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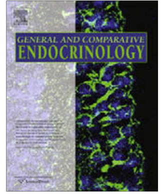

Gene structure, recombinant expression and functional characterization of grass carp leptin

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ABSTRACT

Leptin is an important hormone for the regulation of food intake, energy expenditure and reproduction in mammals, but information regarding its role in teleosts remains scant. In the present study, the gene structures of grass carp (*Ctenopharyngodon idellus*) and silver carp (*Hypophthalmichthys molitrix*) leptins were characterized. Recombinant grass carp leptin (rgc-LEP) was expressed in *Escherichia coli* and purified, and identified by mass spectrometric analysis. A strong anorexic effect on food intake was observed in grass carp on the first day after intraperitoneal (IP) injection of rgc-LEP, but not during the following days. Body weight of the leptin group (LEP group) and the pair-fed group (PF group) showed no difference throughout the experimental period. The acute and chronic effects on the expression of key genes correlating to food intake, energy expenditure, lipid metabolism and digestion were further characterized by real-time PCR. Accordingly, the mRNA levels of neuropeptide Y (NPY), Stearoyl-CoA desaturase 1 (SCD1) and lipoprotein lipase (LPL) were significantly reduced whereas the mRNA levels of uncoupling protein 2 (UCP2), bile salt-activated lipase (BSAL) and fatty acid elongase (ELO) were significantly elevated on the first day after injection. No effect on the expression of these genes (except LPL) was observed on day 13. In contrast to the down-regulation by exogenous leptin in mammals, the mRNA level of grass carp leptin was elevated 5.76-fold on the first day after rgc-LEP treatment. Our results suggest that leptin has an acute effect on the regulation of food intake, energy expenditure and lipid metabolism in grass carp, but the effect can be rapidly counteracted through mechanisms that are currently unknown.

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1. Introduction

Leptin, the protein product of the *obese* gene, is a type-I cytokine secreted primarily by adipocytes in mammals, which plays a key role in the regulation of food intake, energy expenditure and reproduction (Magni et al., 2000; Zhang et al., 1994). One of the most important roles of leptin is its involvement in the regulation of energy balance by decreasing food intake and increasing energy expenditure. Leptin influences the various hypothalamic orexigenic (such as NPY) and anorexigenic (such as proopiomelanocortin, POMC) neuropeptides to regulate food intake (Schwartz et al., 2000). It regulates energy balance by specific regulation of some enzymes (such as Stearoyl-CoA desaturase 1, SCD1), to increase fatty acid oxidation and reduce fatty acid synthesis in mammals (Cohen et al., 2002).

Since the discovery of leptin in mouse by Zhang et al. (1994), a good deal of work had been done on leptin orthologs in non-mammalian species with limited success before 2005. The first putative leptin homolog of non-mammalian species was isolated from chicken (Taouis et al., 1998), but recently Sharp et al. (2008) demonstrated that this nucleotide sequence had not been found in the chicken genome nor in approximately 0.5 million chicken ESTs in public data bases. Kurokawa et al. (2005) identified cDNA coding for a homolog to mammalian leptin in pufferfish, *Takifugu rubripes*, using genomic synteny around the human leptin gene, but it shares only 13.2% amino acid identity with human leptin. Crespi and Denver (2006) isolated a leptin homolog in African clawed frog *Xenopus laevis*, which shares only 13% amino acid identity with pufferfish leptin, and 35% with human leptin. The low amino acid identity of leptin between mammalian and non-mammalian was also observed in tiger salamander *Ambystoma tigrinum* (Boswell et al., 2006) and several teleost species including common carp *Cyprinus carpio*, zebrafish *Danio rerio* (Huising et al., 2006), medaka *Oryzias latipes*, green puffer *Tetraodon nigroviridis* (Kurokawa et al., 2005), rainbow trout *Oncorhynchus mykiss*, and Atlantic salmon *Salmo salar* (Murashita et al., 2008). Very recently, Gorissen et al. (2009) finished the cloning and characterization of two divergent leptin paralogs in zebrafish, coding for leptin-a and leptin-b. Zebrafish leptin-a and leptin-b are orthologs of human leptin, and they share merely 24% amino acid identity with each other (Gorissen et al., 2009). Despite the low amino acid sequence similarity

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between the hitherto identified leptin, the various orthologs seem to have a highly conserved tertiary structure and gene arrangement (Boswell et al., 2006; Crespi and Denver, 2006; Gorissen et al., 2009; Huising et al., 2006; Kurokawa et al., 2005; Murashita et al., 2008).

Previous studies have examined the role(s) of leptin in fish with inconclusive results, using mammalian leptin. For many fish species including coho salmon *Oncorhynchus kisutch* (Baker et al., 2000), catfish *Ictalurus punctatus* (Silverstein and Plisetskaya, 2000), and greensunfish *Lepomis cyanellus* (Londraville and Duvall, 2002), administration of mammalian leptin did not affect food intake or body weight. On the other hand, Volkoff et al. (2003) and de Pedro et al. (2006) reported that administration of mammalian leptin to goldfish (*Carassius auratus*) reduced both food intake and body weight. It is interesting to speculate why mammalian leptin has effects in some fish species but not in others. In common carp, mRNA expression of leptin was found to change acutely after food intake, but involvement of leptin in the long-term regulation of food intake and energy metabolism was not evident from fasting for days or weeks or long-term feeding to satiation (Huising et al., 2006).

Considering the difference of leptin between mammals and non-mammals, as well as among different non-mammalian species, preparation of species-specific leptin is therefore a key step for characterizing the function of leptin in non-mammals, especially in ectothermic vertebrates. Yacobovitz et al. (2008) reported that recombinant pufferfish leptin was biologically active in promoting proliferation of BAF/3 cells stably transfected with the long form of human leptin receptor, but no physiological effects were characterized. To date, only two studies have used a homologous leptin to explore its biological functions in ectothermic vertebrates. The first was performed on African clawed frog, where acute and chronic injections of recombinant frog leptin were shown to be potent anorexigenic effects in the midprometamorphic tadpole and juvenile frog but not in the early prometamorphic tadpole (Crespi and Denver, 2006). The other was performed on rainbow trout, where short-term (8 h) IP injection of recombinant trout leptin had a strong anorexic effect on the feeding behavior of rainbow trout and led to transient reduction of NPY mRNA levels while the mRNA levels of POMCs A1 and A2 were elevated (Murashita et al., 2008). To our knowledge, information regarding the effects of chronic injection of species-specific leptin on the food intake and energy metabolism in teleosts has never been reported.

In this article, we report the cloning and characterization of grass carp and silver carp leptin genes, which are orthologous to known mammalian, amphibian and teleost *obese* genes. In order to explore the biological functions of grass carp leptin, rgc-LEP was expressed in *Escherichia coli* and purified, and its acute and chronic effects on food intake and energy expenditure were examined in grass carp. We isolated the genes of NPY, LPL, ELO, UCP2, BSAL, hepatic lipase (HL), fatty acid desaturase (FAD), hormone sensitive lipase (HSL), pancreatic amylase (AMY), pancreatic trypsin (TRY) and uncoupling protein 1 (UCP1) from grass carp, and examined the effects of acute and chronic rgc-LEP treatments on the expression of these genes, as well as leptin (LEP) and SCD1 genes.

2. Materials and methods

2.1. Experimental animals

Grass carp (body weight 1000 g) and silver carp (body weight 1000 g) for cloning the leptin and other genes were obtained from Guangdong Freshwater Fish Farm, Panyu, Guangdong Province, China. Fish were anesthetized and sacrificed by decapitation. Brain, liver and mesenteric fat were dissected immediately for RNA isolation.

2.2. Cloning of grass carp and silver carp leptin cDNA sequences

Total liver RNA was isolated using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. Reverse transcription was performed with oligo $(dT)_{18}$ primer using ReverTra Ace-α-Kit (ToYoBo, Japan). PCR was performed using primers LEP01F/LEP02R (Table 1) with rTaq polymerase (TaKaRa, Japan). The PCR parameters were 30 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min, with an additional initial 3-min denaturation at 94 °C and a 3-min final extension at 72 °C. PCR products of the expected length (280 bp) were purified from agarose gel, and cloned into the pGEM-T Easy vector (Promega, USA). Inserts were sequenced using an ABI PrismTM 377 (Perkin Elmer, USA). Gene-specific primers were designed in the cloned PCR fragments of grass carp and silver carp LEP cDNAs for 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE (Table 1). First-strand cDNA libraries of liver with 3′ and 5′ adaptors added were synthesized using SMART RACE cDNA Amplification Kit (Clontech, USA) for 3′-RACE and 5′-RACE PCR, respectively. In the first PCR, the cDNA was amplified with two primer sets, LEP5′01R (for 5′-RACE) or LEP3′01F (for 3′-RACE) and Universal Primer Mix (UPM, provided in the kit) using Advantage® 2 PCR Kit (Clontech, USA). In the second PCR, primer sets LEP5′02R (for 5′-RACE) or LEP3′02F (for 3′-RACE) and NUP (Nested Universal primer, provided in the kit) were used. The PCR parameters were 30 cycles at 94 °C for 1 min, 63 °C for 30 s, and 72 °C for 2 min, with an additional initial 3-min denaturation at 94 °C and a 3-min final extension at 72 °C. The RACE products were cloned and sequenced as described above.

