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Construction and activity of a novel GHRH analog, Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys[1]

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KEY WORDS growth hormone; human; *in vitro*; peptide fragments; anterior pituitary gland; rats; somatotropin-releasing hormone; structure-activity relationship

ABSTRACT

AIM: To construct another growth hormone releasing hormone (GHRH) analog, Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide and to compare its activity with that of Pro-Pro-hGHRH(1-44)OH peptide. **METHODS:** The *pro-pro-hGHRH(1-44)-gly-gly-cys* DNA fragment was synthesized by polymerase chain reaction. The recombinant protein was expressed to high levels in *Escherichia coli* BL21 (DE3). The Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptide was purified to homogeneity by cell disruption, washing, ethanol precipitation, acid hydrolysis, and SP-Sephadex C-25 and Sephadex G-25 column chromatography. The peptide molecular mass was determined by electrospray ionization mass spectroscopy (ESI-MS). Its purity was determined by SDS-PAGE. The concentration of growth hormone (GH) stimulated by GHRH and its analogs was determined with antiserum kit against human GH. The other human hormones as hTSH, hFSH, hLH, and hPRL were determined with a paramagnetic particle chemiluminescent immunoassay kit. **RESULTS:** The molecular weight of Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys was 5455.4 kDa which was coincident with the theoretical calculations. All the three peptides at 5 mg/L stimulated GH release from the human fetal pituitary but only the difference between Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys group and blank group was significant ($P<0.05$)**.** Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys 0.01, 0.1, and 1 mg/L and Pro-Pro-hGHRH (1-44)OH 0.1 and 1 mg/L stimulated GH release from rat pituitary in a concentration-dependent manner ($P<0.05$, $P<0.01$). At the same concentration Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys stimulated more GH release than Pro-Pro-hGHRH (1-44)OH. Pro-Pro-hGHRH (1-44)OH 0.01 mg/L and hGHRH (1-40)OH 2 mg/L did not stimulate GH release from rat pituitary ($P>0.05$). Pro-Pro-hGHRH (1-44)OH and Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptides at 5 mg/L did not stimulate hTSH, hFSH, hLH, and hPRL release from human fetal pituitary *in vitro.* **CONCLUSION**: Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys had a better activity than that of Pro-Pro-hGHRH(1-44)OH. Variation at C-terminus of GHRH could modulate its GH releasing activity.

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INTRODUCTION

Experimental and clinical evidence suggested that the secretion of growh hormone (GH) by the pituitary gland is under the regulation of stimulatory and inhibitory factors produced by the hypothalamus[1]. Human growth hormone releasing hormone (hGHRH) was purified from GHRH-hypersecreting pancreatic tumor tissue in 1982[2,3]. The sequence analysis of the hGHRH showed that the hormone existed in three types (37, 40, or 44 amino acid residues) in the hypothalamus[4]. Ling *et al* discovered that the amino acid sequence of human hypothalamic GHRH showed identities of 93 %, 89 %, and 86 % compared with those of hypothalamic GHRHs from the porcine, bovine, and caprine species, respectively[5].

The *N*-terminal amino acid residue of hGHRH was tyrosine and the one of rGHRH (rat) was histidine. Structure–activity relationships showed that the *N*-terminal ^{1}Tyr was required for the high *in vitro* bioactivity of hGHRH. Many compounds with GH-releasing activity need very specific configuration of selected aromatic rings to stimulate GH release[6].

After deletion of amino acid at the C-terminus of hGHRH (1-44), the activity of hGHRH analog was gradually decreased. hGHRH (1-29)NH_2 remained 51 % activity of the parent hGHRH (1-44)NH_2, so functional hGHRH needed at least 1-29 amino acid residues. But the activity of their amino-hGHRH analogs was about 2 times higher than that of the parent carboxyl-hGHRH analogs[7].

We have reported that Pro-Pro-hGHRH(1-44)OH peptide had enhanced activity compared with that of hGHRH (1-44)OH[8]. To get another GHRH analog with enhanced activity and prolonged half-life, we designed Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys based on Pro-Pro-hGHRH (1-44)OH and measured its activity.

