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RESEARCH ARTICLE

Effects of insulin and its related signaling pathways on lipid metabolism in the yellow catfish *Pelteobagrus fulvidraco*

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ABSTRACT

The influence of insulin on hepatic metabolism in fish is not well understood. The present study was therefore conducted to investigate the effects of insulin on lipid metabolism, and the related signaling pathways, in the yellow catfish *Pelteobagrus fulvidraco*. Hepatic lipid and intracellular triglyceride (TG) content, the activity and expression levels of several enzymes and the mRNA expression of transcription factors (PPARα and PPARγ) involved in lipid metabolism were determined. Troglitazone, GW6471, fenofibrate and wortmannin were used to explore the signaling pathways by which insulin influences lipid metabolism. Insulin tended to increase hepatic lipid accumulation, the activity of lipogenic enzymes (6PGD, G6PD, ME, ICDH and FAS) and mRNA levels of FAS, G6PD, 6PGD, CPT IA and PPARγ, but down-regulated PPARα mRNA level. The insulin-induced effect could be stimulated by the specific PPARγ activator troglitazone or reversed by the PI3 kinase/Akt inhibitor wortmannin, demonstrating that signaling pathways of PPARγ and PI3 kinase/Akt were involved in the insulin-induced alteration of lipid metabolism. The specific PPARα pathway activator fenofibrate reduced insulin-induced TG accumulation, down-regulated the mRNA levels of FAS, G6PD and 6PGD, and up-regulated mRNA levels of CPT IA, PPARα and PPARγ. The specific PPARα pathway inhibitor GW6471 reduced insulin-induced changes in the expression of all the tested genes, indicating that PPARα mediated the insulin-induced changes of lipid metabolism. The present results contribute new knowledge on the regulatory role of insulin in hepatic metabolism in fish.

KEY WORDS: Fish, Insulin, Hepatic metabolism, Signaling pathway

INTRODUCTION

Insulin is a peptide hormone that has been shown to be a very important regulator of lipid metabolism (Navarro et al., 2002). Studies have demonstrated the anabolic role of insulin in adipocytes and myocytes (Harmon and Sheridan, 1992; Sánchez-Gurmaches et al., 2011). However, data on the action of insulin on hepatic metabolism, particularly at the molecular level, are scarce in fish (Zhuo et al., 2014). Besides adipose tissue and muscle, the liver is the center of intermediary metabolism and considered as one of the major targets of insulin action (Gutiéres et al., 2003; Jin et al., 2014). In addition, the literature concerning the effects of insulin on lipid metabolism in fish has mainly focused on changes in enzyme activities involved in lipid storage and mobilization. In this sense, the results are often contradictory. Several studies have suggested the anti-lipolytic effect of insulin (Harmon and Sheridan, 1992; Plisetskaya et al., 1989) and an insulin-induced increase of lipid storage in many fish (Cowley and Sheridan, 1993; Plagnes-Juan et al., 2008; Ellesat et al., 2011). In contrast, some authors found that this pathway remained unaffected by insulin (Plagnes-Juan et al., 2008; Warman and Bottino, 1978). Recently, we found that insulin could stimulate triglyceride (TG) accumulation in yellow catfish hepatocytes *in vitro* (Zhuo et al., 2014), but the effect of insulin on lipid metabolism in yellow catfish *in vivo* is still unknown. Unlike in an *in vitro* study, *in vivo*, insulin would potentially interact with other endocrine mediators, which would in turn influence target gene expression. Thus, when using an isolated cell preparation and cell lines, caution should be taken when extrapolating the *in vitro* results to whole fish. However, to date, information on the related signaling pathways involved in the insulin-induced change of lipid metabolism is absent for any fish species.

Several signaling pathways play an important regulatory role in lipid homeostasis, by orchestrating the gene transcription of the enzymes involved in these pathways (Spiegelman and Flier, 2001). PPARα and PPARγ are members of the nuclear receptor superfamily, and are involved in the gene regulation of lipid homeostasis (Schoonjans et al., 1997): PPARα is involved in the regulation of fatty acid catabolism, and PPARγ participates in the regulation of fatty acid storage. PPARα can be activated by the specific ligand fenofibrate and inhibited by GW6471 (Miura et al., 2005). PPARγ can be activated by troglitazone (Van-Oort et al., 2008). In contrast, the phosphatidylinositol 3-kinase (PI3K) pathway is the main pathway involved in the metabolic actions of insulin (Shepherd et al., 1995, 1998; Catalucci et al., 2009), and inhibition of the PI3K pathway by wortmannin blocks most metabolic actions of insulin (Saltiel and Kahn, 2001; Okada et al., 1994). Recently, in our laboratory, studies have suggested that bovine insulin incubation significantly influences the mRNA expression of PPARγ and PPARα in yellow catfish (Zhuo et al., 2014; Zheng et al., 2015b). However, in fish, detailed information of the effects of these signaling pathways (PPARα, PPARγ and PI3K) on the action of insulin on lipid metabolism remains unknown. Given the importance of insulin in regulating lipid metabolism, it is critical to understand the signaling pathways and mechanisms by which this occurs in fish.

