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Research Paper

Signal transduction mechanism for glucagon-induced *leptin* gene expression in goldfish liver

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Abstract

Leptin is a peripheral satiety hormone that also plays important roles in energy homeostasis in vertebrates ranging from fish to mammals. In teleost fish, however, the regulatory mechanism for leptin gene expression still remains unclear. In this study, we found that glucagon, a key hormone in glucose homeostasis, was effective at elevating the *leptin*-AI and *leptin*-AII transcript levels in goldfish liver via both *in vivo* intraperitoneal injection and *in vitro* cells incubation approaches. The responses of *leptin*-AI and *leptin*-AII mRNA to glucagon treatment were highly comparable. In contrast, blockade of local glucagon action could reduce the basal and induced *leptin*-AI and *leptin*-AII mRNA expression. The stimulation of leptin levels by glucagon was caused by the activation of adenylate cyclase (AC)/cyclic-AMP (cAMP)/ protein kinase A (PKA), and probably cAMP response element-binding protein (CREB) cascades. Our study described the effect and signal transduction mechanism of glucagon on leptin gene expression in goldfish liver, and may also provide new insight into leptin as a mediator in the regulatory network of energy metabolism in the fish model.

Key words: leptin, glucagon, gene expression, signal transduction, goldfish.

Introduction

Leptin, the protein product of the *obese* gene [1], is one of the most prominent adipose tissue-derived hormones and plays a key role in the regulation of appetite and metabolism in vertebrates ranging from fish to mammals [2-4]. In mammals, the adipose mRNA and serum protein levels of leptin are positively correlated with body adipose mass [5, 6]. The mammalian serum leptin levels are decreased prior to the depletion of adipose tissue mass by fasting [7], rapidly restored by re-feeding [8], and increased by overfeeding [9]. A number of peptide and non-peptide hormones have been found to mediate leptin production and secretion in mammalian models. Cholecystokinin [10] and ghrelin [11], two gastrointestinal appetite-regulated hormones, are effective at stimulating leptin secretion in rats. The sexual steroid hormones estrogen and androgen may play opposite roles, which are positive and negative, respectively, in the regulation of leptin gene expression in rat fat cells [12] and human placental cells [13]. In addition, insulin is another major stimulator of leptin production and secretion in rat and mouse adipocytes [14, 15], while its homologous hormone insulin-like growth factor-I (IGF-I) may suppress leptin secretion in rats [16].

The anorexigenic effects of leptin have been described in teleost fish [17, 18] as well as in mammals [19]. The nutritional status is shown highly correlated to leptin levels in fish but controversial between different species [4]. Following food deprivation, the plasma leptin content and/or hepatic *leptin* transcript levels are elevated in rainbow trout [20], salmon [21], flounder [22], grouper [23] and minnows [24]. Re-feeding may rapidly eliminate the up-regulation of

leptin protein and/or mRNA levels in flounder [22], grouper [23] and minnows [24] that is caused by starvation. In contrast, neither short- nor long-term fasting nor subsequent re-feeding affected the hepatic *leptin* expression in common carp [25], and the hepatic *leptin* mRNA level increased at a long time after feeding in goldfish [26]. However, the regulatory mediator that links the nutritional status and leptin level in fish is still unclear.

Glucagon is a key metabolism-regulated hormone that is produced in α-cells of the pancreas in mammals. It works to raise the concentration of glucose in plasma, and its effect is opposite to that of insulin, which lowers the circulating glucose levels [27]. During fasting, glucagon is a primary regulator of hepatic glucose production and release [27]. The *proglucagon* [28] and *glucagon receptor* [29] cDNAs have been previously identified in goldfish. Glucagon is effective at the stimulation of glucose production in goldfish hepatocytes, and both goldfish and human glucagons can bind the goldfish glucagon receptor and activate its intracellular signal pathways [29]. In mammalian and non-mammalian species, glucagon can regulate the production and/or secretion of other hormones, e.g., ghrelin in rats [30], somatostatin in rainbow trout [31] and fibroblast growth factor in rats [32]. However, as reported in sheep, glucagon has no effects on blood leptin levels [33, 34]. To our knowledge, the effect of glucagon on leptin regulation in fish has not yet been examined previously.

Phylogenetically, the aspect of *leptin* genes in teleost fish is more complicated than that in mammals [4, 35]. This is partially due to the fish-specific genome duplication (FSGD or 3R) that occurred in teleosts [36]; duplicated *leptin* genes (*leptin*-A and *leptin*-B) have been reported in some fish species, whereas only a single *leptin* gene is found in mammals [1, 4, 37]. The receptor binding affinity of fish leptin-A is higher than that of leptin-B [38, 39], indicating that leptin-A is the dominant form of leptin in fish. Moreover, another genome duplication event, also called tetraploidization, in cyprinids [25] and salmonids [40] resulted in up to four *leptin* paralogs in these species. In goldfish, two *leptin* genes (GenBank: FJ534535 and FJ854572) have been reported. They are both phylogenetically clustered with other Cyprinidae *leptin*-A and therefore named goldfish *leptin*-AI and *leptin*-AII, respectively [26, 41]. However, the *leptin*-B has not yet been reported in goldfish. Recombinant goldfish leptin-AI and leptin-AII protein were effective at inhibiting feeding behavior and reducing food consumption of goldfish via mediating of the transcript levels of some central appetite regulators [17]. The liver is the metabolic center in fish, and it is also the major site for leptin expression in fish [25, 42]. To date, studies on the hormonal regulation of leptin in fish remain limited, except for finding that prolactin suppresses leptin-A synthesis in tilapia [43], and the estrogen stimulates *leptin*-A gene expression in minnows [24]. To shed light on the regulatory mechanism of leptin in fish, using goldfish as a model, we detected the effects of glucagon on goldfish hepatic *leptin*-AI and *leptin*-AII gene expression using both an *in vivo* approach with intraperitoneal (IP) injection and an *in vitro* approach with primary hepatocyte incubation. To further elucidate the signaling mechanisms in this pathway, the functional roles of adenylate cyclase (AC), cyclic-AMP (cAMP), protein kinase A (PKA) and cAMP response element-binding protein (CREB) in gulcagon-regulated goldfish *leptin* mRNA expression were also examined in primary cultured hepatocytes using pharmacological approaches.

