Pharmacological analyses of two naturally occurring porcine melanocortin-4 receptor mutations in domestic pigs

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Abstract

The melanocortin-4 receptor (MC4R) is critical in regulating mammalian food intake and energy expenditure. Numerous mutations in the MC4R gene have been identified from obese humans. So far two naturally occurring porcine MC4R (pMC4R) mutations, D298N and R236H, have been identified from various strains of pigs and D298N is being utilized as a genetic marker to screen performance traits of pigs. In this study, we performed functional analyses of pMC4R D298N and R236H, including their ligand binding and signaling properties in transiently transfected HEK293T cells. Ligand binding assays showed that both D298N and R236H pMC4Rs had similar binding capacities and affinities for the natural agonist α-MSH and the natural antagonist Agouti-related protein as wild-type pMC4R. In signaling assays, both mutants had normal EC50 and maximal signaling to α-MSH. In summary, pMC4R mutants D298N and R236H do not have any overt functional defects; therefore we suggest caution using these mutations as selection markers in breeding programs.

Keywords: Melanocortin-4 receptor; Ligand binding; Signaling; Porcine; Naturally occurring mutations

1. Introduction

The melanocortin-4 receptor (MC4R) is a member of the superfamily of G protein-coupled receptors (GPCRs) consisting of the hallmark seven transmembrane domains (TMs) connected by alternating extracellular and intracellular loops, with an extracellular NH2 terminus and intracellular COOH terminus. Upon hormone stimulation, MC4R couples to the stimulatory heterotrimeric G protein (Gs) and then activates adenylyl cyclase to promote the intracellular accumulation of cAMP. The endogenous agonist of MC4R, α-melanocyte stimulating hormone (α-MSH), is derived from proopiomelanocortin (POMC) via tissue-specific posttranslational processing. Agouti-related protein (AgRP) is the endogenous antagonist of the MC4R.

During the past decade, ample evidences demonstrated the central role of the MC4R in regulating energy homeostasis of rodents (reviewed in [1]). MC4R or POMC knockout mice have increased food intake and obesity [2,3]. Mice over-expressing AgRP are hyperphagic, obese and hyperinsulinemic [4,5] whereas mice administered with α-MSH or melanotan II, an analogue of α-MSH, had decreased food intake [6,7]. Furthermore, mice co-administrated melanotan II and the MC4R antagonist SHU9119 showed a normal food intake, sug-

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gesting that SHU9119 inhibits the effect of melanotan II on food intake [6,7]. MC4R activation also increases energy expenditure [8,9].

Human genetic studies also showed that the MC4R plays a critical role in controlling food intake and body weight in humans. So far more than 110 distinct human MC4R (hMC4R) mutations have been identified from obese patients of various ethnic populations [10]. These mutants are caused by frameshift, in-frame deletion, nonsense and missense mutations. Although some mutants do not have obvious functional defects as determined by in vitro assays and might represent rare polymorphic variants, others are defective in cell surface expression, ligand binding or G protein coupling/activation (reviewed in [11]). Up to 6% of patients with severe early-onset obesity are caused by mutations in MC4R gene in some cohorts, thus representing the most common monogenic form of obesity [12].

In 2000, Rothschild and co-workers identified a missense mutation in the porcine MC4R (pMC4R) gene that change a highly conserved Asp in TM7 in melanocortin receptors (MCRs) to Asn [13]. This Asp, designated 7.49 according to the numbering scheme of Ballesteros and Weinstein [14], lies in the highly conserved N/DPxxY motif. The majority of Family A (rhodopsin-like) GPCRs has an Asn at this position. MCRs, together with gonadotropin-releasing hormone receptor and prostanoid receptors, are some of the few receptors that have an Asp at position 7.49. Within the MCR subfamily, however, this Asp is fully conserved in all the MCRs cloned to date, pointing to its potential functional importance. In our structure-function studies on the hMC4R, we generated D298N hMC4R and found that the mutant receptor has relatively normal function. However, the functional properties of this mutant were not investigated in the original study.