As a part of our ongoing research into the way in which insulin influences lipid metabolism, the present study was conducted to investigate the effects of intraperitoneal bovine insulin administration and its mechanism of action on hepatic lipid metabolism in yellow catfish. Additionally, we used primary hepatocytes of yellow catfish to explore the signaling pathways by which insulin influences lipid metabolism. The increased TG content, and the reduced activity and mRNA expression of lipogenic

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List of abbreviations

6PGD	6-phosphogluconate dehydrogenase
CPT I	carnitine palmitoyltransferase I
FAS	fatty acid synthase
FF	fenofibrate
G6PD	glucose 6-phosphate dehydrogenase
GW	GW6471
ICDH	isocitrate dehydrogenase
ME	malic enzyme
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
T	troglitazone
TG	triglyceride
W	wortmannin

enzymes following insulin treatment were observed in the present *in vivo* study, in agreement with the results obtained in our recent *in vitro* study (Zhuo et al., 2014). However, we also found an increase in CPT I mRNA levels (an important lipolytic enzyme), in contrast to the results in the *in vitro* study (Zhuo et al., 2014). Furthermore, the findings of the present study indicate that signaling pathways of PPARα, PPARγ and PI3K/Akt are involved in the insulin-induced alteration of lipid metabolism.

RESULTS

Lipid deposition

In the present study, the survival rate of yellow catfish was 100% (data not shown) and showed no significant differences among the treatments. Oil red O staining indicated that hepatic lipid content tended to increase with increasing insulin concentration (Fig. 1).

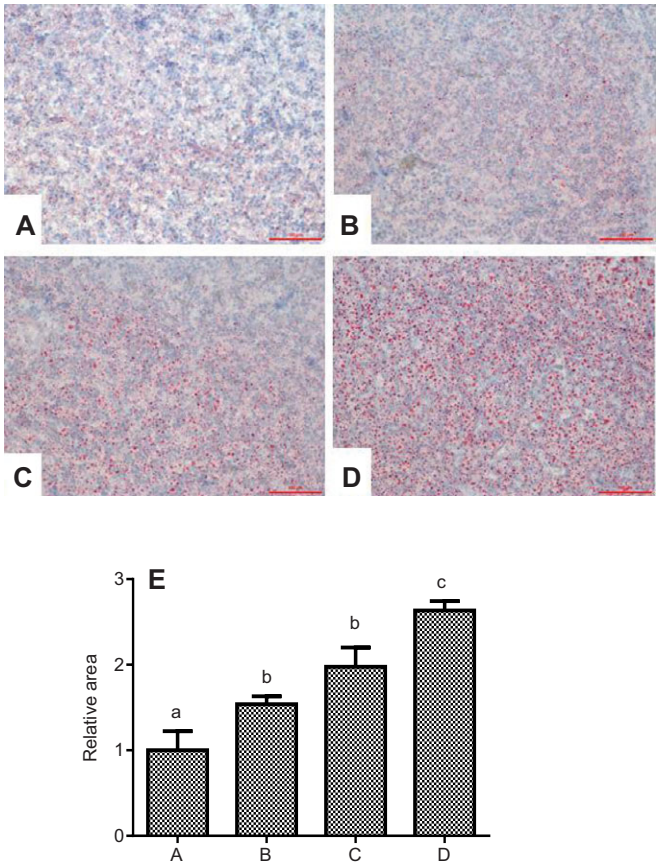

Fig. 1. Liver histochemistry of yellow catfish following insulin injection. Insulin was administered at the following doses: 0 (A), 0.01 μg g^{-1} (B), 0.1 μg g^{-1} (C) and 1μg g^{-1} (D). Scale bars, 100 μm. Images display oil red O staining for lipids and hematoxylin staining for nuclei. (E) The relative areas of oil red O staining in A–D, analyzed by Image-Pro Plus 6.0. Values are expressed as means+s.e.m. (*N*=3). Different letters indicate significant differences among groups ($P<0.05$).

In vivo study: enzyme activity and gene expression

Fatty acid synthase (FAS) activity increased with increasing insulin concentration. Insulin treatment also increased the activity of glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and isocitrate dehydrogenase (ICDH). However, no significant difference was observed in the activity of malic enzyme (ME) and carnitine palmitoyltransferase I (CPT I) among the treatments (Fig. 2A).

Insulin tended to up-regulate mRNA levels of FAS and G6PD. 6PGD mRNA expression was highest with 0.01 and 0.1 μg g^{-1} insulin. CPT IA expression was highest with 0.1 μg g^{-1} insulin and showed no significant differences among the other three groups. PPARα expression was highest for the control and the lowest for the 0.01 μg g^{-1} insulin group. PPARγ expression was highest for the 0.1 μg g^{-1} insulin group, followed by the 1 μg g^{-1} insulin group and showed no significant differences among the other two groups (Fig. 2B).