Materials and Methods

Animals

Goldfish (*Carassius auratus*) with body weights ranging from 25-30 g were acquired from local suppliers and maintained individually in 9 L tanks at 20-25 °C under a 12:12-h dark-light photoperiod with a regular feeding schedule (1 g food pellets per fish, once every day at 10:00 AM) for 14 days prior to the experiments. During the process of tissue sampling, the fish were anesthetized by 0.05% tricaine methanesulfonate (MS222, Sigma) prior to be being killed by spinosectomy according to procedures approved by the Ethics Committees of the South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Test substances

Human glucagon was purchased from Phoenix Pharmaceuticals. Glucagon antagonist (as reported by [29, 44] were synthesized by Sangon Biotech with purity >98% as determined by analytical HPLC. The amino acid sequences of human glucagon and glucagon antagonist are listed in Supplementary table 1. For pharmacological study, IBMX, forskolin, CPT-cAMP, 8-Br-cAMP, MDL 12330A and H89 were purchased from Calbiochem, NKH477 and SQ22536 were purchased from Sigma, and KT5720 was purchased from Tocris. Test substances were prepared as 1 mM or 10 mM frozen stocks in small aliquots and diluted with pre-warmed culture medium to appropriate concentrations 15 min prior to drug treatment.

Intraperitoneal injection and *in vivo* sample collection

The *in vivo* effects of glucagon on *leptin*-AI and

leptin-AII mRNA expression in goldfish livers were analyzed with an IP injection approach. After deep anesthesia with 0.05% MS222, 100 μL of glucagon solution in a concentration of 300 ng/g body weight (bwt) dissolved in freshwater fish physiological saline (FFPS, [45]) was injected into the peritoneal cavity using a 23-gauge needle attached to a 1 mL syringe, and injection of FFPS only was used as a control. The fish were killed at 0, 1.5, 3, 6, 12 and 24 h after injection, and the liver samples were collected, frozen in liquid nitrogen and stored at -80°C for RNA extraction and reverse transcription.

Isolation, primary culture and static incubation of goldfish hepatocytes

The regulation of *leptin*-AI and *leptin*-AII transcript levels by glucagon was further examined in goldfish primary hepatocytes. To prepare the goldfish hepatocytes, goldfish livers (n=5) was excised and washed three times in ice-cold Ca^{2+}/Mg^{2+}-free HBSS (Gibco). The liver fragments were diced to 0.5 mm in thickness by means of a McILwain tissue chopper (Ted Pella) and incubated in Ca^{2+}/Mg^{2+}-free HBSS with EDTA (1 mM) at room temperature for 5 min and digested with Ca^{2+}/Mg^{2+}-free HBSS containing collagenase type IV (1 mg/mL, Invitrogen) and DNase II (0.01 mg/mL, Sigma) at 28 °C for 30 min. Next, the liver fragments were mechanically dispersed into single cells by gentle pipetting. The dispersed hepatocytes were then separated from the remaining fragments by filtration through a sterile 30 μm mesh and harvested by centrifugation at 100×g for 5 min at 4 °C. The hepatocytes obtained were resuspended in DMEM/F-12 (Gibco). The viability of the cells was assessed using a Trypan blue exclusion assay and only preparations with more than 95% viability were used in subsequent experiments. The cells were diluted to 0.4×10^6 cells/mL/well in DMEM/F-12 and seeded onto 24 well-plates precoated with PEI (5 μg/mL, Sigma), then incubated in 5% CO_2 and saturated humidity at 28 °C overnight for recovery. On the second day after cell preparation, test substances prepared in DMEM/F12 medium were gently overlaid onto hepatocytes after the removal of old culture medium. The cells were incubated with the test substances for another 1.5 to 48 h for time course studies, or for 6 h for other dose-dependent or pharmacological studies. Finally, the cells were harvested by dissolving in TRIzol reagent (Invitrogen).

Measurement of goldfish *leptin*-AI and *leptin*-AII mRNA levels

Total RNA from *in vivo* liver samples and *in vitro* hepatocytes samples was isolated by using TRIzol, digested with DNase I (Invitrogen), and reverse transcribed with a PrimeScript™ RT kit (TaKaRa). Transcriptional expression of *leptin*-AI and *leptin*-AII, *leptin receptor*, *preproinsulin*, *IGF*-I and *IGF*-II were detected using SYBR Premix Ex Taq™ II (TaKaRa) in the RotorGene RG-3000 Real-Time PCR System (Qiagen) with primers and PCR conditions as shown in Supplementary table 2. Serially diluted plasmid DNAs containing the ORF sequences for the target genes were used as the standards for the real-time PCRs. After reactions, the identity of the PCR products was routinely confirmed by analysis of melting curve. In this case, the PCR primers for *leptin*-AI or *leptin*-AII amplification were specific, without cross-interaction of the other isoform.

RT-PCR for goldfish *leptin*-AI, *leptin*-AII, *glucagon* and *glucagon receptor* transcripts

For the detection of *leptin*-AI (FJ534535), *leptin*-AII (FJ854572), *proglucagon* (U65528) and *glucagon receptor* (AY584244) transcripts, cDNA samples from goldfish liver and primary hepatocytes were subjected in RT-PCRs with specific primers (listed in Supplementary table 3) for these genes, and *β-actin* (AB039726) was used as an internal control.

Measurement of cAMP production

After an overnight incubation, the old DMEM/F12 medium for the goldfish hepatocytes was replaced with 0.9 mL HHBSA medium (Gibco) supplemented with IBMX (0.1 mM) and incubated at 28 °C for 15 min before adding 0.1 mL of 10×stock solutions of glucagon. The duration of the drug treatment was routinely fixed at 20 min. After that, the culture medium was harvested for the measurement of cAMP release, and cellular cAMP was extracted by adding lysis buffer. The cAMP content was quantified using a Cyclic-AMP XP® Assay Kit (Cell Signaling). cAMP production was defined as the sum of cAMP release and cellular cAMP content.

