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PPARα activation enhances the ability of nile tilapia (*Oreochromis niloticus*) to resist *Aeromonas hydrophila* infection

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A R T I C L E I N F O

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A B S T R A C T

Peroxisome proliferator-activated receptor α (PPARα) plays critical physiological roles in energy metabolism, antioxidation and immunity of mammals, however, these functions have not been fully understood in fish. In the present study, Nile tilapia (*Oreochromis niloticus*) were fed with fenofibrate, an agonist of PPARα, for six weeks, and subsequently challenged with *Aeromonas hydrophila*. The results showed that PPARα was efficiently activated by fenofibrate through increasing mRNA and protein expressions and protein dephosphorylation. PPARα activation increased significantly mitochondrial fatty acid β-oxidation efficiency, the copy number of mitochondrial DNA and expression of monoamine oxidase (MAO), a marker gene of mitochondria. Meanwhile, PPARα activation also increased significantly the expression of NADH dehydrogenase [ubiquinone] 1α subcomplex subunit 9 (NDUFA9, complex I) and mitochondrial cytochrome *c* oxidase 1 (MTCO1, complex IV). The fenofibrate-fed fish had higher survival rate when exposed to *A. hydrophila*. Moreover, the fenofibrate-fed fish also had higher activities of immune and antioxidative enzymes, and gene expressions of anti-inflammatory cytokines, while had lower expressions of pro-inflammatory cytokine genes. Taken together, PPARα activation improved the ability of Nile tilapia to resist *A. hydrophila*, mainly through enhancing mitochondrial fatty acids β-oxidation, immune and antioxidant capacities, as well as inhibiting inflammation. This is the first study showing the regulatory effects of PPARα activation on immune functions through increasing mitochondria-mediated energy supply in fish.

1. Introduction

Bacterial and viral diseases are among the major threats in intensive aquaculture throughout the world [1]. In recent years, infectious diseases have caused huge economic loss in the aquaculture industry and pose risks to consumer health [2]. Therefore, the mechanisms for improving immune activities in aquatic animals to resist pathogenic infections have attracted much research attention. In fish, as with all vertebrates, immune defense relies on innate immunity and adaptive immunity [3]. Current evidence has demonstrated that maintaining a competent immune system and mounting an immune response are energy demanding processes [4,5]. Energetic metabolism is an important process in resisting infections, and immune function has been reported to tightly correlate with energy metabolism in mammals [6,7]. In fish, the activation of the immune response by infection was also reported to be an expensive energetic process, and pathogenic infection would increase the energetic deficit status [8]. Therefore, the regulation of energy metabolism could also affect immune activities in fish.

In general, the maintenance of mitochondrial function is an important guarantee for the organism's energy supply and immune function [9,10]. Recent studies indicated that mitochondria are not only the powerhouses of cellular physiological processes, but also represent the powerhouses of immunity [11]. Mitochondria are the main site for fatty acid degradation through β-oxidation [12], and mitochondrial fatty acid β-oxidation is also one of the most important routes to producing energy in organisms [13,14]. In mammals, accumulating evidence has highlighted the central role of mitochondria on both the innate and adaptive immune systems, and mitochondrial dysfunction or impaired of integrity is closely related to immune function decline [11]. Some mammalian studies have indicated that the immunity was improved by activation of mitochondrial fatty acid β-oxidation [15,16], suggesting mitochondrial fatty acid β-oxidation is an important regulatory process

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in pathogenic infections in mammals. Similarly, our recent study was the first to indicate that the inhibition of mitochondrial β-oxidation in Nile tilapia by suppressing the endogenous synthesis of L-carnitine, which is an essential factor in transporting fatty acid from cytosol to mitochondria, significantly impaired the resistance of fish to *A. hydrophila* infection [17]. However, increasing mitochondrial β-oxidation through dietary supplementation of L-carnitine improved the survival rate of fish after pathogenic infection in Nile tilapia and black seabream [18,19]. Therefore, mitochondrial β-oxidation could be a potential biochemical process connecting immune response and energy metabolism. Accordingly, the upstream regulator of mitochondrial β-oxidation is also hypothesized to play roles in affecting immune activities.

Peroxisome proliferator-activated receptor-α (PPARα) belongs to the nuclear receptor superfamily, which is the central regulator of mitochondrial fatty acid oxidation [20]. It plays a key role in metabolic remodeling in hepatocytes by regulating the expression of some important genes involved in mitochondrial fatty acid oxidation [21]. PPARα can be activated through pharmacological methods, such as fibrate administration [22]. Among a series of fibrate compounds, fenofibrate as a PPARα agonist has been widely used in clinical medicine, and is commonly used in metabolism studies in mammals and fish [23,24]. In rodents, the fenofibrate-induced PPARα activation largely stimulates fatty acid β-oxidation by increasing the number and size of mitochondria [25,26]. Similar to what occurs in mammals, our previous study showed that fenofibrate activates PPARα in Nile tilapia, and therefore causes increases in the number of mitochondria and in mitochondrial fatty acid β-oxidation activities, and finally leads to a reduction in lipid deposition in tissues [24]. The increased mitochondrial fatty acid β-oxidation activities have also been reported in fenofibrate-treated rainbow trout and grass carp [27,28]. Considering the efficient regulation of PPARα towards mitochondrial fatty acid β-oxidation, we hypothesized that the activation of PPARα could also regulate immune activities and improve the resistance to pathogens in fish. However, this has not been verified yet.

Nile tilapia (*Oreochromis niloticus*) is an important economic fish species and also a good fish model for researches because of the available genome information, rapid growth, and high resistance to environmental stresses [29,30]. In order to verify the possible regulatory function of PPARα activation in improving immune activities, in the present study, juvenile Nile tilapia were firstly fed with fenofibrate-supplemented diets for six weeks to allow PPARα activation. Afterwards, the fish were exposed to the pathogenic bacteria *Aeromonas hydrophila* for 14 d to test the resistance ability to the bacteria. To the best of our knowledge, this is the first study to investigate the function of PPARα in regulating immune activity in fish.

