Dehydroepiandrosterone alleviates oleic acid-induced lipid metabolism disorders through activation of AMPK-mTOR signal pathway in primary chicken hepatocytes

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ABSTRACT The incident of lipid metabolism disorders has obviously increased under the undue pursuit of efficiency, which had seriously threatened to the health development of poultry industry. As an important cholesterol-derived intermediate, though dehydroepiandrosterone (**DHEA**) has the fat-reduction effect in animals and humans, but the underlying mechanism still poorly understood. Herein, the present study aimed to investigate the regulatory effects and its molecular mechanism of DHEA on disturbance of lipid metabolism induced by oleic acid (**OA**) in primary chicken hepatocytes. The hepatocytes were treated with 0, 0.1, 1, 10 μM DHEA for 4 h, and then supplemented with 0 or 0.5 mM OA stimulation for another 24 h. Our findings demonstrated that DHEA treatment effectively reduced TG content and alleviated lipid droplet deposition in OA-induced hepatocytes. DHEA inhibited the lipogenesis related factors (*ACC, FAS, SREBP-1c*, and *ACLY*) mRNA level and increased the lipolysis key factors (*CPT-1* and *PPARα*) mRNA levels. In addition, DHEA obviously elevated the protein levels of CPT-1A, p-ACC, and ECHS1; whereas decreased the protein levels of FAS and SREBP-1 in hepatocytes stimulated by OA. Furthermore, DHEA promoted the phosphorylation of AMP-activated protein kinase (**AMPK**) and inhibited the phosphorylation of mammalian target of rapamycin (**mTOR**). Mechanistically, the hepatocytes were pre-treated with AMPK inhibitor compound C or AMPK activator AICAR before addition of DHEA treatment, and the results certified that DHEA activated cAMP/AMPK pathway and which subsequently led the inhibition of mTOR signal, which finally reduced the fat excessive accumulation in OA-stimulated hepatocytes. Collectively, our study unveiled that DHEA protects against the lipid metabolism disorders triggered by OA stimulation through activation of AMPK-mTOR signaling pathway, which prompts the value of DHEA as a potential nutritional supplement in regulating the lipid metabolism and its related disease in poultry.

Key words: Dehydroepiandrosterone, lipid metabolism disorders, hepatocytes, AMPK signaling pathway

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INTRODUCTION

As one mainly sources of edible protein in people, chicken had been concerned by consumers due to its character of high nutritional quality and low cost (Xiong et al., 2020). At present, excessive pursuit the growth rate significantly disturbed the nutritional metabolism in broiler (Singh et al., 2021), which led the immune dysfunction and the decreasing of carcass quality. Metabolic disorders can increase the incidence of liver steatosis which represents as the large amount of lipid accumulation and liver dysfunction (Rozenboim et al., 2016). More important, over consumption of high-fat meat products will lead to the occurrence of metabolism-related diseases in humans, such as obesity, diabetes and atherosclerosis. It reported that lipid metabolism-related diseases, such as fatty liver hemorrhagic syndrome and nonalcoholic fatty liver disease, presented as the excessive fat deposition, liver steatosis and oxidative stress (Ke et al., 2020; Wu et al., 2021). Many studies showed that most bioactive substance or metabolic intermediates exhibits a variety of potential biological effects; and recently most of researchers deemed that dietary supplemental with bioactive ingredients maybe an effective way to control the lipid metabolism disorder and it related diseases in poultry.

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In poultry, the liver acts an important role in maintaining the lipid metabolic homeostasis (Preidis et al., 2017). Meanwhile, the fat accumulation depends on the dynamic balance of fat production and decomposition in liver, and which is regulated by AMP-activated protein kinase (**AMPK**) that a key factor for energy sensing and regulation in body (Fullerton, 2016; Fang et al., 2022). Our previous study had found that oleic acid (**OA**) treatment markedly caused the lipid metabolism disorders and inhibited phosphorylated AMPK protein level in primary chicken hepatocytes; supplemental with (-)-hydroxycitric acid, a bioactive substance extracted from *Garcinia cambogia*, can alleviate the disturbance of lipid metabolism induced by OA stimulation through activation of AMPK signal (Li et al., 2020). Recently, Zhang et al. reported that the AMPK expression level was obviously reduced in liver steatosis model induced by FA (containing OA, palmitic acid in proportions of 2:1)-treatment (Zhang et al., 2021). In addition, it reported that AMPK expression level was markedly decreased in the liver of laying hens with fatty liver hemorrhagic syndrome (Gao et al., 2019) and in mice with nonalcoholic fatty liver disease (Inamdar et al., 2019). Accordingly, AMPK acts an importantly role in maintaining the lipid metabolic homeostasis; thus, activated AMPK with active substances might be a potential target for the prevention of lipid metabolic disorder and it-related diseases.

Dehydroepiandrosterone (**DHEA**), the most abundant circulating steroid hormone in human (Rutkowski et al., 2014), has numerous biological effects, such as antiobesity (Teixeira et al., 2020), antioxidation (Patel et al., 2014), anti-inflammation (Li et al., 2019) and even prolonging life span (Traish et al., 2011). Previous study reported that DHEA can activate AMPK and suppress the lipid accumulation in 3T3-L1 cells (Yokokawa et al., 2020). Our recently study also found that DHEA alleviates lipid metabolic disorders through activation of AMPK-PGC-1α-NRF-1 signaling pathway in rat fed with high-fat-diets (Li et al., 2020). DHEA has been reported to regulate serum and hepatic lipid metabolism related genes expression in embryo chickens (Chen et al., 2010). Herein, present study aimed to investigate the protection effects of DHEA on the lipid metabolism disorders induced by OA stimulation in primary chicken hepatocytes, and further evaluated these beneficial effects of DHEA whether associated with the activation of AMPK-mTOR signaling pathway in hepatocytes. These results will illustrate the molecular mechanism of DHEA in regulating lipid metabolism disorders, and support its use as a nutritional supplement to protect against metabolic-related diseases in poultry and even humans.

MATERIALS AND METHODS

Reagents

DHEA, dimethyl sulfoxide (**DMSO**), penicillin-streptomycin, transferrin, trypsin, 3-(4,5-dimethylthiozol-2-yl)-2,5 diphenyl tetrazolium bromide (**MTT**) and Nile red staining solution were obtained from Sigma-Aldrich (St Louis, MO). Medium 199 was purchased from Hyclone Laboratories (Logan, UT) and L-glutamine was obtained from Amresco (Solon, OH). TRIzol reagent kits were purchased from Invitrogen (Carlsbad, CA); RNase inhibitor was obtained from Promega (Madison, WI). SYBR Green PCR Master Mix and reverse transcriptase kits were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). The assay kits of triglyceride (**TG**) and enzyme-linked immunosorbent assay (**ELISA**) for cyclic adenosine 3′,5′ monophosphate (**cAMP**) were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). The BCA assay kits were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Rabbit antibodies recognized the AMPKα, phospho (p)-AMPKα, ACCα, p-ACCα, FAS, SREBP-1, CPT-1A, ECHS1, GAPDH, and Tubulin were purchased from Cell Signaling Technology (Boston, MA). Mouse antibodies recognized the mTOR and p-mTOR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase conjugate were purchased from Boster Biological Technology Co. Ltd. (Wuhan, China). AICAR (AMPK activator), compound C (AMPK inhibitor), and Rapamycin (RAPA, mTOR inhibitor) were purchased from Selleck Chemicals (Houston, TX).

