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Inhibited autophagy impairs systemic nutrient metabolism in Nile tilapia

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ABSTRACT

Autophagy is a conserved cellular degradation process through which intracellular components are degraded by the lysosome, but its roles in fish metabolism have not been studied in depth. Therefore, the present study aimed to investigate whether autophagy plays a key role in maintaining metabolic homeostasis in fish. In an 8-week feeding trial, Nile tilapia were fed either a control diet with medium fat and medium carbohydrate (Control), or a control diet supplemented with a classic autophagy inhibitor (chloroquine, CQ). CQ supplementation significantly inhibited autophagy and impaired fish growth and protein synthesis, and the glycolysis was stimulated, accompanied by fat accumulation, high oxidative stress and inflammation. Physiological status and gene expressions suggested that impaired autophagy might be at least one cause of the metabolic diseases which has been commonly seen in aquaculture. These results indicate that inhibition of autophagy could significantly affect the metabolism of lipid, carbohydrate and protein in fish; hence, autophagy could play important roles in maintaining homeostasis of nutrient metabolism in cultured fish.

1. Introduction

Autophagy is an evolutionarily conserved pathway that involves the sequestration of cytoplasmic components and their delivery into lysosomes for degradation (Levine and Klionsky, 2004). Except for the functions of autophagy to clean misfolded proteins and to destroy organelles and infecting pathogens(Levine and Kroemer, 2008), the range of autophagy substrates has been extended to nutrients storage, and increasing evidence indicates that autophagy is involved in regulation of metabolism(Ding et al., 2010; Amir and Czaja, 2011; Ward et al., 2016; Schulze et al., 2017). In particular, lipophagy is known to play a critical role in energy metabolism. This biological process could degrade excess lipid droplet (LD) to produce free fatty acids (FFAs) for β-oxidation and then generate acetyl coenzyme A for tricarboxylic acid (TCA) cycle to produce energy (Singh et al., 2009; Skop et al., 2012). Accordingly, in hepatocytes, triglycerides were accumulated and the rate of β-oxidation was decreased when autophagy was genetically ablated (Singh et al., 2009; Liu and Czaja, 2013) or biochemically inhibited by chloroquine (CQ), a classic autophagic inhibitor that blocks functions of the lysosome (Ouimet et al., 2011). Furthermore, recent studies indicate that autophagy is involved in the interactions among lipid, glucose and protein metabolism, and is related to the progression of diabetes (Kuma et al., 2004; Onodera and Ohsumi, 2005; Lim et al., 2014; Farah et al., 2016). For example, autophagy-related genes 7 haploinsufficient ($Atg7^{+/-}$) mice were used to explain their progressions facilitated from obesity to diabetes, as intracellular lipid content and insulin resistance were demonstrated to increase in $Atg7^{+/-}$-ob/ob mice (Lim et al., 2014). Moreover, in the situation of glucose-6-phosphatase deficiency (known as von Gierke's disease or glycogen storage disease type 1A), genetic or pharmacological stimulation of autophagy led to improved hepatic lipid metabolism and reduced hepatic steatosis (Farah et al., 2016). In autophagy-deficient cellular and animal models, the amount of total free amino acids (FAA) or several specific FAAs, such as histidine, methionine, and glutamine/glutamate were largely decreased and bulk protein synthesis was substantially reduced under fasting conditions (Kuma et al., 2004; Onodera and Ohsumi, 2005). All these studies with mammalian models have indicated that autophagy plays an important role in maintaining metabolic homeostasis. However, the physiological functions of autophagy in fish metabolism have seldom been reported.

Currently, the global shortage and the increasing costs of several traditional dietary protein sources, such as fish meal, has been a serious problem in aquaculture. In order to decrease the portion of the dietary protein which is used for energy supply, the diets which contain high non-protein energy, such as high fat and/or high carbohydrate diets (HFD and HCD), have been popularly used in aquaculture to play “protein sparing effects” (Ackman et al., 1999; Xu et al., 2001). However, the popular usage of these HFD and HCD also caused some adverse effects in increasing fat deposition and impairing fish health and growth (Du et al., 2010; Lu et al., 2013a; Sagada et al., 2017; Deng

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et al., 2018). Therefore, an urgent task for fish nutritionists is to understand the mechanisms of metabolic diseases and to identify effective methods to maintain metabolic homeostasis. Excess fat accumulation in fish, caused by a HFD and/or HCD, correlated with impaired fatty acid β-oxidation and lipolysis (Du et al., 2006; Liu et al., 2014; Lei et al., 2017), and inhibited lipid transport activities (Lu et al., 2013b) or lowered lipid catabolism-related nuclear receptor sensitivities (Ning et al., 2016; Ning et al., 2017). Yet the correlation between autophagy and metabolic diseases in fish has not been well established. Recently, for the first time, we reported that lipophagy exists in zebrafish: the inhibition of lipophagy by CQ and 3-methyladenine (3-MA), another autophagy inhibitor that blocks the formation of autophagosomes, caused systemic depression of lipid metabolism and an accumulation of lipid in zebrafish liver (Wang et al., 2018). In addition, Wei et al. reported that dietary zinc could reduce hepatic fat accumulation through activating lipophagy in yellow catfish (Wei et al., 2018). Thus, the results of both studies suggested that autophagy in fish is involved in lipid metabolism. Therefore, we hypothesized that impaired autophagy could be an important cause of excess fat accumulation.

