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Characterization of murine melanocortin receptors mediating adipocyte lipolysis and examination of signalling pathways involved

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ABSTRACT

The melanocortin receptors (MCRs) belong to the G-protein coupled receptors (family A). So far, 5 different subtypes have been described (MC1R–MC5R) and of these MC2R and MC5R have been proposed to act directly in adipocytes and regulate lipolysis in rodents. Using ACTH and α-melanocyte stimulating hormone (α-MSH) generated from proopiomelanocortin (POMC), as well as synthetic MSH analogues to stimulate lipolysis in murine 3T3-L1 adipocytes it is shown that MC2R and MC5R are lipolytic mediators in differentiated 3T3-L1 adipocytes. Involvement of cAMP, phosphorylated extracellular signal-regulated kinase (ERK) 1/2, protein kinase B (PKB), adenosine 5′ monophosphate activated protein kinase (AMPK) and Jun-amino-terminal kinase (JNK) in MCR mediated lipolysis were studied. Interestingly, results obtained in 3T3-L1 cells suggest that lipolysis stimulated by α-MSH, NDP-α-MSH, MT-II, SHU9119 and PG-901 is mediated through MC5R in a cAMP independent manner. Finally, we identify essential differences in MCR mediated lipolysis when using 3T3-L1 cells compared to primary adipocytes.

1. Introduction

The melanocortin system acts through multiple pathways relevant for the prevention of obesity and obesity-related complications (Cone, 1999; Marks et al., 2002; Spiegelman and Flier, 2001). The system is acknowledged to have an important function in regulation of satiety and energy expenditure (Yaswen et al., 1999; Spiegelman and Flier, 2001; Cheung et al., 1997; Azzara et al., 2002). MC4R knockout mice exhibit a well-described phenotype defined by increased lean body mass and fat mass, hyperphagia and disturbances in the metabolic response to overnutrition (Mountjoy et al., 1994; Huszar et al., 1997; Tschop and Heiman, 2001). Furthermore, loss of MC4R function is the most frequent monogenetic alteration in severely obese humans (Krude et al., 1998) and in severe, early onset childhood obesity the frequency of mutations in the MC4R locus is 4–6% (Farooqi et al., 2003). Besides the effect on satiety and energy expenditure, the melanocortin system is believed to influence insulin release and insulin sensitivity (Fan et al., 2000; Huo et al., 2009). The melanocortin peptides and their receptors are also suspected to have a direct lipolytic effect on adipose tissues in rodents (Cho et al., 2005; Boston, 1999; Spirovski et al., 1975). However this effect is controversial in humans (Hoch et al., 2007). The effect of melanocortins on adipocytes have by some researchers been explained by neuronal regulation of lipolysis (Brito et al., 2007), since MC4R mRNA has been identified in sympathetic neurons connected to white adipose tissue (WAT), indicating that central MC4R might stimulate lipid mobilization in the periphery (Song et al., 2005). However, studies

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in differentiated murine 3T3-L1 adipocytes suggest a direct lipolytic effect stimulated by melanocortin peptides ACTH and α-MSH (Bradley et al., 2005; Cho et al., 2005), which supports the earlier identification of MC2R and MC5R mRNA in this cell line (Boston and Cone, 1996).

