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New Potent hGH-RH Analogues with Increased Resistance to Enzymatic Degradation

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Abstract: Four hGH-RH analogues containing homoarginine (Har) and/or D-Arg were obtained by solid-phase methodology using Boc-chemistry. To introduce Har residues, a Lys(Fmoc) protected Lys derivative was incorporated in the appropriate positions (11, 12, 20, 21 or 29); after assembly of the peptide chain the Fmoc group was removed and the peptide-resin was guanidinylated by treatment with *N*, *N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea. The peptides were cleaved from the resin by treatment with liquid HF, and the products were purified by RP-HPLC. The peptides were subjected to digestion by trypsin, and the course of the reaction was followed by HPLC and ESI-MS. It was found that peptide bonds formed by the carboxyl group of Har are completely stable to trypsin. The course of cleavage at Lys or Arg residues depends on the position of Har in the sequence. All the analogues investigated stimulate the release of GH in rats after subcutaneous administration, and were about 50–100 times as potent as rGH-RH itself. The analogues had no effect on PRL, LH and FSH levels.

Keywords: hGH-RH analogues; rGH secretion; homoarginine; guanidine formation; guanidinylation solid-phase peptide synthesis; tryptic digestion

INTRODUCTION

The wide availability of human growth hormone (GH) produced by recombinant technology has made its use practicable and possible in the treatment of GH-deficient patients. However, GH-deficiency is in most cases due to hypothalamic dysfunction in the regulation of GH production and secretion, rather than to pituitary impairment. Thus, a therapy based on the hypothalamic growth hormone-releasing hormone (hGH-RH) that stimulates hGH secretion could also be used in most patients [1]. Native hGH-RH has a length of 44 residues, but synthetic hGH-RH(1–29)-NH_2 displays full biological activity although it is the shortest fragment endowed with this property [2]. Several therapeutic applications have been suggested for these peptides, such as growth stimulation in children, treatment of geriatric patients to reduce loss of muscle and bone; promotion of the healing of wounds, burns and bone fractures and as non-steroidal anabolic agents in chronic debilitating diseases [3–8].

Although the therapeutic usefulness of hGH-RH and its shorter analogue has been demonstrated, susceptibility to enzyme cleavage limits their use as effective drugs. It has been demonstrated that degradation at the site of injection after subcutaneous administration is extremely rapid. The estimated amount in the circulation after subcutaneous injection was only 4% of that

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obtained after intravenous administration [9]. There is a great demand for more stable analogues which would allow a reduction in the doses and frequency of administration. Numerous analogues of hGH-RH have been synthesized and tested to obtain compounds of increased metabolic stability and potency [10–17]. Summaries of the cumulative efforts of various laboratories in this quest have been published [18,19].

It has been reported that hGH-RH(1–29)-NH_2 is rapidly metabolized in the plasma, as a result of Ala^2-Asp^3 peptide bond cleavage by dipeptidylpeptidase IV leading to the inactive hGH-RH(3–29)-NH_2 [20]. The other metabolites observed were characteristic of trypsin-like cleavages between Arg^{11}-Lys^{12} and Lys^{12}-Val^{13} [21]. It was recently demonstrated that a hGH-RH(1–29)-NH_2 analogue was cleaved by trypsin at all basic amino acid residues, including the *C*-terminal amide bond, but an analogous sequence that differed in that it contained Orn instead of Lys was cleaved only at Arg residues [22]. This finding is consistent with the exceptionally high biological activity *in vivo* of analogues containing Orn in positions 12 and 21 [17], which are, however, susceptible to rapid cleavage at Arg. Thus, in order to increase metabolic stability further modifications at positions occupied by Arg residues are needed. So far, there has been no successful solution to this problem.

The aim of the present work was to examine the possibility of obtaining biologically active analogues by replacing Arg residues with homoarginine, Har (side chain extended).

