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Ghrelin and cannabinoids require the ghrelin receptor to affect cellular energy metabolism

Chung Thong Lim [a], Blerina Kola [a], Daniel Feltrin [a], Diego Perez-Tilve [b], Matthias H. Tschöp [b,c], Ashley B. Grossman [a,d], Márta Korbonits [a,*]

[a] Centre for Endocrinology, William Harvey Research Institute, Barts and the London, London EC1M 6BQ, UK
[b] Metabolic Disease Institute, Department of Internal Medicine, University of Cincinnati, Cincinnati, OH 45237, USA
[c] Institute for Diabetes and Obesity, Helmholtz Centre Munich, Department of Medicine, Technische Universität München, Munich, Germany
[d] Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford OX3 7LE, UK

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ABSTRACT

Introduction: Ghrelin is a potent orexigenic brain-gut peptide with lipogenic and diabetogenic effects, possibly mediated by growth hormone secretagogue receptor (GHS-R1a). Cannabinoids also have orexigenic and lipogenic effects. AMPK is a regulator of energy homeostasis and we have previously shown that ghrelin and cannabinoids stimulate hypothalamic AMPK activity while inhibiting it in the liver and adipose tissue, suggesting that AMPK mediates both the central appetite-inducing and peripheral effects of ghrelin and cannabinoids.

Aims: Using GHS-R KO mice, we investigated whether the known ghrelin receptor GHS-R1a is required for the tissue-specific effects of ghrelin on AMPK activity, and if an intact ghrelin signalling pathway is necessary for the effects of cannabinoids on AMPK activity.

Methods: Wild-type and GHS-R KO mice were treated intraperitoneally with ghrelin 500 ng/g bodyweight or CB1 agonist HU210 20 ng/g and hypothalamic, hepatic and adipose AMPK activity was studied using a functional kinase assay.

Results: Ghrelin and HU210 significantly stimulated hypothalamic AMPK activity in wild-type animals (mean ± SEM, 122.5 ± 5.2% and 128 ± 11.6% of control, $p < 0.05$) and inhibited it in liver (55.1 ± 4.8% and 62.2 ± 14.5%, $p < 0.01$) and visceral fat (mesenteric fat (MF): 54.6 ± 16% and 52.0 ± 9.3%, $p < 0.05$; epididymal fat (EF): 47.9 ± 12.1% and 45.6 ± 1.7%, $p < 0.05$). The effects of ghrelin, and interestingly also HU210, on hypothalamic, visceral fat and liver AMPK activity were abolished in the GHS-R KO mice (hypothalamus: 107.9 ± 7.7% and 87.4 ± 13.3%, liver: 100.5 ± 11.6% and 116.7 ± 5.4%, MF: 132.1 ± 29.9% and 107.1 ± 32.7%, EF: 89.8 ± 7.3% and 91.7 ± 18.3%, $p > 0.05$).

Conclusions: Ghrelin requires GHS-R1a for its effect on hypothalamic, liver and adipose tissue AMPK activity. An intact ghrelin signalling pathway is necessary for the effects of cannabinoids on AMPK activity.

1. Introduction

Ghrelin is a circulating growth hormone-releasing and appetite inducing brain-gut peptide (Cummings, 2006) that is predominantly synthesised by the endocrine X/A-like cells in the gastric mucosa (Korbonits et al., 2004). It is the endogenous ligand for the previously 'orphan' growth hormone secretagogue-receptor (GHS-R), a G protein-coupled receptor with seven transmembrane domains. There are two splicing variants of the GHS-R gene: the functioning full-length product GHS-R1a and a truncated variant GHS-R1b.