2.3. Amplification of intronic DNA of leptin gene

Genomic DNA samples of grass carp and silver carp were isolated using Blood & Cell Culture DNA Kit (QIAGEN, USA) according to the manufacturer's recommendations. Primers for PCR amplification of introns 1 and 2 of leptin gene are listed in Table 1, i1LEP01F and i1LEP02R for intron 1, and i2LEP01F and i2LEP02R for intron 2. PCR was performed in 50 μl reactions containing 200–300 ng genomic DNA. The following cycle was used for amplification: denaturation for 1 min at 94 °C, annealing for 30 s at 57 °C, and extension for 1 min at 72 °C. These steps were repeated for 30 cycles with an additional 3-min initial denaturation at 94 °C and a 3-min final extension at 72 °C. The genomic PCR products were sequenced after being cloned into pGEM-T Easy vector.

2.4. Cloning of 5′-flanking region of leptin gene

Universal Genome Walker Kit (Clontech, USA) was used for cloning the sequence of 5′-flanking region of leptin gene. Genomic DNA (2.5 μg) was digested at 37 °C overnight with DraI, and the digested blunt end DNAs were purified by phenol extraction and ethanol precipitation. DNA was then ligated to the Genome Walker adaptor at 16 °C overnight. To stop the reactions, the sample was incubated at 70 °C for 5 min and 72 μl TE (pH 7.5) was added. After the library was constructed, two touchdown PCR amplifications of the library were conducted. Primary PCR was performed using the following cycle procedure. The genomic library was amplified for 7 cycles at 94 °C for 25 s and 72 °C for 3 min, and 32 cycles at 94 °C for 25 s and 67 °C for 3 min in the presence of an outer adaptor primer (AP1, provided in the kit) and the outer gene-specific primer frLEP01R (Table 1), followed by additional treatment at 67 °C for 5 min. The primary PCR mixture was then diluted 50 times with

Table 1
Primer sequences for PCR.

Primers	Sequence
Primers for partial fragment	
LEP01F	GTCAAACTGCAGGCAGACACNATHAT
LEP02R	ATTAACTATCAGCTTTTGCATRAAYTGYTT
Primers for 5′-RACE PCR	
Gc-LEP5′01R	CTGGAAGGTAGTTAGGGT
Gc-LEP5′02R	ATAGGTTTATCAGCAGGAA
Sc-LEP5′01R	CAATAAGGATCTTTGGAGACAG
Sc-LEP5′02R	GTTCCTTAATTCTGTGGATG
Primers for 3′-RACE PCR	
Gc-LEP3′01F	GGAGC CAGC CAAT GGGAAGT
Gc-LEP3′02F	ACGCCACCCACCACATTA
Sc-LEP3′01F	GGAGC CAGC CAAT GGGAAGT
Sc-LEP3′02F	ACGCCACCCACCACATTA
Primers for intron l	
Gc-i1LEP01F	TCATACAATCCTCACATCTCA
Gc-i1LEP02R	GTTCCTTAATTCTGTGGATG
Sc-i1LEP01F	TCATACAATCCTCACATCTCA
Sc-i1LEP02R	GTTCCTTAATTCTGTGGATG
Primers for intron 2	
Gc-i2LEP01F	CATCCACAGAATTAAGGAAC
Gc-i2LEP02R	CAATGAGGATCTTTGGAGACAG
Sc-i2LEP01F	CATCCACAGAATTAAGGAAC
Sc-i2LEP02R	CAATAAGGATCTTTGGAGACAG
Primers for 5′-flanking region	
Gc-frLEP01R	GTTCCTTAATTCTGTGGATG
Gc-frLEP02R	TGAGATGTGAGGATTGTATGA
Sc-frLEP01R	GTTCCTTAATTCTGTGGATG
Sc-frLEP02R	TGAGATGTGAGGATTGTATGA
Primers for recombinant expression	
reLEP01F	CGCAGTCATATGCGTTCGATCCCTATTCATCAG
reLEP02R	GCACGTGGATCCTCATTAGCAGCTCTTCAACTGGTC
Primers for real-time PCR	
rtLEP01F	CAGGCAGACACCATCATCC
rtLEP02R	CTGGAAGGTAGTTAGGGTGTC
rtNPY01F	GCGCTAAGACACTACATCAACC
rtNPY02R	GATGACACATGGGCATGGAC
rtHL01F	CCAGCACTATCCTATAGCAG
rtHL02R	GATATATGAGCGCCAAGG
rtLPL01F	ATTGTGGTGGACTGGTTG
rtLPL02R	CTACATGAGCACCAAGACTG
rtSCD101F	ACTGGAGCTCTGTATGGAC
rtSCD102R	CGTAGATGTCATTCTGGAAG
rtFAD01F	AGCATCTGCCTTACAACC
rtFAD02R	ACACAGGAAGTATCGAACG
rtELO01F	ATCCTGAGGAAGAACAACC
rtELO02R	AGGACATGGATGAAGCTG
rtHSL01F	TGGAACGTTACTGAGTCTGG
rtHSL02R	AAGCGCACGTTGACTGG
rtBSAL01F	GAGCAACTACCTGTATGACG
rtBSAL02R	CGTGCTGATCCCATAGG
rtAMY01F	GACTGAGTTCAAGTATGGTGC
rtAMY02R	TCCAGCACCATGTCCTC
rtTRY01F	CTGGACCATTGACAGTGAC
rtTRY02R	CTCCAGACACTGAAGCTTG
rtUCP101F	GTGGACGTGGTGAAGACTC
rtUCP102R	GACACGAACATCACCACG
rt UCP201F	CGTGGTTTGTGGAAAGG
rt UCP202R	GCTCCAAATGCAGATGTG
rt βACT 01F	CGTGACATCAAGGAGAAG
rt βACT 02R	GAGTTGAAGGTGGTCTCAT

Mixed bases: N, A/T/G/C; R, A/G; Y, C/T; H, A/T/C.

deionized water, and used as a template for a secondary PCR with the nested adaptor primer (AP2, provided in the kit) and the nested gene-specific primer frLEP02R (Table 1). The secondary PCR parameters were the same as primary PCR. The final PCR products were analyzed on agarose gel electrophoresis, cloned into pGEM-T Easy vector and sequenced. Putative transcription regulatory regions were predicted with TFBIND (http://tfbind.ims.u-tokyo.ac.jp/) (Tsunoda and Takagi, 1999).

2.5. Recombinant expression and purification of grass carp leptin

A cDNA fragment encoding the mature peptide of grass carp leptin (gcLEP) was prepared with primers reLEP01F (including a NdeI restriction-enzyme site and an initiator methionine codon immediately upstream from the mature leptin)/reLEP02R (including a TGA termination codon and a BamHI restriction-enzyme site immediately after the final codon) (Table 1) using *Ex* Taq polymerase (TaKaRa, Japan). The PCR product was digested with NdeI and BamHI before it was cloned into the same restriction-enzyme sites in pET3c. The ligation mixture was used to transform *E. coli* JM109 using heat shock transformation. Positive transformants were selected, and inserts were sequenced to confirm the proper sequence. The recombinant pET3c-gcLEP plasmid was transformed into the competent BL21(DE3)pLysS *E. coli* strain for protein expression.