In the present study, Nile tilapias were fed with two purified experimental diets: a diet containing medium contents of carbohydrate and fat (Control diet), and the control diet supplemented with CQ (+CQ) for 8 weeks. The use of CQ in the control diet was intended to inhibit autophagy, and thus verify whether impaired autophagy would cause the metabolic diseases in fish. In the present study, the protein expressions of microtubule associated light chain 3(LC3), Beclin1 and p62 were measured to describe the biological process of autophagy, especially for the LC3, which is a gold marker in the detection of autophagy. The LC3 proteins are synthesized in a precursor form (pro-LC3), which is processed to become LC3-I, and after being covalently bound to phosphatidylethanolamine (LC3-II) through the regulation of Atg5/Atg16/Atg12 complex, which is attached to the phagophore membrane to catalyze membrane elongation and closure (Mizushima et al., 2011). Therefore, the genes expression of LC3 (LC3a/ LC3b, two subtypes) and Atg12 were also detected in fish liver and muscle. Then lipid metabolism-related genes in three tissues were tested, including lipogenesis (fatty acid synthase, acetyl-coA carboxylase α, diacylglycerol o-acyltransferase), lipid catabolism (acyl-coA oxidase, peroxisome proliferator activated receptor alpha, adipose triglyceride lipase, and lipoprotein lipase) and transport (CD36 , fatty acid-binding protein 4). The molecular parameters of glycolysis (pyruvate kinase, glycerol kinase, phosphofructokinase), gluconeogenesis (glucose-6-phosphatase, fructose-1,6-bisphosphatase), glucose uptake (glut2, P-IRβ), protein synthesis (mechanistic target of rapamycin, P-mTOR, P−S6) and decomposition(aminopeptidase n, glutamate dehydrogenase 1, asparagine synthetase, P-EIF2α) were also investigated to better understand the role of autophagy in nutrient metabolism in fish.

2. Materials and methods

2.1. Animal ethics

All experiments were conducted strictly under the Guidance of the Care and Use of Laboratory Animals in China. This study was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.2. Fish, diets and experimental design

Before the experiment, > 100 Nile tilapias (around 10 g) were transported from Guangzhou tilapia farm and acclimated in 200-liter tanks in our lab for 2 weeks. During this acclimating period, fish were fed with the same commercial tilapia diets as fish ate in fish farm. The water parameters were the same as those in the formal experiment. After acclimation, healthy Nile tilapias with similar weights (13.57 ± 0.15 g) were selected and randomly distributed into two groups with 30 fish per tank. Each fish was embedded with a PIT-tag (HID Global, Austin, Texas, USA) using unique radio-frequency identification (RFID) code to precisely track its growth, and the tracking tag could be recognized by a RFID machine (Boise, ID, USA) as described previously(Voulodimos et al., 2010). Treatment groups were set as: control group (a purified diet containing medium contents of carbohydrate and lipid, protein 35%, lipid 5%, carbohydrate 30%) and CQ group (the control diet supplemented with CQ, an well-known autophagy inhibitor). The formulation of the control diet is presented in Supplemental Table 1. All ingredients were ground into powder and mixed thoroughly. During the mixing, the oil and distilled water were added to make a dough. Then the dough was pelleted (2.0 mm dimeter) by using a screw-press pelletizer (F-26, South China University of Technology, Guangzhou, China). The pellets were air dried at room temperature for approximately 48 h until the moisture content was lower than 10%. After drying, all diets were packed and stored at −30 °C until usage. CQ, purchased from the Sigma Chemical Co, was mixed into the control diet to the final dose of 100 mg/kg diet, and the feeding rate was set as 4% body weight (BW). The dosage of CQ was evaluated by our preliminary study, and was verified to have metabolic regulatory functions without obvious toxic effects (see Supplemental Fig. 1). During the 8-week trial, treated fish were fed at 9:00, 14:00 and 20:00 with an equal portion of diet. The weight of each fish was recorded and tracked every 2 weeks and the feeding amount was adjusted accordingly. Water temperature was maintained at 27 ± 1 °C by an automatic heater with a 12 h light-dark cycle for 8 weeks. Continuous aeration was provided by an air blower. At every morning, before feeding, aeration was temporally stopped and the feces were removed by a pumping tube. Afterwards, half water in the tanks was replaced by stock water which had the same water quality as the water in culture system. During the feeding trial, the dissolved oxygen, pH and total ammonia nitrogen were maintained at ranges from 4.8–6.4 mg/L, 7.5 to 7.9 and < 0.02 mg/L, respectively.

2.3. Sampling and the measurements of biochemical parameters

At the end of trial, all fish were fasted overnight, six fish of each group were euthanized (MS-222 at 20 mg/L) and sampled to collect tissues to measure the molecular, protein and biochemical indexes. Hepatic triglyceride (TG), glycogen, malondialdehyde (MDA), superoxide dismutase (SOD), and serum TG, free fatty acid (FFA), and glucose were assessed by commercial kits (Jiancheng Biotech Co. China). The serum insulin was detected by ELISA kits (Hengyuan Biotech Co. China). Briefly, the total lipid of the whole fish body, liver and muscle was extracted by using chloroform/methanol (2:1, *v*/v) as previously described (Bligh and Dyer, 1959). Briefly, the samples were homogenized in the mixed chloroform-methanol 2:1 (vol/vol), and the samples were stored at 4 °C for 24-h extraction. Afterwards, the chloroform phase was carefully moved to a clean glass tube and dried using nitrogen, and the extracted total lipid was weighed and recorded. Whole fish protein and muscle protein were measured by Kjeltec™ 8200 (FOSS, Sweden).

2.4. Mitochondrial and peroxisomal [1-^{14}C] palmitate oxidation in liver

After the feeding trial, the whole liver of 4 fish collected from each group were weighted and homogenized in the ice-cold 0.25 M-sucrose medium containing 2 mM-EGTA and 10 mM-Tris-Cl, pH 7.4,(1:40, *w/v*) by using a drill-driven Teflon glass homogenizer with 4–6 strokes. The samples of homogenate were used for the immediate measurement of total [1-^{14}C] palmitate β-oxidation as previously reported (Degrace et al., 2006; Du et al., 2006). Palmitate oxidation rates were detected at 28 °C using two media as already described (Veerkamp et al., 1983), the first media allowing both mitochondrial and peroxisomal β -oxidation to occur, and the second one allowing peroxisomal β -oxidation only. After 0.5 h, the radio activity initially held by [1-^{14}C] palmitate was

recovered on labelled short molecules released from the β-oxidative cycle and soluble in perchloric acid (acid-soluble products, ASP). The pure radioactive ASP medium was collected using 0.45 μm membrane filters and measured after mixing with the scintillation cocktail in a liquid scintillation spectrometer MicroBeta2 Plate Counter (Perkin, USA).

