Adropin induction of lipoprotein lipase expression in tilapia hepatocytes

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Abstract

The peptide hormone adropin plays a role in energy homeostasis. However, biological actions of adropin in non-mammalian species are still lacking. Using tilapia as a model, we examined the role of adropin in lipoprotein lipase (LPL) regulation in hepatocytes. To this end, the structural identity of tilapia adropin was established by 5'-3'-rapid amplification of cDNA ends (RACE). The transcripts of tilapia adropin were ubiquitously expressed in various tissues with the highest levels in the liver and hypothalamus. The prolonged fasting could elevate tilapia hepatic adropin gene expression, whereas no effect of fasting was observed on hypothalamic adropin gene levels. In primary cultures of tilapia hepatocytes, synthetic adropin was effective in stimulating LPL release, cellular LPL content, and total LPL production. The increase in LPL production also occurred with parallel rises in LPL gene levels. In parallel experiments, adropin could elevate cAMP production and up-regulate protein kinase A (PKA) and PKC activities. Using a pharmacological approach, cAMP/PKA and PLC/inositol trisphosphate (IP3)/PKC cascades were shown to be involved in adropin-stimulated LPL gene expression. Parallel inhibition of p38MAPK and Erk1/2, however, were not effective in these regards. Our findings provide, for the first time, evidence that adropin could stimulate LPL gene expression via direct actions in tilapia hepatocytes through the activation of multiple signaling mechanisms.

Key Words
- adropin
- lipoprotein lipase
- tilapia hepatocytes
- signal transduction pathway

Introduction

Adropin is a peptide hormone encoded by the energy homeostasis-associated (ENHO) gene. This peptide is highly conserved across different mammalian species. Among non-mammalian vertebrates, a homologue to mammalian adropin has not been identified. Although its receptor has not been identified in mammals, the main function of adropin is to maintain energy homeostasis and insulin response (Kumar et al. 2008). Transgenic over expression or systemic adropin treatments markedly attenuate insulin resistance and glucose intolerance in diet-induced obese mice (Kumar et al. 2008). In line with these observations, adropin knockout mice were associated with increased hepatosteatosis, adiposity and insulin resistance (Ganesh Kumar et al. 2012). Even though the
linkage between circulating adropin levels and other various pathophysiological conditions in mammals have been reported, the exact link with adropin remains elusive. Some studies showed that plasma adropin levels were linked with several metabolic diseases, including insulin resistance (Butler et al. 2012) and nonalcoholic fatty liver disease (Sayin et al. 2014) and endothelial dysfunction (Gozal et al. 2013), whereas other studies found that plasma adropin levels were not correlated with endothelial dysfunction in humans with heart failure (Lian et al. 2011) or with BMI in patients with type 2 diabetes mellitus (Topuz et al. 2013). Besides, adropin may be involved in the metabolic adaptation to fasting, although these data are still controversial. In fasting mice, adropin expression is attenuated compared with control values (Kumar et al. 2008), whereas plasma adropin levels in humans do not change in response to fasting (Ganesh Kumar et al. 2012). Unlike its mammalian counterparts, the physiological significance of adropin is still poorly understood in fish.

Lipoprotein lipase (LPL) is a secreted lipolytic enzyme that facilitates the hydrolysis of triglycerides (TG) in chylomicrons to release fatty acids for energy or storage (Davies et al. 2012). In mammals, LPL is expressed in neonate but not adult liver (Merkel et al. 1998). However, there is a re-expression of LPL mRNA in the adult rat liver after partial hepatectomy (Sabugal et al. 1996). In contrast, LPL is expressed in the liver of adult fish (Liang et al. 2002, Feng et al. 2014). Liver LPL targets circulating TG to the liver and increases ketone body production, which can be used as energy as an alternative to glucose (Merkel et al. 2002). The regulations of LPL expression in fish were reported to be affected by various factors, including, but not limited to, hormones (Albalat et al. 2007, Saera-Vila et al. 2007), fasting (Oku et al. 2006, Han et al. 2011) and feeding conditions (Oku et al. 2006, Benedito-Palos et al. 2013). Additional studies of LPL expression in the piscine liver will further our understanding of the mechanisms controlling hepatic lipid regulation and the formation of fatty liver. Recently, a transgenic study has revealed that adropin over-expression could reduce LPL gene expression in white adipose tissues of mice (Kumar et al. 2008). It raises the possibility that adropin may serve as an endocrine/paracrine factor to regulate LPL expression in fish. However, no information is available on the relationship between adropin and LPL gene expression in fish hepatocytes. To test the hypothesis, tilapia hepatocytes were used as a model to examine the hepatic actions and post-receptor signaling mechanisms for the adropin regulation of LPL gene expression.