In vitro study: cell viability and TG accumulation

Cell viability showed no significant difference among treatments (Fig. 3A). Compared with the control, troglitazone alone did not markedly influence TG accumulation, whereas pre-treatment with troglitazone significantly influenced the insulin-induced TG increment (Fig. 3B). Wortmannin alone reduced TG accumulation compared with the control. Pre-treatment with wortmannin significantly reduced insulin-induced TG accumulation. Fenofibrate alone had no significant effect on TG accumulation, whereas fenofibrate pre-treatment significantly reduced insulin-induced TG accumulation. GW6471 alone showed no significant effect on TG accumulation, whereas GW6471 pre-treatment tended to reduce the insulin-induced TG increment.

Effect of insulin signaling pathways on lipid metabolism

Compared with the control, troglitazone addition alone did not significantly influence the mRNA expression of FAS, G6PD, 6PGD or CPT IA, but up-regulated the expression of PPARα and PPARγ. Compared with insulin treatment alone, troglitazone pre-treatment significantly up-regulated the mRNA levels of FAS, 6PGD, CPT IA and PPARγ, but did not significantly influence the mRNA level of G6PD (Fig. 4A).

Wortmannin treatment alone did not significantly influence the mRNA levels of FAS, G6PD, 6PGD, PPARα or PPARγ, compared with the control, but down-regulated CPT IA mRNA expression. Furthermore, wortmannin pre-treatment significantly reduced the insulin-induced up-regulation of G6PD, 6PGD and PPARγ expression, but did not significantly influence the mRNA levels of FAS and CPT IA (Fig. 4B).

Compared with the control, fenofibrate treatment alone did not significantly influence the mRNA expression of FAS, G6PD and 6PGD, but up-regulated the expression of CPT IA, PPARα and PPARγ. Fenofibrate pre-treatment significantly reduced the insulin-induced up-regulation of mRNA expression of G6PD and 6PGD,

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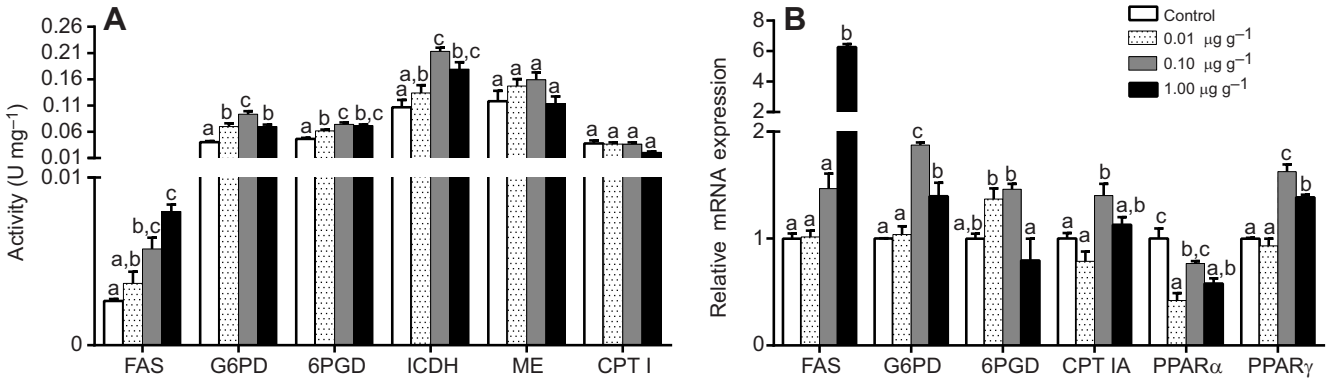

Fig. 2. Effect of insulin injection on enzymatic activity and gene expression in the liver of yellow catfish. Insulin was injected at different concentrations and the effects on enzyme activity (A) and mRNA expression (B) were assessed. Values are expressed as means+s.e.m. (*N*=3). Different letters indicate significant differences among groups (*P*<0.05). FAS, fatty acid synthase; G6PD, glucose 6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; ICDH, isocitrate dehydrogenase; ME, malic enzyme; CPT I, carnitine palmitoyltransferase I; PPAR, peroxisome proliferator-activated receptor.

and increased the mRNA expression of CPT IA, PPARα and PPARγ above levels with insulin alone (Fig. 5A).

Compared with the control, GW6471 alone did not significantly influence mRNA levels of FAS, G6PD, 6PGD and PPARγ, but down-regulated the mRNA expression of CPT IA and PPARα. GW6471 pre-treatment significantly reduced the insulin-induced up-regulation of expression of FAS, G6PD, 6PGD and PPARγ, but showed no significant effect on insulin-induced changes of CPT IA and PPARα expression (Fig. 5B).

