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Short communication

Two faces of PPARα/NFκB signaling pathway in inflammatory responses to adipocytes lipolysis in grass carp *Ctenopharyngodon idella*

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ABSTRACT

Adipose tissue plays an important role in energy reservation, also be considered as vital immunological organ in animals. Adipocytes are the basic unit of adipose tissue, while little is known about the relationship between lipid metabolism and inflammatory response in fish adipocytes so far. In this study, forskolin was used to induce adipocyte lipolysis, and 5 μM forskolin and 30 μM forskolin both triggered lipolysis by increasing ATGL expression. Consequently, 30 μM Forskolin instead of 5 μM Forskolin induced the expression of NF-κB and its target pro-inflammatory cytokine genes including MCP-1, IL-6 and TNF-α. Further study found that low grade rate of lipolysis activated PPARα gene, and its inhibitory effect on the mRNA expression of NF-κB and its target genes inhibited the adipocyte inflammation. On the contrary, high grade rate of lipolysis increased the expression levels of NF-κB and its target genes, while their expression were attenuated by inhibition of reactive oxygen species (ROS) using α-tocopherol, suggesting that ROS generated due to the PPARα-mediated oxidation of released fatty acids from lipolysis may contribute to adipocyte inflammation. These results indicated that PPARα has dose effect in inflammatory responses to adipocyte lipolysis in grass carp. Taken together, grass carp adipocytes have immune activity. The inflammatory response is linked to the grade rate of adipocyte lipolysis in grass carp adipocytes, and excessive adipocyte lipolysis may promote a dynamic immune response in adipose tissue. This is the first study showing the regulatory effects of lipolysis on immune functions in fish adipocytes.

1. Introduction

Adipose tissue has been characterized as a form of connective tissue that involves in nutrient homeostasis and functions as an endocrine organ at the center of energy homeostasis [1]. Moreover, perivisceral adipose in mammals is increasingly seen as playing an important role in immune function [2–4]. In fish, visceral adipose tissue is also considered as an important immune tissue [5,6]. However, adipocytes constitute the major component of adipose tissue [7], and the metabolic regulation in adipocytes correlation with immune responses has not been well established in fish. (see Table 1)

Adipose tissue inflammation is now considered as a key process underlying metabolic diseases in obese individuals [8]. An early characteristic of obesity is enhanced adipocyte lipolysis [9]. This induces the research interests of the relationship between adipocyte lipolysis and inflammatory responses. Activation of lipolysis in cultured 3T3-L1 adipocytes by isoproterenol induces an acute inflammatory state marked by the expression of inflammatory cytokines [10]. Subsequent research found that excessive adipocyte lipolysis activates the JNK/NFκB pathway leading to the up-regulation of COX-2 expression and recruitment of inflammatory macrophages [11]. Perilipin 1 knockout adipocytes promotes lipolysis and secretion of inflammatory responses lipid metabolites such as prostaglandins, which potentiated monocyte migration [12]. These results and mounting evidence indicate that increasing fatty acid mobilization in adipocytes contributes to local and systemic inflammation.

In teleosts, fatty acids (FA) stored in the form of TAGs are also the main energy source [13]. Excessive fat deposited in farmed fish contributes to metabolic disease in fish. The research of fish lipolysis could shed light on mechanisms that control metabolic disorders in farmed fish and give a hint how these disorders could be treated. High fat diet can induce lipase genes expression, including adipose triacylglycerol lipase, hormone-sensitive lipase in adipose tissue of Nile tilapia [14]. Oka et al. [15], found that immune molecules occur in obesity pathways in both zebrafish and mammals. These results indicate that fish and mammals share common pathophysiological pathways in condition of high fat diet [16]. Therefore, understanding the mechanisms how adipocyte lipolysis contribute to inflammatory responses may provide novel strategies to prevent metabolic disease in fish.

Grass carp (*Ctenopharyngodon idella*), a herbivorous freshwater fish,

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Table 1
Specific Primers used for qPCR in this study.