Materials and methods

Animals

Sexually mature male tilapia (standard length: 11±0.5 cm, body weight: 50±5.0 g) were maintained in freshwater aquaria at 28 °C under a 14 h light:10 h darkness photoperiod for at least 7 days prior to tissue sampling and hepatocytes preparation. The fish were fed a commercial diet (40% protein, 12% fat, 2% fiber, 8.5% moisture, 8% ash; Tongwei, China) to satiety twice a day at 10:00 and 16:00 h. During the process of tissue sampling, the fish were sacrificed by spinosectomy after anesthesia with 0.05% MS222 (Sigma) according to the procedures approved by the Animal Ethics Committee of Sichuan University.

Test substances

The tilapia adropin mature peptide was synthesized by GL Biochem (Shanghai, China) using the standard procedures for solid phase peptide synthesis. As revealed by HPLC analysis, the purity of adropin was ≥95% and the homogeneity of the peptide was further confirmed by mass spectrometry. A synthetic peptide was dissolved in double-distilled deionized water and stored frozen in small aliquots at −80 °C as 0.1 mM stocks. Pharmacological agents, including H89, MDL12330A, 3-isobutyl-1-methylxanthine (IBMX), forskolin, 8cpt-2me-cAMP, 12-O-tetradecanoylphorbol-13-acetate (TPA), 2-APB, U73122 and GF109203X were obtained from Calbiochem (San Diego, CA, USA). The PKA peptide inhibitor and the myristoylated PKC peptide inhibitor were purchased from Promega. Similar to the peptide hormones, these pharmacological agents were prepared as 10 mM frozen stocks in DMSO. Stock solutions of test substances were diluted with prewarmed (28 °C) culture medium to appropriate concentrations 15 min prior to drug treatment. The final dilutions of DMSO were <0.1% and had no effects on LPL expression in tilapia hepatocytes.

Molecular cloning of tilapia adropin

Total RNA was extracted from tilapia liver using RNAzol (MRC, Cincinnati, OH, USA) and reversely transcribed using M-MLV (TaKaRa, Dalian, China). Based on the predicted cDNA sequence of tilapia adropin, gene-specific primers were designed and 5′/3′-RACE was conducted using a GeneRacer Kit (Invitrogen) and sequenced by ABI3100 Genetic Analyzer (BGI, Shanghai, China). The full-length cDNA sequences of tilapia adropin were

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subtracted to GenBank under an accession number KP890303. The amino acid sequence of tilapia adropin was aligned with that of other species by using the ClustalW program and MacVector V.9.5.2 programs (Accelrys, San Diego, CA, USA). Motifs were predicted using the Motif Scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Genomic Southern for tilapia adropin

To determine the gene copy number for adropin, a Southern blot was performed with genomic DNA prepared from tilapia as described previously (Jiang et al. 2008a). Briefly, the genomic DNA obtained was digested overnight at 37 °C with restriction enzymes including Hind III, Stu I, Kpn I and Sca I, respectively. On the following day, the digested products were size-fractionated in a 0.7% agarose gel, transblotted onto a positively charged nylon membrane and hybridized with the DIG-labeled probes for tilapia adropin. After that, hybridization signals were detected using a DIG Luminescent Detection Kit (Roche).

Tissue distribution of tilapia adropin

The tissue distribution of adropin was examined by using quantitative real-time PCR. After the RNA extraction and reverse transcription, the samples obtained were subjected to real-time PCR with primers specific for tilapia LPL (forward primer: 5' GTGCTGCTCGGTCTTACTA 3' and reverse primer: 5' CTGGGTTTCTGTTTACTA 3'). The PCR reactions were conducted with the following thermal cycling parameters: 94 °C for 3 min, followed by 35 cycles of amplification with denaturation at 94 °C for 5 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 s and then fluorescent signal collection at 80 °C for 1 s. Serial dilutions of plasmid DNA carrying the open reading frame (ORF) of adropin were used as the standards for data calibration. As an internal control, real-time PCR for 18S RNA was conducted using the primers specific for 18S RNA (forward primer: 5' GGACACGGGAAAGATGCCAG 3' and reverse primer: 5' GTGCTGCTCGGAATTAACCCAGAC 3'). In these experiments, no significant changes were observed for 18S RNA expression. The quantitative results were normalized as a ratio of the target gene/18S RNA expression level.