Although the most potent analogues reported so far contain agmatine (1-amino-4-guanidinobutane) at position 29 [15–17] we rejected this modification. The use of agmatine in this position complicates the course of synthesis, since a special support must be prepared in which agmatine is attached to the resin *via* the guanidine group. Moreover, it is likely that the increased activity of peptides containing agmatine is mainly due to protection against enzymatic degradation at position 29. To obtain the same protection D-Arg or Har was introduced there. Further replacements made were: Dat (desaminotyrosine) at position 1, Ala at position 15, Nle at position 26 and Asp at position 28. The following analogues were synthesized and their activity in *vivo* was examined:

Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr^{10}-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg^{20}-Lys-Leu-Leu-Gln-Asp-Ile-Nle-Asp-D-Arg-NH_2 **1** *(DK-1)*

Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr^{10}-Arg-Har-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg^{20}-Har-Leu-Leu-Gln-Asp-Ile-Nle-Asp-D-Arg-NH_2 **2** *(DK-1G)*

Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr^{10}-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Har^{20}-Lys-Leu-Leu-Gln-Asp-Ile-Nle-Asp-Har-NH_2 **3** *(OW-5)*

Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr^{10}-Har-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Har^{20}-Lys-Leu-Leu-Gln-Asp-Ile-Nle-Asp-Har-NH_2 **4** *(OW-1)*

MATERIALS AND METHODS

N, N′-Bis(*tert*-butoxycarbonyl)-S-methylisothiourea

S-Methylisothiouronium sulphate (2.78 g, 0.01 mol) was dissolved in water (30 ml), and dioxane (30 ml), 1 M NaOH (20 ml, 0.02 mol) and Boc_2O (11.6 g, 0.05 mol) were added. The mixture was stirred at room temperature for 24 h. The solid obtained was filtered, washed with water (50 ml) and suspended in water (200 ml). The suspension was shaken vigorously for 5 min. The product was isolated by filtration and dried over P_2O_5 under reduced pressure to give white crystals. The yield was 4.76 g (82%); mp 125°–126 °C (lit. [23] 122°–123 °C). A sample of the product (323 mg, 1.25 mmol was dissolved in methylene chloride (2 ml) and *n*-propylamine (20 µl, 0.25 mmol) was added and it was allowed to stand for 24 h. TLC on a silica gel plate (hexane: chloroform; 8 : 2) failed to detect Boc-*n*-propylamine, confirming the absence of residual $(Boc)_2O$ in the product.

Peptide Chain Assembly

All amino acids were of L-configuration. α-Amino functions were Boc protected, and the side chains were blocked with the following groups: Asp, cyclohexyl; Lys, Fmoc (if a Lys to Har conversion was planned) or Z; Orn, Z; Ser and Thr, Bzl; and Tyr, Z(2-Br). MBHA resin (0.818 g, 0.4 meq; Bachem California, 0.52 meq/g) was used after swelling in DCM for 30 min, treating with 5% DIEA (1 × 1 min, 1 × 20 min) and washing with DCM (6 × 1 min). The synthesis of the protected peptide-resin was carried out according to the standard procedures: (a) deprotection with 55% TFA in DCM (1 × 1 min, 1 × 20 min); (b) washing with DCM (3 × 1 min); (c) washing with 30% dioxane in

DCM (2 × 1 min); (d) washing with DCM (3 × 1 min) (e) neutralization with 5% DIEA in DCM (1 × 1 min, 1 × 5 min); (f) washing with DCM (6 × 1 min); (g) coupling of Boc-amino acid (1.2 mmole) in the presence of DIPC (1.2 mmole) in DCM for 2 h; washing with DCM (6 × 1 min). Boc-Gln and Boc-Asn were introduced by the DIPC/HOBt method (1.2 mmole). (h) washing with DCM (6 × 1 min).

Guanidinylation

Method 1. Protected peptide-resin was treated with 50% piperidine in DMF (1 × 10 min, 1 × 2 h) to remove the fluorenylmethoxycarbonyl protection, then washed with DMF (3 × 1 min), 50% DMF in DCM (2 × 1 min), 50% methanol in DCM and DCM (3 × 2 min). The resulting protected peptide-resin was treated with *N*, *N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (5 fold molar excess) and DNAP (70 mg) in DMF for 4 days. The product was washed with DMF (3 × 1 min), DCM (3 × 1 min), treated with 55% TFA in DCM (1 × 1 min, 1 × 20 min, 1 × 40 min) and washed with DCM (3 × 1 min), 50% DMF in DCM (2 × 1 min) and DCM (2 × 1 min). The yield was 2.0–2.5 g.