2. Materials and methods

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

2.1. Animals, diets and experimental design

About 450 juvenile male Nile tilapia were obtained from Shanghai Ocean University (Shanghai, China). Before the formal experiment, fish were maintained in three 200-L tanks (each about 150 fish) at 27 ± 1 °C for two weeks. During this acclimating period, fish were fed with a commercial diet (protein 33%, lipid 5%) (Chengdu, China) three times per day. After acclimation, 150 visually healthy fish with relatively similar weights (4.16 ± 0.07 g) were randomly distributed into two groups (three replicates per group, 25 fish per replicate): control group and fenofibrate group. All fish were hand-fed twice daily (8:30 a.m. and 17:30 p.m.) at a feeding rate of 4% body weight, which is lower than the satiation level and could allow fish to eat all these diets within 15min after each feeding. Fenofibrate (Sigma, USA) was mixed into the control diet to the final dose of 200 mg fenofibrate/kg BW per day. This dose of fenofibrate has been used in our previous Nile tilapia study [24]. The formulations of the control and fenofibrate diets and their composition are listed in Table 1. Diets were extruded into 2 mm pellets, air-dried and then stored at −30 °C until for the usage. The total weight of fish in each tank was recorded at each week, and the feeding amount was adjusted accordingly. During the trial, the water temperature was maintained at 27 ± 1 °C with a 14 h light/10 h dark cycle.

Table 1
Formulation and proximate composition of the experimental diets.

Ingredients	NC	NCF
Casein	320	320
Gelatin	80	80
Soybean oil	70	70
Corn starch	300	300
Vitamin premix[a]	25	25
Mineral premix[b]	20	20
$Ca(H_2PO_4)_2$	10	10
Carboxy methyl cellulose CMC	40	40
Cellulose	128	128
Choline chloride	5	5
Phagostimulant	2	2
Butylated hydroxytoluene BHT	0.25	0.25
Fenofibrate	0	5.0
Total quantity	1000	1000
Proximate composition (% dry weight)		
Dry matter	87.29	87.06
Protein	36.60	36.57
Fat	7.16	7.14

[a] Mixed vitamin (mg or IU/kg): 500,000 I.U. Vitamin A, 50,000 I.U. Vitamin D3, 2500 mg Vitamin E, 1000 mg Vitamin K3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 mg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Cholin, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C.

[b] Mixed minerals (g/kg): 147.4 g $MgSO_4 \cdot 7H_2O$; 49.8 g NaCl; 10.9 g Fe (II) gluconate; 3.12 g $MnSO_4 \cdot H_2O$; $ZnSO_4 \cdot 7H_2O$; 0.62 g $CuSO_4 \cdot 5H_2O$; 0.16 g KI; 0.08 g $CoCl_2 \cdot 6H_2O$; 0.06 g NH_4 molybdate; 0.02 g $NaSeO_3$.

2.2. Sampling collection

At the end of six-week trial, before weighing and sampling, all fish in each tank were counted and deprived of feed for 12 h. Nine fish of each group (three per tank) were euthanized (MS-222 at 20 mg/L) (tricaine methanesulfonate, Western Chemicals, Inc., Ferndale, Washington) and sampled to collect tissues to perform biochemical and molecular biological assays. Blood was collected from the caudal vein and centrifuged for serum preparation (3000 rpm, 10 min). The serum was immediately frozen at −80 °C for further analysis. The WG, survival rate, hepatosomatic index (HSI), mesenteric fat index (MFI), spleen body index measurements were calculated using the following formulae:

Weight gain (WG, %) = 100 × (Final fish weight-Initial fish weight) / Initial fish weight

Survival rate (SR, %) = 100 × (Final fish number / Initial fish number)

Hepatosomatic index (HIS, %) = 100 × (Liver weight / body weight)

Mesenteric fat index (MFI, %) = 100 × (Mesenteric fat weight / body weight)

Spleen body index (%) = 100 × (Spleen weight / body weight)

2.3. Aeromonas hydrophila challenge test

Aeromonas hydrophila was purchased from China General Microbiological Culture Collection Center and prepared by culturing them in a Luria Broth (LB) at 37 °C for 16 h with constant shaking (250 rpm). Then bacteria were harvested by centrifugation (3000 rpm, 10 min), washed and re-suspended in phosphate buffer saline (PBS) (pH 7.4). The bacterial count was determined by standard dilution and plating methods, and then the bacterial fluid was diluted to the density of 2×10^8 CFU/ml. At the end of feeding trial, the experimental fish were randomly divided into two groups (three replicates/group, fifteen fish/replicate). All fish were intraperitoneally injected with *A. hydrophila* at LD50 (3.0×10^6) as described by Lu et al. (2019) [18]. Mortality was monitored daily and the challenge test was lasted for 14 d.

2.4. Biochemical parameters analysis

The activities of lysozyme (LZM), acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD) and catalase (CAT), and malondialdehyde (MDA) concentration in sampled tissues were measured using commercial kits (Jiancheng Biotech Co., China). All measurements were performed according to the relevant kit protocol.

2.5. Mitochondrial and peroxisomal [1–^{14}C] palmitate oxidation in liver and muscle

At the end of the feeding trial, liver tissues from six fish collected in each group were weighed and homogenized as described previously [17,24]. The homogenate samples were used for immediate measurements of mitochondrial and peroxisomal [1–^{14}C] palmitate β-oxidation. The total palmitate β-oxidation reaction was initiated by adding 100 μM palmitate (supplemented with 1 μCi [1–^{14}C], specific activity 60 Ci mmol^{-1}) bound to fatty-acid-free bovine serum albumin (BSA). After 90 min of incubation, the samples were treated with 10% (w/v) perchloric acid, which precipitated proteins, and the supernatant containing the acid-soluble products was collected. To measure peroxisomal palmitate oxidation, the mitochondrial β-oxidation activity was inhibited by preincubating the samples by using 10 μM rotenone and 250 μM potassium cyanide (KCN). The rate of total or peroxisomal palmitate oxidation was calculated from the radioactivity of the acid-soluble products. The rate of mitochondrial palmitate oxidation was expressed as the difference between the total palmitate oxidation (without inhibitors) and the peroxisomal oxidation rate. The final reaction media were filtered using Millipore filters (0.45 μm pore size) and the filtrates containing the acid-soluble products (the short metabolites from FA oxidation) were mixed with Ultima Gold XR (Packard) for radioactivity measurements.

2.6. Isolation of RNA, quantitative real-time PCR and western blot analyses

Total RNA from liver tissues was isolated by using a Tri Pure Reagent (Takara, Japan) according to the manufacturer's protocol. The quality and quantity of total RNA were tested by using NANODROP 2000 Spectrophotometer (Thermo, USA). First-strand cDNA was synthesized using a Primer Script™ RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Takara, Japan) by using S1000TM Thermal Cycler (Bio-Rad, USA) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis for genes were performed by mixing 1 μl synthesized cDNA, 1.6 μl forward and reverse primers specific for genes, 10 μl 2 × Ultra SYBR Mixture (CWbio, China), and 7.4 μl nuclease-free water, and run in the CFX Connect Real-Time System (Bio-Rad) according to the manufacturer's protocol. Double reference genes, elongation factor 1 alpha (EF1α) and β-actin, were used to ensure the stability of reference genes in different treatments. The primers sequences used for the qRT-PCR analysis of genes are listed in Table 2. During the study, the qRT-PCR efficiency was between 95% and 105% and the correlation coefficient was above 0.98 for each gene. Each qRT-PCR run was performed in triplicate and negative controls (no cDNA) were conducted. The relative gene expressions were calculated by using the $2^{-\Delta\Delta ct}$ method.