MATERIALS AND METHODS

Construction of the fusion protein gene The *pro-pro-hGHRH(1-44)-gly-gly-cys* DNA fragment was produced by PCR amplification with 5´ primer (CCC CGG ATC CAT ACG CCA TTC GAT GTC TA) and 3´ primer (GGG AAG CTT AGC AAC CAC CCA GAC GAG CTC GAG CAC CA) in the presence of template pED-pro-pro-GHRH vector and pfu-*Tag* DNA polymerase (Promega). The reaction was carried out through 30 cycles with 1 min at 94 ºC, 1 min at 60 ºC, and 1.5 min at 72 ºC. The resulting DNA fragment was digested with restriction endonucleases *BamH*I and *Hind*III, and then the 3´-end of the fusion partner gene of the vector pED was inserted. The novel fusion protein gene was transformed into *E coli* BL2 (DE3) to form engineering bacteria.

Expression and purification of the Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide[8] The engineering bacteria with *pro-pro-hGHRH-gly-gly-cys* gene were inoculated into 200 mL of Luria-Bertani (LB) medium containing kanamycin (50 mg/L) and incubated at 37 ºC. The induction of the fusion protein expression was carried out with 50 mmol/L lactose for 3 h.

About 75.6 g of the recombinant *E coli* cells was resuspended in 226.8 mL of the lysis buffer containing lysozyme 0.3 g/L, DNAase I 7 mg/L, and 1 % Triton X-100 (v/v) and continuously stirred for 5 h at 37 ºC. After the bacteria were entirely disrupted in the lysis buffer, the suspension was centrifuged. The precipitate was washed three times in a total of 600 mL of the lysis buffer containing 1 % Triton X-100 followed by two washes in a total of 400 mL of 1 % Triton X-100 water solution and finally once in 200 mL of water. The last precipitate was re-dissolved in urea solution 8 mol/L (urea dissolved in Tris/HCl 50 mmol/L, pH 8.5) to form a suspension with the final concentration of 4 % (wet weight/volume) while it was continuously stirred overnight at room temperature. The suspension was fractionally precipitated by ethanol saturation and the fraction precipitated between 65 % and 75 % ethanol concentration was collected. The precipitate was dissolved in HCl 60 mmol/L to form a final concentration of 6 % (w/v) and incubated at 50 ºC for 44 h, and pH was adjusted to 9.5 by adding 10 % ammonium hydroxide. The suspension was centrifuged at 6000 r/min for 20 min to collect supernatant. The supernatant was applied to the column chromatography of SP-Sephadex C-25 (Pharmacia). Equilibrium and subsection eluents were ammonium hydroxide with pH 9.5 and pH 11, respectively. The sample from the fraction 2 of SP Sephedex C-25 was applied to Sephadex G-25 (Pharmacia) column and eluted with water. The pH value of the obtained sample was adjusted to 7.4 by 30 % acetic acid.

Determination of the purity of fusion protein and peptide[9] A polyacrylamide gel (T:C=12:2.66) was prepared to analyze the purity of the fusion protein and peptide polyacrylamide gel (T:C=20:9) for Pro-Pro-

hGHRH(1-44)-Gly-Gly-Cys peptide.

Determination of the molecular weight of Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptide The molecular weight of Pro-Pro-GHRH (1-44)-Gly-Gly-Cys was determined by the method of ESI-MS (Electrospray Ionization Mass Spectroscopy, Agilent 1100 LC/MSD). The sample was dissolved in 1 % glacial acetic acid.

***In vitro* activity assays of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys and Pro-Pro-hGHRH(1-44)OH peptides**[10-12]

