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Adropin inhibited tilapia hepatic glucose output and triglyceride accumulation via AMPK activation

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Abstract

Adropin plays a role in the maintenance of energy homeostasis, insulin resistance prevention, and impaired glucose tolerance. However, the molecular mechanisms by which adropin affects hepatic glucose and lipid metabolism *in vitro* are not entirely understood. This study intended to examine the roles and underlying mechanisms of adropin in glucose and lipid metabolism in Nile tilapia. In primary cultured tilapia hepatocytes, adropin significantly attenuated oleic acid (OA)-induced glucose output and reduced the activities and mRNA expression of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which are involved in gluconeogenesis. In contrast, adropin facilitated glucose uptake activity via glucose transporter 1 (*Glut1*) upregulation in OA-treated hepatocytes. One-week of adropin treatment reduced the hepatic total lipid accumulation in OA-fed tilapia without changes in body weight. Subsequent studies revealed that adropin suppressed OA-induced intracellular triglyceride accumulation and decreased the expression of genes and proteins involved in lipid metabolisms such as sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase α (ACC α) and CD36, but upregulated peroxisome proliferator-activated receptor α (PPAR α) levels. In parallel studies, however, adropin had no detectable effects on fatty acid-binding protein 4 (*Fabp4*) and carnitine palmitoyltransferase 1 α (*Cpt1 α*) mRNA expression. Furthermore, adropin treatment dose-dependently increased the phosphorylation level of AMP-activated protein kinase (AMPK). Suppression of AMPK by compound C or AMPK α 1 siRNA blocked adropin-induced decreases in the mature form of SREBP-1c expression, glucose output, and intracellular triglyceride content in OA-treated hepatocytes. These findings suggest that teleost adropin could suppress hepatic gluconeogenesis and triglyceride accumulation via a mechanism dependent on AMPK signalling.

Key Words:

- ▶ adropin
- ▶ glucose utilization
- ▶ triglyceride accumulation
- ▶ hepatocytes
- ▶ tilapia

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Introduction

Adropin, a product of the energy homeostasis-associated (*Enho*) gene, plays an important role in metabolic regulation and insulin sensitivity (Jasaszwili *et al.* 2020). Adropin is primarily synthesized in the liver and brain

tissues of mice (Kumar *et al.* 2008) and fish (Lian *et al.* 2016). The liver is an essential tissue for maintaining glucose and lipid homeostasis, and its metabolic processes are tightly regulated by neuronal and hormonal systems (Rui 2014).

Although the expression of hepatic adropin also appears to depend on the nutritional state, the effect of fasting on adropin expression is still controversial. In mice, fasting reduces the hepatic expression of adropin (Kumar *et al.* 2008), whereas plasma adropin levels do not change in response to fasting in humans (Butler *et al.* 2012). In tilapia, fasting increases and refeeding decreases hepatic adropin gene expression (Lian *et al.* 2016). The causes of these discrepancies could be explained two-fold. One possible explanation for this difference could be related to species-specific variations. Alternatively, additional mechanisms could differentially affect adropin mRNA levels during nutritional changes, such as specific effects on mRNA stability or the involvement of additional transcription factors. The specific receptor for adropin has not yet been identified, but the existence of multiple putative adropin receptors has been suggested. Adropin-mediated activation of the orphan G protein-coupled receptor 19 (GPR19) exerts its biological functions in rat brain tissues (Stein *et al.* 2016), breast cancer cells (Rao & Herr 2017) and cardiac cells (Thapa *et al.* 2018), whereas the central effect of adropin is mediated through the activation of the transient receptor potential cation channel subfamily V 4 (TRPV4) to reduce water consumption in male rats (Yang *et al.* 2017).

Emerging evidence indicates that adropin plays an important role in the regulation of glucose and lipid homeostasis. In HepG2 cells, adropin expression is suppressed following activation of liver X receptors, suggesting sensitivity to carbohydrate and lipid metabolism (Kumar *et al.* 2008). In humans and male rats, associations between plasma adropin concentrations and low-density lipoprotein cholesterol suggest a link with hepatic lipid metabolism (Ghoshal *et al.* 2018). Adropin-knockout mice were associated with increased adiposity, hepatosteatosis, and insulin resistance (Kumar *et al.* 2008), while transgenic overexpression or systemic adropin treatments markedly attenuates insulin resistance and glucose intolerance in diet-induced obese mice (Kumar *et al.* 2008, Gao *et al.* 2015). Furthermore, transgenic overexpression of adropin markedly reduced plasma triacylglycerol level by downregulating the expression of hepatic stearyl-CoA desaturase-1 and fatty acid synthase in mice (Kumar *et al.* 2008). In high-fat diet mice, *in vivo* administration of adropin suppressed hepatic glucose production and improved hepatic insulin sensitivity by decreasing the protein levels of glucose-6-phosphatase (G6Pase), CD36 and PDH kinase 4 (PDK4) expression (Thapa *et al.* 2019). In line with these observations, adropin has been found to regulate pyruvate dehydrogenase

via mitogen-activated protein kinase (MAPK)-PDK4 signalling pathway in cardiac cells (Thapa *et al.* 2018). More recent *in vivo* work has demonstrated that adropin treatment suppresses hepatic gluconeogenesis via the cAMP/PKA/CREB and SREBP-1c signalling pathways in diet-induced obese mice (Gao *et al.* 2019). Also, adropin could directly decrease glucose production via PKA-mediated phosphorylation in primary mouse hepatocytes (Gao *et al.* 2019). AMP-activated protein kinase (AMPK), a heterotrimeric enzyme complex, is the key regulator of energy metabolism in cells, which contributes to the regulation of most metabolic pathways, including glucose metabolism and lipid metabolism (Kahn *et al.* 2005). Recent studies have suggested that adropin triggered anti-dipsogenic effects via calmodulin-dependent protein kinase kinase-AMPK signalling in rats (Yang *et al.* 2017). However, whether activation of the AMPK pathway was linked to adropin-mediated glucose and lipid metabolism in primary cultured hepatocytes remains unknown.

