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Research paper

Cytoskeleton-related regulation of primary cilia shortening mediated by melanin-concentrating hormone receptor 1

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ABSTRACT

Primary cilia are specialized microtubule-based organelles. Their importance is highlighted by the gamut of ciliary diseases associated with various syndromes including diabetes and obesity. Primary cilia serve as signaling hubs through selective interactions with ion channels and conventional G-protein-coupled receptors (GPCRs). Melanin-concentrating hormone (MCH) receptor 1 (MCHR1), a key regulator of feeding, is selectively expressed in neuronal primary cilia in distinct regions of the mouse brain. We previously found that MCH acts on ciliary MCHR1 and induces cilia shortening through a Gi/o-dependent Akt pathway with no cell cycle progression. Many factors can participate in cilia length control. However, the mechanisms for how these molecules are relocated and coordinated to activate cilia shortening are poorly understood. In the present study, we investigated the role of cytoskeletal dynamics in regulating MCH-induced cilia shortening using clonal MCHR1-expressing hTERT-RPE1 cells. Pharmacological and biochemical approaches showed that cilia shortening mediated by MCH was associated with increased soluble cytosolic tubulin without changing the total tubulin amount. Enhanced F-actin fiber intensity was also observed in MCH-treated cells. The actions of various pharmacological agents revealed that coordinated actin machinery, especially actin polymerization, was required for MCHR1-mediated cilia shortening. A recent report indicated the existence of actin-regulated machinery for cilia shortening through GPCR agonist-dependent ectosome release. However, our live-cell imaging experiments showed that MCH progressively elicited cilia shortening without exclusion of fluorescence-positive material from the tip. Short cilia phenotypes have been associated with various metabolic disorders. Thus, the present findings may contribute toward better understanding of how the cytoskeleton is involved in the GPCR ligand-triggered cilia shortening with cell mechanical properties that underlies clinical manifestations such as obesity.

1. Introduction

Primary cilia are antenna-like sensory organelles that protrude from the surface of most growth-arrested vertebrate cells. These structures require large protein complexes and motors for distal addition of tubulin and extension of the ciliary membrane (Malicki and Johnson, 2017). Primary cilia transduce sensory and neuroendocrine signals from the extracellular milieu and play critical roles in the development and homeostasis of many organ systems (Louvi and Grove, 2011). Deficits in ciliogenesis cause diverse genetic disorders, such as Bardet-Biedl syndrome, that are collectively known as ciliopathies and have a complex set of symptoms, including obesity, polycystic kidney disease, retinal degeneration, and neurological defects (Hildebrandt et al., 2011). The cilia on a particular cell type have a normal length range at the right time. Abnormal cilia lengths have been observed in several neurological disorders (Chakravarthy et al., 2012; Formichi et al., 2017), suggesting the importance of maintaining the optimal cilia length in terminally differentiated neurons. In addition, stunted cilia have

Abbreviations: AcTub, acetylated α-tubulin; CytD, cytochalasin D; GPCR, G-protein-coupled receptor; hRPE1 cells, hTERT-RPE1 cells; IFT, intraflagellar transport; MCH, melanin-concentrating hormone; MCHR, melanin-concentrating hormone receptor; PTX, pertussis toxin; TIP, 2,4,6-triiodophenol.

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been found in metabolic disorders. For example, the cilia lengths in particular brain subregions were significantly reduced in obese mice with leptin or *BBS4* deficiency (*ob/ob* and *BBS4*−/− mice) (Agassandian et al., 2014; Han et al., 2014), and an increased frequency of short cilia in the hypothalamus was observed in high-fat-diet-induced obese mice compared with lean mice (Han et al., 2014). Therefore, much attention has been focused on the mechanisms that control cilia length, and extensive studies have identified many factors and processes that participate in cilia assembly and disassembly (Avasthi and Marshall, 2012). Among these, the importance of the large interconnected cytoskeletal networks, particularly microtubules and actin filaments, that provide both the main structural components and intraflagellar transport (IFT) has been noted. In ciliated cells, two types of microtubules can be categorized: cytoplasmic and axonemal. During cilia assembly, ciliary components including cytosolic tubulin dimers are transported by IFT from the cell body into the cilia and incorporated into the ciliary axoneme. Previous data showed that production of cytoplasmic soluble tubulin led to increased cilia length, while a limited supply of soluble tubulin after treatment with microtubule-stabilizing drug taxol led to shortened cilia or no cilia at all (Sharma et al., 2011). Other studies revealed that actin cytoskeleton dynamics are involved in cilia formation and length. A high-throughput RNA interference screen identified several actin regulatory factors, including the actin filament nuclear factor ARP2/3 complex as a positive regulator of ciliogenesis and cilia extension (Kim et al., 2010). Another study reported that microRNA mir-129-3p promoted both ciliogenesis and cilia elongation by repressing branched F-actin formation (Cao et al., 2012). Likewise, treatment of cells with cytochalasin D (CytD), an inhibitor of actin filament polymerization, increased the length of cilia (Kim et al., 2015, 2010; Li et al., 2011). These findings suggest that a polymeric state of the cytoskeleton in the cell body may be a prerequisite for regulating cilia length by facilitating ciliary structure assembly and disassembly.