2.5. Quantitative real-time PCR

Total RNA was isolated by using a Tri Pure Reagent (Aidlab, China). The quality and quantity of total RNA were tested by NANODROP 2000 Spectrophpto (Thermo, USA). cDNAs of tissues total RNA were synthesized using a PrimerScript™ RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Tiangen, China) by S1000TM Thermal Cycler (Bio-Rad, USA). β -actin were used as the reference gene. The primers of β-actin and target genes (Supplemental Table 2) for Quentitative PCR (qPCR) were designed to overlap intron. qPCR (20 μL) was carried out by using 2 × Ultra SYBR Mixture (Aidlab, China) in 12 × 8 well plates in a CFX Connect Real-Time System (Bio-Rad), containing 10 μL of SYBR Mixture, 2 μL cDNA, 1.6 μL of qPCR primers (4 μ M), and 7.4 μL nuclease-free water. The program of qPCR reaction included 95 °C for 10 min, 40 cycles of 95 °C for 5 s and 60 °C for 15 s. The melting curves of amplified products were generated to ensure the specificity of assays at the end of each PCR. qPCR efficiency was between 98% and 102% and the correlation coefficient was over 0.97 for each gene. The method of $2^{-\Delta\Delta Ct}$ was used for estimating the relative cDNA abundance (control CONTROL group as control).

2.6. Western blotting

Liver homogenates were prepared by using the RIPA lysis buffer (Beyotime Biotechnology, China) for western blot (WB) analysis. Proteins were separated by using 7% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes. Membranes were incubated for 1 h in a blocking solution containing 5% milk in TBST. Membranes were washed briefly in TBST and incubated overnight at 4 °C with the anti-LC3A/B(1:800, catalogue no.4108, CST), anti-Beclin1(1:800, catalogue no.11306–1-AP, Proteintech) , anti-P62(1:800, catalogue no.5114, CST), anti- P-mTOR (Ser2448) (1:800, catalogue no.2971,CST), anti-p-AMPKα(Thr172)(1:800, catalogue no.2535, CST), anti-P-S6(Ser235/236) (1:800, catalogue no.4856, CST), anti-P-EIF2α(1:800 catalogue no.3398, CST), anti- p-IRβ(Tyr1345)(1:800, catalogue no.3026, CST), anti-INSR(1:800, catalogue no.20433–1-AP, Proteintech), and anti-GAPDH(1:3000, catalogue no. AB0036, Abways) antibodies. After washing blots to remove excessive primary antibody binding, blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. The WB images were obtained by using the Odyssey CLx Imager (Licor, USA).

2.7. Statistical analyses

Values are expressed as mean ± SEM ($n = 6$). Independent-samples *t*-test was performed to evaluate the significant difference ($P < .05$) of variables between control and + CQ group. All data was conducted using the SPSS Statistics 19.0 software (IBM, USA).

3. Results

3.1. The effect of autophagy inhibition on growth, fat deposition and fatty acid β-oxidation in tilapia

To confirm the efficiency of the CQ reagent, autophagy-related proteins were measured in fish liver. Compared with the control group, CQ supplementation had effectively increased expression of microtubule associated light chain 3(LC3) (Fig. 1A, B), indicating that CQ inhibited lysosomal degradation and caused deposition of LC3-II protein in autolysosomes. CQ supplementation also increased the protein expression of beclin1, which contributes to the activation of downstream autophagy components, and it likewise increased the expression of p62, a protein that tags the autophagy substrates and reflects their degradation. Besides, autophagy related genes such as LC3a, LC3b and Atg12 (Fig. 1C) were decreased in both liver and muscle tissues in CQ treated group. Together, these results showed that autophagy in the tilapia was inhibited by the CQ supplementation. Fig. 1D shows the effect of autophagy inhibition on fish growth and at the end of the feeding trial, the CQ group had the lowest body weight. There were no significant difference in the liver and mesenteric fat mass (Fig. 1E, F) between two groups but rising trends could be seen in the CQ group.

The total lipid content in whole fish, muscle and liver are shown in Fig. 1G. Compared with the control group, fish treated with CQ supplementation tended to display greater total lipid content in whole body, liver and muscle. A similar pattern was observed for triglyceride content in liver (Fig. 1H). CQ supplementation significantly reduced the serum concentration of free fatty acids (FFA) as compared with control diet (Fig. 1I). Moreover, serum concentrations of ALT, a marker of liver injury, were significantly higher in the CQ group (Fig. 1J). Furthermore, a [1–^{14}C]-palmitate β-oxidation assay indicated that CQ tended to reduce mitochondrial β-oxidation activity while increase peroxisomal β-oxidation as compensation (Fig. 1K).

3.2. The effect of autophagy inhibition on the mRNA expression of lipid metabolism genes

The effects of CQ on systemic expressions of lipid metabolism-related genes in liver, muscle and adipose tissues of the Nile tilapia are presented in Fig. 2. In liver, four lipogenesis-related genes (FAS, ACCα, DGAT and SREBP-1C) were downregulated by dietary CQ as compared with the controls, and significance was observed for FAS and SREBP-1C (Fig. 2A). For lipid catabolism-related genes, CQ significantly reduced ATGL and PPARα expressions. Besides, the expressions of lipid intake and transport genes, LPL, CD36 and FABP4 were all decreased in the CQ group. In muscle, the overall tendency was similar with that in liver. (Fig. 2B). In adipose tissue, CQ did not significantly affect expressions of the lipid metabolism-related genes. Together, data for the three tissues indicated that liver was much more sensitive than muscle and adipose tissue to the CQ diets, and autophagy inhibition could cause adverse impacts on lipid metabolism.

3.3. The effect of autophagy inhibition on glycogen and glycometabolism in Nile tilapia

Lower glycogen content in liver and muscle was seen in the CQ treatment group (Fig. 3A, B) Serum glucose and insulin showed a similar pattern and CQ treatment significantly lowered serum insulin as compared with the control group (Fig. 3C, D). Expression of the genes related to glucose metabolism was measured in liver, muscle, and adipose tissues. In liver (Fig. 3E), the expressions of GK and GS were increased in the CQ group while CQ significantly reduced expressions of PK and G6Pase. In muscle (Fig. 3F), CQ significantly increased expressions of most of the glycometabolism-related genes, including PK, GK, G6Pase and Glut4. In adipose tissue (Fig. 4G), CQ significantly increased expressions of PK and PFK. This result indicates that CQ activated glucose metabolism in muscle and adipose tissue.