Western blot of hepatocyte lysates

For western blots, goldfish hepatocytes were seeded in PEI-coated 35 mm dishes at a density of 2.0×10^6 cells/mL/well. After an overnight incubation, the culture medium was replaced with new DMEM/F12 medium containing the appropriate concentrations of test substances and cultured at 28 °C. The glucagon incubation lasted from 0 to 240 min, and for other pharmacological studies, the treatment time was fixed at 30 min. After that, the cells were rinsed with PBS (pH 7.4) and cell lysate were prepared in RIPA buffer (Sangon Biotech) with complete protease and phosphatase inhibitor cocktails (Roche). The cell lysates were resolved on a 15% gel

by SDS-PAGE and transblotted onto a PVDF membrane (Roche) at 20 V for 1 h with a Trans-Blot SD Electrophoretic Cell (Bio-Rad). The membrane was then blocked with 3% BSA in TBST (Sangon Biotech) and incubated overnight at 4 °C with antibodies for phospho-CREB (1:500, Abcam), total CREB (1:500, Abcam) and *ß*-actin (1:2000, Sangon Biotech). On the following day, HRP-conjugated goat anti-rabbit or rabbit anti-mouse IgG (1:2000, Calbiochem) was added and Immobilon Western (Pierce) was used as a HRP substrate for signal development.

Data transformation and statistical analysis

For mRNA expression, the raw data were expressed in terms of fmol target transcript per tube, and then were routinely normalized as the ratio of target gene to *β-actin* mRNA detected in the same sample. Given that no significant differences were noted for *β-actin* mRNA expression in our experiments, the raw data were simply transformed as a percentage of the mean values in the control group for statistical analysis. The data expressed as mean ± SE and were analyzed by using one-way ANOVA followed by Fisher's least significant difference (LSD) test with SPSS (IBM Software).

Results

In vivo regulation of *leptin*-AI and *leptin*-AII transcripts by glucagon in goldfish livers

To investigate the effect of glucagon on *leptin* gene expression in goldfish liver, the approach of IP injection was used. Glucagon mediated a time-dependent increase in hepatic *leptin*-AI (Fig. 1A) and *leptin*-AII (Fig. 1B) transcript expression. The maximal stimulatory responses for *leptin*-AI and *leptin*-AII mRNA to glucagon administration were observed at 12 h (~3.1-fold) and 6 h (~3.9-fold), respectively.

In vitro regulation of *leptin*-AI and *leptin*-AII transcripts by glucagon in goldfish primary hepatocytes

To further confirm whether the regulation of *leptin* expression was directly affected by glucagon, glucagon was routinely added to goldfish primary hepatocytes for static incubation. Similarly, in *in vitro* primary cell culture, the basal levels of *leptin*-AI and *leptin*-AII mRNA were stimulated by glucagon administration in time-dependent manners (Fig. 2A). The maximal effects of glucagon on mRNA expression for both genes were observed at 6 h after treatment. Additionally, increasing concentrations of glucagon could elevate transcriptional levels of *leptin*-AI and *leptin*-AII in dose-dependent manners (Fig. 2B), and the maximal responses to glucagon for both *leptin* isoforms were observed with a dose of 1000 nM. In parallel experiments, high dosage (1000 nM) of glucagon treatment significantly reduced the mRNA levels of *leptin receptor* and *preproinsulin*, but showed no effect on *IGF*-I and *IGF*-II transcripts (Fig. 2C).

Blockade of local glucagon action on *leptin*-AI and *leptin*-AII mRNA expression

Using RT-PCR, the transcripts for *leptin*-AI, *leptin*-AII, *proglucagon* and *glucagon receptor* could be detected in goldfish livers and primary hepatocytes samples (Fig.3A). Additionally, incubation with a glucagon antagonist (1 μM) could reduce the basal mRNA levels of *leptin*-AI and *leptin*-AII, and abolish the increases in both genes induced by glucagon treatment (Fig. 3B).

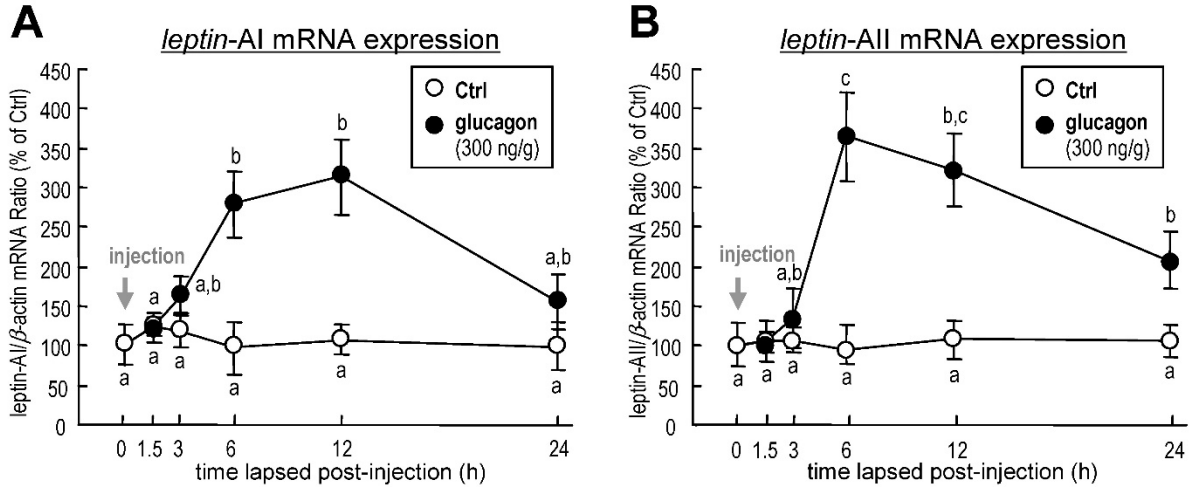

Figure 1. Changes in *leptin*-AI (A) and *leptin*-AII (B) mRNA expression in goldfish liver after IP injection of glucagon (300 ng/g bwt) at selected time points (0, 1.5, 3, 6, 12 and 24 h). In this study, the expression level at time 0 was used as the control group, and real-time PCR for *β-actin* was used as the internal control. The data obtained (n=10) at various time points were then normalized as a percentage of the control group at time 0 h. The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$).

Involvement of AC in glucagon-induced *leptin*-AI and *leptin*-AII expression

To examine the functional role of adenylate cyclase (AC) in glucagon-induced *leptin*-AI and *leptin*-AII gene expression, goldfish hepatocytes were incubated with increasing concentrations of the AC activator forskolin (1-100 nM) or NKH477 (1-100 nM). Similarly to glucagon stimulation, both forskolin and NKH477 could dose-dependently stimulate *leptin*-AI and *leptin*-AII transcript expression (Fig. 4A). In parallel experiment, the glucagon-induced *leptin*-AI and *leptin*-AII gene expression could be blocked by co-treatment with the AC inhibitor MDL12330A (20 μM) or SQ22536 (50 μM, Fig. 4B).

