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Selective Antagonist for the Melanocortin 4 Receptor (HS014) Increases Food Intake in Free-Feeding Rats

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Recently, we discovered a cyclic analogue of MSH (melanocyte stimulating hormone), HS014, which is the first selective antagonist of the MC4 receptor. We have here studied the effects of this peptide on food intake in non-deprived male rats. Vehicle or five doses of HS014 (0.1-10 nmol) were administered ICV at midday. HS014 (0.33-3.3 nmol) significantly and in a dose-dependent manner increased food intake for the first 1 h. At 4 h after the injections, food intake was also significantly increased in rats treated with 1 and 3.3 nmol of HS014, whereas the lowest dose tested (0.1 nmol) was without effect. Cumulative food intake increased to 100% at 4 h after the injections. The highest dose of HS014 (10 nmol) induced sedation and inhibited feeding for first hour of testing. However, this dose also increased food consumption later. These data demonstrate that attenuation of central melanocortinergic tone with HS014 induces disinhibition of feeding and provides additional evidence for the hypothesis that activation of the MC4 receptor inhibits food intake. HS014 may be a useful tool for elucidating the role of the MC receptor subtypes *in vivo*. This is the first report demonstrating an increase in daytime food intake in free-feeding animals caused by a MC receptor active agent. © 1998 Academic Press

The melanocortin (MC) receptors are members of the superfamily of G-protein coupled receptors. Five MC receptor subtypes are cloned; MC1-MC5 [1–5]. The MC1 receptor is expressed in melanocytes and leukocytes, where it has a functional role for pigmentation and inflammation, respectively [6–7]. The MC2 receptor (i.e. the ACTH receptor) is only expressed in the adrenal gland [8], where it mediates glucocorticoneogenesis. The MC3 receptor is mainly expressed in a few brain areas [3,9], and its functional role is much less defined. The MC4 receptor is exclusively found in the brain where it is widely distributed [10]. This subtype has recently been related to control of weight homeostasis [11]. The MC5 receptor is found in a variety of peripheral tissues, and is believed to participate in regulation of exocrine gland function [12].

Each of the MC receptors have a distinct pharmacological profile in regards to the natural MSH peptides; α-MSH, β-MSH, γ-MSH and ACTH. The MC1 receptor has very high and selective affinity for α-MSH, whereas the MC2 receptor only binds ACTH but not the other melanocortins [13–15]. The newly discovered MC3, MC4 and MC5 receptor have comparatively lower affinity for the melanocortic peptides and none of the natural hormones have enough selective properties which would be useful to distinguish between the physiological functions of these subtypes of receptors.

Dominant alleles of the agouti locus, causing ectopic over-expression of the agouti protein, give rise to an obesity syndrome. The agouti protein containing 131 amino acids, is an antagonist for the MC receptors, including the MC3 and MC4 receptors [16,17]. It shares no structural homology to the melanocortic peptides. Recently, it was shown that disruption of the MC4 receptor in a knockout mice caused obesity that showed similar characteristics as that found for the agouti mice [11]. Moreover, ICV injection of the nonselective MC3/MC4 receptor agonist (MT-II) and the nonselective antagonist (SHU9119) influenced deprivation-induced and nocturnal food intake in mice providing further evidence to the hypothesis that melanocorticergic neurones exert a tonic inhibition of feeding behaviour [18].

Our recent discovery of the first MC4 receptor selective antagonist, HS014 [19], prompted us to investigate its putative ability to affect feeding behaviour in rats. HS014 has an about 3 nM K_d for the MC4 receptor, which corresponds to an about 300-fold higher affinity than the natural hormone α-MSH. Moreover, HS014 has an about 20-fold selectivity for the MC4 receptor,

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compared to the MC3 receptor, as well as even higher selectivity in regard to the other MC receptor subtypes.

MATERIALS AND METHODS

Animals. 20 male Wistar rats (National Laboratory Animal Center, Kuopio, Finland) weighing 290-320 g at the time of surgery, were housed individually in hanging wire mesh cages (45×37×19) with free access to food and water in a temperature controlled room at 20±1°C with a 12:12 h light:dark cycle (lights on at 08.00 h). The rats had free access to food pellets (Lactamin R35, Sweden) and tap water.

