The hexapeptide KP-102 (D-Ala-D-B-Nal-Ala-Trp-D-Phe-Lys-NH₂) stimulates growth hormone release in a cichlid fish (*Oreochromis mossambicus*)

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Abstract

Studies in mammals have shown that synthetic Met-enkephalin derivatives, called growth hormone-releasing peptides (GHRPs), stimulate growth hormone (GH) release. The present study was conducted to determine whether the GHRP, KP-102, specifically stimulates GH release in a teleost. Tilapia (*Oreochromis mossambicus*) were given a single intraperitoneal injection of KP-102 (D-Ala-D-B-Nal-Ala-Trp-D-Phe-Lys-NH₂) or bovine GHRH₁₋₂₉-amide or vehicle and blood was sampled at 1, 6 and 12 h after injection. KP-102 was administered at two doses of 1 ng/g and 10 ng/g body weight, whereas GHRH (positive control) was administered at a single dose of 10 ng/g body weight. Plasma levels of tilapia GH and

prolactins (tPRL₁₇₇ and tPRL₁₈₈) were determined by radioimmunoassay. As expected, GHRH injection significantly ($P < 0.001$) elevated plasma GH levels (ng/ml) in tilapia at 6 h post-injection. KP-102 also significantly elevated GH levels (at the low dose) at 6 ($P < 0.05$) and 12 ($P < 0.01$) hours post-injection. There were no significant effects on plasma PRL(s) levels, although mean levels of both PRLs were elevated at 6 h post-injection. These results show for the first time that GHRPs stimulate GH release in teleosts and suggest that the GHRP receptor and possibly a "Ghrelin-like" ligand are also present in lower vertebrates.

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Introduction

Growth hormone (GH) and prolactin (PRL) are structurally related hormones that are secreted by the vertebrate anterior pituitary, and their secretion is principally under the control of neuroendocrine factors (e.g., growth hormone-, gonadotropin- and thyrotropin-releasing hormones and somatostatin and dopamine). In addition to these regulatory factors, synthetic Met-enkephalin derivatives (hexapeptides) have also been shown to stimulate GH, and to some extent, PRL release in mammals (Bowers *et al.* 1990, Elias *et al.* 1995, Bowers 1998). These hexapeptides, also termed growth hormone-releasing peptides (GHRPs), have been extensively studied and have been shown to safely stimulate pulsatile GH release and consequently growth in mammals and humans (Baker *et al.* 1984, Thorner *et al.* 1997, Bowers 1998, Camanni *et al.* 1998, Mericq *et al.* 1998).

Studies in mammals have shown that GHRPs stimulate GH secretion and growth by binding to a novel receptor (Howard *et al.* 1996). Recently, a new endogenous protein termed "Ghrelin", which stimulates GH secretion by binding to the GHRP receptor, has been reported (Kojima *et al.* 1999).

These findings argue for the presence of novel neuroendocrine pathways that control growth-regulating hormones in higher vertebrates. By contrast, in non-mammalian vertebrates, GHRP(s) administration, alone, has not been shown to be active *in vivo* (Bowers *et al.* 1984). Information on the activity of GHRPs in lower vertebrates will provide valuable insight into the common regulatory mechanisms that control vertebrate neuroendocrine physiology (Bowers 1998).

Against this background, we examined whether the GHRP, KP-102, would stimulate GH and/or PRL levels in a teleost, the tilapia (*Oreochromis mossambicus*). In addition to GH, the tilapia pituitary secretes two forms of prolactin (tPRL₁₇₇ and tPRL₁₈₈), one of which contain 177 amino acid residues, and the other, 188 amino acid residues. These two prolactins, to which unique functions have been ascribed (Rubin & Specker 1992, Oshima *et al.* 1996, Sakamoto *et al.* 1997, Shepherd *et al.* 1997), are encoded by separate genes and share only 69% sequence identity (Specker *et al.* 1985, Rentier-Delrue *et al.* 1989, Yamaguchi *et al.* 1991). In this study, we report the effects of KP-102 treatment on circulating GH and PRL(s) levels in a cichlid teleost.

Materials and Methods

Animals

Tilapia (*Oreochromis mossambicus*) were reared in circular 6000-liter tanks in fresh water (FW) and under natural photoperiod at the Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, Hawaii. Animals were fed Purina Trout Chow twice daily (ration was approximately 2% of body weight per day). Water temperature was $25 \pm 2^\circ\text{C}$.

Experimental Approach

Prior to the study, animals were netted, anesthetized with bicarbonate-buffered MS-222 (100 mg/liter) and transferred to oval 60-liter tanks ($n=6/\text{tank}$) supplied with recirculated fresh water as previously described (Sakamoto *et al.* 1997, Shepherd *et al.* 1997). Animals were allowed to acclimate to these conditions for 10 days prior to the study. During this period, animals were fed once daily to satiety; food was withheld 24 h prior to the experiment. After acclimation, animals were given a single intraperitoneal injection of vehicle (sterile saline) alone or vehicle containing KP-102 (D-Ala-D-B-Nal-Ala-Trp-D-Phe-Lys-NH₂) (Kaken Pharmaceuticals, Tokyo, Japan) or bGHRH₁₋₂₉-amide (Sigma Chemical, St Louis, MO). KP-102 was administered at two doses of 1 ng/g (low) and 10 ng/g (high) body weight, whereas bGHRH (positive control) was administered at a single dose of 10 ng/g body weight. Control animals were injected with vehicle alone and injection volume was 1 $\mu\text{l/g}$ body weight in all groups.

