Molecular and Cellular Endocrinology 442 (2017) 165–181

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Ghrelin modulates gene and protein expression of digestive enzymes in the intestine and hepatopancreas of goldfish (*Carassius auratus*) via the GHS-R1a: Possible roles of PLC/PKC and AC/PKA intracellular signaling pathways

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ARTICLE INFO

Article history:
Received 16 September 2016
Received in revised form
28 December 2016
Accepted 28 December 2016
Available online 29 December 2016

Keywords:
Fish
Ghrelin
Sucrase-isomaltase
Aminopeptidase
Trypsin
Lipoprotein lipase

ABSTRACT

Ghrelin, a multifunctional gut-brain hormone, is involved in the regulation of gastric functions in mammals. This study aimed to determine whether ghrelin modulates digestive enzymes in goldfish (*Carassius auratus*). Immunofluorescence microscopy found colocalization of ghrelin, GHS-R1a and the digestive enzymes sucrase-isomaltase, aminopeptidase A, trypsin and lipoprotein lipase in intestinal and hepatopancreatic cells. *In vitro* ghrelin treatment in intestinal and hepatopancreas explant culture led to a concentration- and time-dependent modulation (mainly stimulatory) of most of the digestive enzymes tested. The ghrelin-induced upregulations of digestive enzyme expression were all abolished by pre-incubation with the GHS-R1a ghrelin receptor antagonist [D-Lys3]-GHRP-6, and most of them by the phospholipase C inhibitor U73122 or the protein kinase A inhibitor H89. This indicates that ghrelin effects on digestive enzymes are mediated by GHS-R1a, partly by triggering the PLC/PKC and AC/PKA intracellular signaling pathways. These data suggest a role for ghrelin on digestive processes in fish.
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1. Introduction

Ghrelin is a peptide hormone originally discovered from the rat stomach (Kojima et al., 1999). It is typically found as a 28-amino acid peptide in mammals, but several different isoforms were reported in many animals, especially non-mammalian vertebrates. In goldfish (*Carassius auratus*), 11 molecular forms of ghrelin consisting of 14-, 17-, 18- or 19- amino acid residues were identified (Miura et al., 2009). Stomach is the major tissue source of circulating ghrelin, although the endocrine pancreas, kidney, pituitary and hypothalamus also contribute to the ghrelin pool (Date et al., 2000; Gnanapavan et al., 2002; Kojima et al., 1999; Mori et al., 2000; Wierup et al., 2002). A unique aspect of this hormone is the presence of a post-translational acyl modification, essential for its activation, which is catalysed by ghrelin O-acyl transferase (GOAT)

Abbreviations: AC, Adenylyl cyclase; AgRP, Agouti-related peptide; AMPK, AMP-activated protein kinase; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; [D-Lys3]-GHRP-6, [D-Lys3]-Growth hormone releasing peptide-6; GH, Growth hormone; GHS-R, Growth hormone secretagogue receptor; GOAT, Ghrelin O-acyl transferase; GRL, Ghrelin; IHC, Immunohistochemistry; MAPK, Mitogen-activated protein kinase; mTOR, Mechanistic target of rapamycin; NPY, Neuropeptide Y; OD, Optical density; PBS, Phosphate-buffered saline; PFA, Paraformaldehyde; PKA, Protein kinase A; PKC, Protein kinase C; PLC, Phospholipase C; RT-qPCR, Real-time quantitative PCR.

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http://dx.doi.org/10.1016/j.mce.2016.12.027
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(Gutierrez et al., 2008; Yang et al., 2008). Ghrelin has been involved in a wide array of physiological functions in both mammals and fish (Delporte, 2013; Kaiya et al., 2013, 2008; Müller et al., 2015). These include the promotion of growth hormone release from pituitary (Kamegai et al., 2004; Unniappan and Peter, 2004), stimulation of food intake, carbohydrate utilization and adiposity (Asakawa et al., 2003; Kang et al., 2011; Unniappan et al., 2004; Wren et al., 2001), regulation of water intake (Hashimoto et al., 2007; Kozaka et al., 2003), and modulation of insulin secretion (Cruz et al., 2010; Dezaki, 2013; Yada et al., 2008). Additionally, a role for ghrelin in the circadian system has been suggested (Nisembaum et al., 2014; Yannielli et al., 2007).

Ghrelin acts by binding to G-protein coupled receptors known as growth hormone (GH) secretagogue receptors (GHS-R), or ghrelin receptors (Kaiya et al., 2013; Kojima et al., 1999). Unlike mammals and other tetrapod vertebrates with only one GHS-R gene, teleosts have two paralog GHS-R genes (GHS-R1 and GHS-R2) as a result of the ancestral genome duplication that this group of fish underwent. Additionally, some fishes, including goldfish, have experienced another genome duplication leading to the presence of four subtypes of GHS-Rs (GHS-R1a1, GHS-R1a2, GHS-R2a1 and GHS-R2a2) (Blanco et al., 2016b; Kaiya et al., 2010). Among the different subtypes of GHS-R described, GHS-R1a seems to be the most relevant and the one mediating most of the physiological functions of ghrelin (Gnanapavan et al., 2002; Kaiya et al., 2013; Yin et al., 2014). GHS-R1a is mainly coupled to phospholipase C (PLC)/protein kinase C (PKC) or adenylyl cyclase (AC)/protein kinase A (PKA) intracellular signal transduction pathways (Castañeda et al., 2010; Grey and Chang, 2011; Yin et al., 2014), although it can also act via other molecular pathways, including the AMP-activated protein kinase (AMPK) (Hardie, 2004), the mitogen-activated protein kinase (MAPK) (Kim et al., 2004; Mazzocchi et al., 2004) and the mechanistic target of rapamycin (mTOR) (Li et al., 2014) pathways.