ICV cannulation and injection. Rats were anaesthetised with chloral hydrate (350 mg/kg/10 ml i.p.) and fixed in a stereotaxic frame. Stainless steel cannulae (11 mm in length, 0.7 mm o.d.) were implanted just above the left lateral ventricle (co-ordinates from bregma A:+0.7; L:-1.4; with tooth bar at +3.0). The guide cannula was lowered until its end was 3.2 mm below the skull and fixed to the bone with stainless steel screws and dental acrylic. The cannulae were closed with dummy stylets when not in use. Rats were frequently handled and weighed during the recovery period to habituate them to partial restraint during ICV injections and testing procedure (introduction of Petri dishes with food into their home cages). Two days before the experiment the rats were sham injected to test the correct placement of cannulae. This was done using gravity infusion of Ringer's fluid. When the length of the injection cannula was 12.5 mm fluid flew freely confirming the correct position of cannula in ventricles. One rat did not regain it preoperative weight and was excluded from the study.

Drugs. Cyclic MSH analogue HS014 (Cys-Glu-His-D-Nal-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH_2, the amino acid residues that make up the ring closure are shown underlined) was synthesised using solid phase approach and purified by HPLC as described earlier [19]. The correct molecular weight of the peptide were confirmed by mass spectrometry. HS014 was dissolved in distilled water to a concentration of 2 nmol/μl and stored in aliquots at −20°C. Final dilution were made with Ringer's fluid.

Experimental protocol and injection. The feeding experiments were performed starting from 7th day after the surgery. On the day of the experiment, the food was removed from wire baskets and the rats were injected ICV with vehicle or HS014 (0.1, 0.33, 1.0, 3.3 and 10 nmol) over 1 min using 31 gauge injector connected to the 50 μl Hamilton syringe and infusion pump (World Precision Instruments, Sarasota, USA). The movement of an air bubble inside the PE20 polyethylene tubing confirmed the drug flow. The needle was left in place for 30 second, then the cannula was closed with stylet, rats were returned to home cage and 7 pre-weighted pellets (20−25g) were presented on clean plastic Petri dishes. All injections were carried out between 12.00−13.00 every third day and were given in randomised order in a such way that none of the rats received the same dose of HS014 twice. There were 7-8 rats in each treatment group (except for vehicle where there were 12 rats and at the highest dose of HS014 where there were 4 rats, the injections were stopped because short behavioural activation followed by sedation and loss of body tonus was observed at this dose of HS014). Food intake was measured after 1, 2, and 4 h following the ICV injection by weighting remaining pellets and spillage using Mettler balance to the nearest 0.1g.

Verification of injection sites. Upon the completion of the study, the rats were overdosed with chloral hydrate (600 mg/kg) and methylene blue dye was infused to mark the injection site. The brains were removed and the distribution of the dye was examined. In 18 rats from 19, the dye was uniformly distributed in ventricles confirming the correct placement of cannulae. Only these rats were used in data analysis.

Statistical evaluation. All results are expressed as mean± s.e. mean. The cumulative food intake data and the amount of food consumed during specific time periods were analysed by one way analysis of variance (ANOVA) for repeated measures, followed by multiple comparisons using LSD test where it was appropriate.

Animal ethics. Experimental procedures were carried out in accordance with guidelines of the European Community, local laws and policies and were approved by Ethics Committee of Animal Experiments at the University of Tartu.

RESULTS

In this study, we injected the MC4 receptor selective antagonist HS014 ICV in rats and measured the food intake under controlled conditions during 4 h of daytime feeding. The primary structure of HS014 is shown in Fig. 1. The effects of HS014 (0.1-10 nmol) on food intake are shown in Fig. 2. The cumulative food intake over 4 h was significantly modified by ICV HS014 (time × treatment − $F_{10,84} = 3.19$ $P<0.005$). Individual comparisons revealed that at 1, 2 and 4 h after the injection, HS014 in doses 0.3-3 nmol significantly increased cumulative food intake. The food intake in rats treated with 10 nmol of HS014 was different from controls and all other treatment groups at 1 h, as this dose of HS014 inhibited food intake. Subsequently, the rats treated with 10 nmol started to eat and after 2 h, cumulative food intake was comparable to that seen in controls. At 4 h the cumulative food intake was also increased in rats treated with 10 nmol of HS014. The amount of food consumed for specific time periods is shown in Fig. 3. The rats treated with 1 and 3.3 nmol of HS014 consumed significantly more food than controls during 1-2 h after injection. 10 nmol dose of HS014 tended to increase the amount of food consumed between 1 and 2 h after injection ($P = 0.107$). However, this effect was not significant at conventional level of statistical significance with the number of rats tested. During the next 2 h period (2−4 h) the food intake was significantly increased only in rats treated with 0.1 and 10 nmol HS014.

