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Dehydroepiandrosterone activates cyclic adenosine 3′,5′-monophosphate/protein kinase A signalling and suppresses sterol regulatory element-binding protein-1 expression in cultured primary chicken hepatocytes

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Dehydroepiandrosterone (DHEA), a steroid hormone that is secreted by the adrenal cortex in mammals, has an array of biological actions, including inhibition of fat synthesis, decreasing the number of adipocytes, and a reduction in mammalian metabolic efficiency. Recent studies showed that DHEA may decrease fat deposition in poultry, but the mechanism of action is unclear. In the present study, we demonstrate that DHEA stimulates intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation in chicken hepatocytes during a 30 min incubation period. Increases in intracellular cAMP are evoked by as low as 0·1 μM-DHEA. The cAMP induced by DHEA, while suppressing cAMP-specific phosphodiesterase activity, also activates cAMP-dependent protein kinase A (PKA) in chicken hepatocytes. In addition, the activation of PKA leads to down-regulation of sterol regulatory element-binding protein-1 (SREBP-1). These findings demonstrate that direct action by DHEA leads to activation of the cAMP/PKA signalling system in the modulation of lipid metabolism by repressing SREBP-1, thereby providing a novel explanation for some of the underlying effects proposed for DHEA in the prevention of fat deposition in poultry.

Dehydroepiandrosterone: Hepatic lipid metabolism: Lipogenesis: Cultured primary chicken hepatocytes

Cyclic adenosine 3′,5′-monophosphate (cAMP) is an integral constituent of the kinase cascade that links a number of extracellular signals to a variety of cellular functions. Under physiological conditions, the biosynthesis of lipids in the liver is negatively regulated, at least in part, by the elevated intracellular level of cAMP[1–3]. Glucagon, the adrenalines, and other reagents raise the cellular cAMP concentration level and reduce the activity or level of hepatic lipogenic enzymes[4,5] such as fatty acid synthase, stearoyl-CoA desaturase, and glycerol-3-phosphate acyltransferase. The cAMP-dependent kinase, protein kinase A (PKA), which is a major protein kinase activated by cAMP, has been shown to be involved in lipid metabolism as well[6,7]. Hepatic fatty acid synthesis and fatty acid synthesis in cultured hepatocytes, mediated by sterol regulatory element-binding protein (SREBP)-1, a transcription factor that regulates genes controlling intracellular lipid metabolism, have been shown to be negatively affected by cAMP[8]. It has also been demonstrated that cAMP inhibits insulin-regulated SREBP-1 mRNA levels and the expression of the SREBP-1-regulated genes involved in lipogenesis[9]. However, the molecular mechanism underlying this inhibitory function is still unclear.

Dehydroepiandrosterone (DHEA), the most abundant steroid hormone in circulation[10], is a major secretory product of the human adrenal gland. It has been shown to exert numerous beneficial effects, including anti-obesity, antidiabetic and anticarcinogenic actions in various animal models and in man[11]. Many reports[12,13] have described the effects of DHEA on lipid metabolism, including the induction of hepatic peroxisome proliferation, peroxisome β-oxidation enzymes and several other actions associated with fatty acid metabolism in rodents. Even though some research has been conducted on the regulation of DHEA on lipid metabolism in rats and mice[14,15], there has been a paucity of data generated on the effects of DHEA on lipid metabolism in broiler chickens. The oral administration of DHEA, a potential therapy for fat deposition reduction in humans, may be a practical way to modify carcass lipid deposition and reduce excessive carcass fat in the poultry industry. Therefore, further investigations in this area of research are warranted.

Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine 3′,5′-monophosphate; DHEA, dehydroepiandrosterone; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; LXR, liver X receptor; PDE, phosphodiesterase; PKA, protein kinase A; SREBP, sterol regulatory element-binding protein.

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Recent studies in our laboratory[16,17] and in others[18] have characterised DHEA as a fat-reducing agent in broiler chickens. Chickens fed DHEA exhibited decreased hepatic TAG synthesis and plasma transport, and accelerated liver lipid mobilisation through the regulation of the hepatic lipid metabolic pathways and the expression of the relevant genes involved. It was demonstrated that SREBP-1 mRNA expression was significantly lower than it was in the control group after DHEA treatment in broiler chickens during embryonic development[15]. Although these data suggest that DHEA may have a protective role against fat deposition in avian species, the mechanism underlying these beneficial effects is still unclear. Thus, our interest has focused on whether DHEA decreases SREBP-1 *in vitro* and, if so, how this is accomplished.