Activity assay using rat pituitary Female Sprague–Dawley rats (200-250 g, grade II) from the Experimental Animal Center of Chinese Pharmaceutical University were housed at 25 ºC in 14-h light and 10-h darkness. The rats were fed *ad libitum*. Rat pituitaries must be removed in 30 min. Each rat pituitary was cut into 4 pieces. The peptide concentrations of Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys and Pro-Pro-hGHRH (1-44) were 0.01, 0.1, and 1.0 mg/L and hGHRH (1-40) OH was 2 mg/L. The tissue pieces including 3 pituitaries were incubated in 1 mL Krebs-Ringer's buffer in 1 cm×10 cm glass tube while being shaken every 5 min. The pituitaries were incubated for a total of 5 h (P_1, P_2, I_3, I_4, and I_5). The buffer was removed each hour for radio immuno assay (RIA) of GH and then fresh buffer was added. After pre-incubation periods (P_1 and P_2) of the first 2 h, in the subsequent 3 h the peptide was added alone to the incubation buffer to stimulate the release of GH by the pituitaries (I_3, I_4, and I_5). The *in vitro* hormone release is recorded as the Δvalue, calculated by subtracting the amount of the hormone released in the periods of I_3, I_4, and I_5 from that released in the period of P_2. The *in vitro* studies were performed in three groups. hGHRH (1-40)OH (Sigma) was used as a standard and the GH content of P_2 without any peptide was as blank. The human GH RIA kit (the antiserum against human GH was used) was purchased from Beijing Northern Biotechnology Laboratory.

Activity assay using human fetus pituitary Pituitaries of female abortive fetus of 18-28 weeks old were from Zhongda Hospital, Southeast University, Nanjing. The experiment was permitted by the Ethical Committee of Zhongda Hospital according to Helsinki Declaration. Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys, Pro-Pro-hGHRH (1-44)OH, and hGHRH (1-44)OH peptides 5 mg/L was used to stimulate GH release. The sample included 1 pituitary in each group. The following procedure was the same with that of rat pituitary.

The other hormones (hTSH, hFSH, hLH, and hPRL) released from human fetus pituitary were determined by a paramagnetic particle chemi-luminescent immunoassay kit (American Beckman Co).

Statistics *P* values were determined by comparison of the Δmean value obtained with and without addition of the peptide to the buffer and calculated by *t* test.

RESULTS

Construction and expression of the fusion protein An asparaginase C-terminal fragment from *E coli*

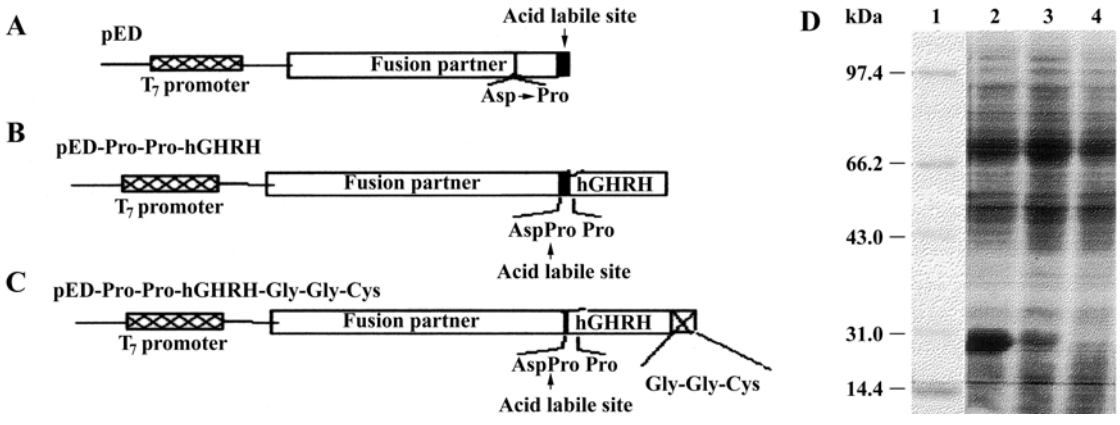

Fig 1. Construction of the fusion partner gene (A) from pED vector and the fusion protein genes with *pro-pro-hGHRH(1-44) OH* gene (B) and *pro-pro-hGHRH(1-44)-gly-gly-cys* gene (C) expressed in *E coli*. T_7 promoter is represented in hatched boxes, recombinant fusion protein gene by open boxes, and the acid labile site by single letter amino acid residue. The unique Pro of *L*-asparaginase was mutated by Ala on Fig 1A. (D) SDS-PAGE (T:C=15:2.66) analysis of fusion protein with Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptide expressed in *E coli*. Lane 1: molecular marker. Lane 2-3: the recombinant *E coli* at 3 and 1 h after induction; Lane 4: recombinant *E coli* before induction.

was tailored to serve as a fusion partner for expressing the peptides. The truncated asparaginase with unique acid labile Asp-Pro (315-316) bond had been mutated into Asp-Ala bond (unpublished). An extra acid labile Asp-Pro linker was inserted between the fusion partner (Fig 1A) and Pro-Pro-hGHRH(1-44)OH (Fig 1B) or Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys (Fig 1C). The fusion proteins were easily expressed to high level in *E coli* (Fig 1D) and accumulated in inclusion body. After cleavage with hydrochloric acid, the peptides were released from the fusion proteins.