Cell Isolation, Culture and Treatment

The isolation and culture of primary chicken hepatocytes was based our previous reported (Li et al., 2017). Briefly, 9-day-old chicken embryos were sterilized and the livers were collected under sterile conditions. Then, the livers were cut into small fragments (about 1−2 mm^3), washed with PBS at 4°C for 3 to 5 times, and then digested with 0.25% trypsin at 37°C for 10 to 15 min. After that, the trypsin was inactivated by additional with M199 medium containing 10% fetal bovine serum (**FBS**). Subsequently, the hepatocytes suspension was filtered with 100-mesh, 200-mesh, and 400-mesh cell sieves, and the hepatocytes were collected through centrifugation and suspended in serum-free M199 medium. The primary chicken hepatocytes were seeded in 6-well or 96-well plastic culture plates (Corning) at a density of 2×10^6 cells/well with 2 mL or 1×10^5 cells/well with 100 μL M199 medium (containing 5 μg/mL transferrin and 1% penicillin-streptomycin); and then incubated at 37°C with an atmosphere of 95% air and 5% CO_2.

The hepatocytes were treated with 0, 0.1, 1, 10 μM DHEA for 4 h, and then supplemented with 0 or 0.5 mM OA stimulation for another 24 h; After treatment, the hepatocytes were collected for subsequent experiment.

Cell Viability Assay

Hepatocytes were cultured in 96-well plates at 1×10^5 cell/well, and stimulated with 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mM OA stimulation for 3, 6, 12, 24, 36, or 48 h, respectively. After that, MTT was

added into each cell well and treated for 4 h, then the supernatant was discarded and DMSO was added to dissolve the purple Formazan crystals. The absorbance was measured at 490 nm by a 550 Microplate Reader Instrument (Bio-Rad, CA) to analyze the optimal concentration and time of OA stimulation. Meanwhile, hepatocytes were treated with 0, 0.1, 1, 10 μM DHEA for 4 h, and then added 0 or 0.5 mM OA stimulation for another 24 h; after that, the MTT assay was also used to determine the effect of DHEA on cell viability.

Nile Red Staining

After treatment, 100 μL/well of 1 μM Nile red dye were added into each well and incubated in a dark environment at room temperature for 30 min. Then, the fluorescence was measured using a laser scanning confocal microscope (Nikon, Tokyo, Japan).

Triglyceride Content Assay

Hepatocytes were collected and triglyceride (**TG**) content was detected using commercial assay kits according to manufacturer's instructions. The results were standardized by protein concentration using BCA assay kits by 550 Microplate Reader Instrument (Bio-Rad, CA).

Detection of cAMP Level

Intracellular levels of cAMP were measured using commercial kits according to the manufacturers' instructions. After incubation, the supernatant was removed and the cAMP levels were determined using enzyme-linked immunosorbent assay kits according to manufacturer's instructions. The results were normalized to the protein concentration, as determined using BCA assay kits.

Real-Time Quantitative PCR (RT-qPCR)

Hepatocytes were collected and total RNA was extracted using Trizol reagent. Total RNA (1 μg) was revered into cDNA using the Superscript II kit. The sample (2 μL) was mixed with 18 μL SYBR Green PCR Master Mix with 2 μL primers for acetyl CoA carboxylase (***ACC***), fatty acid synthase (***FAS***), sterol regulatory element binding protein-1c (***SREBP-1c***), ATP-citrate lyase (***ACLY***), peroxisome proliferators-activated receptor α (***PPARα***), carnitine palmitoyl transferase-1 (***CPT-1***) and GAPDH (as the standard). Samples were analyzed in duplicate using the ABI 7500 Real-time Detection System (Applied Biosystems), and the mRNA level were calculated with the $2^{-\Delta\Delta Ct}$ method. All primers were designed by Primer Premier 5 (Premier Biosoft International, Palo Alto, CA) and synthesized by Tsingke Biotech Co., Ltd. (Shanghai, China) (Table 1).

Western Blot Assays

Total protein in hepatocytes was extracted with RIPA lysate, and the protein concentration was determined using the BCA kits. Extracted protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in 5% bovine serum albumin (**BSA**) and incubated with different antibodies overnight at 4°C. After that, the membranes were incubated with horseradish peroxidase conjugated secondary antibody at room temperature. Protein bands were visualized by an enhanced chemiluminescent substrate (ECL-plus, Thermo Scientific); and the relative quantitative of protein bands was performed with Image J software. GAPDH or Tubulin was used as the loading control protein, and all target protein abundance were normalized to GAPDH or Tubulin content.

Immunofluorescence Staining

Sterile coverslips were placed in 24−well plates and hepatocytes were treated with 0, 0.1, 1, 10 μM DHEA for 4 h, and then added 0 or 0.5 mM OA stimulation for another 24 h. After that, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with

Table 1. Primer sequence of targeted genes and GAPDH.

Gene	GenBank accession Number	Primer sequences (5'−3')	Orientation	Product size (bp)
ACC	NM_205505	GTTGTGGTTGGCAGAGCAAG	Forward	284
		GCACCAAACTTGAGCACCTG	Reverse	
FAS	NM_205155	TGAAGGACCTTATCGCATTGC	Forward	96
		GCATGGGAAGCATTTTGTTGT	Reverse	
SREBP-1c	AY029224	GTCGGCGATCCTGAGGAA	Forward	105
		CTCTTCTGCACGGCCATCTT	Reverse	
ACLY	AJ245664	CACCCAGAGGTGGATGTTCT	Forward	188
		GTTGCAGGCCCAATGTTAGT	Reverse	
PPARα	AF470455	CAAACCAACCATCCTGACGAT	Forward	64
		GGAGGTCAGCCATTTTTTGGA	Reverse	
CPT-1	AY675193	GGGTTGCCCTTATCGTCACA	Forward	151
		TACAACATGGGCTTCCGTCC	Reverse	
GAPDH	NM_204305	AGAAGACGGTGGATGG	Forward	80
		AGTGGACGCTGGGAT	Reverse	

Abbreviations: ACC, acetyl CoA carboxylase; ACLY, ATP-citrate lyase; CPT-1, carnitine palmitoyl transferase-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAS, fatty acid synthase; PPARα, peroxisome proliferator-activated receptor α; SREBP-1c, sterol regulatory element binding protein-1c.

0.5% Triton X−100 for 20 min and then blocked with 5% BSA for 1 h. Then, the cells were incubated with anti-ACCα, p-ACCα, AMPKα, p-AMPKα, p-mTOR, and mTOR diluted with 5% BSA buffer at 1:1000 overnight at 4°C for 24 h; and then incubated with a fluorescent secondary antibody away from light at 37°C for 1 h. The nuclei were revealed by DAPI staining and fixed with glycerin, and the fluorescence images were collected under a laser scanning confocal microscope (Nikon, Tokyo, Japan).

Data Analysis and Statistics

Data were expressed as the mean value ± standard error of the mean (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA) (SPSS 21.0; SPSS, Chicago, IL, USA), and the differences were considered significant at $P<0.05$.

RESULTS

Impact of OA-Stimulated on the Viability of Hepatocytes

In order to investigate the cytotoxic effect of OA stimulation on primary chicken hepatocytes, the cell viability was analyzed in hepatocytes treatment with 0-1 mM OA stimulation for 3 to 48 h, respectively. As shown in Table 2, no significant differences were observed in cell viability when hepatocytes were exposed with 0.1-0.5 mM OA stimulation for 3 to 48 h ($P > 0.05$). However, cell viability was decreased as hepatocytes were stimulated with 0.6 mM OA for 24-48 h; meanwhile, the cell viability prominently declined under presence of 0.7-1.0 mM OA stimulation for 3-48 h in hepatocytes ($P < 0.05$) (Table 2). These results suggested that there is no cytotoxicity hepatocytes treatment with 0.5 mM OA stimulation for 3-48 h. Thus, we selected 0.5 mM OA stimulation for 24 h as the most appropriate condition for modeling of lipid metabolism disorders in subsequent experiments.