Expression of adropin under fasting conditions

Fifty tilapia were distributed in ten 70 liter tanks with five replicates each. The fish were allowed to acclimate to the tanks for 2 weeks prior to the beginning of the experiment. Ten fish, five fed and five fasted/re-fed, were periodically sampled in buckets containing 0.05% MS222 (Sigma) at days 0, 7, 14, and 21 of fasting (F0, F7, F14, and F21, respectively) and days 7 of re-feeding (RF7). Both liver and hypothalamus tissues were extracted and snap frozen in liquid nitrogen. After the RNA extraction and reverse transcription, real-time PCR was performed to determine the transcripts levels of adropin. Values were expressed as a percentage of the control-fed fish at each sampling time.

LPL gene measurement in cultured tilapia hepatocytes

Tilapia hepatocytes were prepared by the collagenase digestion method as described previously (Chow et al. 2004). Briefly, the liver fragments were digested in Ca²⁺/Mg²⁺-free Hank’s balanced salt solution (HBSS) medium with 0.5 mg/ml type IV collagenase (Sigma) for 30 min with constant shaking at 28 °C. After that, the cells were harvested by centrifugation at 50 × g for 5 min and resuspended in M199 medium (Invitrogen). Tilapia hepatocytes were seeded at a density of ~ 0.8 × 10⁶ cells/ml per well in polyethylenimine (PEI) (5 μg/ml) precoated 24-well culture and treated with hormones or drugs for the duration as indicated in individual experiments. After the RNA extraction and reverse transcription, the samples obtained were subjected to real-time PCR with primers specific for tilapia LPL (forward primer: 5' GTGTGGCCCA-CTGCTTAATG 3' and reverse primer: 5' AAGGTCTCCCGG-CAATTC 3'). Real-time PCR for LPL was performed with initial denaturation at 94 °C for 3 min followed by 35 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s and then fluorescent signal collection at 80 °C for 1 s. As an internal control, real-time PCR for 18S RNA was also conducted in each experiment. The quantitative results were normalized as a ratio of the target gene/18S RNA expression level.

Measurement of hepatocytes viability

Viability of hepatocytes was estimated using MTT assay as previously described (Fujii et al. 1995). Briefly, hepatocytes were seeded in 48-well plates at a density of 0.4 × 10⁶ cells/well and treated with different concentrations or time periods of adropin followed by MTT assay. Cell viability was estimated by determining the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The absorbance corresponding
to that of untreated control cells was assumed as 100% cell viability.

Lipoprotein lipase ELISA

LPL concentrations in conditioned medium or lysates were immediately determined by a colorimetric ELISA. The assay was optimized in our laboratory utilizing a monoclonal antibody against zebrafish LPL (Abmart, Shanghai, China) and a zebrafish LPL–HRP conjugate (GenScript, Nanjing, China). Briefly, tilapia hepatocytes prepared by collagenase digestion were seeded in 24-well plates at \( \sim 0.8 \times 10^6 \) cells/ml per well and incubated with test substances for the duration as indicated. After treatment, conditioned media were collected for monitoring LPL release, and the cells were rinsed with cold PBS before being lysed in a buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl) for the measurement of cell content. The total production of LPL in individual wells was deduced pro rata based on the protein data for release and cell content. The release and cell content were normalized to the cell numbers after culture.

Measurement of cAMP production

The tilapia hepatocytes were seeded at a density of \( \sim 3 \times 10^6 \) cells/ml per dish in PEI precoated 35-mm dishes and cultured overnight at 28°C as previously described (Jiang et al. 2008b, Jiang & Wong 2014). On the following day, culture medium was replaced with 0.9 ml HEPES-buffered HBSS with 0.1% BSA and 0.1 mM IBMX. IBMX, the inhibitor for phosphodiesterases, was included to prevent cAMP degradation in hepatocytes. Drug treatment was initiated with adropin with the increasing concentrations (1–1000 nM) and the cells were allowed to incubate at 28°C for 30 min. These cAMP samples were quantified by using a cAMP ELISA kit (EIAab Science Co., Ltd, Wuhan, China).

Measurement of PKA and PKC activity

Tilapia hepatocytes were seeded at a density of \( \sim 3 \times 10^6 \) cells/ml per dish in PEI precoated 35-mm dishes and cultured overnight at 28°C in M199. Drug treatment was initiated by adding 0.1 ml 10×stock solution of adropin with the increasing concentrations (1–1000 nM) and the cells were allowed to incubate at 28°C for 30 min. To assay protein kinase A (PKA) and PKC activity, the supernatant (0.3 μg) were incubated at 30°C for 20 min with 10 μM ATP, 0.5 μCi of \([\gamma-32P]\)ATP as previously described (Jiang et al. 2014) and a peptide substrates mixture from SignaTECT PKA and PKC assay systems (Promega). PKA and PKC activities were detected as described by the manufacturer.

