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Role of a liver fatty acid-binding protein gene in lipid metabolism in chicken hepatocytes

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ABSTRACT. This study investigated the role of the chicken liver fatty acid-binding protein (L-FABP) gene in lipid metabolism in hepatocytes, and the regulatory relationships between L-FABP and genes related to lipid metabolism. The short hairpin RNA (shRNA) interference vector with L-FABP and an eukaryotic expression vector were used. Chicken hepatocytes were subjected to shRNA-mediated knockdown or L-FABP cDNA overexpression. Expression levels of lipid metabolism-related genes and biochemical parameters were detected 24, 36, 48, 60, and 72 h after transfection with the interference or overexpression plasmids for L-FABP, PPARα and L-BABP expression levels, and the total amount of cholesterol, were significantly affected by L-FABP expression. L-FABP may affect lipid metabolism by regulating PPARα and L-BABP in chicken hepatocytes.

Key words: Chicken hepatocytes; L-BABP; L-FABP; PPARα; Total cholesterol

INTRODUCTION

Liver fatty acid-binding protein (L-FABP) is a member of the fatty acid-binding protein family (FABPs), and accounts for 2 to 5% of the soluble protein present in hepatocytes. Studies on mammals have indicated that L-FABP plays a key role in the dietary intake of fatty acids, and in the homeostasis of fatty acids and cholesterol (CHOL) in hepatocytes (Martin et al., 2003; Newberry et al., 2003; Storch and Corsico, 2008). L-FABP also participates in lipid β-oxidation and the secretion of very-low-density lipoproteins (Spann et al., 2006; Newberry et al., 2009). Studies in poultry have found that L-FABP is only highly expressed in the liver and intestinal tissues (Shi et al., 2008), which suggests that the gene has a similar role in poultry; similar results have been found in mice and humans (Veerkamp et al., 1991; Schroeder et al., 1998). Studies have also shown that L-FABP is an important candidate gene that regulates lipid metabolism in poultry, and plays an important role in lipid metabolism in hepatocytes, through peroxisome proliferator-activated receptor alpha (PPARα) regulating lipid metabolism-related genes (He et al., 2013). L-FABP is an important candidate gene for traits of intramuscular and abdominal fat in poultry; an L-FABP polymorphism is significantly associated with the deposition of intramuscular and abdominal fat in poultry (He et al., 2012, 2013).

To further explore the functions of L-FABP in lipid metabolism in poultry, and reveal the molecular mechanism of L-FABP regulation of lipid metabolism in hepatocytes, we examined the expression levels of genes involved in lipid synthesis and metabolism [acetyl coenzyme A carboxylase (ACC) and PPARα], lipid catabolism [long-chain acyl-CoA synthetase 4 (ACSL4)], lipid transportation and balancing lipid metabolism [PERILIPIN, carnitine palmitoyltransferase I (CPTI), apolipoproteins (APOAI and APOB), and sterol regulatory element binding protein I (SREBPI)], and another member of the FABP family found in hepatocytes, liver bile acid-binding protein (L-BABP). We measured these genes' expression levels and lipid metabolism-related biochemical parameters when L-FABP was upregulated or downregulated, and analyzed the relationships between these genes in the regulation of lipid metabolism in hepatocytes.

MATERIAL AND METHODS

Bacterial strains, plasmids, and laboratory animals

An *Escherichia coli* DH5α, an L-FABP eukaryotic expression vector (pcDNA3.1-L-FABP), which were constructed and stored by our lab, a short hairpin RNA (shRNA) interference vector (pGenesil-L-FABP1), and the corresponding unrelated interference vector (pGenesil-L-FABP0) that were synthesized by Invitrogen Biotechnology Co., Ltd., (Shanghai, China) were used. Chickens (Arbor Acres) that were 16-18 days old were obtained from the Northeast Agricultural University, Harbin, China.