DHEA Relieves Lipid Metabolism Disorders Induced by OA Stimulation in Hepatocytes

As shown in Figure 1A, no significant differences were observed in cell viability in OA-stimulated hepatocytes after 0.1-10 μM DHEA treatment ($P > 0.05$). OA stimulation markedly enhanced the TG content in hepatocytes compared with control group; and 0.1-10 DHEA treatment significantly inhibited the increasing of TG content induced by OA stimulation in hepatocytes ($P < 0.05$) (Figure 1B). Meanwhile, the massive production of lipid droplets induced by OA stimulation was also dramatically reversed in hepatocytes treatment with different dose of DHEA ($P < 0.01$) (Figure 1C-1D). These results indicated that DHEA alleviates the excessive fat accumulation induced by OA stimulation in hepatocytes.

DHEA Modulates Lipid Metabolism-Related Factors Expression in OA-Stimulated Hepatocytes

OA stimulation markedly increased the lipogenesis related factors (*ACC, FAS, SREBP-1c* and *ACLY*) mRNA level and inhibited the lipolysis key factors (*CPT-1* and *PPARα*) mRNA levels when compared with control group ($P < 0.01$) (Figure 2). However, DHEA treatment obviously blocked the increasing of lipogenesis related factors and decreasing of lipolysis key factors induced by OA stimulation in hepatocytes ($P < 0.05$) (Figure 2). For further certified the regulation effect of DHEA on lipid metabolism-related factors, the protein levels were analyzed using western bolt and immunofluorescence. Results showed that the decreasing of p-ACC, CPT-1A, ECHS1 protein levels and increasing of FAS, SREBP-1 protein level induced by OA stimulation obviously were reversed in hepatocytes after DHEA treatment ($P < 0.05$) (Figure 3A-3F). Meanwhile, immunofluorescence also confirmed that DHEA improved p-ACC protein levels in OA-stimulated hepatocytes (Figure 3G). These results implied that DHEA attenuates lipid metabolism disorders induced by OA stimulation through inhibiting lipogenesis gene

Table 2. The effect of OA on cell viability in primary chicken hepatocytes.

	OA treatment time/h					
$C_{[OA/(mM)]}$	3	6	12	24	36	48
0	0.599 ± 0.024	0.611 ± 0.038	0.709 ± 0.069	0.813 ± 0.043	0.677 ± 0.049	0.461 ± 0.056
0.1	0.570 ± 0.038	0.638 ± 0.075	0.730 ± 0.058	0.815 ± 0.031	0.638 ± 0.013	0.427 ± 0.078
0.2	0.611 ± 0.057	0.608 ± 0.027	0.681 ± 0.037	0.769 ± 0.039	0.645 ± 0.013	0.460 ± 0.032
0.3	0.564 ± 0.037	0.583 ± 0.047	0.670 ± 0.068	0.764 ± 0.053	0.622 ± 0.048	0.498 ± 0.076
0.4	0.538 ± 0.079	0.558 ± 0.028	0.669 ± 0.038	0.763 ± 0.047	0.584 ± 0.058	0.439 ± 0.060
0.5	0.579 ± 0.058	0.580 ± 0.063	0.689 ± 0.057	0.750 ± 0.071	0.560 ± 0.094	0.372 ± 0.078
0.6	0.548 ± 0.023	0.558 ± 0.037	0.619 ± 0.028	0.685 ± 0.036*	0.466 ± 0.013**	0.248 ± 0.079**
0.7	0.405 ± 0.029**	0.428 ± 0.049**	0.468 ± 0.038**	0.507 ± 0.036**	0.381 ± 0.012**	0.282 ± 0.044**
0.8	0.412 ± 0.028**	0.409 ± 0.036**	0.380 ± 0.029**	0.368 ± 0.050**	0.329 ± 0.006**	0.241 ± 0.047**
0.9	0.386 ± 0.038**	0.397 ± 0.017**	0.368 ± 0.028**	0.302 ± 0.045**	0.283 ± 0.012**	0.166 ± 0.018**
1.0	0.388 ± 0.024**	0.278 ± 0.028**	0.289 ± 0.037**	0.259 ± 0.012**	0.138 ± 0.012**	0.123 ± 0.006**

The cell viability of primary chicken hepatocytes was determined after treatment with 0 to 1 mM OA for 3 to 48 h, respectively. Data are presented as means ± SEM.

*$P < 0.05$.

**$P < 0.01$, compared with the control group.

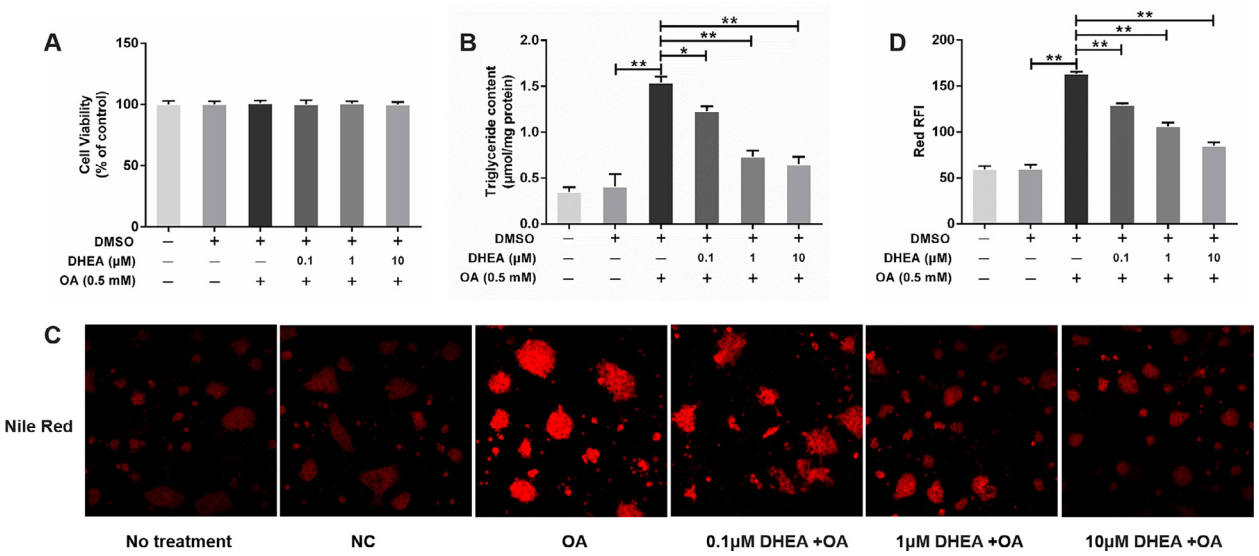

Figure 1. Effect of DHEA on lipid accumulation in OA-stimulated hepatocytes. The primary chicken hepatocytes were treated with 0 to 10 μM DHEA for 4 h, and then added 0 or 0.5 mM OA stimulation for another 24 h. A: Cell viability; B: Triglyceride content; C: Representative photomicrographs of Nile red staining (200 ×); D: Relative fluorescence intensity (RFI) of Nile red staining. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

expression and enhancing lipolysis gene expression in hepatocytes.

DHEA Activates AMPK Signal through Improving cAMP Level in OA-Stimulated Hepatocytes

As shown in Figure 4A, the cAMP content was significantly inhibited in hepatocytes when exposed to OA ($P < 0.01$), whereas the inhibition effect on cAMP content caused by OA stimulation was partly overcame in hepatocytes treatment with 1-10 μM DHEA ($P < 0.05$). Meanwhile, 0.1-10 μM DHEA treatment markedly inhibited the reduction of p-AMPK protein level caused by OA stimulation in hepatocytes ($P < 0.05$) (Figure 4B-4C). Immunofluorescence analysis also confirmed that DHEA improved the p-AMPK protein levels in OA-stimulated hepatocytes (Figure 4D). These results implied that DHEA reactivates cAMP-AMPK signaling pathway that had been inhibited in hepatocytes caused by OA stimulation.

