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Molecular cloning and pharmacological characterization of giant panda (*Ailuropoda melanoleuca*) melanocortin-4 receptor

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ABSTRACT

The melanocortin-4 receptor (MC4R) is critical in regulating mammalian food intake and energy expenditure. Giant panda (*Ailuropoda melanoleuca*), famous as the living fossil, is an endangered species endemic to China. We are interested in exploring the functions of the giant panda MC4R (amMC4R) in regulating energy homeostasis and report herein the molecular cloning and pharmacology of the amMC4R. Sequence analysis revealed that amMC4R was highly homologous (>88%) at nucleotide and amino acid sequences to several mammalian MC4Rs. Western blot revealed that the expression construct myc-amMC4R in pcDNA3.1 was successfully constructed and expressed in HEK293T cells. With human MC4R (hMC4R) as a control, pharmacological characteristics of amMC4R were analyzed with binding and signaling assays. Four agonists, including [Nle^4, D-Phe^7]-α-melanocyte stimulating hormone (NDP-MSH), α- and β-MSH, and a small molecule agonist, THIQ, were used in binding and signaling assays. We showed that amMC4R bound NDP-MSH with the highest affinity followed by THIQ, α-MSH, and β-MSH, with the same ranking order as hMC4R. Treatment of HEK293T cells expressing amMC4R with different concentrations of agonists resulted in dose-dependent increase of intracellular cAMP levels, with similar EC_{50}s for the four agonists. The results suggested that the cloned amMC4R encoded a functional MC4R. The availability of amMC4R and its binding and signaling properties will facilitate the investigation of amMC4R in regulating food intake and energy homeostasis.

1. Introduction

The melanocortin-4 receptor (MC4R) belongs to the superfamily of G protein-coupled receptors (GPCRs) consisting of the hallmark seven transmembrane domains (TMDs) connected by alternating extracellular and intracellular loops, with an extracellular N-terminus and intracellular C-terminus (Gantz et al., 1993). The endogenous agonist of MC4R, α-melanocyte stimulating hormone (MSH), is a small peptide derived from post-translational processing of pro-opiomelanocortin (reviewed in (Smith and Funder, 1988)). Agouti-related protein is the endogenous antagonist of the MC4R (Ollmann et al., 1997). Upon agonist stimulation, MC4R couples to the stimulatory heterotrimeric G protein (Gs) and then activates adenylyl cyclase to promote the intracellular accumulation of cAMP (Gantz et al., 1993). Other signaling pathways, including calcium and mitogen-activated protein kinase, are also activated by the MC4R (Mountjoy et al., 2001; Vongs et al., 2004), although the relevance of these signaling pathways in mediating the diverse functions of the MC4R are not fully elucidated yet (reviewed in (Tao, 2010, 2014)).

Since the cloning of human MC4R (hMC4R) in 1993 (Gantz et al., 1993), MC4R has been cloned from a number of other mammals, including rat (Mountjoy et al., 1994), pig (Kim et al., 2000), cattle (Haegeman et al., 2001), and dog (Skorczyk et al., 2007). In recent years, with increasing interests in the role of MC4R in energy homeostasis of lower vertebrates, MC4Rs of some non-mammalian species including trout (Haitina et al., 2004), flounder (Kobayashi et al., 2008), goldfish (Cerda-Reverter et al., 2003),

Abbreviations: amMC4R, giant panda (*Ailuropoda melanoleuca*) melanocortin-4 receptor; GPCR, G protein-coupled receptor; MC4R, melanocortin-4 receptor; MSH, melanocyte-stimulating hormone; NDP-MSH, [Nle^4, D-Phe^7]-α-MSH; PCR, polymerase chain reaction; THIQ, [(N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine; TMD, transmembrane domain.

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spiny dogfish (Ringholm et al., 2003), and cavefish (Aspiras et al., 2015), among others, were also cloned.

During the past decade, anatomical, pharmacological, and rodent genetic studies have shown that MC4R plays a critical role in regulating energy homeostasis (reviewed in (Cone, 2005; Tao, 2005)). It regulates both energy intake and expenditure (Huszar et al., 1997), with the effect of food intake accounting for 60% of the effect on body weight (Balthasar et al., 2005). Human genetic studies provided further supporting evidence that the MC4R is also critical in maintaining energy homeostasis in humans, playing major roles in the development of both monogenic and polygenic obesity (reviewed in (Hinney et al., 2013; Tao, 2009)). Mutations in *MC4R* are the most common cause of early-onset obesity, with up to 6% of severely obese Caucasian children harboring *MC4R* mutations (Farooqi et al., 2003). Mutations in cavefish *mc4r* have been shown to contribute to the adaptation to nutrient availability (Aspiras et al., 2015).

