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Pharmacological characterization of canine melanocortin-4 receptor and its natural variant V213F

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Abstract

Dogs have become one of most important companion animals in the modern society. However, it is estimated that 20–40% of owned dogs are obese suggesting that obesity has become one of the most important canine health problem. In addition, obesity in dogs also leads to type II diabetes. Since the melanocortin-4 receptor (MC4R) has been demonstrated to be essential in maintaining energy homeostasis in several different species, including rodents and human, we initiated studies towards elucidating the roles of MC4R in obesity pathogenesis in dogs. Recently, canine MC4R was cloned and a missense variant V213F was identified. We designed primers and successfully cloned canine MC4R and generated the variant V213F by site-directed mutagenesis. The objective of this study was to investigate the pharmacological properties of canine MC4R and its natural variant V213F. We measured ligand binding and signaling properties using both natural and synthetic ligands. Human MC4R was also included in the experiments for comparison. Both wild type canine MC4R and its natural variant V213F functioned normally in terms of binding and signaling. Of the ligands we used, [Nle⁴, D-Phe⁷]- α -melanocyte stimulating hormone is the most potent ligand. We conclude that the cloned canine MC4R is a functional receptor and the natural variant V213F does not have any functional defect and therefore is not likely to cause obesity in dogs.

1. Introduction

The melanocortin system is essential for regulation of energy homeostasis and body weight [1]. This system includes neurons that express pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) as well as melanocortin receptors [2]. Tissue-specific posttranslational processing of POMC generates various peptides, including α -, β -, and γ -melanocyte-stimulating hormone (MSH) produced in the hypothalamus and the neurointermediate lobe of pituitary as well as other peripheral tissues [3]. There are five subtypes of melanocortin receptors (MC1R-MC5R) [2]. The melanocortin-4 receptor (MC4R) is stimulated by its endogenous agonists α - and β -MSHs and inhibited by the endogenous antagonist AgRP [4].

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Disclosure

None.

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The MC4R is a member of G-protein coupled receptor (GPCR) superfamily [5]. It consists of seven transmembrane domains (TMs) connected by alternating extracellular and intracellular loops, with an intracellular COOH terminus and an extracellular NH₂ terminus. Upon agonist stimulation, MC4R couples to the stimulatory heterotrimeric G protein (Gs) and activates adenylyl cyclase to promote the intracellular production of the second messenger cAMP [4].

Studies in the last decade have shown MC4R plays a central role in regulating energy homeostasis in rodents [1]. Both *Pomc*- and *Mc4r*-null mice developed a marked obesity syndrome associated with hyperphagia, hyperinsulinemia, hyperglycemia and an increase in linear growth [6, 7]. In addition, mice that over-express AgRP demonstrate a hyperphagic, obese and hyperinsulinemic phenotype [8, 9] while mice that were administered α -MSH or its analog melanotan II have decreased food intake [10, 11]. Therefore, either genetic inactivation of MC4R or pharmacological inhibition through over-expression of its endogenous antagonist can increase food intake and develop obesity.

Human genetic studies also unraveled the important role of MC4R in regulation of food intake and body weight. Since 1998, when the first frameshift mutation was identified in *MC4R* gene associated with dominant-inherited morbid obesity in humans [12, 13], more than 150 distinct *MC4R* mutations have been identified in obese patients from various ethnic populations [4]. These mutations include frameshift, inframe deletion, nonsense and missense mutations, scattered throughout the coding region of *MC4R* gene. Functional analysis of human MC4R showed that *MC4R* mutations could lead to receptor defects including receptor biosynthesis, cell surface expression, ligand binding or signaling [14, 15]. In one large study, 5.8% of subjects with severe early onset obesity were identified to have mutations in *MC4R* [16]. Therefore, *MC4R* deficiency represents the most common form of monogenic obesity.

In 2007, canine MC4R (cMC4R) was cloned and three single nucleotide polymorphisms (SNPs) were identified, one of which (G637T) is a missense variant, valine to phenylalanine at position 213 (V213F) [17]. Of the 31 dogs genotyped in this study, 84% had 637G. It was suggested that the variant might be functioning normally because both valine and phenylalanine are hydrophobic [17]. This variant was identified in another more recent study [18]. In preliminary analysis of 187 Golden Retrievers, no association between the polymorphisms and morphological measures were found. However, these authors suggested that the variant will likely affect receptor function, because this missense variant changed an amino acid residue that is highly conserved and under strong purifying selection. Furthermore, PolyPen program predicted that this polymorphism is likely damaging, although the authors conceded that this prediction needed to be experimentally verified by in vitro assays [18].