Western blot analysis

Western blot analysis was performed as previously described elsewhere (Jiang et al. 2008b). The blots were incubated overnight at 4°C with primary antibodies. The antibodies that can detect the phosphorylated form and total content of PKA (1:1000; Abcam, MA, USA) and PKCβ (1:1000; Abcam, Cambridge, MA, USA) were obtained from commercial sources and used at the dilutions recommended by the manufactures. Finally, the immunoreactive bands were visualized by a chemiluminescence reagent (Pierce, Rockford, IL, USA).

Data transformation and statistics

Data presented, expressed as means ± S.E.M., are the results pooled from four to five experiments. A one-way ANOVA was conducted to test the differences of LPL expression. Two-way ANOVA was conducted to test the effects of the treatments (feeding and fasting/re-feeding) on adropin gene expression in the liver and hypothalamus. Multiple comparisons were conducted using the Fisher’s least significant difference test. The level of statistical significance was set at \( P<0.05 \).

Results

Molecular cloning of tilapia adropin cDNA

The tilapia adropin encoding sequence was found in the chromosome, scaffold GL831146.1 (location 7018079–7018143). Chromosomal synteny analysis revealed that the neighborhood genes around the tilapia adropin, including DNAI1 and FAM219A, are conserved in other vertebrates of adropin genes (Fig. 1A). To confirm that the predicted coding sequence of tilapia adropin is correct, the full-length cDNA for adropin was isolated from the tilapia liver using 5′/3′-RACE. The adropin cDNA is 427 bp in size with one putative polyadenylation signal in 3′UTR and an ORF of 210 bp encoding a 69 a.a. precursor for tilapia adropin (Fig. 1B). The tilapia adropin precursor is composed of a 37 a.a. signal peptide followed by a 32 a.a. mature protein with a deduced molecular weight of 7.4 kDa. Using the Motif Scan program, there are an N-myristoylation pattern (GLSTAA) and two casein kinase II phosphorylation sites (positions 42–45, SSPE; 53–56, SEAE). As shown in Fig. 1C, the sequences alignment at the...
protein level reveals that the a.a. sequence of tilapia adropin shares relatively high amino acid sequence identities with that of platyfish (87%), molly (82%) and stickleback (82%) but has limited identities with that of chicken (46%), alligator (44%), cow (36%), mouse (35%) and human (35%).

Gene copy number and tissue distribution of adropin expression

The gene copy number of adropin was examined in tilapia using Southern blot (Fig. 2A). As revealed by the hybridization signals using digoxigenin (DIG)-labeled cDNA probes for adropin, a single band was consistently observed in tilapia genomic DNA with prior digestion of Hind III, Stu I, Kpn I and Sca I respectively. These results indicate that the newly cloned adropin is a single-copy gene in the tilapia genome. To characterize the tissue expression profiles of adropin, real-time PCR was performed in RNA samples prepared from selected tissues. The expression patterns of adropin were ubiquitously detected in various tissues and brain areas. High levels of adropin expression were located in the liver and hypothalamus, to a lower extent in the cerebellum, intestine, kidney, medulla oblongata, muscle, olfactory bulbs, optic tectum, pituitary, spinal cord, spleen and telencephalon, and with low levels in the fat and stomach (Fig. 2B).

Effect of long-term fasting on the expression of adropin in the liver and hypothalamus

To assess the effects of prolonged nutrient restriction (fasting) and subsequent restoration (re-feeding) on the adropin gene expression, we investigated the gene

Figure 1

Molecular cloning and sequence alignment of tilapia adropin. (A) Comparison of adropin gene characterization between tilapia and other vertebrates. Adropin gene was located in a syntenic region conserved between humans, mice, chicken and tilapia. To determine the synteny, adropin and their flanking genes were mapped on the genome database of representative species using BLAT with a default setting on the ENSEMBL genome browser (http://www.ensembl.org). Dashed lines denote the genes of interest. Dotted lines indicate the syntenic genes identified in these species. Chr, chromosome. (B) Nucleotide and deduced a.a. sequences of tilapia adropin. Signal peptide was underlined. A putative polyadenylation signal in the 3' UTR was underlined in bold type. In the amino acid sequence, the potential N-myristoylation site (light gray boxed area) and two casein kinase II phosphorylation sites (empty boxed area) are indicated. An asterisk (*) represents the stop codon at the end of the ORF sequence (GenBank accession no. KP890303). (C) Alignment of tilapia adropin a.a. sequence with the corresponding adropin sequences reported in other species. Sequence alignment was conducted using the Clustal W algorithm with MacVector V. 9.5.2. The conserved a.a. residues in these sequences were boxed in gray. The sequences of adropin for other species were downloaded from the GenBank and/or by searching Ensembl genomes. GenBank accession numbers: human (XP_351809), mouse (NM_027147), cow (predicament adropin, NP_001157125), chicken (predicament adropin, NP_001289120) and alligator (predicated adropin, XP_006270011).
expression profiles of adropin by real-time PCR in the liver and hypothalamus of tilapia. Our results showed that the hepatic adropin gene was elevated approximately three-fold higher than that of fed controls after 7, 14, and 21 days of fasting (Fig. 3A). Following re-feeding for 7 days, the adropin gene in the liver returned to the continuously fed control level. By contrast, the abundance of the hypothalamic adropin gene was not affected by fasting and was similar to the fed control throughout the starvation studies. Also, there was no obvious change in hypothalamic adropin gene levels after 7 days of re-feeding (Fig. 3B).

