Intrahypothalamic Perfusion of KP102 Stimulates Growth Hormone Release in Goats

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Abstract. The hypothalamic actions of KP102 (also called GHRP-2) on the release of GH were studied in female goats. KP102 (10^{-5} M) was perfused into the goat hypothalamus through a microdialysis probe (CMA/10 probe with a 4 mm membrane length) at a rate of 4 μ l/min for 90 min, and plasma GH concentrations before and after perfusion were measured. The intrahypothalamic perfusion of 10^{-5} M KP102 significantly stimulated GH release in goats ($P < 0.05$). The GH levels began to rise after commencement of perfusion, and reached a maximum mean value at 180 min. The concentrations of GH at 165, 180, 195, 210, 225 and 240 min after commencement of perfusion of KP102 were significantly higher than the corresponding values for control animals ($P < 0.05$). KP102 had no effect on GH pulse frequency, but it significantly increased the GH pulse amplitude after the perfusion ($P < 0.05$). In contrast to KP102, intrahypothalamic perfusion of 10^{-5} M GHRH had no effect on the stimulation of GH release in goats even if intravenous injection of 10^{-5} M GHRH significantly stimulated GH release ($P < 0.05$). These results suggest that KP102 may act partly on the hypothalamus to stimulate GH release in goats.

Key words: KP102, GHRP-2, GH, Goat, Hypothalamus

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KP102 (also called GHRP-2), a new second generation GH-releasing peptide (GHRP), has potent GH-releasing activity *in vitro* [1, 2] and *in vivo* [2–8] in several animal species [1, 2, 5–8] as well as humans [3, 4]. The GH-releasing activity of KP102 has been reported to be more potent than GHRP-6 and GHRP-1 [1, 3], and at present KP102 seems to be the most potent member of the family of GHRPs. The actions and mechanisms of KP102 on the release of GH are being clarified [1–8]. KP102 stimulates GH release by directly acting on the pituitary cells [1, 2], but a central action of KP102 has also been suggested [3, 9, 10] by the following evidence: (1) the family of GHRPs is

considered to have both direct and indirect actions on the pituitary to stimulate GH release [3, 9, 10], (2) synergistic or additive GH release is observed when GH-releasing hormone (GHRH) and KP102 are administered together *in vivo* [2, 4, 5], (3) GH secretagogue-receptor mRNA has been identified in the hypothalamus [11], but the precise *in vivo* mechanism of GH release by KP102 remains to be clarified. Recently Nakagawa *et al.* [7] reported that injection of KP102 into the arcuate nucleus or intra-third cerebral ventricle stimulated GH release in pentobarbital-anesthetized rats. This result was consistent with that of Fairhall *et al.* [12] who showed that intracerebroventricular (i.c.v.) injection of GHRP-6 stimulated GH release in urethane-anesthetized guinea pigs, but i.c.v. injection of GHRP-6 suppressed GH release in freely moving conscious rats [13]. Since anesthesia modifies GH release [14–16], further precise *in vivo* experiments on conscious animals are necessary. We have examined the GH-releasing action of KP102 in ruminant animals [2, 8]. In the present study,

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considering these controversial results, the hypothalamic site of action of KP102 on the release of GH was examined in conscious goats by a microdialysis technique [17–20]. The action of KP102 was compared with that of GHRH.

Materials and Methods

Animals

Seven female Saanen goats in the luteal phase (age, 2–3 years; mean body weight, 45 kg) were used. The goat was loosely tied to a stanchion, and natural light was allowed to enter through the windows. The goats were fed hay and concentrate from 0830 h to 0930 h and from 1630 h to 1730 h daily. Water was available continuously. On the day of the experiment they were not fed either before or during the experiment, but only after the experiment.