One of the most important established roles of ghrelin is the regulation of appetite and energy homeostasis (Kojima et al., 2004; Korbonits et al., 2004). Both central and peripheral administrations of ghrelin cause an increase in appetite and body weight (Kamegai et al., 2001; Tschop et al., 2000; Wren et al., 2001a). Ghrelin acts mainly in the hypothalamus by binding to its receptors in areas that are important for appetite regulation, namely the arcuate nucleus, paraventricular nuclei, dorsomedial region, central nucleus of amygdale and the nucleus of solitary tract (Mano-Otagiri et al., 2006; Olszewski et al., 2003). Ghrelin stimulates the secretion of orexigenic neuropeptide Y, agouti-related protein and orexin, and inhibits the release of anorexigenic

* Corresponding author. Address: Endocrinology and Metabolism, Queen Mary University of London Barts and the London School of Medicine and Dentistry, Department of Endocrinology, Charterhouse Square, London EC1M 6BQ, UK. Tel.: +44 20 7882 6238; fax: +44 20 7882 6197.
E-mail address: m.korbonits@qmul.ac.uk (M. Korbonits).

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compounds such as pro-opiomelanocortin, cocaine- and amphetamine-regulated transcript and α-melanocyte-stimulating hormone, thus resulting in an increase in appetite (Gao and Horvath, 2007; Kohno et al., 2003).

In the liver, ghrelin has opposite effects to insulin on the expression of the enzyme PEPCK, thereby promoting gluconeogenesis and also causing a decrease in glucose uptake and fatty acid oxidation (Rigault et al., 2007). Ghrelin also stimulates hepatic lipogenesis *de novo* in a GH-independent fashion (Sangiao-Alvarellos et al., 2009). Ghrelin promotes adipose tissue deposition by reducing the use of fat as metabolic fuel (Tschop et al., 2000). Intracerebroventricular infusion of ghrelin into neuropeptide-Y (NPY) neurons promotes adiposity, thereby suggesting that the effects of ghrelin on adiposity could be driven through central NPY pathways (Baran et al., 2002). Ghrelin has also recently been shown to play a crucial role in blood glucose maintenance during prolonged extreme hypocaloric conditions via an increase in growth hormone release, and possibly via a direct action (Zhao et al., 2010). All these central and peripheral effects of ghrelin are suggested to be via an effect on the GHS-R.

We have previously shown that ghrelin increases AMP-activated protein kinase (AMPK) activity in the hypothalamus and inhibits it in the liver and adipose tissue (Kola et al., 2005), suggesting that AMPK mediates at least some of the central and peripheral metabolic effects of ghrelin. AMPK is a key enzyme regulator in energy homeostasis both at cellular and whole body energy levels (Kola et al., 2006; Xue and Kahn, 2006). Activated AMPK inhibits anabolic pathways as well as stimulating catabolic pathways and appetite, thus conserving the ATP levels (Xue and Kahn, 2006).

Cannabinoids are also orexigenic compounds that have been shown to increase food intake (Williams et al., 1998) via the cannabinoid (CB)-1 receptor (Jamshidi and Taylor, 2001; Williams and Kirkham, 1999). Peripherally, cannabinoids promote lipogenesis in the liver (Osei-Hyiaman et al., 2005). We have also shown that cannabinoids increase AMPK activity in the hypothalamus and inhibit it in the liver and adipose tissue (Kola et al., 2005).

It has been shown that the effects of ghrelin on food intake and AMPK activity are absent in CB1 knock-out (KO) or CB1 receptor antagonist rimonabant-treated rodents (Kola et al., 2008). Ghrelin also increased the hypothalamic endocannabinoid content in wild-type (WT) mice, but not in CB1 KO or rimonabant-treated mice (Kola et al., 2008). These results suggested that an intact cannabinoids signalling system is necessary for the effects of ghrelin on AMPK activity.

By using GHS-R KO mice model, we aimed to investigate whether the GHS-R is required for the effects of ghrelin on AMPK activity of different tissues, and if an intact ghrelin signalling pathway is necessary for the effects of cannabinoids on AMPK activity. We hypothesised that ghrelin requires the presence of GHS-R for its effects on AMPK activity of different tissues. We also hypothesised that the effects of cannabinoids on AMPK activity of different tissues require the presence of an intact ghrelin–GHS-R signalling pathway.