Chicken primary hepatocyte culture

This study was approved by the Northeast Agricultural University Animal Ethics Committee. The chickens were fasted for 3 h prior to surgery. The abdominal cavity was opened under sterile conditions, and the intestine was gently pulled to the left of the body to

expose the mesenteric veins. A cannula was positioned in one hepatic portal vein; the other vein was cut. The liver was perfused with preheated (42°C) perfusion buffer A (500 mL) and buffer B (100 mL). The liver tissue was digested in a preheated (37°C) buffer C for 40 min, and was shaken once every 5 min (see Table 1 for the perfusion buffer recipes) Medium (10% fetal bovine serum, 10,000 IU/L penicillin, and 10,000 µg/mL streptomycin, pH, 7.4) was added to terminate digestion, and the digested tissue was resuspended and filtered using 200-mesh stainless steel filters.

The filtrate was centrifuged at 500 *g* for 5 min, and the supernatant was discarded. The pellet was resuspended in red cell lysis buffer, centrifuged at 500 rpm for 5 min, resuspended in William's E medium (GIBCO, Shanghai, China), and centrifuged again at 500 rpm for 5 min. The pellet was then resuspended in phosphate-buffered saline (PBS), and centrifuged at 500 rpm for 5 min. The hepatocytes were inoculated at a density of 5 x 10^5 cells/mL in medium (10% fetal bovine serum, 10-6 M insulin, 10-6 mol dexamethasone, 10 mg/L vitamin C, 10,000 IU/L penicillin, and 10,000 µg/mL streptomycin, pH 7.4) and cultured in an incubator at 37°C. The medium was changed to wash away nonadherent substances every 24 h. The cells were used for L-FABP gene interference and overexpression tests when the passaged cells reached 80 to 90% confluence.

Table 1. Compositions of the perfusion buffers A, B, and C.

	Buffer A (g/L)	Buffer B (g/L)	Buffer C (g/L)
NaCl	8.006	8.006	8.006
KCl	0.224	0.224	0.224
HEPES	2.383	2.383	2.383
$Na_2HPO_4 \cdot 12H_2O$	1.074	1.074	1.074
EDTA	1.861	-	-
$CaCl_2$	-	-	0.6
Collagenase IV	-	-	0.4

"-" indicates that nothing was added.

Transfection

Transfection was conducted according to manufacturer instructions, using Lipofectamine 2000® (Invitrogen, Shanghai, China). Briefly (using a 24-well plate as an example), both the plasmid DNA and the Lipofectamine 2000® were separately diluted in serum-free medium and incubated at room temperature for 5 min. The Lipofectamine 2000® solution was then mixed with the DNA solution and incubated at room temperature for 20 min. One hundred microliters of this mixture was added to the cell culture medium. The cells were cultured at 37°C in 5% CO_2, and the medium was changed after 4 to 6 h to remove the transfection reagents.

Total protein extraction

The culture medium was removed, and cells were washed once with PBS and digested by trypsin. Medium was added to terminate the digestion, and cells were transferred into 1.5-mL Eppendorf tubes and centrifuged at 2000 rpm for 3 min. The superna-

tant was discarded, and the pellet was resuspended in PBS and centrifuged again at 2000 rpm for 3 min. Cell lysis buffer (100-200 μL) containing 1 mM phenylmethylsulfonyl fluoride was added to each well of the six-well plates. The cells were lysed completely, and the lysates were mixed by shaking for 30 min at room temperature and centrifuged at 12,000 rpm for 5 min. The total protein concentration in the supernatant was determined using an Enhanced BCA Protein Assay Kit (Beijing, China), and samples were stored at -80°C.

Western blot analysis

Fifty-microgram protein samples were loaded per lane, and after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane was blocked with skim milk for 3 h at room temperature, washed with PBS Tween (PBST), incubated with chicken L-FABP antibody (1:3000) (Shi et al., 2008) for 2 h at room temperature with shaking, and incubated with the secondary antibody [horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000; ZSGB-Bio, Beijing, China)] for 1 h at room temperature. After washing with PBST, the immunoreactive protein on the membrane was visualized using enhanced chemiluminescence, and exposed using a chemiluminescence imaging system (Beijing Sage Creation Science Co. Ltd., Beijing, China).