Primers	Sequences (5′-3′)
NF-κB F	GAAGAAGGATGTGGGAGATG
NF-κB R	TGTTGTCGTAGATGGGCTGAG
TNFα F	GCTGCTGTCTGCTTCACGCT
TNFα R	TGCCTGGTCCTGGTTCACTCT
IL-6 F	CCCTGGTCAACGACATCAA
IL-6 R	GTCCACCCTTCCTCTTGCT
MCP1 F	GACTGTCCCAGACGAGCTAT
MCP1 R	CTTTAGCAACATGTCCGCTCG
β-actin-F	TCCACCTTCCAGCAGATGTGGATT
β-actin-R	AGTTTGAGTCGGCGTGAAGTGGTA

is an important farmed fish in China for its delicious meat and high market value [17]. It is considered as a good model for the study of lipid metabolism because grass carp store excess fat in liver and adipose tissue; it is however not known how this affects fish health. A better understanding of the molecular mechanisms governing the immune activities of adipocytes may provide a therapeutic approach for the treatment of metabolic disorders in farmed fish. Hence, the aim was to establish the possible relationship between adipocyte lipolysis and immune in grass carp. In this study, different dose of forskolin was used to trigger grass carp adipocyte lipolysis. Then, we used Nile red staining and gene expression analyses with real-time qPCR to examine effects of lipolysis on immune functions of grass carp adipocytes. Our study indicated that increasing lipolysis could lead to inflammatory responses due to the activation of NF-κB by ROS generated from PPARα-mediated oxidation. To the best of our knowledge, this is the first report establishing the correlation between adipocyte lipolysis and inflammatory responses in fish.

2. Materials and methods

2.1. Adipocyte isolation and treatments

The grass carp pre-adipocytes were cultured as described by Liu et al. [18], with minor modifications. Briefly, the adipose tissue (~ 180g) was isolated by sterile dissection from the abdominal cavity of 4–5 fishes. The tissue was washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated in 0.1% Type I collagenase (Sigma, USA) with 2% BSA (Sigma) at room temperature for 30 min. The cell suspension was filtered through a 200-μm nylon filter and centrifuged at 590 g for 10 min. The cell pellet was incubated in erythrocyte lysing buffer for 10 min at room temperature, washed twice and resuspended in growth medium (GM), containing Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. The resuspended cells were seeded in gelatin pre-coated plates at a density of approximately 10 g tissue/25 cm^2. To reach 80–90% confluency, the cells were incubated at 28 °C with 5% CO_2 for one week. The differentiation was induced in adipogenic medium (AM) containing GM supplemented with 10 μg/mL insulin, 10 nmol/L triiodothyronine, 1 μmol/L dexamethasone and 0.5 mmol/L 3-Isobutyl-1-Methylxanthine (IBMX). The medium was changed every 2 days.

Following treatment, the culture medium was discarded and replaced with medium containing different concentrations of forskolin (Beyotime Biotechnology, Shanghai), the PPARα agonist WY14643 and inhibitor GW6471 or α-Tocopherol (Sigma-Aldrich, St. Louis, MO, USA) at day 8 of differentiation. The cells were collected at 24h after the administration. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Three replicate samples were analyzed for each treatment group. Three independent experiments were performed for each treatment and control.

2.2. NEFA content measurement

At day 8 of differentiation, adipocytes cultured in 24-well plates were treated 24 h. After the treatment, medium in each well was collected for measurement of NEFA content concentration using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacture's protocol. Briefly, a mixture of 4 μL medium and 250 μL determination working liquid was added to the 96-well plate, which was then incubated at 37 °C for 15 min, after which the OD value was read in a microplate spectrophotometer at 546 nm.

2.3. Nile Red staining

Adipocytes were washed twice with PBS (pH 7.4) and fixed with 10% formaldehyde for 30 min at 4 °C. After washing with distilled water two times, a stock solution of 1 mg/ml Nile Red (Sigma, St. Louis, MO) was prepared in acetone and added to the cell preparation to a final concentration of 1 μg/ml. Cells were incubated at 25 °C for 30 min in the dark. Nile red fluorescence was viewed at 530 nm on a fluorescent microscope.

2.4. Detection of ROS

ROS production was detected using the probe 2,7-dichlorofluorescein diacetate (DCFH-DA), which can be oxidized to the fluorescent 2′,7′-dichlorofluorescein in the presence of ROS (Nanjing Jiancheng Bioengineering Institute, China). Adipocytes were incubated in 100 μM DCFH-DA for 1 h at 28 °C. Cells were then washed twice with PBS (pH 7.4) and examined with a fluorescence microplate reader using Gen 5 software (Bio Tek Instruments, Winooski, USA). The fluorescence was measured at an emission wavelength of 530 nm, using an excitation wavelength of 485 nm. Data were normalized to the control values.