In addition, several proteins associated with glucose metabolism-related signal pathway were examined in liver (Fig. 3H, I). As compared with the control group, the expression of insulin receptor β phosphorylation (p-IRβ) was weakened in the CQ group, which demonstrating that insulin sensitivity was impaired in the CQ group. The expression ratio of p-IRβ/insulin receptor (INSR) was also significantly less in the CQ group. Similarly, the AMP-activated protein kinase phosphorylation (p-AMPK), which is an important metabolic regulator with the function

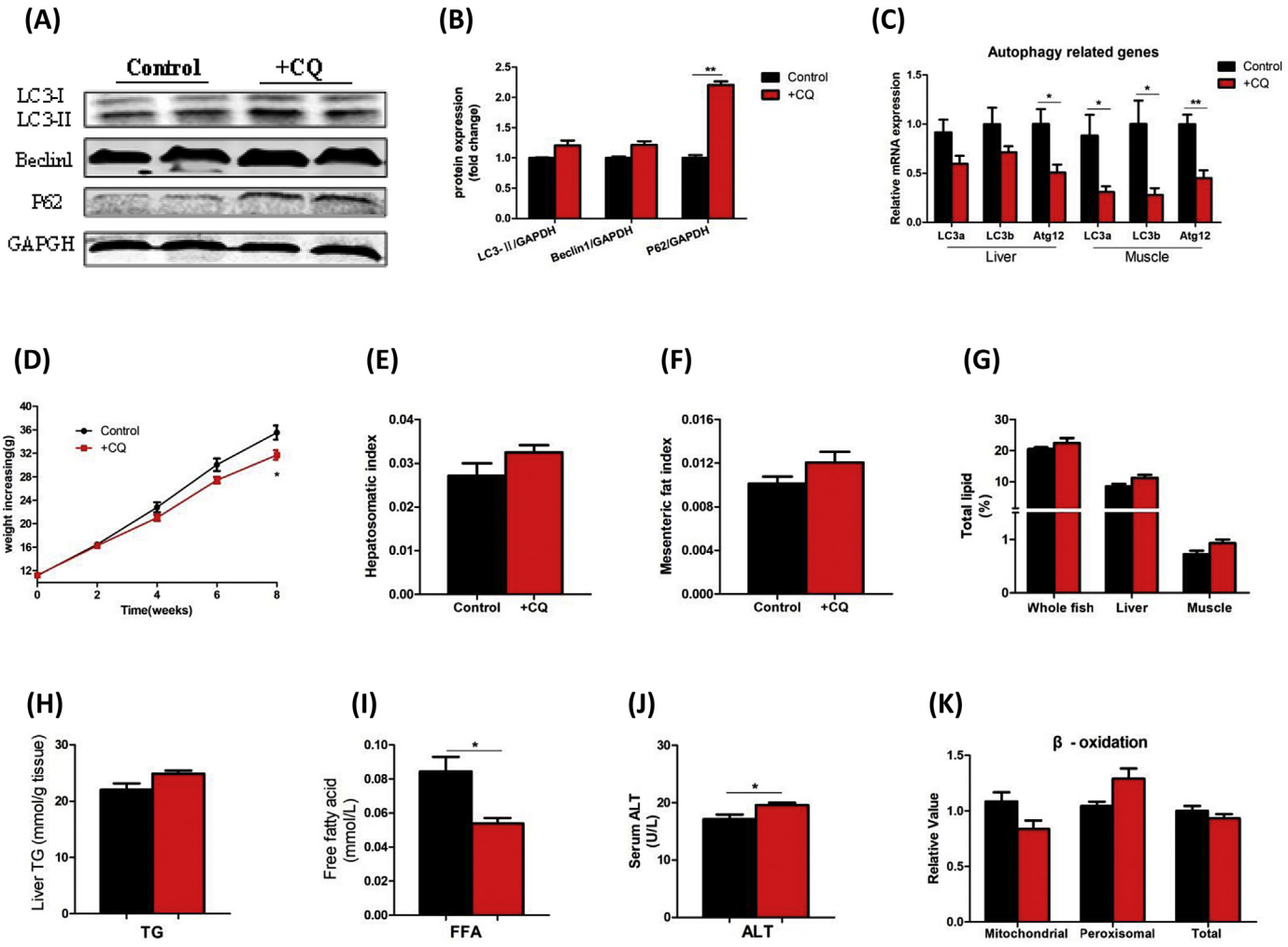

Fig. 1. The effect of autophagy inhibition on Tilapia's growth, fat deposition and fatty acid β-oxidation in the 8-week trial. (A, B) autophagy related proteins in fish liver were measured by western blotting; (C) autophagy related genes in fish liver and muscle; (D) growth curve; (E) hepatic–somatic index (HSI): liver weight / body weight; (F) mesenteric fat index: mesenteric fat weight / body weight; (G) the total lipid content of whole fish, liver and muscle; (H) hepatic triglyceride (TG) content; (I) free fatty acids (FFAs) in serum; (J) alanine aminotransferase (ALT) in serum;. (K) β-oxidation capability of [1-^{14}C] palmitate in the homogenates of liver; Values are means ± SEM (n = 6). Values with *,**statistically differ at $P < .05$, $P < .01$.

of inhibiting the anabolic pathway and simultaneously activating the catabolic pathway for energy homeostasis, showed the same expression trend as p-IRβ/INSR. From all the above data, we summarize that different tissues regulated glycometabolism differently based on the CQ treatment, and CQ caused abnormal expressions of signal proteins and metabolism genes in liver.

3.4. The effect of autophagy inhibition on protein metabolism in Nile tilapia

The protein content in the muscle tended to decrease in the CQ group (Fig. 4A), and a significant difference was seen in the whole fish. Some genes related to protein metabolism were further measured in liver and muscle (Fig. 5B, C). CQ significantly reduced mRNA expression of mTOR in both tissues. The mRNA expression of aminopeptidase N (APN), which plays a role in protein and amino acid digestion and absorption, was found no difference between two groups. Glutamate dehydrogenase (GDH1) and asparagine synthetase (ASNS), which are essential in amino acid catabolism, were significantly less in liver of the CQ group. A similar trend of GDH1 and ASNS was seen in muscle. The protein-synthesis-related signal proteins were further assayed in liver. The phosphorylation of mTOR and S6, which occurs downstream of mTOR phosphorylation, was markedly diminished in the CQ group (Fig. 4D, E). But EIF2α phosphorylation, which functions in protein catabolism, displayed no obvious differences. In summary, CQ supplementation tended to reduce protein synthesis.

