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Ectopic Co-expression of Growth Hormone Releasing Hormone and Pituitary Adenylate Activating Peptide in Skeletal Muscle Enhance Animal Growth*

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Abstract Growth hormone (GHRH) and pitutary adenylate cyclase activating peptide (PACAP) are the members of the PACAP/Glucagon superfamily, who are related in both sequence and function. Their stimulation of GH secretion and animal growth is concerned. A series of expression plasmid, pIRES1-GHRH-PACAP (P-G-P), pIRES1-GHRH (P-G) and pIRES1-PACAP(P-P), were constructed, extracted and purified, then transfected into CHO cell line with Lipofectamine. The expression was examined by RT-PCR, dot-ELISA and Western blotting. The biological activity of expression products was detected in rats. At 8 h after injection of transfection supernatant, serum IGF-Ⅰ concentrations in P-G-P group were significantly higher than that in other groups($P < 0.05$). PLGA encapsulating plasmid microspheres were prepared and injected intramuscularly into rabbit legs. Growth behavior and IGF-1 level were measured at day 0, 15, 30 and 45 after injection. Greater body weights gain and higher serum IGF-Ⅰ levels were observed in three plasmid microsphere injection groups, compared with control group. At day 30, the body weight gain in P-G-P group was greater than saline group (81%, $P < 0.01$), P-G microsphere group (15%, $P < 0.05$) and P-P microsphere group (7%, $P > 0.05$), serum IGF-Ⅰ concentration in P-G-P microsphere group showed a 16.68% increase to P-G microsphere ($P > 0.05$), a 17.14% increase to P-P microsphere($P > 0.05$) and a 50.46% increase to control ($P < 0.05$). These results suggest that co-expression of GHRH and PACAP in one expression plasmid might exert an additive stimulation of GH secretion and growth when delivered into rabbit skeletal muscle with PLGA microsphere. The results may provide a new approach to regulate animal growth.

Key words GHRH, PACAP, PLGA microspheres, animal growth
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Growth hormone releasing hormone (GHRH) and pituitary adenylate cyclase-activating peptide (PACAP) are members of the PACAP/Glucagon superfamily. They are related in both sequence and function [1]. In sockeye salmon and several other vertebrates, both hormones are involved in growth hormone release from the pituitary [1, 2]. GHRH and PACAP are encoded by the same gene in the invertebrate, but by separate genes in mammals, GHRH is encoded alone and PACAP is coded together with PRP. The structures of the GHRH/PACAP and PRP/PACAP genes are strikingly similar, thus the mammalian PRP/PACAP gene probably arose by duplication of an ancestral GHRH/PACAP gene.

Growth hormone (GH) produced by somatotrophs of the adenohypophysis is the anabolic hormone crucial for long bone growth, muscle accretion, energy homeostasis and the metabolism of proteins, sugars, fats and minerals in mammals. A series of stimulatory and inhibitory releasing hormones of hypothalamic and peripheral origins controls the pulsatile release of GH from somatotrophs. GHRH is an earlier discovered positive regulator to GH secretion. PACAP is a potent

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GH secretagogue, and has been proposed as the ancestral releasing factor for GH [3]. PACAP27 and PACAP38 stimulate GH release *in vitro* from bovine and porcine and chicken somatotrophs[4~6], and *in vivo* in cattle [7]. The relationship between the two neuropeptides on GH secretion stimulation has not been clearly described. Since GHRH and PACAP have their own individual receptors in pituitary cells, we propose that GHRH and PACAP may function simultaneously on pituitary cells and stimulate GH secretion additively, while exogenous GHRH and PACAP are provided.

We have delivered GHRH (1～32) expression plasmid into animal skeletal muscle with PLGA microsphere and GHRH gene was well expressed in our former research [8]. In the present study, GHRH (1～32) and PACAP (1～38) are cloned into one expression vector, then expressed both *in vitro* and *in vivo* to see if GHRH and PACAP had an additive function in stimulation of GH secretion and animal growth.

1 Materials and methods

1.1 Animals

Wistar rats (male, 180～220 g), New Zealand White rabbits (male, 0.8～1.25 kg) were provided by experimental animal center of Jilin University. All animals care and performance were strictly following the institutional Guide for the Care and Use of Experimental Animals.