In the Western blot assays, the homogenates of liver tissues were cell lysed by using ice-cold RIPA lysis buffer (Beyotime Biotechnology, China) in the presence of 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology, China) for 30 min. After 10,400 g centrifugation for 12 min, the resulting supernatant was mixed with 5 × SDS loading buffer and boiled at 100 °C for 15 min. Proteins (40 μg) were loaded, separated on SDS-PAGE gels and transferred to nitrocellulose filter membranes. The membranes were blocked for 1 h by using 5% bovine serum albumin (BSA) in PBS supplemented with 0.1% Tween 20. The antibodies against total PPARα and PPARα phosphorylation (phospho Ser12) (Abcam, USA) were used. Bound antibodies were detected using goat anti-rabbit IgG (Li-Cor Biotechnology, USA) by using the Odyssey CLx Imager (Li-Cor). All antibodies were tested in preliminary studies and found to be specific for PPARα in Nile tilapia samples.

2.7. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Data were tested for normality and homogeneity of variance by using Shapiro-Wilk test and Levene's test, respectively. Significant differences ($P < 0.05$) between two groups were performed by using two-tailed independent *t*-test. Analyses were performed using IBM SPSS Statistics 19.0 software (IBM, USA).

3. Results

3.1. Growth performance and body indices parameters

After the six-week feeding trial, fenofibrate treatment did not affected significantly the WG, survival rate, MFI, and spleen body index of Nile tilapia compared to the control group (Fig. 1). It decreased significantly the relatively liver weight of the treated fish (Fig. 1; $P < 0.05$). These results indicate that fenofibrate might have no effects on growth performance, however, liver could be a main target organ for fenofibrate treatment in Nile tilapia.

3.2. The activation of PPARα by fenofibrate

As seen from Fig. 2, quantitative real-time PCR and western blot analyses showed that fenofibrate significantly increased the mRNA and protein expression of PPARα in liver, as well as significantly decreased PPARα phosphorylation (phospho Ser12) as compared to the control group ($P < 0.05$).

3.3. Effect of fenofibrate on fatty acid β-oxidation and mitochondrial function

We measured [1–^{14}C] palmitate metabolism in tissue homogenates to investigate the effect of fenofibrate on FA β-oxidation. From Fig. 3, the results showed that fenofibrate significantly increased mitochondrial and peroxisomal β-oxidization in liver and muscle tissues ($P < 0.05$). Similarly, fenofibrate treatment increased significantly the mRNA expression of carnitine palmitoyltransferase 1a (CPT1a), which is a speed-limiting enzyme in fatty acid β-oxidation ($P < 0.05$). Furthermore, the quantity of mitochondrial cytochrome *b* DNA, which is a commonly used marker of the number of mitochondria, increased significantly in the fenofibrate group. Fenofibrate also significantly increased the mRNA expression of monoamine oxidase (MAO), which is the marker gene of mitochondrial ($P < 0.05$). We also measured the mRNA expressions of mitochondrial respiratory chain proteins and found that, fenofibrate treatment increased significantly the mRNA

Table 2
Nucleotide sequences of the primers used to assay gene expressions by real-time PCR.

Usage	Gene name	Sense and antisense primer (5′-3′)	Notes
RT-PCR cDNA	EF1α	F: CTACGTGACCATCATTGATGCC R: AACACCAGCAGCAACGATCA	AB075952
	β-actin	F: AGCCTTCCTTCCTTGGTATGGAAT R: TGTTGGCGTACAGGTCCTTACG	KJ126772
	PPARα	F: GTTCCTCAAGAGTCTCCGCC R: AAAGAGCTAGGTCGCTGTCG	NM_001290066.1
	AMPK	F: CTGCGTGTGAGAAGGAAGAATC R: CGGAAGTCAAGGAGGTAGGTT	KP296728
	CPT1a	F: TTTCCAGGCCTCCTTACCCA R: TTGTACTGCTCATTGTCCAGCAGA	XM_003440552
	CPT1b	F: AAGGGACGTTACTTCAAGGTG R: TCCGACTTGTCTGCCAAGAT	GQ395696
	MAO	F: CAACAGTACGCGTCCAGGAT R: TTAGTTCTGCAGTCCAGGCG	XM_003440238.4
	NDUFA9	F: ACCTTTTGTGCCCTACCCTC R: TTTGTCTGGGGTTGTCCAGG	XM_003447056.4
	SDHA	F: GGTATTCCGTACCGGCTCTG R: GTCGGTGTTCCACACAATGC	XM_003443687.5
	MTCO1	F: CTGTTTATCCCCCACTCGCA R: AATAGATGACACCCCGGCCA	LC189956.1
	CAT	F: AGAACTTGGCCGGGTTTCTA R: GCCTCCGCATTGTACTTCTTG	XM_003447521.5
	IL1β	F: GAGCACAGAATTCCAGGATGAAAG R: TGAACTGAGGTGGTCCAGCTGT	XM_019365841.1
	TNFα	F: CAGAAGCACTAAAGGCGAAGAACA R: TTCTAGATGGATGGCTGCCTTG	NM_001279533
	IL10	F: CAGCAGCAGGAGCATCAGCATT R: CACAGGAGGACGGTCTGAGAAGT	KP645180.1
	TGFβ	F: AAGAGGAGGAGGAATACTTTGCCA R: GAAGCTCATTGAGATGACTTTGGG	NM_001311325.1
	SOD	F: TGCCTTTGTCCAGACCGTAG R: GTGTCCAACGCTGTCATCAC	XM_003446807.4
	GST	F: CGTCGTCGTCTCTGTTGCC R: CATCGTCGTGGTGGTCATAGC	NM_001279635.1
	GSH-PX	F: ACGACAACCAGGGACTACAC R: GTCCAAACTGATTGCAGGGC	NM_001279711.1
	Caspase3	F: GGAGTGGACGATACAGACGCAAA R: TGAAGCTGTGTGACTGGGGCTT	NM_001282894.1
	Caspase9	F: ATACTTGAGGAAAACGCTGCCACT R: GAACCAGGCATTTGTTTGTAGAGC	XM_003455320.4
	TLR2	F: GTATCTCAGTGCTCGTCGCTCA R: TTTCATTATCGTCTCCAGTGCG	XM_019360109.2
	MYD88	F: TTTACGCTTCCTCACCATTTGT R: CCGCCTGCTCCACAGTTAT	NM_001311322.1
	COX2	F: GGAGCTCGAAGTAAACGCCT R: GGCCGGGTGTAGTCACAAAT	XM_003445052
	NF-κB	F: CGACCACTACCTACACGCTC R: GATGTCGTTTGAGGCATCGC	XM_019363515.2
RT-PCR mtDNA	CYTb	F: CATACACTATACTTCCGACATC R: CGAATGAGTCAGCCGTAG	MtDNA primer
	EF1α	F: TTGGCGGTGAGAACTGTCG R: GTGCGTGAATGGGTGAATG	Genome DNA, intron