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted as described above. The extracted and purified total RNA was treated with RNase-free DNase to prevent the genomic DNA amplification. One microgram of total RNA was used for reverse transcription with First Strand cDNA Synthesis Kit (ToYoBo, Tokyo, Japan). Real-time RT-PCR was performed using a CFX96TM Real-Time PCR Detection System (Bio-Rad, USA). The amplification was performed in a final volume of 20 μL containing 0.6 μLof each primer (0.5 μM), 1 μL of the diluted cDNA (10-fold), 10 μL of 2 × SYBR®Premix Ex Taq™II (TaKaRa Bio, Inc., Shiga, Japan) and 7.8 μL of sterilized double-distilled water. The real-time PCR contained an initial activation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. The expression levels were determined by the delta-delta CT method ($2^{-\triangle\triangle CT}$). β-Actin mRNA was used as the internal control.

2.6. Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA). Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Forskolin induced adipocytes lipolysis in grass carp

Forskolin is an activator of lipolysis in adipocytes [19]. So we used forskolin to trigger lipolysis in grass carp adipocytes. The formation of small lipid droplets in adipocytes (Fig. 1A) and the increased NEFA content in cell culture media (Fig. 1B) as well as up-regulation of ATGL gene expression (Fig. 1C) suggested that forskolin induced lipolysis in

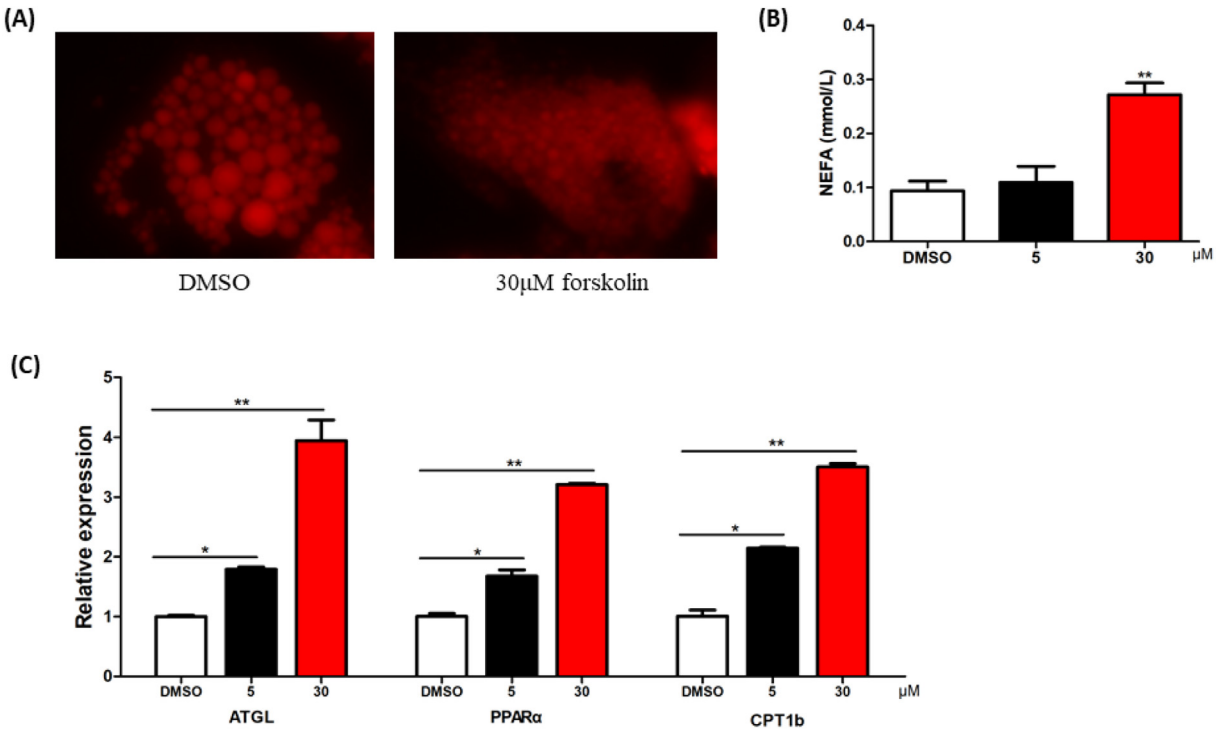

Fig. 1. Adipocytes lipolysis in grass carp was triggered by forskolin. (A) Nile-red staining of lipid droplets. (B) NEFA content in cell culture media (n = 3). (C) Gene expression in adipocytes (n = 3). Differentiated cells at day 8 pretreated with forskolin or vehicle. Data are mean ± SD of biologically independent samples; unpaired two-sided Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

