Domestic Animal Endocrinology 54 (2016) 68–75

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Pharmacologic analyses of four chicken melanocortin-4 receptor mutations

Z.-Q. Wang [a,b,*], J.-S. Huang [b], J.-H. Zhou [a], L. Shi [a], X.-F. Jiang [a], Y.-X. Tao [b,**]

[a] College of Veterinary Medicine, Yangzhou University, Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu 225009, People's Republic of China
[b] Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form 12 May 2015
Accepted 24 June 2015

Keywords:
Melanocortin-4 receptor
Ligand binding
Signaling
Chicken
Mutation

ABSTRACT

The melanocortin-4 receptor (MC4R) is a critical regulator of mammalian food intake and energy expenditure, with receptor activation resulting in decreased food intake and increased energy expenditure. Recently, studies on role of MC4R in regulation of food intake have been extended to other species, such as chicken. Functional study of mutant MC4Rs is important in proving the causal link between *MC4R* mutation and production traits. Herein, we cloned chicken *MC4R* (cMC4R) complementary DNA and generated 4 mutant cMC4Rs (Q18H, G21R, S76L, and L299P) by site-directed mutagenesis and measured their expression by flow cytometry. Pharmacologic characteristics were analyzed with binding and signaling assays using 3 agonists. We showed that G21R had decreased cell surface and total expression ($P < 0.05$), whereas the other 3 mutants had similar total and cell surface expression levels as wild-type cMC4R. The 4 mutants had either decreased (Q18H, G21R, S76L; $P < 0.05$) or no (L299P) binding to radiolabeled [Nle^4, D-Phe^7]-α-melanocyte-stimulating hormone (MSH). In signaling assays, Q18H was constitutively active. Q18H, G21R, and S76L had decreased responses to α-MSH stimulation ($P < 0.05$). L299P had decreased basal and ligand-stimulated signaling ($P < 0.01$). Nle^4, D-Phe^7-MSH was the most potent agonist for cMC4R and therefore would be better suited for further in vivo studies. We conclude that the cloned cMC4R was a functional receptor and provided detailed functional data for these mutations, contributing to a better understanding of cMC4R variants associated with production traits.

1. Introduction

During the past 2 decades, the melanocortin-4 receptor (MC4R) was found to regulate energy homeostasis and play critical roles in the development of both monogenic and polygenic obesity [1,2]. The MC4R regulates both food intake and energy expenditure [3], with the effect of food intake accounting for 60% of the effect on body weight [4]. Human genetic studies provided further supporting evidence that the MC4R is important in maintaining energy homeostasis in humans. Mutations in *MC4R* are the most common cause of early onset obesity [5] and more than 170 distinct mutations have been identified [6,7]. Although some of these MC4R mutants do not have obvious functional defects and might represent rare polymorphic variants (Class V), others are defective in expression (Class I), cell surface trafficking (Class II), ligand binding (Class III) or signaling (Class IV), including defect in mitogen-activated protein kinase signaling [8] (reviewed in [9,10]). These functional studies provide the missing link between mutation identification and causality of the mutation in obesity pathogenesis.

Based on the expression patterns of melanocortin system genes in tissues, it is hypothesized that avian

* Corresponding author. Tel: +86 51487979224; fax: +86 51487972218.
** Corresponding author. Tel.: +1 3348445396; fax: +1 3348445388.
E-mail addresses: zqwang@yzu.edu.cn (Z.-Q. Wang), taoyaxi@auburn.edu (Y.-X. Tao).

0739-7240/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.domaniend.2015.06.003

melanocortins may serve as local mediators regulating a variety of functions in the brain and peripheral tissues as it does in mammals [11–13]. Results from studies on Japanese quail and broiler breeder hens strongly indicate an association of melanocortin system with control of appetite, similar to the effects in mammals [12,13]. It was suggested that some polymorphisms of *MC4R* might be associated with higher body weight or improved feed conversion in chickens [14]. Two missense polymorphisms of the chicken *MC4R* (*cMC4R*) gene, Q18H, and S76L, were detected, and the S76L heterozygote is associated with greater body weight than the L76 homozygote in female broilers [14]. Similarly, Qiu et al [15] found a missense mutation G21R in *cMC4R* gene and indicated that the mutation has close relationship with body weight, eviscerated weight, and weight of thigh meat. However, no functional characterization of these mutations has been performed to demonstrate the causality between *MC4R* mutations and production traits.