expression of NDUFA9 (complex I; NADH dehydrogenase [ubiquinone] 1a subcomplex subunit 9) and MTCO1 (complex IV; mitochondrial cytochrome *c* oxidase 1) ($P < 0.05$). Although the fenofibrate diet did not affect significantly the SDHA (complex II; succinate dehydrogenase complex subunit A) mRNA expression, it induced an increasing tendency compared to the control group. Moreover, fenofibrate treatment increased significantly the mRNA expression of 5’ adenosine monophosphate-activated protein kinase (AMPK), which is a critical regulator of mitochondrial biogenesis and cellular energy sensor ($P < 0.05$).

3.4. Survival rate of nile tilapia after A. hydrophila infection challenge

In order to investigate whether the PPARα activation would affect the stress resistance ability of Nile tilapia, after 6-week feeding trial, the fish were challenged by *A. hydrophila* infection. The results clearly indicated that the survival rate of fenofibrate group was significantly higher than the control group under the bacterial infection situation from 7 to 14 days (Fig. 4), indicating that the activation of PPARα markedly enhanced the resistance of Nile tilapia to *A. hydrophila* infection.

3.5. Influence of fenofibrate on immunity and anti-oxidative enzymatic activities in serum and liver

In serum, fenofibrate increased significantly the LZM, AKP and ACP activities compared to the control group (Fig. 5; $P < 0.05$), however, fenofibrate did not affect significantly the SOD activity and the level of MDA in serum compared to the control group ($P > 0.05$). In liver, although the fenofibrate treatment did not affect lysozyme activity, it showed an increasing tendency in the activities of other immune enzymes, such as AKP and ACP, compared to control group (Fig. 5). However, fenofibrate increased significantly the SOD and CAT activities, and decreased significantly the content of MDA in liver compared

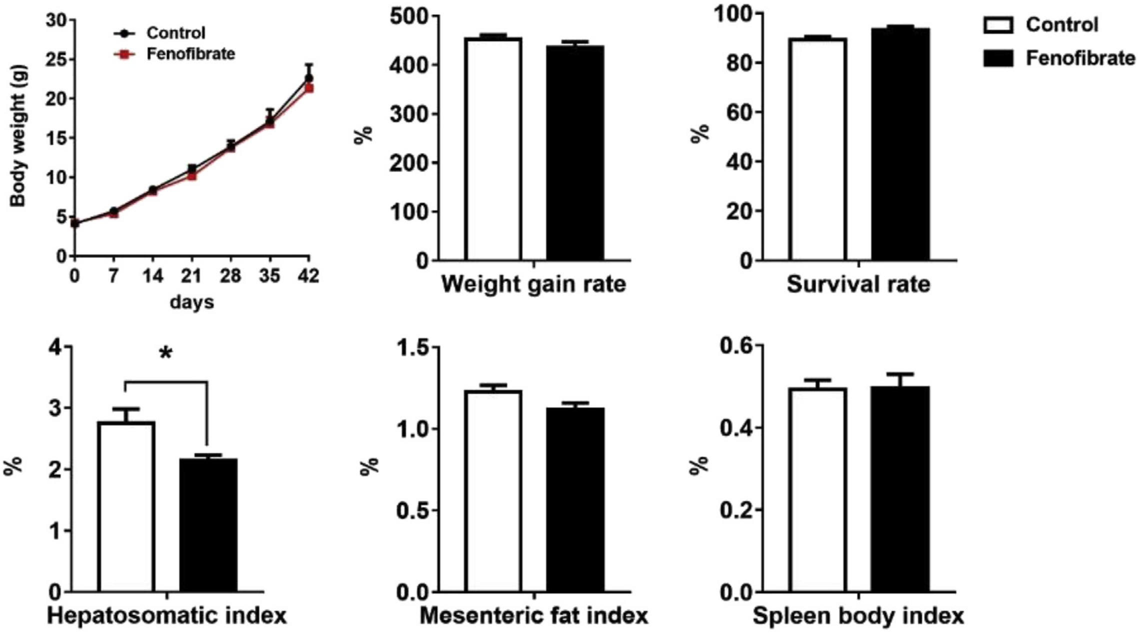

Fig. 1. The effects of fenofibrate on growth performance and tissue parameters. Data are expressed as mean ± SEM (n = 3–9). The values with * statistically differ at $P < 0.05$.

to the control group (Fig. 5; $P < 0.05$). These results suggest that, fenofibrate could improve the antioxidant and immune abilities in Nile tilapia.

3.6. Effects of fenofibrate on expressions of genes related to antioxidation and apoptosis in liver

The hepatic expressions of some genes related to antioxidative enzymes, such as SOD, phospholipid hydroperoxide glutathione peroxidase (GSH-Px), glutathione S-transferase (GST) and CAT, are shown in Fig. 6. There were significantly higher gene expressions of SOD, GSH-Px, and CAT in the fenofibrate group than those in the control group (Fig. 6; $P < 0.05$). The fish fed with fenofibrate had a significant lower expression of caspase3 than the control ($P < 0.05$), and also tended to decrease expression of caspase 9 ($P > 0.05$).