Against this background, although it is well known that the control of glucose and lipid metabolism is closely linked with adropin, the mechanism by which adropin affects hepatic carbohydrate lipid metabolism *in vitro* are still poorly defined. This study intended to examine the hypothesis that adropin promotes glucose and lipid homeostasis in oleic acid (OA)-induced tilapia hepatocytes by activation of the AMPK signalling pathway. Literature data confirmed that OA is used to induce insulin resistance model in cultured primary hepatocytes of rat (Ochs and Harris 1986), mice (Malhi *et al.* 2006, Liu *et al.* 2007), human (Sharma *et al.* 2011), goose (Liu *et al.* 2018) and fish (Du *et al.* 2018). Nile tilapia (*Oreochromis niloticus*) is an important aquaculture species cultured worldwide. Since tilapia has well-developed digestive and metabolic organs compared with zebrafish, tilapia could be used as a better fish model for nutrition and metabolism studies (He *et al.* 2015). In our present study, we tested our hypothesis by (1) examining the roles of adropin on hepatic glucose utilization and lipid metabolism and (2) investigating the potential role of the AMPK signalling pathway in mediating adropin effects on hepatic glucose utilization and triglyceride content in OA-treated hepatocytes.

Materials and methods

Animals

Male tilapia was reared as previously described (Lian *et al.* 2016). Briefly, fish were maintained in three 300 L tanks (eighty fish per tank) filled with aerated freshwater at

28°C under 10 h dark/14 h light photoperiod for at least 7 days before tissue sampling and hepatocytes preparation. After anaesthesia with 0.05% MS222 (Sigma), the fish were killed by spinosectomy according to the procedures approved by the Animal Ethics Committee of Sichuan University.

Test substances

The 32-amino acid peptide, originally derived from 38–69 fragment of tilapia adropin, 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 $\geq 98\%$, and the homogeneity of the peptide was further confirmed by mass spectrometry. A synthetic peptide was dissolved in fish physiological saline (FPS) (Wolf 1963) and stored at -80°C as 0.1 mM stocks. OA was purchased from Sigma-Aldrich. To prepare the 5 mM OA, OA was mixed with free fatty acid-free BSA (Sigma) at a molar ratio of 3:1 (Oliveira *et al.* 2015). The stock solutions were then diluted into the pre-warmed culture medium to achieve a final solution of 250 μM . Pharmacological agents, both STF-31 and compound C were obtained from Calbiochem, and were prepared as 10 mM frozen stocks in dimethyl sulfoxide (DMSO). In these studies, the final dilutions of DMSO were always $<0.1\%$ (v/v) and had no effects on target gene expression in primary cultures of tilapia hepatocytes (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article).

Isolation and culture of primary hepatocytes

Tilapia hepatocytes were pooled from four individual fish in each batch of experiments and were prepared by collagenase digestion method as described previously (Chow *et al.* 2004, Yanhong *et al.* 2008). Briefly, tilapia livers were perfused *in situ* by a pre-warmed (28°C) $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS medium to wash out the blood, and then the liver fragments were digested in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS medium with 0.5 mg/mL type IV collagenase (Sigma) and 0.01 mg/mL DNase II (Sigma) for 30 min with constant shaking at 28°C . Hepatocytes were dispersed by gentle pipetting and filtered through a sterile 40 μm mesh metal screen (Sigma) followed by a nylon mesh with a pore size of $\sim 20 \mu\text{m}$ to remove the undigested fragments/debris. After that, hepatocytes were harvested by centrifugation at 50 $\times g$ for 5 min and resuspended in M199 medium (Invitrogen) containing 0.5% BSA (Sigma). Hepatocytes were seeded at a density of $\sim 0.7 \times 10^6$ cells/well in PEI

(5 $\mu\text{g}/\text{mL}$) precoated 24-well plate and incubated overnight at 28°C in a humidified atmosphere of 95% air and 5% CO_2 . On the following day, hepatocytes were incubated with control medium or the high-fat medium containing 250 μM OA, followed by vehicle (FPS) or 100 nM adropin treatment for 24 h in the absence or presence of OA. The co-incubation time and concentration with the tested agents were designed based on preliminary experiments.

Cell viability assay

The viability of hepatocytes was measured using MTT assay as previously described (Fujii *et al.* 1995). Briefly, liver cells were seeded in 48-well plates at a density of $\sim 0.4 \times 10^6$ cells/well. After incubation in medium with 100 nM adropin or vehicle for 24 h in the absence or presence of OA (250 μM), cell viability was determined by the reduction of MTT to formazan. The absorbance of vehicle control cells was assumed as 100% cell viability.

Hepatic glucose output

Hepatocytes were incubated with 100 nM adropin or vehicle for 24 h in the absence or presence of OA as described above. After that, hepatocytes were washed twice with FPS to remove glucose and then incubated for 3 h in glucose production assay medium (glucose- and phenol red-free M199 containing gluconeogenic substrates). At the end of this incubation, media solution was used for measuring the glucose concentration using a colourimetric glucose assay kit (BioVision, Milpitas, CA, USA). Glucose production was normalized to total protein concentration as measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific Inc.) with BSA as a standard to correct for potential variation in cell number.

PEPCK and G6Pase activity analysis

The activities of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) (EC4.1.1.32) was determined based on the method of Bentle and Lard (Bentle & Lardy 1976). One unit of cytosolic PEPCK activity was expressed as nanomoles NADH oxidized/min/g protein. The G6Pase (EC3. 1. 3. 9) activity was estimated using the method described by (Alegre *et al.* 1988). One unit of G6Pase activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μM of glucose-6-phosphate/min/g protein. The protein concentration of hepatic homogenates was determined by using the BCA protein

assay kit (Pierce) with BSA as a standard. Enzyme-specific activity was expressed as unit/g soluble protein.