Detection of gene expression by real-time polymerase chain reaction (PCR) analysis

The mRNA expression of the lipid metabolism-related genes (ACC, PPARα, ACSL4, PERILIPIN, CPTI, APOAI, APOB, SREBPI, and L-BABP) was detected using real-time reverse transcription PCR (RT-PCR). Using a FastStart Universal SYBR Green Master (ROX) kit (Roche, Shanghai, China) in accordance with manufacturer instructions, the reaction system was as follows: 5 μL ROX, 0.1 μL 30 μM forward primer, 0.1 μL 30 μM reverse primer, 3.8 μL water, 1 μL template, and 10 ng cDNA, in a total volume of 10 μL. The mixture was added to an ABI 7500 detection system (Applied Biosystems, Beijing, China) with the following reaction steps and conditions: activation at 50°C for 2 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and extension at 60°C for 60 s, for a total of 40 cycles, with triplicates for each sample. Non-POU domain-containing octamer-binding (NONO) was used as an internal control. The primers used for the real-time PCR are listed in Table 2. All the primers were synthesized by Invitrogen Biotechnology Co. Ltd. (Invitrogen, Shanghai, China).

Detection of biochemical parameters

The levels of total CHOL, triglycerides (TG), high-density lipoproteins (HDL), and low-density lipoproteins (LDL) in the cell culture medium of the interference group, the interference control group, the overexpression group, and the overexpression control group were detected using total CHOL, TG, HDL, and LDL kit from BioSino Biotechnology and Science Inc. (BioSino, Beijing, China).

Table 2. Gene-specific primers used for the real-time polymerase chain reaction.

Gene	GenBank accession No.	Primer sequence (5'-3')
L-FABP	AY563636	F: 5'-GCAGAATGGGAATAAGTT-3' R: 5'-TTGTATGGGTGATGGTGT-3'
NONO	NM_001031532.1	F: 5'-AGAAGCAGCAGCAAGAAC-3' R: 5'-CCTCCATCCTCCTCAGT-3'
ACC	NM205505	F: 5'-TTCCTACCAAGACTCCCTAT-3' R: 5'-GGTTTCTACGGCAACTACTC-3'
PPARα	NM_001001464	F: 5'-TTTAACGGAGTTCCAATCGC-3' R: 5'-AAACCCTTACAACCTTCACAA-3'
ACSL4	XM_420317	F: 5'-GCACTACTAGGAGGGAACAT-3' R: 5'-CTCTAGGATTAGGCTTGTCT-3'
PERILIPIN	NM_001127439	F: 5'-GGGGTGACTGGCGGTTGTA-3' R: 5'-GCCGTAGAGGTTGGCGTAG-3'
CPTI	NM_001012898	F: 5'-AGAGGGCGTGGACCAATAA-3' R: 5'-CTGGGATGCGGGAGGTATT-3'
APOAI	M96012	F: 5'-GCATTCGGGATATGGTGG-3' R: 5'-CTCAGCGTGTCCAGGTTGT-3'
APOB	DQ630943	F: 5'-GACTTGGTTACACGCCTCA-3' R: 5'-TAACTTGCCTGTTATGCTC-3'
SREBPI	AY029224	F: 5'-GGTCCGGGCCATGTTGA-3' R: 5'-CAGGTTGGTGCGGGTGA-3'
L-BABP	AF380998	F: 5'-CCTCCATAATGGCATTCAGT-3' R: 5'-AGTAGTAATGTCAGCCTCTTT-3'

RESULTS AND DISCUSSION

Detection of L-FABP gene interference and overexpression

The differences in L-FABP expression between cells in the interference and control groups and the overexpression and control groups were analyzed using western blot. The results show that L-FABP expression was significantly lower at 24, 36, 48, 60, and 72 h after transfection with the L-FABP shRNA interference vector than in the control group. L-FABP expression was significantly higher ($P < 0.05$) at 24, 36, 48, 60, and 72 h after transfection with the eukaryotic expression vector than in the control group (Figures 1 and 2).

