

Responses of Plasma IGF-1, IGFBPs and Hepatic GH Receptor to Growth Hormone Releasing Peptides (GHRP)-2 Administration and Energy Level in Wethers

Hong Gu Lee, Young Cheng Jin¹, Hisashi Hidari², Yun Jaie Choi¹, Seon Ku Kim, Teak Soon Shin, Byung Uuk Cho, Yong Gyun Kim, Keun Ki Kim, Hong Joo Son, Sang Mong Lee, Hyun Chul Park and Han Seok Kang*

Department of Bio-Resources and Applied Life Science, Pusan National University, Miryang 627-706, Korea

¹Department of Agricultural Biotechnology, Seoul National University, Seoul 441-744, Korea

²Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

Received June 1, 2008 / Accepted July 22, 2008

The purpose of this study was to determine the effect of energy supplement on responses of plasma insulin-like growth factor (IGF)-1 and IGF binding proteins (IGFBPs) to growth hormone-releasing peptide-2 (GHRP-2) administration in normal protein-fed wethers, and to observe the effect of GHRP-2 treatment on hepatic growth hormone (GH) receptor in well-fed wethers. Plasma IGF-1 and 39-42 kDa IGFBP-3 during the HENP (CP, crude protein 0.34 and TDN, total digestible nutrients 1.83 kg/day DM, dry matter intake) treatment period were higher than in the LENP (CP 0.32 kg and TDN 0.87 kg/day DM intake) period ($P < 0.05$). The response of GH was stimulated by GHRP-2 (12.5 µg/kg body weight/day) administration during both of the feed treatment periods ($P < 0.05$). The area under curve (AUC) increment and average concentration of GH (0-180 min) with GHRP-2 administration was higher during HENP treatment than LENP treatment ($P < 0.01$). During the HENP treatment period from day 1 to day 7 of twice daily GHRP-2 treatment, the plasma IGF-1 increment was increased on days 2, 6 and 7 of GHRP-2 administration ($P < 0.05$). On the basis of ligand blotting, the proportions of plasma 39-43 kDa IGFBP-3 during the HENP treatment period only showed a significant difference on days 6 and 7 with GHRP-2 administration. No significant difference in the specific binding of ¹²⁵I-labeled oGH to hepatic membranes was detected between the saline and GHRP-2 treatments of the HENP-fed wethers. These results suggest that the nutritional balance between energy and protein may affect the endogenous GH / IGF-1 axis as well as plasma IGFBP-3 levels.

Key words : Dietary energy, growth hormone-releasing peptide-2 (GHRP-2), insulin-like growth factor-1 (IGF-1), IGF binding proteins (IGFBPs), wether

Introduction

Along with GH, nutritional status is the principal regulator of IGF-1 concentrations in serum. The effect of nutrition and GH on circulating IGF-1 is related to changes in IGFBPs in the blood. Numerous studies have observed the relationship between GH and nutrition and circulating IGF-1 and IGFBPs in ruminants [15,35,38]. Dietary protein is a very important nutrient in circulating IGF-1 and IGFBPs in humans and rats [34,36].

In cattle, however, the IGF-1 response to dietary protein was affected by available dietary energy [11], because the interaction between dietary energy and protein might influence digestion and protein utilization in ruminants. This

may correspond to the close interrelationships between dietary protein and energy metabolism that occur in ruminal fermentation. In cows, responses to plasma concentrations of IGF-1 to bGH administration were altered by manipulated energy and protein intake [25]. Our previous study observed that increased protein intake did not affect plasma IGFBPs despite increased plasma IGF-1 concentration during GHRP-2 administration in low dietary energy steers [20]. Although, high nutrition level increased in plasma 38-43 kDa IGFBP-3 and 24 kDa IGFBP-4 with an increased plasma IGF-1 level during growth hormone (GH)-releasing peptide 2 (D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH₂; GHRP-2 or KP102) administration [19].

Therefore, manipulation of energy and dietary protein may affect the response of plasma IGF-1 to GHRP-2 administration as well as regulation of IGFBPs. In addition, the study of how the regulation mechanism affects hepatic

*Corresponding author

Tel : +82-55-350-5512, Fax : +82-55-350-5519

E-mail : knghs@pusan.ac.kr

GH receptors and stimulated endogenous GH with GHRP-2 administration, may be important to understanding the effect of GHRP-2 administration on plasma IGF-1 and IGFBPs in ruminants. It is important to remember that specific hepatic ^{125}I -oGH binding was enhanced by chronic bGH treatment with respect to increased plasma IGF-1 concentration [31].

The objectives of this study were two-fold: 1) to determine the effect of energy supplement on responses of plasma IGF-1 and IGFBPs to GHRP-2 administration in wethers with normal dietary protein intake and 2) to observe the effect of GHRP-2 administration on hepatic GH receptors in well-fed wethers.

Materials and methods

Animals and experimental procedures

Eight castrated adult male sheep were used (64.5 ± 4.8 kg body weight, mean \pm SEM). The experimental animals were housed in metabolic cages and fed alfalfa, fish meal and a concentrate diet with 3.0% body weight of DM from 0830 hr to 0930 hr and from 1700 hr to 1800 hr daily for 4 weeks. Water was available ad libitum. The animals were divided into saline (S; n=4) and GHRP-2 groups (KP; n=4) of the same average body weight and were gradually adapted to low energy and normal protein in comparison with the NRC [28] requirement (LENP; CP 0.32 kg and TDN 0.84 kg/day DM intake) for 10 days twice daily before the start of saline or GHRP-2 administration. The LENP group received a concentrate diet, alfalfa cubes and fish meal at 1.82% DM of body weight per day. The concentrate was a fattening diet based on commercial fattening diet (CP 14.7%, EE, ether extract 3.53%, CF, crude fiber 3.63% and Ash 3.85%), the alfalfa hay cube (CP 15.70%, EE 3.21%, CF 30.4% Ash 12.97%) and the commercial fish meal diet (CP 57.96%, EE 8.78 Ash 2.59%). Wethers in each group (S or GHRP-2) received saline or GHRP-2 (KP; Kaken Pharmaceutical Co., Ltd. Japan) for 7 days after the 10-day adaptation period with LENP. After the LENP treatment period, each group (saline or GHRP-2) began receiving high energy and normal protein in comparison with NRC (American national research council) [28] (HENP; CP 0.34 and TDN 1.7 kg/day DM intake, n=4) for 10 days twice daily before the start of GHRP-2 administration. The HENP group received concentrates, alfalfa hay cubes and fish meal at 3.34% DM of body weight