grass carp adipocytes. Interestingly, the NEFA content was no significantly difference in 5 μM forksolin treatment compared with control. Fatty acids hydrolyzed from lipolysis fuel energy production via mitochondrial β-oxidation [20,21]. The expression of PPARα and CPT1b were both significantly up-regulated in 5 μM and 30 μM forksolin, indicating that FFAs released from lipolysis were used for fatty acid β-oxidation in grass carp adipocytes in low grade rate of lipolysis.

3.2. Lipolysis has a dose-dependent differential impact on inflammatory response in adipocytes

By triggering adipocyte lipolysis, 30 μM Forksolin instead of 5 μM Forksolin induced the expression of NF-κB and its target genes including MCP-1, IL-6 and TNF-α, indicating that excess lipolysis can lead to adipocyte inflammation (Fig. 2).

3.3. PPARα is a transcriptional inhibitor of NF-κB in adipocytes

PPARα ameliorates inflammation by inhibiting NF-κB. Therefore, we investigate whether PPARα mediated NF-κB signaling pathway. NF-κB and its target genes were inhibited using PPARα agonist, while PPARα inhibitor activated NF-κB signaling pathway (Fig. 3). These results indicated that PPARα inhibited NF-κB signaling pathway in grass carp.

3.4. Inhibiting PPARα has a differential impact on inflammatory response induced by different dose of lipolysis in adipocytes

In the state of low rates of lipolysis, the mRNA expression of NF-κB and its target genes including MCP-1, IL-6 and TNF-α was up regulated by PPARα inhibitor (Fig. 4A). This indicated that PPARα inhibitor can activate NF-κB signaling pathway, inducing adipocyte inflammation. Hence, Low rates of lipolysis did not induce adipocyte inflammation by activating PPARα. However, in the state of high rates of lipolysis, the mRNA expression of NF-κB and its target genes including MCP-1, IL-6 and TNF-α recovered to the normal levels by PPARα inhibitor (Fig. 4B). Hence, inhibiting PPARα can ameliorate excess lipolysis induced-adipocyte inflammation.

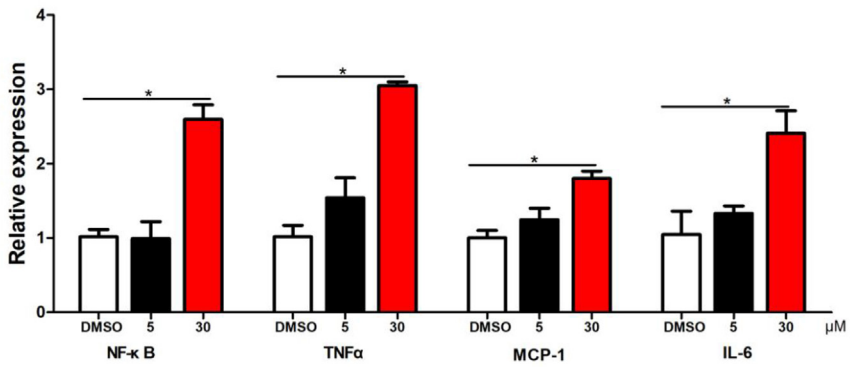

Fig. 2. Excess lipolysis induced adipocyte inflammation. The expression levels of NF-κB and its target genes in differentiated adipocytes at day 8 after treated with forskolin or vehicle were determined by quantitative Real-Time PCR. Data (mean ± SD, n = 3) were referred to the control treatment using β-actin as a control. *$P < 0.05$ indicated a significant difference as determined by Student's t-test.