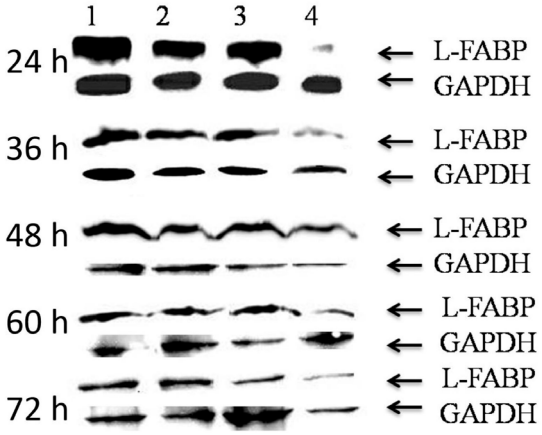

Figure 1. Analysis of L-FABP interference and overexpression effects using western blot. *Lane 1* = overexpression group; *lane 2* = overexpression control group; *lane 3* = interference control group; *lane 4* = interference group.

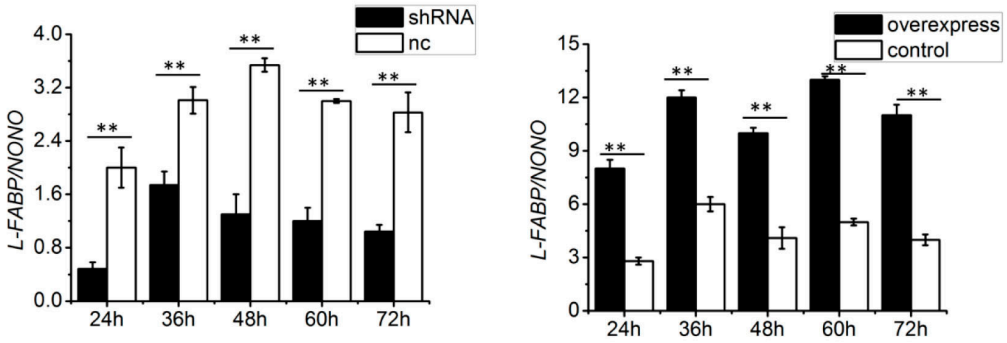

Figure 2. Analysis of L-FABP interference and overexpression effects using a real-time polymerase chain reaction. *P < 0.05, **P < 0.01.

Changes in the mRNA expression of lipid metabolism-related genes after L-FABP interference

The expression levels of the lipid metabolism-related genes (ACC, PPARα, ATGL4, PERILIPIN, CPTI, APOAI, APOB, SREBPI, and L-BABP) in the cells of the interference and the control groups were detected using real-time RT-PCR (Figure 3). The results show that ACC expression at 24 and 36 h in the L-FABP interference group was significantly lower than ACC expression in the control group (P < 0.01). PPARα and L-BABP expression in the L-FABP interference group was significantly lower at the 24-, 48-, and 60-h time points (P < 0.01). PERILIPIN expression in the L-FABP interference group was significantly lower at the 24-, 48-, 60-, and 72-h time points (P < 0.05 or P < 0.01). CPTI expression in the L-FABP interference group was significantly lower at the 60- and 72-h time points (P < 0.01). APOB expression in the L-FABP interference group was significantly lower at the 36-, 48-, and 60-h time points (P < 0.01). ACSL4, APOAI, and SREBPI expression exhibited inconsistent differences in the interference and control groups at each time point.

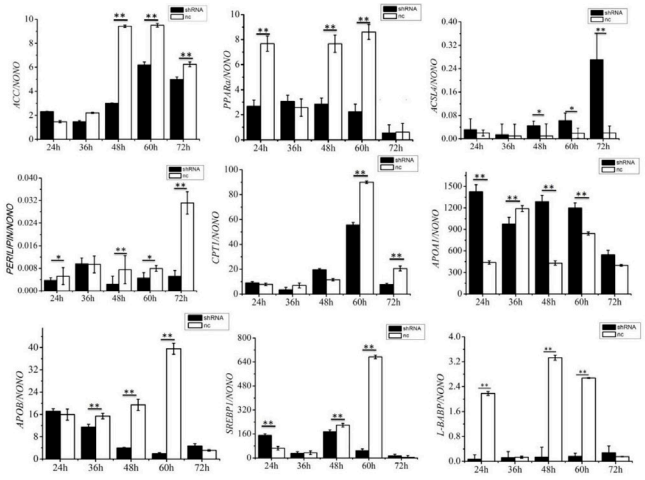

Figure 3. mRNA expression of lipid metabolism-related genes after L-FABP interference; *P < 0.05, **P < 0.01.

