of cAMP levels have been associated with HO-1 induction in rat hepatocyte cultures (Immenschuh et al., 1998) and more recently in RAW264.7 cells after incubation with MTII or adrenocorticotropin (Lam et al., 2005). Peritoneal MØ were removed at different time points, induction was observed at 4 h for HO-1, and this effect was long-lasting and fully evident even at the 24-h time point. We next investigated whether this induction of HO-1 was a prerequisite for the anti-inflammatory effects of [D-Trp^8]-γ-MSH in a model of urate peritonitis, using a protocol recently validated (Lam et al., 2005). The importance of the role of HO-1 was determined by the fact that the HO-1 inhibitor ZnPPIX blocked the anti-inflammatory effects of [D-Trp^8]-γ-MSH. Therefore, [D-Trp^8]-γ-MSH-induced HO-1 expression is instrumental for its specific antimigratory actions.

In conclusion, we report here that the selective MC3R agonist [D-Trp^8]-γ-MSH modulates the host inflammatory response in mice bearing a nonfunctional MC1R, highlighting the pivotal role MC3R plays in modulating the host inflammatory response.

Acknowledgments

We thank Drs. R. de Médicis and A. Lussier (University of Sherbrooke, Sherbrooke, QC, Canada) for the supply of MSU crystals.

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Address correspondence to: Stephen J. Getting, PhD, Senior Lecturer in Pharmacology, School of Biosciences, Department of Human and Health Sciences, University of Westminster, 115 New Cavendish Street, London, W1W 6UW United Kingdom. E-mail: s.getting@westminster.co.uk

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