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Gamma-melanocyte stimulating hormone regulates the expression and cellular localization of epithelial sodium channel in inner medullary collecting duct cells

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ABSTRACT

Gamma$_2$-melanocyte-stimulating hormone (γ_2MSH) is a peptide hormone released by the pituitary gland which is thought to act directly on the renal inner medulla to promote increased sodium excretion into urine (natriuresis). The aim of this study was to determine if a stable analog, [Nle^3, D-Phe^6]-γ_2MSH (NDP-γ_2MSH), of the native peptide regulated the activity, expression and cellular localization of epithelial sodium channel (ENaC) in a murine inner medullary collecting duct (mIMCD-3) cell line. Our results indicate that expression of the γ_2MSH receptor, melanocortin receptor 3 receptor (MC3R), is up-regulated by culturing the cells in media with an increased osmolality (~400 mOsm/kg). Furthermore, stimulation of cAMP signaling and sodium transport by 1 nM NDP-γ_2MSH occurs only in cells cultured in the high osmolality media. Finally, treatment of mIMCD-3 cells cultured in high osmolality medium for 1 h with 1 nM NDP-γ_2MSH causes a reduction in expression of serum- and glucocorticoid-induced kinase (sgk1) and a reduction in expression and cell surface abundance of the alpha subunit of ENaC. Collectively, this data suggest that γ_2MSH directly regulates both ENaC expression and cellular localization in the inner medulla to exert its natriuretic effect.

1. Introduction

γ-Melanocyte-stimulating hormone (γMSH) comprises a family of three peptides (γ_1, γ_2 and γ_3MSH) that are synthesized in the pituitary gland by post-translational processing of the precursor pro-opiomelanocortin protein by prohormone convertase PC2. All MSH peptides exert their functions by interacting with a family of five melanocortin receptors (MC_1–MC_5), all of which are G-protein-coupled receptors which activate the cAMP/protein kinase A signaling pathway [6]. Of the five melanocortin receptors, the γMSH family has the highest affinity for the MC_3 receptor (MC3R) and is considered to be the natural ligand for this receptor. It has been reported that γ_2MSH is a natriuretic peptide which acts in an endocrine fashion to increase sodium excretion via a direct effect on the kidneys [6]. In rats fed a high-salt (HS) diet, plasma concentrations of γ_2MSH increase by approximately 100% and expression of the MC3R is upregulated in the inner medulla [2,4,14]. Collectively, these findings suggest that γ_2MSH may play a central role in the regulation of sodium homeostasis. It is well established that defects in the renal handling of sodium are involved in the development and maintenance of hypertension. In support of an involvement for γ_2MSH/MC3R in blood pressure regulation, it has been shown that mice lacking either the mc3r or proconvertase 2 (pc2) genes develop salt-sensitive hypertension. The hypertension in pc2−/−, but not mc3r−/−, mice was rapidly corrected by both intravenous and intracerebroventricular infusion of picomolar doses of exogenous γ_2MSH [13]. The biologically stable analog [Nle^{3}D-Phe^6]-γ_2MSH (NDP-γ_2MSH) has been also shown to prevent salt-sensitive hypertension in rats deficient in endogenous γ_2MSH [15] and a recent study by our group also showed that the analog induced a much more potent natriuresis/diuresis in HS fed rats compared to NS fed rats [4]. Therefore, the in vivo effects of both the native peptide [14] and its stable analog [4] are greatly enhanced in high salt fed rats.

How γ_2MSH acts at a cellular and molecular level to trigger natriuresis is unclear. The upregulation of MC3R expression in the inner medulla in response to an HS diet suggests that γ_2MSH may act directly on the inner medullary collecting duct (IMCD) to modulate the rate of sodium reabsorption. The activity and cellular localization of epithelial sodium channel (ENaC), which is the apical sodium transporter in the collecting duct is tightly regulated

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to control sodium reabsorption in this section of the nephron [5] An attractive hypothesis is that binding and activation of MC3R on IMCD epithelial cells may result in down-regulation of ENaC resulting in increased urinary excretion of sodium. The objective of this study was to investigate this hypothesis.

2. Materials and methods

2.1. Materials

Unless stated otherwise, all reagents were purchased from Sigma–Aldrich Ireland Ltd., Arklow, Ireland. The [YV(Nle)GH(D-Phe)RWNRFG] (NDP-γ_2MSH) peptide used in this study was synthesized by Sigma–Genosys Ltd., Cambridge, UK.

2.2. General cell culture and preparation of lysates

The murine inner medullary collecting duct-3 (mIMCD-3) was obtained from LGC Standards, Middlesex, UK. These cells were grown on 3450-clear transwells (0.4 μm pore size) (Corning B.V. Life Sciences, Netherlands) at 37 °C and 5% CO_2. Three types of culture media were used in this study: normal sodium medium [145 mM Na^+] consisted of DMEM/F12, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin; high sodium medium [200 mM Na^+] consisted of DMEM/F12, 55 mM $NaHCO_3$, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and mannitol medium consisted of DMEM/F12, 110 mM mannitol, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were grown in these media to confluency and were then made quiescent by incubation in serum-free medium for 24 h prior to experimentation.