Purification of the Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide The fusion protein was obtained by cell disruption, washing, and ethanol precipitation. The peptide was purified by acid hydrolysis (Fig 2A), SP-Sephadex C-25 column (Fig 3A), and Sephadex G-25 column (Fig 3B) chromatography. The purities of fusion protein (Fig 4A), the Pro-Pro-hGHRH(1-44)OH peptide (Fig 4B), and Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide (Fig 4C) were demonstrated by SDS-PAGE.

Determination of the molecular weight of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide The molecular weight of Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys was 5455.4 kDa which was in coincident with the theoretical calculations (Fig 2B).

***In vitro* release of GH** The basal GH release from human fetal pituitary was stable after pre-incubation for 2 h (P_2). In the subsequent three hours (I_3, I_4, and I_5) GH from the pituitaries could be measured at each hour after stimulation with Pro-Pro-hGHRH (1-44)OH, hGHRH (1-44)OH, and Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptide (Fig 5).

All the three peptides at 5 mg/L stimulated GH release in the human fetal pituitary but only the difference between Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys group and blank group was significant (P<0.05, Tab 1).

Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys 0.01-10 mg/L and Pro-Pro-hGHRH (1-44)OH 0.1-10 mg/L increased GH release from rat pituitary in a concentration-dependent manner (P<0.05, P<0.01). But Pro-Pro-hGHRH

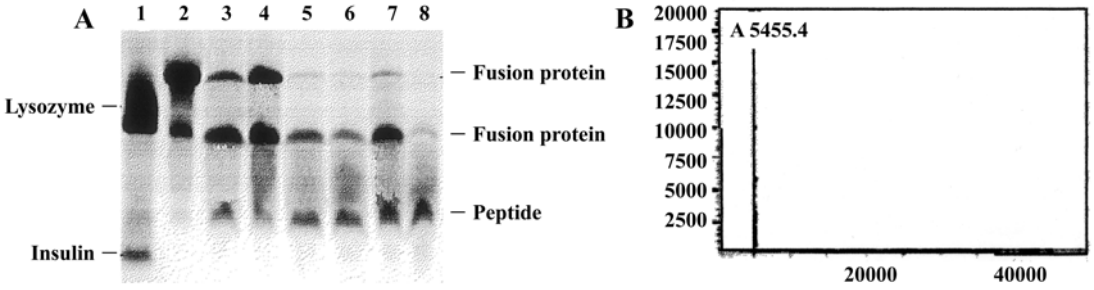

Fig 2. (A) SDS-PAGE (T:C=20:4.9) analysis of cleavage of fusion protein in hydrochloric acid 60 mmol/L. Samples were prepared by dissolving proteins in Laemmli sample buffer and boiling for 5 min in the presence of β- mercaptoethanol and SDS. Lane 1, markers (lysozyme 14400 Da and insulin 5733 Da); Lane 2-8: samples at 7, 17, 24, 48, 54, 60, and 72 h after cleavage of the fusion protein. (B) ESI-MS result of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide.