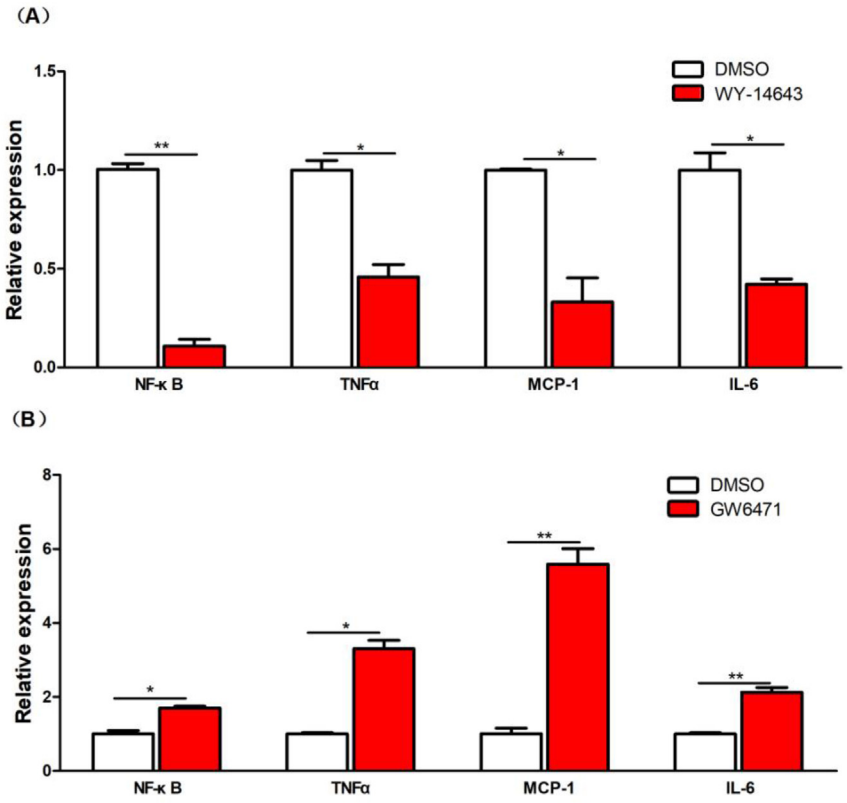

Fig. 3. NF-κB signaling pathway is regulated by PPARα. The expression levels of NF-κB and its target genes in differentiated adipocytes at day 8 after treated with WY14643 or GW6471 were determined by quantitative Real-Time PCR. Data (mean ± SD, n = 3) were referred to the control treatment using β-actin as a control. $*P < 0.05$, $**P < 0.01$ indicated a significant difference as determined by Student's t-test.

3.5. High grade rate of lipolysis mediates NF-κB and its target pro-inflammatory cytokine genes upregulation via a ROS-dependent mechanism in adipocytes

Why excess lipolysis induced adipocyte inflammation? Some study found that enhanced FAs oxidation causes sustained mitochondrial ROS [22], so we suppose that excess lipolysis may produce ROS to lead to adipocyte inflammation according to the up-regulation of PPARα and CPT1b. ROS was detected by fluorescent probe DCFH-DA assay, and we found that 30 μM Forksolin can significantly induce ROS accumulation (Fig. 5A). To determine whether ROS mediated the effects of excess lipolysis on adipocyte inflammation, we used α-tocopherol to scavenge ROS. As shown in Fig. 5A, the expression of NF-κB and its target genes including MCP-1, IL-6 and TNF-α recovered to the normal levels (Fig. 5B). These results indicated that excess lipolysis produces ROS that contributes to adipocyte inflammation.