DISCUSSION

Recently, we demonstrated that insulin could stimulate TG accumulation in yellow catfish hepatocytes (Zhuo et al., 2014). Considering the obvious physiological differences between primary isolated hepatocytes and fish liver tissues, caution should be exercised when extrapolating these *in vitro* data to the *in vivo* situation. The results presented here constitute the first *in vivo* study into the effects of intraperitoneal insulin administration on lipid metabolism at both the enzymatic and molecular level in fish. Furthermore, for the first time, we investigated the related signaling pathways by which insulin influences target genes involved in lipid metabolism.

In the present study, intraperitoneal insulin administration increased hepatic lipid content, and the activity and the mRNA level of FAS, G6PD and 6PGD, in agreement with our *in vitro* study (Zhuo et al., 2014). Increased lipid accumulation after insulin treatment has also been observed in other studies (Plagnes-Juan et al., 2008; Ellesat et al., 2011). In general, fat accumulation results from the balance between *de novo* synthesis of fatty acids and fat catabolism via β-oxidation, and many key enzymes are involved in the process (Leavens and Birnbaum, 2011). 6PGD, G6PD, ME, ICDH and FAS are the key enzymes involved in fatty acid biosynthesis (Carvalho and Fernandes, 2008). In the present study, the increased enzymatic activity and mRNA level of these lipogenic genes paralleled the increase in hepatic lipid content, which supports the lipogenic role of insulin, as suggested by Polakof et al. (2010a,b). Caruso and Sheridan (2011) also suggested that insulin increases lipogenesis and inhibits fatty acid oxidation. Moreover, our study indicates that the increased activity of FAS, G6PD and 6PGD was attributable to the increase in the mRNA expression of the genes encoding them, indicating that these enzymes were regulated mainly at the transcriptional level by insulin. Previous studies have also shown that bovine insulin stimulates FAS activity and gene expression in trout hepatocytes (Plagnes-Juan et al., 2008; Cowley and Sheridan, 1993), and G6PD and FAS transcript levels in rainbow trout (Polakof et al., 2010a,b).

However, it is generally known that insulin exhibits an anti-lipolytic function by inhibiting fatty acid catabolism (Leaver et al., 2008). In the present study, we found that CPT I activity showed no significant difference after insulin treatment, in agreement with our previous *in vitro* study (Zhuo et al., 2014), probably indicating that

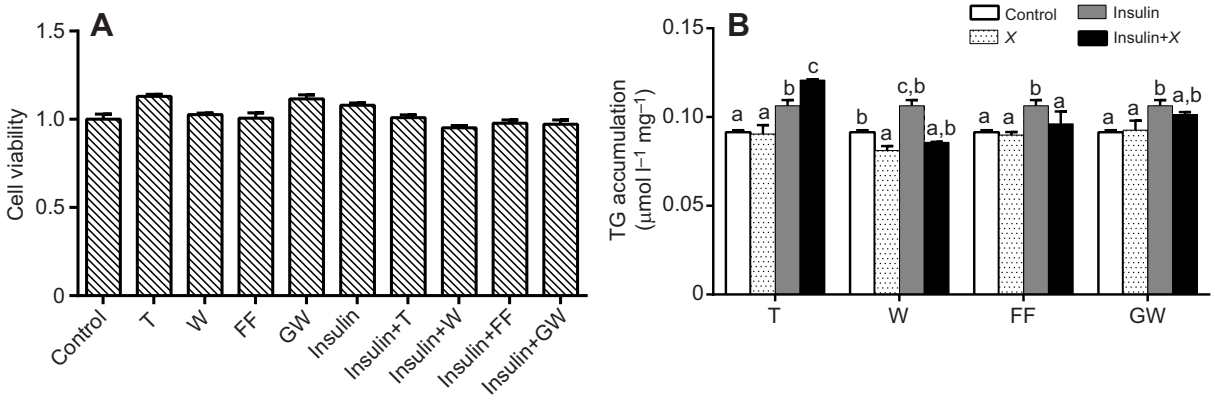

Fig. 3. Effects of insulin and pathway activators/inhibitors on cell viability and triglyceride content in primary hepatocytes from yellow catfish. (A) Cell viability. (B) Triglyceride (TG) accumulation; here, *X* represents troglitazone (T), wortmannin (W), fenofibrate (FF) or GW6471 (GW), as indicated. Values are expressed as means+s.e.m. (*N*=3). Different letters indicate significant differences among groups (*P*<0.05).