Hepatic glucose uptake assays

The glucose uptake ability was analyzed using a glucose uptake colourimetric assay kit according to the manufacturer's instructions (BioVision). After treatment, hepatocytes were washed with glucose uptake medium (glucose-free M199) two times and resuspended with 90 μ L of glucose uptake medium. The cells were incubated for 40 min followed by the addition of 10 μ L of 10 mM 2-deoxyglucose and cultured for 1 h at 37°C, and then the cells were lysed with 100 μ L of assay buffer (supplied in glucose uptake colourimetric assay kit). The absorbance was measured at a 412 nm wavelength on a microplate reader (BioTek Instruments, Inc.).

Animal experiments

After 2 weeks acclimating period, the fish were randomly assigned to the four groups ($n=7-9$ /group) as follows: administered with vehicle (FPS), administered with adropin (450 nmol/kg/day), OA fed administered with a vehicle, and OA fed administered with adropin (450 nmol/kg/day). The OA-fed group was treated with OA (4 mg/kg of body weight) daily by oral gavage for 7 days, and the adropin-treated group was given once daily by an intraperitoneal injection of 450 nmol/kg/day adropin comparable with the concentrations previously reported (Gao *et al.* 2019), whereas the vehicle group received FPS (0.1 mL). After the 1-week trial, the fish were fasted for 18 h and then killed for the collection of tissue samples. The liver tissues were frozen at -80°C until subsequent analysis.

Measurements of total hepatic lipid and intracellular triglyceride content

Total liver lipids were extracted in 20 volume of chloroform: methanol (2:1, v/v) containing 0.01% of butylated hydroxytoluene as anti-oxidant according to Folch's method (Folch *et al.* 1957). The extracts were evaporated in a tared rotary evaporator flask and then frozen at -80°C until subsequent analysis. Cellular triglyceride content was measured using a triglyceride assay kit (JianCheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions and normalized to total protein concentration.

Oil Red O staining of liver tissue

For Oil Red O staining, frozen liver tissues were cut into 7 μ m with Probe-On-Plus Slides (Thermo Fisher Scientific) and fixed to microscope slides. Sections were reacted with Oil Red O solution buffer for 7 min at 60°C and then incubated with 85% propylene glycol for 3 min. After rinsing with distilled water, sections were stained with haematoxylin for counterstaining.

Quantitative real-time PCR assays

Total RNA was extracted from tilapia hepatocytes by TRIzol reagent (Life Technologies) followed by chloroform/isopropanol extraction. RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific), and 1 μ g of total RNA was reverse transcribed using iScriptTM RT Supermix (Bio-Rad). qPCR assays were performed on a Mastercycler[®] ep realplex system (Eppendorf) with SYBR Green Mix (Bio-Rad) and analyzed using the software Realplex 2.2 (Eppendorf). The cycling conditions were the following: 2 min incubation at 95°C, 45 amplification cycles (95°C for 30 s, 60°C for 30 s, and 45 s at 75°C, with fluorescence detection at the end of each cycle), followed by melting curve of the amplified products obtained by ramped increase of the temperature from 55 to 95°C to confirm the presence of single amplification product per reaction. Primer sequences are provided in Table 1, and the specificity of each primer pair was verified by the previous study in tilapia (He *et al.* 2015, Lian *et al.* 2016). β -Actin was used as an internal control for normalizing genes of interest, and transcript levels were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.

Western blot analysis

Western blot was performed as described previously (Jiang & Wong 2013). Proteins were separated on SDS-PAGE gels, transferred to PVDF (polyvinylidene difluoride, Millipore), and incubated with antibodies against SREBP-1c (1:1000, Santa Cruz Biotechnology), ACC α (1:1000, Santa Cruz Biotechnology), CD36 (1:1000, Santa Cruz Biotechnology), PPAR α (1:1000, Santa Cruz Biotechnology), phospho-AMPK α (1:1000, Cell Signaling Technology), AMPK α (1:1000, Cell Signaling Technology), Lamin b (1:1000, Cell Signaling Technology) and β -actin (1:5,000; Cell Signaling Technology) for overnight at 4°C. These antibodies have been used previously in zebrafish (Liu *et al.* 2017), tilapia (Ning *et al.* 2017) and bream (Shi *et al.* 2018),

Table 1 Real-time primer sequences for genes of interest.

Primer name	Sequence (5' to 3')
<i>Pepck1</i> forward	TGCCAGCCAGACTCCATTCA
<i>Pcpck1</i> reverse	ACTCTCCACACGAGCCACATCC
<i>G6pca</i> forward	ATCGGAGACTGGCTCAACTTGGT
<i>G6pca</i> reverse	TGGCATGACCTGAAGGACTTCT
<i>Glut1</i> forward	GTTGGAAGTGCAGGATGAGCT
<i>Glut1</i> reverse	ATAGCAACAGCGATGGACACAC
<i>Glut2</i> forward	GGCACTCTAGCTCTGGCTGTGT
<i>Glut2</i> reverse	GGGTGGTACCTGGGTCTTCT
<i>Srebp1c</i> forward	ATTCAGACTCAGCTCCAAGGG
<i>Srebp1c</i> reverse	TCAGGCTTCAAAGTGGTCAG
<i>Acca</i> forward	GGAGTTCGGACAGCACCTATGAA
<i>Acca</i> reverse	AGCAGGAGAAGCAACAGTGAAGT
<i>Cd36</i> forward	GCAGCAGTACCAGCGATTCT
<i>Cd36</i> reverse	TCTCCAGTACGCAGCCAGGA
<i>Fabp4</i> forward	AAGCTGGGAGAGGAGTTTGATGAA
<i>Fabp4</i> reverse	TCTCTTTGCCGTCCCCTTCT
<i>Pparα</i> forward	CTGATAAAGCTTCGGGCTTCCA
<i>Pparα</i> reverse	CGCTCACACTTATCATACTCCAGCT
<i>Cpt1a</i> forward	TTTCCAGGCCTCCTTACCCA
<i>Cpt1a</i> reverse	TTGTACTGCTCATTGTCCAGCAGA
<i>β-actin</i> forward	GTGATGGTGGGTATGGGT
<i>β-actin</i> reverse	GGCAACTCTCAGCTCGTT