3.5. The effect of autophagy inhibition on antioxidant capability and inflammatory gene expression

To evaluate for possible oxidative stress caused by altered autophagy, we determined the content of malondialdehyde (MDA) and superoxide dismutase (SOD) activities in serum and liver (Fig. 5A–D). CQ increased MDA content in serum and liver, and the SOD value for the CQ group was significantly lower than that of the control (Fig. 5B, D). Two inflammatory factors, TNF-α and IL-1β, were further assayed in liver, muscle and adipose tissue (Fig. 5E, F). Data show that the expressions of both inflammatory cytokines increased in tissues of the CQ group. All the above data show that CQ could induce oxidative stress and inflammation.

4. Discussion

4.1. Autophagy inhibition causes lipometabolic disturbance

In the present study, CQ was used to inhibit autophagy by blocking its final step-lysosomal degradation, as reported in mammals (Skop

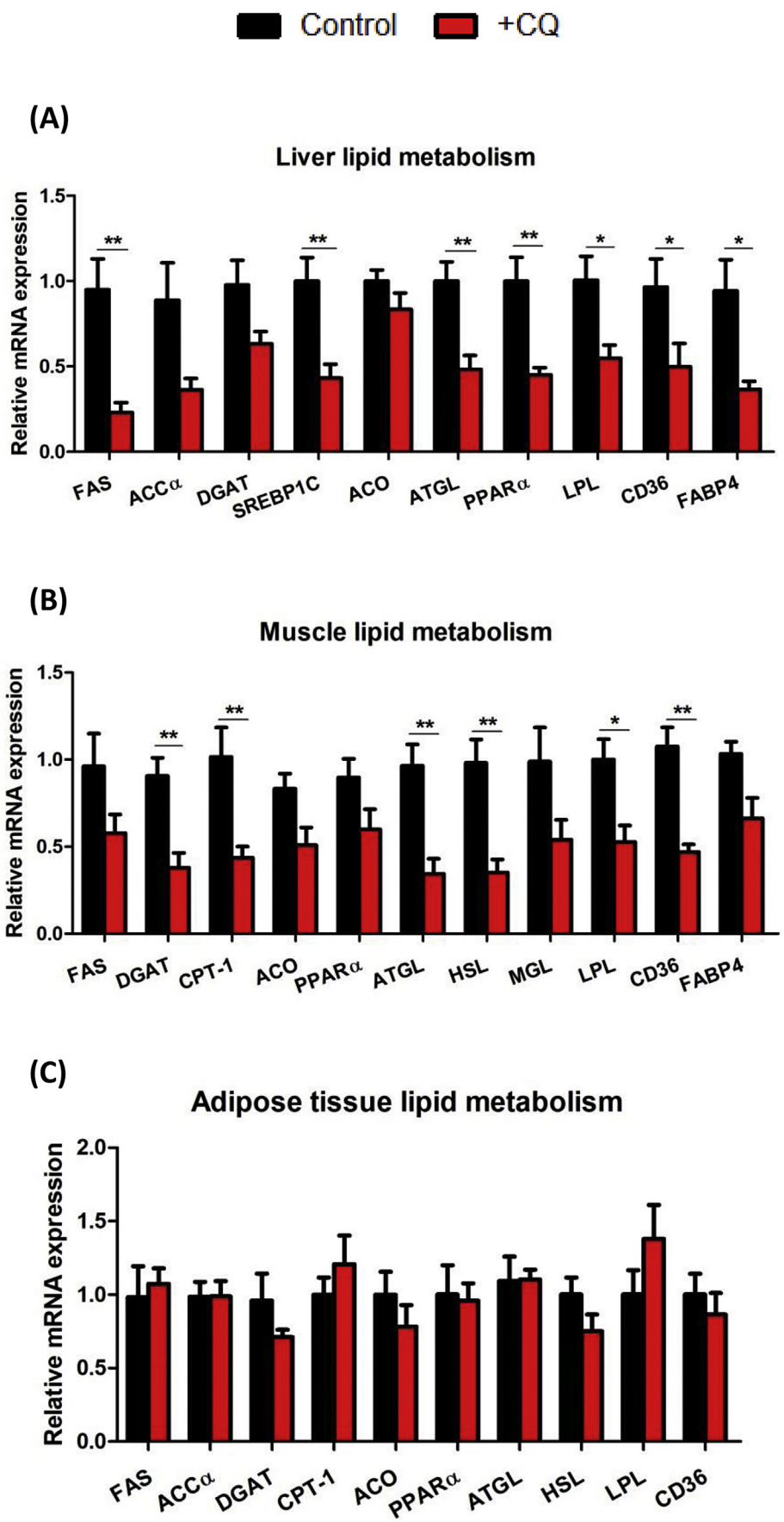

Fig. 2. The effect of autophagy inhibition on the mRNA expression of lipid metabolism genes in liver, muscle and adipose tissue. All values are means ± SEM (n = 6). Values with *,**statistically differ at $P < .05$, $P < .01$.