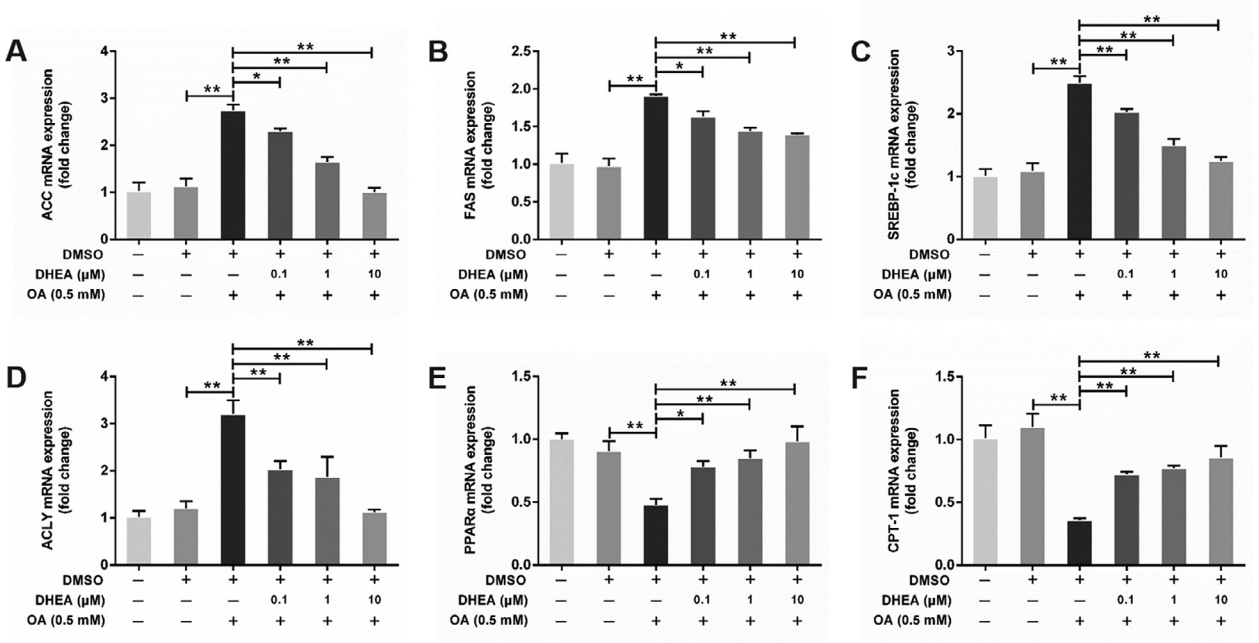

Figure 2. Effect of DHEA on lipid metabolism related factors mRNA level in OA-stimulated hepatocytes. The primary chicken hepatocytes were treated with 0 to 10 μM DHEA for 4 h, and then added with 0 or 0.5 mM OA stimulation for another 24 h. A: Acetyl-CoA carboxylase (ACC) mRNA level; B: Fatty acid synthase (FAS) mRNA level; C: Sterol regulatory element binding protein-1c (SREBP-1c) mRNA level; D: ATP-citrate lyase (ACLY) mRNA level; E: Peroxisome proliferator-activated receptor α (PPARα) mRNA level; F: Carnitine palmitoyl transferase-1 (CPT-1) mRNA level. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

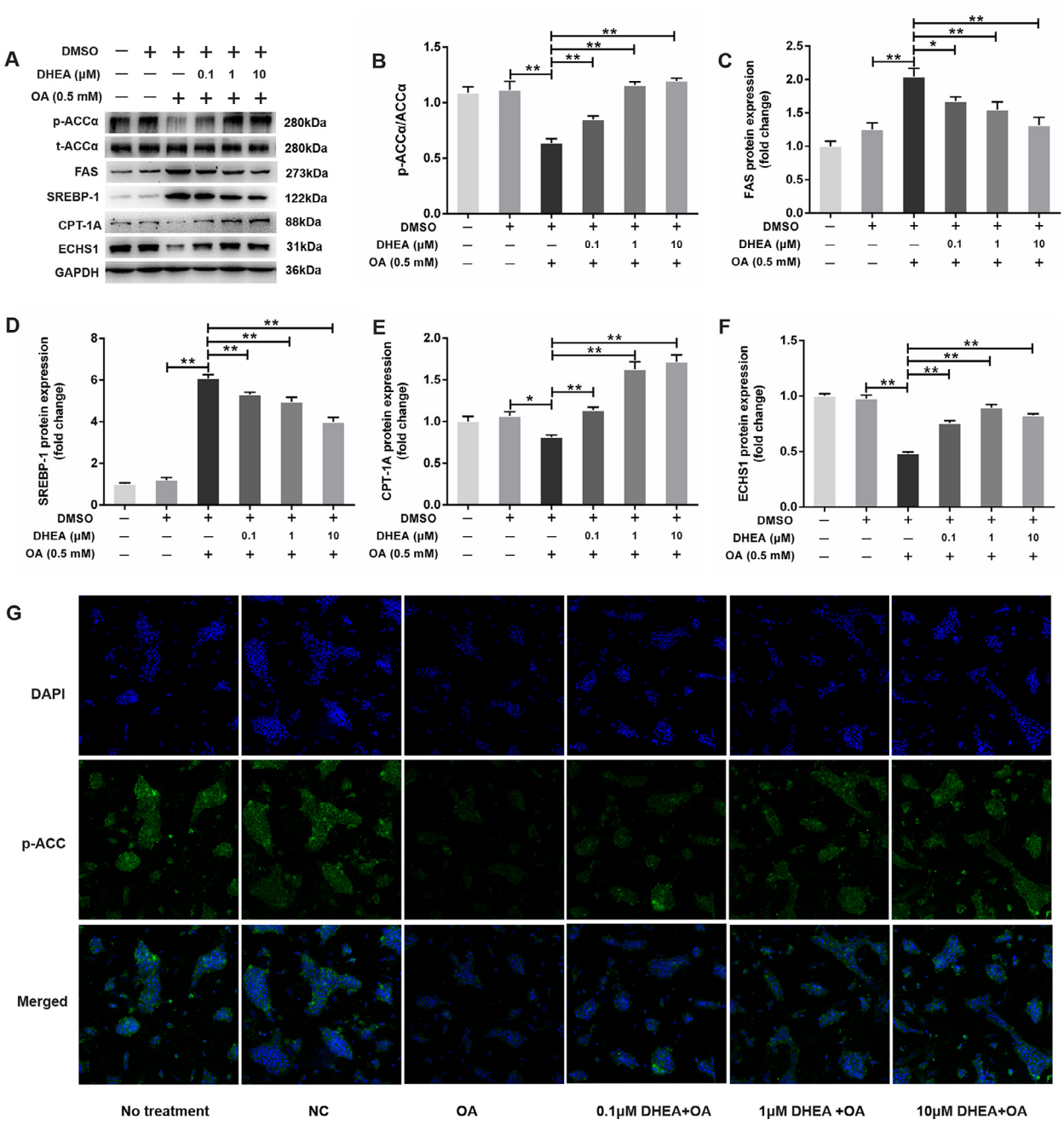

Figure 3. Effect of DHEA on lipid metabolism key factors protein level in OA-stimulated hepatocytes. The primary chicken hepatocytes were treated with 0 to 10 μM DHEA for 4 h, and then treated with 0 or 0.5 mM OA stimulation for another 24 h. A: Immunoblot of phospho (p)-ACCα, FAS, SREBP-1, CPT-1A and ECHS1 protein; B: p-ACCα protein level; C: FAS protein level; D: SREBP-1 protein level; E: CPT-1A protein level; F: ECHS1 protein level; G: p-ACC protein level was analyzed by immunofluorescence. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