Therefore, a study was carried out to investigate whether DHEA activates the cAMP-dependent signalling system in cultured primary chicken hepatocytes and, subsequently, whether DHEA protects against fat deposition by activation of the cAMP/PKA pathway.

Experimental methods

Materials

DHEA, dimethyl sulfoxide, poly-L-lysine solution, 3-isobutyl-1-methylxanthine (IBMX), forskolin, penicillin, streptomycin and trypsin were purchased from Sigma (St Louis, MO, USA); transferrin, L-glutamine and HEPES were obtained from Amresco Inc. (Solon, OH, USA); M199 medium and fetal bovine serum (FBS) were from Gibco Laboratories (Grand Island, NY, USA); ATP and GTP were purchased from MBI Fermentas (Vilnius, Lithuania); the cell-permeable PKA inhibitor peptide PKI was obtained from Calbiochem (San Diego, CA, USA); pyruvate kinase, myokinase, phosphoenolpyruvate and protease inhibitor cocktail were from Merck Chemical Ltd (Darmstadt, Germany); H89 was purchased from Alexis Biochemicals (Lausen, Switzerland); a cAMP RIA kit was obtained from Beifang Biotechnology Corp. (Beijing, People's Republic of China); a PKA assay kit and cAMP were from Upstate Biotechnology (Lake Placid, NY, USA); SREBP-1 antibody (MA1-38 651) was purchased from ABR-Affinity BioReagents, Inc. (Golden, CO, USA); goat anti-mouse IgG (γ-chain specific, peroxidase conjugate) was from Boster Biological Technology, Ltd (Wuhan, People's Republic of China); nuclear and cytoplasmic protein extraction kit and a bicinchoninic acid (BCA) protein determination kit were obtained from Galen Biopharm International Co., Ltd (Beijing, People's Republic of China); protein assay kits were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, People's Republic of China); all other reagent details are indicated in the text.

Isolation of hepatocytes

Animal care and use protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. Fertilised avian chicken eggs were purchased from a commercial hatching factory and incubated at 38·5°C and at a relative humidity of 60 %. Primary culture of embryonic hepatocytes was conducted based on the method described by Kennedy *et al.*[19]. The embryos were killed by decapitation on incubation day 9, after being anaesthetised in cold PBS, and the livers were removed under sterile conditions. After washing in M199 medium, the livers were asceptically minced into small fragments (about 1 mm^3) by eye scissors and suspended in fresh medium for 1–2 min. Then the supernatant fraction was aspirated. Most erythrocytes can be removed after the above procedure has been repeated three to four times[20]. The liver tissues were incubated in PBS (Ca^{2+}- and Mg^{2+}-free) trypsin solution (0·25 mg/ml) in a vibrating water-bath (ninety cycles/min, 37°C) for 10–15 min. Meanwhile, the solution was pipetted frequently in order to facilitate cell dissociation, until it consisted of three to five cells in each aggregate. The hepatocytes were collected by centrifugation (100 ***g***, 5 min) and filtrated through a 150 μm mesh. The material was washed three times with fresh M199 medium, and the cell number was determined using a haemocytometer. Cell survival was determined by the trypan blue exclusion test and resulted in a survival rate of >90 %.

Primary culture of chicken hepatocytes

Hepatocytes were seeded in poly-L-lysine-coated six-well plastic culture plates (Nunc Company, Roskilde, Denmark) with a density of 10^6 per well in 2 ml M199 medium. Supplements were added using 10 % FBS, transferrin (5 μg/ml), 2 mM-L-glutamine and 1·75 mM-HEPES. The culture medium also contained penicillin (100 IU/ml) and streptomycin (100 μg/ml). Hepatocytes were incubated at 37°C in an atmosphere of 95 % air and 5 % CO_2. Following 24 h acclimatisation to the culture environment, cells were cultured for 24 h in phenol-red and FBS-free M199 medium.