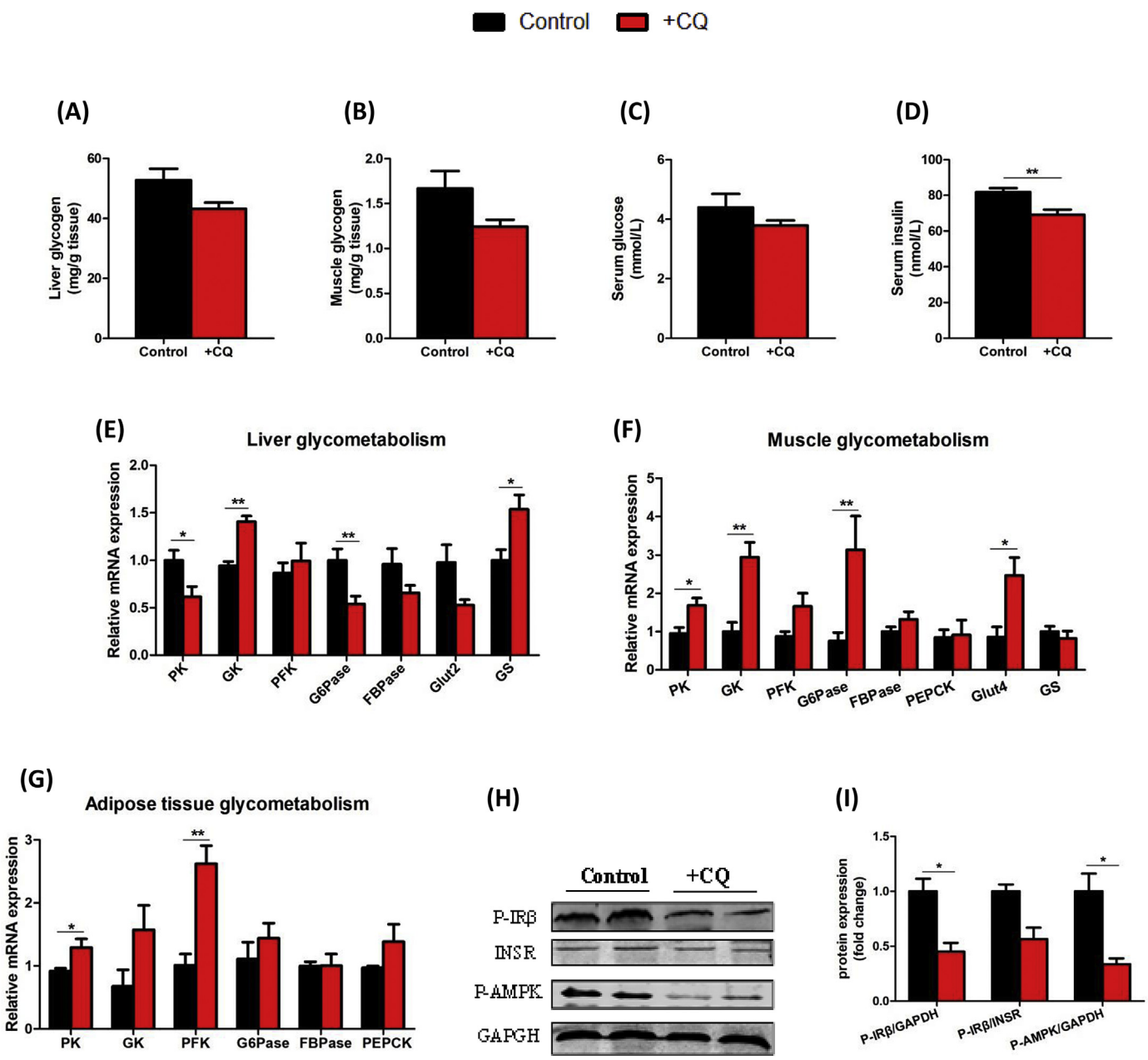

Fig. 3. The effect of autophagy inhibition on glycogen and glycometabolism in Tilapia. (A) glycogen content in liver; (B) glycogen in muscle; (C) glucose in serum; (D) insulin in serum; (E–G) the genes related to glycometabolism in three tissues; (H,I) the protein expression of P-IRβ, INSR and P-AMPK in liver. Values are means ± SEM (n = 6). Values with *,**statistically differ at $P < .05$, $P < .01$.

et al., 2012). Fish fed with CQ supplementation showed poor growth, increased fat deposition in tissues, suppressed lipogenesis genes in liver and muscle, all accompanied by higher oxidative stress and inflammation. More specifically, the lipogenesis genes in liver were significantly inhibited by CQ treatment, which might be a negative feedback because of fat deposition. In muscle, DGAT and the lipid catabolism genes were consistently downregulated by CQ, and in adipose tissue, CQ did not influence these genes. Therefore, our results reveal that: compared with the control, CQ supplementation generated lipometabolic disturbance, which had significant effects on lipid metabolism genes, and the order of reaction sensitivity from strong to weak was liver, muscle and adipose tissue. In mammalian models, mice with hepatic-specific ablation of the autophagy gene Atg7 secreted significantly less triglycerides, with a concomitant increase in liver lipid content (Ouimet, 2013). Recent work has also shown that hepatocytes lacking autophagy were sensitised to galactosamine and lipopolysaccharide (GalN/LPS) injury and death from TNF (Amir et al., 2013). In absence of Atg7/autophagy, the resistance of keratinocytes to intrinsic and environmental oxidative stress was severely impaired and resulted in DNA damage, cell cycle arrest, and a disturbed lipid phenotype (Song et al., 2016). In addition, autophagy blockage has been reported to potentiate inflammasome activity, whereas stimulating autophagy limited it (Shi et al., 2012). Above all, we concluded that autophagy plays essential roles in fish lipid metabolism and homeostasis.

4.2. Autophagy regulation is a promising strategy to maintain lipid homoeostasis

According to the classic fish nutrition theory, the proportion of dietary protein which is used for energy consumption could be partly replaced by dietary carbohydrate and/or lipid, and this is demonstrated