It is well known that many gastrointestinal peptides, including ghrelin (Peeters, 2005; Schubert, 2010), participate in the regulation of gastric functions and digestive processes in mammals. Ghrelin has been shown to stimulate gastric emptying (Dornonville de la Cour et al., 2004; Masuda et al., 2000), and enhance gastric acid secretion (Date et al., 2001; Masuda et al., 2000; Mori et al., 2007; Yakabi et al., 2008). Ghrelin also modulates the expression of pepsin in the stomach and duodenum of rats (Warzecha et al., 2006), the activity of pepsin in gastric mucosal cells of piglets (Du et al., 2016), and the activity of some lysosomal hydrolases in rabbits (Witek et al., 2005). Additionally, a role for ghrelin in the regulation of the pancreatic exocrine secretion has been reported in mammals, although contradictory observations were reported in relation to this action. Thus, pancreatic amylase secretion and pancreatic fluid and protein output in rats are increased by intraduodenally and intracerebroventricularly administered ghrelin respectively (Nawrot-Porąbka et al., 2007; Sato et al., 2003). However, intravenous administration of ghrelin inhibits the pancreatic protein output in rats (Zhang et al., 2001), as well as it reduces the volume of pancreatic-biliary juice protein and trypsin outputs (Kapica et al., 2008, 2006). Ghrelin was found to produce no changes in amylase release from dispersed rat pancreatic acinar cells (Zhang et al., 2001). Thus, ghrelin effects on digestive enzymes appear to be species specific and are dependent on the mode of administration.

While ghrelin seems to be an important modulator of gastrointestinal functions in mammals, whether this function is conserved across vertebrates, especially in fish has been scarcely investigated. A role for ghrelin in the modulation of intestinal motility has been suggested in fish, particularly zebrafish, in which ghrelin was reported to cause a significant contraction of both circular and longitudinal muscle in the intestinal bulb and the mid/distal intestine (Olsson et al., 2008). A contractile effect for ghrelin on the longitudinal muscles of the intestinal bulb and stomach was also reported in goldfish and rainbow trout (respectively), although the magnitude of the observed contractions was considerably less (Kitazawa et al., 2012). Recently, we reported that the expression levels of ghrelin and other components of the ghrelinergic system, including GOAT and GHS-R-1a, is differentially modulated by the macronutrient composition of the diet (Blanco et al., 2016a), suggesting that ghrelin is implicated in digestive functions.

In this study, we aimed to determine the possible role of ghrelin on some of the main digestive enzymes in goldfish: the glucosidase enzyme sucrase-isomaltase, the proteolytic enzymes aminopeptidase A and trypsin, and the lipolytic enzyme lipoprotein lipase (Sarbahi, 1951). First, we characterized whether ghrelin or GHS-R1a are present in the same endocrine cells expressing one of the above mentioned digestive enzymes within the goldfish intestine and hepatopancreas. Then, using an *in vitro* approach, we studied the possible modulation of ghrelin on the abundance of these digestive enzymes in cultured intestine and hepatopancreas explants. Our results show that ghrelin modulates the expression of sucrase-isomaltase, aminopeptidase A and lipoprotein lipase in intestine in a time- and concentration-dependent manner, and that it exerts a mainly stimulatory effect on sucrase-isomaltase and lipoprotein lipase in hepatopancreas after short exposure times. Based on these results, we investigated whether the GHS-R1a ghrelin receptor antagonist [D-Lys3]-Growth hormone releasing peptide-6 ([D-Lys^3]-GHRP-6) counteracts the modulatory action of ghrelin. Finally, we attempted to determine if the PLC/PKC and AC/PKA intracellular signal transduction pathways contribute to ghrelin effects on digestive enzyme expression.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus*) of the common variety, with a body weight of 5 ± 1 g (for immunohistochemistry) or 32 ± 8 g (for tissue culture), were obtained from a commercial supplier (Aquatic Imports, Calgary, AB, Canada). Fish were housed in 300 L aquaria with filtered fresh water at 20 ± 2° C and continuous aeration, and maintained under a 12 h light:12 h darkness (12L:12D) photoperiod (lights on at 07:00 h). Food from a commercial pellet diet for goldfish (Goldfish granules, Aqueon, Franklin, WI, USA) was offered daily at 10:00 h until visually apparent satiety. Sampling is detailed below. All fish studies adhered to the Canadian Council of Animal Care guidelines, and research protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number, 2012-0082).

2.2. Reagents

The acylated 19 amino acid, full-length form of goldfish ghrelin (GTS(Octanoyl)FLSPAQKPQGRRPPRM) was obtained from GenScript (Piscataway, NJ, USA). Peptide was reconstituted in distilled water at a stock concentration of 100 μM. Stock solutions of the GHS-R1a ghrelin receptor antagonist [D-Lys^3]-GHRP-6 and the PLC inhibitor U73122 (both from Sigma-Aldrich, Oakville, ON, Canada) were prepared at a concentration of 10 mM in absolute ethanol. The PKA inhibitor H89 (Sigma-Aldrich) stock solution was prepared in distilled water at a concentration of 10 mM. All the stock solutions were diluted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 44 mM sodium bicarbonate, 1% penicillin-streptomycin and 0.05% gentamicin (DMEM+) to reach the required experimental concentrations just before use. The

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