We hypothesize that the central melanocortin system is also critical for regulating energy homeostasis in dogs. Therefore, as the initial step for our long-term goal of understanding the melanocortin regulation of energy balance in dog, we studied the pharmacology of cMC4R and its natural variant V213F. Herein, we performed functional studies on cMC4R and its natural variant V213F using two endogenous ligands, α -MSH and β -MSH, and the superpotent analog [Nle⁴, D-Phe⁷]- α -MSH (NDP-MSH). Ligand binding and signaling properties of these receptors were determined.

2. Materials and methods

2.1. Peptides and supplies

NDP-MSH was obtained from Peptides International (Louisville, KY). α - and β -MSH were purchased from Bachem (King of Prussia, PA). ^{125}I -iodinated NDP-MSH was purchased from the Peptide Radioiodination Service Center at The University of Mississippi (University, MS) with a specific activity of 2176 Ci/mmol. Tissue culture plastic wares were purchased from Corning (Corning, NY). Dulbecco's modified Eagles media (DMEM) and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Site-directed mutagenesis

Wild type (WT) cMC4R was cloned by the same strategy as described previously in our cloning of porcine MC3R and MC4R [19, 20] by Zhenchuan Fan. Nucleotide sequencing of the cloned cMC4R showed that the sequence matched that in GenBank (accession number DQ084210). The natural variant V213F was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol described previously [21].

2.3. Cells and transfection

Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were grown at 5% CO_2 in DMEM supplemented with 10% newborn calf serum, 10mM HEPES, and 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. For transient expression of the MC4Rs, cells were plated on gelatin-coated 35-mm 6-well plates and transfected using the calcium precipitation method [22]. Four-microgram plasmid in 2ml media was used per 35-mm well. The transfection cocktail included 86 μl water, 10 μl 2.5M CaCl_2 , 4 μl of plasmid DNA, and 100 μl of 2 $\times$ BSS (consisting of 280mMNaCl, 1.5mMNa 2HPO_4 , 50mM N,N-bis[2-hydroxyl]-2-aminoethane sulfonic acid, pH 6.95). After 15 min incubation in the hood at room temperature, 1.8 ml of growth media was combined with the cocktail and put into a well in 6-well clusters. Forty-eight hours after transfection, cells were used for measuring ligand binding and hormone stimulation of cAMP generation.

2.4. Radioligand binding assay

Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) modified to contain 1 mg/ml BSA (referred to as Waymouth/BSA). One milliliter of fresh Waymouth/BSA was added to each well, and then 100,000 cpm of ^{125}I -NDP-MSH (50 μl) was added to each well, with or without different concentrations of unlabeled α -, β -, or NDP-MSH. The final concentration of unlabeled ligands ranged from 10^{-10} to 10^{-5} M (for α - and β -MSH) or 10^{-11} to 10^{-6} M (for NDP-MSH). After incubation at 37 °C for 1 h, cells were placed on ice, washed twice with cold Hanks' balanced salt solution (Sigma-Aldrich) modified to contain 1 mg/ml BSA (referred as HBSS/BSA). Then cells were lysed with 0.5N NaOH and lysates were collected using cotton swabs, and counted in a gamma counter. Binding capacity and IC_{50} were calculated using Prism software.

2.5. Ligand stimulation of intracellular cAMP generation

Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well. After incubation at 37 °C for 15 min, either buffer alone or different concentrations of α -, β -, or NDP-MSH were added. The final concentrations ranged from 10^{-12} to 10^{-6} M (for NDP-MSH) or 10^{-11} to 10^{-5} M (for α - and β -MSH). After incubation at 37 °C for 1 h, cells were then placed on ice, media aspirated, and intracellular

cAMP extracted by the addition of 0.5 N perchloric acid containing 180 $\mu\text{g/ml}$ theophylline, and measured using radioimmunoassay [20]. All determinations were performed in triplicate. Maximal responses (R_{max}) and EC_{50} values were calculated using Prism 4 (GraphPad Software).