Method 2. In the synthesis of **2,** which was prepared from a peptide-resin containing Lys[Z(2-Br)] protected with 2-chlorobenzyloxycarbonyl group, the peptide was cleaved from the resin as described below. The crude product (5–8 mg) was then dissolved in DMF (0.5 ml), NEt_3(two drops) and *N*, *N*′-bis-butyloxycarbonyl-*S*-methylisothiourea (20 fold molar excess) was added and stirred for 16 h at 37 °C. Water (3 ml) was added and the solution was lyophilized. The solid residue was washed with EtOAc (3 × 5 ml) and then treated with TFA for 15 min. The TFA was removed by evaporation, and the crude peptide was dissolved in acetic acid and lyophilized. Pure peptide was obtained using HPLC as described below.

Cleavage of the Peptide from the Resin

A sample (500 mg) of protected peptide-resin was treated with liquid HF (10 ml) in the presence of anisole (1 ml) for 1 h at 0 °C. The HF was removed under reduced pressure, and the residue was treated with cold Et_2O, extracted with 50% acetic acid and lyophilized. The yield was 240–260 mg.

Purification of Peptides

The crude peptides (20 mg samples) were purified using a Knauer HPLC system with a Vertex column Nucleosil-300 C_{18} (8 × 200 mm, 5 μm); solvent system: A, 0.1% TFA in water, B, 80% MeCN in A. Elution: 20%–55% B in 30 min, then 55% B in 30 min. Flow rate 2 ml/min. Fractions were analysed on a Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 μm) using a gradient of 25%–70% in 30 min; flow rate 1 ml/min; detection at 220 nm. Homogeneous fractions containing a single peak were combined, diluted with water and lyophilized. Fractions were pooled for maximum purity rather than yield. The yield of homogeneous product was 1.0–4.3 mg. Structures were confirmed by mass spectra using a ADM-604 apparatus. LSIMS: **1** ($C_{151}H_{249}N_{43}O_{43}$), M calcd 3367, (M + 1) obtained 3368; **2** ($C_{153}H_{253}N_{47}O_{43}$)M calcd 3450, obtained for (M + 1) 3451; **3** ($C_{154}H_{253}O_{43}N_{43}$) M calcd 3395, obtained for (M + 1) 3396; **4** ($C_{155}H_{255}O_{43}N_{43}$) M calcd 3409, obtained 3411.

Tryptic Hydrolysis of Peptides

A sample of peptide (1.2 mg) was dissolved in ammonium acetate buffer (2.9 ml, 0.05 M, pH 8.5) and incubated at 37 °C for 20 min. Then a solution of trypsin (100 μl, 0.02 mg/ml; Serva, 36 U/mg) was added. The resulting solution was incubated at 37 °C for 15 min. A sample (500 μl) of the solution was diluted with 0.5 M acetic acid (1 ml) and lyophilized. Analysis of the resulting material was performed by HPLC using a Knauer system with a Eurospher 100 C_{18} (4 × 250 mm, 5 μm) column; solvent system: 0.1% TFA in water, B: 80% MeCN in A; linear gradient 25%–70% B; flow rate 1 ml/min; detection at 220 nm. Fractions were collected and peptides were identified by ESI-MS using a Finnigan MAT 95S spectrometer, Bremen, Germany (Figure 1 and Table 1).

Bioassays

Female Wistar-Kyoto rats (in dioestrus) weighing 180–200 g were anaesthetized with vetbutal and rat GH-RH (Sigma, product no G 6646) in doses of 50 and 150 μg/kg of body weight, and analogues in doses of 1 and 3 μg/kg of body weight were administered subcutaneously in saline. Control groups were injected with saline only. Blood samples were drawn from cannulated rats at 0, 15 and 30 min. The serum rGH, rLH, rFSH and rPRL concentrations were measured with RIA methods using commercial kits (RIA BIOTRAK, Amersham Life Science: GH, product no RPA 551, sensitivity 1.6 ng/ml; PRL, product no RPA 553, sensitivity 0.7 ng/ml; LH, product no 552, sensitivity 1.0 ng/ml; FSH, product no 550, sensitivity 1.7 ng/ml).