As a key regulator of lipid metabolism, activated AMPK can decrease intracellular lipid droplets accumulation. Thus, in order to investigate the role of AMPK in alleviating lipid metabolism disorders by DHEA treatment, the hepatocytes were pre-treated with AMPK inhibitor compound C or AMPK activator AICAR before addition of DHEA treatment. Immunofluorescence analysis showed that the enhancing effect of DHEA or AICAR treatment on the p-AMPK and p-ACC protein levels was markedly reversed in OA-stimulated hepatocytes pretreated with compound C (Figure 5); and similar results were also confirmed by western blot ($P < 0.01$) (Figure 6A-6C). Meanwhile, DHEA or AICAR treatment markedly decreased SREBP-1 protein levels in OA-stimulated hepatocytes ($P < 0.01$), which also was reversed as the cells pre-treated with compound C ($P < 0.01$) (Figure 6D). Importantly, pre-treatment with compound C markedly reversed the reduction effect of DHEA on lipid droplet accumulation in OA-stimulated hepatocytes (Figure 6E-6F). These results indicated that DHEA regulates the lipid metabolism related factors expression through enhancing the cAMP content and then improving p-AMPK protein level, and which alleviates the lipid metabolism disorders induced by OA stimulation in hepatocytes.

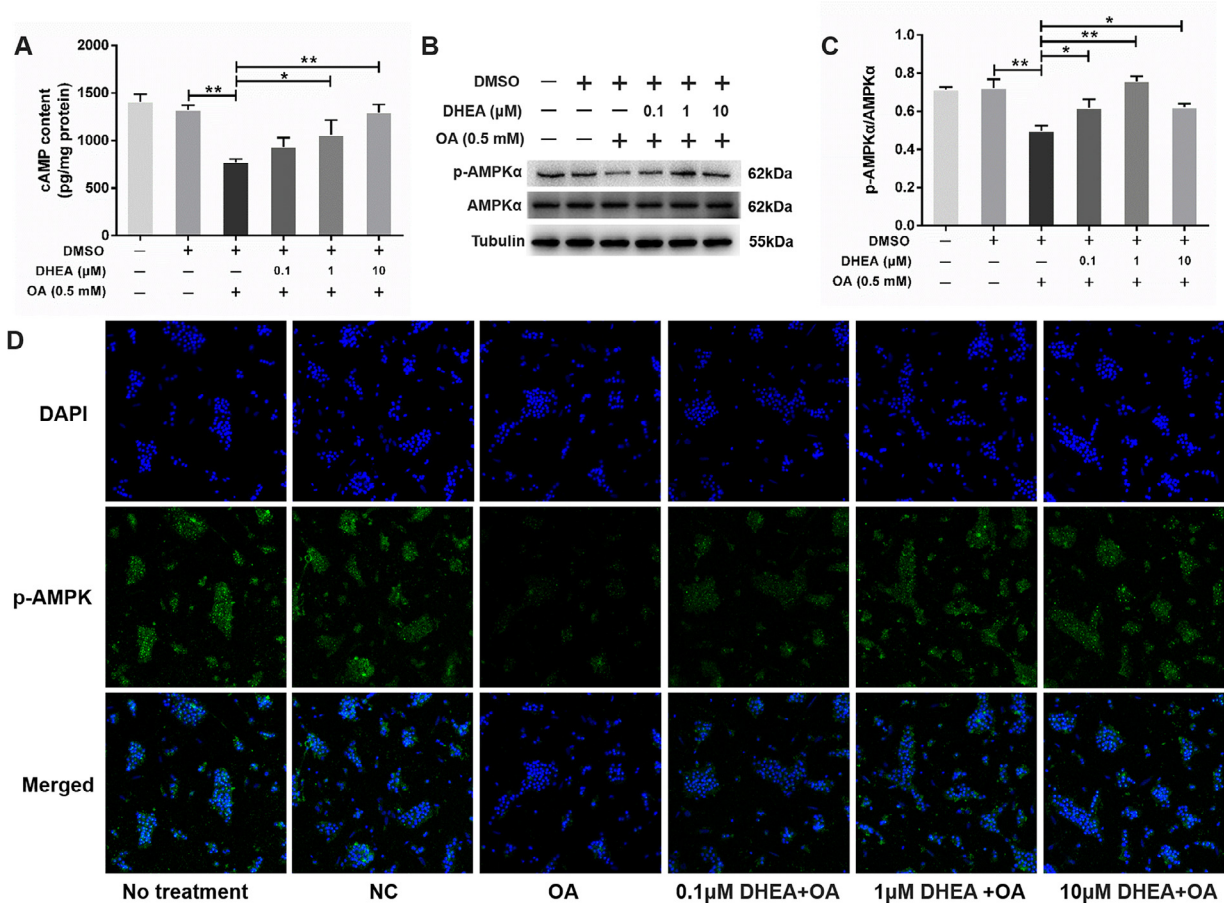

Figure 4. Effect of DHEA on cAMP content and p-AMPK protein level in OA-stimulated hepatocytes. The primary chicken hepatocytes were treated with 0-10 μM DHEA for 4 h, and then added with 0 or 0.5 mM OA stimulation for another 24 h. A: cAMP content; B: Immunoblot of phospho (p)-AMPKα and AMPKα protein; C: p-AMPKα protein level; D: p-AMPK protein level was analyzed by Immunofluorescence. Tubulin was used as loading control. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

DHEA Relieves Lipid Metabolism Disorders by Activating AMPK-mTOR Signal Pathway in OA-Stimulated Hepatocytes

OA stimulation obviously increased p-mTOR protein level when compared with the control group ($P < 0.01$); and 0.1-10 μM DHEA treatment significantly blocked the increasing of p-mTOR protein level induced by OA stimulation in hepatocytes ($P < 0.05$) (Figure 7A−B). In addition, DHEA or AICAR treatment markedly decreased the p-mTOR in OA-stimulated hepatocytes ($P < 0.01$), and this effect also was markedly reversed in cells pretreated with compound C ($P < 0.01$) (Figure 7C-7D). These results indicated that DHEA inhibited mTOR signal activation mediated by activating AMPK in OA-stimulated hepatocytes.

For further investigate the role of mTOR in alleviating lipid metabolism disorders by DHEA treatment, the hepatocytes were pretreated with mTOR inhibitor RAPA before addition of DHEA treatment. The results showed that DHEA treatment decreased the p-mTOR protein level, and this inhibition effect was obviously reinforced in OA-stimulated hepatocytes with RAPA and DHEA synergetic treatment ($P < 0.01$) (Figure 7E−G). Importantly, DHEA or RAPA and DHEA synergetic treatment obviously reduced the upregulation of SREBP-1 protein levels and lipid droplet accumulation caused by OA stimulation in hepatocytes ($P < 0.05$) (Figure 7H−J). These results demonstrated that DHEA alleviates lipid metabolism disorders caused by OA stimulation through activating AMPK and which subsequently inhibiting mTOR signal in hepatocytes.