confirming that epitopes detected by these antisera are highly homologous to that of the fish counterparts. After washing blots to remove excessive primary antibody binding, the blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. Blots were developed by SuperSignal WestPico chemiluminescent substrate (Pierce) using the FluorChem FC2 detection system (Biozym Scientific). ImageJ was used to analyze the densitometric values.

RNA interference

The siRNA constructs against AMPK α 1 mRNA (the predominant isoform of AMPK in tilapia liver (Xu *et al.* 2002)), as well as scrambled control siRNA, were designed and synthesized by Gene Pharma (Shanghai, China). RNAi was introduced into primary hepatocytes by reverse transfection with siRNA-Mate (GenePharma) according to the manufacturer's protocol. Briefly, the siRNA transfection mixture was applied to PEI-coated six-well plates right before the plating of primary hepatocytes in culture media without antibiotics. The experiments were performed 24 h after transfection. Gene silencing was verified by detecting protein with immunoblot analysis after transient transfection of hepatocytes with siRNA. The siRNA sequences are as follows: AMPK α 1 (sense 5'-GGAGAGUCCUUAAGAACAAdTdT-3' and anti-sense 5'-UUGUUCUUAAGGAACUCUCCdTdT-3'); and scrambled

siRNA (sense 5'-GGAUUGUCCGAUAAGACAAdTdT-3' and anti-sense 5'-UUGUCUUAUCGGACAAUCCdTdT-3').

Statistics analysis

Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n=3$). Data were analyzed using ANOVA followed by *post hoc* Bonferroni's test that was used for the multiple comparisons. A value of $P<0.05$ was considered statistically significant. All analyses were performed with GraphPad Prism Software, Inc.

Results

Adropin reduced glucose output and inhibited gluconeogenesis in tilapia hepatocytes

To determine whether adropin regulates hepatic gluconeogenesis, glucose output analysis was performed on OA-treated tilapia primary hepatocytes. The level of glucose output was significantly increased in hepatocytes with 250 μ M OA treatment, which was more than 1.5-fold higher than vehicle control, whereas these stimulatory effects were not seen when the cells were co-incubated in the presence of 100 nM adropin. In contrast, no significant changes were found in the level of glucose production in hepatocytes treated with vehicle control compared to adropin alone (3.05 ± 0.26 μ g/mg protein vs 2.80 ± 0.33 μ g/mg protein; $P>0.05$) (Fig. 1A). To rule out the possibility that the inhibitory effect is due to a decreased cell number, we tested the effects of adropin and OA on hepatocyte viability. The results showed that adropin, OA, or the combined treatment exhibited no toxic effects at the indicated dosages (Fig. 1B). PEPCK and G6Pase are rate-limiting enzymes regulating hepatic gluconeogenesis (Yabaluri & Bashyam 2010). To determine whether adropin-reduced glucose output was attributed to decreases in the rate of the hepatic gluconeogenesis, effects of adropin on PEPCK and G6Pase activities were examined in OA-treated tilapia hepatocytes. As shown in Fig. 1C and D, OA treatment enhanced PEPCK ($\sim 80\%$ increase, $P<0.05$) and G6Pase ($\sim 50\%$ increase, $P<0.05$) activities compared to vehicle control. In contrast, adropin was effective in negating the stimulation of PEPCK and G6Pase activities in OA-treated hepatocytes. In parallel studies, adropin could negate OA-increased cytosolic *pepck1* and *g6pca* gene expression (Fig. 1E and F), suggesting that the inhibition of adropin on PEPCK

Figure 1

Adropin treatment suppresses glucose production and the key enzymes of gluconeogenesis in OA-treated tilapia hepatocytes. (A) The effect of adropin inhibition on OA-induced glucose output. (B) The effect of adropin treatment on hepatocytes viability. Cell viability was determined by MTT assay. (C) and (D) Inhibitory effects of adropin on PEPC and G6Pase activities. (E) and (F) Inhibitory effects of adropin on *pepck1* and *g6pca* mRNA expression. Genes were measured by qPCR and normalized to β -actin levels. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, two-way ANOVA).

and G6Pase activities is due to decreases in mRNA levels of these enzymes.

Adropin increased glucose uptake via the activation of GLUT1 gene expression in OA-treated hepatocytes

To further investigate the effects of adropin on glucose utilization, glucose uptake assay was performed in OA-treated hepatocytes. As shown in Fig. 2A, the level of glucose uptake significantly decreased in response to OA compared to that in the vehicle control, whereas

co-treatment with adropin at 100 nM significantly increased glucose uptake compared to cells treated with OA only. To investigate whether the glucose transporters (GLUTs) are involved in elevated glucose uptake in hepatocytes, we analyzed the mRNA expression of *glut1* and *glut2*. *Glut4* was excluded because it was known that *glut4* mRNA was found to be scarcely expressed in the liver tissue of tilapia (Chen *et al.* 2017). The gene expression of hepatic *glut1* and *glut2* decreased in OA-treated cells, whereas the transcription of *glut1* but not *glut2* mRNA was upregulated by co-treatment with adropin (Fig. 2B and C). Since the expression level of *glut1* appeared to correspond to the glucose uptake ability, we predicted that GLUT1 might play a central role in the glucose uptake in hepatocytes. To evaluate the involvement of GLUT1 in adropin-mediated glucose uptake, the stimulatory effects of adropin (100 nM) on glucose uptake were examined with co-treatment of the STF-31 (5 μ M), a selective GLUT1 inhibitor. Consistently, glucose uptake was inhibited by