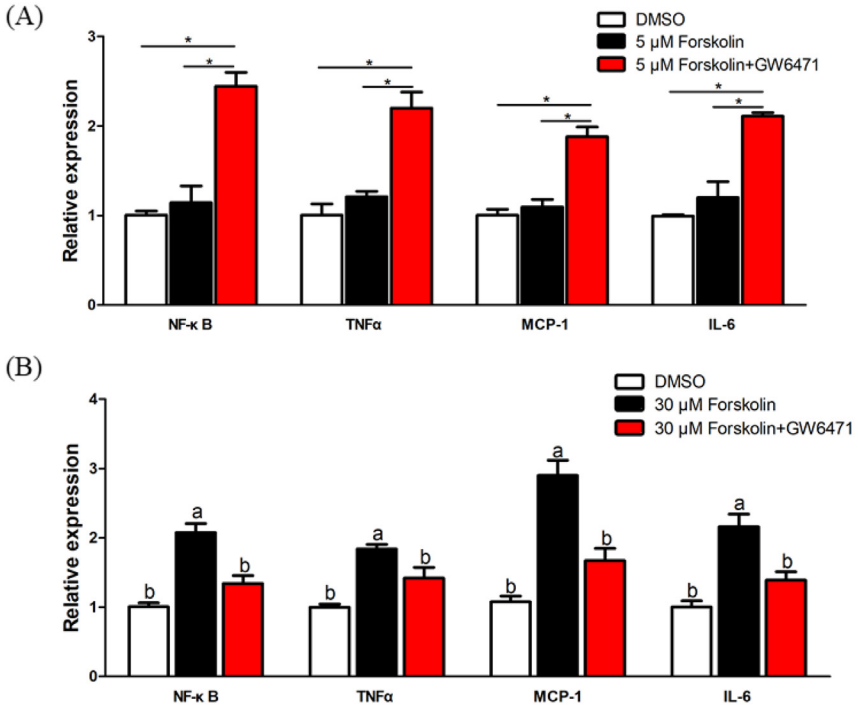

Fig. 4. Effects of Inhibiting PPARα on the NF-κB inflammatory signaling pathway in 5 μM and 30 μM forskolin-induced adipocyte lipolysis. The expression levels of NF-κB and its target genes in differentiated adipocytes at day 8 after treated with forskolin or GW6471 were determined by quantitative Real-Time PCR. Data (mean ± SD, n = 3) were referred to the control treatment using β-actin as a control. Different letters indicate significant differences at $P < 0.05$. $*P < 0.05$ indicated a significant difference as determined by Student's t-test.

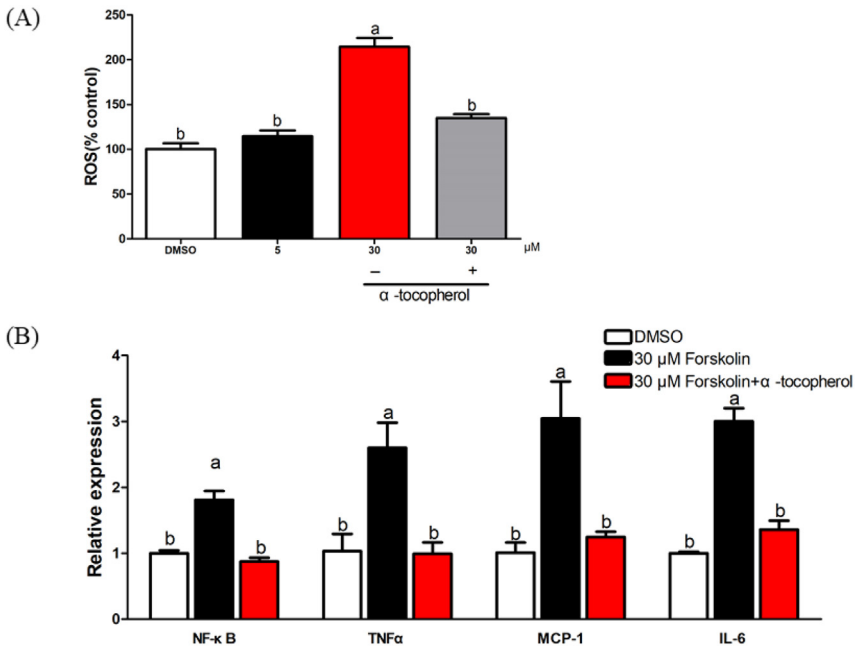

Fig. 5. ROS activate NF-κB signaling pathway in grass carp adipocytes. (A) ROS was detected by fluorescent probe DCFH-DA assay in fully differentiated. (B) Quantitative analysis of the relative mRNA expression levels of NF-κB and its target genes expression in adipocytes. Fully differentiated adipocytes at day 8 were treated with 5 μM and 30 μM forskolin or α-tocopherol for 24 h. The gene expression levels were determined by quantitative Real-Time PCR. Data (mean ± SEM, n = 3) were referred to the control treatment using β-actin as a control. Different letters indicate significant differences at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$ indicated a significant difference as determined by Student's t-test.

4. Discussion

Researchers are paying more and more attention to lipolysis in fish, because the promotion of lipid catabolism for energy production will be largely helpful to increase dietary protein efficiency and decrease body lipid deposit. Therefore, it is taken for granted that the more lipolysis was increased, the better. However, in this study, we found that the inflammatory response is related to the grade rate of adipocyte lipolysis in grass carp adipocytes, and the excessive lipolysis triggers adipose inflammation by increasing the production of inflammatory cytokines.

