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Goldfish kisspeptin: Molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level

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ABSTRACT

Kisspeptin, the product of Kiss1 gene, is a novel regulator of the gonadotropic axis. In mammals, its stimulatory effect on gonadotropin secretion is well documented and mediated mainly by hypothalamic release of gonadotropin-releasing hormone. Although the pituitary actions of kisspeptin have been reported, the effects of kisspeptin on gonadotropin release via direct action on pituitary cells are still controversial. Using goldfish as a model, here we examined the direct actions of kisspeptin on pituitary functions in modern-day bony fish. As a first step, the structural identity of goldfish Kiss1 was established by 5′/3′RACE and Kiss1 transcript was shown to be widely expressed in various tissues in goldfish. At the pituitary level, Kiss1 receptor (Kiss1r) expression was detected in immuno-identified gonadotrophs, lactotrophs, and somatotrophs. Kiss1 transcript was also located in goldfish somatotrophs but not in lactotrophs or gonadotrophs. In parallel studies, goldfish kisspeptin-10 was synthesized and used to test the pituitary actions of kisspeptin *in vitro*. In goldfish pituitary cell cultures, 30-min incubation with kisspeptin-10 increased basal release of luteinizing hormone (LH), prolactin (PRL), and growth hormone (GH). Transcript expression of LH, PRL, and GH were also elevated by prolonging kisspeptin-10 treatment to 24 h. These results taken together suggest that kisspeptin via Kiss1r activation can act directly at the pituitary level to trigger LH, PRL, and GH secretion and gene expression in goldfish. Our finding of Kiss1 expression in somatotrophs also rises the possibility that kisspeptin may be produced locally in the fish pituitary and serve as an autocrine/paracrine regulator.

1. Introduction

Kisspeptin, a member of the RFamide peptide family, was first identified by its inhibitory effect on cancer metastasis (Lee et al., 1996). In mammals, variants of kisspeptin including kisspeptin-54, kisspeptin-14, kisspeptin-13, and kisspeptin-10 have been identified (Bilban et al., 2004). These variants are the products of Kiss1 gene caused by differential processing of Kiss1 preprohormone (Ohtaki et al., 2001). They all share the common C-terminal 10 amino acid (a.a.) core sequence kisspeptin-10, which allows them to bind to their cognate receptor Kiss1r (or GPR54) with high affinity (Kotani et al., 2001). Kisspeptin, through activation of Kiss1r, plays an important role in the timing of puberty (Colledge, 2004), maintenance of gonadal functions (Roa et al., 2008a), photoperiod control of seasonal breeding (Greives et al., 2007), metabolic gating of fertility (Castellano et al., 2009), and trophoblast invasion during pregnancy (Bilban et al., 2004). In recent years, kisspeptin has been proposed to be a novel gatekeeper for the gonadotropic axis mainly by its potent stimulation on gonadotropin secretion (Roa et al., 2009; Roseweir and Millar, 2009). In the rat, the stimulatory actions on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are mediated by the Kiss1/Kiss1r systems within the periventricular and arcuate nuclei (Gottsch et al., 2004), which can activate gonadotropin-releasing hormone (GnRH) neurons located in the preoptic area (POA) of the hypothalamus (Roseweir and Millar, 2009). In the same animal model, kisspeptin-induced GnRH release has been documented both *in vivo* (Messager et al., 2005) and *in vitro* (Nazian, 2006; Quaynor et al., 2007) and is involved in steroid feedback during preovulatory LH surge (Adachi et al., 2007). In ovariectomized cows, sex steroids can also modulate the sensitivity of LH secretion *in vivo* to kisspeptin stimulation (Whitlock et al., 2008). These findings taken together support the idea that kisspeptin is an integral component of the neuroendocrine control for reproduction.