Figure 2

Adropin induces glucose uptake in hepatocytes. (A) The effects of adropin on the glucose uptake in OA-treated hepatocytes. (B) and (C) The effects of adropin on the mRNA expression of *glut1* and *glut2* in OA-treated hepatocytes. Genes were measured by qPCR and normalized to β -actin levels. (D) The effect of GLUT1 inhibition on adropin-induced glucose uptake. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, two-way ANOVA).

OA exposure, whereas adropin-stimulated glucose uptake was abrogated by STF-31 in the presence of OA (Fig. 2D).

Adropin ameliorated the OA-mediated hepatic lipid accumulation

Since hepatic glucose utilization and lipid content are associated, the effect of adropin on hepatic lipid metabolism was then assessed by *in vivo* and *in vitro* experiments. The *in vivo* effects of adropin were assessed in the OA-fed tilapia. As shown in Fig. 3A, there were no significant differences in weight gain between OA-fed tilapia and OA-fed adropin-administered tilapia. However, OA feeding increased the hepatic total lipid content markedly when compared with vehicle control, and adropin treatment significantly reduced lipid content under basal or OA-fed conditions (Fig. 3B). Histological examination of Oil Red O staining of the cryosectioned liver tissue showed the accumulation of lipid droplets in OA-fed tilapia liver, whereas only a small number of lipid droplets were observed in the livers of normal diet-fed tilapia. Lipid droplets were greatly reduced in the livers of OA-fed adropin-administered tilapia (Fig. 3C). To explore the effect of adropin on hepatic triglyceride accumulation *in vitro*, hepatic triglyceride contents were measured in OA-induced hepatocytes. Analysis of intracellular lipids

revealed a dramatic increase in triglyceride content in OA-treated hepatocytes. As expected, a decrease in triglyceride content was observed in cells treated with adropin in comparison with the only OA-treated cells ($P < 0.001$, OA vs OA+Ad). Of note, adropin treatment alone also could reduce triglyceride content in hepatocytes ($P < 0.05$, Vehi. vs Ad) (Fig. 3D).

Adropin altered the expression of lipid metabolic genes in OA-treated hepatocytes

To address the molecular mechanism underlying the suppressive effect of adropin on lipid accumulation in hepatocytes, we examined the effects of adropin on the expression of genes associated with lipid metabolism. As shown in Fig. 4A and B, we found that adropin treatment at 100 nM for 24 h slightly, but significantly, decreased the gene expression levels of sterol regulatory element-binding protein-1c (*srebp-1c*) and acetyl-CoA-carboxylase alpha (*acca*) in the absence of OA ($P < 0.05$, Vehi. vs Ad). Furthermore, OA treatment led to a 2.1-fold and 1.6-fold increases in *srebp-1c* and *acca* mRNA levels when compared with that in the vehicle control, respectively, and these increases could be prevented by co-treatment with adropin. Furthermore, adropin suppressed the OA-induced increase in mRNA levels of *cd36* (Fig. 4C),

Figure 3

Effects of adropin on hepatic lipid accumulation both *in vivo* and *in vitro*. (A) The effect of adropin on body weight of OA-fed tilapia. (B) Adropin suppressed hepatic total lipid content in OA-fed tilapia. (C) Representative histological section analysis of inhibitory effects of adropin on lipid droplet formation in liver stained by Oil Red O staining. The tissue slides were examined under a light microscope at a magnification of $\times 200$. (D) Inhibitory effects of adropin on triglyceride content in hepatocytes with OA treatment. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, two-way ANOVA). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0077>.

Figure 4

Adropin regulates the expression of lipid metabolism-related genes in tilapia hepatocytes. The effects of adropin on mRNA expression levels of lipogenic genes: (A) *Srebp-1c* and (B) *Accα*, fatty acid transporter genes: (C) *Cd36* and (D) *Fabp4*, and fatty acid oxidation genes: (E) *Ppara* and (F) *Cpt1α*. Lipid metabolism-related genes were measured by qPCR and normalized to β -actin levels. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ s.e.m. ($n = 3$) and expressed as percentages of the vehicle control values. Different superscript letters in each column show significant differences ($P < 0.05$, two-way ANOVA).

whereas adropin rescued the OA-inhibited peroxisome proliferator-activated receptor alpha (*ppara*) gene expression (Fig. 4E). However, a similar dose of adropin did not alter fatty acid-binding proteins 4 (*Fabp4*) and carnitine palmitoyltransferase 1 α (*Cpt1α*) mRNA levels in OA-treated hepatocytes (Fig. 4D and F).