Transformed cells were grown in 1 L of Luria-Broth (LB) medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When absorbance at 600 nm reached 0.6, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.25 mM. Cells were cultivated for another 4 h and then were harvested by centrifugation at 6000*g* for 15 min at 4 °C. The cells were resuspended in 100 ml of cell lysis buffer (50 mM Tris–HCl, 0.1 M NaCl, 1 mM EDTA; pH 7.5, with 1 mM fresh PMSF), and disrupted by sonication for 30 min at 50% output. Inclusion bodies (IBs) were separated from the cell lysate by centrifugation at 14,000 *g* for 30 min at 4 °C. The pellet was washed twice with 1% Triton X-100 in cell lysis buffer and twice with double-distilled water to remove any contaminants. The pellet was resuspended in 100 ml of denaturation buffer containing 8 M urea in 25 mM Tris–HCl (pH 7.5). The suspension was gently shaken for 4 h. After centrifugation at 14,000 *g* for 30 min at 18 °C to remove the undissolved material, the urea in the supernatant was diluted by 4 M urea for 12 h then by further twofold diluted urea buffer for every 12 h. When the urea concentration became to 1 M or below, reduced glutathione and oxidized glutathione were added to a final concentration of 2.5 and 0.25 mM, respectively, and the recombinant proteins were further stepwise dialyzed against a lowered concentration of urea until the urea concentration reached 0.25 mM. The solution was then dialyzed against PBS (PH 7.5) for 12 h with three buffer exchanges and finally sterile filtered through a 0.22-μm cellulose acetate syringe filter (Whatman, England).

The purity and homogeneity of the purified rgc-LEP were detected by SDS–PAGE under reducing and non-reducing conditions. Protein concentration of rgc-LEP was determined with a BCA protein assay kit (Beyotime, PR China) using BSA as a standard.

2.6. Identification of rgc-LEP by mass spectrometric analysis

A band of rgc-LEP cut from polyacrylamide gel which stained with Coomassie blue, was collected. The protein SDS–PAGE band was destained with 25 mM NH_4HCO_3 in 50% acetonitrile. After dehydration of the gel, the protein was digested with trypsin (Promega, USA) in 50 mM NH_4HCO_3 at 37 °C overnight. The derived peptide was eluted, desalted and freeze-dried. The freeze-dried protein was dissolved in 0.1% TFA, and then analyzed by 4800 plus MALDI-TOF-TOF Analyzer (AppliedBiosystems, USA). The data obtained from first and second mass spectrometric analysis were analyzed by GPS Explore v3.6 software (AppliedBiosystems, USA). The protein was then identified by searching in the local database using MASCOT v2.1 (Matrix Science, UK) software.

2.7. Effects of chronic IP rgc-LEP administration on food intake and body weight

Juvenile grass carp were obtained from and reared in Guangdong Freshwater Fish Farm (Panyu, PR China). Fish were randomly

distributed into nine cages (1 × 1 × 1 m) in a tank (4 × 4 × 1 m) with a constant flow of filtered water. Grass carp were fed by hand using a commercial floating diet (Haiwei, Guangzhou, PR China) at 10:00, 12:00 and 14:00 every day. The feeding rate was 2% of their body weight per feeding. Waste diet was removed at 11:59, 13:59 and 15:59, and dried for feed intake corrections. Feces were cleaned every day. Animals were acclimated to these conditions for at least 15 days prior to experimental use, showing a normal feeding pattern during this acclimation period. Food was withheld for 24 h prior to the start of the experiment.

For the experiment, all fish were mixed and weighed. Grass carp with similar body weight (body weight 14.26 ± 0.22 g) were selected and randomly distributed into nine groups ($n = 15$ for each group). Fish were anesthetized in water containing tricaine methanesulfonate (MS-222, 1:10,000). Immediately after loss of equilibrium, fish were injected daily between 8:30 and 9:00. The IP injections were performed with a 1 ml syringe and 0.3 mm Microlance needle close to the ventral midline posterior to the pelvic fins. Grass carp (three groups) were IP injected with 50 μl PBS and fed in excess (PBS group, 14.26 ± 0.26 g BW), or IP injected with 50 μl PBS containing 30 μg rgc-LEP (total 2.1 μg/g BW) and fed in excess (LEP group, 14.26 ± 0.23 g BW), or IP injected with 50 μl PBS and fed with the same amount of food as that consumed by LEP group (pair feeding group, PF group, 14.25 ± 0.20 g BW). The feeding time of PBS group and LEP group lasted for 6 h (10:00–16:00, daily). Wasted diet was then removed and dried for feed intake corrections. In order to ensure the complete intake of the diet, the feeding time of PF group was not limited. Both PBS and LEP groups were fed in excess and they ingested freely within the given feeding time (6 h), whereas the PF group was fed with the same amount of food (limited) as that consumed by LEP group and its feeding time was not limited. Thus, the PBS group is more suitable as the control than the PF group for studying the effects of rgc-LEP on feeding behavior and NPY gene expression, whereas the PF group is more suitable as the control than the PBS group for studying the effects of rgc-LEP on body weight and expression of genes correlating to energy expenditure, lipid metabolism and digestion. The injection dose of rgc-LEP was based upon our preliminary experiments and reports on trout (Murashita et al., 2008) and goldfish (de Pedro et al., 2006).

Fish were maintained for 12 days as described above. Throughout the experimental period, water temperature was 26 ± 2 °C. Body weight was recorded every 4 days. At the end of the experiment (13th day, 2 h after injection without feeding), two fish of each group were randomly selected and killed with 2-phenoxyethanol. Liver, brain and mesenteric fat of the LEP group, liver and mesenteric fat of the PF group, and brain of the PBS group were dissected and frozen immediately in liquid nitrogen, followed by storage at −80 °C until RNA extraction.

2.8. Acute and chronic effects of rgc-leptin on the expression of selected genes

An acute experiment was performed using the same stock of grass carp. Two groups of grass carp (body weight 14.51 ± 0.31 g, $n = 15$ for each group) were IP injected with 50 μl PBS (PBS group) or 50 μl PBS containing 30 μg rgc-LEP (LEP group). Fish were then sampled at 2 h after injection without feeding ($n = 6$ for each group). Liver, brain and mesenteric fat of the PBS group and the LEP group were dissected for RNA isolation. Total RNA was extracted using SV Total RNA Isolation System. One microliter of total RNA was used for reverse transcription using ReverTra Ace qPCR RT Kit (ToYoBo, Japan) according to the manufacturer's recommendations.

In order to assess the effect of rgc-LEP on genes expression, genes of NPY, FAD, ELO, LPL, HL, HSL, BSAL, AMY, TRY, UCP1 and UCP2 from grass carp were isolated using RT-PCR method. PCR was performed by degenerate primers (data not shown), which were designed based on the sequences available in GenBank, with rTaq polymerase (TaKaRa, Japan). PCR products of the expected length were sequenced, and identified by analyzing in the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers of SCD1 (available in GenBank, Accession No. AJ243835), NPY, LEP, FAD, ELO, LPL, HL, HSL, BSAL, AMY, TRY, UCP1, UCP2 and β-actin (βACT, the control gene) for the real-time PCR were designed in Table 1.

Real-time PCR was performed by Mini Opticon Two-Color Real-Time PCR Detection System (Bio-Rad, USA) using Real-time PCR Master Mix (ToYoBo, Japan) according to the manufacturer's recommendations. The PCR parameters were 40 cycles at 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 45 s, with an additional initial 1-min denaturation at 95 °C. We used a relative quantification method to calculate the gene expression values as described by Livak and Schmittgen (2001).

2.9. Statistical analysis

Statistical analysis was performed with SPSS 13.0 software. Significant differences between control and exposure groups were performed using one-way analysis of variance (ANOVA) followed by the LSD (least significant difference) post hoc test, after checking for data normality and homogeneity of variances. Differences were considered significant if $p < 0.05$. Results are presented as the means ± SE ($n = 6/15$).

3. Results

3.1. Gene structures and characterization of grass carp and silver carp leptins

Full-length cDNA sequences of grass carp and silver carp leptins were obtained by RT-PCR and RACE methods (GenBank Accession Nos. EU719623 and EU719624). Grass carp (silver carp) leptin was 1096 bp (1144 bp) in length with a 70 bp (73 bp) 5′-UTR, a 504 bp (552 bp) 3′-UTR, and an ORF of 173 (172) amino acids, including the 20 (20)-residue putative signal peptide and 153 (152)-residue putative mature peptide (Fig. 1).

To understand the regulation of grass carp (silver carp) leptin gene, a 265 bp (265 bp) fragment upstream of the transcription initiation site was amplified using genome walker method. Analysis of the promoter regions of grass carp and silver carp leptin genes both revealed a typical TATA box, an activator protein 1 (AP1) site, a cAMP response element binding (CREB) site, a CCAAT/enhancer-binding protein (C/EBP) site, an acute myeloid leukemia 1 (AML1) site, a upstream stimulatory factor (USF) site and a special protein 1 (SP1) site, located at 29 bp, 50 bp, 70 bp, 150 bp, 199 bp, 214 bp and 253 bp upstream of the transcriptional start site, respectively (Fig. 1).