Fragments	Peptides			
	1 [$Arg^{11,\ 20}Lys^{12,\ 21}$D-$Arg^{29}$]*	**2** [$Arg^{11,\ 20}Har^{12,\ 21}$D-$Arg^{29}$]*	**3** [$Arg^{11}Lys^{12,\ 21}Har^{20,\ 29}$]*	**4** [$Har^{11,\ 20,\ 29}Lys^{12,\ 21}$]*
(1-29)-NH_2	-	+	-	-
(1-11)-OH	+	+	+	-
(12-29)-NH_2	-	-	+	-
(1-12)-OH	+	-	+	+
(13-29)-NH_2	-	-	+	+
(21-29)-NH_2	+	+	-	-
(1-21)-OH	-	-	-	+
(22-29)-NH_2	+	-	+	+
(12-20)-OH	+	+	-	-
(13-20)-OH	+	-	-	-
(12-21)-OH	-	-	+	-
(13-21)-OH	-	-	+	+

Figure 1 Identified products of tryptic digestion. Dibasic amino acid residues in positions 11, 12, 20, 21, 29 are shown*.

Table 1 Identification of Degradation Products by ESI MS

Peptide	Fragment	Ion $[M + zH]^{z+}$		
		z	Calculated	Found
1	(1–11)-OH	2	653.7	653.7
	(1–12)-OH	2	717.8	717.7
	(21–29)-NH_2	2	557.2	556.8
	(22–29)-NH_2	1	985.2	984.4
		2	493.1	492.7
	(12–20)-OH	1	985.1	985.7
		2	493.1	493.3
	(13–20)-OH	1	856.9	857.4
		2	429.0	429.3
2	(1–29)-NH_2	4	864.0	864.2
		5	691.2	691.2
	(1–11)-OH	2	653.7	653.9
	(21–29)-NH_2	2	578.2	577.8
	(12–20)-OH	2	514.6	514.3
3	(1–11)-OH	2	653.7	653.1
	(12–29)-NH_2	3	703.2	703.5
		4	527.7	527.7
	(1–12)-OH	2	717.8	717.3
	(13–29)-NH_2	3	660.1	660.5
		4	495.4	495.7
	(22–29)-NH_2	1	999.2	998.7
		2	500.1	499.8
	(12–21)-OH	2	564.7	564.4
		3	376.8	376.5
	(13–21)-OH	2	500.6	500.3
		3	334.1	333.8
4	(1–12)-OH	2	724.8	724.4
	(13–29)-NH_2	2	989.7	990.1
		3	660.1	660.8
		4	495.7	495.7
	(1–21)-OH	3	810.3	810.5
		4	608.0	608.1
	(22–29)-NH_2	2	500.1	499.8
	(13–21)-OH	2	500.6	500.1

RESULTS AND DISCUSSION

Four novel analogues of hGH-RH(1–29)-NH_2 containing homoarginine (Har) or D-Arg residues instead of Arg and Lys were synthesized by solid-phase methodology. These replacements were made to

