Sex steroid hormones do not enhance the direct stimulatory effect of kisspetin-10 on the secretion of growth hormone from bovine anterior pituitary cells

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ABSTRACT
The aims of the present study were to clarify the effect of kisspeptin-10 (Kp10) on the secretion of growth hormone (GH) from bovine anterior pituitary (AP) cells, and evaluate the ability of sex steroid hormones to enhance the sensitivity of somatotrophic cells to Kp10. AP cells prepared from 8–11-month-old castrated calves were incubated for 12 h with estradiol (E2, $10^{-8}$ mol/L), progesterone (P4, $10^{-8}$ mol/L), testosterone (T, $10^{-8}$ mol/L), or vehicle only (control), and then for 2 h with Kp10. The amount of GH released in the medium was measured by a time-resolved fluoroimmunoassay. Kp10 ($10^{-6}$ or $10^{-5}$ mol/L) significantly stimulated the secretion of GH from the AP cells regardless of steroid treatments ($P < 0.05$), and E2, P4, and T had no effect on this response. The GH-releasing response to growth hormone-releasing hormone (GHRH, $10^{-8}$ mol/L) was significantly greater than that to Kp10 ($P < 0.05$). The present results suggest that Kp10 directly stimulates the release of GH from somatotrophic cells and sex steroid hormones do not enhance the sensitivity of these cells to Kp10. Furthermore, they suggest that the GH-releasing effect of Kp10 is less potent than that of GHRH.

Key words: bovine, GH, Kisspeptin-10, somatotropes.

INTRODUCTION
Kisspeptin is a neuropeptide hormone encoded by KISS-1 (Gottsch et al. 2009) in the hypothalamus, and acts as a functional ligand of a G-protein-coupled receptor (GPR54) (Kotani et al. 2001; Ohtaki et al. 2001). The Kiss-1/GPR54 system has become the most important signals potentially involved in controlling the reproductive axis (Navarro et al. 2004; Shahab et al. 2005; Smith et al. 2006). However, kisspeptin has also been suggested to play a role in not only the gonadotrophic axis but also the somatotrophic axis for several reasons, including: (i) its secretion in the hypothalamic portal circulation (Smith et al. 2008); (ii) the expression of GPR54 by human (Kotani et al. 2001; Ohtaki et al. 2001), rat (Gutierrez-Pascual et al. 2007), and ovine (Smith et al. 2006) pituitary cells; and (iii) the secretion of growth hormone (GH) in response to kisspeptin both in vivo (Kadokawa et al. 2008a; Whitlock et al. 2008) and in vitro (Kadokawa et al. 2008b).

The secretion of GH is closely related to levels of sex steroid hormones (Bourguignon 1988). Estradiol (E2) plays an important role in the secretion (Muller et al. 1999). Several studies in vivo have found a strong positive correlation between the secretion of testosterone (T) and that of GH (Giustina et al. 1997; Bondanelli et al. 2003). Furthermore, a recent study in vivo found that kisspeptin stimulated the secretion of GH in ovariectomized cows given progesterone (P4) and/or E2 (Whitlock et al. 2008).

Our group has recently reported that kisspeptin-10 (Kp10: a shorter variant of kisspeptin retaining full biological activity) stimulated the secretion of GH directly from bovine pituitary cells (Kadokawa et al. 2008b). However, we have not evaluated the role of sex steroid hormones and Kp10 in the secretion of GH from the pituitary cells. The aims of the present study were to evaluate the ability of sex steroid hormones to enhance the sensitivity of somatotrophic cells to Kp10 in cattle.

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MATERIALS AND METHODS

All experimental procedures undertaken were approved by the Animal Care and Use Committee of Iwate University, Morioka, Japan.

Peptides

Human Kp10 amide (amino acid sequence: YNWNSFGLRF-NH₂) was synthesized in our laboratory (Ezzat Ahmed et al. 2009; Hashizume et al. 2010). The peptide has been confirmed to stimulate the release of gonadotropins in goats (Hashizume et al. 2010) and cattle (Ezzat Ahmed et al. 2009) in our previous studies. Human growth hormone-releasing hormone (GHRH) was purchased from Peptide Institute Inc., Osaka, Japan.