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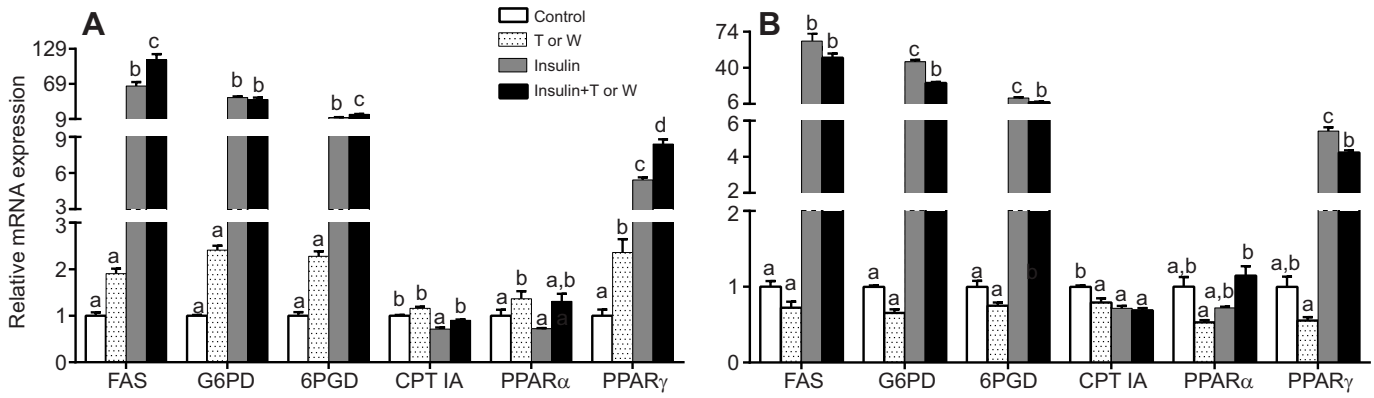

Fig. 4. Effects of insulin, troglitazone and wortmannin on mRNA expression of enzymes involved in lipid metabolism in yellow catfish. Incubation was carried out with troglitazone (T) and insulin alone and in combination (A), or with wortmannin (W) and insulin alone and in combination (B). Values are expressed as means+s.e.m. (*N*=3). Different letters indicate significant differences among groups (*P*<0.05).

CPT I did not constitute the limiting step for hepatic lipolysis in yellow catfish. However, some differences were observed in CPT IA expression between the present *in vivo* study and the *in vitro* study by Zhuo et al. (2014). For example, our *in vivo* study indicates up-regulation of the CPT IA mRNA level by insulin, in contrast to the results of Zhuo et al. (2014) using primary yellow catfish hepatocytes. Different results have also been found by Polakof et al. (2011), Sánchez-Gurmaches et al. (2011), Jin et al. (2014) and Plagnes-Juan et al. (2008), with stimulation, no significant difference and inhibition of CPT IA expression after insulin treatment. This inconsistency may be due to differences in the specific tissue, the dose of insulin administered and the investigation period. Besides, the possibility that differences in feeding habits influence the effects of insulin cannot be disregarded, as the number of insulin receptors is regulated by nutritional state and diet composition. For example, in rainbow trout and European seabass it was observed that high carbohydrate diets caused an up-regulation of insulin receptor number compared with fish fed on low carbohydrate diets (Gutiérrez et al., 1991; Baños et al., 1998). Adaptation of carnivorous fish to a high carbohydrate diet improves their insulin response (Mazur et al., 1992). However, studies have also indicated that *in vivo* administration of pharmacological doses of insulin to rainbow trout inhibits CPT IA expression (Polakof et al., 2010a, 2011), although *in vitro* studies did not support this (Plagnes-Juan et al., 2008). Different findings between the *in vivo* and *in vitro* studies imply that the effects observed *in vivo* may not be a direct action of insulin, but instead represent indirect action which occurs via other metabolic or hormonal changes following insulin injection.

Lipid metabolism in fish is controlled by some nuclear transcription factors, and PPARs are the most important ones (Cruz-Garcia et al., 2012). PPARγ is critical for the regulation of lipogenesis and lipid storage, and PPARα has been implicated in fatty acid catabolism (Ribet et al., 2010; Minghetti et al., 2011). The findings of the present study indicate that insulin injection significantly influences mRNA levels of PPARα and PPARγ, suggesting that they mediate the insulin-induced change of lipid metabolism, in agreement with other studies (Zhuo et al., 2014; Cruz-Garcia et al., 2015; Zheng et al., 2015b). However, we also found some differences between our *in vivo* study and the *in vitro* study (Zhuo et al., 2014) in the insulin-induced change in mRNA levels of these transcription factors. For example, PPARα gene expression was highest for the control and the lowest for the 0.01 μg g^{-1} insulin group for our *in vivo* study, whereas PPARα gene expression tended to decline with increasing insulin incubation concentration for the *in vitro* study (Zhuo et al., 2014). Therefore, other factors might influence the regulation of PPAR transcription following *in vivo* insulin injection.