For western blotting of MC3R, cell lysates were prepared after incubation in serum-free medium. In the total αENaC and serum- and glucocorticoid-induced kinase (sgk1) studies, NDP-γ_2MSH to a final concentration of 1 nM in serum-free medium was added to the basolateral compartment of the transwell for various time intervals, at which point all medium was removed and cells were washed in ice-cold PBS. Cells were scraped from transwells in 100 μl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% (sodium dodecyl sulphate) SDS, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)), transferred into 1.5 ml microfuge tubes and placed on ice for 30 min. Samples were then centrifuged at 20,000 *g* for 10 min and supernatants were transferred to fresh 1.5 ml microfuge tubes and were stored at −80 °C prior to analysis.

2.3. Western blot analysis

Protein concentrations were assessed using the BCA assay (Pierce, Rockford, IL) as described in manufacturer's protocol and samples were diluted in 6× SDS-PAGE loading buffer (300 mM Tris-HCl pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromophenol blue and 60% glycerol) to a loading concentration of 25 μg in 20 μl. Protein extracts were resolved on 12% SDS-polyacrylamide gels and were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk and were incubated overnight with antibodies to the following proteins: MC3R (Sigma–Aldrich) (1:500 dilution); αENaC (Abcam, Cambridge, UK) (1:2000 dilution); SGK1 (Abcam, Cambridge, UK) (1:1000 dilution) and actin (Millipore, Billerica, MA) (1:20,000 dilution) in 5% non-fat dried milk/TBST. The membrane was then incubated for 1 h at room temperature with a 1:1000 dilution of HRP-linked anti-rabbit secondary antibody (Cell Signaling Technology, Beverly MA) in 5% non-fat dried milk/TBST. Bands were visualized using enhanced chemiluminescence (ECL Plus, GE Healthcare, Bucks, UK). Resultant films were digitally photographed and densitometric analysis of bands was performed (UviPhotoMW, Solartech, Harrison, MI).

2.4. $[Na+]_i$ measurement

mIMCD-3 cells were grown on 35 mm glass-bottom dishes (10 mm grade '0′ cover-slip) (MatTek Corporation, Ashland, MA) in the normal sodium, high sodium and mannitol media described in Section 2.2 to ∼60% confluency. The cells were then incubated in normal sodium, high sodium and mannitol serum-free media without phenol red for 24 h. Cells were then loaded for 30 min at 37 °C and 5% CO_2 with the cell permeant sodium dye, sodium green tetraacetate (5 μm, Molecular Probes, Eugene, OR, USA), in serum-free medium without phenol red followed by washing with PBS three times and addition of fresh serum-free medium without phenol.

The plate was placed on the stage of an Olympus IX51 inverted fluorescence microscope within an encapsulating incubator (Solent Scientific, Segensworth, UK) maintained at 37 °C and excited at a wavelength of 480 nm (Cairn Monochromator and 75-W Xenon lamp, Cairn, Faversham, Kent, UK). Fluorescence was measured for every 20 s via an Olympus UplanF1 1.3 NA 100x oil-immersion objective, filtered through a dichroic mirror (515 nm cut-off) and recorded using a Hamamatsu ORCA-ER CCD video-camera (Hamamatsu Photonics Ltd., Hertfordshire, UK), set at exposure time of 500 ms per channel. Andor IQ v1.9 software (Andor, Belfast, Northern Ireland), was employed to control the hardware. At $t = 1.5$ min, either vehicle control or NDP-γ_2MSH to a final concentration of 1 nM was added to the plate and fluorescence was recorded for another 60 min. Following each experiment, calibration of the intracellular sodium green dye fluorescence was done using ionophores (5 μm nigericin and 5 μM monensin) to permeabilize the cell membrane and equilibrate $[Na^+]_i$ with extracellular $[Na^+]$ over a range of 0–140 mM.

2.5. $[cAMP]_i$ MEASUREMENT

mIMCD-3 cells were grown on HTS transwells (0.4 μm pore size) in a 96 well plate (Corning B.V. Life Sciences, Netherlands) at 37 °C and 5% CO_2 in the normal sodium, high sodium and mannitol media described in Section 2.2 until fully confluent. Following serum-starvation for 24 h, the basolateral compartments of the transwells were treated with vehicle control, 10 μM forskolin or 1 nM NDP-γ_2MSH for 30 min. Other wells were pre-treated with 1 nM SHU9119 (MC3R/MC4R antagonist) (Tocris Biosciences) for 30 min prior to treatment with vehicle or NDP-γ_2MSH. Intracellular cAMP concentrations were measured in the 96 well plate using a HitHunter cAMP Chemiluminescence Assay kit for adherent cells (GE Healthcare, Bucks, UK) as described by the manufacturer's protocol. cAMP standards provided with the kit was assayed simultaneously. Luminescence was quantified using a Wallac 1420 VICTOR2 plate reader (PerkinElmer, Bucks, UK). Intracellular cAMP concentrations of the mIMCD-3 cells were calculated from the standard curve and were expressed as pmole/well.

2.6. Biotinylation of mIMCD-3 cells

mIMCD-3 cells were cultured in T-125 flasks (Sarstedt, Nümbrecht, Germany) in high sodium culture medium as described in Section 2.2 until ∼95% confluent. Following serum starvation for 24 h, cells were treated with either vehicle or 1 nM NDP-γ_2MSH for 60 min. Cell surface biotinylation was performed with Sulfo-NHS-SS-Biotin using a Cell Surface Protein Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL USA) as described in the manufacturer's