Adenohypophysial cells and culture

The pituitary glands were obtained from three castrated Holstein calves (age: 8–11 months). Anterior pituitary (AP) cells were enzymatically dispersed by a method described previously (Hashizume et al. 1994; Hashizume & Kasuya 2009). Cell viability as determined by trypan blue assay exclusion was greater than 90%. The dispersed cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% horse serum (Gibco), 100 IU/mL of penicillin and 50 mg/mL of streptomycin. The cells (2.5 × 10⁵ cells/mL) were then plated in Costar 24-well dishes and cultured for 2.5 days at 37°C in a humidified atmosphere of 5% CO₂. After 2 days of culture, E2 (10⁻⁶ mol/L), P₄ (10⁻⁶ mol/L), T (10⁻⁵ mol/L), or vehicle only as a control was added to the wells. Other wells were left without steroids and treated with Kp10 only. Twelve hours later, the cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with DMEM alone (control), or DMEM containing 10⁻⁶ or 10⁻³ mol/L of Kp10. The experimental protocol for steroid treatments (Hashizume et al. 2002) and the concentrations of Kp10 (Kadokawa et al. 2008b) were as in our previous study. After a 2-h incubation period, the medium was removed from cells and stored at −30°C until the assay of bovine GH. In the present study, GHRH (10⁻⁸ mol/L) was also added to the non-steroid-treated AP cells.

Hormone assay

Concentrations of bovine GH in media were measured by a time-resolved fluorimunoassay (TR-FIA) procedure with slight modifications to a system described previously (Kaneko et al. 2002). The standard GH preparation and the hormone for labeling europium (Eu) were USDA-bGH-B-1. The antiserum to bovine GH was AFPB55. Eu-labeled GH tracer was prepared by DELFIA Eu-Labeling kit (PerkinElmer Las, Inc., Boston, MA, USA). An antibody to GH (diluted 1:50 000) was added to wells that were coated with anti-monkey γ-globulin antiserum (Bethyl Laboratories, Inc., Montgomery, TX, USA) and incubated overnight. After washing the wells, serially diluted GH standard (0.016 to 4 ng/well) and media samples were added and incubated overnight. After washing the wells, Eu-labeled GH (40 000 counts per sec/well) was distributed in all wells and incubated for 4 h. After washing the wells, enhancement solution was added to the wells and fluorescence was measured using the time-resolved fluorimeter (multilabel counter, 2030 A/V/LCO X, PerkinElmer). The specificity of the assay was validated by comparing displacement curves for serial dilutions (2.5, 5 and 10 μL/well) of pooled bovine plasma and culture media with the displacement curve of the reference preparation of GH (Fig. 1). Samples were assayed in a single run. The intra-assay coefficient of variation and the least detectable value were 6.5%, and 3.2 ng/mL, respectively.

Statistical analysis

Four wells were used for each treatment and the experiment was repeated three times with three different pituitary glands. All data from the experiments are presented as the mean ± SEM. Statistical comparisons across different doses of Kp10 (0, 10⁻⁶ and 10⁻³ mol/L) in each steroid treated group were performed using one-way analysis of variance (ANOVA), and Newman-Keuls test was used as a post-hoc test. Comparisons across both different doses of Kp10 and steroid treatments between the steroid treated and un-treated groups were evaluated by two-way ANOVA, and the Bonferroni test was used as a post-hoc test. All data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Results were considered significant at the P < 0.05 level.