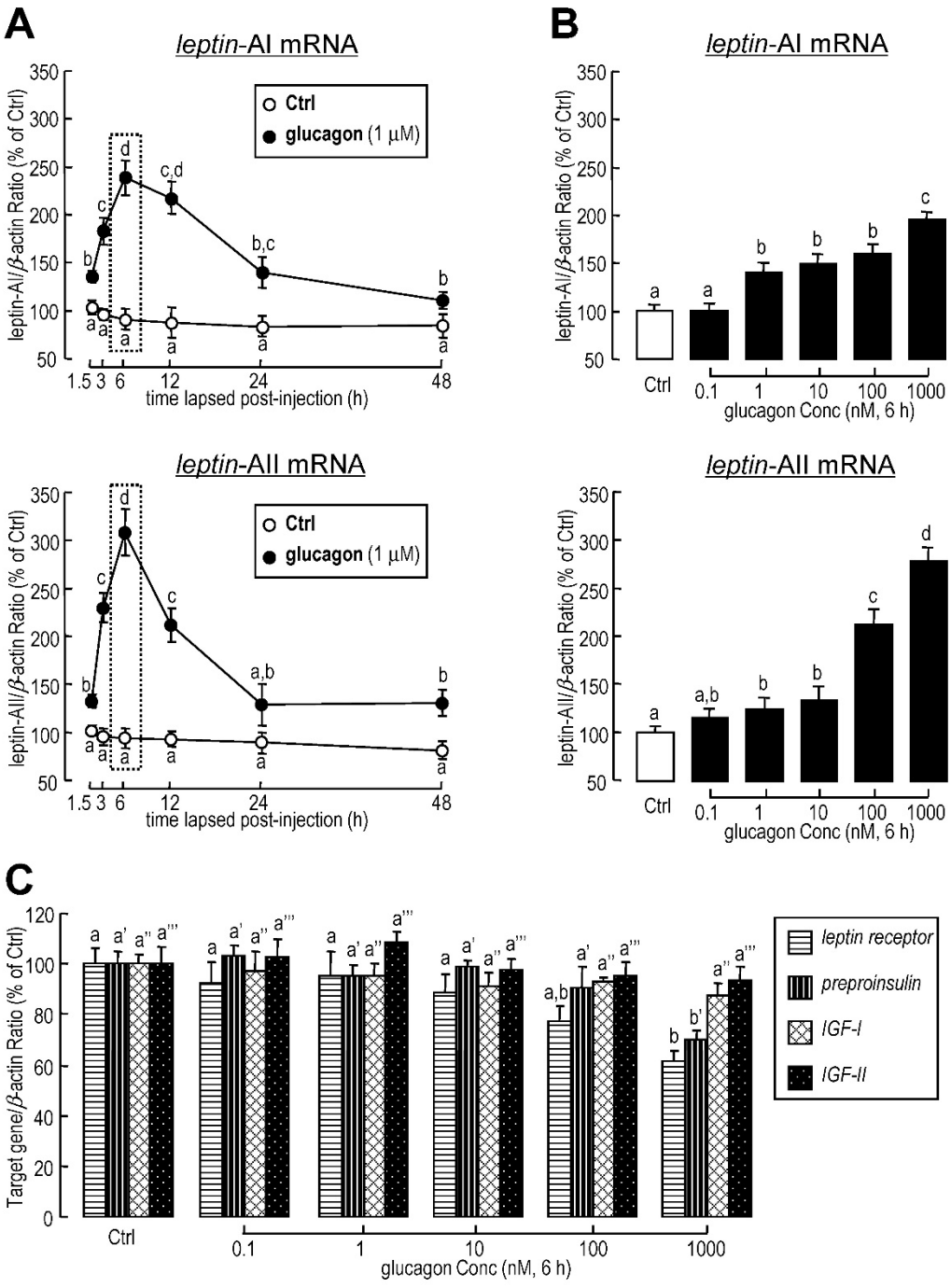

Figure 2. Effects of glucagon treatment on *leptin*-AI and *leptin*-AII transcripts in goldfish primary hepatocytes. A: Time course of glucagon treatment on *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocytes culture. Using static incubation, hepatocytes were exposed to human glucagon (1 μM) for the time indicated. The data obtained (n=4) at various time points were then normalized as a percentage of the control group at time 1.5 h. B: Dose-dependent effects of glucagon treatment on *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte culture. C: Dose-dependent effects of glucagon treatment on *leptin receptor*, *preproinsulin*, *IGF*-I and *IGF*-II mRNA expression in goldfish hepatocyte culture. Goldfish hepatocyte cultures were incubated for 6 h with increasing levels of human glucagon (0.1-1000 nM). The data obtained (n=4) with various concentrations of glucagon treatment were then normalized as a percentage of the control groups without glucagon. The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$).