2. Materials and methods

2.1. In vivo study

Male WT C57BL/6 J mice and GHS-R KO mice (Sun et al., 2004) weighing approximately 35 g and age-matched were used in the study, each equally divided into three groups. Animals were housed individually under standard environmental conditions (light from 6 AM to 6 PM, temperature 22 ± 1 °C). The mice were handled regularly and were given intraperitoneal (ip) injection for 4 days before the study day with saline or the CB1 agonist HU210 (2, 5 or 20 ng/g, Tocris Bioscience), to get them accustomed to injection and to the cannabinoid effect, as suggested previously (Kirkham and Tucci, 2006). The average food intake of chow diet after 1, 2, 3 and 4 h post-injection were measured on the day before the study day.

The mice were again fed *ad libitum* the night before. On the day of Experiment 2, they were treated with vehicle (saline), ghrelin 500 ng/g or HU210 20 ng/g. The doses were chosen for their established effectiveness in earlier studies to promote increases in food intake (Kirkham et al., 2002; Osei-Hyiaman et al., 2005). Food was removed immediately upon treatment, leaving them with water only. One hour later the mice were killed and the hypothalamus, liver, fat pads (inguinal, mesenteric and epididymal fat: IF, MF and EF respectively) and pituitary gland were removed. Tissue samples were frozen in liquid nitrogen and stored at −80 °C directly. The experimental procedures carried out in this study were in compliance with the UK Animals (Scientific Procedures) Act 1986.

2.2. GHS-R and CB1 receptors expression study

To confirm the absence of GHS-R expression in GHS-R KO mice and to study the expression of CB1 receptor in both WT and GHS-R KO mice, RNA was extracted from the pituitary glands of WT and GHS-R KO mice using Qiagen RNA extraction kit. RNA was quantified using Nanodrop spectrophotometer and RT-PCR was performed to obtain cDNA. Each reaction contained 1 μg of DNase I-treated total RNA isolated from different mouse tissues and primers for GHS-R1a (forward primer 5′ GTATGGGTGTCGAGCGTCTT 3′, reverse primer 5′ CTTCTGGTGTGGAGCAATGA 3′) or CB1 receptor (forward primer 5′ CTGGTTCTGATCCTGGTGGT 3′, reverse primer 5′ TGTCTCAGGTCCTTGCTCCT 3′) respectively. The cycling parameters for 35 cycles were set as 94 °C, 30 s; 60 °C, 1 min; and 72 °C, 30 s. Samples were loaded onto 2% agarose gel.

2.3. AMPK activity assay

The kinase assay for AMPK activity has been described previously (Hawley et al., 2003; Kola et al., 2005). Briefly, the tissues of interest (hypothalamus, fat pads and liver) were weighed and homogenised with Precellys 24 using CK14 tubes containing ceramic beads (Stretton Scientific, Stretton, UK) at 6000 rpm for 1 cycle of 20 s in tissue lysis buffer containing 50 mM Tris–HCl, 50nM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethane sulfonyl fluoride (PMSF), 5 μg/ml soybean trypsin inhibitor (SBTI) and protease inhibitor (for hypothalamus, hypothalamic lysis buffer containing 50 mM Tris–HCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 250 mM sucrose, 1% Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride, 5 mg/ml soybean trypsin inhibitor and protease inhibitor was used). Protein content was determined using BCA assay (Pierce, Rockford, USA). AMPK was immunoprecipitated with an equal mixture of α1-AMPK and α2-AMPK antibodies (Hawley et al., 2003) and AMPK activity was determined by the entity of phosphorylation of SAMS (Pepceuticals Ltd., Nottingham, UK), a synthetic peptide substrate of AMPK.

2.4. Statistical analysis

Data were analysed using the ANOVA followed by the Newman–Keuls test or the Kruskal–Wallis test followed by Conover–Inman comparison, as appropriate. $p < 0.05$ was considered significant and data were expressed as means ± standard error in each treatment group.