3.7. Effects of fenofibrate on expressions of immunity and inflammation-related genes in liver

The results on the expressions of the genes related to immunity and inflammation in the liver are given in Fig. 7. The fenofibrate treatment decreased significantly the expressions of pro-inflammatory genes (tumor necrosis factor alpha, TNFα and cyclooxygenase 2, COX2) compared to the control group ($P < 0.05$). On the contrary, the

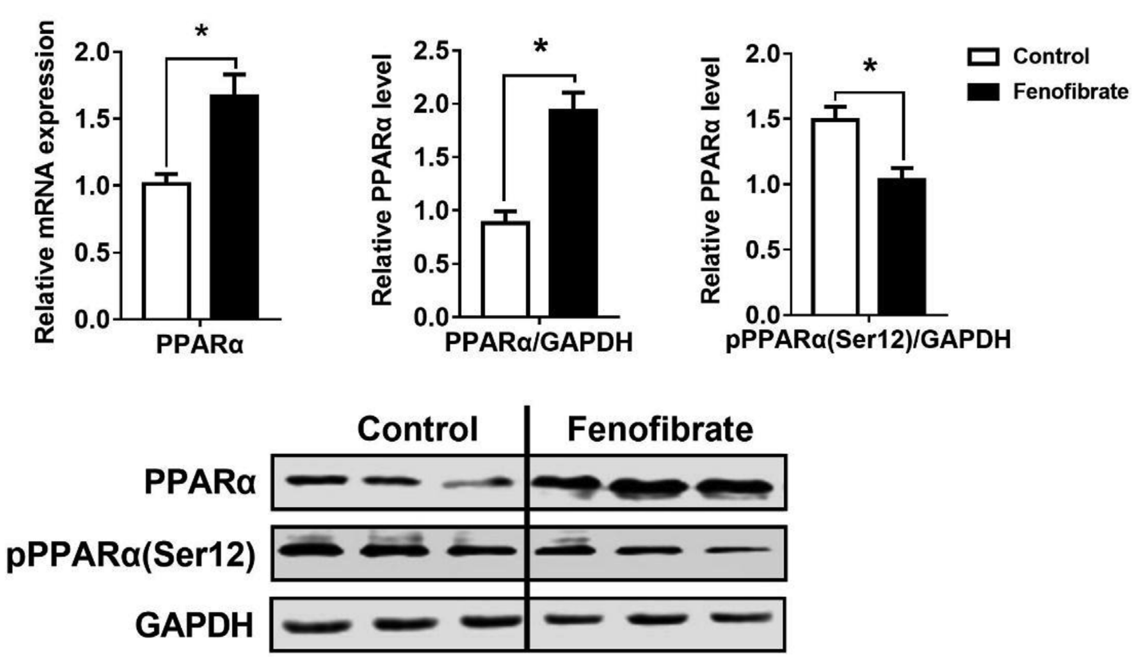

Fig. 2. The effects of fenofibrate on the mRNA, protein expression and the phosphorylation level of peroxisome proliferator activated receptor-α (PPARα) in liver during the six-week trial. Data are expressed as mean ± SEM (n = 3). The values with * statistically differ at $P < 0.05$.

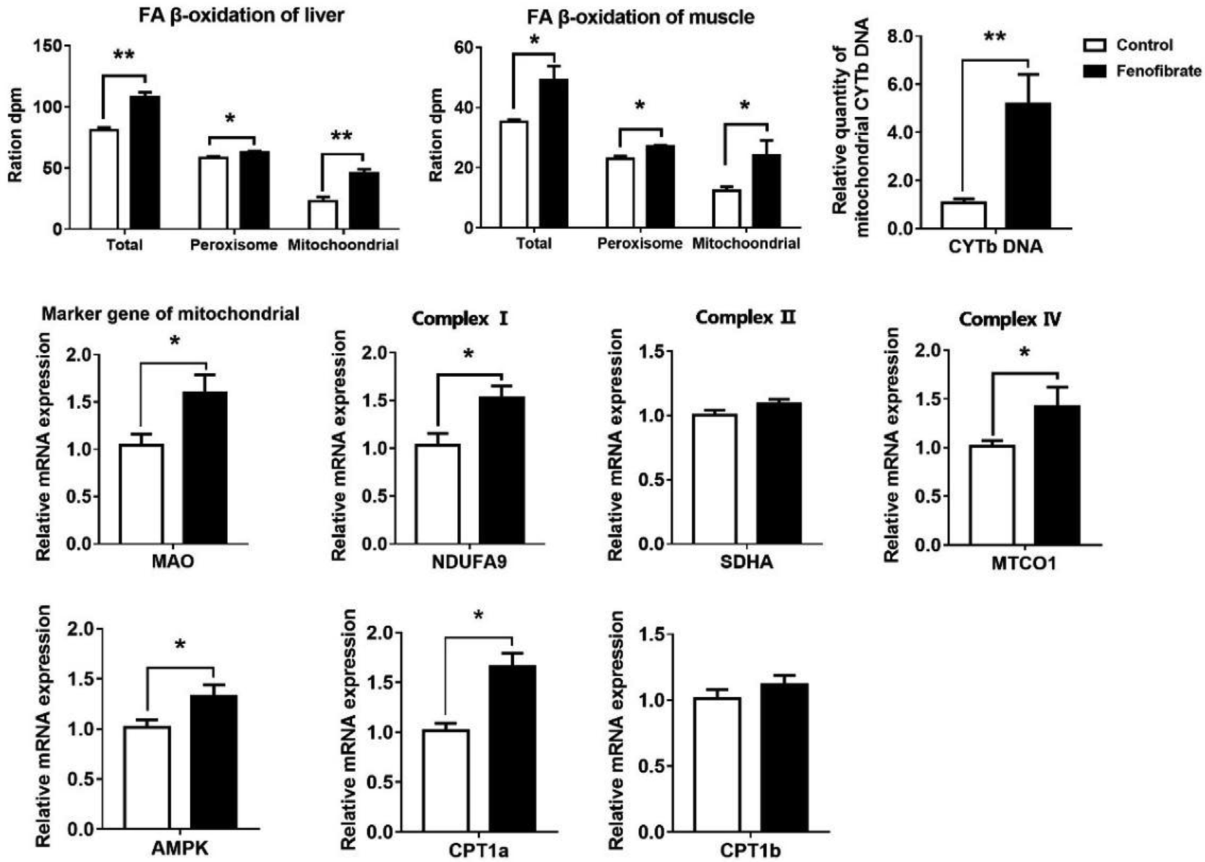

Fig. 3. The effect of fenofibrate on fatty acid β-oxidation (mitochondrial and peroxisomal [1-^{14}C] palmitate oxidation, CPT1a and CPT1b), mitochondrial functions (mitochondrial proliferation and mitochondrial respiratory chain activity) and energy metabolism (AMPK) during the six-week trial. Data are expressed as mean ± SEM (n = 6). The values with * statistically differ at $P < 0.05$, and ** statistically differ at $P < 0.01$.