Mammalian melanin-concentrating hormone (MCH) is an orexigenic neuropeptide that shows restricted expression to the lateral hypothalamus and the zona incerta (Bittencourt et al., 1992). In rodents, MCH acts through the sole receptor MCH receptor 1 (MCHR1), a class A G-protein-coupled receptor (GPCR) (Chambers et al., 1999; Saito et al., 1999). When transfected into non-ciliated CHO or HEK293 cells, MCHR1 can elicit calcium mobilization, inhibit forskolin-induced cAMP generation, and stimulate extracellular signal-regulated kinase phosphorylation via Gαi/o- and Gαq-mediated signaling (Hamamoto et al., 2015; Hawes et al., 2004). MCH neurons send their projections across the brain, and MCHR1 mRNA is expressed at high levels in several brain regions such as the hippocampus and nucleus accumbens (Saito et al., 2001). Growing evidence from genetic and behavioral studies suggests that the MCH-MCHR1 system regulates distinct aspects of feeding and energy metabolism in rodents (Chen et al., 2002; Marsh et al., 2002; Shimada et al., 1998). The neuronal cilia as signaling hubs detect and amplify extracellular neurotransmission by harboring membrane receptors including a limited set of class A GPCRs (Schou et al., 2015). Indeed, MCHR1 was shown to be enriched in a subset of motile ependymal cilia (Conductier et al., 2013) and primary cilia (Berbari et al., 2008a). Of note, neurons from obese mice lacking *BBS2* or *BBS4* protein failed to accumulate MCHR1 in the ciliary membrane (Berbari et al., 2008b). Instead, the receptor was found in cytoplasmic punctate areas in neurons. As short cilia phenotypes were also detected in BBS4 knockout mice (Han et al., 2014), it is possible that MCHR1 may regulate energy homeostasis through primary cilia. In this regard, we recently identified the novel biological phenomenon that MCH mediates cilia shortening through ciliary MCHR1 using hTERT-RPE1 (hRPE1) epithelial cells (Hamamoto et al., 2016). To the best of our knowledge, our report provided the first example of effective neuropeptide-induced cilia length reduction. Although ciliogenesis is usually tightly coupled to the cell cycle, cell cycle reentry was not a determinant for MCH-induced cilia shortening. Our study further showed the importance of a Gi/o-dependent Akt pathway for MCH-induced cilia shortening as the initial signaling pathway. However, the underlying mechanism for how structural changes in cytoskeletal elements are involved in the cilia shortening induced by MCH was not elucidated. In the present study, we addressed this issue using a newly isolated clonal cell line, and provide data that increased soluble tubulin in the cell body is accompanied by MCHR1-mediated cilia shortening. Furthermore, we show evidence that actin-polymerizing activity is positively involved in MCH-induced cilia shortening.

2. Materials and methods

2.1. Cell culture and cloning

hRPE1 cells stably expressing MCHR1:EGFP were established as described previously (Hamamoto et al., 2016). The cells were grown in Dulbecco's modified Eagle's medium/F12 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 0.5 mM sodium pyruvate, 15 mM HEPES (pH 7.5), 10 μg/ml hygromycin B, and 1% penicillin/streptomycin at 37 °C under 5% CO_2. As heterogeneous cell populations were observed during cell passages of the original stable cells, 15 individual clones were selected and seeded on Lab-Tek 8-well plates. To induce ciliogenesis in confluent cultures, the complete medium was replaced with serum-free medium, and the cells were cultured for a further 24 h, fixed, and observed by BX51 fluorescence microscopy (Olympus, Tokyo, Japan). Next, five individual clones were chosen using a mean cilia length of 3–6 μm as an index under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The five clones were further tested for the efficiency of MCH-induced cilia shortening by culture in serum-free medium containing MCH (Peptide Institute, Osaka, Japan) for 6 h. After fixation, the cells were processed for enhanced immunofluorescence detection by double-staining with anti-GFP and anti-acetylated-tubulin (AcTub) antibodies. Finally, one clone, named clone 7, was selected to analyze the time- and dose-dependency, effects on cilia length of pharmacological compounds, and live-cell imaging.

2.2. Effect of drug treatment on MCH-induced cilia length control

The following drugs were used in this study: pertussis toxin (PTX; List Biological Laboratories Inc., Campbell, CA); taxol, nocodazole (Sigma, St. Louis, MO); LY294002, CK-666, SMIFH2 (Merck Millipore, Darmstadt, Germany); CytD, latrunculin A, blebbistatin (Wako, Osaka, Japan); 2,4,6-triiodophenol (TIP; Tokyo Kasei, Tokyo, Japan); Akt1/2, BTP2 (Abcam, Cambridge, UK).

Clone 7 cells were pretreated with 10 ng/ml PTX for 24 h in serum-free medium. For other reagents, cells were serum-starved for 24 h prior to the pretreatment. The final concentrations of the reagents and the pretreatment periods were: 10 μM LY294002 (30 min); 3 μM Akt1/2 (30 min); 1 μM taxol (2 h); 100 nM nocodazole (2 h); 30 nM CytD (2 h); 300 nM latrunculin A (2 h); 50 μM CK-666 (30 min), 6 μM SMIFH2 (15 min); 10 μM blebbistatin (15 min); 10 μM TIP (15 min); 10 μM BTP2 (1 h). For LY294002 and Akt1/2, the cells were pretreated and incubated with or without MCH for 1 h. After MCH/drug removal by washing with serum-free medium, the cells were incubated without MCH for 5 h. Other drugs were incubated with or without MCH for 6 h after pretreatment. The treated cells were fixed and immunostained.