Although the hypothalamus represents the primary site of action for kisspeptin regulation of reproduction, the direct effect of kisspeptin at the pituitary level is still unclear (Richard et al., 2009). Kiss1r mRNA expression have been reported in the pituitary of the rat

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(Richard et al., 2008) and human (Ohtaki et al., 2001). In sheep, kisspeptin immunoreactivity can be detected in hypophysial portal blood, despite the fact that its levels do not exhibit noticeable changes during reproductive cycle and preovulatory LH surge (Smith et al., 2008). Although these findings raise the possibility that kisspeptin may also act as a hypophysiotropic factor, *in vitro* studies on pituitary actions of kisspeptin have yielded conflicting results. In static cultures of rat pituitary fragments (Thompson et al., 2004) and pituitary cells (Matsui et al., 2004; Navarro et al., 2005a), kisspeptin treatment did not alter LH and FSH secretion at the pituitary level. In other studies with rat pituitary cell cultures, however, kisspeptin not only could induce LH release but also stimulate growth hormone (GH) secretion. These hormone-release responses were of lower magnitude compared to that caused by the primary regulators GnRH (for LH) and GHRH (for GH) and occurred with concurrent rises in cytosolic Ca^{2+} in gonadotrophs and somatotrophs (Gutierrez-Pascual et al., 2007). Recently, kisspeptin-induced prolactin (PRL) release have also been reported in bovine pituitary cells (Kadokawa et al., 2008), suggesting that lactotrophs may also serve as the pituitary target for kisspeptin actions.

Similar to mammals, Kiss1 has been cloned in fish models (Biran et al., 2008) and its involvement in gonadotropin release (Felip et al., 2008) and signaling of sexual maturation/puberty (Filby et al., 2008) have been confirmed (e.g., medaka and zebrafish). Besides Kiss1, a second Kiss gene, namely Kiss2, was also identified in lower vertebrates including the bony fish. Apparently, the two Kiss genes arose early in vertebrate evolution by gene duplication and Kiss2 gene might have been lost recently in the mammalian lineage (Felip et al., 2008). Apart from the core sequence for kisspeptin-10, the sequence homology between Kiss1 and Kiss2 is rather low (~20–25%). When comparing kisspeptin-10 sequences encoded by the two genes, a distinct pattern of a.a. substitutions at position 1 and 10 can also be noted, with the "Y–Y form" (YNL/WNSFGLRY) for Kiss1 and "F–F form" (FNY/FNPFG LRF) for Kiss2, respectively (Kitahashi et al., 2009). In zebrafish, Kiss1 and Kiss2 are expressed in different locations within the hypothalamus (Kitahashi et al., 2009) and may exert differential actions on LH and FSH secretion/expression (Felip et al., 2008; Kitahashi et al., 2009). At present, the studies on pituitary actions of kisspeptin are restricted to mammals and no information is available in fish species. In this study, using goldfish as a model, we seek to examine the pituitary actions of kisspeptin in regulating pituitary hormone secretion and gene expression in modern-day bony fish. The goldfish was used due to the availability a well-established pituitary cell culture system and ample information for endocrine signaling at the pituitary level (Popesku et al., 2008). As a first step, goldfish Kiss1 was cloned by 3′/5′RACE and its tissue expression profile was characterized by Northern blot and RT-PCR. To provide the initial evidence for the hypothesis that the goldfish pituitary indeed can serve as a target for kisspeptin action, RT-PCR for Kiss1r was performed in goldfish lactotrophs, somatotrophs, and gonadotrophs isolated by laser capture microdissection. The results obtained were then confirmed by testing the effects of goldfish kisspeptin-10 on LH, GH, and PRL release and transcript expression in primary cultures of goldfish pituitary cells. To our knowledge, the present study represents the first report in fish models on pituitary hormone regulation by kisspeptin via direct actions at the pituitary cell level.