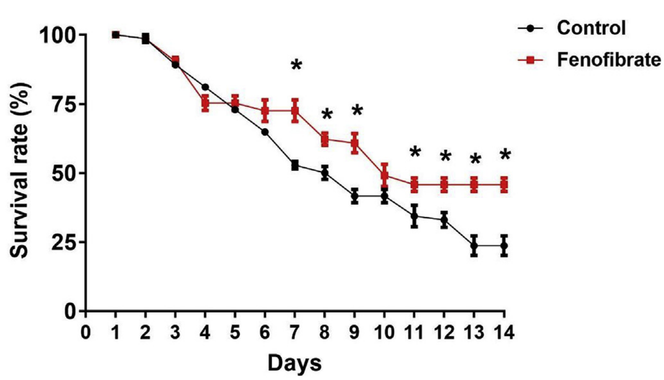

Fig. 4. The effect of fenofibrate on the survival rate of Nile tilapia after *A. hydrophila* infection. Data are expressed as mean ± SEM (n = 3). The values with * statistically differ at $P < 0.05$.

fenofibrate-fed fish had elevated expressions of anti-inflammatory genes (interleukin 10, IL10 and transforming growth factor β, TGFβ) ($P < 0.05$). The fenofibrate treatment also increased significantly the expressions of toll like receptor 2 (TLR2) and nuclear factor κB (NF-κB) compared to the control group ($P < 0.05$). The fenofibrate also tended to decrease the expression of myeloid differentiation factor 88 (MYD88), which is a down-stream target gene of TLR, but there was no significant difference between the fenofibrate and control groups.

4. Discussion

4.1. Fenofibrate efficiently activates PPARα in nile tilapia

PPARα is a ligand-activated transcription factor [31]. A range of synthetic compounds, such as fibrate drugs, are regarded as PPARα ligands. Fenofibrate, regarded as PPARα ligand in mammals and fish, is one type of fibrate drug [27,32]. In the present study, our results showed that fenofibrate increased PPARα expression at the transcriptional and protein levels. Meanwhile, fenofibrate remarkably decreased the phosphorylation level of PPARα (Ser12). This agrees with our recent work in which fenofibrate activated PPARα by decreasing phosphorylation in Nile tilapia *in vivo* and *in vitro* [24]. In mammals, the mechanisms for ligand-induced PPARα activation have been identified through increasing PPARα expression at the transcriptional, protein or phosphorylation levels [24,33]. A number of mammalian studies have shown that PPARα is activated through increasing phosphorylation [34,35]. However, other animal or cell studies indicated that dephosphorylation of PPARα upregulated hepatic FA β-oxidation [33,36]. Although the underlying mechanisms of PPARα activation have still not been fully identified even in mammals, it is commonly accepted that increased mRNA, protein expression and the modification of phosphorylation of the PPARα protein are the main activating mechanisms. Our results further support the indication that dephosphorylation may be a main mechanism of PPARα activation in fish.

4.2. PPARα activation enhances immune ability by increasing mitochondria-mediated energy supply

Recent studies indicate that energy metabolism plays a key role in

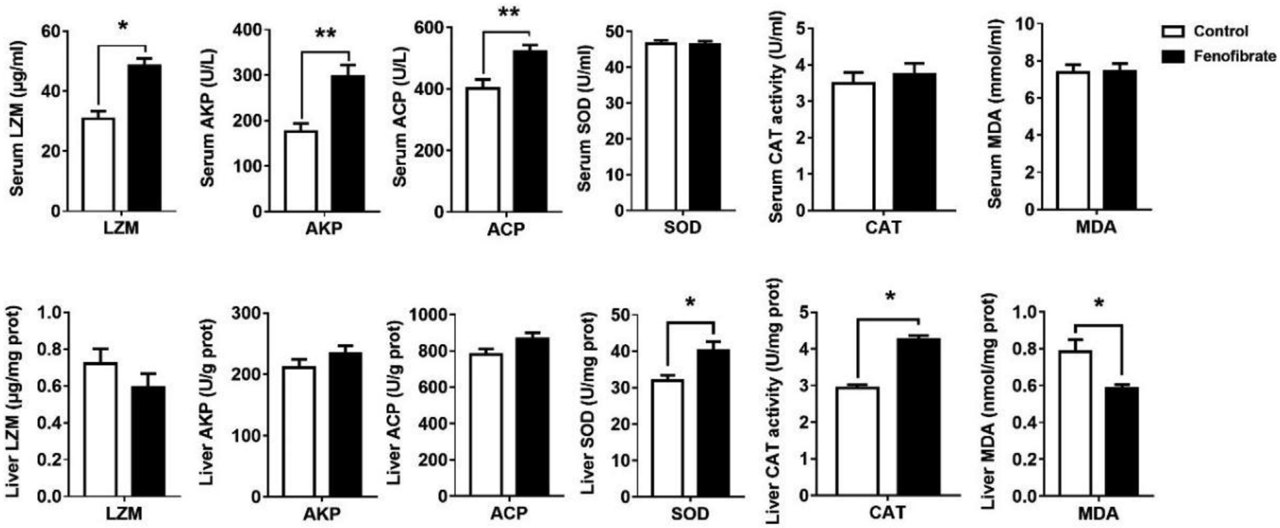

Fig. 5. The effect of fenofibrate on the activities of immune enzymes (Lysozyme, ACP and AKP), antioxidant enzymes (SOD and CAT), and lipid peroxidation marker (MDA) content in the serum and liver. Data are expressed as mean ± SEM (n = 6). The values with * statistically differ at $P < 0.05$, and ** statistically differ at $P < 0.01$.

supporting immunity maintenance [37,38], thus the relationship between energy metabolism and immunity has become a hot topic. PPARα is a key regulator of energy homeostasis. Establishing the correlation between PPARα and immunity in Nile tilapia was the main purpose of the present study. The present results showed that PPARα activation significantly increased the survival rate of Nile tilapia when exposed to *A. hydrophila*, and this improved antibacterial ability was related to the increased activity of some immune enzymes (LZM, AKP, ACP) and activated the TLR2 signaling pathway. This suggested that activation of PPARα systemically improved the ability of fish to resist infection. Some recent research has showed that at pathogen exposure, maintaining normal immune functions and/or enhancing synthesis and activity of humoral components, are energy-expensive [4,39]. Mitochondria, the powerhouse of the cells, play a pivotal role in the final oxidation of metabolites such as fatty acids (FA). Mitochondrial oxidative phosphorylation (OXPHOS) is responsible for the production of the total energy of most cells [40]. Moreover, almost every single enzymatic step in mitochondrial uptake and subsequent oxidative breakdown of FA is regulated by PPARα [41]. PPARα stimulates acyl-CoA import into the mitochondria to produce ATP by upregulating expression of CPT1a and CPT1b [42,43]. In the present study, PPARα activation increased the mitochondrial FA β-oxidation efficiency and upregulation of the expression of speed-limiting enzyme (CPT1a) in mitochondrial FA β-oxidation. This agrees with some previous fish studies in which PPARα activation improved mitochondrial FA β-oxidation in Nile tilapia, grass carp and rainbow trout [24,27,44]. Our another study also showed that the inhibition of mitochondrial fatty acid β-oxidation significantly impaired the immune functions in Nile tilapia [17]. In contrast, dietary supplementation of L-carnitine improved the immunity of Nile tilapia and zebrafish by enhancing