Giant panda (*Ailuropoda melanoleuca*), famous as the "living fossil", is an ancient species with a history of about 7 million years and an endangered species endemic to China (Lindburg and Baragona, 2004). Due to its highly specialized feeding habits and limited reproductive capability, giant panda typically has a low fertility and high neonatal mortality, contributing to the endangered status of giant panda. There are only about 1600 giant pandas surviving in the wild. Previous studies suggested that there is a close relationship between the nutritional status and reproduction of the mammals (reviewed in (Tena-Sempere, 2013)). The MC4R participate in the control of energy homeostasis as well some reproductive function (reviewed in (Tao, 2010)). Hence cloning the panda MC4R is important for understanding nutritional problems and field starvation of giant panda. So far there are few studies on the giant panda at the molecular level.

As the central melanocortin system has been suggested to play an important role in regulation of energy balance from fish to humans, we propose that it is also critical for energy homeostasis of giant panda. To begin to understand the roles MC4R might play in regulating energy homeostasis and other physiological processes in the giant panda, we report herein the molecular cloning and pharmacology of the giant panda MC4R (amMC4R). Based on the sequence deposited at NCBI (reference sequence number XM002924679.1), we designed primers and successfully cloned amMC4R cDNA and inserted it into pcDNA 3.1, a mammalian expression vector. We also analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those of human and other species reported. With hMC4R as a control, the binding and signaling properties of amMC4R were investigated using several agonists of the MC4R, including [Nle^4, D-Phe^7]-α-MSH (NDP-MSH), α- and β-MSH, and a small molecule agonist of the MC4R, THIQ ([(N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-yl carbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine) (Sebhat et al., 2002). This study provided data for inquiring into the hereditary traits of the gene from giant panda and basis for formulating protection strategy for the giant panda.

2. Materials and methods

2.1. Hormones and supplies

NDP-MSH was purchased from Peptides International (Louisville, KY). α-MSH was purchased from Pi Proteomics (Huntsville, AL). β-MSH was purchased from CHI Scientific (Maynard, MA). THIQ was purchased from Tocris Bioscience (Ellisville, MO). ^{125}I-NDP-MSH was iodinated as previously described (Mo et al., 2012). ^{125}I-cAMP was iodinated with chloramine T method (Steiner et al., 1969). Tissue culture plastic wares were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, and other reagents for cell culture were obtained from SunShine (Nanjing SunShine Biotechnology Co., Ltd., Nanjing, China).

2.2. Materials and DNA preparation

Genomic DNA was isolated from about 200 μl of giant panda blood collected and donated by colleagues at Yangzhou Zoo (Yangzhou, China) using the Genomic DNA Preparation Kit according to the manufacturer's instructions (TransGen Biotech, Beijing, China). The genomic DNA extracted were dissolved in water, and kept at −70 °C.

2.3. Molecular cloning of amMC4R

The amMC4R coding region was amplified directly from giant panda genomic DNA using a primer pair (sense primer: 5′-AAGAATTCATGAACTCGACGCTCCACCATGGGATG-3′ and anti-sense primer: 5′-CCTCTAGATTAATATCTGCTAGACAAGTCACAAAGGCC-3′) designed based on the published nucleotide sequence of amMC4R (NCBI reference sequence number: XM002924679.1) incorporating *Eco*RI and *Xba*I restriction sites in sense and anti-sense primers (underlined), respectively. PCR amplification was performed in a 25 μl mixture containing 100 ng of giant panda genomic DNA, 0.4 μM of each primer, 12.5 μl 2× Easy Taq PCR SuperMix (TransGen Biotech) with the following cycling parameters: 2 min at 95 °C for one 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.

After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1× TAE buffer, stained with ethidium bromide and visualized under UV light. The expected size fragments of PCR products were harvested and purified from gel using Axygen PCR purification kit (Axygen, Beijing, China), and then double digested with *Eco*RI and *Xba*I (Promega, Shanghai, China). The double digested PCR fragment was ligated into the expression vector pcDNA3.1(+) using T4 DNA ligase (TransGen Biotech) at 16 °C for 12 h. The recombinant molecules were transformed into *Escherichia coli* competent cells (DH5α), and then spread on the LB-agar plate containing 50 μg/mL ampicillin. Plasmid DNA was extracted with Axygen mini-preparation kit to screen clones with insert of expected size after digestion with *Eco*RI and *Xba*I. The nucleotide sequence of the cloned amMC4R was determined by sequencing three independent plasmids performed at Sangon Biotech Co. Ltd (Shanghai, China). After verifying that the entire coding region was achieved by automated DNA sequencing, amMC4R tagged at its N terminus (after the initiating Met) with myc tag was generated by Sangon Biotech (Shanghai) Co. Ltd. Plasmid DNA containing a myc epitope tag and the amMC4R of correct sequence (myc-amMC4R-pcDNA3.1) was prepared with Axygen Plasmid Maxi kit for transfection as described below.

2.4. Homology and phylogenetic analysis of amMC4R

Homology and phylogenetic analyses at nucleotide and amino acid levels were performed between different species including giant panda (*A. melanoleuca*), human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), pig (*Sus scrofa*), cattle (*Bos taurus*), dog (*Canis lupus*), cat (*Felis catus*), chicken (*Gallus gallus*) and zebrafish (*Danio rerio*) using DNASTAR Lasergene v7.1.0 program, according to the manufacturer's protocols.