1.2 Construction of expression vectors: pIRES1-GHRH (P-G), pIRES1-PACAP (P-P) and pIRES1-GHRH-PACAP(P-G-P)

The cloning process involved standard molecular cloning methods. Briefly, GHRH (1～32) with signal peptide sequence was amplified by PCR from pcDNA3-GHRH (constructed in our group), the forward primer was CTTGTGGGTCTCGTCGTA-CTTAAGGC (with *Eco*R Ⅰ site) and the reverse primer was CGGGATCCTTATTACCCCTGCTGCC-TGCTGAG (with *Bam*H Ⅰ site). PCR was carried out according to established procedures (30 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s). Resulting PCR products were purified on agarose gel elecphoreses with Agarose gel DNA fragments recovery kit (TAKARA), then digested with *Eco*R Ⅰ and *Bam*H Ⅰ (2U) at 37℃ for 1 h, and subcloned into plasmid pIRES1 by the process of ligation, transformation and positive clone selection. Thus P-G was successfully constructed. PACAP (1～38) gene was chemically synthesized (finished by TAKARA) according to published cDNA sequence with the addition of the same signal peptide sequence as that of GHRH. *Xba* Ⅰ digestion site was designed and added to the 5′ upstream end and *Sma* Ⅰ site to the 3′ end of PACAP DNA, respectively. The PACPA cDNA expression plasmid (P-P) was constructed by replacing *neo* gene in plasmid pIRES1 after digestion of synthesized fragment and pIRES1 plasmid with *Xba* Ⅰ and *Sma* Ⅰ, followed by ligation, transformation and positive clone selection.The same strategy was used to construct expression plasmid pIRES1-GHRH-PACAP (P-G-P) by inserting PACAP DNA into pIRES1-GHRH to replace *neo* gene. Three kinds of constructed plasmid were finally identified by DNA sequencing.

1.3 Expression of GHRH and PACAP DNA in CHO cell line

1.3.1 Transfection. P-G, P-P and P-G-P plasmids were extracted and purified with Plasmid Extraction Kit (TAKARA) according to protocol provided by manufacturer for transfection purpose, then quantified with DNA GeneQuant (Pharmacia Biotech). CHO cells (preserved in our laboratory) seeded in 5 ml culture bottle were cultivated in 1640 medium with 10% bovine fetal serum at 37℃/5% CO_2, then splited in six-well, tissue-culture grade plates and maintained at 37℃/5%CO_2 until 60%～70% confluency in their respective growth media. 0.1 μg (2 μl) each plasmid DNA and then 25 μl 1640 medium were added into clean Eppendorf, 4 μl Lipofectamine (GibcoBRL) was mixed with 20 μl 1640 medium and then transferred into plasmid DNA containing Eppendorf, place the Eppendorf at 37℃/5% CO_2 for 30 min. Each plasmid preparation was in triplicate. Cells were then washed with PBS (pH 7.4) and the medium replaced with 2 ml 1640 medium without addition of fetal bovine serum. Mixture of plasmid and Lipofectamine prepared above were pipetted into plate well. Cultivate at 37℃/5% CO_2 for 24 h, followed by replacing the medium with 2 ml 1640 with 10% fetal bovine serum and cultivate for another 24 h. Collect the supernatant and stored at –20℃ for further analysis. Cells were used for RNA extraction.

1.3.2 RT-PCR detection of expression. Total RNA was extracted by adding 1ml Trizol reagent (Promega) to plate well and following the procedure provided by manufacturer. The extracted total RNA samples were digested by RNAase free DNAase. Total RNA was

evaluated in formaldehyde denaturation agrose gel electrophoresis. Reverse transcription was performed by using the total RNA as the template, and Oligo dT_{12} as the primer. Incubate each RNA (10 μl) sample at 70℃ for 5 min with 1 μl (20 μmol/L) Oligo dT_{18} primers. The samples were then cooled on ice for 2 min and centrifuged briefly. To the mRNA/primer solution the following was added: 5× MMLV buffer, 4 μl, RNasin 0.4 μl (40U, Promega), dNTP 1 μl, MMLV 1 μl(8U, Promega). Add RNase free water to total volume of 20 μl, the reagents were mixed and incubated at 37℃ for 1.5 h. The reverse transcriptase enzyme was then heat inactivated at 95℃ for 10 min. A negative reverse transcriptase reaction was performed according to the above directions. However, no RNA template was added. This negative reaction was carried through to the polymerase chain reaction and used as a negative control. The cDNA was used in the polymerase chain reaction. Primers for GHRH were described in **2.2**; primers for PACAP were GCCCCGGGATGACCATGTGTAGCGGAGCGAG-GCTGG and CGTCTAGATTATTTGTTTTTAA-CCCTTTGTTTATAC. The conditions for the PCR were as follows, 2.5 μl 10×PCR buffer, 0.2 mmol/L dNTPs, 0.4 mmol/L each primer , 2.5 U Taq polymerase (Gibco BRL), 6 μl reverse transcriptase cDNA product and 12 μl sterile filtered water. The PCR reactions were run for 30 cycles of 95℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min, on a DNA thermal cycler (PE 9600). Following the completion of the reaction, 10 μl PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide for visualization. Five PCR reactions were designed for GHRH detection of P-G and P-G-P transfection, PACAP detection of P-P and P-G-P transfection, and negative control.