DISCUSSION

The recent discovery that melanocorticergic neurones may play a role for feeding behaviour has opened the possibility that eating disorders and abnormal body weight can be treated by MC receptor active agents. The elucidation of the physiological effects of the MC receptor subtypes has been hampered by the lack of subtype selective antagonists. Moreover, none of the known natural melanocortic peptides are selective for the MC4 receptor. Development of a non-selective MC3/MC4 receptor antagonist SHU9119 [20] and the new selective MC4 receptor antagonist HS014 [19] has opened the possibility to explore the role of MC receptor subtypes in physiological processes including the control of feeding behaviour.

Our data show that HS014 is a potent orexigenic agent giving up to a 90 % and 100 % increase in cumulative daytime food in take at 1 nM dose after 2 and 4 h, respectively. The effect of HS014 is dose dependent, giv-

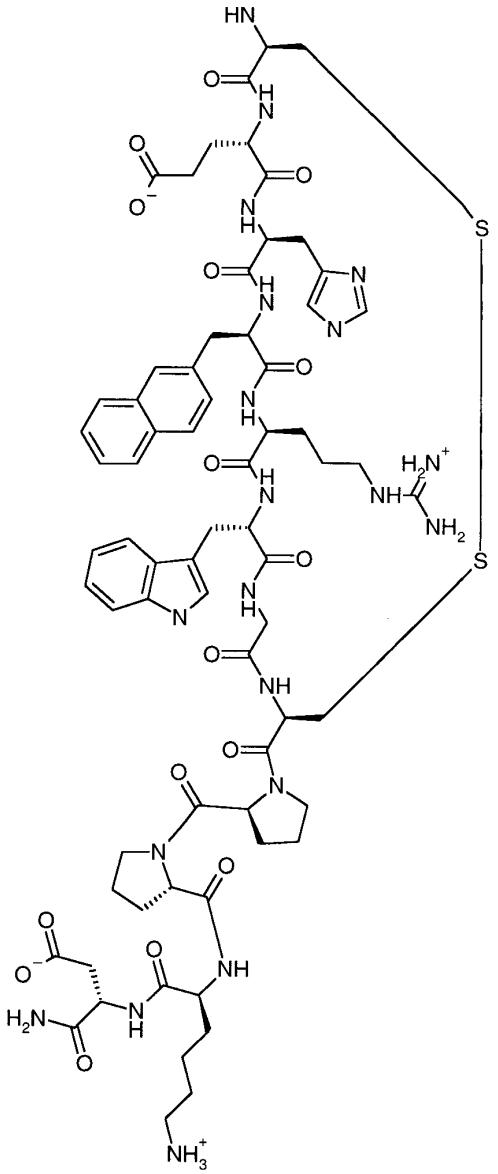

FIG. 1. Primary structure of the MC4 receptor selective MSH analogue; HS014 (cyclic [AcCys[11], D-Nal[14], Cys[18], Asp-NH_2[22]]-β-MSH(11-22)).

ing a bell shaped dose response curve. HS014 had no effect at the lowest dose tested. The highest dose tested decreased the food intake at 1 h. We conclude that the inhibition of food intake was unspecific, caused by the coincidal behavioural depression as 10 nmol of HS014 induced sedation lasting around 40 min at this high dose. However, after 4 h, all the doses showed increase in cumulative food intake. Thus, to the best of our knowledge, this is a first report showing that attenuation of melanocortinergic-tone increases feeding in rats during the daytime when food intake is generally low.

We have earlier shown that HS014 is a potent antagonist *in vitro* by tests on expressed human MC4 receptors using cAMP measurements [19]. The orexigenic

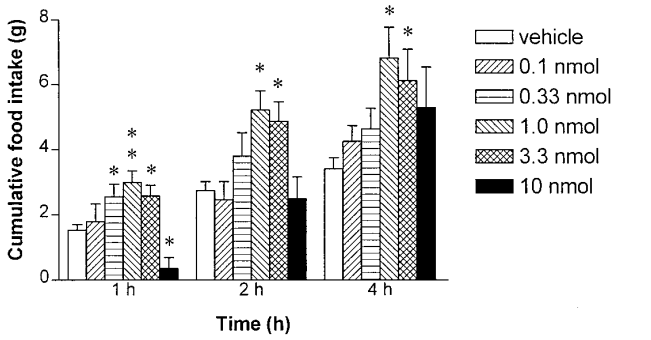

FIG. 2. Cumulative food intake at 1, 2 and 4 hours in free-feeding rats after ICV injection of vehicle (n=15) or HS014 [0.1 (n=7); 0.33 (n=7); 1.0 (n=7); 3.3 (n=7) and 10.0 nmol (n=4)]. Drug or vehicle was injected ICV in a volume of 5.0 μl immediately before the test. Data are presented as mean±s.e.mean. *$P<0.05$- significantly different from vehicle treatment (LSD test).