We next addressed the question of the insulin-mediated signaling pathway, which might be involved in the effects of insulin on gene transcription related to lipid metabolism. Wortmannin is a potent inhibitor of PI3K. In our study, wortmannin pre-treatment

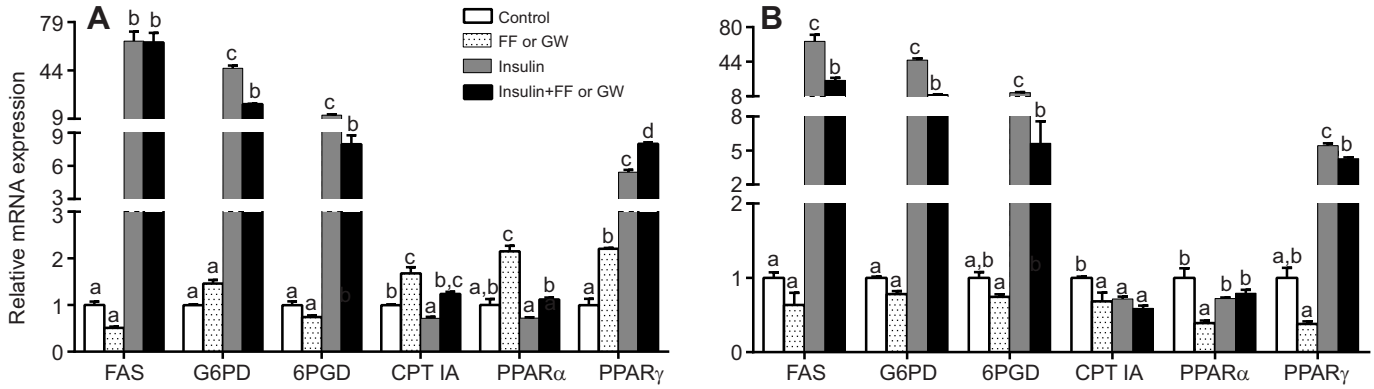

Fig. 5. Effects of insulin, fenofibrate and GW6471 on mRNA expression of enzymes involved in lipid metabolism in yellow catfish. Incubation was performed with fenofibrate (FF) and insulin alone and in combination (A), or with GW6471 (GW) and insulin alone and in combination (B). Values are expressed as means+s.e.m. (*N*=3). Different letters indicate significant differences among groups (*P*<0.05).

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Table 1. Primers used for qPCR analysis

Gene	GenBank accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-actin	EU161066	GCACAGTAAAGGCGTTGTGA	ACATCTGCTGGAAGGTGGAC
18S rRNA	GQ4652361	CTGCCGGTGGTCTTCTTCCA	ATTCAGCGGGTCGTCTCGTC
GAPDH	KP938521	TTTCAGCGAGAGAGACCCAG	ATGACTCTCTTGGCACCTCC
HPRT	KP938523	ATGCTTCTGACCTGGAACGT	TTGCGGTTCAGTGCTTTGAT
FAS	JN579124	AACTAAAGGCTGCTGGTTGCTA	CACCTTCCCGTCACAAACCTC
G6PD	JX992744	CAGGAATGAACGCTGGGATG	TCTGCTACGGTAGGTCAGGTCC
6PGD	JX992745	GCTCTGATGTGGCGAGGTGG	CGTAGAAGGACAGTGCAGTGGTAAA
CPT IA	JQ074177	ATTTGAAGAAGCACCCAGAGTATGT	CCCTTTTATGGACGGAGACAGA
PPARα	JX992740	CGAGGATGGGATGCTGGTG	CGTCTGGGTGGTTCGTCTGC
PPARγ	JX992741	ACGCCCCGTTCGTTATCC	TGAGCAGAGTCACCTGGTCATTG

significantly reduced the insulin-induced up-regulation of G6PD, 6PGD and PPARγ expression, but did not significantly influence the mRNA levels of FAS and CPT IA. This suggests that the effect of insulin is mediated through the PI3K pathway. Similarly, Wang and Sul (1998) found that the effect of insulin on FAS gene expression was mediated by the PI3K pathway in 3T3-L1 adipocytes. Troglitazone is a high affinity ligand for PPARγ, and several studies have identified PPARγ-binding sites in the regulatory regions of various genes involved in lipid metabolism (Castelein et al., 1994; Lehmann et al., 1995; Lenhard et al., 1997). In the present study, troglitazone significantly stimulated insulin-induced PPARγ expression, accompanied by the up-regulation of expression of genes involved in lipogenesis and TG accumulation, indicating that insulin-mediated regulation of genes linked to lipid metabolism and TG accumulation was, at least partially, through the PPARγ pathway. Similarly, troglitazone is known to regulate the expression of adipocyte-specific genes through PPARγ (Harris and Kletzien, 1994; Lehmann et al., 1995). Lenhard et al. (1997) and Bouraoui et al. (2012) also found that the simultaneous presence of troglitazone and insulin increased the content of triglycerides in adipocytes. Our study found that fenofibrate pre-treatment significantly reduced the insulin-induced up-regulation of mRNA expression of G6PD and 6PGD, but increased the mRNA expression of CPT IA, PPARα and PPARγ above levels obtained with insulin alone. Zheng et al. (2015a) found that PPARα was highly expressed in the liver in yellow catfish, and fenofibrate exerted lipid-lowering activity via activation of PPARα, leading to altered expression of genes involved in lipid metabolism. Fenofibrate addition also down-regulated the mRNA levels of G6PD and 6PGD, and reduced the insulin-induced TG accumulation, in agreement with other reports (Idzior-Walus et al., 2000; Furuhashi et al., 2002). Here, GW6471 pre-treatment significantly reduced the insulin-induced up-regulation of expression of FAS, G6PD and 6PGD, further indicating that the PPARα signaling pathway is involved in the insulin-induced change of lipid metabolism in yellow catfish.