1.3.3 Detection of expressed GHRH and PACAP with Dot-ELISA and Western blotting. The collected supernatants of transfected CHO cells were concentrated 10-fold with vacuum concentrator. 1 μl of each concentrated culture supernatant was dotted on a strip of nitrocellulose membrane (0.2 μm, Amersham) with pipette. 1 μl (0.1 μg) hGHRH(1～29)(Sigma) and 1 μl(0.1 μg) hPACAP(1～38)(Sigma) were dotted as positive control, and CHO culture supernatant without any plasmids transfection was used for negative control. The strip was dried at room temperature, blocked for 1 h at 37℃ with 5% defatted milk in PBS, washed 3 times with PBS and incubated with antibody in block buffer (goat anti-human GHRH (1 ～29) for GHRH detection, rabbit anti-human PACAP for PACAP detection, Sigma. 1∶1 000 diluted with PBS) for 1 h at 37℃ in Hybridizer (HB-1000, Gene Company). After washing three times with PBST (PBS plus Tween 20, 0.05% final concentration) for 20 min, the strips were incubated with the second antibody in block buffer (horse anti-goat horseradish peroxidase-conjugate for GHRH detection, goat anti-rabbit horseradish peroxidase-conjugate for PACAP detection, Sigma. 1∶1 000 dilution) for 1 h at 25℃ in Hybridizer (HB-1000, Gene Company). Following 30 min washing 3 times with PBST, the reaction was developed with 3, 3′-diaminobenzidine dihydrochloride and hydrogen peroxide in PBS and then stopped by washing in tap water. The concentrated culture supernatants were also subjected to Western blotting as a further check for the presence of the GHRH and PACAP band. Firstly the concentrated culture supernatants were separated with SDS-PAGE (15%), then transferred electrophoretically to nitrocellulose membrane, followed with immune detection described as dot-ELISA. The primary and secondary antibodies used for developing the nitrocellulose blot were the same as described in the dot-ELISA.

1.3.4 Biological activity assay of GHRH (1～32) and PACAP expressed in CHO cells using rats *in vivo*. Twenty four health Wistar rats (male, 180～220 g,) were grouped at random after 3 days regular feeding. Each group received one of the following treatments on the experiment day: (1) group Ⅰ, injection of culture supernatant transfected with P-G; (2) group Ⅱ, injection of culture supernatant transfected with P-P; (3) group Ⅲ, injection of culture supernatant transfected with P-G-P; (4) group Ⅳ, injection of normal culture media. All injections were muscular in leg. Blood was sampled by tail tip removal under ethyl ether anaesthesia at 0, 2, 4 and 8 h post-injection. Serum sample was collected and stored at –20℃ until IGF-Ⅰ estimation. Rat plasma IGF-Ⅰ concentrations were measured by radioimmunoassay (RIA) with rat IGF-Ⅰ RIA kit (Diagnostic Systems Laboratories, Inc.) in triplet (CV<10%).

1.4 Effects of skeletal muscle expression of PLGA encapsulating P-G, P-P and P-G-P on rabbit weight gain and IGF-Ⅰ levels

1.4.1 Preparation of PLGA microspheres encapsulating plasmids. The plasmid was extracted with standard

alkaline denaturation method and quantified with GeneQuant (Pharmacia Biotech). PLGA microspheres encapsulating plasmids were prepared by double emulsion-in liquid evaporation process[9, 10]. 1 mg plasmid dissolved in 2 ml of water was as the inner phase, and 10 mg PLGA was dissolved in 10 ml methanyl dichloride and acetone as the oil phase. The mixture was sonicated at 40℃ for 50 s (15 W). The entire mixture was then cooled to 15℃ in an icebath after which 4% PVA was added and the entire mixture was sonicated for an additional 150 s (15 W), then quickly transferred to a beaker containing 10 ml 0.4% PVA. The mixture was subsequently stirred at room temperature for 18 h to evaporate the organic solvent and stabilize the microspheres and the microshperes were then separated by centrifugation (12 000 r/min for 10 mins). Blank microspheres were prepared using the same method but the plasmid was replaced with water.

1.4.2 Animal treatments.

Rabbits (New Zealand White rabbits, male, 0.8～1.25 kg) were divided into four groups, 8 in each group, followed by different injections in tibialis muscle: (1) group Ⅰ, blank PLGA microspheres injection, 1 ml, 0 mg plasmids/ml; (2) group Ⅱ, P-G PLGA microspheres injection, 1 ml, 0.5 mg plasmids/ml; (3) group Ⅲ, P-P PLGA microspheres injection, 1 ml, 0.5 mg plasmids/ml; (4) group Ⅳ, P-G-P PLGA microspheres injection, 1 ml, 0.5 mg plasmids/ml .

Body weight was measured and recorded at day 0 (just before injection), 15, 30 and 45 post-injection, and at the same time blood samples were collected and serum was extracted. IGF-Ⅰ concentrations were measured with a rat RIA kit (Diagnostic Systems Laboratories, Inc.) according to manufacturer's protocol. All serum analyses were conducted in triplicate.

1.5 Statistical analysis

The results are presented as $\bar{x} \pm s$. All data were analyzed with Duncan's multiple range tests using SSPS 10.0 software. P value was for overall comparison. Results were considered significant when $P < 0.05$.

2 Results

2.1 Construction of expression vectors: pIRES1-GHRH(1～32)(P-G),pIRES1-PACAP(1～38)(P-P) and pIRES1-GHRH(1～32)-PACAP(1～38)(P-G-P)

Three expression vectors, P-G, P-P and P-G-P were successfully constructed by the methods described in **2.2**. Results representing vector construction are not shown here in detail, such as the PCR amplification of GHRH (1～32) and PACAP, transformation selection and identification with enzyme digestion, PCR and sequencing.