Intracellular cyclic adenosine 3′,5′-monophosphate assay

The accumulation of cAMP in chicken hepatocytes under basal or stimulated conditions was determined using a RIA kit. After culturing for 24 h in phenol-red and FBS-free M199 medium at 37°C, cells were incubated with a dimethyl sulfoxide solution of DHEA (0·01 μM, 0·1 μM, 1 μM, 10 μM or 100 μM) for 20 min or with 0·1 μM-DHEA for varying lengths of time in phenol-red and FBS-free M199 medium at 37°C. Control cultures received an equal volume of dimethyl sulfoxide, which in any experiment did not exceed 0·25 % (v/v). After incubation, the supernatant fraction was rapidly removed, and the intracellular cAMP extraction and quantification were performed according to the manufacturer's instructions. Data were normalised to the protein concentration in samples as determined by a protein assay kit.

Adenylate cyclase and cyclic adenosine 3′,5′-monophosphate-specific phosphodiesterase assay

Chicken hepatocytes pre-incubated with 0·2 mM-IBMX or vehicle for 5 min, followed by the addition of 0·1 μM-DHEA, 20 μM-forskolin or vehicle for varied lengths of time at 37°C were tested for adenylate cyclase (AC) activity. Cell lysates isolated from hepatocytes exposed to 0·1 μM-DHEA, 0·2 mM-IBMX or vehicle were tested for phosphodiesterase (PDE) activity. The methods were as described previously[21]. One unit of AC enzyme activity represents 1 mg of tissue protein converting 1 pmol cAMP to ATP per min, and the

PDE activity was determined as the amount of cAMP hydrolysed during the reaction time.

Protein kinase A activity assay

Analysis of PKA activity was based on its ability to phosphorylate its specific substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), using the PKA assay kit. Briefly, chicken hepatocytes treated with 0·1 μM-DHEA or vehicle as described above were scraped and collected in PBS supplemented with protease and phosphatase inhibitor cocktail. Cytoplasmic and nuclear proteins were harvested by sonication and centrifugation. Cell lysate (200 μg) was added to a mixture of cAMP, Kemptide, protease inhibitor mixture and inhibitor peptide in the assay dilution buffer. The reaction was started by adding Mg/ATP mixture containing [γ-^{32}P]ATP (specific activity, 3448 counts per min/pmol). After incubating for 10 min at 30°C with constant shaking, samples were sampled onto P81 phosphocellulose squares and washed in 0·75 % phosphoric acid to stop the reaction. Furthermore, the squares were washed in acetone, added to scintillation vials containing Ecolite (ECN, Costa Mesa, CA, USA) and quantified on a Tri-Carb 2900TR Liquid Scintillation Counter (Packard, Meriden, CT, USA).

Western blot analysis

Chicken hepatocytes cultured in media as described above were exposed to 0·1 μM-DHEA, 20 μM-forskolin or vehicle for 1 h at 37°C. The cells were also exposed to 10 μM-H89, 2 μM-PKA inhibitor peptide (PKI) or vehicle for 30 min at 37°C. Then, 0·1 μM-DHEA was added, the cells were cultured for another 1 h, and scraped for the subsequent determination of protein levels. Nuclear extracts were prepared by using a nuclear and cytoplasmic protein extraction kit. Protein levels were measured using a bicinchoninic acid (BCA) protein determination kit. A quantity of 60 μg nuclear extract was separated on a 10 % SDS–polyacrylamide denaturing gel. Following SDS–PAGE, proteins were electrophoretically transferred to Immobilon™-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked at 25°C for 2 h in blocking buffer (2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-buffered saline (TBS; pH 7·6; Tris base 2·42 g/l, NaCl 8 g/l), 0·1 % Tween 20, 5 % non-fat dry milk). Proteins were then incubated at 4°C overnight with SREBP-1 antibody (MA1-38 651). After washing with TBS (pH 7·6) containing 0·1 % Tween 20, goat anti-mouse IgG peroxidase conjugate (1:4000) in washing solution was added and incubated for 60 min. The immunoreactive proteins were detected by superSignal chemiluminescence. The protein bands were digitally imaged for densitometric quantification with a software program (Eastman Kodak Company, Rochester, NY, USA).