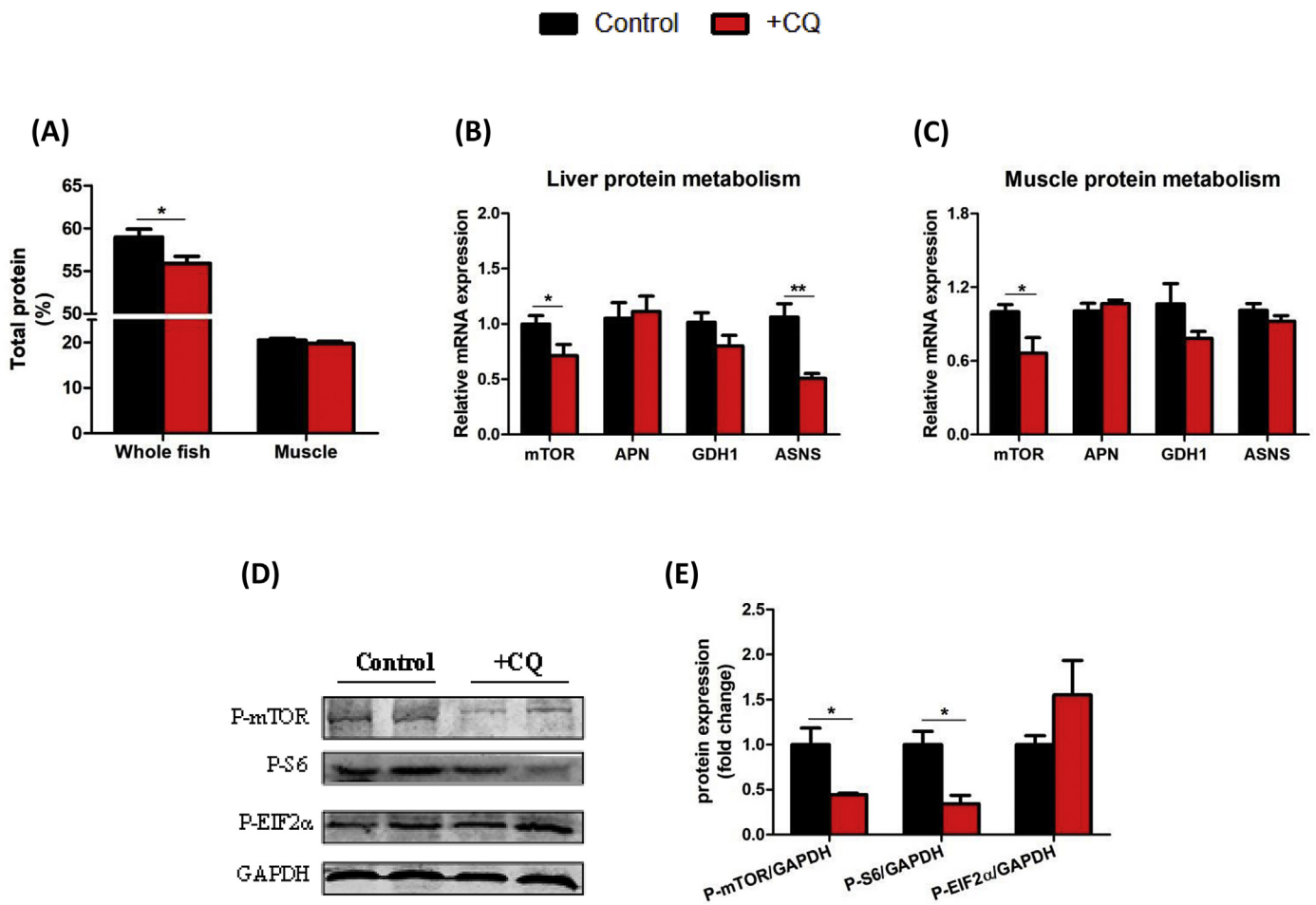

Fig. 4. The effect of autophagy inhibition on protein metabolism in Tilapia. (A) the total protein content of whole fish and muscle; (B, C) the genes related to protein metabolism in liver and muscle; (D, E) the protein expression of P-mTOR, P-S6 and P-EIF2α in liver. Values are means ± SEM (n = 6). Values with *,**statistically differ at $P < .05$, $P < .01$.

as "protein sparing effects", and has been proved by many literatures (Beamish and Medland, 1986; Shiau and Lin, 2001; Li et al., 2012). Because of the shortage of good sources of dietary protein (Watanabe, 2002), such as fish meal, the cost of dietary protein is increasing. In the current aquaculture industry, based on cost-effective approach, and the intention trying to maximize protein-sparing effect, a high proportion of dietary fat and/or carbohydrate has been commonly seen in aquafeeds. In recent years, adverse effects from a HFD and/or HCD in fish have been commonly reported, including excess fat accumulation in tissues (Wang et al., 2015; Zhang et al., 2017), high oxidative stress (Lu et al., 2017), impaired immunity (Jia et al., 2017), destruction of mitochondria functions (Lu et al., 2014), and abnormal gene expression patterns (Zhang et al., 2014; Prisingkorn et al., 2017). Our study indicated that impaired autophagy after feeding with a normal diet could also cause phenotypes similar to those seen with the HF and/or HC diet. Although the CQ treatment were not exactly the same as those with the HFD or HCD treatment, for example, the effects of CQ in adipose tissue were relatively low, but high fat diet largely induced accumulation of mesenteric adipose tissue (Liu et al., 2018). Therefore, the impaired autophagy might be a result of long-term unbalanced nutrient diet feeding, which needs to be further confirmed in fish.

With the functions and mechanisms of autophagy being gradually disclosed, several natural compounds, as an alternative to drugs or biochemical reagents, have been screened for their potential to modulate autophagy. For instance, epigallocatechin3-gallate, found in green tea, has lipophagic effects in the mice liver and in primary bovine aortic endothelial cells (Kim et al., 2013a; Staff, 2014). Dietary polyphenol bergamot has been proven to attenuate hepatic steatosis in mice (Parafati et al., 2015). Additionally, small molecules such as resveratrol and caffeine are capable of exerting protective effects on fatty liver disease via the autophagy-lysosomal pathway in mice and human hepatoma HepG2 cells (Sinha et al., 2014; Zhang et al., 2015; Tang et al., 2016). These studies potentially pave the way for a promising strategy that maintaining lipid homoeostasis through the regulation of autophagy.