**Up-regulation of hepatic LPL expression by synthetic adropin**

To examine the direct effect of adropin on LPL expression in hepatocytes, tilapia hepatocytes were incubated for 24 h with increasing levels (1–1000 nM) of adropin. After drug treatment, LPL protein expression was monitored by ELISA using an antibody for zebrafish LPL. Based on our validation, the antibody cross-reacts with tilapia LPL (data not shown). As shown in Fig. 4A, tilapia adropin was effective in elevating LPL release and cellular LPL content, as well as total LPL production. To test if the rise in LPL total production could be correlated with a corresponding elevation in the LPL gene expression, LPL gene levels in tilapia hepatocytes were also quantified by real-time PCR. In this case, LPL gene expression in tilapia hepatocytes was increased in a time-related manner with fixed concentrations of tilapia adropin (100 nM), and the maximal induction on LPL gene expression was observed at 24 h (Fig. 4B). In tilapia hepatocytes, adropin treatment could also be effective in inducing LPL gene expression in a dose-related fashion (Fig. 4C). The minimal effective doses for adropin stimulation could be observed at 10 nM level, while the maximal responses were noted in the 100–1000 nM dose range. In parallel studies,
Effects of adropin on LPL release, synthesis and gene expression in tilapia hepatocytes. (A) Dose-dependence of 24-h treatment with increasing levels of adropin (1–1000 nM) on LPL release, cell content and total production. After drug treatment, culture medium was harvested for the measurement of LPL release and cell lysate was prepared for monitoring LPL content in hepatocytes. In the data presented, means ± s.e.m. (n = 4) and groups denoted by the same letter represent a similar magnitude of LPL expression (P < 0.05, ANOVA followed by Fisher’s LSD test). (B) Effect of time course and (C) dose-dependence of adropin treatment on LPL gene expression in primary cultured tilapia hepatocytes. Hepatocytes were incubated with adropin (100 nM) for the duration as indicated or with increasing levels (1–1000 nM) of adropin for 24 h and subjected to quantitative real-time PCR for LPL gene measurement. The 18S rRNA acts as an internal control, and all quantitative results were normalized to the ratio of the target gene/18S expression. Data presented for LPL gene expression are expressed as means ± s.e.m. (n = 4), and groups denoted by the same letter represent a similar magnitude of LPL gene expression (P < 0.05, ANOVA followed by Fisher’s LSD test). (D) Effect of time course and (E) dose-dependence of adropin treatment on hepatocytes viability. MTT assay analysis showed that treatment with adropin did not affect hepatocytes viability (Fig. 4D and E).

MTT assay analysis showed that treatment with adropin did not affect hepatocytes viability (Fig. 4D and E).

Functional role of cAMP/PKA-dependent mechanisms in LPL gene expression

To examine the functional role of the cAMP-dependent pathway in adropin-induced LPL gene expression, tilapia hepatocytes were incubated with increasing concentrations of adropin (n = 4). In this case, adropin was effective in elevating the CAMP production in a dose-dependent manner using primary cultured tilapia hepatocytes (Fig. 5A). Consistent with these findings, PKA phosphorylation in hepatocytes was augmented consistently in a concentration-related fashion with the treatment of adropin (Fig. 5B). In parallel studies, forskolin-induced PKA activities were sensitive to the blockade by the PKA peptide inhibitor (PKI, 100 µM, n = 4) (Fig. 5B, small inset). In tilapia hepatocytes, adropin induction could trigger the phosphorylation of PKA without changing in the total amount of protein, suggesting that the increase in the phosphorylation rate was not due to a change in the total protein levels of these kinases (Fig. 5C, gel bands). To evaluate the functional role of Epac in adropin-stimulated LPL gene expression, the effects of the Epac agonist on LPL gene expression were also examined. In this case, this stimulatory action could not be mimicked by the Epac agonist 8cpt-2me-cAMP (1 and 10 µM, data not shown). To obtain further support for the specific involvement of cAMP/PKA pathway in the adropin induction of LPL gene expression, tilapia hepatocytes were challenged with adropin (100 nM, n = 4) in the presence or absence of the angiotensin-converting (AC) inhibitor MDL 12330A (10 µM) or PKA inhibitor H89 (10 µM). As shown in Fig. 5D, the two inhibitors abolished the stimulatory effect of adropin on LPL gene expression in primary cultured tilapia hepatocytes.