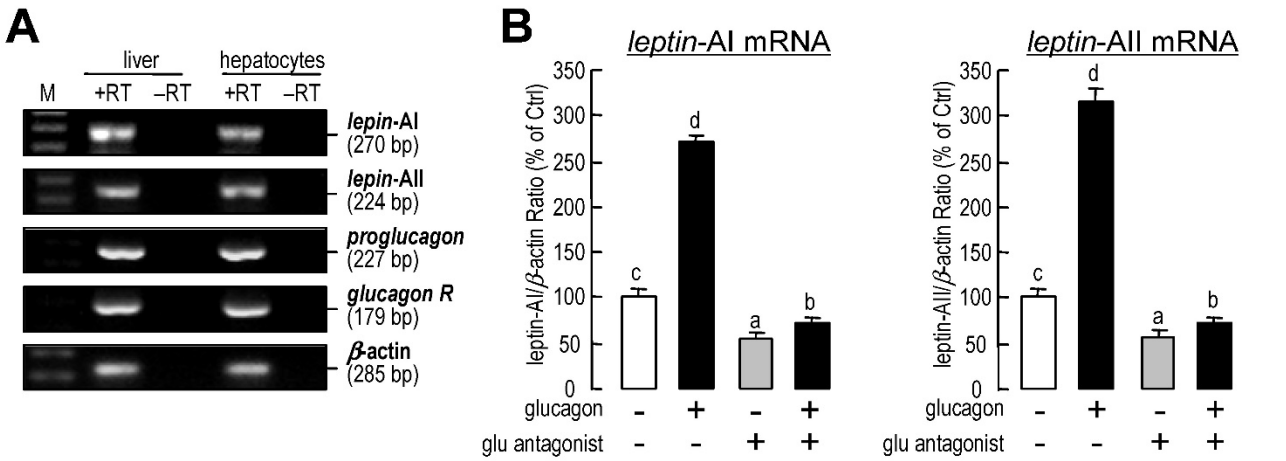

Figure 3. Autocrine/paracrine actions of glucagon on *leptin*-AI and *leptin*-AII transcripts in goldfish liver. A: Detection of *leptin*-AI, *leptin*-AII, *proglucagon* and *glucagon receptor* transcripts in goldfish livers. cDNA samples from goldfish livers and primary hepatocytes were used for detection and RNA samples reverse transcribed without the enzyme were used as negative controls. B: Effects of glucagon antagonist on glucagon-stimulated *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte cultures. The hepatocytes were incubated for 6 h with glucagon (1 μM) in the presence or absence of glucagon antagonist (1 μM). In this study, the data presented are expressed as the mean ± SE (n=4). The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$).

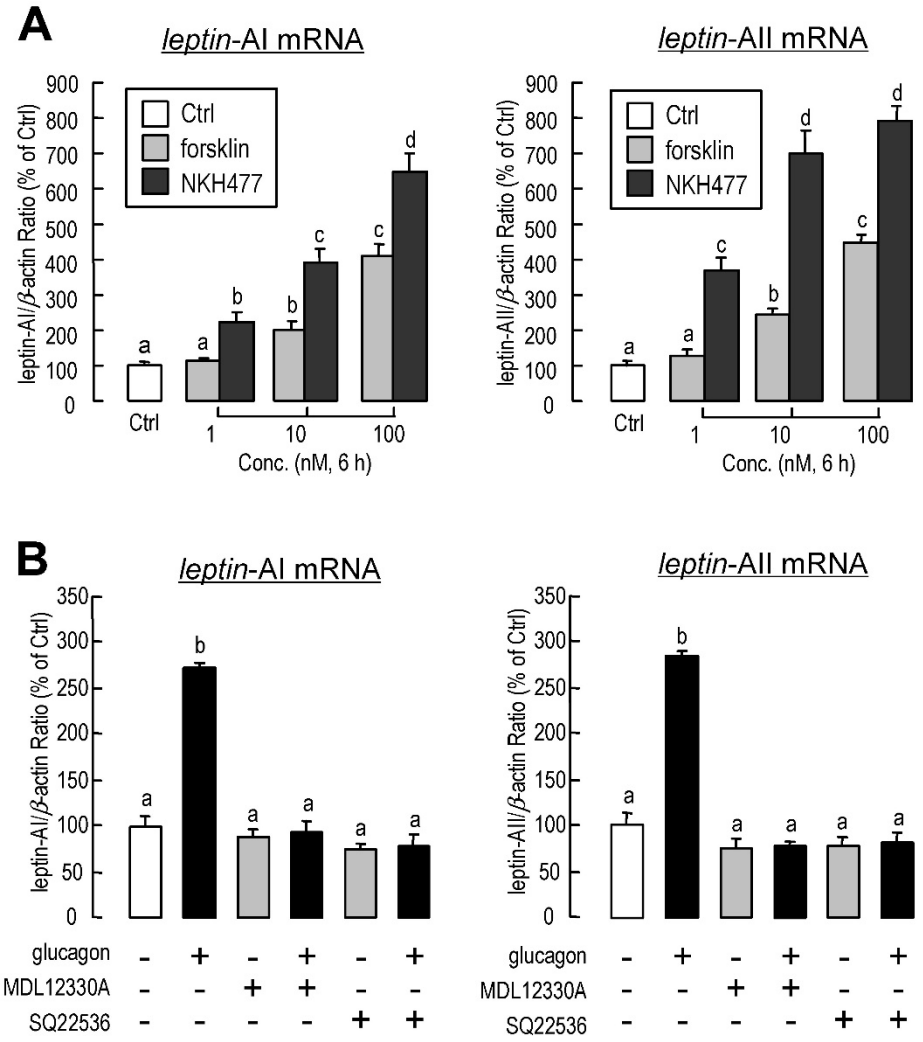

Figure 4. Involvement of AC in glucagon-induced *leptin*-AI and *leptin*-AII expression in goldfish livers. A: Effects of AC activators on *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte cultures. The hepatocytes were incubated for 6 h with increasing doses of forskolin (1-100 nM) or NKH477 (1-100 nM). B: Effects of AC inhibitors on glucagon-stimulated *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte cultures. The hepatocytes were incubated for 6 h with glucagon (1 μM) in the presence or absence of MDL12330A (20 μM) or SQ22536 (50 μM). In this study, the data presented are expressed as the mean ± SE (n=4). The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$).