Like the leptin genes of mammals, African clawed frog and other fishes, the grass carp (silver carp) leptin gene consisted of three exons and two introns (Figs. 1 and 2). The sizes of intron 1 and intron 2 of grass carp (silver carp) leptin gene were 948 bp (951 bp) and 111 bp (111 bp). The two introns appeared at similar positions as in the leptin genes of frog and human, as well as those of other fishes (data not shown). DNA sequence identity of leptin intron 1 and intron 2 between grass carp and silver carp were 89.6% and 100%, respectively.

Multiple alignment was carried out based on the amino acid sequences of leptin from grass carp, silver carp, other fish species, frog, mouse and human. The grass carp leptin had 87.9%, 73.4% and 57.2% amino acid identity with silver carp leptin, common carp

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A
                                                                                SP1
1      cgacggcccgggctggtaaaagt                                                                       23
                               USF              AML1
24     tataaatccatagtgaattgtaacacacgatcacatggtaactgtggtggatgtattacaatgacaaatatttgtagtacgtgtgtgt     113
         C/EBP                                                                        CREB
114    gtgaggtgggtttgtgttccgctcgaggtttgtgttgtgtccccctcatgctgttctgcactgggagaatcagggaaggaatggctgag     203
                   AP1              TATA
204    gtgattcacacatgagtcaacagctatcagctatataagcctgaccactcatggagcctgggTATCATACAATCCTCACATCTCAAAGgt     293
294    aagtccccttcgctcgctctctctctctactcatcactaccctcacactatctgcagctctgagcactggcattctcgctaataatgtgt     383
384    gactttgtcatctaattttatagcctttaaaagggtattttattatttatattatagagaaactaagtttacattcaaacacggaa     473
474    taaaaaaaaattagagtgcttttggaatgaagcaatcattgtttatacaagggcaacatgttctttggggaatttaccagatattgaaat     563
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1014   taaattgcactcaggaatgcagatatagttgcactttctttcagtaggagccagttagtttcttgagcaccaaatcagcatatggaatga    1203
1204   tttctgaagatttagctttgccataacaggaataaatgaactgtatttctaatcaaataaattcagccttgataaacagttacatttaaa    1293
1294   agatttttagtagacattcagaataatgcttattttatttcagcagGTATTGCAGTATTGCCAAGATAAAGACCACCATATACAAGAACC    1383
1384   ATGTATTCTCCAGTTCTTCTCTACACCTGCTTTTTGAGCATTCTTGGTATGATTGATGGCCGTTCGATCCCTATTCATCAGGATAACCTC    1473
1       M  Y  S  P  V  L  L  Y  T  C  F  L  S  I  L  G  M  I  D  G  R  S  I  P  I  H  Q  D  N  L       30
1474   AAAAACTTGGTCAAACTACAGGCAGACACCATCATCCACAGAATTAAGGAACACAATGAGAAGgtaatgggttagaactcaatgaactag    1563
31      K  N  L  V  K  L  Q  A  D  T  I  I  H  R  I  K  E  H  N  E  K                                 51
1564   ttgcatggcttctgttctgttaaaggttaacacgggtgaacctcatggagatctggtcatgctcattttgtcttctctcacagCTAAAA    1653
52                                                                                            L  K    53
1654   CTGTCTCCAAAGATCCTCATTGGGGATTCAGAGCTTTACCCCGAGGTTCCTGCTGATAAACCTATCCAAGGGCTTGGGTCCATCGTGGAC    1743
54      L  S  P  K  I  L  I  G  D  S  E  L  Y  P  E  V  P  A  D  K  P  I  Q  G  L  G  S  I  V  D       83
1744   ACCCTAACTACCTTCCAGAAGATTCTTCAAACACTGCCCAAGGGGCATGTGAGCCAGTTACACAATGATATGTCCACCCTTTTGGAATAC    1833
84      T  L  T  T  F  Q  K  I  L  Q  T  L  P  K  G  H  V  S  Q  L  H  N  D  M  S  T  L  L  E  Y      113
1834   TTTAAAGACAGGATGACATTTATGCGTTGCACACTAAAGGAGCCAGCCAATGGAAGTCACTGGACACTTTCATAGAGAAGAACGCCACC    1923
114     F  K  D  R  M  T  F  M  R  C  T  L  K  E  P  A  N  G  K  S  L  D  T  F  I  E  K  N  A  T      143
1924   CACCACATTACTTTTGGGTACATGGCTTTAGACAGACTGAAACAGTTCATGCAAAAGCTGATAGCTAATCTTGACCAGTTGAAGAGCTGC    2013
134     H  H  I  T  F  G  Y  M  A  L  D  R  L  K  Q  F  M  Q  K  L  I  A  N  L  D  Q  L  K  S  C      173
2014   TAATCTGCCACTATTATAGCATTACGGTATACTATTTATTATATTTATTTAAAACCCTGTATATTTATAGGCCAAAGTATTTTTTGGCAC    2103
       ***
2104   ATTTTAATGTACAAACAAATTTAAAAATTATTTCCTGCTATTAAACAGAAAGCCAGAAATGTTGCTTGCGACATCATGGTGCGCCAATCA    2193
2194   ATTTGTCCTTACGATGTAAACAAACACTAGCAATCTGATACCTGCACACATTGCCATGTCATACTTCATGCTGTTTGCAGGATGACATCA    2283
2284   GCACTTGCTGCGCTTGCTCAATGATGTCACTGTTTACCAGAAGCAGCGAGGTCAGACTCTGCTGGGAAGCTGGAATTATGTTCCCACTTC    2373
2374   TTGCTGCAACACCAGCAATGTAGGACACACACCAAAATATATATATATTTTCCTACACAGTTTGAATCTATGCACTTTAAATATCCTGTG    2463
2464   ATTGTAAATAGTTTTGTATTTGTATTCTTCCAAAAAAAAAAAAAAAAAAAAAAAAAA                                    2420