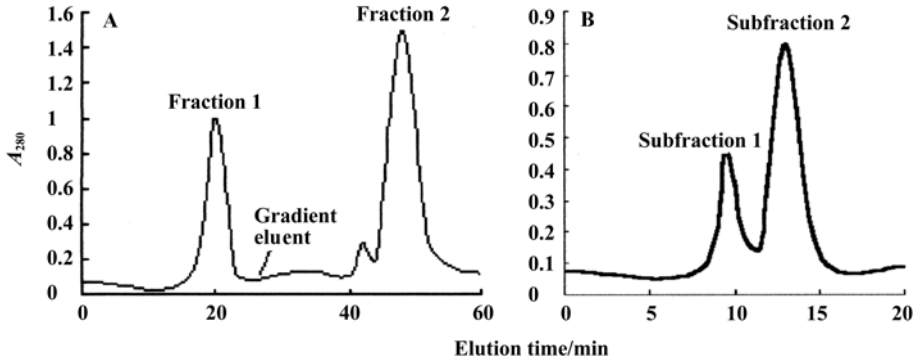

Fig 3. (A) Column chromatography on SP-Sephadex C-25 of the supernatant from acid-hydrolysis product. Equilibrium eluent: ammonium hydroxide (pH 9.5; 0-25 min); subsection gradient eluent: ammonium hydroxide (pH 11; 26-60 min). (B) Column chromatography on Sephadex G-25 of the fraction 2 of SP-Sephadex C-25 column chromatography and its eluent is water. The effluent was monitored continuously at 280 nm at room temperature with Beijing UV Detector.

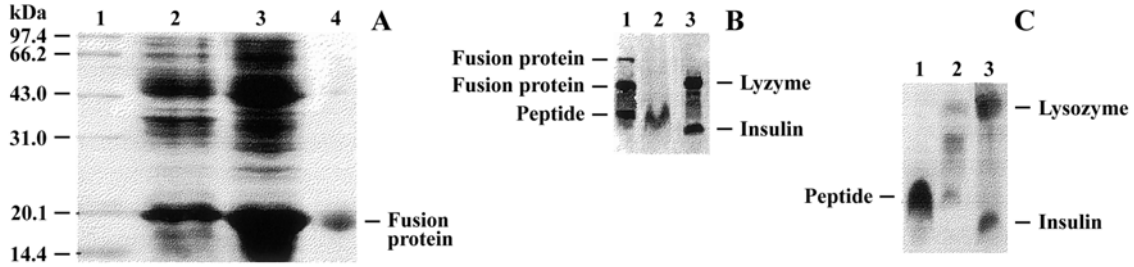

Fig 4. (A) SDS-PAGE analysis (T:C=15:2.66) of the fusion protein in purifying procedure. Lane 1: standard protein markers; Lane 2: recombinant bacteria; Lane 3: coarse inclusion body; Lane 4: pure inclusion body precipitated by 75 % ethanol. (B) SDS-PAGE analysis (T:C=20:4.9) of the Pro-Pro-hGHRH(1-44)OH peptide in the purification procedure. Lane 1: acid hydrolysis product; Lane 2: pure Pro-Pro-hGHRH(1-44)OH peptide from Sephadex G-25 column chromatography; Lane 3: protein markers (lysozyme 14400 Da and insulin 5733 Da). (C) SDS-PAGE analysis (T:C=20:4.9) of the Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide in the purification procedure. Lane 1: Pure Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys peptide from Sephadex G-25 column chromatography; Lane 2: acid hydrolysis product; Lane 3: protein markers (lysozyme 14400 Da and insulin 5733 Da).