2.6. Statistic analysis

Statistical calculations were performed using Prism 4. One sample t-test was used for comparisons on maximal binding and signaling. For comparisons on IC_{50} and EC_{50} , an unpaired t-test was used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ligand binding and signaling properties of the WT cMC4R and its natural variant V213F using NDP-MSH as the ligand

HEK293T cells have been used extensively as an *in vitro* system for evaluation of functional properties of MC4Rs from various species. Herein, WT cMC4R and the natural variant V213F cMC4R were transiently transfected into HEK293T cells and their ligand binding and signaling properties were analyzed. Human MC4R (hMC4R) was used in these experiments for comparison. As it is widely used in MCR studies, the superpotent analogue of α -MSH, NDP-MSH, was used in these experiments [4]. As shown in Table 1 and Figure 1, WT hMC4R, WT cMC4R and V213F cMC4R bound NDP-MSH with an IC_{50} of 55.59, 37.21, and 37.68 nM, respectively. Therefore these three receptors had similar binding affinities to NDP-MSH. Maximal binding (B_{max}) of WT and V213F cMC4Rs were reduced to 54% and 68% of that of WT hMC4R, that were statistically significant.

The signaling properties of these receptors were analyzed upon stimulation with NDP-MSH in HEK293T cells transiently transfected with these receptor constructs. Results showed that dose-dependent increases of intracellular cAMP were induced by NDP-MSH for all the receptors. As shown in Table 1, NDP-MSH stimulated cAMP production with similar EC_{50} of 0.92, 1.12, and 1.13 nM for WT hMC4R, WT cMC4R and V213F cMC4R, respectively. The maximal responses (R_{max}) of WT cMC4R and V213F cMC4R were 78% and 84% of that of WT hMC4R.

3.2. Ligand binding and signaling properties of the WT cMC4R and its natural variant V213F using α -MSH as the ligand

Although NDP-MSH is widely used in MCR research [4], it is a superpotent long-lasting analog of the natural agonist α -MSH [23]. Therefore, distinct pharmacological characteristics of the mutant receptors might be observed using α -MSH. Thus, we also measured ligand binding and signaling properties of these receptors using α -MSH. As shown in Figure 2 and Table 1, the three receptors bound to α -MSH with similar IC_{50} s. EC_{50} and R_{max} are also shown in Table 1. WT and V213F cMC4R had similar EC_{50} s. R_{max} of WT and V213F cMC4Rs were reduced to 70% and 80% of that of WT hMC4R, respectively.

3.3. Ligand binding and signaling properties of the WT cMC4R and its natural variant V213F using β -MSH as the ligand

In research on body weight regulation, α -MSH has been the focus of the majority of studies because rodents lack the N-terminal cleavage site for β -MSH and are therefore deficient in β -MSH [24]. However, the amino acid sequence of β -MSH is highly conserved across species and most species, including dogs, contain both cleavage sites for β -MSH, therefore β -MSH may have a key role in the control of energy homeostasis in dogs. Hence, we also investigated the ligand binding and signaling properties of these MC4R receptors using β -

MSH. As shown in Table 1 and Figure 3, WT hMC4R, WT and V213F cMC4Rs bound β -MSH with an IC_{50} of 883.13, 481.07, and 90.37 nM, respectively. The three receptors showed similar signaling efficiency with similar EC_{50} values (Table 1). R_{max} was similar for WT and V213F cMC4Rs. The R_{max} of WT cMC4R was reduced to 60% of that of WT hMC4R (Table 1).

4. Discussion

Although obesity is a multifactorial disease associated with genetic, behavioral and environmental components, studies have shown that humans can become severely obese directly resulting from genetic disruption of a single element of a homeostatic system regulating energy balance [25].

Ever since human MC4R was cloned in 1993 [5], MC4R have been cloned in several other mammalian species and some non-mammalian species, including fishes and birds [4]. In addition to the extensive studies in rodents and humans, several studies addressed the roles of the MC4R in regulating energy homeostasis in pigs. Barb and colleagues showed that although NDP-MSH treatment decreases food intake, antagonists including AgRP do not increase food intake, which is different from studies in other species [26]. The first natural variant identified in porcine *MC4R* gene, D298N, was initially reported to be associated with certain traits such as fatness, growth, and feed intake [27]. Some subsequent studies supported the association whereas others did not support the association. Our functional study showed that the porcine D298N MC4R has normal function [19] therefore not consistent with it being a cause for the association with the traits originally identified.

Recently, canine MC4R was cloned and a missense variant V213F was identified [17]. The variant was also identified in another independent study [18]. The latter study tried to identify association of the variant with several phenotypes, such as weight, length, height, or body index score (weight divided by (length $\times$ height)). However, no association was found. The authors suggested that several reasons might account for this lack of association, including small sample size and age of the animals. Two other possibilities were also put forth. One is that the variant might not affect receptor function or MC4R might play a less important role in the regulation of energy balance in dogs than in humans.