B
                                                                           SP1
1      cgacggcccgggctggtaaaagctacaaa                                                                 29
                           USF              AML1
30     tctataatgaattgtaacacaacgatcacatggtgacggtggcgggaagtattacaatgacaaatatttgtagtacgtctgtgtgtgaga     119
C/EBP                                                                             CREB
120    tgggtttgtgttccgctcgaggtttgtgttgtgtccccctcatgctgttctgcactgggagaatcagagaaggaatggctgaggtgatt     209
              AP1              TATA
210    cacacatgagtcaacagctatcagctatataagcctgaccactcatggagcctgggTATCATACAATCCTCACATCTCAAAGgtaagtcc     299
300    ccttcgctctctctctctactcatcactactctcacacaatctgcagctctgagactcttgctaataatgtgtgattttgtcatctaatt     389
390    tttttatcttttaaaagggtatttttattatttatattatggagaaactaagtttacattccacacagggagtaaaaaaagattagag     479
480    tgcttttggaatgaagcaatcattgtttatacaagggcaacatgttctttggggaaatgaccagatattgaaatccaaatgtaggcacag     569
570    aggctgtcattttgttcatttagaagtgagattgaacatagcagaactcttaattgcttgatgttgcagactggagaggcatggggttca     659
660    atgacatgctcattgtgaagactacaggcaacatcacattagagctcccttagatgtatttccatgtgcaaggaaccctcttttaaatgt     749
750    tgtattttgttggacaaactgttcttaatttgaattggaagtatttggaggatctgatatcggagattaaatgtcatgtaaaatcaactg     839
840    aaatatcaagcctggattgcaagattgtgcaattagacacagcttcagtgatttacagtttttcgatagaaaatattctttattatttga     929
930    atttactacaagtgatttcttaatgcagtttgggagaatgtgaatatggaagaatgaagcttttcgccaaaaaattgcattcaggaatgc    1019
1020   agatatagtctaaattgcactttctttcagtaggggccagttagtttcttgagcaccaaatcagcatatggaatgatttctaaagattca    1109
1110   gctttgccataacaggaataaattaactgcatttctaatctaataaattcagccttgaaaagcattagctatttaaacaattacatttaa    1199
1200   aagattttttgtagacattcagaataatgtttatttctttcagCAGGTATTGCACTATTGCCAAGATAAAGACCACCATATACAGGAACC    1289
1290   ATGTATTTTCCAGTTCTTCTCTACACCTGCTTTTTGAGCATTCTTGGTCTGATTGATGGCCGTTCGATCCCCTTTCATCCGGAGAGTCTC    1379
1       M  Y  F  P  V  L  L  Y  T  C  F  L  S  I  L  G  L  I  D  G  R  S  I  P  F  H  P  E  S  L       30
1380   AAAAGCTTAAAACAGCAGGCAGACACCATCATCCACAGAATTAAGGAACACAATGAGAAGgtaatgggttagaactcaatgaactagttg    1469
31      K  S  L  K  Q  Q  A  D  T  I  I  H  R  I  K  E  H  N  E  K                                    50
1470   catggcttctgttctgttaaaggttaacacgggtgaacctcatggagatctggtcatgctcattttgtcttctctcacagCTAAAACTG    1559
51                                                                                         L  K  L     53
1560   TCTCCAAAGATCCTTATTGGGGATTCAGAGCTTTACCCTGAGGTTCCTGCTGATAAACCCATCCAAGGGCTTGGGTCCATCATAGACACC    1649
54      S  P  K  I  L  I  G  D  S  E  L  Y  P  E  V  P  A  D  K  P  I  Q  G  L  G  S  I  I  D  T       83
1650   CTAACTACCTTCCAGAAGGTTCTTCAAACACTGCCCAAGGGGCATGTGAGCCAGTTACACAGTGATGTGTCCACCCTTTTGGACTACTTT    1739
84      L  T  T  F  Q  K  V  L  Q  T  L  P  K  G  H  V  S  Q  L  H  S  D  V  S  T  L  L  D  Y  F      113
1740   AAAGTTTGGATGACATTTATGCGTTGCACACCAAAGGAGCCAGCCAATGGGAAGTCACTGGACACTTTCATACAGAAGAACGCCACCCAC    1829
114     K  V  W  M  T  F  M  R  C  T  P  K  E  P  A  N  G  K  S  L  D  T  F  I  Q  K  N  A  T  H      143
1830   CACGTTACTTTTGGGTACATGGCTTTAGACAGACTGAAACAGTTCATGCAAAAGCTGATAGCTAATCTTGACCAGGTGAAGAGCTGCTAA    1919
144     H  V  T  F  G  Y  M  A  L  D  R  L  K  Q  F  M  Q  K  L  I  A  N  L  D  Q  V  K  S  C ***   172
1920   TCTGCCACTATTATAGCATTATGGTATACTATTTATTATATTTATTTAAAACTCTCTATATTTATAGACCAAAGCAGCTTTTTTTGGCAC    2009
2010   ATTTTTGGCACAAATAGATTTTAAAATTATTCCCTGCTATTAAACAAAGCCAGAAATGTTGCTTGTGACATCATGGTGCACCAATCAATC    2099
2100   TGTCCTTACGATGCAAACAAACACTAGCAATCTGATACCTGCACACATTGCCATGTCATACTTCATGCTGTTTTACAGGATGACATCAGC    2189
2190   ACTTGCTGCACTTGCTCAATGATGTCACTGTGTTTACCAGAAGAAGCGAGGTCAGACTCTGCTGGGAAGCTGGAATTATGTTCCCATTTT    2279
2280   ACTTCTTGCTGCAACACCAGCAATGTAGGACACATACCAAAATATATATATATTTTCCTACACAGTGTGAATCTATGCACTTTAAATATCCT    2369
2370   GTGATTGTAAATAGTTTTGTATTTTGTGTAATGATGCATTTTTCCATAAAAACAAACCAATAAAATAAATTTATTGTATACTTTTGTCAA    2459
2460   AAAAAAAAAAAA                                                                                 2471
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Fig. 1. Gene characterization of grass carp (A) and silver carp (B) leptins. The lengths of 5′-flanking region, intron 1 and intron 2 of grass carp leptin are 265 bp (1–265 bp), 948 bp (292–1239 bp) and 111 bp (1437–1547 bp), respectively. The lengths of 5′-flanking region, intron 1 and intron 2 of silver carp leptin are 265 bp (1–265 bp), 951 bp (292–1242 bp) and 111 bp (1440–1550 bp), respectively. Polyadenylation signal is darkened. Putative regulatory elements identified in the 5′-flanking regions are boxed. Putative transcription regulatory regions were predicted with TFBIND (http://tfbind.ims.u-tokyo.ac.jp/). SP1, special protein 1; USF, upstream stimulatory factor; AML1, acute myeloid leukemia 1; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP response element binding; AP1, activator protein 1; TATA, TATA box. GenBank accession numbers: grass carp, FJ373293; silver carp, FJ373294.

leptin-a-I and zebrafish leptin-a, whereas only 20.0%, 28.8%, 28.7%, 23.0% and 23.0% amino acid identity with pufferfish, rainbow trout, African clawed frog, mouse and human leptins, respectively (Fig. 3A). Both grass carp and silver carp leptins comprised of four helices and had two cysteine residues for the disulfide bonds of α-helices C and D (Fig. 3A). The predicted tertiary structures (modeled by the ProModII program at the SWISS-MODEL automated protein modeling server, based upon human leptin Protein Data Bank structure file 1AX8.pdb) of grass carp and silver carp leptins were highly conserved with human leptin (Fig. 3B), in spite of considerable divergence in the primary structures (Fig. 3A). The phylogenetic analysis showed that all the vertebrate leptins cluster together. Within the leptin cluster, all teleost leptins formed an independent cluster, while amphibian leptins and mammalian leptins formed another cluster. In the teleost leptin cluster, zebrafish leptin-b formed an independent clade with medaka leptin-b, while grass carp and silver carp leptins formed another clade with zebrafish leptin-a and other available teleost leptins (Fig. 4).

3.2. Recombinant expression and purification of grass carp leptin

DNA sequence encoding the mature peptide of grass carp leptin (gcLEP) was amplified by PCR and subcloned into the pET3c expression vector based on the NdeI and BamHI restriction-en-

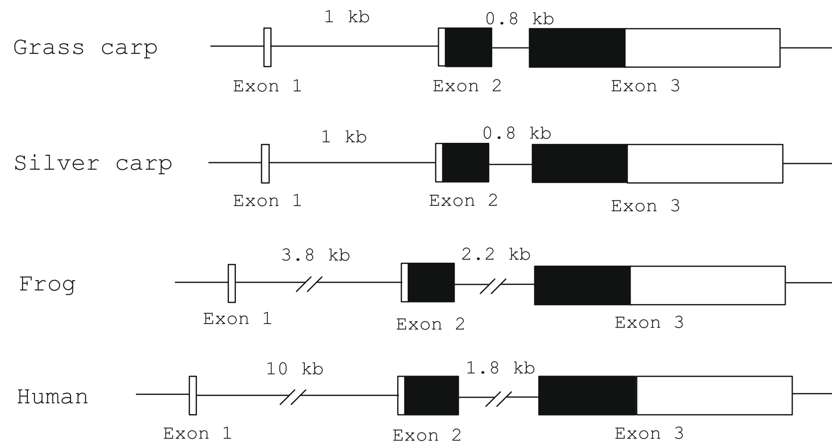
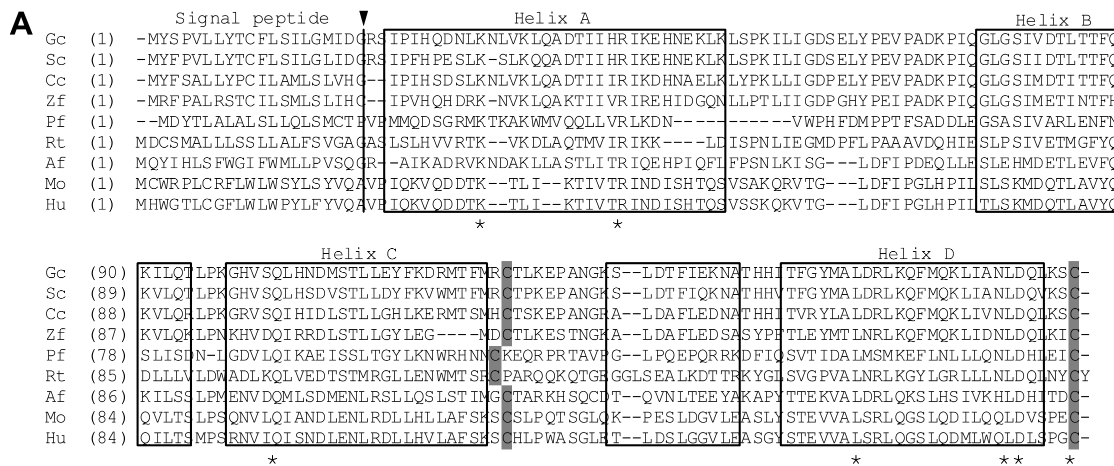


Fig. 2. Gene structures of grass carp, silver carp, African clawed frog and human leptins. Dark shading shows coding regions. The gene structure of African clawed frog leptin was quoted from the report by Crespi and Denver (2006). The genomic DNA sequence of human leptin was obtained from the human genomic database at NCBI (http://www.ncbi.nlm.nih.gov/Genomes/). GenBank accession numbers: grass carp, FJ373293; silver carp, FJ373294.