protect the molecules from digestion by trypsin-like proteases. Arg and Lys residues are known to be processing sites for cleavage by trypsin-like proteases in the native peptide. We recently demonstrated that the introduction of Orn instead of Lys decreases the number of degradation products after treatment with trypsin [22]. It was our hope that the Har position would not be susceptible to trypsin. The introduction of a D-Arg residue in position 29 was based on the assumption that configuration of this residue is not important for biological activity. This is supported by the fact that analogues containing agmatine, which does not contain a chiral carbon atom, are among the most active GH-RH analogues obtained so far. Moreover, for the synthesis of agmatine-containing analogues, the preparation of an agmatine-resin conjugate, in which agmatine is attached to the polymer via its guanidine group, is needed. Additional replacements were: Dat in position 1 to prevent digestion at the *N*-terminus, and Nle in position 26 instead of Met to protect from chemical oxidation during synthesis. A distinctive feature of this synthesis was the use of commercially available Boc-Lys(Fmoc)-OH. Fmoc protection was removed after the peptide chain had been assembled, by treatment with piperidine, and the peptide-resin was then guanidinylated. Guanidinylation was performed using *N*, *N*′-bis(*tert*-butoxycarbonyl)-S-methylisothiourea with a catalytic amount of DMAP. This process was controlled using the Kaiser colour test, and stopped when free amino groups were no longer detected. The peptide was deprotected and cleaved from the resin by treatment with liquid HF. The crude product was purified to homogeneity by RP-HPLC. Peptide **2** was obtained using BocLys[Z(2-Cl)]-OH. In this case the peptide-resin was treated with liquid HF, the peptide was isolated and then guanidinylated. The final yield was lower than that obtained when guanidinylation was carried out with the peptide-resin. The peptides were subjected to digestion by trypsin under conditions in which an analogue containing unmodified Arg and Lys had been studied [22]. The products of digestion were separated using HPLC and characterized by ESI-MS. It was observed that after digestion for 60 min, all peptide bonds formed by the carboxyl groups of Lys and Arg were completely cleaved, but those at Har remained unchanged. Also, the amide bond formed with Har and D-Arg at position 29 was stable. However, an analysis of products obtained after 15 min digestion allowed the observation of intermediate peptides. Examination of these structures indicates (see Figure 1 and Table 1) that the rate of hydrolysis depends on the sequence of the peptides e.g. a 1–21 fragment was found in the products obtained after digestion of **3** (OW-5), but not in those from **4** (OW-1). It also can be seen that although peptides **2** and **4** contain two peptide bonds that are vulnerable to proteolysis, the former is still present in the reaction mixture after 15 min of digestion, while the latter is completely digested. This effect could be even higher in physiological conditions where the concentration of trypsin-like enzymes is lower: after digestion of hGH-RH(1–29)-NH_2 in plasma for 60 min, a substantial amount of the intact peptide remained [20]. The peptides were tested for their ability to stimulate GH release in rats by injecting them subcutaneously. The potency was compared to that of rGH-RH. Doses of 1 and 3 μg of the analogues per kg of body weight, and doses of 50 and 150 μg of rGH-RH per kg of body weight were injected. The results are shown in Table 2. It is clear that all these analogues are very potent, analogue **2** being about 100 times as potent as rGH-RH itself.

It is interesting to compare the results of digestion by trypsin with the ability to promote the release of GH. The principles that govern peptide hormone–receptor interaction and the interaction of enzymes with their substrates are similar. Both peptide hormones and enzymes bind to their targets of action specifically to express their activity. In this case elongation of the side chain of Arg by a single methylene group resulted in the formation of analogues which contained a peptide bond completely resistant to cleavage by trypsin. Interaction of the analogues with the receptor is less sensitive to this particular change. However substitution of Arg by Lys, which does not change susceptibility of the analogues to trypsin digestion, resulted in the loss of ability to release GH [24]. It appears from our work that a guanidino group in the side chain of residues 11 and 20 is needed to express GH releasing activity.

None of the analogues on injection had any effect upon the secretion of rPRL, rLH or rFSH (Tables 3, 4 and 5). It is worth noting that the injection of rGH-RH itself increased the rPRL plasma level. This is consistent with the finding that hGH-RH mediated oPRL release by 20%–25% more than in controls [25]. All the presently reported analogues were specific: doses that promoted high levels of rGH had no effect on rPRL secretion.

CONCLUSIONS

Four analogues of human GH-RH-(1–29)-NH_2 were synthesized by solid-phase methodology. The

Table 2 Effects of Subcutaneous Administration of hGH-RH Analogues on rGH Release in Rats

Compound	Dose (μg/kg)	n[a]	Serum concentration (ng/ml) ± SEM		
			0 min	15 min	30 min
Saline		10	62.3 ± 2.5	65.1 ± 2.8	63.8 ± 1.6
rGH-RH	50	10		245.6 ± 26.2	73.6 ± 2.0
	150	10		437.2 ± 47.9	99.4 ± 5.9
1 (DK-1)	1.0	9		263.3 ± 28.0	138.0 ± 15.7
	3.0	9		734.0 ± 44.7	109.0 ± 14.1
2 (DK-1G)	1.0	10		654.0 ± 38.4	188.5 ± 14.4
	3.0	10		752.5 ± 40.5	105.0 ± 6.0
3 (OW-5)	1.0	8		116.9 ± 7.6	95.6 ± 6.4
	3.0	8		617.5 ± 42.7	95.0 ± 6.3
4 (OW-1)	1.0	8		156.2 ± 8.6	100.6 ± 7.2
	3.0	8		221.8 ± 28.4	98.1 ± 5.3

[a] Number of animals.