Preparation of KP102 and hGRF(1-29)-NH₂

The KP102 preparation was generously supplied by Kaken Pharmaceutical Co. Ltd., Urayasu, Japan. The hGRF(1-29)-NH₂ (GHRH) was supplied by Dr. S. Ohashi, National Institute of Bioscience and Human Technology, Japan. These preparations were dissolved in sterilized artificial cerebrospinal fluid (artificial CSF) [20] before use, and perfused into the goat hypothalamus at a concentration of 10^{-5} M through a microdialysis probe. A concentration of 10^{-5} M was chosen because the GH releasing activity of 10^{-5} M KP102 had already been confirmed by our preliminary study in the goat. 10^{-5} M GHRH was also prepared by 2 ml of sterilized saline for an experiment on intravenous injection (i.v.) of GHRH.

Intrahypothalamic perfusion system and experiments

The microdialysis system was previously described by Hashizume *et al.* [17, 20]. Briefly, unilateral guide tubes were stereotactically implanted into the hypothalamus in each goat at least 2 weeks before the first experiment. On the day of the experiment, the microdialysis probe (CMA/10 probe with a 4 mm membrane length;

Carnegie Medicine, Stockholm, Sweden) was inserted into the implanted guide tube. Artificial CSF was pumped through the probe tip at a rate of 4 μ l/min with a battery-driven syringe pump which was strapped to the goat's back. After the artificial CSF was perfused for 4 h, the CSF was replaced with a solution including 10^{-5} M KP102 or GHRH for 1.5 h, and then the artificial CSF was perfused again for 4 h. Perfusate fractions were discarded, and the microdialysis system was used only as a local delivery technique. In the controls, the artificial CSF was not replaced with a solution including KP102 or GHRH, and only artificial CSF was perfused throughout the experiments. The experiment was performed at the same site in the hypothalamus at 4- or 5-day intervals in each goat. KP102 was perfused in 7 goats, and GHRH was perfused in 4 of 7 goats. At the end of all perfusion experiments, 5 of 7 goats were given a single i.v. injection of 10^{-5} M GHRH or 2 ml of saline (as a control). The injection was given 4 h after commencement of blood collection, and the blood was collected 4 h after the injection. The experiments were carried out from September to November.

At the end of the experiments, each goat was anesthetized with intravenous pentobarbital sodium, and the placement of the probe in the brain was histologically confirmed [20].

Blood sampling method

The jugular venous blood of the experimental animals was taken from the indwelling catheter previously inserted into one of the external jugular veins. Blood samples (one ml each) were collected into centrifuge tubes containing heparin and were immediately chilled with ice. Blood samples were drawn at 15-min intervals throughout the experiment. Individual plasma samples were obtained after centrifugation and stored at -20°C until assayed.

Radioimmunoassay (RIA)

Plasma GH concentrations were measured by a double-antibody RIA [21]. The GH standard preparation and hormone for iodination were USDA-bGH-B-1. Antiserum to bGH prepared in monkeys was supplied by Dr. T. Johke (National

Institute of Animal Industry, Japan) and goat-monkey IgG serum (2nd antibody) was supplied by Dr. K. Wakabayashi (Institute of Endocrinology, Gunma University, Japan). The parallelism between goat plasma GH and bGH was described previously [22]. The intra- and inter-assay coefficients of variation were 5.9% and 5.8%, respectively. The least detectable value was 0.1 ng/tube.

Statistical analysis

All data are shown as the mean $\pm$ SEM. GH pulses before, during and after the perfusion of KP102 were identified by the pulse analysis program [23]. The G parameters used for pulse identification in this study were G(1)=4.4, G(2)=2.6, G(3)=1.92, G(4)=1.46 and G(5)=1.13, respectively. These parameters were derived from the values reported by Clarke *et al.* [24]. The areas under the GH response curve (AUC) per hour before, during and after the perfusion of KP102 were also calculated. The statistical significance of differences between groups was determined by Student's *t*-test or the Cochran-Cox test after analysis of uniformity of the variances by Bartlett's test [25].

Results were considered significant at the $P < 0.05$ level.