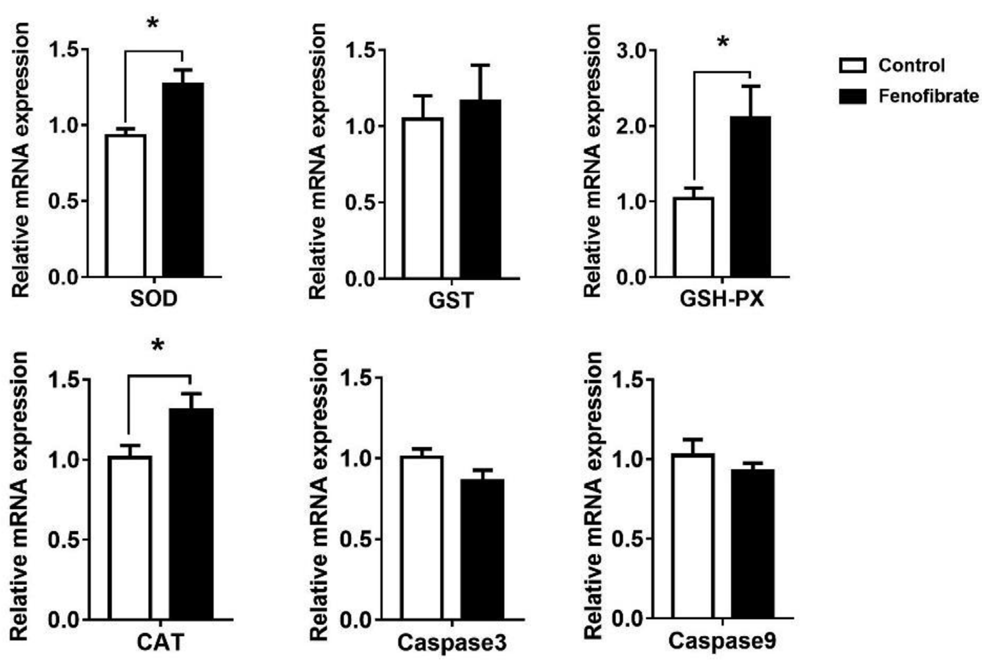

Fig. 6. Expressions of the genes related to antioxidation (SOD, GST and GSH-Px) and apoptosis (Caspase 3 and Caspase 9) in liver during the six-week trial. Data are expressed as mean ± SEM (n = 6). The values with * statistically differ at $P < 0.05$.

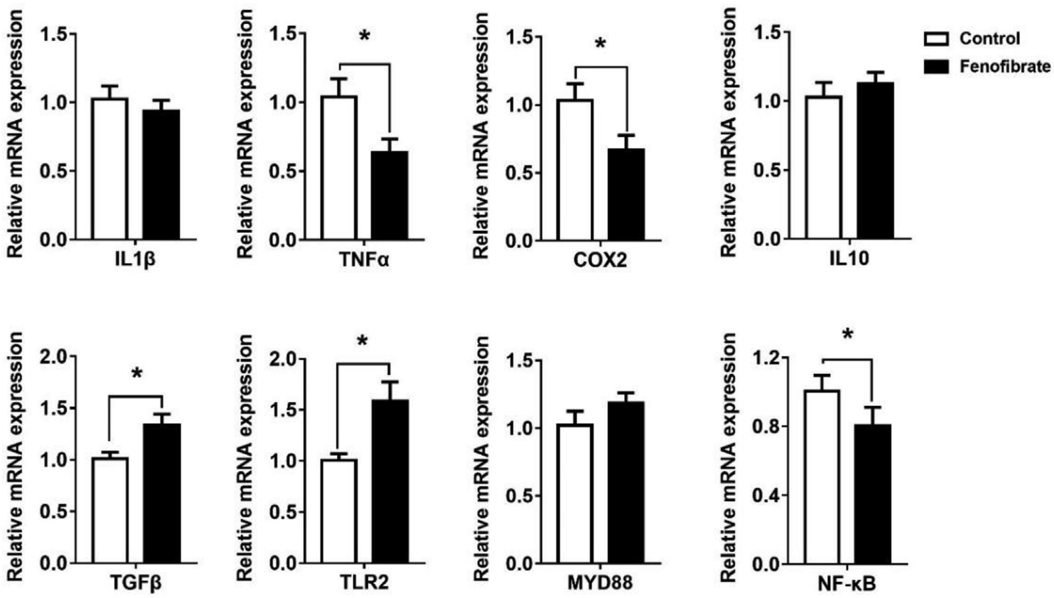

Fig. 7. Expressions of the genes related to pro-inflammation (IL1β, TNFα and COX2), anti-inflammation (IL10 and TGFβ) and immune functions (TLR2, MYD88 and NF-κB) in liver during the six-week trial. Data are expressed as mean ± SEM (n = 6). The values with * statistically differ at $P < 0.05$.