Functional role of PLC/IP3/PKC-dependent mechanisms in LPL gene expression

To test the possible involvement of the phospholipase C (PLC)/inositol trisphosphate (IP3)/PKC pathways in adropin-stimulated LPL gene expression, tilapia hepatocytes were challenged with adropin (100 nM, n = 4) in the presence or absence of the PLC inhibitor U73122 (10 µM), IP3 receptor inhibitor 2APB (100 µM) and PKC inhibitor GF109203X (10 nM). As shown in Fig. 6A, adropin consistently induced a significant increase in LPL gene expression, and this stimulatory effect of adropin was blocked by the inhibitors for PLC, PKC and IP3 receptors respectively. To further confirm that PKC activation is involved in LPL expression, hepatocytes were exposed to increasing concentrations of the PKC activator 12-O-tetradecanoylphloroglucinol 13-acetate (TPA; 1–100 nM, n = 4). In this case, LPL gene expression was increased in a dose-dependent manner with TPA stimulation (Fig. 6B). In parallel experiments, PKC
phosphorylation was also elevated in a concentration-related fashion by adropin treatment (Fig. 6C) and TPA-induced PKC phosphorylation activity could be blocked by the PKC peptide inhibitor (PKCI, 100 μM) (Fig. 6C, gel bands). Moreover, immunodetection of PKCβ phosphorylation was also increased by adropin treatment without affecting the total levels of the PKCβ (Fig. 6D, small inset).

Figure 6
Functional role of PLC/IP3/PKC-dependent mechanisms in LPL gene expression. (A) Effects of PLC inhibitor U73122, IP3 receptor inhibitor 2APB and PKC inhibitor GF10923X on adropin induction of LPL gene expression. Hepatocytes are exposed to adropin (100 nM) for 24 h with or without simultaneous treatment of the PLC inhibitor U73122 (10 μM), IP3 receptor inhibitor 2APB (100 μM) and PKC inhibitor GF10923X (10 nM). (B) Effects of increasing doses of the PKC activator TPA (1–100 nM) on LPL gene expression in tilapia hepatocytes. (C) PKC activity is increased by adropin in tilapia hepatocytes. Hepatocytes are exposed to adropin with increasing concentrations (1–1000 nM). In parallel studies, TPA (100 nM) and PKC activator forskolin (FSK, 1 μM) is used as a positive control for stimulation and inhibition respectively. Data presented are pooled results from four separate experiments, and treatment groups denoted by the same letter represent a similar level of expression (P<0.05; ANOVA followed by Fisher’s LSD test). Parallel blotting of total PKA was performed to serve as the internal control. Blots represent prototypical examples of experiments replicated at least three times. (D) Effects of MDL12330A and H89 on adropin-induced LPL gene expression. Hepatocytes are incubated for 24 h with adropin (100 nM) in the presence or absence of the AC inhibitor MDL12330A (MDL, 10 μM) or PKA inhibitor H89 (10 μM). After drug treatment, total RNA was isolated for LPL gene measurement using real-time PCR. Data presented are pooled results from four separate experiments, and treatment groups denoted by the same letter represent a similar level of expression (P<0.05; ANOVA followed by Fisher’s LSD test).
blocking the stimulatory effects of adropin (Fig. 7A).

expression in tilapia hepatocytes.

mediate the stimulatory effects of adropin on LPL gene expression in tilapia hepatocytes (Fig. 7A). These results suggested that p42/44MAPK and p38 MAPK pathways obviously do not mediate the stimulatory effects of adropin on LPL gene expression in tilapia hepatocytes.

Effects of p38MAPK and p42/44MAPK pathways inhibition

To examine whether MAPK cascades are involved in adropin-induced LPL gene expression, the effects of p38MAPK and p42/44MAPK pathways inhibition on adropin-stimulated LPL gene expression were also tested in tilapia hepatocytes (n = 4). In this case, the blockade of MEK inhibitor PD98059 (10 μM) was not effective in blocking the stimulatory effects of adropin (Fig. 7A). Similar results were obtained with p38MAPK inhibitor SB203580 (10 μM, Fig. 7B). These results suggested that p42/44MAPK and p38MAPK pathways obviously do not mediate the stimulatory effects of adropin on LPL gene expression in tilapia hepatocytes.