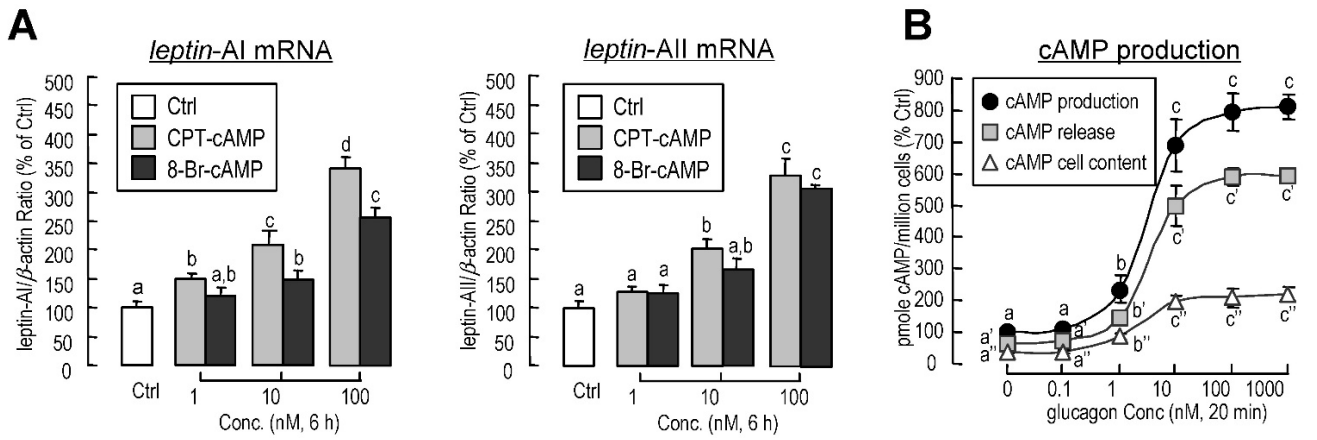

Figure 5. Involvement of cAMP in glucagon-induced *leptin*-AI and *leptin*-AII expression in goldfish livers. A: Effects of cAMP analogs on *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte cultures. The hepatocytes were incubated for 6 h with increasing doses of CPT-cAMP (1-100 nM) or 8-Br-cAMP (1-100 nM). B: Effects of glucagon on cAMP release, cellular cAMP content, and total cAMP production in goldfish primary hepatocytes. The hepatocytes pretreated with IBMX (0.1 mM) were then incubated for 20 min with increasing doses of glucagon (0.1-1000 nM). In this study, the data presented are expressed as the mean ± SE (n=4 and 3 for mRNA and cAMP measurement, respectively). The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$).

Involvement of cAMP in glucagon-induced *leptin*-AI and *leptin*-AII expression

To determine the participation of cyclic-AMP (cAMP) in glucagon-induced *leptin*-AI and *leptin*-AII mRNA expression, goldfish hepatocytes were incubated with increasing concentrations of the membrane-permeant cAMP analog CPT-cAMP (1-100 nM) or 8-Br-cAMP (1-100 nM). In this case, both CPT-cAMP and 8-Br-cAMP could elicit *leptin*-AI and *leptin*-AII mRNA expression in a dose-dependent manner (Fig. 5A). In addition, increasing concentration of glucagon was effective at stimulating cAMP release, cAMP cell content, and total cAMP production in goldfish hepatocytes in a dose-dependent manner (Fig. 5B).

Involvement of PKA and CREB in glucagon-induced *leptin*-AI and *leptin*-AII expression

The involvement of protein kinase A (PKA) and cAMP-response element binding protein (CREB)-related signal pathways in *leptin*-AI and *leptin*-AII regulation in goldfish hepatocytes was also evaluated in this study. Following co-incubation with the PKA inhibitor H89 (20 μM) or KT5720 (60 nM), the stimulatory effects of glucagon on *leptin*-AI and *leptin*-AII mRNA expression were abolished (Fig. 6A). On the other hand, glucagon was effective at triggering the phosphorylation of CREB in a time-dependent manner without affecting the total CREB content (Fig. 6B), and this stimulatory effect could be mimicked by incubation with the AC activator forsklin (1-100 nM) or the cAMP analog CPT-cAMP (1-100 nM, Fig. 6C), but blocked by co-treatment with the AC inhibitor MDL12330A (20 μM) or the PKA inhibitor H89 (20 μM, Fig. 6D).

Discussion

In mammals, the hyperglycemia-derived hormone insulin is one of the major stimulators for leptin production and secretion [15, 46]. In contrast, our current study showed that the hypoglycemia-derived hormone glucagon could induce *leptin* gene expression in goldfish. It is well known that glucagon and insulin exert opposite effects on serum glucose level through the differential regulation of glycogenolysis and gluconeogenesis [27]. The circulating insulin level has been found to increase with high serum glucose levels and decrease with low serum glucose levels, while the circulating glucagon level acts opposite to the insulin level [47]. The opposite regulatory mechanism for *leptin* gene expression between mammals and fish may be contributed by the fundamental difference in the metabolism system between endothermic and ectothermic vertebrates. Very interestingly, incubation of insulin or its paralog hormones, IGF-I and IGF-II, may suppress *leptin* mRNA level in the goldfish hepatocytes (Yan *et al*. unpublished data), suggesting that glucagon and insulin also exert opposite effects on *leptin* gene expression in this Cyprinidae species. In fish, leptin has been considered more as a metabolic regulator than a satiety factor. In tilapia, leptin may convert the hepatic glycogen into plasma glucose [48]. In *leptin receptor* knockout medaka, largely deposits of visceral fat were observed [49]. In *leptin*-A knockdown zebrafish embryos, the metabolic rate and oxygen consumption are significantly lower than in un-treated embryos [50]. In *leptin receptor* knockout zebrafish, it has been shown that leptin is not required for adipostasis, food intake, or reproduction, but retains a role in the regulation of

glucose homeostasis [51]. Additionally, the mRNA levels of *insulin* and *glucagon* are higher in *leptin receptor*-deficient zebrafish [51], implying an important link between leptin and the insulin/glucagon-controlled glucose homeostasis system.

Due to genome/single gene duplication events, teleost fish possess more abundant *leptin* genes than mammals. There is only one *leptin* gene in mammals, whereas duplicated *leptin* genes, namely *leptin*-A and *leptin*-B, have been identified in at least the *Beloniformes*, *Cypriniformes*, *Salmoniformes* and *Perciformes* lineages in fish [4]. In addition, the tetraploidization events within the carp and salmon genomes are likely the events that, independently from one another, gave rise to paralogous *leptin*-AI and *leptin*-AII in these species [25, 40]. The amino acid sequence homology shared between leptin-A and leptin-B are very low, e.g., 24.5% in zebrafish [37], 17.1% in medaka [52], and 19.5% in tilapia [39]. Likewise, the regulatory modules for fish *leptin*-A and *leptin*-B gene expression are distant. In grouper, only *leptin*-A, but not *leptin*-B, hepatic mRNA levels may increase after fasting [23]. In minnows, estrogen administration induced only *leptin*-A, but not *leptin*-B, hepatic mRNA expression [24]. In contrast, the amino acid sequence homology shared between leptin-AI and leptin-AII are much higher, e.g., 82.0% in