Blood Sampling

For sampling, animals from each group (control, GHRH and GHRP) were anesthetized in bicarbonate-buffered MS-222 (100 mg/liter) and blood was withdrawn by caudal puncture using heparinized syringes. Animals (control, GHRH and GHRP low and high) were sampled for blood at 1, 6 and 12 h after initial injection. Plasma was separated by centrifugation and stored at -80°C for later hormone analyses. Plasma levels of tilapia GH and prolactins (tPRL₁₇₇ and tPRL₁₈₈) were determined by radioimmunoassay as described by Yada *et al.* (1994). Values are reported in nanograms per milliliter (ng/ml) plus or minus the standard error of the mean (S.E.M.).

Statistics

Differences among groups were evaluated by two-way analysis of variance (two-way ANOVA) with treatment and sampling time as independent variables (main effects) (Minitab Statistical Software Package, State College, PA). Where significant differences occurred ($P<0.05$) with the main effects, comparisons between group means were performed using Fisher's Least Significant Test (FPLSD) for predetermined pairwise comparisons (Steele & Torrie 1980).

Results

There was a significant ($P<0.001$) main effect of sampling time on plasma GH levels. By contrast, there was no significant ($P>0.05$) effect of treatment on plasma GH levels; however, there was a significant ($P<0.001$) interaction of treatment $\times$ time on plasma GH levels (Figure 1 A). Given this treatment $\times$ time interaction, we proceeded to examine pairwise differences between treatment groups and controls at the various sampling time points. These comparisons show that there was no significant ($P>0.05$) effect of sampling time on plasma GH or prolactin(s) levels in the control groups (Figures 1 A-C). Compared with the corresponding control value, GHRH injection significantly elevated plasma GH levels (6.5 ± 2.3 vs 0.6 ± 0.3 ng/ml; $P<0.001$) in tilapia at 6 h post-injection (Figure 1A). Compared with corresponding control values, the low dose of KP-102 significantly elevated GH levels at 6 (3.2 ± 1 vs 0.6 ± 0.3 ng/ml; $P<0.05$) and 12 h (6.4 ± 2 vs 2.2 ± 1 ng/ml; $P<0.01$) post-injection. At the high dose of KP-102, there was a significant effect on plasma GH levels only at 12 h (7.1 ± 3 vs 2.2 ± 1 ng/ml; $P<0.001$) post-injection (Figure 1A).

There were no statistically significant ($P>0.05$) treatment or sampling time effects on plasma tPRL₁₇₇ or tPRL₁₈₈ levels (Figures 1 B-C), although mean levels of both prolactins in this study were elevated at the 6 h time point.

Discussion

Our results show that the GHRP, KP-102, stimulates GH release in the cichlid teleost, *Oreochromis mossambicus*. This finding suggests that the GHRP receptor may also be present in tilapia. This hypothesis is supported by the recent molecular cloning of a gene, in the Pufferfish (*Spherooides nephelus*), which shares significant (58%) identity to the human GHRP receptor (Palyha *et al.* 2000).

Mean levels of plasma PRL(s) were elevated at the 6-h sampling time point, although these differences were not statistically significant. We interpret this to suggest that the stimulatory effects of KP-102 treatment may be specific to GH secretion. Our present observations that circulating PRL(s) levels in tilapia were not significantly affected by KP-102 treatment, or sampling time, is indeed interesting since studies in mammals have shown that GHRP administration can stimulate increases in PRL levels (Bowers *et al.* 1990, Elias *et al.* 1995, Bowers 1998). This difference may be attributed to bi-hormonal pituitary cells (mammotomatotropes), that are present in mammals (Frawley & Boockfor 1991), but not in teleosts (Nishioka *et al.* 1993, Specker *et al.* 1993), and which secrete both GH and PRL.

The finding that KP-102 stimulates GH levels in a teleost is particularly exciting, since recent findings in mammals (i.e., high-affinity GHRP receptors) strongly suggest that GHRP(s) mimic, or in fact are, as yet, unidentified, endogenous,

Figure 1 (A-C): Effects of intraperitoneal injection of KP-102, bGHRH or vehicle on plasma levels of GH (panel A), tPRL₁₇₇ (panel B) and tPRL₁₈₈ (panel C) sampled at 1, 6 and 12 h after initial injection. Values are mean $\pm$ SEM ($n=5-6$ per group). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with the corresponding control value (FPLSD).

vertebrate neuroendocrine ligand(s) (Bowers 1998, Camanni *et al.* 1998). In this regard, Kojima and colleagues (Kojima *et al.* 1999) recently isolated an acylated peptide (termed "Ghrelin") of 28 amino acids (from rat stomach) that binds to the GHRP receptor, consequently stimulating GH release *in vitro* and *in vivo*. In addition, Yang and colleagues (Yang *et al.* 1998) recently demonstrated that tripeptide derivatives of GHRPs exhibit GH secretagogue activity in the rat pituitary, and (Geris *et al.* 1998) found that the non-peptidyl

(benzolactam) GH secretagogue (L-692,429) stimulates GH secretion in an avian species.

Collectively, the discoveries of new GH secretagogue(s) (Ghrelin and GHRP tripeptide derivatives), that GHRPs are active in lower vertebrates (Figure 1A), and a new prolactin-releasing peptide (Hinuma *et al.* 1998), which functions in a wide range of vertebrates, opens new avenues to the study of vertebrate pituitary neuroendocrine physiology. Furthermore, our results show that the role of KP-102, in stimulating GH release, is physiologically conserved among taxonomically distant vertebrate groups, and suggest the presence of a "Ghrelin-like" ligand in lower vertebrates as well. Comparative studies of how GHRPs/Ghrelin work *in vivo* will add significantly to our understanding of the common neuroendocrine mechanisms that regulate pituitary physiology, and consequently the growth, development and metabolism of economically important vertebrates.

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