Results

Intrahypothalamic perfusion of 10^{-5} M KP102 stimulated GH release in 7 goats. Representative responses to KP102 in individual animals are shown in Fig. 1. The GH releasing pattern was a little different in each goat. Perfusion of KP102 had no effect on GH pulse frequency, but the GH pulse amplitude tended to increase during perfusion of

Table 1. Characteristics of GH pulses before, during and after intrahypothalamic perfusion of 10^{-5} M KP102 in goats

	Perfusion of KP102		
	Before	During	After
Pulse frequency (pulse/h)	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Pulse amplitude (ng/ml)	5.5 ± 0.6^b	$11.4 \pm 3.3^{a,b}$	13.9 ± 1.7^a

Each value represents the mean $\pm$ SEM for 7 animals. ^{a,b}Values with different superscript letters indicate significant differences ($P < 0.05$).

Fig. 1. Typical changes in plasma GH levels before and after intrahypothalamic perfusion of 10^{-5} M KP102 in goats. Four individual results (#236, #416, #417, #420) obtained from 7 goats are shown. KP102 was perfused through the microdialysis membrane for 90 min. The period of perfusion is indicated by the hatched bar.

Fig. 2. Average concentrations of plasma GH before and after intrahypothalamic perfusion of 10^{-5} M KP102 in goats. Each value represents the mean $\pm$ SEM for 7 animals. * $P < 0.05$ compared with the corresponding values for controls. a: Significant differences ($P < 0.05$) from pre-KP102 concentrations of GH.

Fig. 3. Average concentrations of plasma GH before and after intrahypothalamic perfusion of 10^{-5} M GHRH (hGRF(1-29)-NH₂) in goats. Each value represents the mean $\pm$ SEM for 4 animals (#219, #236, #328, #421). Other explanations are the same as in Fig. 1.

KP102, and it was significantly ($P < 0.05$) increased after the perfusion (Table 1).

Figure 2 shows the averaged concentrations of plasma GH before and after intrahypothalamic perfusion of 10^{-5} M KP102 in 7 goats. The mean plasma GH concentration in control goats varied within the range of 4.3 to 7.9 ng/ml, and no significant changes in the plasma GH levels were observed throughout the experiment. The GH levels began to rise after commencement of perfusion of KP102 from a value of 4.1 ± 1.1 (mean $\pm$ SEM) ng/ml at just before perfusion, and reached

Fig. 4. Plasma GH responses to the intravenous injection of 10^{-5} M GHRH or saline in goats. Each value represents the mean $\pm$ SEM for 5 animals (#219, #236, #328, #416, #421). * $P < 0.05$ compared with the corresponding values for controls. An arrow indicates the time of GHRH or saline injection.

a maximum mean value of 17.7 ± 3.5 ng/ml after 180 min of perfusion. The concentrations of GH at 165, 180, 195, 210, 225 and 240 min after commencement of perfusion of KP102 were significantly ($P < 0.05$) higher than the corresponding values for controls (Fig. 2). The concentrations of GH at 120, 165, 180, 195, 210, 240 and 255 min after commencement of perfusion of KP102 were also significantly ($P < 0.05$) higher than the pre-KP102 concentrations (Fig. 2). The AUC per hour before, during and after the perfusion of KP102 were 246 ± 39 , 379 ± 79 and 768 ± 108 ng $\cdot$ min $\cdot$ ml $^{-1}$, respectively, and the AUC after the perfusion was significantly ($P < 0.05$) higher than that either before or during the perfusion.

Figure 3 shows the average concentrations of plasma GH before and after intrahypothalamic perfusion of 10^{-5} M GHRH in 4 goats. Intrahypothalamic perfusion of 10^{-5} M GHRH had no effect on GH release in goats (Fig. 3), but in contrast to this result, i.v. injection of 10^{-5} M GHRH significantly ($P < 0.05$) stimulated GH release in the goat (Fig. 4).

Each site of perfusion in the hypothalamus was examined histologically in serial sections of the brain. The areas into which we perfused the test agent in 7 goats were 0 to 4 mm lateral to the midline of the third ventricle, from 1 to 5 mm above the base of the hypothalamus. These areas included the preoptic area (#417, #421), the anterior hypothalamic area (#328), the arcuate nucleus (#236), the ventromedial nucleus (#219) and the

dorso- and ventromedial nucleus (#416). The inside of the third ventricle was also perfused in a goat (#420).