DISCUSSION

Chicken has become one of the main meat products for the daily diet of people due to it with the characteristics of high protein content and low fat (Lin et al., 2021). Over the past decades, genetic breeding and nutrition manipulation have significant raised the production performance of chickens, while excessive pursuit of the growth rate also led the dramatic increasing of metabolism disorders in broiler (Griffin and Goddard, 1994). Metabolism disorders, especially the disturbance of lipid metabolism, not only reduces the carcass quality and feed utilization, but also increases the incidence of metabolic diseases in poultry (Mirderikvandi et al., 2019). In addition, the incidence of obesity, diabetes, atherosclerosis and other cardiovascular diseases will increase in people who overconsumed of low-quality meat products (Wan et al., 2016); and

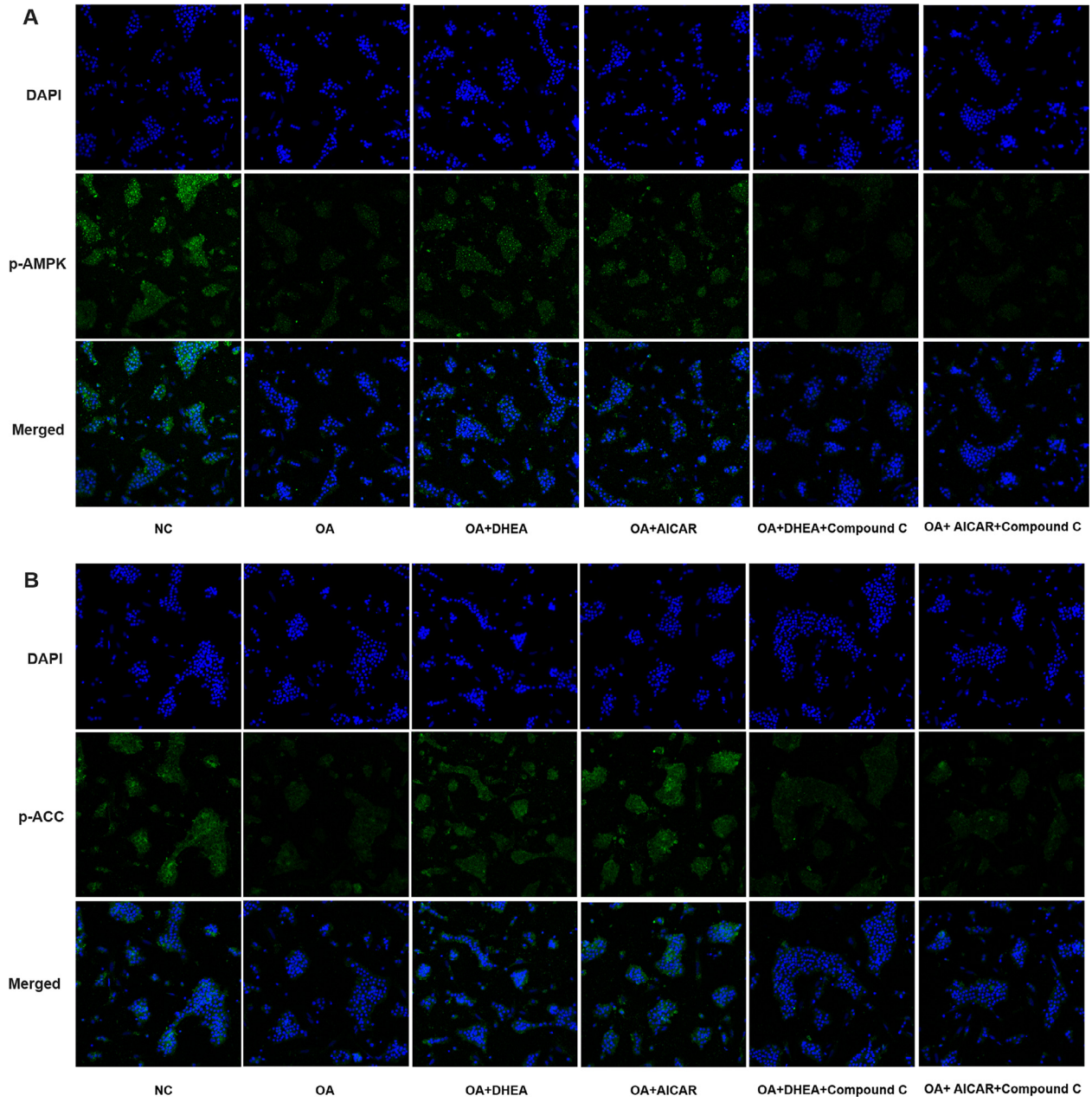

Figure 5. DHEA increases p-ACC protein level through activation of AMPK in OA-stimulated hepatocytes. The primary chicken hepatocytes were pretreated with 10 μM AMPK inhibitor compound C for 1 h, followed by treatment with 10 μM DHEA or 1mM AMPK activator AICAR for 4 h; after that, the cells were added with 0 or 0.5 mM OA stimulation for another 24 h. A: p-AMPK protein level was analyzed by immunofluorescence; B: p-ACC protein level was analyzed by immunofluorescence.

which attracted the consumers pay more attention to the livestock and poultry meat quality. Moreover, imbalance of nutrients caused the liver steatosis, which presents as in a large amount of lipid accumulation and liver dysfunction, had seriously threatened the health of people (Guo et al., 2021). Therefore, there has a great theoretical significance and application value to reveal the effect and mechanism of physiological regulators in controlling the lipid metabolism disorders in poultry and humans.

It reported that excessive free fatty acids including oleic acid (**OA**) or palmitic acid (**PA**) can induce the lipid metabolism disorder in cell and even reliably reproduce the key features of liver steatosis (Zhang et al., 2021). Our recently study also showed that 0.6 mM OA-stimulated treatment for 24 h had caused the steatosis and inflammation response in primary chicken hepatocytes (Li et al., 2020). In the current study, no significant differences were observed on the viability in hepatocytes by 0.5 mM OA-stimulated for 24 h; meanwhile, 0.5 mM OA stimulation markedly enhanced the TG content and lipid droplets accumulation, which indicated that 0.5 mM OA stimulation for 24 h can cause the lipid metabolism disorders in primary chicken hepatocytes. DHEA, a cholesterol-derived intermediate, has been approved by FDA as a multifunctional nutritional supplemental for improving the health of human (Chimote and Chimote, 2018). Previous study showed that DHEA can reduce the fat deposition in human or mice (Aoki and Terauchi, 2018). In addition, our recent