Table 3 Effects of Subcutaneous Administration of hGH-RH Analogues on rPRL Release in Rats

Compound	Dose (μg/kg)	n[a]	Serum concentration (ng/ml) ± SEM		
			0 min	15 min	30 min
Saline		10	30.6 ± 0.8	30.4 ± 0.6	31.5 ± 0.9
rGH-RH	50	10		47.0 ± 1.9	50.7 ± 2.5
	150	10		58.5 ± 1.7	54.3 ± 2.2
1 (DK-1)	1.0	9		30.5 ± 1.0	26.3 ± 2.9
	3.0	9		32.6 ± 1.9	30.7 ± 1.1
2 (DK-1G)	1.0	10		37.8 ± 4.3	37.9 ± 1.0
	3.0	10		34.8 ± 2.8	42.7 ± 3.2
3 (OW-5)	1.0	8		38.3 ± 2.8	34.4 ± 1.9
	3.0	8		37.2 ± 1.1	37.4 ± 3.0
4 (OW-1)	1.0	8		32.4 ± 1.8	33.2 ± 3.0
	3.0	8		38.2 ± 3.7	36.7 ± 2.4

[a] Number of animals.

modifications introduced were intended to increase resistance to trypsin-like degradation: Har was introduced in the positions occupied by dibasic amino acids, and D-Arg was introduced at the *C*-terminal position. The characteristic feature of the syntheses was that Har residues were introduced indirectly by guanidinylation of Lys side chains exposed after peptide assembly. The introduction of Har at one of the endo positions occupied by Lys or Arg in endo positions (11,12, 20 and 21), and Har or D-Arg in position 29 abolished cleavage by trypsin at these positions and, in some cases, slightly increased overall stability.

The analogues described are very potent after subcutaneous administration. The increment in potency is probably caused by increased enzymatic stability. It seems that these modifications of hGH-RH could be a promising lead in the development of clinically useful analogues for the therapy of patients with growth hormone deficiency.

Table 4 Effects of Subcutaneous Administration of hGH-RH Analogues on rLH Release in Rats

Compound	Dose (μg/kg)	n[a]	Serum concentration (ng/ml) ± SEM 0 min	15 min	30 min
Saline		10	2.3 ± 0.5	2.1 ± 0.3	1.8 ± 0.6
rGH-RH	50	10		2.2 ± 1.2	2.3 ± 0.9
	150	10		3.6 ± 0.5	5.1 ± 0.7
1 (DK-1)	1.0	9		1.5 ± 0.5	2.1 ± 0.2
	3.0	9		2.1 ± 0.2	1.3 ± 0.1
2 (DK-1G)	1.0	10		2.0 ± 0.4	1.8 ± 0.4
	3.0	10		2.5 ± 0.5	2.0 ± 0.6
3 (OW-5)	1.0	8		2.1 ± 0.2	2.6 ± 0.2
	3.0	8		2.4 ± 0.1	2.4 ± 0.2
4 (OW-1)	1.0	8		3.2 ± 0.5	2.3 ± 0.3
	3.0	8		3.3 ± 0.2	2.7 ± 0.1

[a] Number of animals.

Table 5 Effects of Subcutaneous Administration of hGH-RH Analogues on rFSH Release in Rats

Compound	Dose (μg/kg)	n[a]	Serum concentration (ng/ml) ± SEM 0 min	15 min	30 min
Saline		10	2.3 ± 0.2	2.5 ± 0.2	2.4 ± 0.1
rGH-RH	50	10		2.1 ± 0.2	2.2 ± 0.1
	150	10		2.3 ± 0.4	2.0 ± 0.1
1 (DK-1)	1.0	9		1.5 ± 0.2	1.9 ± 0.1
	3.0	9		2.7 ± 0.8	2.1 ± 0.3
2 (DK-1G)	1.0	10		1.9 ± 0.1	1.8 ± 0.05
	3.0	10		2.4 ± 0.04	2.1 ± 0.06
3 (OW-5)	1.0	8		1.8 ± 0.2	1.8 ± 0.1
	3.0	8		1.6 ± 0.1	1.5 ± 0.2
4 (OW-1)	1.0	8		1.7 ± 0.2	2.0 ± 0.3
	3.0	8		3.4 ± 0.5	1.3 ± 0.1

[a] Number of animals.

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