per day. The wethers in each group received saline (S) or GHRP-2 (KP; Kaken Pharmaceutical Co., Ltd. Japan) for 7 days after the 10-day adaptation period with HENP. The animals were fed twice daily at 0830 hr and 1700 hr. The GHRP-2 was administered twice daily at 1000 hr and 1800 hr for 6 days by i.v. injection ($12.5 \mu\text{g}/\text{kg}$ body weight dissolved in 10 ml of saline) 90 min after feeding through an indwelling catheter inserted into a jugular vein in each steer. Water was available ad libitum. All animal-based procedures were conducted in accordance with the "Guidelines for the Care and Use of Experimental Animals of Pusan National University," which were formulated from the "Declaration of Helsinki and Guiding Principles in the Care and Use of Animals."

Blood and liver sampling

Blood samples were collected at -30, -20, -10, 0, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120 and 180 min after injection of GHRP-2 and saline solution on day 1 of treatment for plasma GH assay. Additional samples were taken twice daily for analysis of plasma urea nitrogen (PUN), IGF-1 and IGFBPs. The collected blood was centrifuged and plasma was stored at -30°C until it was assayed.

After 7 days of GHRP-2 treatment for wethers fed on normal protein intake with energy supplement, the eight wethers were slaughtered within 2 hr of arrival at the abattoir to minimize the nutritional and GHRP-2 effects on the hepatic GH receptor (approximately 3 hr after the last injection of GHRP-2). The livers were removed within 5 min of death, washed in saline, and weighed. The samples were immediately frozen in liquid nitrogen and stored at -80°C .

Analysis of plasma GH, IGF-1, insulin and PUN

Plasma GH and IGF-1 were assayed by radioimmunoassay as previously described by Roh et al. [30]. Briefly, plasma GH was assayed by the double antibody method using ovine anti serum (NIDDK-anti-oGH-2, lot AFPC0123080), and ovine GH (NIDDK-bGH-1-5, lot AFP-12855B) was used for GH standard and iodinated by the chloramine T method for radioimmunoassay and radioreceptorassay; the sensitivity was 0.13 ng/ml, and the intra-assay coefficients of variation (CV) were less than 6.8%, respectively. The plasma IGF-1 level was measured using double antibody RIA with human anti-IGF-1 (NIDDK, lot AFP4892898). Human IGF-1 (Amersham, lot

30) was used for IGF-1 standard and iodinated using the chloramine T method for radioimmunoassay and western ligand blotting. The plasma samples were first extracted according to the method of Daughaday et al. [9]. The sensitivity of the IGF-1 assay was 0.78 ng/ml, and inter- and intra-assay CV was 15.3 and 9.4%, respectively.

Analysis of plasma IGFs

Western ligand blotting

12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (15×15×0.15 cm) electrophoresis was performed under non-reducing conditions according to the procedure of Laemmli [18]. Detection of the IGFs was performed in accordance with Hossenlopp et al. [16]. The intensities on the autoradiographs were analyzed using NIH-image software (NIH: National Institutes of Health, USA). The data from the IGF bands were expressed as a percentage of the relative absorbency of each sample IGF band compared to the total IGFs of the standard plasma pool as determined by scanning (NIH-image) the autoradiogram from the Western ligand blot [19].

Analysis of hepatic GH binding

Membrane preparation

Analysis of Hepatic GH binding was performed according to Sauerwein et al. [31]. For membrane preparation the liver samples were thawed at 4°C, cut into small pieces and washed in cold (4°C) 0.025M Tris, 0.01 M CaCl₂, pH 7.6. The tissue was then weighed and homogenized (1:2 w/v) in 0.025 M Tris, 0.01 M CaCl₂, pH 7.6, containing aprotinin (Sigma, lot 75H7180) with Physcotron homogenizer (Japan). Homogenization was performed in an ice bath with 15-30 sec bursts at maximum speed with 45-sec intervals of cooling between each burst. The homogenate was then centrifuged at 1,000× g for 60 min at 4°C. The supernatant was collected and centrifuged at 40,000× g for 60 min at 4°C. The resulting pellet was incubated with 4 M MgCl₂ for 20 min on ice to remove endogenous bound GH. After a 120-min centrifugation (4°C, 40,000× g), the pellet was resuspended in 0.025 M Tris, 0.01 M CaCl₂, pH 7.6, aliquoted and frozen at -80°C.

Radioreceptor assay

The hepatic receptors were rehomogenized and then added in brief succession to the test tubes containing ¹²⁵I-oGH and unlabeled ligand. All reagents were prepared in 0.025 M Tris, 0.01 M CaCl₂, 0.5% (W/V) bovine serum albumin,

pH 7.6. The assay was performed with approximately 300 µg protein per tube, and the protein concentration of the liver was measured by protein-dye binding method [3]. The protein concentration in membrane preparations was not different in the experimental groups (Table 5). Preliminary experiments have shown that the binding of ¹²⁵I-oGH is proportional with the amount of membrane preparation added in the range of membrane concentration used in the present studies. The receptor preparations were incubated with 50,000 cpm ¹²⁵I-oGH and with various concentrations of unlabeled oGH (0-3 µg/tube) in 0.5ml volume for 22 hr at room temperature. Equilibrium was reached under these conditions and the observed binding was fully reversible by adding excess unlabeled oGH. Nonspecific binding was determined by adding 1-3 µg oGH/tube. The incubation was terminated by the addition of 2 ml cold 0.025 M Tris 0.01 M CaCl₂, pH 7.6. Bound and free ¹²⁵I-oGH was separated by centrifugation at 3,000× g at 4°C. The bound ¹²⁵I-oGH was counted by gamma-counter.