2.2 Expression of GHRH and PACAP in CHO cell line

After transfection of plasmids, CHO cells were collected and total RNA was extracted. Electrophoresis of RT-PCR products showed(Figure 1) that GHRH (156 bp, including 60 bp signal peptide sequence) was amplified in CHO cells transfected with P-G and P-G-P plasmids; PACAP (174 bp, including 60 bp signal peptide sequence) was observed in samples transfected with P-P and P-G-P plasmids. The results indicate the transfected genes had been successfully transcribed to their respective mRNA.

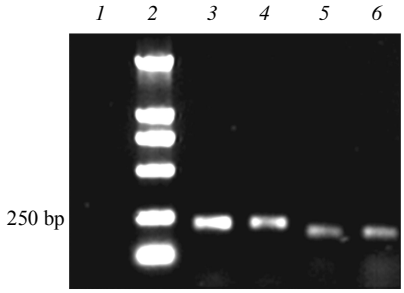

Fig. 1 RT-PCR of total RNA from transfected CHO cells

Lane *1* represents negtive Control with no observed PCR products. Lane *2* is DL2000 marker. Lane *3*, *4* are RT-PCR amplification of PACAP gene, lane *3* is sample from P-P transfection and lane *4* is from P-G-P transfection CHO cells, both of which show specific band for PACAP. Lane *5*, *6* are RT-PCR amplification of GHRH gene, lane *5* represents sample from P-G transfection and lane *6* is from P-G-P transfection CHO cells, both of which exhibit specific band for GHRH.

We measured GRRH and PACAP expression in the transfected CHO cells *via* dot-ELISA and Western blotting. In dot-ELISA examination, immuno-reactive PACAP peptide was successfully detected in culture supernatants from P-P and P-G-P transfected CHO cells; immuno-reactive GHRH peptide was detected in both culture supernatants of P-G and P-G-P transfected CHO cells (Figure 2). Western blotting shows rational molecular mass of expressed peptides (Figure 3).

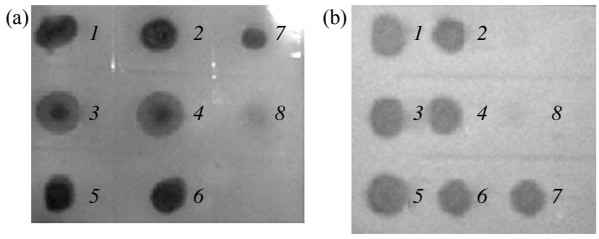

Fig. 2　Dot-ELISA of CHO expressed GHRH and PACAP

(a) GHRH detection, dot *1*～*3* are for culture supernatants of P-G transfected CHO cells and dot *4*～*6* are for P-G-P transfected CHO cells, all of which show positive results, dot *7* is positive and dot *8* is negtive control. (b) PACAP detection, dot *1*～*3* are for culture supernatants of P-P transfected CHO cells and dot *4*～*6* are for P-G-P transfected CHO cells, all of which show positive results, dot *7* is positive and dot *8* is negative control.

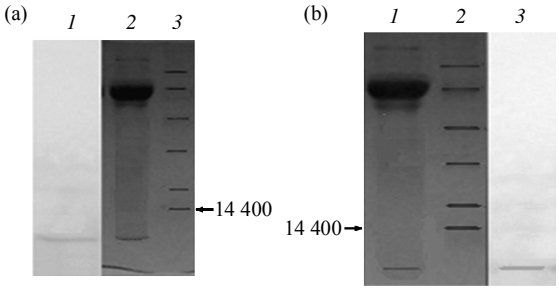

Fig. 3　Expressed GHRH and PACPA detection with Western blotting in CHO cells

(a) is results for GHRH and (b) for PACAP. Lane *3* in (a) and lane *2* in (b) represent protein markers. Lane *2* in (a) represents CHO culture supernatant transfected with P-G plasmid separated by SDS-PAGE, lane *1* is immuno-blotting with GHRH antibody. Lane *1* in (b) represents CHO culture supernatant transfected with P-P plasmid separated by SDS-PAGE, lane *1* is immuno-blotting with PACAP antibody.

To measure the biological activity of expressed peptides, culture supernatants of P-G, P-P and P-G-P transfected CHO cells were injected muscularly into rats and serum IGF-Ⅰ levels were monitored to indicate change of GH secretion. The variations of serum IGF-Ⅰ levels after infusion of different culture supernatants are shown in Figure 4. Rises of IGF-Ⅰ level, as the IGF-Ⅰ secretary response to culture supernatants injection, were observed in 3 groups injected with culture supernatants of plasmid transfection CHO cells through the experiment period. But no significant increase of IGF-Ⅰ concentration ($P > 0.05$) was found before 4 h post-injection relative to control group. At 8 h, significant increase of the IGF-Ⅰ level was observed in the group of P-G-P transfection GHO cells supernatant, higher than control group, P-G group and P-P group by 37.52%, 23.25% and 22.46%, respectively ($P < 0.05$ for all individual comparisons). Even though IGF-Ⅰ level in both P-G group and P-P group was higher than that in control group at 8 h post-injection, no significant difference emerged ($P > 0.05$) yet. It seemed clear that GHRH and PACAP might exert stronger stimulation of GH secretion, leading to IGF-Ⅰ level increase, than GHRH and PACAP alone in rats.