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2. Materials and methods

2.1. Peptides and supplies

[Nle4, D-Phe7]-α-MSH (NDP-MSH), a superpotent analogue of α-MSH, was obtained from Phoenix Pharmaceuticals (Belmont, CA). α-MSH was purchased from Bachem (King of Prussia, PA). Human AgRP(86-132) was obtained from Peptides International (Louisville, KY). 125I-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 medium (DMEM) and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

2.2. Molecular cloning of pMC4R

The pMC4R coding region was amplified directly from porcine genomic DNA (Novagen, San Diego, CA) using a primer pair (sense primer 5′-AAGAATTCTGAACTCAACCCATCAC-3′ and anti-sense primer 5′-CCTCTAGATATTATCTGCT-AGACAAATC-3′) designed based on the published nucleotide sequence of pMC4R (GenBank access no. AB021664) incorporating EcoRI and XbaI restriction sites in sense and anti-sense primers, respectively (underlined). PCR amplification was performed in a 50 μl mixture containing 100 ng of the porcine genomic DNA, 0.25 mM dNTPs, 0.4 μM of each primer, 1 × pfu DNA polymerase buffer, 1.5 mM of MgCl2, and 2.5 U pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with the following cycling parameters: 2 min at 95 °C for one cycle and 1 min at 95 °C, 45 s at 56 °C, and 90 s at 72 °C for 35 cycles followed by a final cycle of extension at 72 °C for 10 min. The PCR products of expected size as visualized by agarose gel electrophoresis were purified with Qiagen PCR purification kit (Qiagen, Valencia, CA) and double digested with EcoRI and XbaI (New England Biolabs, Beverly, MA). The PCR fragment was further purified, ligated into the expression vector pcDNA3.1, and transformed into JM109 competent cells. Cells were grown overnight on LB agar plates containing ampicillin and 8 clones were selected for growing in LB medium. Plasmid
DNA was extracted with Qiaprep spin miniprep kit (Qiagen) to screen clones with the insert of expected size after digestion with EcoRI and XbaI. The nucleotide sequence of the cloned pMC4R was determined by sequencing three independent plasmids performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center. Plasmid DNA containing the pMC4R of correct sequence was prepared with Qiagen plasmid maxi kit (Qiagen) for site-directed mutagenesis or transfection as described below.

2.3. Site-directed mutagenesis

Nucleotide sequencing of the cloned pMC4R showed that it is D298N pMC4R. Wild-type (WT) pMC4R was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol described previously [23]. R236H pMC4R was made using the WT pMC4R as the template by the same method. Wild-type hMC4R attached with a myc epitope at its N terminus (after the initiating Met) has been described previously [23]. The hMC4R mutant D298N was generated using this construct as template by the same mutagenesis method.

2.4. Cells and transfection

Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were grown in 5% CO2 in DMEM supplemented with 10% newborn calf serum, 10 mM HEPES, and 100 units/ml of penicillin and 100 µg/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 [24]. Four-microgram plasmid in 2 ml media was used per 35-mm well. The calcium precipitation method was used to precipitate the antibody-bound fraction of cAMP extracted by the addition of 0.5 N NaOH, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramines T method. Polyclonal antibody against cAMP was obtained from Strategic BiosoLutions (Newark, DE). The radioimmunoassay was performed as described before [25] except that polyethylene glycol 8000 was used to precipitate the antibody-bound fraction of cAMP instead of a second antibody in the original publication. Maximal responses (Rmax) and EC50 values were calculated using Prism software version 4 (GraphPad Software, San Diego, CA).