Statistical analysis

The AUC of GH was calculated for 180 min after

Table 1. The effect of energy supply on plasma IGF-1 and IGFs in normal protein-fed wethers

	Pre-treatment	LENP	HENP	Significance
IGF-1 (ng/ml) ^a	162.1 ± 26.4	144.7 ± 21.2	164.9 ± 21.1	*
Incre (%) ^c	100	91.4 ± 13.1	101.7 ± 13.0	*
38-43 kDa IGFBP-3 (%) ^b	66.0 ± 1.0	61.4 ± 2.4	70.6 ± 1.4	*
Incre (%) ^c	100	93.0 ± 2.6	107.0 ± 2.6	*
34 kDa IGFBP-2 (%) ^b	10.3 ± 1.0	10.5 ± 1.8	10.1 ± 0.8	NS
Incre (%) ^c	100	100.3 ± 8.1	99.7 ± 8.1	NS
24 kDa IGFBP-4 (%) ^b	5.7 ± 0.5	5.4 ± 0.7	6.0 ± 0.5	NS
Incre (%) ^c	100	94.5 ± 9.3	105.5 ± 9.3	NS

Values are the mean±SEM for 4 wethers.

^{a,b)} are single sample collected after 10 day of feed treatment period.

^{b)} was observed by ligand blotting. One µl plasma was run on a 12.5% PAGE gel, transferred to nitrocellulose and blotted with 100,000 cpm ¹²⁵I-IGF-1/ml buffer. The absorbance of each IGFBP band was expressed as a relative percentage of the absorbance of that IGFBP band for absorbance of pooled stand.

^{c)} was expressed as a relative percentage of after for before feed treatment.

* P<0.05, NS is not significant.

LENP: Low energy and normal protein treatment

HENP: High energy and normal protein treatment

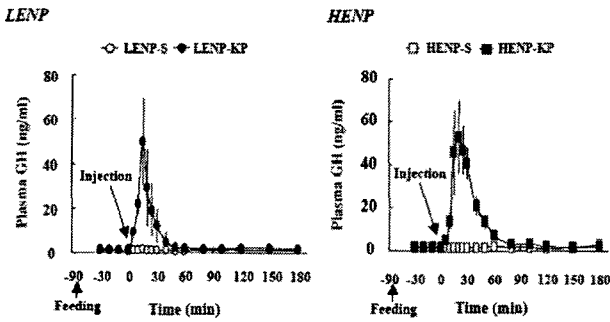


Fig. 1. Response of plasma GH to GHRP-2 administration in high protein fed wethers on different planes of energy. Data points represent the mean±SEM of 4 wethers. LERP-S: Low energy and normal protein group treated with saline, LERP-KP: Low energy and normal protein group treated with GHRP-2, HENP-S: High energy and normal protein group treated with saline, HENP-KP: High energy and normal protein group treated with GHRP-2

Table 2. Profile of bovine plasma GH with GHRP-2 administration on day 6 in high-protein fed wethers on different planes of energy

	LERP-S	LERP-KP	HENP-S	HENP-KP
AUC (ng/min.ml) ¹	305.8 ± 74.9 ^c	1254.1 ± 150.4 ^b	272.5 ± 33.9 ^c	2496.3 ± 556.9 ^a
AVG (ng/ml) ²	1.3 ± 0.1 ^c	9.0 ± 1.0 ^b	1.5 ± 0.1 ^c	14.6 ± 3.3 ^a
Max Peak (ng/ml) ³	2.4 ± 0.6 ^b	50.0 ± 1.1 ^a	3.1 ± 0.4 ^b	58.5 ± 15.0 ^a

Values are the mean±SEM for 4 wethers.

¹) area was corrected for the 180 min (between 0 and 180) periods

²) was calculated average of concentration for 180 min.

³) is maximum peak after saline or GHRP-2 administration
^{a,b,c}) significant differences between treatment were analyzed by analysis of variance, using the Duncan's multiple range test after General Linear Model.

LERP-S: Low energy and normal protein group treated with saline, LERP-KP: Low energy and normal protein group treated with GHRP-2, HENP-S: High energy and normal protein group treated with saline, HENP-KP: High energy and normal protein group treated with GHRP-2

GHRP-2 administration and corrected for the basal AUC (-30, -20, -10 and 0 min), which was evaluated by analysis of variance and the Duncan's multiple range test using the General Linear Model (GLM) procedure of the SAS program package (SAS Institute, Cary, NC). The mean of the GH concentration during the period of feed treatment was calculated for 180 min. The binding parameters of the oGH receptors were analyzed using Scatchard analysis [32]. Significant differences noted during the feed adaptation period, and the differences between GHRP-2 treated and

untreated animals noted during the period of GHRP-2 administration were analyzed by paired t-tests. Other analyses were conducted by one- or two-way analysis using the GLM procedure.

Results

The effect of protein and energy nutrition on plasma IGF-1 and IGFBPs levels

Plasma IGF-1 and 39-42 kDa IGFBP-3 during the HENP treatment period were higher than in the LERP period (P<0.05), but plasma 34 kDa IGFBP-2 and 24 kDa IGFBP-4 were not significantly different in the LERP period compared to the HENP period (Table 1).

The response of plasma GH to GHRP-2 treatment (Fig. 1, Table 2)

The response of GH was stimulated by GHRP-2 administration during both feed treatment periods (P<0.05). Although amplitude of the maximum GH peaks responding to GHRP-2 injection did not show any significant difference between the LERP and HENP treatments, the AUC increment and average concentration of GH (0-180 min) with GHRP-2 was higher during HENP treatment than during LERP treatment (P<0.05).

Response to plasma IGF-1 and IGFBPs levels to GHRP-2 treatment

During the period from day 1 to day 7 of twice daily GHRP-2 treatment in the HENP, plasma IGF-1 increment was increased at days 2, 6 and 7 of GHRP-2 administration (P<0.05). In the LERP treatment period, the GHRP-2 treatment increased the plasma IGF-1 increment on day 3 (P<0.05) (Fig. 2, Table 3). On the basis of immunoblotting with bovine IGFBP-2 and IGFBP-3 antiserum, we observed the band of IGFBP-2 at approximately 34 kDa, and IGFBP-3 was detected at 38-43 kDa in a previous study [19]. On the basis of ligand blotting, when the data from the IGFBPs bands were expressed as a percentage of the relative abundance of each sample IGFBP band compared to the total IGFBPs of the standard plasma pool, the proportions of plasma 34 kDa IGFBP-3 during the HENP treatment period showed a significant difference only on day 7 (P<0.05), other IGFBPs were not changed with GHRP-2 administration during either the HENP or the LERP treatment periods (Fig. 3, Table 4).