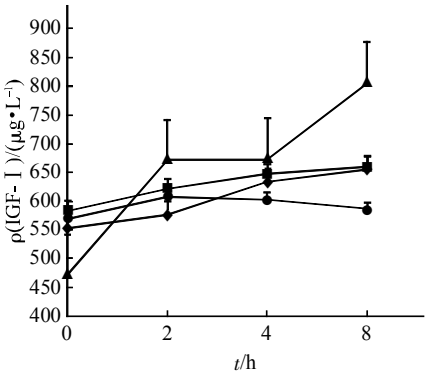

Fig. 4　Rats serum IGF-Ⅰ levels before and post-injection of culture supernatants of CHO cells transfected with different plasmids

The datum of each point was from average of six rats in the same group. IGF-Ⅰ level of one serum sample was the average of three data ($CV < 10\%$), since it was detected in triplet. Vertical bars represent the *s*; when the *s* is small, the bar is masked by the symbol. Supernatants of P-G-P transfection CHO, containing expressed GHRH and PACAP bolus, exhibit more robust IGF-Ⅰ levels than the other groups. ♦—♦: P-G; ■—■ : P-P; ▲—▲: P-G-P; ●—●: Control.

2.3　Effects of skeletal muscle expression of PLGA encapsulating P-G, P-P and P-G-P on rabbit growth and IGF-Ⅰ levels

Intramuscular injection of GHRH expression plasmid led to ectopic GHRH expression in skeletal muscle, thus causing stimulation of native GH synthesis and release and greater weight gain in tested animals. We have tried a method of PLGA microspheres encapsulating GHRH expression plasmid (pcDNA3-GHRH) to enhance GHRH expression[8]. In this study, PLGA microspheres encapsulating P-P, P-G and P-G-P were prepared, as described in **2.4.1**. The average diameter of PLGA microspheres was 2.8 μm, 1 mg PLGA microspheres loaded 7.9 μg plasmids. Then PLGA microspheres were injected intramuscularly into rabbits to observe their effects on weight gain and IGF-Ⅰ levels.

Rabbits weight gain post-injection in 4 groups are shown in Figure 5. Weight gain per day in each of 3 groups injected with PLGA microspheres encapsulating P-G, P-P or P-G-P plasmids was significantly greater than control group from 0 to 15 days post-injection, higher by 62.56% in P-G-P group ($P < 0.05$), 61.43% in P-P group ($P < 0.05$) and 52.92% in P-G($P < 0.05$), respectively. Interestingly, from 15 to 30 days post-injection, three plasmid injection groups had even greater weight gain per day than control group, respectively. During this period, weight gain per day increased by 120.23% relative to control in P-G-P group ($P < 0.05$), by 84.70% in P-P group ($P < 0.05$) and by 72.61% in P-G group ($P < 0.05$). In the last 15 days, significant difference was not observed between control and each of plasmid injection groups($P > 0.05$). Regarding comparison between P-G-P group and P-G group or P-P group, significant difference was not found through the test period, though weight gain per day in P-G-P group was greater than P-P group (19.34%, $P > 0.05$) or P-G group(27.58%, $P > 0.05$) in 15～30 days period. It is clear to note that injection of P-G-P plasmids encapsulated with PLGA microspheres achieved greatest weight gain in rabbits.

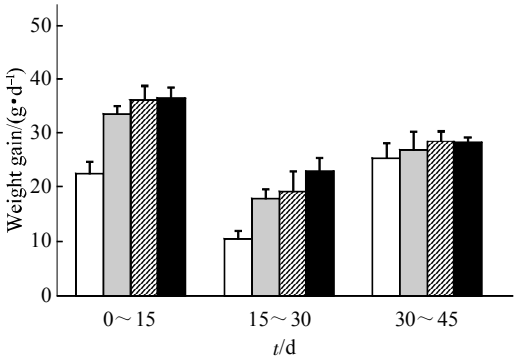

Fig. 5 Rabbit weight gain per day in deferent test periods

Mean values for 8 rabbits in the same group are shown. Vertical bars represent the s. Three PLGA microspheres encapsulating plasmids injection groups show significant greater weight gain per day than control group during 0 ～15 and 15 ～30 days post injection, respectively. Compared with P-G or P-P injection group, P-G-P injection group gained non-significantly greater weight per day through the test period. □: Control; ▨: P-G; ▨: P-P; ■: P-G-P.

The serum IGF-Ⅰ levels from different treated groups at day 0, 15, 30 and 45 post injection are shown in Figure 6. No differences were observed in IGF-Ⅰ levels between groups prior to injection. An increase of serum IGF-Ⅰ concentration at day 15 post-injection was observed mainly in P-G-P group compared with other groups ($P > 0.05$). Importantly, at day 30 post-injection, a significant increase of serum IGF-Ⅰ level in P-G-P group was observed relative to control group (75.2%, $P < 0.05$), P-G group(9.24%, $P < 0.05$) and P-P group(13.89%, $P < 0.05$). At day 45 post-injection, no significant difference was apparent among groups. The results show that at day 15 and 30 post-injection, serum IGF-Ⅰ levels in P-G-P group maintained the highest among 4 groups. These findings are consistent with the increase in weight gain per day.