In conclusion, bovine insulin increased TG accumulation by up-regulating hepatic lipogenesis and down-regulating lipolysis. Some differences were also observed between the present study and our recent *in vitro* study, indicating that caution should be taken when extrapolating *in vitro* results to the *in vivo* condition. Our study also indicated that the insulin-induced changes of lipid metabolism could be mediated, at least in part, by the modulation of signaling pathways of PPARα, PPARγ and PI3K. Considering the potential differences between bovine insulin and fish insulin, there might be limits to the use of bovine insulin to explore the effect of insulin on fish. However, as fish insulin is not widely available and high amino acid identity was observed between fish and bovine insulin, the findings from the present study still shed new light on the molecular basis for the effect of insulin on lipid deposition and metabolism in fish.

MATERIALS AND METHODS

Reagents

Bovine insulin, reagents involved in enzyme activity assays, and specific pathway activators and inhibitors were purchased from Sigma-Aldrich (St Louis, MO, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (USA). Reagents involved in cell culture were purchased from Gibco/Invitrogen, UK. Other reagents were bought from SCRC (Shanghai, China).

Experimental treatments

Two experiments were carried out. Experiment 1 was conducted to explore the effect of intraperitoneal injection of bovine insulin on lipid deposition and metabolism in yellow catfish. Experiment 2 was conducted to determine the signaling pathways of insulin influencing lipid metabolism by using hepatocytes of yellow catfish. Both experiments continued for 48 h. The experiments followed the ethical guidelines of Huazhong Agricultural University for the care and use of laboratory animals and animal cells.

Experiment 1: *in vivo* study with intraperitoneal insulin injection

Yellow catfish were obtained from a local fish pond (Wuhan, China) and transferred to indoor fiberglass tanks (300 l water volume) for a 2 week acclimation period. During this time, the fish were fed to apparent satiation twice daily with a commercial pellet diet (Haida, Ezhou, China). At the beginning of the trial, 180 uniform-sized fish (mean mass, 21.6±1.4 g, mean ±s.e.m.) were held in 12 fiberglass tanks, with 15 fish in each tank. Prior to insulin injection, they were fasted for 24 h. They were then anesthetized (10 mg l^{-1} MS-222) and intraperitoneally injected with phosphate-buffered saline (1 µl g^{-1} fish body mass, M_b; control) or with different doses of insulin (0.01, 0.1 and 1 µg g^{-1} M_b). After injection, fish were returned to their corresponding tanks. Fish were not fed after injection and no mortality was observed. Sampling occurred at 48 h. For each insulin treatment, three fish per tank were randomly collected and the liver was immediately sampled for the assessment of mRNA levels; other fish from each tank were sampled to assess histochemistry and enzyme activity in the liver.

The experiment was carried out in a static aquarium system, which was continuously aerated to maintain dissolved oxygen near saturation. During the experiment, fish were subjected to the ambient environment and normal photoperiod at 14 h light:10 h dark. Water temperature was 22.2±2.1°C. Dissolved oxygen and pH were 6.87±0.12 mg l^{-1} and 7.83±0.52, respectively.

Experiment 2: *in vitro* study of the signaling pathways involved in insulin-induced lipid metabolism

Yellow catfish (M_b, 12.5±0.5 g) were killed and the primary hepatocytes were isolated according to Zhuo et al. (2014). Hepatocytes were counted using a hemocytometer based on the Trypan Blue exclusion method, and hepatocytes with more than 95% viability were used for the present experiment. The hepatocyte cell suspension was plated onto 25 cm^2 flasks at

the density of 1×10^6 cells ml^{-1}. In order to explore the insulin signaling pathways influencing lipid metabolism, 11 groups were designed: control (containing 0.1% DMSO); troglitazone (5 µmol l^{-1}); wortmannin (1 µmol l^{-1}); fenofibrate (10 µmol l^{-1}); GW6471 (5 µmol l^{-1}); insulin (0 nmol l^{-1}); insulin (100 nmol l^{-1}); insulin (100 nmol l^{-1})+troglitazone (5 µmol l^{-1}); insulin (100 nmol l^{-1})+wortmannin (1 µmol l^{-1}); insulin (100 nmol l^{-1})+fenofibrate (10 µmol l^{-1}); and insulin (100 nmol l^{-1}) +GW6471 (5 µmol l^{-1}). Each treatment was performed in triplicate, and three independent experiments were carried out. The activators or inhibitors were added 1 h prior to the addition of insulin. The concentration of insulin, specific inhibitors and activators was selected according to our preliminary experiment and previous *in vitro* studies carried out in other fish and mammals (Wang and Sul, 1998; Bouraoui et al., 2012; Zhuo et al., 2014). Sampling occurred after 48 h incubation.