MC1R action is primarily connected to melanocytes (Wikberg et al., 2000) but it is also expressed in human subcutaneous fat where the receptor has been found to elicit cell proliferation (Hoch et al., 2007). MC2R is primarily expressed within the adrenal cortex, but has also been identified in mouse white adipose tissue (WAT) (Boston, 1999) and is found to induce lipolysis in WAT from both mouse and rat (Bradley et al., 2005; Boston, 1999). Indeed, the MC2R was suggested to stimulate lipolysis through activation of hormone sensitive lipase in 3T3-L1 adipocytes (Cho et al., 2005). The expression of MC2R in human adipose tissue remains controversial (Abdel-Malek et al., 2000; Wikberg et al., 2000). The receptor has by some researchers been identified in human subcutaneous WAT (Smith et al., 2003), although the lipolytic function remains unclear. MC3R is located in the brain where it is expressed in the hypothalamus (Marks et al., 2006) especially in the arcuate nucleus (Roselli-Rehfuss et al., 1993; Mountjoy et al., 1994; Mountjoy and Wild, 1998). MC3R knockout mice display reduced lean body mass, an increased fat mass and are slightly hypophagic compared to wild type controls (Marks et al., 2006), but the receptor has not been ascribed lipolytic importance. MC4R is widely located throughout the CNS and high levels have been found especially in hypothalamic regions involved in regulation of feeding behaviour and energy expenditure, such as the paraventricular nucleus of the hypothalamus (Mountjoy et al., 1994; Mountjoy and Wild, 1998). MC4R mRNA has also been identified in human adipose tissue, where it might contribute to the control of energy balance and body weight (Chagnon et al., 1997) although this involvement remains controversial. The function of MC5R is poorly understood despite its extensive distribution in the periphery (Bednarek et al., 2007). MC5R has been identified in exocrine gland tissue and are involved in glandular secretion (van der Kraan et al., 1998). Besides the expression in 3T3-L1 cells, MC5R has also been identified in human adipose tissue and associated with obesity phenotypes (Chagnon et al., 1997).

The G_s transduction pathway is established to be a validated path of signalling for all MCRs (Barrett et al., 1994; Mountjoy et al., 1992) all though other signalling pathways have been suggested (Buch et al., 2009; Konda et al., 1994; Daniels et al., 2003; Rodrigues et al., 2009). The natural occurring melanocortin peptide family consists of α-, β- and γ-melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). These peptides are generated from a common precursor proopiomelanocortin (POMC) and function as MCR agonists. It has previously been shown, that MC2R binds ACTH and none of the other POMC derived peptides (Baumann et al., 1986; Boston and Cone, 1996). When a MCR is stimulated, it couples to $G\alpha_s$, which activates intracellular adenylyl cyclase and increases cAMP production. α-MSH stimulated phosphorylation of extracellular signal-regulated kinase (ERK) has previously been linked to lipolysis in 3T3-L1 cells and MC5R has been established to activate phosphatidylinositol 3-kinase dependent ERK after stimulation by α-MSH (Rodrigues et al., 2009). In addition, cAMP dependent AMPK has been linked to MC5R mediated fatty acid oxidation stimulated by α-MSH in skeletal muscle (An et al., 2007). The essential function of central MC4R in regulation of satiety and energy expenditure raises questions of other potential metabolic effects of the melanocortin system. So far, the attention of this system in metabolic research has been focused on the CNS. A systemic abundance of other melanocortin peptides than ACTH has been suggested by several researchers (Yaswen et al., 1999; Shishioh-Ikejima et al., 2010; Zemel and Shi, 2000). Furthermore, peripheral effects of melancortin receptor antagonist agouti-related protein (AgRP) supports the assumption that a systemic melanocortin system indeed exists (Doghman et al., 2007). However, it is not fully known where and how the melanocortin peptides act peripherally. Identification of pharmacological selective ligands with physiological relevance in the periphery is an essential aspect not yet clarified. MC5R has been shown to be expressed in various peripheral tissues relevant for lipid and glucose metabolism (An et al., 2007; Boston, 1999), for which reason identification of peripheral MC5R selective agonists are highly relevant. Different non-selective and selective MCR agonists and antagonists have been described in various studies, addressing the role of MCR receptor pharmacology. However, one single focussed study which correlates binding affinities of these known MCR ligands on mouse receptors with their ability to induce lipolysis in adipocytes has not previously been shown. In this study, mouse MCR binding affinities of known human agonists are linked to MCR mediation of lipolysis in murine 3T3-L1 cells and the signalling pathways involved. We find that lipolysis in 3T3-L1 cells stimulated by α-MSH, NDP-α-MSH, MT-II, SHU9119 and PG-901 are mediated through MC5R independent of cAMP signalling. Finally, we identify essential differences in MCR mediated lipolysis between 3T3-L1 cells and primary adipocytes from isolated mouse epididymal adipose tissue.