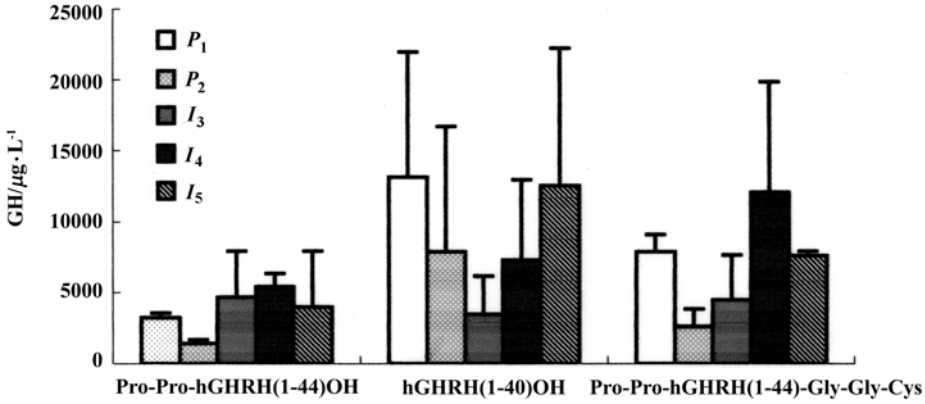

Fig 5. *In vitro* GH release from female fetus pituitaries in different periods by Pro-Pro-hGHRH(1-44)OH, hGHRH(1-44)OH, and Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys. The pituitaries were incubated for a total of 5 h (P_1, P_2, I_3, I_4, and I_5). The buffer was removed each hour for RIA of GH and then fresh buffer was added. After pre-incubation periods (P_1 and P_2) of the first 2 h, in the subsequent three hours (3, 4, and 5 h) the peptide was added alone to the incubation buffer to stimulate the release of GH by the pituitaries (I_3, I_4, and I_5). n=3. Mean±SD.

Tab 1. Effect of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys, Pro-Pro-hGHRH(1-44)OH, and hGHRH(1-40)OH peptides at 5 mg/L on GH release from human fetal pituitary *in vitro*. n=3. Mean±SD. $^bP<0.05$ *vs* Blank.

Peptide	GH concentration/μg·L^{-1}		
	Blank (P_2)	Total GH release (I_3+I_4+I_5)	Net
Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys	2624±1231	24164±5017^b	21540±6720
Pro-Pro-hGHRH (1-44) OH	1417±288	14036±4667	12619±4893
hGHRH (1-40) OH	7868±8861	23301±15094	15433±6573

The pituitaries were incubated for a total of 5 h (P_1, P_2, I_3, I_4, and I_5). The buffer was removed each hour for radio immuno assay of GH and then fresh buffer was added. After pre-incubation periods (P_1 and P_2) of the first 2 h, in the subsequent three hours (3, 4, and 5 h) the peptide was added alone to the incubation buffer to stimulate the release of GH by the pituitaries (I_3, I_4, and I_5).

Tab 2. Effect of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys, Pro-Pro-hGHRH(1-44)OH, and hGHRH(1-40)OH peptides on GH release from rat pituitary *in vitro*. *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* Blank.

Peptide	Concentration/mg·L^{-1}	GH concentration/μg·L^{-1}		
		Blank (P_2)	Total	GH (1-44)OH
Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys	0.01	2.7±0.9	8.5±1.7^{b}	5.8±0.8
	0.1	6.9±3.6	18.1±7.9^{b}	11.1±4.3
	1	4.1±1.8	26.8±3.4^{c}	22.7±1.7
Pro-Pro-hGHRH(1-44)OH	0.01	0.5±0.8	0.12±0.14	0
	0.1	4.3±4.6	12.5±7.2^{b}	8.1±2.7
	1.0	4.1±2.6	16.6±5.8^{b}	12.5±3.2
hGHRH(1-40)OH	≤2	0.33±0.24	<1[1)]	0

[1)] Below the sensitivity of the radioimmunoassay kit.

The pituitaries were incubated for a total of 5 h (P_1, P_2, I_3, I_4, and I_5). The buffer was removed each hour for RIA of GH and then fresh buffer was added. After pre-incubation periods (P_1 and P_2) of the first 2 h, in the subsequent three hours (3, 4, and 5 h) the peptide was added alone to the incubation buffer to stimulate the release of GH by the pituitaries (I_3, I_4, and I_5).

Tab 3. Effect of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys and Pro-Pro-hGHRH(1-44)OH peptides at 5 mg/L on other pituitary hormones release from human fetal pituitary *in vitro*. *n*=3. Mean±SD.

Peptide	Hormone	P_1	P_2	I_3	I_4	I_5
Pro-Pro-hGHRH(1-44)OH	hTSH/mU·L^{-1}	577±663	294±289	183±189	146±128	137±131
	hFSH/U·L^{-1}	950±804	638±552	321±247	250±172	204±150
	hLH/U·L^{-1}	693±424	512±379	269±185	208±116	181±110
	hPRL/μg·L^{-1}	382±443	288±388	165±210	135±159	133±163
Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys	hTSH/mU·L^{-1}	118±73	82±55	47±15	46±15	45±20
	hFSH/U·L^{-1}	213±188	130±86	88±48	71±48	67±51
	hLH/U·L^{-1}	451±395	302±208	155±50	126±67	103±70
	hPRL/μg·L^{-1}	123±49	65±8	44±12	40±7	31±10

The pituitaries were incubated for a total of 5 h (P_1, P_2, I_3, I_4, and I_5). The buffer was removed each hour for RIA of GH and then fresh buffer was added. After pre-incubation periods (P_1 and P_2) of the first 2 h, in the subsequent three hours (3, 4, and 5 h) the peptide was added alone to the incubation buffer to stimulate the release of GH by the pituitaries (I_3, I_4, and I_5).