In this study, we investigated the pharmacological characteristics of WT cMC4R and its natural variant V213F. Three ligands were used and hMC4R was included in all experiments for comparison. We were interested in whether cMC4R had similar pharmacology as hMC4R. Therefore, ligands developed for human medicine could potentially be used for treating dog obesity. We were also interested in whether the missense variant might be involved in obesity pathogenesis in dogs.

Our results showed that cMC4R is fully functional as it bound and signaled normally when stimulated by these three agonists, with similar EC_{50} as hMC4R. We also found no overt functional defects for V213F cMC4R upon the stimulation of these three agonists. Therefore it is not likely to cause obesity in dogs. These data also highlighted the critical importance of experimentally verifying *in silico* predictions.

In our signaling experiments, we showed that the rank order in terms of potency for the three agonists was: NDP-MSH > α -MSH = β -MSH for WT hMC4R, WT cMC4R and V213F cMC4R. From these results, we showed that as a commonly used analog for MCR studies, NDP-MSH is more potent than the endogenous ligands α - and β -MSH, therefore would be better for further *in vivo* studies to elucidate the functional importance of cMC4R in energy homeostasis in dogs.

We also observed that cMC4R bound β -MSH with higher affinity compared to α -MSH. Most research on the role of MC4R in regulating energy balance has concentrated on α -MSH, in part because the animal models, rodents, lack the N-terminal cleavage site for β -MSH and are therefore β -MSH deficient. Recently, the identification of a missense mutation within the coding region for β -MSH in *POMC* and its association with early-onset human obesity renewed interest in β -MSH in human energy homeostasis [28, 29]. One study also found that β -MSH has significantly higher affinities than α -MSH at both human and rodent MC4Rs [30]. Therefore β -MSH might also be an important endogenous ligand for activation of MC4R. Here, our data indicated that cMC4R had higher affinity for β -MSH than α -MSH. However, these *in vitro* data should be treated with caution. For instance, previous studies on pMC4R showed pMC4R binds to AgRP and SHU9119 normally *in vitro*. However, these antagonists fail to increase food intake *in vivo* [26].

In conclusion, our results showed cMC4R was fully functional upon stimulation with three MC4R agonists *in vitro*. The natural variant cMC4R V213F did not have any functional defects and therefore is unlikely to cause obesity in dogs. Therefore the lack of association of the variant with body index score is due the normal function of the variant. Our results do not support the hypothesis that the MC4R might play a less important role in canine physiology compared to human physiology. As many analogs targeting MC4R are being developed for human obesity treatment, further studies will focus on whether cMC4R bind and respond to these analogs similarly as hMC4R, thus indicating whether it is practical to use these analogs to treat dog obesity.

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Figure 1.

Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC4R and cMC4R constructs and binding and signaling assays were performed as described in Materials and Methods. In binding assay, different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I -NDP-MSH to MC4Rs on intact cells. Results shown are expressed as the mean $\pm$ SEM from duplicate determinations within one experiment. In signaling assays, HEK293T cells transiently transfected with the indicated MC4R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean $\pm$ SEM of triplicate determinations within one experiment. All experiments were performed three times.

Figure 2. Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using α -MSH as the ligand. See Fig. 1 for details.

Figure 3.
Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using β -MSH as the ligand. See Fig. 1 for details.

Table 1

Ligand stimulated cAMP production and binding at the MC4Rs

MC4R	n	cAMP Generation						Binding			
		NDP-MSH		α -MSH		β -MSH		Bmax	NDP-MSH		β -MSH
		EC ₅₀ (nM)	Rmax	EC ₅₀ (nM)	Rmax	EC ₅₀ (nM)	Rmax		IC ₅₀ (nM)	IC ₅₀ (nM)	
WThMC4R	3	0.92±0.01	100	1.90±0.28	100	3.19±2.03	100	100	55.59±5.36	520.33±196.19	883.13±464.12
WTcMC4R	3	1.12±0.50	78±26	4.20±0.92	70±14	2.26±0.75	60±19	54±3	37.21±1.47	1687.37±281.25	481.07±246.87
V213FcMC4R	3	1.13±0.26	84±38	4.81±1.76	80±11	4.87±2.65	93±15	68±8	37.68±2.31	880.20±363.64	90.37±44.46

The data are expressed as the mean ± SEM of three independent experiments. The maximal response (Rmax) was 3740 ± 672 pmol cAMP/10⁶ cells for WT hMC4R under NDP-MSH stimulation, 1732 ± 553 pmol under α -MSH stimulation, and 2094 ± 709 pmol under β -MSH stimulation.