2.6. 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-MSH 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-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 µg/ml theophylline, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramines T method. Polyclonal antibody against cAMP was obtained from Strategic Biosolutions (Newark, DE). The radioimmunoassay was performed as described before [25] except that polyethylene glycol 8000 was used to precipitate the antibody-bound fraction of cAMP instead of a second antibody in the original publication. Maximal responses (Rmax) and EC50 values were calculated using Prism software version 4 (GraphPad Software).
2.7. Statistical analysis

Statistical calculations were performed using Prism 4 [26]. For comparisons on maximal binding and signaling, one sample t-test was used. For comparisons on IC$_{50}$ and EC$_{50}$, an unpaired t-test was used.

3. Results

3.1. Ligand binding and signaling properties of the MC4R mutants 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 and mutant pMC4Rs were transiently transfected into HEK293T cells and their ligand binding and signaling properties were analyzed. Wild-type and D298N hMC4Rs were included in these experiments for comparison. The superpotent analogue of $\alpha$-MSH widely used in MCR studies, NDP-MSH, was used in these studies. As shown in Fig. 1A and Table 1, WT, D298N and R236H pMC4Rs bound NDP-MSH with an IC$_{50}$ of 2.46, 6.70 and 14.00 nM, respectively. Maximal binding ($B_{\text{max}}$) of the two mutants were similar to that of WT pMC4R (Table 1). Similar results were obtained for hMC4Rs: WT and D298N hMC4Rs bound NDP-MSH with IC$_{50}$ of 7.70 and 14.07 nM, respectively, and the $B_{\text{max}}$ of hMC4R D298N was at the same level as that of WT hMC4R (Fig. 1A and Table 1).

The signaling properties of the WT and mutant MC4Rs were analyzed upon stimulation with NDP-MSH in HEK293T cells transiently transfected with these receptor constructs. The results showed that NDP-MSH induced dose-dependent increases of intracellular cAMP in all groups (Fig. 1B). As shown in Table 1, NDP-MSH stimulated cAMP accumulation with EC$_{50}$ of 2.80, 27.62 and 15.43 nM for pMC4R WT, D298N and R236H, respectively. The maximal responses of pMC4R D298N and R236H were reduced to 76% and 65% of that of WT pMC4R, respectively. NDP-MSH stimulated cAMP generation with EC$_{50}$s of 1.63 and 12.96 nM for hMC4R WT and D298N, respectively. The maximal responses were similar for hMC4R WT and D298N (Table 1). We also measured the basal (constitutive) activities of the expressed receptors. The results showed
Table 1

<table>
<thead>
<tr>
<th>Ligand binding and agonist-stimulated cAMP generation of WT and mutant pMC4Rs and hMC4Rs</th>
<th>n</th>
<th>NDP-MSH-stimulated cAMP</th>
<th>AgRP binding</th>
<th>α-MSH-stimulated cAMP</th>
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<td>Rmax (% wt)</td>
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<td>nDOP-MSH binding</td>
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<tr>
<td>pMC4R WT</td>
<td>3</td>
<td>2.46 ± 0.27</td>
<td>2.90 ± 0.41</td>
<td>100</td>
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<tr>
<td>pMC4R D298N</td>
<td>3</td>
<td>6.70 ± 1.33</td>
<td>7.70 ± 1.56</td>
<td>100</td>
</tr>
<tr>
<td>pMC4R R236H</td>
<td>3</td>
<td>14.00 ± 1.90</td>
<td>14.07 ± 1.16</td>
<td>100</td>
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<tr>
<td>IMC4R D298N</td>
<td>3</td>
<td>7.70 ± 1.56</td>
<td>12.96 ± 2.70</td>
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</table>

The data are expressed as the mean ± S.E.M. of three independent experiments. The maximal binding (Bmax) of intact HEK293T cells expressing WT pMC4R or hMC4R were 15601 ± 1568 and 15646 ± 1214 cpm bound/106 cells, respectively. The maximal responses (Rmax) were 4520 ± 359 and 1513 ± 271 pmol cAMP/106 cells for WT pMC4R and hMC4R, respectively. IC50 is the concentration of NDP-MSH, AgRP or α-MSH that is needed to cause 50% inhibition in the binding assay. EC50 is the concentration of NDP-MSH or hMC4R that results in 50% stimulation of the maximal response.