(1-44)OH 0.01 mg/L and hGHRH(1-40)OH 2 mg/L did not stimulate GH release (P>0.05) (Tab 2).

***In vitro* release of other pituitary hormones** The concentrations of other hormones (hTSH, hFSH, hLH, and hPRL) released from human pituitary decreased with time and were not affected by Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys and Pro-Pro-hGHRH(1-44)OH peptides at 5 mg/L at any time points (Tab 3).

DISCUSSION

At least 29 amino acid residues were essential for functional hGHRH including hGHRH (1-37)OH, hGHRH (1-40)OH, and GHRH (1-44)NH_2. The chemical synthesis of GHRH was too expensive. In this study we successfully prepared Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys in the novel expression system.

In the original design, the Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys should be transformed into hGHRH-Gly-Gly-Cys by a recombinant dipeptidyl aminopeptidase[13].

We observed that Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys peptide had better activity to stimulate GH release from rat pituitary than that of Pro-Pro-hGHRH (1-44)OH and hGHRH (1-40). The GH released by Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys was much more than that of Pro-Pro-hGHRH (1-44)OH at the same concentration. At 0.01 mg/L Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys still had a good activity whereas Pro-Pro-hGHRH (1-44)OH did not. hGHRH (1-40)OH 0.01, 0.05,

0.1, 0.2, 1.0 or 2 mg/L did not show any activity. According to the result from rat pituitary, the activities of the three analogs may be ranked: Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys>Pro-Pro-hGHRH(1-44)OH>hGHRH (1-40)OH. We further observed that GH release from human fetal pituitary was much stronger than that from rat pituitary after three peptides stimulation. It could be partly explained that human GH had 60 % identity with rat GH and the antiserum kit against human GH used in this study was more sensitive to measure hGHRH than rGHRH. Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys and Pro-Pro-hGHRH (1-44)OH did not stimulate other hormones (hTSH, hFSH, hLH, and hPRL**)** release from human pituitary suggesting that the two GHRH analogs had better functional selectivity.

Ling *et al*[7] reported that the activity of hGHRH (1-44)OH was 2.06 times higher than that of hGHRH (1-40)OH, which possibly resulted from the reduction of the positive charge at C-terminus and the change of the molecular conformation. Additionally when the C-terminal carboxyl group of hGHRH analog was amidated, the activity of the amidated analog was almost doubled, which possibly resulted from enhancement of alkalinity and hydrophobicity at C-terminus. Gly-Gly-Cys extension on C-terminal of Pro-Pro-hGHRH (1-44)OH [Pro-Pro-hGHRH (1-44)-Gly-Gly-Cys] was flexible and extrorse so it can possibly change the molecular conformation to expose more active sites to promote GH release. We predicated that the extra active Cys residue which was similar to Ser enhanced the activity of Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys by intensifying the interaction between peptide and the receptor on pituitary, because the four Ser residues in the parental hGHRH (1-44) played a significant role in maintaining the active structure of hGHRH

Although the usefulness of hGHRH(1-44)NH_2 and hGHRH (1-29)NH_2 had been demonstrated, there was still a great demand for more stable analogs which would allow a reduction in the dose and frequency of administration. Examination of enzymatic degradation in serum and homogenate of liver, pituitary, and hypothalamus indicated that hGHRH(1-44) and its shortened fragment were rapidly metabolized to inactive product[14].

The limitations in our study was that only antiserum kit against human GH was used, so we could not detect rGHRH release secreted by cultured pituitary cells and had to use pituitary tissue to release more rGHRH. Species-specific kit should be used in our future study.

In summary, Pro-Pro-hGHRH(1-44)-Gly-Gly-Cys had enhanced bioactivity to stimulate GH release than Pro-Pro-hGHRH (1-44)OH.

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