2. Materials and methods

2.1. Animals

One-year (1+)-old goldfish, *Carassius auratus* (Linnaeus 1758), with body weight from 35 to 45 g were acquired from local pet stores and maintained in 200-L aquaria at 20 ± 2 °C under 12 h dark:12 h light photoperiod for at least 7 days prior to tissue sampling and pituitary cell dispersion. To minimize the effects of sex steroids on kisspeptin's action, fish at late stages of gonadal regression (GSI ⩽ 0.1%) were used in the present study. Given that sexually regressed goldfish do not exhibit sexual dimorphism of external features, goldfish of mixed sexes were routinely used for pituitary cell preparation. During the process, the fish were sacrificed by spinosectomy after anesthesia with 0.05% MS222 (Sigma, St. Louis, MO) according to the procedures approved by the committee of animal use at the University of Hong Kong.

2.2. Molecular cloning of goldfish Kiss1 cDNA

Total RNA was extracted from the brain of goldfish using TRIZOL (Invitrogen, Grand Island, NY) and reversely transcribed with a SuperScript II First Strand cDNA Synthesis Kit (Invitrogen). Using primers designed based on the conserved regions of open-reading frames (ORF) of zebrafish and medaka Kiss1, nested PCR was performed and a partial fragment of goldfish Kiss1 cDNA was isolated. Based on the nucleotide sequence obtained, new primers were synthesized and used in 5′/3′RACE with a GeneRacer™ Kit (Invitrogen) using total RNA from the goldfish brain as the template. PCRs were conducted according the instructions of the manufacturer. PCR products of appropriate sizes were isolated and sub-cloned into the pGEM-T Easy vector (Promega, Madison, WI) for DNA sequencing using a BigDye Sequencing Kit (Applied Biosystems, Foster City, CA). The full-length cDNA for goldfish Kiss1 (GenBank Accession No.: FJ236327) was then compiled using the MacVector 9.5.2 software (Oxford Molecular, Madison, WI). The signal peptide of Kiss1 preprohormone was predicted using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) and phylogenetic analysis of Kiss1 cDNA was conducted using the neighbor-joining method with PHYLIP and TreeView programs provided by Taxonomy and Systematics Server at Glasgow (http://www.taxonomy.zoology.gla.ac.uk/rod/treeview). After that, a DIG-labeled probe for goldfish Kiss1 was prepared for Northern blot and PCR Southern using a PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany).

2.3. RT-PCR and Northern blot for Kiss1 expression

The expression profiles of Kiss1 in various tissues and brain areas of the goldfish were examined using RT-PCR. Briefly, total RNA was isolated from selected tissues and brain areas using TRIZOL. The RNA samples prepared were digested with RNase-free DNase I (Roche) and reversely transcribed using SuperScript II (Invitrogen). RT samples obtained were used as the templates for PCR using the primers specific for goldfish Kiss1 (Forward Primer: 5′-CAAACGCATTAAAAATGAAGCTACT-3′; Reverse Primer: 5′-CACA AGATTTAGCACGACCCAG-3′). PCR was conducted for 34 cycles with 30 s at 94 °C for denaturing, 30 s at 56 °C for annealing, and 60 s at 72 °C for primer extension. Routinely, the reaction was terminated with a final extension step for 5 min at 72 °C. After that, the PCR products obtained were resolved in 1% gel, visualized by staining with ethidium bromide, and transblotted onto a positively charged nylon membrane. To check for the authenticity of PCR products, Southern blot was conducted using a DIG-labeled cDNA probe for goldfish Kiss1. Using similar PCR conditions, RT-PCR for β actin was also performed to serve as an internal control with the primers 5′-CTGGTATCGTGATGGACTCT-3′ and 5′-AGCTCATAGCTCTTCTC CAG-3′.

To further characterize the form(s) and size of Kiss1 transcript expressed in the goldfish, Northern blot was also performed as described previously (Huo et al., 2004). Total RNA was isolated with TRIZOL from selected tissues including the brain, gut, kidney, and liver. These RNA samples were denatured, size-fractionated in 1%