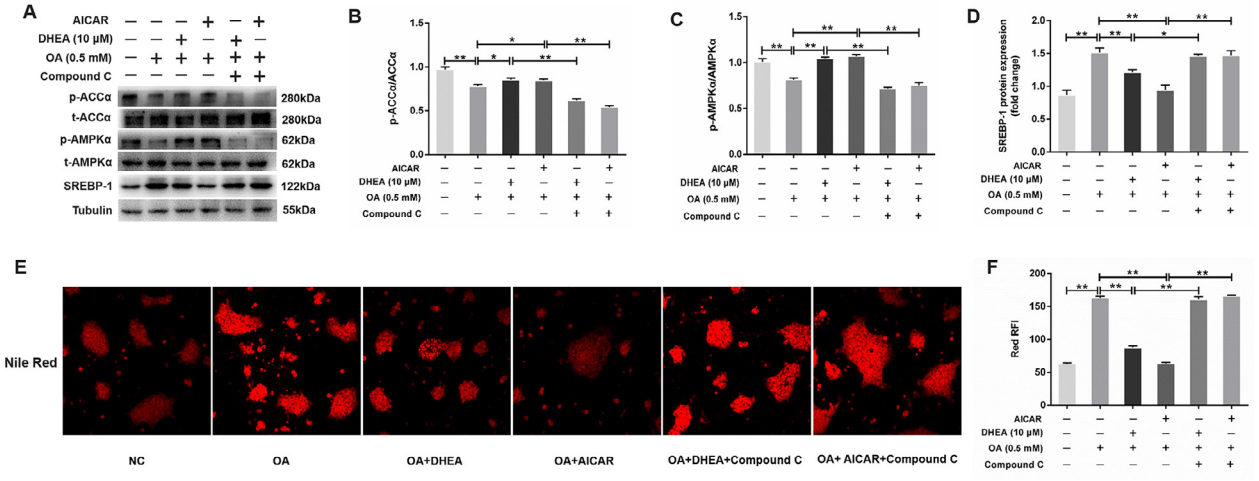

Figure 6. DHEA alleviates lipid droplet accumulation induced by OA stimulation through activating AMPK in hepatocytes. The primary chicken hepatocytes were pre-treated with 10 μM AMPK inhibitor compound C for 1 h, followed by treatment with 10 μM DHEA or 1mM AMPK activator AICAR for 4 h; after that, the cells were added with 0 or 0.5 mM OA stimulation for another 24 h. A: Immunoblot of phospho (p)-ACCα, p-AMPK$\alpha$and SREBP-1 protein; B: p-ACCα protein level; C: p-AMPKα protein level; D: SREBP-1 protein level; E: Representative photomicrographs of Nile red staining (200 ×); F: Relative fluorescence intensity (RFI) of Nile red staining. Tubulin was used as loading control. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

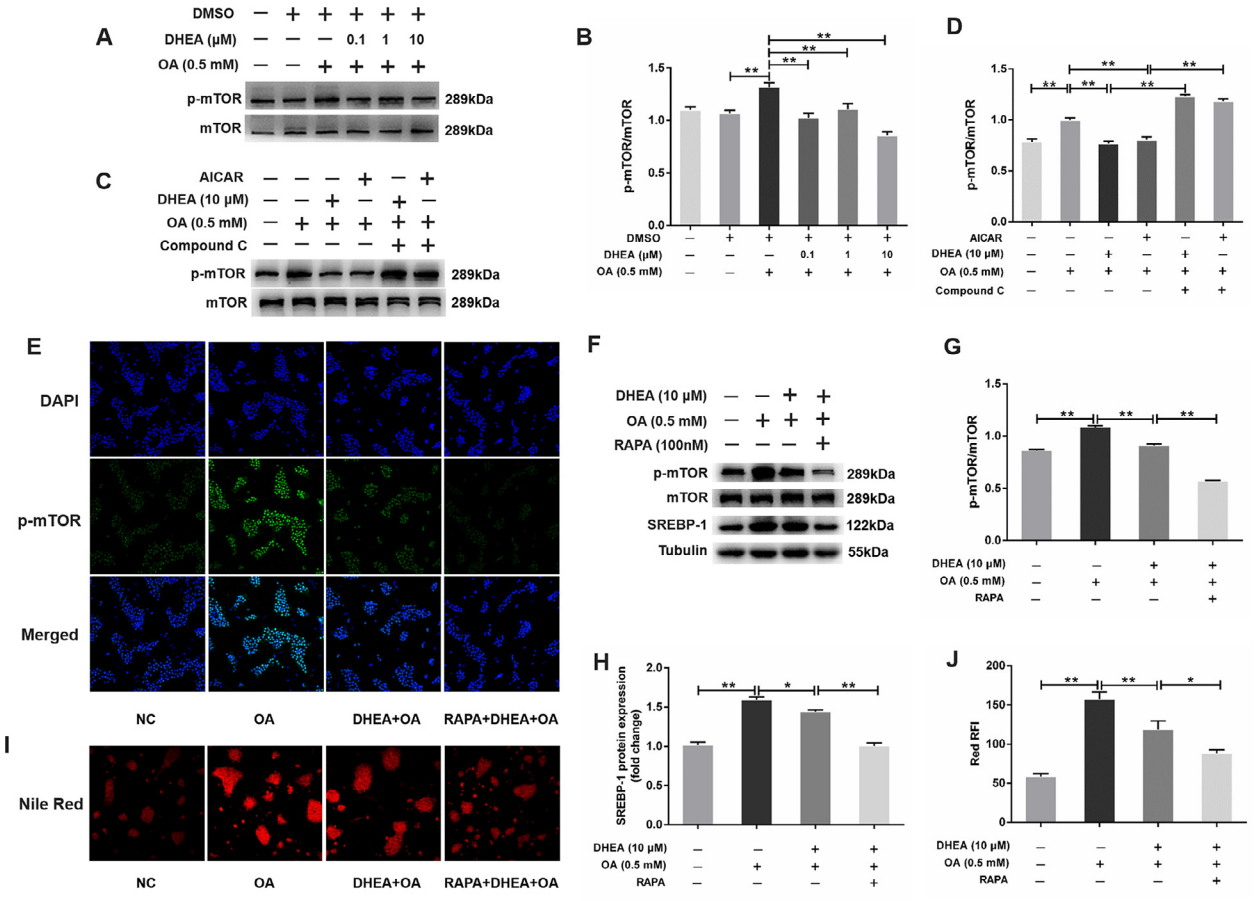

Figure 7. DHEA protects against lipid metabolism disorders triggered by OA stimulation through activation of AMPK-mTOR signaling in hepatocytes. The primary chicken hepatocytes were treated with 0 to 10 μM DHEA for 4 h, and then added with 0 or 0.5 mM OA stimulation for another 24 h. A: Immunoblot of phospho (p)-mTOR and mTOR; B: p-mTOR/mTOR. The primary chicken hepatocytes were pretreated with 10 μM AMPK inhibitor compound C for 1 h, followed by treatment with 10 μM DHEA or 1mM AMPK activator AICAR for 4 h; after that, the cells were added with 0 or 0.5 mM OA stimulation for another 24 h. C: Immunoblot of phospho (p)-mTOR and p-mTOR; D: p-mTOR/mTOR. The primary chicken hepatocytes were pre-treated with 100 nM mTOR inhibitor RAPA for 1 h, followed by treatment with 0 or 10 μM DHEA for 4 h; after that, the cells were added with 0 or 0.5 mM OA stimulation for another 24 h. E: p-mTOR protein level was analyzed by immunofluorescence; F: Immunoblot of p-mTOR and SREBP-1; G: p-mTOR/mTOR; H: SREBP-1 protein level; I: Representative photomicrographs of Nile red staining (200 ×); J: Relative fluorescence intensity (RFI) of Nile red staining. Tubulin was used as loading control. Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the indicated treatment groups.

study also found that DHEA markedly inhibited PA-induced the lipid metabolic disorder in BRL-3A cells (Li et al., 2020). In this study, DHEA treatment inhibited the enhancement of TG content and lipid droplets accumulation caused by OA stimulation in primary chicken hepatocytes, which implied that DHEA attenuates the lipid metabolism disorders caused by OA stimulation in hepatocytes.

Fat deposition is closely related to the dynamic balance of lipogenesis and lipolysis (Casanova et al., 2019), and which is controlled by the key factors of lipid metabolism. In hepatocytes, fatty acid synthesis is mediated by transcription factor SREBP-1 that regulate the genes expression involving into intracellular fatty acid and cholesterol synthesis (Eberlé et al., 2004). In this study, DHEA treatment obviously decreased the SREBP-1 protein level in OA-stimulated primary chicken hepatocytes. Previous study showed that SREBP-1 regulate the lipogenesis factors expression, such as ACC and FAS (Zhu et al., 2019). Present results showed that OA stimulation markedly increased lipogenesis related factors (ACC, FAS, and ACLY) mRNA level, and these enhancing effects induced by OA stimulation were dispelled in hepatocytes by DHEA treatment. Meanwhile, DHEA markedly decreased the FAS protein level and increased p-ACC protein level in OA-stimulated primary chicken hepatocytes, which implied that DHEA inhibits the rate-limiting enzyme expression of de novo synthesis of fatty acids in OA-stimulated hepatocytes. It reported that the inhibition of ACC activity can lead the reduction of intracellular malonyl-CoA levels, which then activates CPT-1 and ultimately increases the fatty acid oxidation (Yuan et al., 2018). As another nuclear transcription factor, PPARα acts an essential role in regulating lipolysis-related gene transcription (Diniz et al., 2021). In this study, we found that DHEA treatment significantly enhanced the PPARα and CPT-1 mRNA levels in OA-stimulated primary chicken hepatocytes. Taken the above results, our data demonstrated that DHEA alleviates lipid metabolism disorders caused by OA stimulation through inhibition of lipogenesis and enhancing of lipolysis in hepatocytes.