Adropin regulated the expression of lipid metabolism-related proteins in OA-treated hepatocytes

Consistent with the role of adropin in the regulation of lipogenic genes expression, we observed that the

mature form of SREBP-1c was dose-dependently reduced in OA-treated hepatocytes with adropin treatment. The maximal inhibition of SREBP-1c response to adropin was estimated to be approximately 41% (Fig. 5A). In parallel experiments, cells treated with adropin also exhibited a strong dose-dependent downregulation of ACC α protein expression in OA-treated hepatocytes. The minimal effective dose for adropin to decrease ACC α expression could be observed at 10 nM. At the highest dose of adropin treatment, the ACC α level was reduced to near control level (Fig. 5B). To further determine how adropin alters lipid metabolism, we measured the protein expression of CD36 and PPAR α , which are essential for lipid uptake and β -oxidation, respectively. After being treated with 250 μ M OA, hepatocytes showed a marked increase in the protein level of CD36 (Fig. 5C) and a significant decrease in the protein levels of PPAR α (Fig. 5D). However, the intervention of adropin dose-dependently

Figure 5

Adropin regulates the expression of lipid metabolism-related proteins in tilapia hepatocytes. Dose-dependence of adropin induction on (A) mature SREBP-1c, (B) ACC α , (C) CD36, and (D) PPAR α protein expression in OA-treated hepatocytes. Representative bands of mature SREBP-1c, ACC α , CD36 and PPAR α were detected by Western blot (gel bands). Parallel blottings of Lamin b and β -actin were used as a loading control for nuclear and cytoplasmic proteins, respectively. Blots represent prototypical examples of experiments. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ s.e.m. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, one-way ANOVA).

decreased the level of CD36 to upregulate the fatty acid translocase (Fig. 5C), but increased the level of PPAR α in a dose-related manner, which regulates genes involved in β -oxidation (Fig. 5D).

AMPK pathway mediated adropin-reduced glucose output and triglyceride accumulation in OA-treated hepatocytes

As a cellular energy regulator, AMPK signalling plays an important role in glucose and lipid metabolism (Ceddia 2013). To investigate whether this signalling mechanism regulates hepatic glucose and triglyceride metabolism induced by adropin in a high-fat environment, we evaluated the AMPK phosphorylation in OA-treated hepatocytes. Western blot analysis indicated that 250 μ M OA suppressed the phosphorylation of AMPK α at Thr-172, which is essential for AMPK activation. In contrast, the addition of adropin significantly enhanced phosphorylated AMPK α levels in a dose-dependent manner. The maximum stimulatory effect on the activation of phosphorylated AMPK α was observed at the dosage range from 10 to 100 nM ($P < 0.05$ vs OA treatment alone) (Fig. 6A). These results indicated the promoting function of adropin on AMPK activity. To further explore the possibility that AMPK is involved in adropin-induced glucose utilization and triglyceride accumulation in OA-treated hepatocytes, compound C, a specific inhibitor of AMPK, was used to block the activation of AMPK. As shown in Fig. 6B, Western blot analysis of the nuclear fractions demonstrated that blockade of AMPK activity by compound C completely attenuated the inhibition of SREBP-1c nuclear translocation induced by adropin in the presence of OA. Furthermore, compound C treatment significantly reversed the adropin-mediated suppression of the glucose output (Fig. 6C) and triglyceride content (Fig. 6E) in the presence of OA. In parallel experiments, however, pretreatment with compound C was not effective in blocking the stimulatory effects of adropin on glucose uptake in OA-treated hepatocytes (Fig. 6D). To further investigate the association between AMPK and adropin-induced glucose output and triglyceride levels in hepatocytes, AMPK α 1 siRNA was administered to block the AMPK signalling. The downregulation of AMPK in siRNA-transfected hepatocytes was confirmed by Western blotting (Fig. 7A). Consistently, knockdown of AMPK α 1 abolished the repression of nuclear SREBP-1c translocation (Fig. 7B), glucose output (Fig. 7C) and triglyceride accumulation (Fig. 7D) evoked by adropin in tilapia hepatocytes.

Discussion

Several lines of evidence have supported that adropin plays a role in the physiological regulation of metabolic homeostasis. It has demonstrated that adropin-knockout mice are insulin resistant, while transgenic overexpression of adropin leads to improved glycemic control (Kumar *et al.* 2008). Under hyperinsulinemic-euglycemic clamp conditions, adropin-knockout mice attenuated suppression of endogenous glucose production by insulin (Ganesh Kumar *et al.* 2012). In complementary studies, adropin treatment of obese mice led to a suppression basal and insulin-stimulated hepatic glucose production

Figure 6

The effect of adropin on gluconeogenesis and triglyceride content is dependent on AMPK in OA-induced hepatocytes. (A) Effects of adropin on AMPK α phosphorylation in hepatocytes cells. After drug treatment, AMPK α phosphorylation was detected by Western blot analysis (gel bands). The ratio of phosphorylated/total form of the proteins of AMPK was used as a measure of activation. Effects of AMPK inhibition on adropin-induced (B) mature SREBP-1c protein expression, (C) Glucose output, (D) Glucose uptake, and (E) Triglyceride content. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, one-way ANOVA).

Figure 7

Knockdown of AMPK α 1 abolished the effects of adropin on glucose output and triglyceride accumulation in tilapia hepatocytes. Hepatocytes were transfected with control (scrambled) or AMPK-targeting siRNA (siAMPK α 1) and stimulated with adropin (100 nM) 24 h later. (A) AMPK protein expression after siRNA transfection was determined by Western blot analysis. Effects of AMPK knock-down on adropin-induced (B) mature SREBP-1c protein expression, (C) Glucose output, and (D) Triglyceride content. Three biological replicates were analyzed, and four technical repeats were performed per sample. All results are expressed as the mean $\pm$ S.E.M. ($n = 3$). Different superscript letters in each column show significant differences ($P < 0.05$, one-way ANOVA).