The organs in the body involved in key metabolic and immune functions have evolved from common ancestral structures in higher organisms [23]. For example, the Drosophila fat body incorporates the mammalian homologues of the haematopoietic and the liver as well as immune systems [24,25], while this site is equal to mammalian adipose tissue due to sharing similar developmental and functional pathways [26,27]. Therefore, metabolism are so crucially linked to inflammatory processes from evolutionary perspectives [23]. Our results that excessive lipolysis triggers adipose inflammation provided strong evidence to support this view. Excessive body fat is considered as a chronic inflammatory disorder. White adipose tissue (WAT) is not only an energy storage organ but also functions as an immune organ [2–4]. In this study, the immune response of grass carp adipocytes indicated that adipocytes should be also considered as immune site, providing evidence of the immune role of WAT in phylogenetically remote vertebrate classe.

PPARα is a ligand-activated transcription factor that regulates lipid catabolism. Besides its main role in regulation of lipid metabolism, PPARα exerts anti-inflammatory effects [28,29]. The anti-inflammatory actions of PPARα are mediated via preventing the generation of inflammatory cytokines partially by negatively regulating NF-κB, which govern innate and adaptive immunity [30,31]. MCP-1, IL-6 and TNF-α are proinflammatory markers that are expressed and released by adipocytes [32] and they are also target genes of NF-κB [33]. Our results showed that NF-κB and these proinflammatory markers were inhibited by PPARα, indicating that the role of PPARα in anti-inflammatory is conserved from fish to mammals. It is known that PPARα interferes with NF-κB involving direct protein-protein interaction with subunit p65, thus diminishing NF-κB DNA binding [34]. However, we found that PPARα is a transcriptional inhibitor of NF-κB in adipocytes (Fig. 3). Thus, the PPARα-mediated transcriptional inhibition of NF-κB activation might be one possible mechanism underlying its inhibitory actions on pro-inflammatory cytokine production in grass carp adipocytes.

The main function of adipocytes is to satisfy energetic demand of other tissues, such as liver and muscle, by releasing FAs via lipolysis, so adipocytes are considered 'altruistic'. In this study, we found that FFAs released from lipolysis were used for fatty acid β-oxidation in grass carp adipocytes in low grade rate of lipolysis, suggesting that adipocytes may be 'egoistic' holding released FAs to satisfy their own energetic requirements, as reported in Barbato et al., [35]. Besides the role of FAs from lipolysis as energy substrates and precursors of other lipids, FAs are directly involved in cellular signaling pathways and regulation of gene transcription [36]. In our work, lipolysis can also induce PPARα signaling. These results emphasize the role of lipolysis in cellular signaling processes.

Although PPARα ameliorated inflammation by inhibiting the transcriptional of NF-κB signaling pathway in low grade of lipolysis in adipocytes, it showed the opposite in high grade of lipolysis in our work. It is known that activation of PPARα is associated with increased fatty acid catabolism; however, enhanced FAs oxidation mainly mitochondrial ROS [22]. ROS can lead to a pro-inflammatory signaling cascade by activating NF-κB [37,38]. Thus, excess lipolysis contributes to adipocyte inflammation by excessive ROS production, which may be responsible for adipose tissue dysfunction. In this study, we showed the different contribution of lipolysis to the physiology and pathophysiology of grass carp adipocyte function. Under normal physiological conditions, lipolysis may play a beneficial role in grass carp adipocytes via providing energy substrates. However, lipolysis will have negative effects on grass carp adipocyte function by inducing inflammation, contributing to WAT dysfunction under pathophysiology conditions (e.g. obesity).

In conclusion, lipolysis is linked to inflammatory processes in grass carp adipocytes. Low grade rate of lipolysis didn't trigger inflammation because of the PPARα inhibitory effect on NF-κB and its target genes. However, increasing PPARα in excessive adipocyte lipolysis produced ROS that induced NF-κB and its target genes, contributing to inflammation in adipocytes. Apart from their established role in energy metabolism, lipolysis also may participate in inflammatory signaling processes in fish. Hence, when we do research on increasing energy utilization by promotion of lipid catabolism, the inflammation induced by lipolysis should be considered. Further study should investigate how

the excessive adipocyte lipolysis promotes a dynamic immune response in adipose tissue *in vivo*, especially in high-fat diet-fed fish.

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