In this study, we found that OA stimulation significantly decreased the cAMP content and p-AMPK protein levels in hepatocytes; however, DHEA treatment obviously increased the cAMP content and p-AMPK protein levels in OA-stimulated hepatocytes, these results is similar with the reported that that DHEA can activate AMPK and then suppresses lipid accumulation in 3T3-L1 cells (Yokokawa et al., 2020). The cAMP system tightly controls the metabolism regulation and energy balance through phosphorylated AMPK and mTOR (Hardie et al., 2003). It well known that the activation of AMPK caused by the cAMP level elevation can inhibit lipogenesis factors expression and enhance lipolysis factors expression (Hardie et al., 2012). Our recently study certified that DHEA protects against hepatic lipid metabolic disorders by activation of AMPK-PGC-1α-NRF-1 signaling pathway in rats fed with high-fat diets (Li et al., 2020). These results implied that DHEA reactivates the cAMP-AMPK signaling pathway that had been inhibited in hepatocytes caused by OA stimulation. For further certified the protection effects of DHEA on lipid metabolism disorders whether is associated with the activation of AMPK, the hepatocytes were pretreatment with the AMPK inhibitor

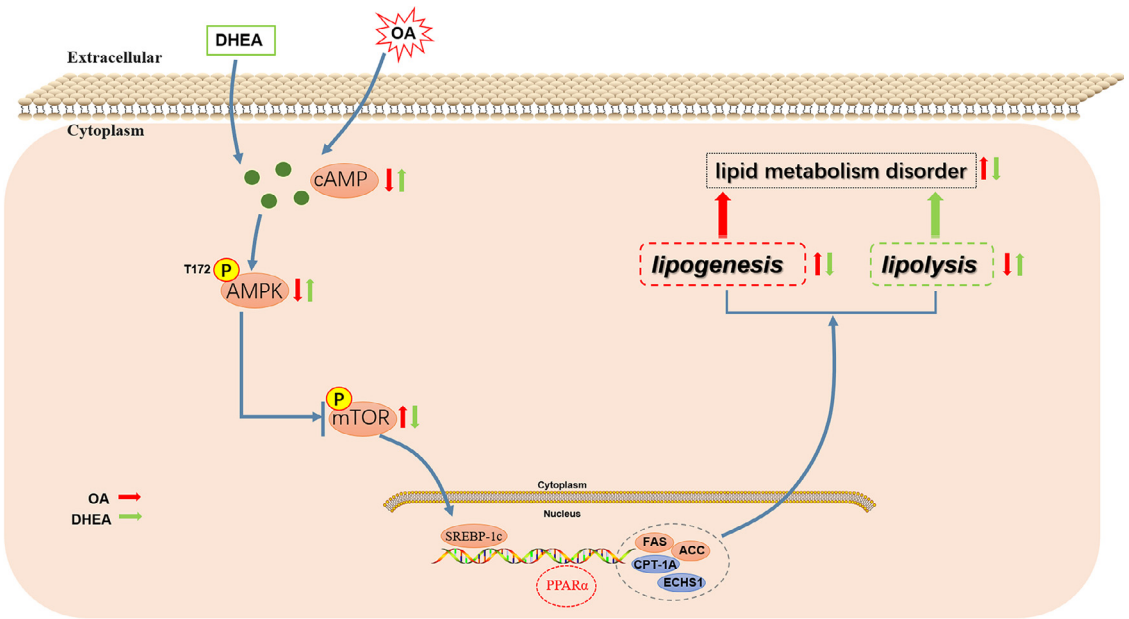

Figure 8. Schematic diagram of possible mechanism about DHEA attenuates lipid metabolism disorders triggered by OA stimulation in hepatocytes. DHEA improves the cAMP content and then improves the phosphorylated AMP-activated protein kinase (AMPK) protein level, and that subsequently blocks the mTOR activation induced by OA stimulation to regulate the lipid metabolism related factors expression in primary chicken hepatocytes. In brief, our data demonstrated that DHEA protects against the lipid metabolism disorder triggered by OA stimulation through activating the cAMP/AMPK-mTOR signaling pathway in hepatocytes, and these data prompts the value of DHEA as a potential nutritional supplement in regulating the lipid metabolism and its related disease in poultry and humans.

compound C or activator AICAR. We found that the increasing of p-AMPK, p-ACC protein levels and the decreasing of SREBP-1 protein level led by DHEA or AICAR treatment were reversed in OA-stimulated hepatocytes pretreatment with compound C. More importantly, the mitigative effect of DHEA on the fat excessive accumulation caused by OA was dispelled as the hepatocytes pretreated with compound C. Thus, the above data suggested that DHEA protects against lipid metabolism disorders caused by OA stimulation in hepatocytes through activation of AMPK signal.

Present study found that DHEA treatment obviously decreased the p-mTOR protein level in OA-stimulated hepatocytes, and this inhibition effect was dispelled when the hepatocytes were pretreated with compound C. The AMPK and mTOR, two interconnected factors, had been revealed to play crucial role in metabolism regulation (Kogut et al., 2016); and the biological activity of mTOR is significantly attenuated by phosphorylated AMPK (Maiuri et al., 2007). These results implied that DHEA inhibits mTOR signaling activation mediated by activating AMPK in OA-stimulated hepatocytes. Numerous studies have shown that the dysregulation of mTOR is closely related to obesity, diabetes and fatty liver disease (Saxton and Sabatini, 2017). It reported that the inhibition of mTOR can decrease the lipogenesis through reduction the SREBP-1 expression in liver and improve lipid metabolism disorders (Chakrabarti et al., 2010). Increased evidence showed that the mTOR-SREBP-1 signaling pathway plays a key role in regulating the hepatic cellular lipid metabolism (Liu et al., 2016). Thus, for further explore the role of mTOR in DHEA alleviating OA-stimulated the lipid metabolism disorders, the hepatocytes were pretreatment with mTOR inhibitor RAPA. We found that DHEA decreased the p-mTOR and SREBP-1 protein level in OA-stimulated hepatocytes, and the inhibition effect of DHEA on p-mTOR and SREBP-1 protein level obviously reinforced in OA-stimulated hepatocytes combined with RAPA treatment. Meanwhile, Nile red staining analysis also showed that the reduction effect of DHEA on massive production of lipid droplet was dramatically reinforced in OA-stimulated hepatocytes with RAPA and DHEA synergetic treatment. These results demonstrated that DHEA attenuates the lipid metabolism disorders triggered by OA stimulation through activating AMPK and which subsequently blocks the activation of mTOR in hepatocytes.

CONCLUSION

In conclusion, we demonstrated that DHEA protects against the lipid metabolism disorders triggered by OA stimulation through inhibiting lipogenesis factors expression and increasing lipolysis factors expression in hepatocytes, and these beneficial effects is achieved by activating the cAMP/AMPK-mTOR signaling pathway (Figure 8). These results revealed the molecular mechanism of DHEA against lipid metabolism disorders, and support it as a potential nutritional supplement in preventing metabolic-related diseases in poultry and even humans.

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DISCLOSURES

The authors declare that they have no competing interests.

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