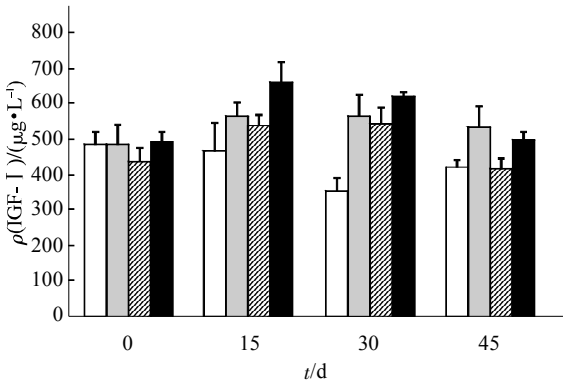

Fig. 6 Rabbits serum IGF-Ⅰ levels at day 0, 15, 30 and 45 post-injection

The datum of each point was from average of 8 rabbits in the same group. IGF-Ⅰ level of one serum sample was the average of three data (CV<10%), since it was detected in triplet. Vertical bars represent the s; when the s is small, the bar is masked by the symbol. At day 15 and 30 post-injection, serum IGF-Ⅰ levels in P-G-P group maintained the highest among 4 groups. □: Control; ▨: P-G; ▨: P-P; ■: P-G-P.

3 Discussion

The pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone releasing hormone (GHRH) belong to the PACAP/glucagon superfamily, which includes nine hormones in humans that are related by structure, distribution, function and receptors. The origin of the ancestral superfamily members is at least as old as the invertebrates. PACAP was found in 1989, its sequence conservation is shown by the complete identity of the amino acid sequence for mammalian PACAP peptides. The chicken and frog forms of PACAP each have only one amino acid change in comparison with the mammalian form, whereas fish PACAP peptides have three or four changes (89% ～92% amino acid identity) with mammalian PACAP. In 1984 the hypothalamic form of the GHRH peptide was sequenced and reported to

be 44 amino acids[11]. Both peptides are encoded in the same gene, and it is supposed that a separate GHRH gene evolved by gene duplication only about 250 million years ago.

It has been well known that the major function of GHRH, released from neurosecretory terminals in the median eminence, is to stimulate the release and synthesis of GH. PACAP is a potent GH secretagogue, and has been proposed as the ancestral releasing factor for GH [3]. PACAP27 and PACAP38 stimulate GH release *in vitro* from bovine and porcine and chicken somatotrophs[4~6], and *in vivo* in cattle[7]. GHRH and PACAP act on different receptors in pituitary cells.

To deliver an expression plasmid containing GHRH gene to animal is effective approach to increase animal GH concentration for certain purpose, i.e. to improve animal growth. Draghia-Akli R, *et al.*[12] reported that injection of psk-GHRH to mouse muscle tissues resulted in GH secretion and enhanced growth. In our previous research, it is proved that injection of pcDNA3-GHRH could stimulated GH secretion in rabbit after intramuscular injection[13, 14]. The similar results have been reported in the pig[15] and sheep[16].

Even though Wolff *et al.*[17] found that gene expression in skeletal muscle could be achieved by a simple intramuscular injection of naked plasmid DNA, yet this method has not been practically used because of its low expression level. Encapsulation techniques, involved in our research, can be used to protect sensitive bioagents, such as plasmid DNA, from degradation. PLGA, which is a biocompatible and biodegradable polymer, has been used for the encapsulation of proteins and peptides[18], and for genes[9, 10]. Much more attention has been drawn to study PLGA nanoparticles or microparticles as a new non-viral gene delivery system due to its controlled release characteristics and protection of DNA from degradation. It has been reported that PLGA could protect plasmid DNA from digestion by DNase Ⅰ *in vitro*[19], and Cohen *et al.*[20] has reported that plasmid DNA encapsulated in PLGA nanoparticles achieved higher AP (a reporter gene) expression level both *in vitro* and *in vivo* compared with cationic-liposome delivery.

PACAP is a multifunctional peptide, but only stimulation of GH secretion is concerned in the present research. In mammals the primary releaser of GH is GHRH, but PACAP also appears to have a role in GH release. PACAP (10^{-11} to 10^{-8} mol/L) increases Ca^{2+} concentrations in rat anterior pituitary cell cultures in a dose-dependent manner, and some of the cells experiencing a rise in Ca^{2+} concentration have been identified as somatotropes[21]. PACAP also stimulates cAMP accumulation in rat static pituitary cell cultures[22]. Several researchers have shown that PACAP induces GH release and synthesis[21,23~25]. The proposed mechanism is that PACAP increases Ca^{2+} levels *via* a cAMP/PKA-mediated pathway[26] and that the release of GH by PACAP does not require PKC activation. This points to the involvement of cAMP in the process of GH release and not IP[21]. Also, it has been suggested that PACAP activates a sodium channel *via* an AC/PKA pathway[26]. The result is membrane depolarization and, in turn, calcium channel activation that triggers the increase in cytosolic calcium necessary for GH release. In addition to stimulating GH release, PACAP induces an increase in rat GH gene transcription in static cell culture[23]. Although the actions of PACAP on GH release in mammals have not been fully elucidated, PACAP functions have been studied across vertebrates and an evolutionary perspective is clear. Therefore, it appears that, at least in some nonmammals, PACAP is a hypophysiotropic releaser of GH.