RESULTS

Figure 2 shows the effect of Kp10 on the secretion of GH from bovine AP cells, treated with steroids or not. In the group not treated with steroids (Fig. 2a), 10⁻⁶ mol/L and 10⁻³ mol/L of Kp10 significantly increased the concentration of GH in the culture medium by 43.0% (109.7 ± 4.9 ng/mL) and 70.2% (130.6 ± 3.3 ng/mL), respectively (P < 0.05), compared to the controls (76.7 ± 5.2 ng/mL). Kp10 significantly stimulated the secretion of GH from bovine AP cells in the steroid-treated groups. In the E₂-treated
10^6 mol/L and 10^5 mol/L of Kp10 significantly increased the concentration of GH in the medium by 23.8% (109.7 ± 4.1 ng/mL) and 42.8% (126.5 ± 3.1 ng/mL), respectively (P < 0.05), compared to the controls (88.6 ± 4.6 ng/mL). In the P4-treated group (Fig. 2c), 10^6 mol/L and 10^5 mol/L of Kp10 significantly increased the concentration of GH in the medium by 59.2% (112.1 ± 8.6 ng/mL) and 59.5% (112.3 ± 2.8 ng/mL), respectively (P < 0.05), compared to the controls (70.4 ± 4.5 ng/mL). In the T-treated group (Fig. 2d), 10^6 mol/L and 10^5 mol/L of Kp10 significantly increased the concentration of GH in the culture medium by 42.3% (124.5 ± 4.1 ng/mL) and 44.5% (126.5 ± 3.1 ng/mL), respectively (P < 0.05), compared to the controls (87.5 ± 4.7 ng/mL). There were no significant differences in the concentration of GH between the steroid treated and untreated groups at either 10^6 or 10^5 mol/L of Kp10. The GH-releasing response to GHRH, that is a 124.5% increase in GH, was significantly greater than that to Kp10 (P < 0.05) (Fig. 3).

**DISCUSSION**

Kp10 (10^6 or 10^5 mol/L) stimulated the secretion of GH from cultured bovine AP cells regardless of the presence of sex steroid hormones. Furthermore, the GH-releasing effect of Kp10 was less potent than that of GHRH. The finding that Kp10 acted directly on the secretion of GH in vitro was consistent with our previous results (Kadokawa et al. 2008b).
GPR54 receptors are expressed in ruminant pituitary somatotropes (Smith et al. 2008). E2 (Wood 2008), P4 (Ashley et al. 2009) and T (Wood & Keller-Wood 2008) receptors are also located in the ruminant pituitary. The experimental protocol of the present study followed that of our previous study, which showed that E2 treatment (10^{-8} mol/L) enhanced the GnRH-stimulated release of LH in bovine pituitary cells (Hashizume et al. 2002). However, our results showed that 10^{-6} mol/L and 10^{-5} mol/L of sex steroid hormones did not directly enhance the GH-secretory response to Kp10 in bovine pituitary cells. Further studies may be needed to evaluate the proper concentrations of sex steroid hormones to modulate Kp10-stimulated GH release. In addition, we may need to examine the expression of sex steroid hormone receptors on bovine pituitary somatotropes. A recent study in vivo has found that Kp10 stimulated the secretion of GH in ovariectomized cows given P4 and/or E2 (Whitlock et al. 2008). However, the present study showed no effect of either E2, P4 or T on pituitary cells in vitro. Kisspeptin-producing neurons in the hypothalamus were found to express receptors for sex steroid hormones (Smith et al. 2005a,b). Therefore, the role of steroids in the somatotrophic axis might be mainly mediated at the hypothalamic level, either by regulation of kisspeptin’s release and/or stimulation of GHRH-producing neurons.

The direct effect of Kp10 on the secretion of GH was less potent than that of GHRH. Kisspeptin may have an additive effect with GHRH, rather than its own effect on the secretion. The peripheral administration of Kp10 to goats and cattle failed to stimulate the release of GH in our recent study (Ezzat Ahmed et al. 2009; Hashizume et al. 2010), supporting the present results of less potent activity of Kp10 in vitro. However, kisspeptin-immunoreactive fibers are located in the external zone of the median eminence in sheep (Franceschini et al. 2006). This allows for the possibility that kisspeptin is secreted into the hypothalamic portal system to reach the somatotropes in ruminants (Smith et al. 2008). Therefore, this peptide may modulate the secretion of GH in the pituitary gland in vivo regardless of the presence of sex steroid hormones.

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