(Thapa *et al.* 2019). The recent *in vitro* and *in vivo* studies indicated that adropin treatment downregulated the expressions of *g6pase*, whereas having no effect of adropin on suppression of *pepck* gene expression in mice (Gao *et al.* 2019, Thapa *et al.* 2019). Consistent with previous findings, the present study provides evidence that adropin inhibited hepatic glucose production. Hepatic glucose production is mainly regulated by PEPCK and G6Pase, which are important to catalyze the rate-limiting steps in gluconeogenesis, and their activities are primarily modulated through hormonal control of transcription (Yabaluri & Bashyam 2010). In our current studies, adropin is effective in suppressing the level of the enzyme activities and transcriptions of PEPCK and G6Pase in OA-induced hepatocytes. Therefore, the decreased gluconeogenesis apparently contributes to

the adropin-induced reduction in glucose production in OA-induced hepatocytes. Also, loss of adropin production reduces glucose uptake in gastrocnemius and diaphragm muscles (Ganesh Kumar *et al.* 2012), whereas adropin treatment significantly decreases in whole-body glucose uptake under hyperinsulinemic conditions (Thapa *et al.* 2019). These previous studies indicate that adropin plays an essential role in controlling glucose uptake in mouse models. However, the exact effects of adropin on hepatic glucose uptake and underlying mechanisms *in vitro* are not fully understood. The current results showed that adropin could enhance OA-reduced glucose uptake in hepatocytes. It is well established that the increased glucose uptake in hepatocytes has been primarily attributed to the upregulation of GLUTs (Karim *et al.* 2012). In search of the mechanism by which adropin enhances glucose uptake, we analyzed the gene expression of different GLUT subtypes. Adropin abrogated OA-reduced *glut1* mRNA level, but did not alter *glut2* gene expression in OA-treated hepatocytes. Since the changes in the gene expression level of *glut1* appeared to correspond to the glucose uptake ability, the effect of STF-31, a selective GLUT1 inhibitor, on the glucose uptake activity was tested. Our results clearly showed that adropin-stimulated glucose uptake in OA-treated hepatocytes was completely suppressed by treatment with STF-31, suggesting that GLUT1 plays a central role in adropin-mediated glucose uptake activity of tilapia hepatocytes. Therefore, adropin exerts its glucose-lowering effect in OA-induced hepatocytes by suppressing gluconeogenesis and facilitating glucose uptake.

Apart from governing glucose utilization, it has been found that circulating adropin is suppressed by obesity in mice (Kumar *et al.* 2008) and humans (Butler *et al.* 2012). Transgenic overexpression of adropin markedly reduced plasma triacylglycerol levels in diet-induced obese mice (Kumar *et al.* 2008). Adropin-knockout mice exhibit increased adiposity, fasting triglycerides and hepatic steatosis (Ganesh Kumar *et al.* 2012). Moreover, intraperitoneal administration of adropin to hyperlipidemic rats for 10 days was extremely effective in decreasing the levels of serum triglycerides (Akcilar *et al.* 2016). In contrast, short-term treatment of adropin failed to result in significant reductions in triacylglycerol levels in diet-induced obese mice (Gao *et al.* 2019). In tilapia, adropin treatment has no significant effect on OA-fed tilapia body weight, which is consistent with previous results from mice studies (Kumar *et al.* 2008), whereas intraperitoneal administration of adropin could significantly attenuate hepatic lipid content in OA-fed tilapia. Studies in Atlantic salmon have shown increased

total hepatic lipid storage with increasing levels of OA in the diet (Stubhaug *et al.* 2005). Oil Red O staining confirmed massive red-stained lipid accumulation in the OA-fed tilapia liver tissue. In contrast, the administration of adropin significantly ameliorated lipid droplet accumulation in the liver. *In vitro* studies further confirmed that OA treatment alone caused a significant increase in triglyceride accumulation in hepatocytes. Especially, pretreatment of adropin inhibited OA-induced triglyceride levels. Our present results demonstrated that adropin could decrease hepatic lipid accumulation *in vivo* and *in vitro* and suggested that adropin has potential hepatoprotective effects in hyperlipidemic fish.

Hepatic lipid accumulation may be primarily caused by increased *de novo* lipid synthesis, enhanced lipid uptake and suppressing of lipid catabolism. SREBP-1c is well known as the transcription factor that induces key hepatic lipogenic enzymes to promote lipogenesis (Horton *et al.* 2002). In cultured rat hepatocytes, insulin increases SREBP-1c transcriptional level, whereas glucagon decreases SREBP-1c gene expression (Foretz *et al.* 1999). Recent studies have provided evidence that the inhibition expression of SREBP-1c has been observed in adropin-treated diet-induced obese male mice (Kumar *et al.* 2008, Gao *et al.* 2019), whereas *srebp-1c* mRNA was increased in adropin knockout mice (Ganesh Kumar *et al.* 2012). However, the regulation of this transcription factor by adropin *in vitro* is not fully clarified. Our results support such a mechanism, showing that adropin inhibited OA-mediated increases in *srebp-1c* gene expression. Moreover, such effects were confirmed by Western blotting in which the protein levels of the nuclear translocation of SREBP-1c in hepatocytes elevated by OA exposure and abrogated by adropin. Activation of SREBP-1c is known to regulate the expression of a battery of lipid metabolic enzymes that include ACC α (Talukdar & Hillgartner 2006), CD36 (Mencarelli *et al.* 2010) and PPAR α (Bougarne *et al.* 2018). ACC α is a cytosolic enzyme that is a key regulatory step in the fatty acid synthesis pathway (Chen *et al.* 2019). Studies in mice support that liver-specific deletion of ACC α reduces hepatic triglyceride accumulation (Mao *et al.* 2006). CD36 is known as fatty acid translocase because it binds long-chain free fatty acids and facilitates their transport into cells (Garcia *et al.* 2019), which elevated expression levels with high-fat diets in human (Miquilena-Colina *et al.* 2011) and mice (Wilson *et al.* 2016). Hepatocyte-specific disruption of CD36 leads to reduced fatty liver and enhances insulin sensitivity in high-fat diet mice (Wilson *et al.* 2016). It has been shown that injected adropin suppressed the protein expression levels