Our results showed that infusion of expressed PACAP in transfected CHO cells caused a non-significant IGF-Ⅰ level increase in rats, which is similar with expressed GHRH in transfected CHO cells. It proves that expressed PACAP may stimulate GH secretion in rats, indicated by serum IGF-Ⅰ level, though the expressed PACAP in CHO cells was not quantitively determined. Intramuscularly injection of PLGA encapsulating PACAP expression plasmid into rabbits led to a significant increase of body weight gain per day (61.32%, $P < 0.05$) compared with control from 0 to 15 days after injection, which indicates that expressed PACAP in rabbit skeletal muscular was able to stimulate rabbit growth.

The relationship between GHRH and PACAP in stimulation of GH secretion has not been clearly elucidated before, especially in one mammalian species. Since PACAP and GHRH have individual receptor in pituitary cell of their own[24], it is reasonable to calculate they have an additive function in stimulating GH secretion. In our study, co-injection of PACAP and GHRH expressed by transfected CHO cells exerted more robust stimulation to increase rat serum IGF-Ⅰ level than individual PACAP and GHRH. In rabbits,

intramuscularly injection of PLGA encapsulating expression plasmid co-expressing GHRH and PACAP had the greatest body weight gain per day among tested groups in 0～15 and 15～30 days after injection, which supposes that expressed GHRH and PACAP had at least an additive function in stimulation of rabbit growth. As observed in rabbit serum IGF-Ⅰ level curve after injection, co-expression of PACAP and GHRH also led to a higher IGF-Ⅰ level than the single PACAP or GHRH expression, which may be an evidence for that GHRH and PACAP had an additive function in stimulation of GH secretion.

IGF-Ⅰ is a down-stream molecule involved in the growth regulation axis (GRF-GH-IGF axis) with stable serum concentration, regulated by GH. We evaluated IGF-Ⅰ levels, rather than strict GRF or GH levels, in this study to investigate the downstream physiological functions of expressing GHRH and PACAP in muscle fibers.

In conclusion, our study has shown that PACAP and GHRH may have an additive function in stimulation of GH secretion for rat and rabbit growth; muscular expression of foreign PACAP can exert a stimulation of rabbit growth.

References

1 Sherwood N M, Krueckl S L, McRory J E. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocrine Reviews, 2000, **21**(6): 619～670

2 Parker D B, Power M E, Swanson P, *et al*. Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide (PACAP) in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology, 1997, **138**(1): 414～423

3 Sherwood N M, Krueckl S L, McRory J E. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr Rev, 2000, **21**(6): 619～670

4 Hashizume T, Soliman E B, Kanematsu S. Effects of pituitary adenylate cyclase-activating polypeptide (PACAP), prostaglandin E2 (PGE2) and growth hormone releasing factor (GHRH) on the release of growth hormone from cultured bovine anterior pituitary cells *in vitro*. Domest Anim Endocrinol, 1994, **11**(4): 331～337

5 Martinez-Fuentes A J, Malagon M M, Castano J P, *et al*. Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 and PACAP27 differentially stimulate growth hormone release and mRNA accumulation in porcine somatotropes, Life Sci, 1998, **62**(26): 2379～2390

6 Peeters K, Langouche L, Vandesande F, *et al*. Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on cAMP formation and growth hormone release from chicken anterior pituitary cells. Ann N Y Acad Sci, 1998, **865**: 471～474

7 Radcliff R P, Lookingland K J, Chapin L T, *et al*. Pituitary adenylate cyclase-activating polypeptide induces secretion of growth hormone in cattle. Domest Anim Endocrinol, 2001, **21**(3): 187～196

8 Ren X H, Zhang Y L, Liu S C, *et al*. The expression of growth hormone release factor gene carried with PLGA microspheres in muscle tissue and its effect on mouse growth. Chin J Biotechnology, 2004, **20**(4): 616～619

9 Luo D, Woodrow-Mμmford K, Belcheva N, *et al*. Controlled DNA delivery systems. Pharm Res, 1999, **16**(8): 1300～1308

10 Jeong J H, Park T G. Poly(L-lysine)-g-poly(D, L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J Control Release, 2002, **82**(1):159～166

11 Ling N, Esch F, Böhlen P, *et al*. Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor. Proc Natl Acad Sci USA, 1984, **81**(14): 4302～4306

12 Draghia-Akli R, Li X, Schwartz R J. Enhanced growth by ectopic expression of growth hormone releasing hormone using an injectable myogenic vector. Nat Biotechnol, 1997, **15**(12): 1285～1289

13 Zhang Y L, Lian J Q, Liu S C, *et al*. Effects of growth hormone-releasing factor expressed in rabbit skeletal muscle on rabbit growth. Chin J Vet Sci, 2003, **23**(1): 56～58