Discussion

In the present study, we demonstrated that KP102 can act at a hypothalamic site to release GH in goats. This was deduced from the following evidence: (1) KP102 administered into the hypothalamus stimulated GH release, (2) Unlike KP102, the administration of GHRH into the hypothalamus failed to stimulate GH release.

The central actions of various substances *in vivo* have been examined mainly by i.c.v. injection methods, but with that method the results obtained may not always be accurate because administered substances can leak out into the bloodstream and reach the pituitary to release hormones directly. And the target site of the brain is obscure even if the administered substances released pituitary hormones by acting indirectly on the pituitary gland. The microinjection method may overcome these drawbacks, but the solution in which the substance is dissolved may affect the cell activity even if the volume of the solution is small, and the substances cannot be administered continuously as in the case of the microdialysis technique. Our microdialysis system allowed us to circumvent these drawbacks, and KP102 was allowed to diffuse gradually into the specific sites.

The GH releasing pattern was a little different in each goat. This was considered to be due to the differences between perfused sites in the goats. GH responses to KP102 in goat #420 and #236 promptly occurred when compared with other goats. Perfusion sites in #420 and #236 were in the third ventricle and the arcuate nucleus, respectively. Therefore, in these goats, there was the possibility that KP102 had easy access to the arcuate nucleus, where GHRH neurons are located. And KP102 may act on the arcuate nucleus to promptly stimulate GH release.

GH responses after i.v. injection of KP102 were a little delayed when compared with GHRH, and the high GH levels gradually decreased to the basal concentrations in the previous study in the goat [8]. Furthermore, some irregular GH peaks appeared after a series of i.v. injections of KP102 [8]. In these respects, our present observations after

perfusion of KP102 might be similar to those in the i.v. injection study [8], but the GH release responses after perfusion of KP102 were more continuous than those after i.v. injection [8].

Our results indicating a hypothalamic site of action of KP102 were contrary to the report of Yagi *et al.* [13] who showed that an i.c.v. injection of GHRP-6 suppressed GH secretion in freely moving male rats. This contradiction might be due to differences between the two experiments in the method of administration. GHRP-6 administered in the 3rd ventricle is diffused into the cerebrospinal fluid, and GHRP-6 might have access to some neurons to increase somatostatin (SS) and reduce GHRH or both, but this contradiction might be due to differences in the animal species used in the experiments.

There are two possible mechanisms by which KP102 could act centrally to stimulate GH release. First, an intrahypothalamic administration of KP102 could stimulate GHRH neurons directly. An i.v. injection of hexarelin, one of a family of GHRPs, caused an increase in GH levels associated with GHRH release into the hypophyseal portal blood in sheep [26]. The i.c.v. or i.v. injection of GHRP-6 into rats increased c-fos expression and firing of neurons in the arcuate nucleus [27]. Moreover, the infusion of GHRP-6 increased GHRH pulse frequency in the hypophyseal portal blood in sheep [28]. Second, KP102 could inhibit the neuroendocrine SS cells, resulting in a decrease in SS release into the portal blood, but it was reported that an i.v. injection of hexarelin had no effect on SS release into the hypophyseal portal blood in sheep [26]. The infusion of GHRP-6 was also reported to have no effect on the SS concentration, pulse frequency or pulse amplitude in the hypophyseal portal blood in sheep [28].

Recently, GH secretagogue (GHS: GHRP-6, MK-0677, KP102)-receptor (GHS-R) mRNA was identified not only in the pituitary gland but also in the arcuate-ventromedial hypothalamus and infundibular hypothalamus [11]. Expression of GHS-R on neurons near the median eminence supports the proposed role of the GHS-R as a critical component of a neuroendocrine pathway involved in pulsatile GH release. In the present study, KP102 was administered the base of the hypothalamus, sites which were not within the arcuate nucleus (except one goat), where GHRH neurons are located, but these sites were close to

the arcuate nucleus and the KP102 administered was considered to be able to reach the nucleus after traversing the hypothalamic tissues. KP102 was therefore supposed to be able to bind the GHS-R and result in an increase in GH release.

In conclusion, our results suggest that KP102 may act partly on the hypothalamus to stimulate GH release in goats.

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