Changes in the mRNA expression of lipid metabolism-related genes after L-FABP overexpression

The expression levels of the lipid metabolism-related genes in the cells of the overexpression and the control groups were detected using real-time RT-PCR (Figure 4). The results show that PPARα expression in the L-FABP overexpression group at 36 and 60 h was significantly higher than L-FABP overexpression in the control group ($P < 0.01$). However, at 72 h, PPARα expression was significantly lower than in the control group ($P < 0.01$). L-BABP expression was significantly higher at the 24-, 36-, 60-, and 72-h time points in the L-FABP overexpression group ($P < 0.01$). ACC, ATGL4, PERILIPIN, CPTI, APOAI, APOB, and SREBPI expression exhibited inconsistent differences in the overexpression and control groups at each time point.

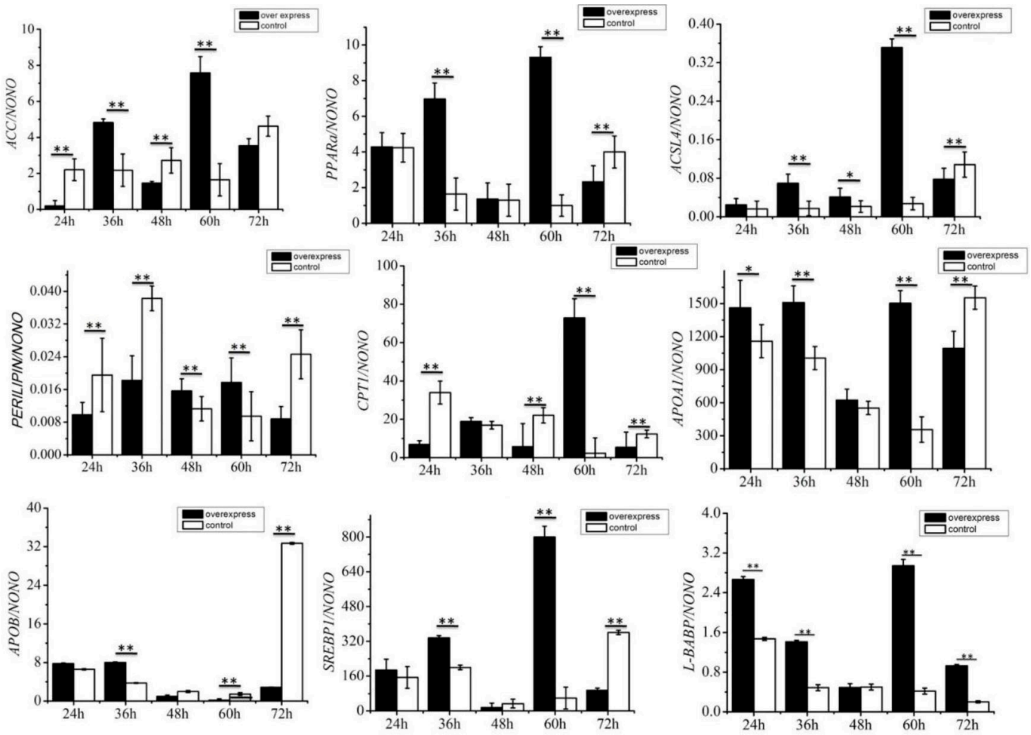

Figure 4. mRNA expression of lipid metabolism-related genes after L-FABP overexpression; *P < 0.05, **P < 0.01.