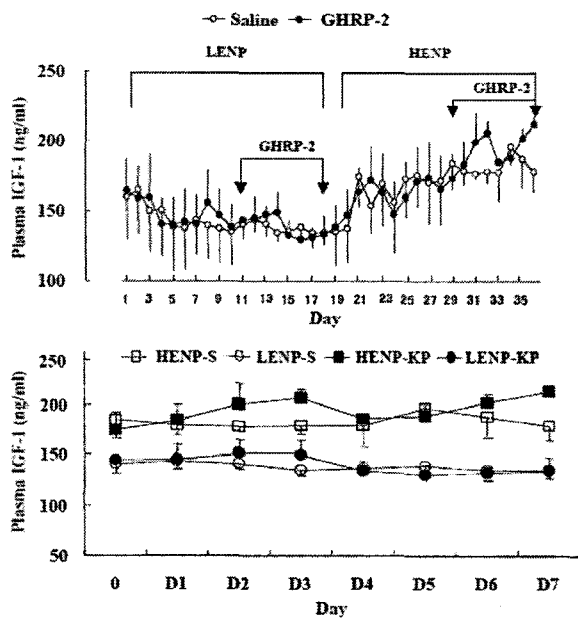


Fig. 2. Profiles of plasma IGF-1 concentration after a single injection of either saline or GHRP-2 (12.5 µg/kg BW) for 7 days in high protein fed wethers on different planes of energy. Data points represent the mean±SEM of 4 wethers. LEMP-S: Low energy and normal protein group treated with saline, LEMP-KP: Low energy and normal protein group treated with GHRP-2, HENP-S: High energy and normal protein group treated with saline, HENP-KP: High energy and normal protein group treated with GHRP-2

Table 3. Daily profile of plasma IGF-1 with GHRP-2 treatment in normal-protein fed wethers on different planes of energy

		Treatment				Significance	
		LENP		HENP		GHRP-2	
		Control	GHRP-2	Control	GHRP-2	LENP	HENP
Day 0	ng/ml ^a	140.3 ± 13.7	138.4 ± 10.5	184.1 ± 15.7	173.2 ± 19.9	NS	NS
	Incre (%) ^b	100	100	100	100	NS	NS
Day 1	ng/ml ^a	143.1 ± 11.5	144.9 ± 15.1	178.5 ± 17.1	190.5 ± 23.3	NS	NS
	Incre (%) ^b	103.4 ± 6.7	104.3 ± 6.5	97.3 ± 4.2	123.6 ± 9.0	NS	NS
Day 2	ng/ml ^a	140.2 ± 16.6	146.6 ± 10.5	176.6 ± 15.7	212.0 ± 24.1	NS	NS
	Incre (%) ^b	100.8 ± 8.9	106.3 ± 4.6	96.2 ± 6.8	110.3 ± 9.2	NS	*
Day 3	ng/ml ^a	131.0 ± 9.3	151.0 ± 9.6	178.0 ± 24.2	193.9 ± 34.0	NS	NS
	Incre (%) ^b	92.4 ± 2.0	110.0 ± 6.9	96.1 ± 5.8	107.1 ± 4.8	*	NS
Day 4	ng/ml ^a	135.5 ± 15.6	132.6 ± 10.3	177.8 ± 23.7	184.7 ± 21.2	NS	NS
	Incre (%) ^b	96.1 ± 3.9	96.7 ± 8.6	95.8 ± 5.0	109.9 ± 9.6	NS	NS
Day 5	ng/ml ^a	138.2 ± 16.1	129.3 ± 8.8	195.9 ± 15.4	187.9 ± 22.9	NS	NS
	Incre (%) ^b	98.8 ± 6.8	94.6 ± 7.3	107.0 ± 6.7	124.0 ± 8.5	NS	NS
Day 6	ng/ml ^a	134.0 ± 14.0	130.9 ± 7.4	187.2 ± 14.8	211.5 ± 19.3	NS	NS
	Incre (%) ^b	96.3 ± 6.8	95.7 ± 6.5	102.5 ± 2.7	126.1 ± 10.9	NS	*
Day 7	ng/ml ^a	134.2 ± 8.7	132.9 ± 13.8	178.3 ± 14.9	211.8 ± 6.4	NS	NS
	Incre (%) ^b	97.2 ± 6.1	97.1 ± 11.0	97.0 ± 4.0	115.9 ± 5.7	NS	*

Values are the mean ± SEM for 4 wethers.

^{a)} is average concentration of two samples collected at 1000 and 1700 hr.

^{b)} was expressed as a relative percentage of after for before feed treatment.

*P<0.05, NS is not significant.