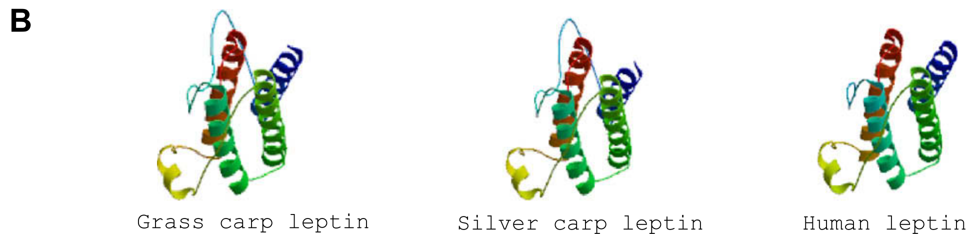


Fig. 3. Molecular characterization of vertebrate leptins. (A) Multiple alignment of amino acid sequences of leptins. The identical amino acid residues are indicated by asterisk. Dashes indicate the amino acid gaps that are necessary to align these sequences. Conserved cysteine residues involved in the formation of disulfide bridges are shaded. The α-helices, inferred from human leptin, are boxed. GenBank accession numbers: grass carp (Gc), EU719623; silver carp (Sc), EU719624; common carp (Cc) leptin-a-I, CAH33828; zebrafish (Zf) leptin-a, BN000830; pufferfish (Pf), ABI93547; rainbow trout (Rt), AB354909; African clawed frog (Af), AY884210; mouse (Mo), NP_032519 and human (Hu), NP_000221. (B) Tertiary structures of grass carp, silver carp and human leptins. Secondary and tertiary protein structures were modeled using the ProModII program at the SWISS-MODEL automated protein modeling server, based upon human leptin (1AX8.pdb) Protein Data Bank structure file.

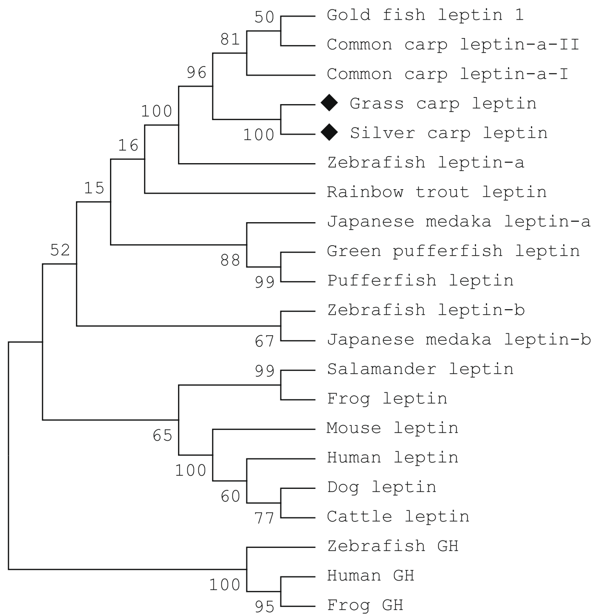


Fig. 4. Phylogenetic tree of vertebrate leptins. The phylogenetic tree was constructed by neighbor-joining method in clustalx 1.83 and MEGA4. Numbers at the branches reflect the confidence level as obtained by bootstrapping (1000 replications). GH: growth hormone. Growth hormone sequences were included as the outgroup. The GenBank accession numbers of the sequences used in the figure were as follows: mouse leptin, NP_032519; human leptin, NP_000221; zebrafish leptin-a, NM_001128576; common carp leptin-a-II, CAH33827; common carp leptin-a-I, CAH33828; goldfish leptin-a-I, ACL68083; grass carp leptin, EU719623; silver carp leptin, EU719624; rainbow trout leptin, BAG09232; Japanese medaka leptin-a, BAD94448; green pufferfish leptin, BAD94451; pufferfish leptin, BAD94444; salamander leptin, 22154139; African clawed frog leptin, AAX77665; zebrafish leptin-b, AM901009; Japanese medaka leptin-b, BN001183; dog leptin, O02720; cattle leptin, P50595; human GH, P01241; zebrafish GH, Q1JQ34; frog GH, NP_001083848.

zyme sites. When this was done, the first amino acid residue of mature gcLEP, Arg, directly followed Met. The recombinant pET3c-gcLEP plasmid was expressed in *E. coli* BL21(DE3)pLysS under the control of a T7 promoter, using IPTG as inductor. The expressive level of rgc-LEP had no difference under different IPTG concentrations (0.25, 0.5 and 1 mM), and almost all of the protein was produced as IBs (data not shown). IBs were resuspended in 8 M urea and then sequentially renatured by step dialysis into physiological buffers.

Concentration of rgc-LEP was determined by BCA method. 75 mg of purified rgc-LEP was obtained from 1 L of bacterial culture. The purity and homogeneity of rgc-LEP were documented by 12% SDS–PAGE. Only one band of ~18 kDa was obtained under reducing condition, while two bands of ~17 kDa (predominant product) and ~35 kDa (minor product) were obtained under non-reducing condition (Fig. 5). The change of the band position (from ~18 kDa under reducing condition to ~17 kDa under non-reducing condition) should result from the formation of a disulfide bond between Cys-123 and Cys-173 of one rgc-LEP molecule (Cys-123 and Cys-173 are the only two Cys residues present in mature grass carp leptin), which produced a more compact conformation and would result in less resistance while moving through the gel (Jeong and Lee, 1999; Park et al., 2001). The minor band of ~35 kDa should result from the formation of a disulfide bond between two rgc-LEP molecules (Yacobovitz et al., 2008). Identification of rgc-LEP was performed by sequencing its partial amino acid sequences. Four regions of rgc-LEP (35% of rgc-LEP amino acids) were identified (data not shown).

3.3. Effects of acute and chronic IP administration of rgc-LEP on food intake and body weight

The chronic effects of rgc-LEP on food intake and body weight in grass carp were presented in Fig. 6. The food intake of the LEP group was significantly ($p < 0.05$) reduced relative to the PBS group on the first day after IP rgc-LEP injection, but not during the following days. On the first day, food intake of the LEP group (about 0.4% of their body weight) reduced 65% compared to the PBS group (about 1.2% of their body weight). However, the anorexic effect

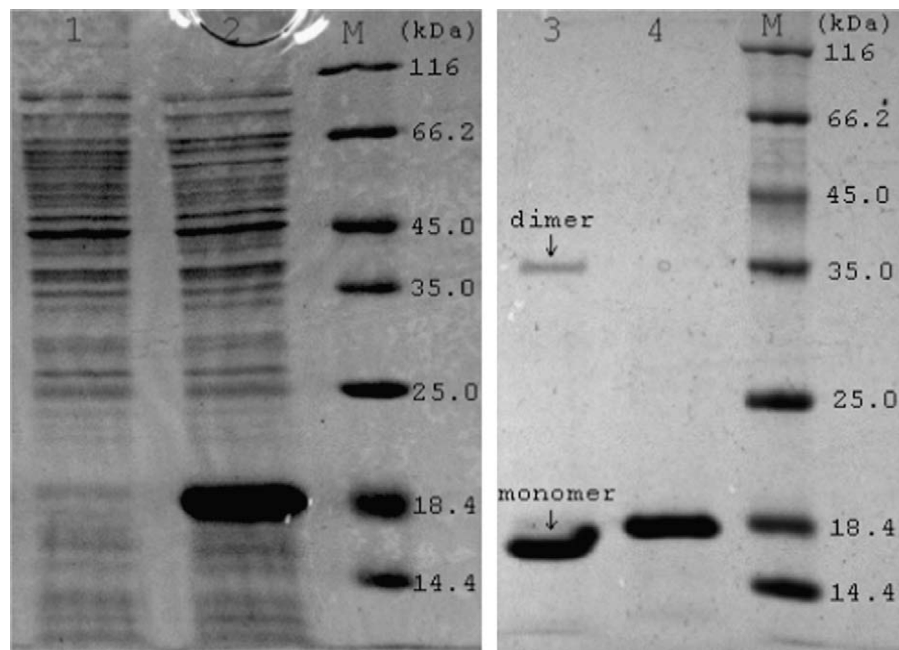


Fig. 5. Recombinant expression and purification of grass carp leptin. Lane 1: uninduced cells, lane 2: IPTG-induced cells, lane 3: purified rgc-LEP under non-reducing condition, lane 4: purified rgc-LEP under reducing condition, M: makers. The refolded monomeric form of rgc-LEP (lane 3, without reducing agents) migrated faster than the unfolded form (lane 4, with reducing agents) because the compact conformation resulted in less resistance while moving through the gel. The band of ~35 kDa only appeared under non-reducing condition indicates that the dimer is formed by disulfide bond.