Discussion

Adropin is a peptide hormone involved in the regulation of energy homeostasis in mammals (Kumar et al. 2008). However, biological functions of adropin have not been identified in other vertebrate classes. In this report, to shed light on the functional role of adropin in fish species, we searched for tilapia adropin in the genome database using genomic synteny of human adropin. In the tilapia genome, FAM219A and DNAI1 genes neighbor adropin in the scaffold GL831146.1, which are in line with the gene arrangement around adropin of vertebrate species examined. Therefore, the gene arrangement around adropin is probably conserved within vertebrates. The structural identity of tilapia adropin was established by molecular cloning. Sequence alignment at the protein level reveals that tilapia adropin is highly homologous to fish species (82–87%). However, the sequence homology drops to lower levels when compared to adropin reported in mammalian, chicken and alligator adropin (35–46%). There are a putative N-myristoylation site (GLSTAA) and two casein kinase II phosphorylation sites (SSPE and SEAE) in tilapia and other fish species. In contrast, two putative N-myristoylation sites (GAAISQ and GALIAI) and a casein kinase II phosphorylation site (SLSE) can be identified in mammals. Unlike mammalian counterparts, a potential tyrosine kinase phosphorylation site (KPSHEGSY) is absent in fish species, chicken and alligator. The result of Southern blot reveals that adropin is a single-copy gene in the tilapia genome. In accordance to the pleiotropic nature of adropin functions (Aydin 2014), adropin transcript was found to be ubiquitously expressed in various tissues and brain areas in tilapia. These results are also in agreement with the recent proposal that adropin immunoreactivity was observed in the sinusoidal cells of the liver and in the vascular space, pia mater, neural cells and neurons of the brain (Aydin et al. 2013).

Lipid depletion during starvation has been demonstrated in many fish species, including marine as well as freshwater (Sargent et al. 1989). In Perciformes, the liver is one of the main sites of lipid storage (Stubhaug et al. 2005). During fasting in sea bass (Dicentrarchus labrax), a significant loss of stored lipids, glycogen and proteins were found in the liver, whereas during re-feeding the hepatic energy reserves were rapidly replenished (Gambardella et al. 2012). In tilapia (Oreochromis niloticus), lipids in the liver are rapidly decomposed for energy during the initial stage of fasting (Liu et al. 2009). Because the highest levels of adropin transcripts can be detected in the liver and hypothalamus of tilapia, these findings promote us to investigate the effect of long-term fasting and re-feeding on adropin gene expression in the liver and hypothalamus of tilapia. In the current studies, adropin expression in response to fasting status change might be tissue specific. The increase in hepatic adropin expression may reflect a mechanism through which energy reserves are preferentially mobilized from the liver during long-term fasting. The return of the hepatic adropin expression levels to the continuously fed controls following re-feeding may indicate a down-regulation of adropin’s lipolytic effects on liver tissue as reserves are deposited during a period of nutrient excess. However, these findings are not consistent with results from a previous report in mice in which hepatic adropin mRNA levels were decreased.
after 10 days of fasting compared to controls that had been fed a low-fat diet (Kumar et al. 2008). The cause for the discrepancy on the hepatic adropin level in response to food deprivation is unclear and may be related to species-specific variations. The hypothalamus, with the highest expression level of adropin, is associated with the regulation of food intake (Lin et al. 2000). The information of hypothalamic adropin expression during long-term fasting is still largely unknown. In our current studies, we observed no effect of fasting and re-feeding on hypothalamic adropin gene expression in tilapia, suggesting that hypothalamic adropin is not directly involved in the regulation of feeding. Whether adropin functions as a neuropeptide in the central nervous system to affect homeostatic regulation of metabolism remains to be investigated.

The hormonal regulation of lipid metabolism in vertebrate is a complex process in which several secreted factors of liver are critical for energy homeostasis (Sheridan 1988, Reitman 2007). In mammals, several observations suggest that adropin has a role in the regulation of energy homeostasis. In mice muscle, adropin has a significant role in regulating substrate oxidation during feeding and fasting cycles (Gao et al. 2014). In diet-induced obese mice, transgenic overexpression adropin exhibit inhibition LPL genes expression in adipose tissue (Kumar et al. 2008). Given that the hepatic LPL gene is substantially expressed in fish (Albalat et al. 2006, Feng et al. 2014, Tian et al. 2015), correlations between adropin and LPL were suspected to play a role in lipid metabolism at the tilapia hepatocytes level. This idea is supported by our in vitro studies with tilapia hepatocytes in which adropin was effective in elevating LPL release, cellular LPL content and total LPL production. In these experiments, the increase in LPL production also occurred with parallel rises in LPL gene expression, suggesting that adropin can not only induce LPL release but also trigger LPL synthesis via up-regulation of LPL gene expression. Previous studies pointed to the effect of LPL on lipid metabolism in tilapia (Oreochromis niloticus) in which hepatic LPL gene expression levels were synchronously increased after fasting (Han et al. 2011, Tian et al. 2013), and further, there was a negative correlation between TG levels and LPL levels (Tian et al. 2013), suggesting LPL is an important lipolytic factor in tilapia. In mice, hepatic adropin gene expression is regulated by liver X receptor (LXR)-α (Kumar et al. 2008), which is a nuclear receptor involved in cholesterol and triglyceride metabolism (Kalaany & Mangelsdorf 2006). The link between the stimulation of hepatic LPL expression by adropin and the response to the activation of LXR may be consistent with a significant role for this peptide in maintaining energy homeostasis.