14 Zhang Y L, Zhang Y J, Ouyang H S. Expression of growth hormone-releasing factor gene in animal skeletal muscle. Chin J Vet Sci, 2000, **20**(4): 360～364

15 Draghia-Akli R, Ellis K M, Hill L A, *et al*. High-efficiency growth hormone releasing hormone plasmid vector administration into skeletal muscle mediated by electroporation in pigs. FASEB J, 2003, **17**(3): 526～528

16 Meng Q Y, Chen Z Q, Yu Z Q, *et al*. Increased body weight *via* injecting myogenic expression growth hormone-releasing hormone (GHRH) plasmid DNA into sheep. Anim Biotech, 2004, **15**(2): 175～192

17 Wolff J A, Malone R W, Williams P, *et al*. Direct gene transfer into mouse muscle *in vivo*. Science, 1990, **247**(4949): 1465～1468

18 Dhiman N, Dutta M, Khuller G K. Poly (DL-lactide-co-glycolide) based delivery systems for vaccines and drugs. Indian J Exp Biol, 2000, **38**(8): 746～752

19 Han S O, Mathato R I, Sung Y K, *et al*. Development of biomaterials for gene therapy. Mol Ther, 2000, **2**(4): 302～317

20 Cohen H, Levy R J, Gao J, *et al*. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Ther, 2000, **7**(22): 1896～1905

21 Yada T, Vigh S, Arimura A. Pituitary adenylate cyclase activating polypeptide (PACAP) increases cytosolic-free calcium concentration in folliculo-stellate cells and somatotropes of rat pituitary. Peptides, 1993, **14**(2): 235～239

22 Wei L, Chan W W S, Butler B, *et al*. Pituitary adenylate cyclase activating polypeptide - induced desensitization on growth hormone release from rat primary pituitary cells. Biochem Biophys Res

Commun, 1993, **197**(3): 1396～1401

23 Velkeniers B, Zheng L, Kazemzadeh M, *et al*. Effect of pituitary adenylate cyclase activating polypeptide 38 on growth hormone and prolactin expression. J Endocrinol, 1994, **143**(1): 1～11

24 Goth M I, Lyons C E, Canny B J, *et al*. Pituitary adenylate cyclase activating polypeptide, growth hormone (GH) releasing peptide and GH-releasing hormone stimulate GH release through distinct pituitary receptors. Endocrinology, 1992, **130**(2): 939～944

25 Yamauchi K, Murakami Y, Koshimura K, *et al*. Involvement of pituitary adenylate cyclase-activating polypeptide in growth hormone secretion induced by serotoninergic mechansims in the rat. Endocrinology, 1996, **137**(5): 1693～1697

26 Miyake T, Koshimura K, Murakami Y, *et al*. Involvement of tetrodotoxin-sensitive Na^+ channel in PACAP-induced increase in intracellular Ca^{2+} concentration in GH_3 cells. Biomed Res, 1996, **17**(2):191～196

共表达生长激素释放激素(GHRH)与垂体腺苷酸环化酶(PACAP)对动物生长的影响 *

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摘要　生长激素释放激素(GHRH)与垂体腺苷酸环化酶(PACAP)在序列及功能方面均相似，且同为 PACAP/ 胰高血糖素超家族成员．研究了这二者对生长激素释放的刺激作用，以及对动物生长的影响．构建了 3 个表达载体，pIRES1- GHRH-PACAP (P-G-P)，pIRES1-GHRH(P-G) 及 pIRES1-PACAP(P-P)，并转染到 CHO 细胞中，进行 RT-PCR，Dot-ELISA 以及 Westen-blot 检测．此外，给大鼠注射细胞上清表达产物，检测其生物学活性．注射 8 h 后，注射表达 P-G-P 上清的大鼠血清中 IGF-Ⅰ浓度显著高于其他组($P < 0.05$)．用 PLGA 微球包裹各种质粒，并注射到家兔后肢胫前肌．观察家兔生长情况，并于注射后 0，15，30，45 天时分别采集家兔血液，检测血液中 IGF-Ⅰ浓度．结果显示，三质粒注射组动物体重变化及血液中 IGF-Ⅰ浓度均高于对照组．注射后 30 天时，P-G-P 组增重较对照组提高 81% ($P < 0.01$)，P-G 组比对照组提高 15%($P > 0.05$)，P-P 组比对照组高 7%($P > 0.05$)．另一方面，P-G-P 组动物血液中 IGF-Ⅰ含量比分别比 P-G、P-P 及对照组提高 16.68% ($P > 0.05$)，17.14%($P > 0.05$)，50.46%($P < 0.05$)．以上结果揭示：给动物注射 PLGA 微球包裹的共表达 GHRH 与 PACAP 质粒，可以增强动物体内生长激素(GH)的分泌，并促进动物生长．通过上述研究发现，肌肉注射 PACAP 表达质粒可以促进家兔的生长，PACAP 和 GHRH 共表达可以起到协同作用．这可能为动物的促生长研究提供新的方法．

关键词　生长激素释放激素, 垂体腺苷酸环化酶, PLGA 微球, 动物生长

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