LENP: Low energy and normal protein treatment

HENP: High energy and normal protein treatment

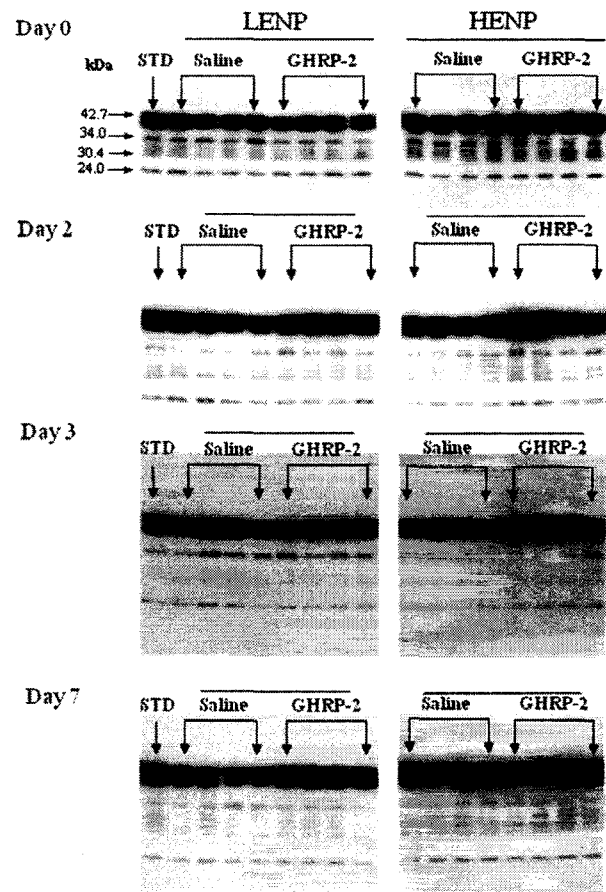


Fig. 3. B: Western ligand blots of plasma samples on day 0, 2, 3 and 7 of from wethers administrated either saline or GH-releasing peptide-2 (GHRP-2; 12.5 µg/kg BW) on different planes of energy for 7 days. One µl plasma was run on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and blotted with 1000,000 cpm [125I]-IGF-1/10ml buffer. The standard plasma samples (STD) were pool for all the plasma samples collected during the experimental periods (lane 1). The molecular weight markers used were BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

HENP: Low energy and normal protein treatment, HENP: High energy and normal protein treatment

Hepatic GH binding

Specific binding of ¹²⁵I-labeled oGH to hepatic membranes showed no significant difference between saline and GHRP-2 treatments in the HENP-fed wethers (Table 5). Analysis of displacement curves yielded linear results with analysis of Scatchard plots in both saline and GHRP-2 treatments.

Discussion

In ruminants, plasma IGF-1, IGFBP-2 and IGFBP-3 have

Table 4. The effect of GHRP-2 administration and energy supply on plasma IGFbps in normal-protein fed wethers ^a

	Treatment				Significance GHRP-2	
	LENP		HENP		LENP	HENP
	Control	GHRP-2	Control	GHRP-2		
Day 0						
38-43 kDa IGFBP-3 (%)	63.8 ± 1.8	59.0 ± 2.4	71.0 ± 0.7	70.1 ± 2.0	NS	NS
34 kDa IGFBP-2 (%)	12.5 ± 2.0	8.5 ± 0.8	10.2 ± 1.0	10.0 ± 1.1	NS	NS
24 kDa IGFBP-4 (%)	4.5 ± 0.8	6.4 ± 0.6	6.3 ± 0.3	5.7 ± 0.8	NS	NS
Day 2						
38-43 kDa IGFBP-3 (%)	70.4 ± 3.2	74.5 ± 1.4	73.7 ± 4.8	88.7 ± 4.3	NS	NS
34 kDa IGFBP-2 (%)	14.5 ± 1.0	11.9 ± 1.3	10.3 ± 0.6	11.2 ± 1.7	NS	NS
24 kDa IGFBP-4 (%)	7.7 ± 0.8	5.8 ± 0.4	6.1 ± 0.8	4.8 ± 0.3	NS	NS
Day 3						
38-43 kDa IGFBP-3 (%)	88.0 ± 3.8	71.9 ± 0.3	71.2 ± 2.3	81.7 ± 3.2	NS	*
34 kDa IGFBP-2 (%)	9.1 ± 0.8	13.5 ± 1.1	11.0 ± 1.4	16.1 ± 3.1	NS	0.44
24 kDa IGFBP-4 (%)	7.6 ± 1.0	6.0 ± 0.3	4.0 ± 0.5	5.1 ± 0.3	NS	0.18
Day 7						
38-43 kDa IGFBP-3 (%)	58.5 ± 4.0	63.6 ± 1.8	70.5 ± 3.8	81.3 ± 1.5	NS	*
34 kDa IGFBP-2 (%)	10.7 ± 1.2	8.1 ± 0.8	10.5 ± 1.4	12.8 ± 0.5	NS	NS
24 kDa IGFBP-4 (%)	2.9 ± 0.2	3.7 ± 0.3	3.1 ± 0.2	3.8 ± 0.3	NS	NS

Values are mean±SEM of 4 wethers.

^a) was observed by ligand blotting. One µl plasma was run on a 12.5% PAGE gel, transferred to nitrocellulose and blotted with 100,000 cpm ¹²⁵I - IGF-1/ml buffer. The absorbance of each IGFBP band was expressed as a relative percentage of the absorbance of that IGFBP band for absorbance of pooled standard plasma.

* P<0.05, NS is not significant.

LENP: Low energy and normal protein treatment

HENP: High energy and normal protein treatment

Table 5. Specific hepatic ¹²⁵I-oGH binding with GHRP-2 treatment in high energy and normal protein fed wethers

Treatment	Liver WT(kg)	Protein (µg/100mg liver) ^a	Specific ¹²⁵ I-oGH binding (%) ^b
Saline	1.20 ± 0.12	319.8 ± 35.0	9.8 ± 1.6
GHRP-2	1.11 ± 0.14	331.5 ± 27.9	11.4 ± 1.5

Values are mean±SEM of 4 wethers

^a) is hepatic membrane protein.

^b) is relatively percentage of ¹²⁵I-oGH specifically bound with hepatic membrane for total ¹²⁵I-oGH.

been shown to be sensitive to feed intake in several studies [11,19,22,25]. Specific effects of dietary protein intake on circulating IGF-1 have been demonstrated in rats [8], pigs [6] and cattle [20], but the response of IGF-1 to dietary protein requires the intake of adequate energy [33].

In the present study, we observed that plasma IGFBP-3 levels, as well as IGF-1 concentrations, were higher during HENP than LENP feed treatment periods in wethers. Elsasser et al. (1989) reported that plasma IGF-1 concentration linearly increased with increasing levels of dietary protein intake (r=0.74) in the higher ME steers, but the enhanced effect of high dietary protein on plasma IGF-1 was limited in low-energy intake cattle [11]. The increased plas-

ma IGF-1 concentration, compared to the energy supply in wethers with normal protein intake, may be related to an increase in hepatic IGF-1 mRNA levels induced by increased hepatic GH binding [36]. GH binding with hepatic GH receptors increased with well-balanced high nutrition [1,4] rather than by only with specific protein supplement under poor energy intake in rats [23].