At present, the post-receptor signaling mechanisms coupled with the adropin receptor are largely unknown. To delineate the molecular mechanisms of adropin function in hepatocytes, we investigate the intracellular signaling pathways involved in the stimulatory effect of adropin on LPL gene expression. In hepatocytes prepared from the tilapia, adropin treatment could dose dependently increase total cAMP production and trigger PKA phosphorylation. In contrast, adropin-induced LPL gene expression was markedly reduced by blocking AC with MDL 12330A or inactivating PKA by H89. It has been demonstrated that cAMP not only could activate PKA but also exchange protein activated by cAMP (Epac) that functions as guanine nucleotide exchange factors for both Rap1 and Rap2, members of the Ras family of small G proteins (Gloerich & Bos 2010). However, there is a possibility that cAMP-dependent Epac activation by adropin-stimulated LPL gene expression could be ruled out, because the selective Epac agonist, 8cpt-2me-cAMP, had no effect on the LPL gene expression. These results indicate that the AC/cAMP/PKA pathway plays a key role in maintaining the adropin induction of LPL gene expression in tilapia hepatocytes. Apart from cAMP/PKA-dependent mechanisms, coupling with the PLC/IP3/PKC pathway was also observed for adropin-stimulated LPL gene expression. In tilapia hepatocytes, inhibiting PLC using U73122, blocking IP3 receptor with 2-APB and inactivating PKC by GF10923X suppressed basal and adropin-simulated LPL gene expression, whereas PKC activation by TPA was found to increase LPL gene expression. Using a direct assay of PKC kinase activity, total PKC enzyme activity was significantly elevated by treatment with adropin in tilapia hepatocytes. Adropin-induced PKCβ phosphorylation could be corroborated by western blot analysis. These results indicate that the PLC/IP3/PKC pathway is involved in both basal maintenance and adropin induction of LPL gene expression. Given that IP3 receptors are Ca 2+ channels responsible for [Ca 2+]i release from IP3-sensitive Ca 2+ stores (Jiang et al. 2008b), we should not rule out the possibility that [Ca 2+]i mobilization may contribute to adropin-stimulated LPL gene expression. In human endothelial cells, adropin can elevate eNOS protein levels and mRNA expression, and these stimulatory effects are mediated through the VEGFR2-Pi3K-Akt and VEGFR2-ERK1/2 pathways (Lovren et al. 2010). This finding prompted us to speculate that other signaling pathways might be involved in adropin-stimulated LPL gene expression. In tilapia hepatocytes, we
show that adropin-induced LPL gene expression was not sensitive to the blockade by p38MAPK inhibitor SB203580 and MEK inhibitor PD98059, suggesting that the MAPK-dependent mechanisms might not be involved in adropin-stimulated LPL gene expression. However, it remains to be determined whether adropin is also coupled with the PI3K/Akt pathway to control LPL gene expression in hepatocytes.

In summary, we have cloned adropin in tilapia and confirmed that it is a single-copy gene. Although fish adropin shares relatively low amino acid sequence identities with mammalian species, the gene arrangement around tilapia adropin was similar to that of other vertebrates. In line with the previous findings, tilapia adropin transcripts were detected mainly in the liver and hypothalamus with low levels of expression in peripheral tissues. Fasting resulted in an increase in liver adropin expression, and hepatic adropin mRNA concentrations were restored after 7 days of re-feeding, whereas hypothalamus-derived tilapia adropin does not appear to be regulated by a nutritional state. Using primary cultures of tilapia hepatocytes, we have shown that adropin could trigger LPL secretion, LPL production and gene expression via direct action at the cellular level. Using a pharmacological approach, cAMP/PKA and PLC/IP3/PKC cascades were shown to be involved in adropin-induced LPL gene expression. Our findings for the first time provide evidence that adropin could stimulate LPL gene expression via direct actions in tilapia hepatocytes through the activation of multiple signaling mechanisms.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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