Sample analysis

Histochemical observation of yellow catfish liver

For histochemical observation, the left lobe of the liver (sliced into 3 mm-thick slabs) was collected and immediately frozen in liquid nitrogen. Frozen liver was cut on a cryostat microtome; 9 µm-thick sections were fixed in 4% formaldehyde for 10 min, stained with oil red O and then prepared for light microscopy, according to Lillie and Fullmer (1965).

Cell viability and TG accumulation assay in hepatocytes

The MTT assay was used to test cell viability following the method described in our previous study (Zhuo et al., 2014). Wells containing medium plus the corresponding reagents without cells were used as a blank control, providing the baseline zero absorbance. The results are presented as percentage cell viability, which was calculated as the ratio of absorbance A in the experimental well to A in the positive control well. For the intracellular TG accumulation assay, the cells were homogenized in PBS. TG was determined by the glycerol 3-phosphate oxidase *p*-aminophenol (GPO-PAP) method, using a commercial kit (Nanjing Jian Cheng Bio-engineering Institute, Nanjing, China), and expressed as µmol l^{-1} TG mg^{-1} cellular protein.

Enzyme activity analysis

For determination of the activity of several lipogenic enzymes, liver samples were homogenized in ice-cold buffer (0.02 mol l^{-1} Tris HCl, 0.25 mol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 0.1 mol l^{-1} sodium fluoride, 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride, 0.01 mol l^{-1} β-mercaptoethanol, pH 7.4), and centrifuged at 20,000 ***g*** at 4°C for 30 min. The supernatant was collected and immediately used for the analysis of enzyme activity. The activity of all five lipogenic enzymes was assayed spectrophotometrically. FAS activity was determined according to the method of Chang et al. (1967) as modified by Chakrabarty and Leveille (1969). G6PD and 6PGD were determined by the method of Barroso et al. (1999), ME activity following Wise and Ball (1964) and ICDH activity according to Bernt and Bergmeyer (1974). One unit of enzyme activity (IU), defined as the amount of enzyme that converted 1 µmol of substrate to product per minute at 28°C, was expressed as U mg^{-1} soluble protein.

For CPT I activity assay, mitochondria were isolated from liver according to Suarez and Hochachka (1981) with modifications by Morash et al. (2008). CPT I activity was determined using the method of Bieber and Fiol (1986), based on measurement of the initial CoA-SH formation by the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) reaction at 412 nm. One unit (IU) of CPT I activity was defined as 1 µmol of product formed per minute per milligram of mitochondrial protein at 25°C.

The soluble protein concentration of homogenates was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. These analyses were conducted in triplicate.

mRNA level determination by quantitative real-time PCR

The primer sequences and the mRNA levels of several genes related to lipid metabolism were determined by the real-time fluorescence quantitative real-time PCR (qPCR) method described in Zhuo et al. (2014). Total RNA extraction and first strand cDNA synthesis were performed according to methods in Zheng et al. (2013). The cDNA synthesis reactions were diluted to 200 µl in water. Real-time PCR was performed in a 20 µl reaction mixture including 10 µl SYBR Premix Ex Taq™ II (2×), 0.4 µl each primer (25 µmol l^{-1}; Table 1), 1 µl diluted first-strand cDNA product and 8.2 µl ddH_2O. Reactions were based on a three-step method as follows: 95°C for 30 s, 40 cycles of 5 s at 95°C, 10 s at 57°C and 30 s at 72°C. All samples were performed in triplicate and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. A non-template control and dissociation curve were included to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA. A set of five housekeeping genes (β-actin, GAPDH, EF1α, 18S rRNA and HPRT) were selected from the literature (Zhao et al., 2011) in order to test their transcription stability. By using geNorm software (Vandesompele et al., 2002), mRNA expression of β-actin and GAPDH proved the most stable across tissue types (M-value of 0.31), while β-actin and 18S rRNA expression were the most stable following insulin treatment (M-value of 0.35). The relative expression levels were calculated using the Pfaffl method (Pfaffl, 2001) when normalizing to the geometric mean of the best combination of two housekeeping genes, as suggested by geNorm software.

Statistical analysis

Statistical analysis was performed with SPSS 19.0 software. Results are presented as means±s.e.m. Prior to statistical analysis, an arcsine transformation was used before processing percentage data. All data were tested for normality of distribution using the Kolmogorov–Smirnov test. The homogeneity of variances among the different treatments was tested using Bartlett's test. They were then subjected to one-way ANOVA followed by the Duncan's test. The minimum significant level was set at 0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.-Q.Z. and Z.L. designed the experiment. M.-Q.Z. performed the majority of the experiments with the help of Y.-X.P., K.W., Y.-F.F., L.-H.Z. and Y.-F.S. M.-Q.Z., Z.L., Y.-X.P. and K.W. analyzed the data. M.-Q.Z. and Z.L. wrote the manuscript. All the authors approved the manuscript.

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