of CD36 in high-fat diet mice (Thapa *et al.* 2019). Here we demonstrated that adropin inhibited OA-mediated increases in the gene and protein expression levels of ACC α and CD36, suggesting that adropin stimulated lipid metabolism through combined inhibition of lipogenesis and lipid uptake. This, however, contradicts the study of Kumar *et al.* (2008), which documented adropin treatment failed to affect ACC α and CD36 mRNA expression in the diet-induced obese male mice liver. This difference could be attributed to different species, dietary fatty acids, as well as genes under consideration. The FABPs gene family comprises a group of high-affinity intracellular fatty acid-binding proteins (Zimmerman & Veerkamp 2002). Expression levels of *Fabp4* in this study were not significantly altered by adropin. Given that different FABP isoforms shared similar functions in lipid homeostasis (Zimmerman & Veerkamp 2002), we could not exclude the possibility that other isoforms of FABP could be involved in adropin-mediated fatty acid transport. PPAR α is involved in promoting gluconeogenesis (Xu *et al.* 2002) and stimulating hepatic fatty acid oxidation (Pawlak *et al.* 2015), which is the predominantly expressed form in the liver. It is worth noting that the treatment of adropin upregulated OA-inhibited PPAR α at the mRNA and protein levels in hepatocytes, which are thought to be involved in the increase in fatty acid oxidation in tilapia. This is contrary to the previous studies by Kumar *et al.* (2008) and Ganesh Kumar *et al.* (2012). In their studies, adropin overexpression or knockout mice could not affect hepatic PPAR α mRNA expression. The cause for this inconsistency is not readily apparent, but might be due to species-specific physiological differences. The enzyme of CPT1 α is essential for fatty acid oxidation (Bonnefont *et al.* 2004). However, this phenomenon is not supported by our results since *Cpt1a* was not significantly different in this study. This is in agreement with the recent study by Thapa *et al.* (2019) where non-significant differences recorded in CPT1 α protein expression in adropin-treated high-fat diet mice. Therefore, adropin exhibits an inhibitory effect on hepatic lipid accumulation through the regulation of key lipid metabolic factors in a fish model.

Then we focused on the mechanism by which adropin modulated glucose utilization and triglyceride metabolism in OA-treated hepatocytes. The role of AMPK as a sensor of metabolism regulates glucose and lipid metabolism, which is a conserved master regulator of metabolism in vertebrates (Garcia & Shaw 2017). Activation of AMPK has the potential to change cells from an anabolic to a catabolic state, leading to inhibition of hepatic lipogenesis and increased fatty acid oxidation

(Kahn *et al.* 2005). AMPK could also modulate SREBP-1c and PPAR α , which plays an important role in the regulation of gene products responsible for the synthesis of cholesterol, fatty acids, and triglycerides in the liver (Trepiana *et al.* 2018). These considerations prompted us to propose AMPK was likely to be targeted by adropin in OA-induced tilapia hepatocytes. However, it remains controversial whether AMPK is required for the metabolic effects of adropin. Some reports have demonstrated that adropin regulates cardiac energy substrate preference through activation of downstream insulin signalling, but not AMPK pathway (Altamimi *et al.* 2019). In line with this report, the administration of adropin fails to alter AMPK activity in the diet-induced obese male mice liver (Gao *et al.* 2019). In contrast, injection of adropin in the third ventricle of the rats increased AMPK activation via calmodulin-dependent protein kinase kinase- β (Yang *et al.* 2017). In the present study, adropin treatment was found to activate AMPK to increase the p-AMPK/AMPK ratio in OA-treated hepatocytes. To further confirm whether the effects of adropin on glucose utilization and triglyceride metabolism were mediated by activation of AMPK, the pharmacological AMPK inhibitor compound-C and AMPK α 1 siRNA were applied to block the AMPK signalling, respectively. We observed that blockade of adropin-induced activation of AMPK reversed its inhibition on SREBP-1c nuclear translocation. In addition, blocking AMPK also were effective in abolishing the inhibition of adropin on glucose output and triglyceride accumulation in hepatocytes. These data suggest that AMPK is required in adropin-induced gluconeogenesis and a marked accumulation of hepatic triglycerides in OA-induced fish hepatocytes. However, AMPK involved in adropin-induced glucose uptake can be ruled out because compound C had no effect on glucose uptake in hepatocytes. This result was surprising given that previous work in rodent models had shown

that AMPK activation stimulates glucose uptake (Hayashi *et al.* 1998, Logie *et al.* 2018). Since adropin directly enhances the hepatic insulin signalling pathway to reduce hyperglycemia in diet-induced obesity mice (Gao *et al.* 2019), the possibility that adropin-stimulated glucose uptake activity through insulin signalling should be concerned. Together with these reports, our current findings suggest that adropin could activate AMPK and/or insulin signalling pathways under certain conditions. However, whether crosstalk was occurring between these two signalling pathways in the presence of adropin is still unclear. Therefore, studies are required to further elucidate how these two signalling pathways collectively contribute to the hypoglycemic effect of adropin.

In conclusion, our data demonstrate that tilapia adropin inhibits gluconeogenesis by downregulation of PEPCK and G6Pase enzyme activities and gene expression, and increases glucose uptake by upregulation of *glut1* gene expression. Meanwhile, adropin attenuates triglyceride accumulation by downregulating several important metabolic regulatory factors involved in lipid metabolism, such as SREBP-1c, ACC α , and CD36 and upregulating PPAR α expression. We also propose that adropin activates AMPK to inhibit hepatic gluconeogenesis and triglyceride content in a high-fat environment (Fig. 8). Our results provide new knowledge of the adropin in Nile tilapia and could help to understand the regulatory mechanism of energy metabolism in fish.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0077>.

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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Figure 8

Schematic diagram showing the downstream signal mechanism of adropin in modulating glucose utilization and triglyceride (TG) accumulation in hepatocytes. Grey arrows represent the changes caused by adropin.

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