The circulating IGFBP-3 closely parallels that of IGF-1 itself. During chronic dietary restriction or deprivation, serum IGFBP-3 shows positive correlation with change in IGF-1 in rats [10,37] and sheep [12,15]. However, the IGFBP-3 was unchanged by dietary protein supplement despite increased plasma IGF-1 in sheep [7] and steers [20]. In addition, the serum IGFBP-3 levels significantly declined during energy restriction but did not change during protein restriction in the offspring [34].

Furthermore, chronic dietary restriction decreased serum IGFBP-3 levels and a parallel change of the liver IGFBP-3 mRNA level was observed in rats [10]. Overall, our results provide evidence for the importance of dietary energy to effect change in plasma IGF-1 and IGFBP-3 when normal protein intake is supplied to wethers.

In this study, the plasma IGFBP-2 level was not changed by energy manipulation. This may be due to the adequate supply of dietary protein during both feed treatment periods, because dietary protein affected serum IGFBP-2 and hepatic IGFBP-2 mRNA abundance in rats [21], humans [34] and in our previous steer study [20].

On the other hand, in the previous study [20], we observed that the response of GH to GHRP-2 administration was the same in steers with high-protein and low-protein intake, but not low dietary energy. The response of plasma GH AUC to GHRP-2 administration showed a significant difference on day 6 of GHRP-2 administration during HENP and LENP periods. This is consistent with the GH response to GHRP-2 administration in low and high intake steers [19]. The GH response to GHRH administration in sheep, however, has been shown to be higher in poorly fed than in well-fed animals [14]. This indicates that the effect of GHRP-2 administration on GH release may be regulated differently in comparison with GRRH in steers on different nutritional planes. GH releasing peptides (GHRPs) stimulate GH release through G-protein-linked receptors that have an endocrine pathway distinct from that described for GHRH [17,29].

The enhanced endogenous GH, with GHRP-2 admin-

istration, only increased IGF-1 on day 4 in LENP wethers, but increased on days 2, 6 and 7 in comparison with the saline group in HENP wethers. The rate of production of IGF-1 was influenced by the concentration and affinity of hepatic GH receptors in steers [4,5] and sheep [1]. The GH-binding sites to ruminant liver membranes were lower in feed-restricted groups than in well-fed groups [4,24]. In addition, the number of hepatic binding sites for GH and the capacity of the high-affinity GH receptors were significantly related to exogenous GH treatment in sheep [31]. In yearling sheep, GH treatment increased hepatic GHRs in a dose-dependent manner [26]. However, our study failed to observe the change in hepatic GH binding as well as the capacity of the high or low-affinity GH receptor in HENP wethers, although plasma IGF-1 concentration increased with GHRP-2 administration. Thus the developed responses of plasma IGF-1 to GHRP-2 administration in HENP wethers might correspond to enhancing the blood GH (Table 3). Plasma IGFBP-3 was increased on days 3 and 7 of GHRP-2 administration in HENP wethers. The enhanced plasma IGFBP-3 seems to cause the plasma IGF-1 concentration to increase in GHRP-2 administrated wethers during HENP periods, because IGFBP-3 provides a long-lived, stable reservoir of circulating IGF-1, which binds more than 90% of IGF-1 in serum [2]. The changes in the serum levels of IGFBP-3 correlate significantly with changes in the serum levels of IGF-1 [27]. On the other hand, in the LENP wether's plasma IGFBP-3 showed no significant variance with GHRP-2 administration despite enhanced plasma IGF-1 on day 3 of treatment, as the results showed in our previous study [25]. Exogenous GH treatment increased IGFBP-3 expression by a pathway independent of IGFs in liver cells in vitro [13]. Overall, the increased IGFBP-3 with GHRP-2 administration may play a role in prolonging increased circulation of IGF-1 under a sufficient intake of dietary energy and protein.

In conclusion, our study demonstrated that a sufficient intake of energy increased plasma IGF-1 and IGFBP-3 levels in normal protein-fed wethers. A change in the plasma IGFBP-3 level, according to the increased plasma IGF-1 with GHRP-2 administration, was shown only in high energy-fed wethers on normal protein. However, the hepatic GH binding was not changed by GHRP-2 administration in HENP wethers. Thus, we believe that the nutritional balance between energy and dietary protein may affect the endogenous GH/IGF-1 axis as well as plasma IGFBP-3 levels.

Acknowledgements

The authors are grateful to the NIDDK program for supplying reagents and procedures for the GH and IGF-I RIA to Kaken Pharmaceutical Co., Ltd. (Japan) for providing GHRP-2. This work was supported for two years by Pusan National University Research Grant.

References

1. Bass, J. J., J. M. Oldham, S. C. Hodgkinson, P. J. Fowke, H. Sauerwein, P. Molan, B. H. Breier and P. D. Gluckman. 1991. Influence of nutrition and bovine growth hormone (GH) on hepatic GH binding, insulin-like growth factor-1 and growth of lambs. *J. Endocrinol.* **128**, 181-186.
2. Baxter, R. C. 1991. Insulin-like growth factor (IGF) binding proteins; the role of serum IGFBPs in regulating IGF availability. *Acta Paediatr. Scand. (Suppl)* **372**, 107-114.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
4. Breier, B. H., P. D. Gluckman and J. J. Bass. 1988b. The somatotrophic axis in young steers: influence of nutritional status and oestradiol-17 β on hepatic high- and low-affinity somatotrophic binding sites. *J. Endocrinol.* **116**, 169-177.
5. Breier, B. H., P. D. Gluckman and J. J. Bass. 1988a. Influence of nutritional status and oestradiol-17 β on plasma growth hormone, insulin-like growth factors- I and -II and the response to exogenous growth hormone in young steers. *J. Endocrinol.* **118**, 243-250.
6. Campbell, R. G., R. J. Johnson, R. H. King, M. R. Taverner and D. J. Meisinger. 1990. Interaction of dietary protein content and exogenous porcine growth hormone administration on protein and lipid accretion rates in growing pigs. *J. Anim. Sci.* **68**, 3217-3225.
7. Clarke, I. J., T. P. Fletcher, C. C. Pomares, J. H. G. Holmes, F. Dunshea, G. B. Thomas, A. J. Tilbrook, P. E. Walton and D. B. Galloway. 1993. Effect of high-protein feed supplements on concentrations of growth hormone (GH), insulin-like growth factor-1 (IGF-1) and IGF-binding protein-3 in plasma and on the amounts of GH and messenger RNA for GH in the pituitary glands of adult rams. *J. Endocrinol.* **138**, 421-427.
8. Dardevet, D., M. Manin, M. Balage, C. Sornet and J. Grizard. 1991. Influence of low- and high-protein diets on insulin and insulin-like growth factor-1 binding to skeletal muscle and liver in the growing rat. *Br. J. Nutr.* **65**, 47-60.
9. Daughaday, W. H., I. K. Mariz and S. L. Blethen. 1980. Inhibition of assay of bound somatomedin to membrane receptor and immunobinding sites: A comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol extraction serum. *J. Clin. Endocrinol. Metab.* **51**, 781-788.