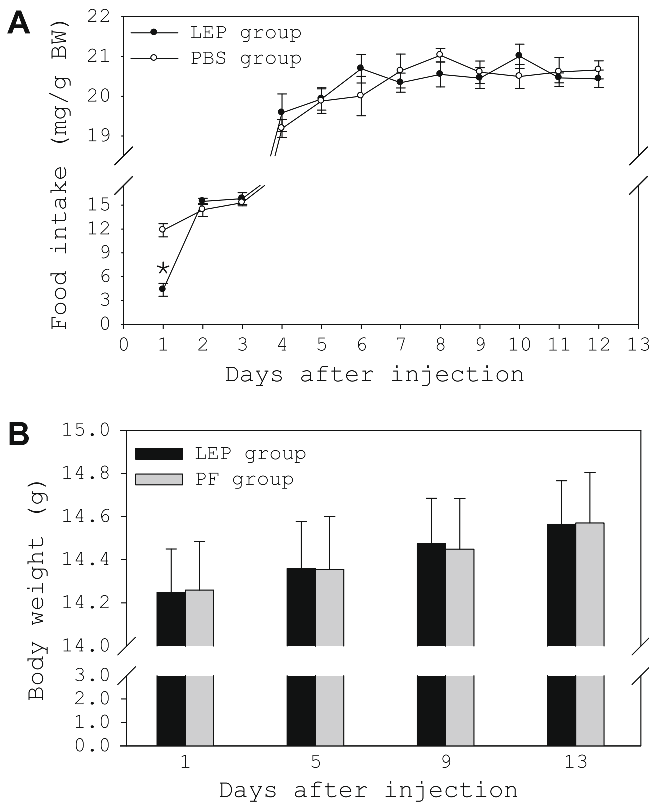


Fig. 6. Effects of IP administration of rgc-LEP (2.1 μg/g BW/day) on food intake (A) and body weight (B) in grass carp ($n = 15$/group). PBS group, PBS injection group ($n = 3$); LEP group, rgc-LEP injection group ($n = 3$); PF group, PBS injection and pair feeding group ($n = 3$). Both PBS and LEP groups were fed in excess, and their feeding time lasted for 6 h. Wasted diet was then removed and dried for feed intake corrections. PF group was fed with the same amount of food as that consumed by LEP group diet. In order to ensure the complete intake of the diet, the feeding time of the PF group was not limited. The results are expressed as mean ± SEM. Significance was compared with the PBS group or the PF group ($^{*}p < 0.05$).

was not observed during days 2–12 (Fig. 6A). No significant differences in the means of body weight were observed between the LEP group and PF group throughout the experimental period (Fig. 6B).

3.4. Effects of acute and chronic IP administrate of rgc-LEP on gene expression

In order to assess the effects of rgc-LEP on the expression of selected genes, partial cDNA sequences of NPY, FAD, ELO, LPL, HL, HSL, BSAL, AMY, TRY, UCP1 and UCP2 were obtained from grass carp by RT-PCR using degenerate primers (data not shown). The confirmed sequences were submitted to GenBank. The GenBank accession numbers were as follows: FJ641971, FJ641974, FJ641973, FJ436077, FJ436064, FJ843081, FJ641972, FJ641975, FJ641976, FJ436059 and AY948546, respectively. The expression of selected genes representing multiple metabolic processes was concurrently measured by real-time PCR technology in the acute (1 day) and chronic (13 days) experiments of rgc-LEP treatment.

In the acute experiment, the mRNA levels of LEP (liver), ELO (liver), BSAL (liver) and UCP2 (mesenteric fat) of the LEP group were significantly ($p < 0.05$) increased compared to those of the PBS group (5.76-fold, 1.49-fold, 1.96-fold and 2.03-fold, respectively), while the mRNA levels of NPY (brain), LPL (liver) and SCD1 (liver) were significantly ($p < 0.05$) decreased (0.43-fold, 0.79-fold and 0.08-fold, respectively). Meanwhile, the mRNA levels of HSL (mesenteric fat), HL (liver) and TRY (liver) tended to increase (1.24-fold, 1.36-fold and 1.28-fold, respectively) but not significantly. Expression of UCP1 (liver), FAD (liver) and AMY (liver) genes were not affected by rgc-LEP injection (Fig. 7A).

In the chronic experiment, rgc-LEP significantly reduced the mRNA levels of LPL (0.86-fold), but no rgc-LEP-inducing effect was observed on the mRNA expression of other genes (Fig. 7B).

4. Discussion

Previous studies had pointed out that the primary sequences of mammalian and non-mammalian leptins were considerably different, but their gene structures and predicted tertiary structures were conserved (Boswell et al., 2006; Crespi and Denver, 2006; Huising et al., 2006; Kurokawa et al., 2005; Murashita et al., 2008). In this study, grass carp leptin had only 28.7% amino acid identity with African clawed frog leptin, and 23.0% with human leptin. However, its gene structure which contained three exons and two introns, and its predicted tertiary structure, and the cysteines for the disulfide bond, were highly similar to those of African clawed frog and human leptins. Similar results were also found in silver carp.

Very recently, Gorissen et al. (2009) finished the cloning and characterization of two divergent leptin paralogs in zebrafish, coding for leptin-a and leptin-b. The duplicate leptin genes were also discovered in Japanese medaka, which represents another teleost lineage, suggesting that fish seem to commonly possess two types of leptin (a and b type) genes (Gorissen et al., 2009; Kurokawa and Murashita, 2009). The observed expression patterns of zebrafish leptin-a and leptin-b were substantially different, and hepatic leptin-a mRNA level showed no significant response to fasting for 1 week whereas hepatic leptin-b mRNA level significantly decreased after 1 week of food deprivation (Gorissen et al., 2009). These results indicate that the functions of leptin-a and leptin-b might be different. In the present study, the degenerate primers we designed for cloning of grass carp and silver carp leptins were based upon the available leptin-a type genes. Phylogenetic analysis also indicates that both grass carp and silver carp leptins we obtained belong to leptin-a type.

In African clawed frog and mammals, acute leptin treatment can suppress food intake. Recombinant frog leptin had potential inhibitory effects on appetite in midprometamorphic tadpoles and juvenile frogs (Crespi and Denver, 2006). In mammals, leptin suppressed food intake mediated by hypothalamic regulation (Schwartz et al., 1996, 2000; Stephens et al., 1995). NPY and POMCs, which are synthesized in the hypothalamus, are important factors for the regulation of feeding behavior in mammals (Hillebrand et al., 2002), as well as in fish (Kiris et al., 2007; Lopez-Patino et al., 1999; Silverstein and Plisetskaya, 2000). Leptin treatment could suppress food intake by decreasing hypothalamic NPY mRNA level and increasing POMCs mRNA levels in rodents (Schwartz et al., 1997; Thornton et al., 1997). Very recently, Murashita et al. (2008) confirmed that acute (8 h) IP injection of homologous leptin suppressed food intake and hypothalamic NPY mRNA level in rainbow trout while the mRNA levels of POMCs A1 and A2 were elevated, suggesting that trout leptin regulated food intake through the hypothalamus as it did in mammals. In the present study, peripheral rgc-LEP significantly ($p < 0.05$) reduced the food intake and brain NPY mRNA level of grass carp on the first day after injection. These results are consistent with the notion that the neurocircuitry pathways that control feeding by leptin were ancient and had been conserved through evolution (Murashita et al., 2008).