effects of HS014 are thus conceivable due to its antagonistic action on the MC4 receptor. These data give further evidence that activation of the MC4 receptor inhibits food intake. The potent effects of HS014 on food intake suggest that this cyclic peptide may be a useful tool to elucidate the role of the MC4 receptor *in vivo*. However, it can not be excluded that the MC3 receptor might also be involved in feeding behaviour as the agouti peptide, SHU9119 and HS014 are all effective antagonists for also the MC3 receptor, albeit HS014 being MC4 receptor selective whereas the other peptides do not show such selectivity [16,17,21].

There are two previous studies where the influence on food intake were tested by injection of a MC3/MC4 receptor antagonist. In the first study it was shown that SHU9119 caused a significant increase in food intake in mice at a 3 nmol dose (about 50 % for nocturnal feeding in *ad libitum* fed mice and 25 % for daytime feeding after fasting) [18]. Although the data were not shown, 6 nmol SHU9119 was claimed to be without effect on daytime food intake in animals fed *ad libitum*.

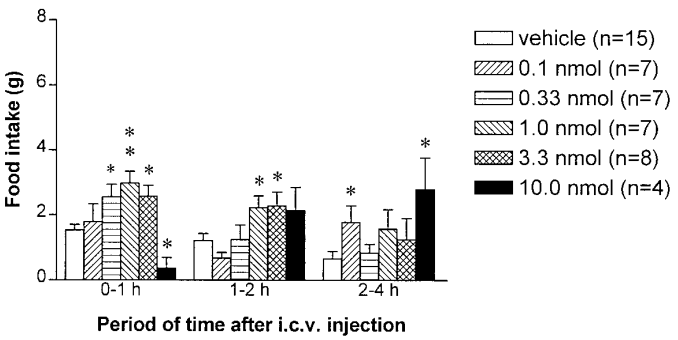

FIG. 3. The effect of HS014 (0.1-10 nmol) on the amount of food consumed during specific time periods (0-1, 1-2 and 2-4 hours after drug administration). See Fig 1, for number of animals in groups. Data are presented as mean±s.e.mean. Drug or vehicle was injected ICV in a volume of 5.0 μl immediately before the test. *$P<0.05$- significantly different from vehicle treatment (LSD test).

This is in apparent contrast with the present study where HS014 significantly increased food intake during daytime. At present there is no good explanation for this discrepancy. Although direct comparisons between these studies and the present one are not possible (i.e. different species and different peptide) it should be noted that the effect of SHU9119 is lower in percentages than what we got for HS014. In the other study, SHU9119 significantly increased food intake also in mice at a dose of 1 nmol (the data were not shown), but at 0.5 nmol it was inactive or even slightly lowered the food intake [22]. This may be due to the fact that the baseline food intake was already elevated in these experiments. However, it should be noted that the increase in food intake caused by HS014 in the present study was lower than food intake in rats after food deprivation or ICV injections of neuropeptide Y [23]. This suggests that other neurotransmitters may affect the response to MC receptor blockage and that the orexigenic effect of HS014 could be potentiated.

It has been known for a while that feeding behaviour is affected by hormones like leptin, neuropeptide Y and galanin [24–26]. Initial studies have not yet clarified how these different systems may interrelate [27,28], but it has been proposed that the MC4 receptor is a important mediator in leptin's effect on food intake [22]. The neurochemical changes associated with weakening of central melanocortic tone resulting in orexigenic response after ICV injection of MC4 antagonists are unknown at present. It has been shown that neuropeptide Y, but not galanin or POMC gene expression is altered in the hypothalamus of genetically obese mice [27]. Studies with selective antagonists of these receptors are warranted to investigate the relationship of MC-ergic neurotransmission with other systems regulating food intake.

In conclusion, we have shown that ICV injections of HS014 result in a dose dependent increase of daytime food intake in free-feeding rats. These data provide additional evidence to the hypothesis that the MC4 receptor mediates an inhibitory tone on food intake. HS014 appears to be a useful compound to study the role of MC receptor subtypes in the mediation of complex effects of melanocortin peptides in the central nervous system.

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