4.3. Autophagy is involved in the regulation of glucose and protein metabolism

Our data show that glycogen content was lower in the CQ group (Fig. 3), indicating that glycogen utilisation was improved by the CQ treatments. Diminished insulin sensitivity as reflected by serum glucose and insulin was consistent with the western blotting results, which suggests that metabolizing glycogen to provide energy is in demand under the condition of blocked-autophagy. In addition, genetic data from the three tissues showed that glycolysis, elevated by CQ treatment, took place mainly in muscle, and CQ also improved glycolysis in adipose tissue; however, CQ mostly reduced the capacity of gluconeogenesis in liver. Therefore, CQ-induced autophagy inhibition seemed to reduce the portion of energy sourced from lipid metabolism, yet enhanced glycolysis compensated on the whole. The results partly correspond to previous transcriptomic data on CQ-treated zebrafish (Wang et al., 2018). In the mammalian model, mice with targeted disruption of an autophagy-related gene in pancreatic beta cells had defective insulin

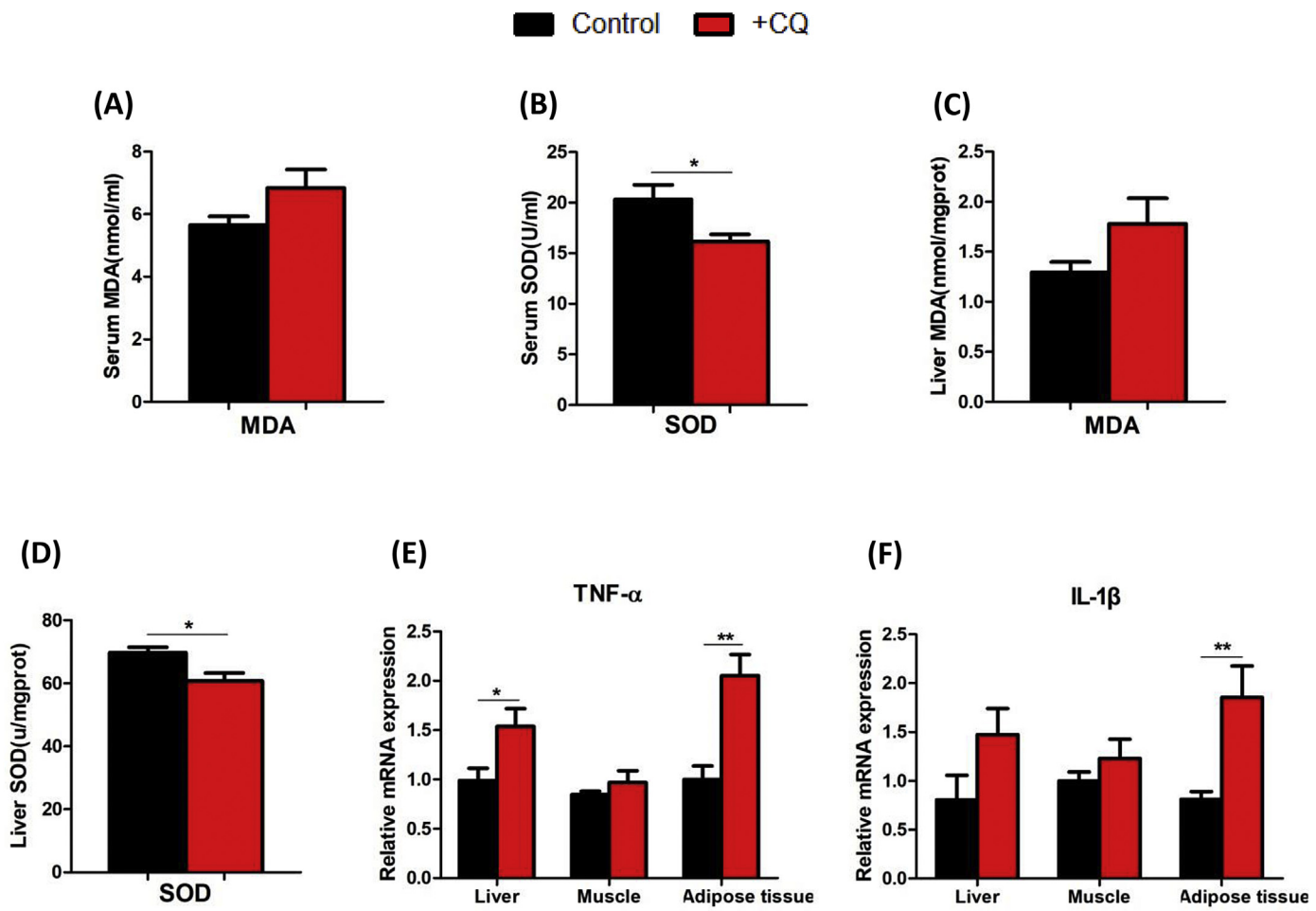

Fig. 5. The effect of autophagy inhibition on antioxidant capability and inflammatory gene expression. (A) malondialdehyde(MDA) in serum; (B) superoxide dismutase(SOD) in serum; (C) MDA in liver; (D) SOD in liver; (E) the relative mRNA expression of TNF-a in three tissue; (F) the relative mRNA expression of IL-1β in liver, muscle and adipose tissue. Values are means ± SEM (n = 6). Values with *,**statistically differ at $P < .05$, $P < .01$.

release (Kim et al., 2013b). During fasting, autophagy was critical for mice to survive and maintain their fat and glycogen stores; indeed, fasted Atg7-null mice died of hypoglycaemia (Kimmelman and White, 2017). Another study showed that liver-specific autophagy plays a role in blood glucose regulation and contributes to the maintenance of blood amino acid levels (Ezaki et al., 2011). Hence, glucose metabolism can be regulated by autophagy to some extent as well (Ha et al., 2015).

In the case of protein metabolism, CQ inhibited mRNA expression of mTOR, thus causing lower body protein content (Fig. 4A). This means that at least impaired autophagy would reduce protein synthesis. One explanation is that the impaired autophagy (lipophagy) blocks lipid catabolism, and higher protein catabolism is required to supply energy to maintain energy homeostasis. In the present study, we point out that inhibited autophagy could inhibit protein synthesis, but no significance was found on protein degradation especially in muscle. However, the details of this mechanism await further investigation. In short, autophagy is likely to be a promising target to adjust the metabolic homeostasis of three major nutrients.

4.4. Conclusions

The present results suggest that autophagy inhibition affects the growth of fish. Moreover, autophagy is conducive to maintaining the homeostasis of the fish, the inhibition of which will worsen the metabolic profiles of otherwise healthy fish, as demonstrated by lipid accumulation, weaker antioxidant capacity and increased inflammation. Furthermore, autophagy influences other forms of nutrient metabolism, such as glycometabolism and proteometabolism, and plays an important role in fuelling energy and nutrient stores. The more intricate mechanism is possibly associated with the AMPK and mTOR signalling pathways.

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Declaration of Competing interests

All authors declare no conflicts of interests.

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