In African clawed frog and mammals, leptin regulates energy expenditure. To date, this effect has not yet been confirmed in fish. In this study, we assessed the expression of several critical genes that are regulated by leptin to investigate the energy expenditure effect of leptin in grass carp. SCD1 is a key enzyme involved in

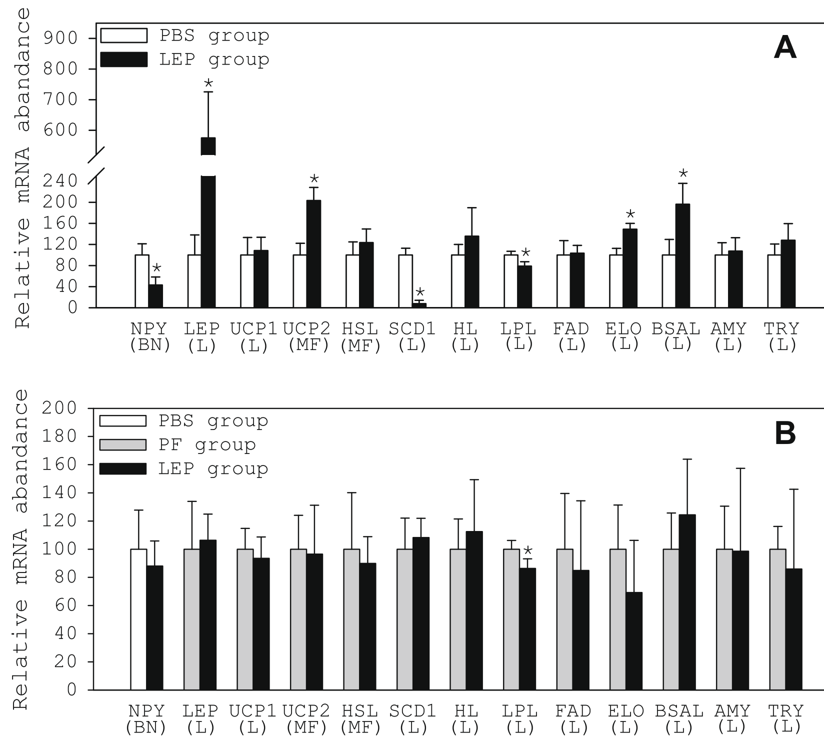


Fig. 7. Acute and chronic effects of IP administration of rgc-LEP on gene expression in grass carp. (A) Acute effect of rgc-LEP (2.1 μg/g BW; sampled at 2 h after injection; $n = 6$) on gene expression. (B) Chronic (13 days) effect of rgc-LEP (2.1 μg/g BW/day; sampled at 13th day, 2 h after injection; $n = 6$) on gene expression. PBS group, PBS injection group; LEP group, rgc-LEP injection group; PF group, PBS injection and pair feeding group. BN, brain; L, liver; MF, mesenteric fat. NPY, neuropeptide Y; LEP, leptin, UCP1, uncoupling protein 1; UCP2, uncoupling protein 2; HSL, hormone sensitive lipase; SCD1, Stearoyl-CoA desaturase 1; HL, hepatic lipase; LPL, lipoprotein lipase; FAD, fatty acid desaturase; ELO, fatty acid elongase; BSAL, bile salt-activated lipase; AMY, pancreatic amylase; TRY, pancreatic trypsin. Gene expression was normalized to the housekeeping gene (β-actin) and the gene expression of control group (PBS group or PF group) was set to 100. The results are expressed as mean ± SEM. Significance was compared with the PBS group or the PF group ($^{*}p < 0.05$).

the biosynthesis of monounsaturated fatty acid. Its mRNA level and enzyme activity were down-regulated by leptin in mice (Cohen et al., 2002). In the present study, the mRNA level of SCD1 was significantly decreased, which is in agreement with the observation in mice. This suggests that grass carp leptin might exert its metabolic effects through inhibition of this enzyme, similar to the case in mammals. Thus, SCD1 seems to be a potentially important target for studying leptin's metabolic actions in grass carp. HSL is the rate-limiting enzyme responsible for the intracellular triacylglycerol hydrolysis and fatty acid mobilization in adipose tissue. Its mRNA level was significantly elevated by leptin treatment in mice (Zhang et al., 2008). In the present study, HSL mRNA level was increased 1.24-fold by leptin treatment. UCP2 is involved in transporting fatty acids, and the up-regulation of this protein following leptin treatment would increase fatty acid transport across the mitochondrial membrane and enhance lipid β-oxidation (Fleury et al., 1997; Liang et al., 2003). Here, the expression of UCP2 mRNA was significantly ($p < 0.05$) increased. Our finding coincides with the observation in rats, which administration of leptin increased inguinal adipose tissue expression of the UCP2 transcript, with no change in mRNA level of UCP1 in liver (Scarpace et al., 1998; Zhang et al., 2008). Taken together, the data presented here suggests that leptin might have a potential effect on energy expenditure in grass carp.

Furthermore, leptin also acts as a regulator of lipid metabolism, which possibly interacts with its effects on food intake and energy expenditure to regulate body fat deposition. In this study, several lipid metabolism-related genes were selected for assessing their expression to evaluate this effect of leptin in grass carp. LPL acts on triacylglycerols in plasma lipoproteins to release fatty acids which are transported to the adipose tissue for storage or to other organs, such as muscle, for oxidation (Robinson, 1970; Auwerx et al., 1992). An inhibitory effect on LPL activity was observed in adipose tissues by leptin treatment in mice (Picard et al., 1998). Our previous studies demonstrated hepatic LPL mRNA level of red sea bream (*Pagrus major*) was regulated by the nutritional conditions (Liang et al., 2002a, 2002b). In the present study, grass carp liver LPL mRNA level of the LEP group was only 79% ($p < 0.05$) compared with that of the PBS group, suggesting that leptin has a suppressive effect on LPL as it did in mice. ELO is a critical enzyme in the pathways for the elongation of C18 polyunsaturated fatty acids to C20/22 highly unsaturated fatty acids, which are crucial to the nutritional health, physiology and reproductivity of higher vertebrates (Simopoulos, 2000). BSAL is an enzyme with pleiotropic biological functions. It participates in the hydrolization of triacylglycerides, phospholipids, esters of cholesterol and of lipid-soluble vitamins, and in the intestinal free cholesterol absorption, acting as a cholesterol transfer protein (review in Lombardo, 2001). To date, the effect of leptin on the expression of ELO and BSAL genes has not been investigated. Here, we found that the mRNA levels of ELO and BSAL were increased 1.49-fold and 1.96-fold after leptin treatment, respectively. No significant changes were observed in the mRNA levels of HL and FAD, the other two lipid metabolism-related genes. Taken together, LPL, ELO and BSAL, which are important enzymes correlating to lipid metabolism, were substantially altered by leptin, implying that leptin can regulate lipid metabolism in grass carp.

For better understanding of rgc-LEP's action in grass carp, the mRNA levels of key digestive enzymes (AMY and TRY) and LEP itself were also assessed. The mRNA levels of grass carp AMY and TRY showed no significant changes between the LEP group and PF group whereas the mRNA level of LEP was elevated 5.76-fold

on the first day after rgc-LEP treatment. In contrast, the expression of leptin gene was down-regulated by exogenous leptin in mice (Zhang et al., 1997, 2008). It is interesting to speculate the reason why leptin injection elevated leptin mRNA level in grass carp. To date, the effect of exogenous leptin on the expression of leptin gene in fish has never been reported. Whether this effect also exists in other fish species or not, awaits future research. However, it should be noted that grass carp is a special fish species with enormous appetite and heavy mesenteric fat deposition. The positive feedback loop of leptin might provide a possible way for grass carp to effectively inhibit its enormous appetite acutely.

In African clawed frog and rodents, chronic administration of leptin significantly reduced food intake and body weight (Crespi and Denver, 2006; Halaas et al., 1995; Zhang et al., 2008). Leptin could significantly reduce body weight within 2 weeks and its actions were persistent (Crespi and Denver, 2006; de Pedro et al., 2006; Zhang et al., 2008). In the present study, we daily IP injected rgc-LEP to grass carp for 13 days. Although strong anorexic effect and regulation the energy expenditure of rgc-LEP were observed on the first day after injection, we did not find differences in the feeding behavior, body weight or the expression of selected genes (except LPL) during the following days or at the end of the experiment. These data suggest that grass carp could resist to the actions of rgc-LEP after repeated injections. In coho salmon, long-term (28 days) peripheral human leptin did not alter growth, energy stores, gonad weight, pituitary content of follicle-stimulating hormone, or plasma levels of insulin-like growth factor-I, insulin, growth hormone, or thyroxine, under both fed and fasted conditions (Baker et al., 2000). On the other hand, chronic administration of murine leptin to green sunfish did not affect body weight or the activity of several enzymes, which are important indicators of intracellular fat metabolism (Londraville and Duvall, 2002). However, it should be noted that both of the experiments used a mammalian leptin and did not examine its acute effects on the feeding behavior or the expression of genes that can be induced by leptin. In addition, leptin mRNA expression in common carp changed acutely after food intake, but the involvement of leptin in the long-term regulation of food intake and energy metabolism was not evident from fasting for days or weeks or long-term feeding to satiation (Huising et al., 2006), indicating that the pattern of regulation of leptin expression in fish is different from that of mammals.

In summary, we have characterized the gene structures of grass carp and silver carp leptins (both belonging to leptin-a type), and studied the acute and chronic effects of rgc-LEP on the food intake and mRNA expression of key genes correlating to food intake, energy expenditure, lipid metabolism and digestion in grass carp. The results suggest that the neurocircuitry pathways that control food intake and energy expenditure by leptin were ancient and had been conserved through evolution. However, in contrast to African clawed frog and mammals, the effect of leptin on food intake and energy expenditure in grass carp is acute, but not chronic.

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