3.2. Ligand binding and signaling properties of the MC4R mutants using α-MSH as the ligand

Although NDP-MSH is widely used in MCR studies, it is a superpotent long-lasting analog of the natural agonist derived from POMC, α-MSH. Different pharmacological properties of the mutant receptors might be observed using NDP-MSH vs. α-MSH. Therefore, we also measured ligand binding and signaling properties of the mutant pMC4Rs using α-MSH as the ligand. These results showed that there is no difference between the WT pMC4R and either of the pMC4R mutants, R236H and D298N, in any of the parameters measured (Fig. 3 and Table 1).

3.3. Ligand binding properties of the MC4R mutants using the antagonist AgRP

Since the balance between the agonist α-MSH and the antagonist AgRP determines the function of the MC4R, it is important to determine whether the mutant receptors have increased affinity for AgRP. Increased affinity for AgRP, even in the face of normal affinity for and response to α-MSH, would result in decreased function that pMC4R D298N, but not R236H, had decreased basal signaling compared with WT pMC4R whereas hMC4R D298N has normal constitutive activity (Fig. 2).
Fig. 3. Ligand binding and signaling properties of the WT and mutant pMC4Rs using α-MSH as the ligand. HEK293T cells were transiently transfected with the indicated pMC4R constructs and binding and signaling assays were performed as described in Section 2. In (A) different concentrations of unlabeled α-MSH were used to displace the binding of 125I-NDP-MSH to pMC4Rs on intact cells. Results shown are expressed as the mean ± S.E.M. from duplicate determinations within one experiment. In (B) HEK293T cells transiently transfected with the indicated pMC4R constructs were stimulated with different concentrations of α-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean ± S.E.M. of triplicate determinations within one experiment. All experiments were performed three times.

of the MC4R. Therefore, we also determined the binding properties of the mutant receptors to AgRP. As shown in Fig. 4, both WT and mutant pMC4Rs bound AgRP with similar high affinity (Table 1). Similarly, WT and D298N hMC4Rs bound to AgRP with the same affinities (Fig. 4 and Table 1).

4. Discussion

More than 110 distinct mutations have been identified from obese humans in the MC4R gene. Functional studies are essential for establishing a causal relationship between the mutations identified and the obesity phenotype [11,27]. This is highlighted by several recent studies where functional studies provided strong support for the genetic studies [12,28,29].

Studies on the involvement of pig MC4R in feed intake and energy balance have resulted in conflicting results. Barb et al. showed that intracerebroventricular administration of NDP-MSH decreased feed intake, but treatments with the synthetic antagonist SHU9119 or AgRP, the natural antagonist of the MC4R, failed to stimulate feed intake [30], which differs from the results obtained in rodents [6,31,32] and sheep [33]. In vitro, these ligands did act as antagonists [30]. We showed herein that WT and D298N pMC4Rs have similar binding affinities for AgRP (Fig. 4 and Table 1). Therefore, these data, while conforming the in vitro data of Barb et al., cannot provide an explanation for the lack of effect of AgRP on feed intake in pigs as observed by Barb et al. [30].

Rothschild and co-workers identified a missense mutation in pMC4R, D298N, which mutated the conserved Asp in N/DPxxY motif in TM7. They showed that this mutation was associated with fatness, growth, and feed intake traits [13]. Subsequent functional studies on this mutant revealed that the mutation did not affect ligand binding but totally abolished ligand-stimulated