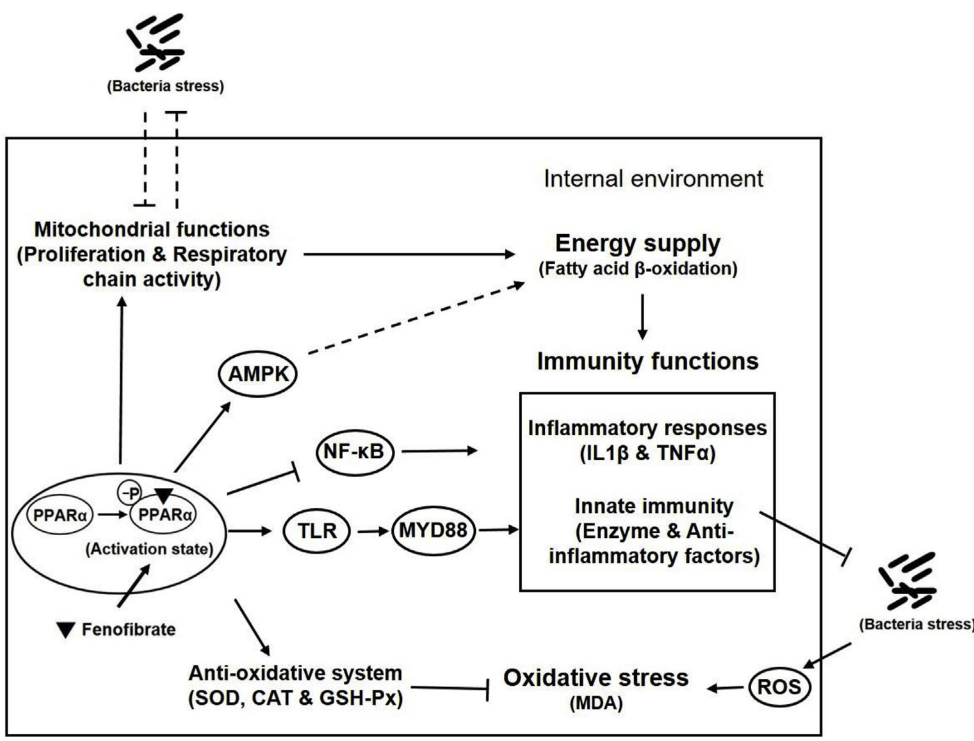

Fig. 8. Summary of the protective mechanisms of PPARα activation in resisting A. hydrophila infection in Nile tilapia.

mitochondrial fatty acid β-oxidation [18,45]. Moreover, the reduction of respiration rates and mitochondrial enzyme activity impaired immune responses in scallop [46]. These results indicate that the mitochondria-mediated energy supply also played important roles in immune activity in fish. In some mammalian studies, PPARα activation has also been proved to increase numerical density and size of mitochondria, and maintain mitochondrial membrane potential [47–49]. In the present results, we also found that PPARα activation increased the number of mitochondria and enhanced mRNA expression of mitochondrial complex enzymes (complex I, II and IV), which are the key components in respiration and ATP production. Additionally, PPARα activation upregulated the expression of AMPK, which is an important energy sensor. All these results suggest that PPARα activation largely improves mitochondrial functions and further increases the mitochondria-mediated energy supply in Nile tilapia. This helps fish to have a stronger ability to maintain or elevate immune activities, even at pathogen exposure. The correlations between PPARα activation, energy supply and improved immune functions are shown in Fig. 8.

4.3. PPARα activation enhances antioxidation and reduces inflammation

A number of studies have shown that the ability of fish to resist infectious diseases is closely related to antioxidative and anti-inflammatory activity [18,50]. Activities of antioxidant enzymes and lipid peroxidation products (such as MDA) are indicators of oxidative cell damage [51]. The present study showed that activation of PPARα increased the mRNA expression and activity of antioxidant enzymes (SOD, CAT, GSH-Px), whereas the level of MDA decreased significantly in fish. This suggests that PPARα activation improves the antioxidant capacity of Nile tilapia. This agrees with some mammalian studies in which PPARα activation significantly improved antioxidant ability *in vivo* and *in vitro* [52,53]. Although the mechanisms of the beneficial effects of PPARα activation on antioxidative action have not been intensively studied in fish, it could been associated with the PPARα activation-induced improvement of mitochondrial function because a number of studies indicated that the impaired mitochondria, which could be caused by pathogens or environmental stresses, is a main source of ROS [54,55]. Therefore, if PPARα activation could help to maintain or improve mitochondrial functions at the time of pathogen exposure, it could also reduce the cellular oxidative stress.

PPARα is not exclusively a metabolic regulator, but also has potent anti-inflammatory activities [56]. In a mammalian study, the anti-inflammatory action of PPARα inhibited NF-κB mediated inflammatory gene expression, which governs innate and adaptive immunity [57]. IL1β, TNFα and COX2 are proinflammatory markers and are also target genes of NF-κB [58]. Our present results showed that NF-κB and a number of proinflammatory markers were inhibited while anti-inflammatory cytokines (IL10 and TGFβ) were upregulated by fenofibrate. Similarly, a very recent fish study indicated that PPARα activation attenuated inflammatory responses by reducing NF-κB expression in grass carp adipocytes [59]. Because both oxidative stress and inflammation negatively correlate with immune functions [60], our results illustrate that PPARα activation improves the immunity of Nile tilapia by enhancing the antioxidation and anti-inflammatory ability. This indicates also that the roles of PPARα in reducing oxidative stress and inflammation should be conserved either in fish or in mammals. The comprehensive correlations among PPARα activation, inflammation, oxidative stress and immune functions are also illustrated in Fig. 8. In addition, PPARα is expressed in a wide variety of tissues and cells of the immune system, including macrophages, dendritic cells (DCs), T cells and B cells [61]. It could also affect immune functions or inflammatory process through directly stimulating immune cells in tissues. Previous study found that PPARα activation promoted differentiation and proliferation of Kupffer cells, T cells and lymphocytes in mammals [62,63]. Furthermore, PPARα could also regulate inflammation and immune response, through the ability of this receptor to regulate DC and T cell cytokine production [61]. However, the regulatory relationship between PPARα and immune cells is still unclear in fish, and it may be one of the research directions in further.

It should be noted that, although the effects of the direct activation of PPARα on fish immunity are rarely investigated, a number of fish studies have illustrated that some n-3 long-chain unsaturated fatty acids (LCUFA), such as EPA and DHA, could play beneficial roles in improving immune functions, antioxidative abilities, and reducing inflammatory factors [64,65]. However, the mechanisms involved in these beneficial effects of n-3 LCUFA are still poorly understood. Of note, some fatty acids, especially n-3 LCUFA and their metabolites, are regarded as PPARα ligands, which could activate the transcriptional activity of PPARα [66]. Therefore, according to the results of our present study, n-3 LCUFA could be beneficial in improving fish immunity by activating the PPARα signaling pathway. This suggests that PPARα could be activated by a series of nutritional factors in practical aquaculture and play positive roles in promoting fish health. The possible effects of dietary components on PPARα activation and the related immunity-stimulating effects should be intensively investigated in future.

5. Conclusion

Our present study indicated that the PPARα activation could enhance immune ability through increasing mitochondria-mediated energy supply, inhibiting inflammation and enhancing antioxidant activities in Nile tilapia. Therefore, PPARα could be a potential new target in regulating immune function in fish. Our present study provides an important route to correlate energy metabolism and immunity in fish.

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