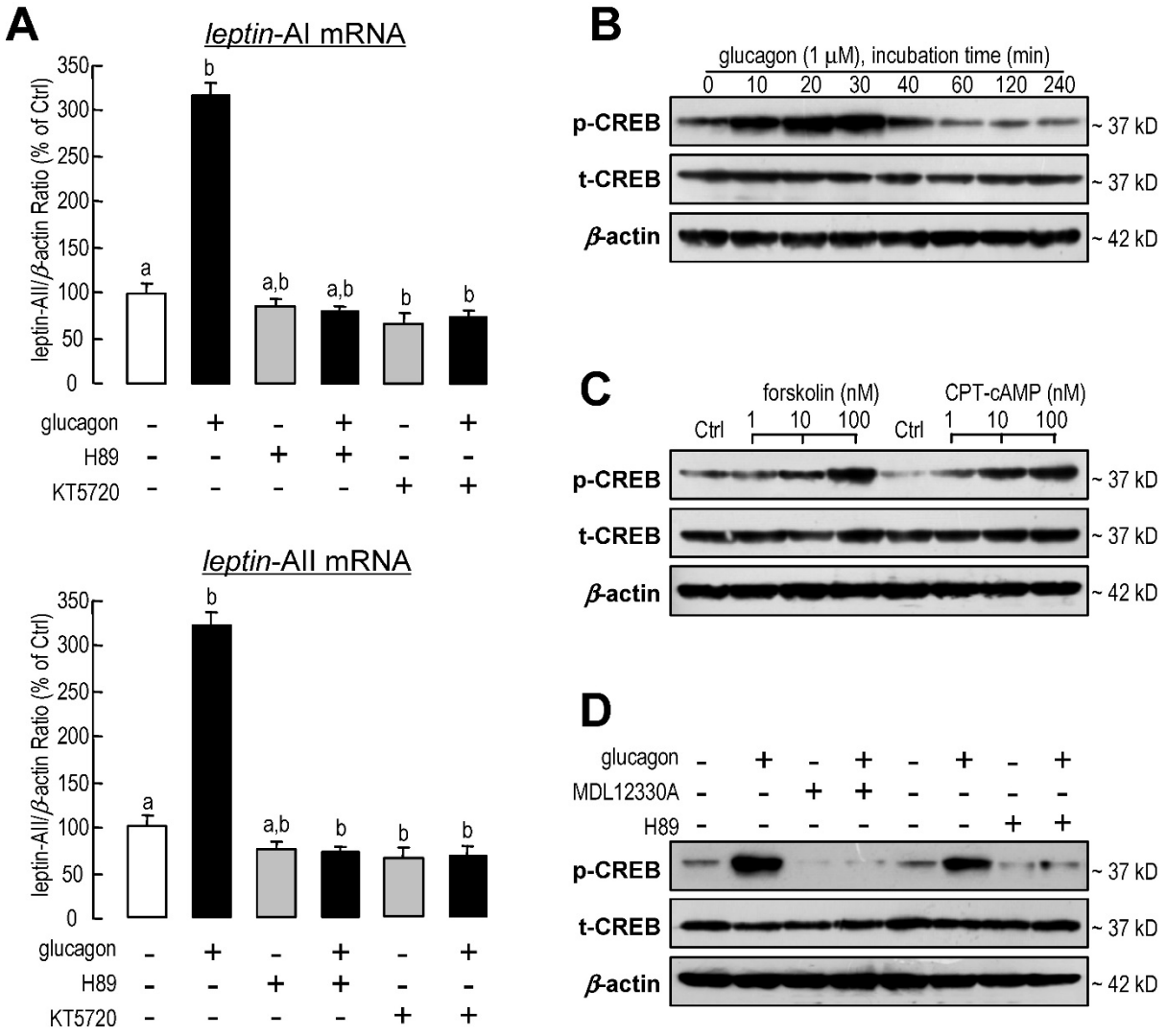

Figure 6. Involvement of PKA and CREB in glucagon-induced *leptin*-AI and *leptin*-AII expression in goldfish livers. A: Effects of PKA inhibitors on glucagon-stimulated *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocyte cultures. The hepatocytes were incubated for 6 h with glucagon (1 μM) in the presence or absence of H89 (20 μM) or KT5720 (60 nM). In this study, the data presented are expressed as the mean ± SE (n=4). The same letter represents a similar level of transcriptional expression ($P > 0.05$), and the different letter represents significant difference in levels of transcriptional expression between two groups ($P < 0.05$). B: Glucagon-induced phosphorylation of CREB in goldfish hepatocytes. The hepatocytes were treated with glucagon (1 μM) for 0, 10, 20, 30, 40, 60, 120 or 240 min. C: Effects of an AC activator and cAMP analog on the phosphorylation of CREB in goldfish hepatocytes. The hepatocytes were incubated for 30 min with increasing doses of forskolin (1-100 nM) or CPT-cAMP (1-100 nM). D: Blockade of AC or PKA on glucagon-induced CREB phosphorylation in goldfish hepatocytes. The hepatocytes were incubated for 30 min with glucagon (1 μM) in the presence or absence of MDL12330A (20 μM) or H89 (20 μM). Cell lysates prepared for western blot were incubated with the antisera for phosphorylated and total CREB, and parallel blotting of *β*-actin was used as an internal control.

common carp [25], and 79.5% in goldfish. Previously studies showed that the anorexigenic effects of goldfish leptin-AI and leptin-AII in were highly comparable [17]. Our present study shows that the regulatory modules for goldfish *leptin*-AI and *leptin*-AII gene expression are also similar. The divergence of *leptin*-A and *leptin*-B is due to the FSGD approximately 296 million years ago (Mya, [53]), while the *leptin*-AI and *leptin*-AII differentiation due to the *Cypriniformes* genome tetraploidization is estimated to have occurred more recently (~16 Mya, [54]). Therefore, it is speculated that the regulatory modules change between *leptin*-A and *leptin*-B during evolution, whereas they remain conserved between *leptin*-AI and *leptin*-AII.

The liver is one of the most abundant sites for *leptin* expression in goldfish [26], and it is also the metabolic center in fish [55]. Glucagon is mainly produced in the gallbladder and intestine of goldfish [28], but its high expression signal could also be detected in the liver (Fig. 3A). Both goldfish and human glucagon display high affinity toward the goldfish glucagon receptor and are able to activate its intracellular signaling [29, 56]. The present study shows that glucagon is effective at stimulating goldfish hepatic *leptin* gene expression, and blockade of the endogenous glucagon by its antagonist could suppress the basal and glucagon-induced *leptin*-AI and *leptin*-AII mRNA levels (Fig. 3B). The opposite effects by glucagon blockade also prove that glucagon is a stimulator for *leptin*-AI and *leptin*-AII gene expression in goldfish liver. In addition, glucagon treatment reduced the goldfish hepatic *preproinsulin* mRNA level (Fig. 2C), similar to that previously reported in the rat pancreatic β-cells [57]. Glucagon administration also suppressed the expression of *leptin receptor* transcript (Fig. 2C), and it is possible a mechanism for eliminating the local actions of the increased leptin. However, given that *leptin receptor* mRNA is predominant expressed in the central nervous system but not liver, how glucagon mediating the leptin signaling is still need to be investigated. Combined with our results, the metabolic hormones insulin, glucagon and leptin may serve as a regulatory network in the goldfish liver to respond different nutrient status.