Changes in lipid metabolism-related biochemical parameters in a cell culture medium after L-FABP interference

Levels of CHOL, TG, HDL, and LDL in the cell culture medium of the interference and the control groups were evaluated (Figure 5). The results show that the level of total CHOL at the 24-, 36-, 60-, and 72-h time points was significantly higher in the L-FABP interference group than in the control group ($P < 0.01$). The levels of TG, HDL, and LDL exhibited inconsistent differences in the interference and control groups at each time point.

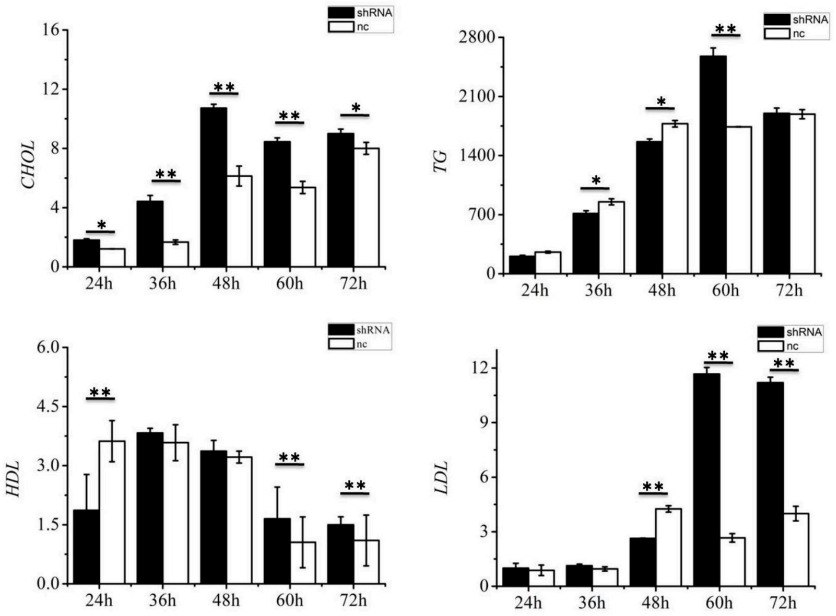

Figure 5. Changes in lipid metabolism-related biochemical parameters in a cell culture medium after L-FABP interference; *P < 0.05, **P < 0.01.

Changes in lipid metabolism-related biochemical parameters in a cell culture medium after L-FABP overexpression

Levels of total CHOL, TG, HDL, and LDL in the cell culture medium of the overexpression group and the control group were evaluated (Figure 6). The results show that the level of total CHOL at the 36-, 48-, 60-, and 72-h time points was significantly lower in the L-FABP overexpression group than in the control group (P < 0.01). TG levels were significantly lower in the L-FABP overexpression group at the 36- and 48-h time points (P < 0.01) than in the control group. LDL levels were significantly higher in the L-FABP overexpression group at the 24-, 48-, and 60-h time points (P < 0.05 or P < 0.01) than in the control group. HDL levels exhibited inconsistent differences in the overexpression and control groups at each time point.

As an important gene in lipid metabolism, L-FABP has attracted much attention. L-FABP plays a significant role in lipid metabolism in mammals, and is associated with type 2 diabetes, obesity, insulin resistance, and fatty liver disease in humans (Jolly et al., 2000; Atshaves et al., 2010), and participates in the regulation of body weight, affects the uptake rate of intracellular fatty acid, and is involved in lipid β-oxidation in the mammalian liver and intestine (Martin et al., 2003; Spann et al., 2006; Newberry et al., 2009). The function of L-FABP in lipid metabolism in poultry has attracted interest from many researchers, because it is associated with traits related to abdominal fat deposition (Zhang et al., 2013), fatty acid transportation, and intramuscular fat content (He et al., 2012, 2013).