2. Materials and methods

2.1. Test compounds

NDP-α-MSH (BACHEM), ACTH (BACHEM), α-MSH (BACHEM), melanotan (MT-II), SHU9119, PG-901, LY2112688 (prepared in-house using standard peptide synthesis protocol) and isoproterenol (Sigma).

2.2. Materials and cell lines

Trizol (Invitrogen) and 1-bromo-3-chloro-propane (Sigma) were employed in the extraction of RNA from adipose tissue. RNeasy mini-kit (Qiagen) and RNase-free DNase set (Qiagen) were used in RNA extraction from cells and tissue. A RNA ladder was used to visualize intact RNA (Invitrogen). iScript™ cDNA synthesis kit (Biorad) was employed when synthesizing cDNA. Platinum qPCR supermix (Invitrogen), Primers (scanprimer/oligonucleotides; DNA-Technology) and LNA probes (Exiqon ProbeLibrary) were used in real time PCR. B16-F12 mouse melanoma cells (ECACC) expressing MC1R, mouse MC3R cDNA transfected CHO-K1 cells (Euroscreen), mouse MC4R cDNA transfected BHK570 cells (in house produced) and mouse MC5R cDNA transfected CHO-K1 cells (PerkinElmer Products) were employed in binding studies. I^{125}-NDP-α-MSH (PerkinElmer) was used as radioligand in binding assays. 97% Ovalbumin (Sigma), Tween-20 (Merck-Schuchardt), HEPES (Sigma) and hydroxypropyl-β-cyclodextrin (HP-β-CD) (Acros Organics) were used in all binding buffers and Bacitracin (Sigma) was used in MC4R binding buffer. Confluent 3T3-L1 cells were harvested using versene (Lonza) and inducers of adipogenesis in 3T3-L1 cells were Troglitazone (Sigma), IBMX (Sigma), dexamethazone (Sigma) and human insulin (Novo Nordisk). Cells were stained using Oil Red O (Sigma). NEFA-HR (2) kit from Wako was employed when measuring release of non-esterified fatty acid (NEFA) as an index of lipolysis. Collagenase Type II (Sigma C-6885) was used in degradation of epididymal WAT. Non-metabolizable adenosine phenylisopropyl adenosine (PIA) (Sigma) and adenosine deaminase (ADA) (Roche) was used when measuring NEFA from isolated adipocytes. FlashPlate® cAMP assay (Life Science Products) was employed to determine cyclase activity in stimulated 3T3-L1 cells. Invitrogen cell extraction buffer, protease inhibitor (Sigma) and 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF) (Calbiochem) was used in protein purification. In immunoblotting Bis Tris gels (4–12%, Invitrogen), NuPAGE MOPS SDS Running buffer (Thermo Scientific), antioxidant (Thermo Scientific), full range rainbow marker (Amersham), iBLOT gel transfer stacks (Invitrogen), starting block T20 buffer (Thermo Scientific), TBS-T (Ambresco) and Amersham detection reagents were used. Primary antibodies for ERK, PKB, AMPK and JNK were purchased from Cell Signalling. Secondary antibodies from Thermo Scientific and MEK inhibitor (U0126) from Calbiochem were used.

2.3. Cell culture

B16-F12 melanoma cells expressing MC1R was cultured in DMEM with glutamax and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Mouse MC3R and MC5R cell lines were cultured in Ham's F12 medium 10% FCS, 1% pen/strep and 0.4 mg/ml G418 at 37 °C and 5% CO_2. Mouse MC4R cell line was cultured in DMEM, 10% FCS, 1 mg/ml G418 and 1% pen/strep at 37 °C and 5% CO_2.