As the initial step for our long-term goal of understanding the melanocortin regulation of energy balance in chicken, we performed detailed functional studies on 4 cMC4R mutations, including Q18H, G21R, S76L, and L299P. L299P was identified in a mouse obesity model and functional implications studied in human MC4R [16,17], therefore introduced into cMC4R in the present study for comparison. Herein, we cloned *cMC4R* complementary DNA (cDNA) and performed functional studies including expression, ligand binding, and signaling.

2. Materials and methods

2.1. Hormones and supplies

[Nle^4, D-Phe^7]- (NDP-), α-, and β-melanocyte stimulating hormones (MSHs) of human origin were purchased from Sigma-Aldrich (Shanghai) Trading Co, Ltd (catalog numbers M8764, M4135, and M6513, respectively). NDP-MSH is a superpotent analog of α-MSH frequently used in melanocortin receptor studies [18]. Chicken and human α-MSHs have the same sequence whereas there are 7 of 22 amino acids that are different between chicken and human β-MSHs. Chicken β-MSH was not commercially available for use in the present study. ^{125}I-NDP-MSH was iodinated as previously described [19]. Tissue culture plasticware were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, and other reagents for cell culture were obtained from Invitrogen (Carlsbad, CA).

2.2. Molecular cloning of cMC4R

The cMC4R coding region was amplified directly from chicken genomic DNA using a primer pair (sense primer: 5′- AAGAATTCATGAATTTCACCCAGCATCGTGGG-3′ and antisense primer: 5′- CCTCTAGACTAATATTTGCCAGGTAAATC-3′) designed based on the published nucleotide sequence of cMC4R (GenBank access no. AB012211) incorporating *EcoR*I and *Xba*I restriction sites in sense and antisense primers, respectively (underlined). Polymerase chain reaction (PCR) amplification was performed in a 50-μL mixture containing 100 ng of the chicken genomic DNA, 0.25-mM dNTPs, 0.4 μM of each primer, 1 × Pfu DNA polymerase buffer, 1.5 mM of $MgCl_2$, and 2.5 U PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) with the following cycling parameters: 2 min at 95°C for 1 cycle and 1 min at 95°C, 60 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 *EcoR*I and *Xba*I (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 Luria-Bertani agar plates containing ampicillin, and 8 clones were selected for growing in Luria-Bertani medium. Plasmid DNA was extracted with QIAprep Spin Miniprep Kit (Qiagen) to screen clones with the insert of expected size after digestion with *EcoR*I and *Xba*I. The nucleotide sequence of the cloned cMC4R was determined by sequencing 3 independent plasmids performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center. Plasmid DNA containing the cMC4R of correct sequence was prepared with QIAGEN Plasmid Maxi Kit (Qiagen) for site-directed mutagenesis or transfection as described in the following section.

2.3. Site-directed mutagenesis

Nucleotide sequencing of the cloned cMC4R showed that it is Q18H cMC4R. Wild-type (WT) cMC4R was obtained by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the protocol. WT cMC4R tagged at its N terminus (after the initiating methionine) with myc tag was generated by Sangon Biotech (Shanghai) Co Ltd. Fourpoint mutations were introduced into the WT cMC4R containing the c-myc epitope tag with QuikChange Site-Directed Mutagenesis Kit. The nucleotide sequence of the mutated cMC4R was determined by sequencing 3 independent clones. Plasmid DNA containing the cMC4R of intended mutations was prepared with QIAGEN Plasmid Maxi Kit for transfection and resequenced.

2.4. Cells and transfections

Human embryonic kidney (HEK) 293T or 293 cells, obtained from American Type Culture Collection (Manassas, VA), were grown at 5% CO_2 in Dulbecco's Modified Eagle's Medium containing 50-μg/mL gentamicin, 10-mM HEPES, 10% newborn calf serum, 100 units/mL of penicillin, and 100-μg/mL streptomycin. For transient expression of the cMC4Rs, cells were plated on gelatin-coated 35-mm 6-well clusters. When the cells reached 50% to 70% confluency, they were transfected using the calcium precipitation method [20]. One microgram plasmid in 2-mL media was used per 35-mm well. The transfection cocktail for each well included 86-μL sterile water, 10-μL 2.5 M $CaCl_2$, 4 μL of plasmid DNA (0.25 μg/μL in sterile H_2O), and 100 μL of 2 × balanced salt solution (consisting of 280-mM NaCl, 1.5-mM Na_2HPO_4, 50-mM *N*, *N*-bis[2-hydroxyl]-2-aminoethanesulfonicacid, pH 6.95). After 15-min incubation in the hood at room temperature, 1.8-mL growth medium for each well was combined with the cocktail and put into a well in 6-well clusters. Cells were