Both the FABP and PPAR family members exhibit tissue-specific expression characteristics, and the two types of gene are mutually regulated. For example, A-FABP and PPARγ are mutually regulated in adipose tissue, and E-FABP and PPARδ are mutually regulated in

epidermal tissue (Adida and Spener, 2002). Similar mechanisms are also present in hepatocytes, in relation to L-FABP and PPARα (Atshaves et al., 2010). The overexpression of L-FABP in human hepatoma cell lines and primary duck hepatocytes results in upregulated mRNA levels of PPARα (Bordewick et al., 1989). A study in ducks found that L-FABP regulated lipid metabolism-related genes (e.g., FAS and LPL) through PPARα (He et al., 2012). In this study, we found a positive correlation between L-FABP and PPARα expression levels, suggesting that L-FABP regulates PPARα expression in chicken hepatocytes.

Studies in mammals have shown that L-FABP not only directly regulates PPARα in the nucleus, but also affects the synthesis of total CHOL, TG, and apolipoproteins in the endoplasmic reticulum (Atshaves et al., 2010). Fluorescence hybridization experiments have confirmed that L-FABP is capable of binding to CHOL and its analogs (Fischer et al., 1985; Sams et al., 1991; Avdulov et al., 1999; Schroeder et al., 2000; Stolowich et al., 1999, 2002; Soccio and Breslow, 2004; Martin et al., 2009). L-FABP in mammalian hepatocytes can regulate the absorption and metabolism of CHOL, and affect its movement in a bilayer membrane (Martin et al., 2005). The present study in chickens obtained similar results as have been obtained in mammal studies, i.e., there is a negative correlation between L-FABP expression levels and the amount of total CHOL secreted from hepatocytes. We suggest that the mechanism in chickens is similar to that in mammals. We hypothesize that L-FABP binds to CHOL and its analogs in hepatocytes, and transports them to the endoplasmic reticulum to synthesize total CHOL.

We also found a positive correlation between L-FABP and L-BABP. L-BABP is only expressed in the liver (QingQiu et al., 2011), and it substantially affects chicken body weight, abdominal fat weight, and abdominal fat percentage (Zhao et al., 2013). L-BABP also exhibited a negative correlation with the amount of total CHOL (Gao et al., 2015). Further work is required to determine the exact method of interaction between these two genes.

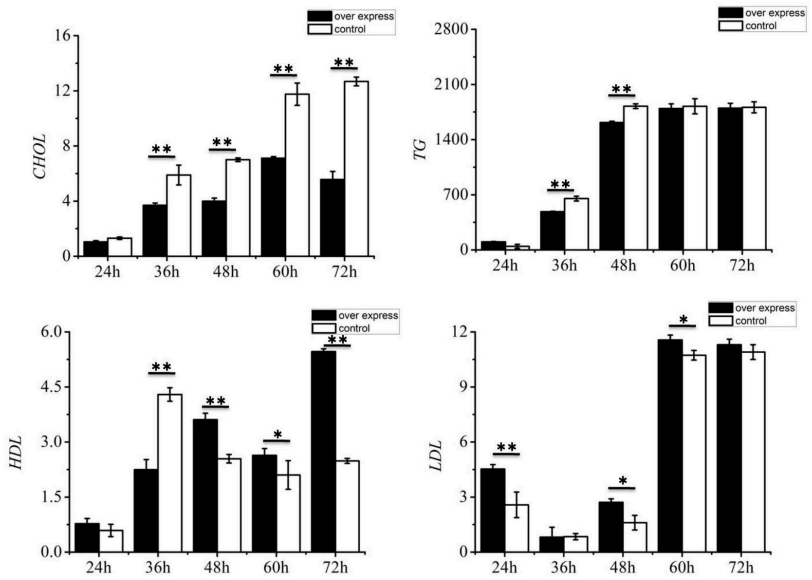

Figure 6. Changes in lipid metabolism-related biochemical parameters in a cell culture medium after L-FABP overexpression; *P < 0.05, **P < 0.01.

CONCLUSIONS

In this study, we investigated the possible functions of L-FABP in chicken hepatocytes, and found that L-FABP expression affects PPARα and L-BABP expression, as well as the total CHOL amount. These results will be valuable for further investigations of the role of L-FABP in chicken lipid metabolism, and the regulatory relationships between lipid metabolism-related genes.

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