Fig. 4. Ligand binding properties of the WT and mutant MC4Rs on AgRP. HEK293T cells were transiently transfected with the indicated pMC4R and hMC4R constructs and binding assays were performed using AgRP as the competitor. Different concentrations of AgRP were used to displace the binding of 125I-NDP-MSH to pMC4Rs or hMC4Rs on intact cells. Results shown are expressed as the mean ± S.E.M. from duplicate determinations within one experiment. All experiments were performed three times.
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larly, in other GPCRs that result in diseases, mutations 
Class V: mutants with no known defects [11,23]. Simi-
defects (reviewed in [11]). We classify these mutants as 
a number of mutations that result in no overt functional 
phan, or glutamine was observed. It should be pointed 
stimulated activities [35]. This arginine is not conserved 
from a patient and found to have normal functions, 
values between our own data on hMC4R and the data on 
the pMC4R. We cloned the pMC4R from genomic DNA 
purchased from a commercial vendor and determined 
that the cDNA we cloned was in fact the mutant D298N. 
We then generated the WT cDNA using site-directed 
tageneesis in response to NDP-MSH stimulation [34]. 
Another mutation in pMC4R, R236H, was recently 
identified in another screening effort [19]. We showed 
here that the mutant had normal ligand binding and sig-
aling [15] therefore belonging to the Class IV cat-
ency according to our classification scheme [11,23]: 
mutants defective in G protein coupling/activation. From 
a structure-function point of view, this mutation is inter-
esting in that a fully conserved Asp in MCRs is mutated to Asn, the most commonly found amino acid in Family 
A GPCRs. Since Asn is found in most of the Family A GPCRs, it can be reasoned that Asn should con-
fer normal functions for the receptor. Our preliminary 
results with hMC4R showed that indeed D298N hMC4R 
is fully functional in terms of both ligand binding and 
signaling. Therefore, we were interested in identifying 
the molecular determinants dictating the dramatic differ-
ces between our own data on hMC4R and the data on 
the pMC4R. We cloned the pMC4R from genomic DNA 
without normal affinity and generate cAMP with similar 
maximal signaling, although the EC_{50}s are increased 
(Fig. 1 and Table 1). The reason for this discrepancy is 
not clear. We noticed that the maximal stimulation in the 
study by Kim et al., about 16 pmol per million cells, is 
extremely low in this transient transfection system using 
HEK293 cells. In our radioimmunoassay for cAMP, this 
is about the lowest level we can detect. Recently, Patten 
et al. also showed that D298N hMC4R retained cAMP 
formation in response to NDP-MSH stimulation [34]. 

Another mutation in pMC4R, R236H, was recently 
identified in another screening effort [19]. We showed 
here that the mutant had normal ligand binding and sig-
aling properties compared to WT pMC4R when the 
natural ligand α-MSH was used for these assays (Fig. 3 
and Table 1), suggesting that it might not cause signifi-
cant changes in feed intake and growth in pigs. The 
corresponding mutation was also identified in hMC4R 
from a patient and found to have normal functions, 
including cell surface expression as well as basal and 
stimulated activities [35]. This arginine is not conserved 
in the cloned MCRs. In other MCRs, histidine, trypto-
phan, or glutamine was observed. It should be pointed 
out that in functional studies of hMC4R mutations iden-
tified from obese patients, several groups have reported 
a number of mutations that result in no overt functional 
defects (reviewed in [11]). We classify these mutants as 
Class V: mutants with no known defects [11,23]. Simi-
larly, in other GPCRs that result in diseases, mutations 
identified from patients that have normal functions were 
observed not infrequently (reviewed in [10]), suggesting 
that these mutations were not the cause of the disease. 
One drawback of these experiments is that the MC4R 
is expressed in neurons. Our experiments in HEK293T 
cells may not reveal any neuron-specific aspects of the 
MC4R function.

Another interesting aspect that is worth mentioning is 
that from all the screening efforts in different strains of 
pigs, only two mutations in pMC4R were identified. In 
humans, numerous mutations were identified from both 
obese patients and the general population. The reason for 
this lower occurrence of pMC4R mutation is unknown 
at this time.

In summary, we reported here that the two mutations 
in pMC4R, R236H and D298N, do not result in overt 
defects in receptor functions, suggesting that they might 
not cause significant increases in feed intake and growth. 
We therefore suggest caution using these mutations as 
selection markers in breeding programs.

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