The molecular mechanisms for glucagon in teleosts are mainly focused on its functions in gluconeogenesis and glycogenolysis [58, 59]. However, the glucagon-mediated signal transduction pathways responsible for *leptin* gene expression are totally unknown. Therefore, we further investigated this question in goldfish hepatocytes using pharmacological approaches. The glucagon receptor is a member of the class-B G-protein coupled receptors (G-PCR) with high expression levels in the goldfish livers [29]. Major consequences of glucagon binding to the glucagon receptor include the stimulation of AC activity, the production of intracellular cAMP and the subsequent activation of PKA and phosphorylation of CREB [27]. The cell signaling events following the activation of glucagon receptor in fish species is well documented to be coupled to the AC/cAMP/PKA cascades [29, 56]. In this study, the induction of goldfish hepatic *leptin*-AI and *leptin*-AII mRNA expression by glucagon could be mimicked by activating AC with its activator forskolin (Fig. 4A), and by blockade via co-treatment of the AC inhibitor MDL12330A (Fig. 4B), indicating the functional role of AC in glucagon-regulated *leptin* genes expression. In addition, cAMP, the down-stream signaling element of AC, was observed during glucagon-induced *leptin* genes expression. The involvement of AC and cAMP was supported by the result that cAMP production in goldfish hepatocytes could be elevated dose-dependently with glucagon treatment (Fig. 5B), and incubation of the membrane-permeant cAMP analog CPT-cAMP was effective at up-regulating *leptin*-AI and *leptin*-AII mRNA levels (Fig. 5A). Furthermore, the PKA inhibitor H89 abolished the stimulatory effects of glucagon on *leptin*-AI and *leptin*-AII mRNA expression (Fig. 6A), indicating that PKA is critical for the glucagon-mediated stimulation of *leptin* genes expression. On the other hand, glucagon treatment could trigger the phosphorylation of CREB in goldfish hepatocytes (Fig. 6B), and this phosphorylation could be mimicked by the AC activator forskolin and cAMP analog CPT-cAMP (Fig. 6C), but was abolished by co-treatment of the AC inhibitor MDL12330A or the PKA inhibitor H89 (Fig. 6D), indicating that AC/cAMP/PKA is an up-stream element of glucagon-induced CREB phosphorylation. Thus, CREB is probably a transcription factor for glucagon-induced *leptin*-AI and *leptin*-AII gene expression. Given that the time of glucagon action on *leptin* mRNA expression is much longer than that on CREB phosphorylation, there may be a more complicated mechanism for *leptin* gene transcription than that initiated by CREB, but further confirmation using promoter assay is needed. These results, as a whole, suggest that the AC/cAMP/PKA cascades, and probably CREB, may play a key role in the glucagon-mediated induction of *leptin* gene expression in the goldfish liver, as shown in Fig. 7.

In summary, we demonstrated using *in vivo* and *in vitro* approaches that glucagon stimulates *leptin*-AI and *leptin*-AII gene expression in goldfish liver. The responses of *leptin*-AI and *leptin*-AII to glucagon treatment are highly comparable, and blockade of

local glucagon action could reduce the basal and induced *leptin*-AI and *leptin*-AII mRNA expression. The stimulatory effects of glucagon on goldfish *leptin* gene expression are mediated by the intracellular AC/cAMP/PKA and probable CREB cascades (Fig. 7). This study exhibits the first evidence for glucagon-mediated regulation of *leptin* expression in lower vertebrates, and may provide new insight into leptin as a mediator in the regulatory network of energy metabolism in the fish model.

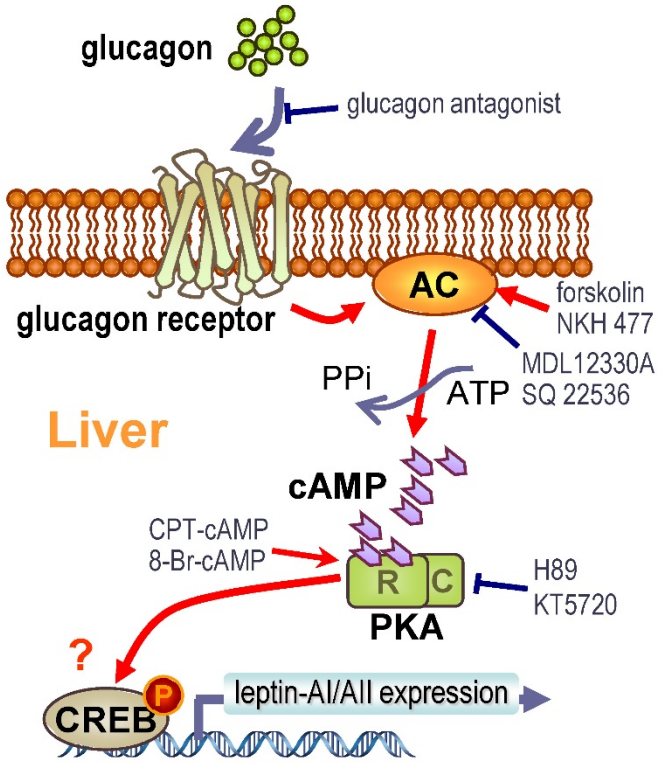

Figure 7. Working model for the signal transduction mechanism of glucagon-induced *leptin*-AI and *leptin*-AII mRNA expression in goldfish hepatocytes.

Supplementary Material

Supplementary data. 1 (Suppl. 1): Amino acid sequences of human glucagon and glucagon antagonist. Supplementary data. 2 (Suppl. 2): Primer sequences and real-time PCR conditions used in this study. Supplementary data. 3 (Suppl. 3): Primer sequences for goldfish *leptin*-AI, *leptin*-AII, *proglucagon* and *glucagon receptor* transcript detection. http://www.ijbs.com/v12p1544s1.pdf

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Author Contributions

TC and SC conceived of and designed the experiments. AFY, TC, SC, XJ and WH performed the experiments. AFY, TC, SC, CHR and FL analyzed the data. TC, CQH and DST contributed reagents/materials/analysis tools. AFY and TC wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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