10. Donovan, S. M., L. C. Atilano, R. L. Hintz, D. M. Wilson and R. G. 1991. Rosenfeld. Differential regulation of the insulin-like growth factor (IGF-I and -II) and IGF binding proteins during malnutrition in the neonatal rat. *Endocrinology* **129**, 149-157.
11. Elsasser, T. H., T. S. Rumsey and A. C. Hammond. 1989. Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-1 in beef cattle. *J. Anim. Sci.* **67**, 128-141.
12. Gallaher, B. W., B. H. Breier, M. H. Oliver, J. E. Harding and P. D. Gluckman. 1992. Ontogenic differences in the nutritional regulation of circulating IGF binding proteins in sheep plasma. *Acta Endocrinol.* **126**, 49-54.
13. Gucev, Z. S., Y. Oh, K. M. Kelley, J. I. Labarta, P. Vorwerk and R. G. Rosenfeld. 1997. Evidence for insuli-like growth factor (IGF)-independent transcriptional regulation of IGF binding protein-3 by growth hormone in SKHEP-1 human hepatocarcinoma cells. *Endocrinology* **138**, 1464-1470.
14. Hart, I. C., P. M. E. Chadwick, A. Coert, S. James and A. D. Simmonds. 1985. Effect of different growth hormone-releasing factors on the concentrations of growth hormone, insulin and metabolites in the plasma of sheep maintained in positive and negative energy balance. *J. Endocrinol.* **105**, 113-119.
15. Hodgkinson, S. C., J. J. Bass and P. D. Gluckman. 1991. Plasma IGF-1 binding proteins in sheep: effect of recombinant growth hormone treatment and nutritional status. *Domest. Anim. Endocrinol.* **8**, 343-351.
16. Hossenlopp, P., D. Seurin, B. Segovia-Quinson, S. Hardouin and M. Binoux. 1986. Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Anal. Biochem.* **154**, 138-143.
17. Howard, A. D., S. D. Feighner, D. F. Cully, J. P. Arena, P. A. Liberator, C. I. Rosenblum, M. Hamelin, D. L. Hreniuk, O. C. Palyha, J. Anderson, P. S. Pares, C. Diaz, M. Chou, K. K. Liu, K. K. McKee, S. S. Pong, L. Y. Chung, A. Elbrecht, M. Dashkevicz, R. Heavens, M. Rigby, D. J. S. Sirinathsinghii, D. C. Dean, D. G. Melillo, A. A. Patchett, R. Nargund, P. R. Griffin, J. A. DeMartino, S. K. Gupta, J. M. Schaeffer, R. G. Smith and L. H. T. Van der Ploeg. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science (Wash DC)* **273**, 974-977.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685.
19. Lee, H. G., R. A. Vega, L. T. Phung, N. Matsunaga, H. Kuwayama and H. Hidari. 2000. The effect of growth hormone releasing peptide-2 (KP102) administration on plasma insulin-like growth factor (IGF)-I and IGF-binding proteins in Holstein steers on different planes of nutrition. *Domest. Anim. Endocrinol.* **18**, 293-308.
20. Lee, H. G., Y. J. Choi, S. R. Lee, H. Kuwayama, H. Hidari and S. K. You. 2005. Effects of dietary protein and growth hormone-releasing peptide (GHRP-2) on plasma IGF-1 and IGFBPs in Holstein steers. *Domest. Anim. Endocrinol.* **28**, 134-146.
21. Lemozy, S., J. B. Pucilowska and L. E. Underwood. 1994. Reduction of insulin-like growth factor-I (IGF-I) in protein-restricted rats is associated with differential regulation of IGF-binding protein messenger ribonucleic acids in liver and kidney, and peptides in liver and serum. *Endocrinology* **135**, 617-623.
22. Ellenberger, M. A., D. E. Johnson, G. E. Carstens, K. L. Hossner, M. D. Holland, T. M. Nett and C. F. Nockels. 1989. Endocrine and metabolic changes during altered growth rates in beef cattle. *J. Anim. Sci.* **67**, 1446-1454.
23. Maes, M., Y. Amand, L. E. Underwood, D. Maiter and J. M. Ketelslegers. 1988. Decreased serum insulin-like growth factor I response to growth hormone in hypophysectomized rats fed a low protein diet: evidence for a postreceptor defect. *Acta Endocrinol. (Copenh)* **117**, 320-326.
24. McFadden, T. B., T. E. Daniel and R. M. Akers. 1990. Effects of plane of nutrition, growth hormone and unsaturated fat on growth hormone, insulin and prolactin receptors in prepubertal lambs. *J. Anim. Sci.* **68**, 3180-3189.
25. McGuire, M. A., J. L. Vicini, D. E. Bauman and J. J. Veenhuizen. 1992. Insulin-like growth factors and binding proteins in ruminants and their nutritional regulation. *J. Anim. Sci.* **70**, 2901-2910.
26. Min, S. H., D. D. Mackenzie, B. H. Breier, S. N. McCutcheon and P. D. Gluckman. 1999. Ontogeny of IGF-I responsiveness to bGH in young lambs. *Domest. Anim. Endocrinol.* **17**, 409-419.
27. Mohan, S., C. Libanati, C. Dony, K. Lang, N. Srinivasan and D. J. Baylink. 1995. Development, validation, and application of a radioimmunoassay for insulin-like growth factor binding protein-5 in human serum and other biological fluids. *J. Clin. Endocrinol. Metab.* **80**, 2638-2645.
28. NRC. 2001. Nutrient requirements of dairy cattle 7th revised edition. National academy press, Washington, DC.
29. Pong, S. S., L. Y. P. Chung, D. C. Dean, R. P. Nargund, A. A. Patchett and R. G. Smith. 1996. Identification of a new G-protein-linked receptor for growth hormone secretagogues. *Mol. Endocrinol.* **10**, 57-61.
30. Roh, S. G., N. Matsunaga, S. Hidaka and H. Hidari. 1996. Characteristics of growth hormone secretion responsiveness to growth hormone-releasing peptide-2 (GHRP-2 or KP102) in calves. *Endocr. J.* **43**, 291-298.
31. Sauerwein, H., B. H. Breier, J. J. Bass and P. D. Gluckman. 1991. Chronic treatment with bovine growth hormone up-regulates high-affinity hepatic somatotrophic receptors in sheep. *Acta Endocrinol. (Copenh)* **124**, 307-313.
32. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51**, 660-675.
33. Seve, B., O. Ballevre, P. Ganier, J. Noblet, J. Prugnaud and C. Obled. 1993. Recombinant porcine somatotropin and dietary protein enhance protein synthesis in growing pigs. *J. Nutr.* **123**, 529-540.
34. Smith, W. J., L. E. Underwood and D. R. Clemmons. 1995.

- Effects of caloric or protein restriction on insulin-like growth factor-1(IGF-1) and IGF-binding proteins in children and adults. *J. Clin. Endocrinol. Meta.* **80**, 443-449.
35. Stanko, R. L., W. S. Cohick, D. W. Shaw, R. W. Harvey, D. R. Clemmons, M. D. Whitacre and J. D. Armstrong. 1994. Effect of somatotropin and/or equine chorionic gonadotropin on serum and follicular insulin-like growth factor I and insulin-like growth factor binding proteins in cattle. *Biol. Reprod.* **50**, 290-300.
36. Straus, D. S. and C. D. Takemoto. 1990. Effect of dietary protein deprivation on insulin-like growth factor (IGF)-I and -II, IGF binding protein-2, and serum albumin gene expression in rat. *Endocrinology* **127**, 1849-1860.
37. Thissen, J. P., L. E. Underwood, D. Maiter, M. Maes, D. R. Clemmons and J. M. Ketelslegers. 1991. Failure of insulin-like growth factor-1 (IGF-1) infusion to promote growth in protein-restricted rats despite normalization of serum IGF-1 concentrations. *Endocrinology* **128**, 885-890.
38. Vanderkool, W. K., M. J. Vandehaar, B. K. Sharma, M. Binelli, H. A. Tucker, R. M. Akers and W. M. Moseley. 1995. Comparison of growth hormone-releasing factor and somatotropin: the somatotropic axis in lactating primiparous cows. *J. Dairy Sci.* **78**, 2140-2149.

초록 : 거세면양에 있어서 에너지수준과 GHRP-2의 투여가 혈장 IGF-1, IGF-BPs 및 hepatic GH 수용체에 미치는 반응

이흥구 · 김영성¹ · Hisashi Hidari · 최윤재¹ · 김선구 · 신태순 · 조병욱 · 김용균 · 김근기 · 손홍주 · 이상몽
박현철 · 강한석*

(부산대학교 생명자원과학대학 생명자원과학부 및 응용생명과학부, ¹서울대학교 농업생명과학대학 농생명공학부, ²일본 오비히로 대학)

본연구는 정상으로 단백질을 급여한 거세면양에 있어서 에너지 첨가가 GHRP-2투여에 대한 혈장 IGF-1 및 IGF-BPs에 대한 반응과, 고에너지 급여에 따른 GHRP-2투여가 hepatic GH 수용체에 미치는 영향을 검증하기 위하여 실시하였다. 시험 결과 HENP (CP 0.34 kg, TDN 1.83 kg/day DM intake)처리기간 동안 혈장 IGF-1 과 39-42kDa IGFBP-3수준은 LENP (CP 0.32 kg, TDN 0.87 kg/day DM intake)기간에 비하여 높게 나타났으나 (P<0.05), 혈장 34 kDa IGFBP-2와 24 kDa IGFBP-4는 영양처리에 의해 영향을 받지 않았다. 각 영양처리 기간 동안 GHRP-2 (12.5 µg/kg body weight/day)투여는 혈장 GH 반응이 촉진되었으며(P<0.05), 혈장 GH 평균 함량과 AUC증가에 있어서는 LENP처리 기간에 비하여 HENP처리기간에서 유의적으로 높게 나타났다(P<0.01). 특히 HENP에서 7일간 GHRP-2투여에 의한 일중 혈장 IGF-1 변화양상을 조사한 결과 투여 2, 6 및 7일에서 뚜렷한 증가양상을 보였다(P<0.05). 이에 반하여 LENP에서는 오직 투여 3일째에서 Saline구에 비하여 유의적인 증가를 확인하였다(P<0.05). IGF-BPs의 ligand blotting 결과 HENP구에서 혈장 39-43 kDa IGFBP-3의 수준의 증가가 투여 6일과 7일에서 관찰되었으나 혈장 IGFBP-2수준은 두 영양처리시기에서 유의적인 차이를 관찰하지 못했다. 아울러 HENP구에 있어서 간세포막에 ¹²⁵I-oGH의 결합력을 측정된 결과 GHRP-2투여에 의한 영향은 관찰되지 않았다. 이와 같은 결과는 거세면양에 있어서 단백질과 에너지 사이의 영양적 균형은 내인성 GH/IGF-1 axis는 물론 혈장 IGFBP-3수준의 변화에 영향을 미치고 있음을 시사한다.