Statistical analysis

Data were analysed with one-way ANOVA and expressed as mean values with their standard errors. Treatment differences were subjected to a Duncan's multiple comparison test. Differences were considered significant at $P<0·05$. All statistical analyses were performed with SPSS 11.0 for Windows (StatSoft, Inc., Tulsa, OK, USA).

Results

Dehydroepiandrosterone stimulates cyclic adenosine 3′,5′-monophosphate in cultured primary chicken hepatocytes

In the present study, we first determined the effect of DHEA on intracellular cAMP in chicken hepatocytes. Cells were incubated with a dimethyl sulfoxide solution of DHEA (0·01–100 μM) or vehicle for 20 min at 37°C. A marked increase in cAMP was found in 0·1–100 μM-DHEA-treated groups. A DHEA concentration of 0·1 μM caused the maximum stimulation of intracellular cAMP accumulation when compared with the control group (Fig. 1(a)). Thus, we used 0·1 μM-DHEA to culture chicken hepatocytes for various time periods in phenol-red and FBS-free M199 medium at 37°C. The levels of cAMP were significantly higher during the experimental period from 5 min to 30 min as compared with the control group, but then decreased, with no further marked increase after 60 min of DHEA incubation (Fig. 1(b)).

Adenylate cyclase and phosphodiesterase activity

To determine whether DHEA elevates intracellular cAMP through the stimulation of cAMP production and/or the inhibition of cAMP hydrolysis, the effect of 0·1 μM-DHEA on

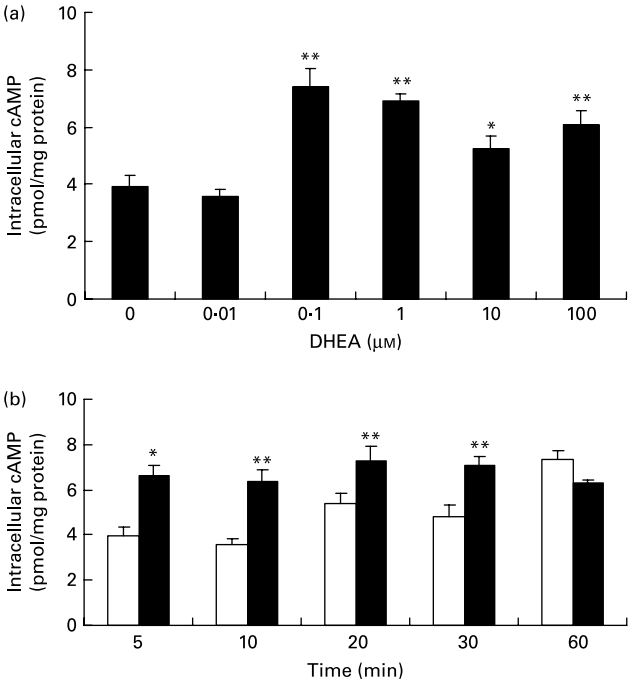

Fig. 1. Effect of dehydroepiandrosterone (DHEA) on intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation. Chicken hepatocytes were incubated in phenol-red and fetal bovine serum-free M199 medium with various concentrations of DHEA (0·01 μM to 100 μM) or vehicle (0 μM) for 20 min (a) or with 0·1 μM-DHEA for various periods of time (b) at 37°C. (□), Vehicle; (■), DHEA. Intracellular cAMP was extracted and measured by RIA and normalised to cellular protein. Values are means (n 6), with standard errors represented by vertical bars. Mean value was significantly different from that of the vehicle-treated group: *$P<0·05$, **$P<0·01$.

AC and PDE activity in chicken hepatocytes was tested. As can be seen in Fig. 2, no significant differences were observed in AC activity in both the DHEA-treated and the control groups at different time periods. As expected, a full stimulation was achieved at 20 μM-forskolin during the incubation period, a powerful agonist of AC activity. In contrast, DHEA markedly suppressed PDE activity at 10 min and 20 min as compared with the control group (Fig. 3). Moreover, the inhibitor IBMX was also potent in suppressing PDE activity for varied lengths of time (Fig. 3).

Dehydroepiandrosterone activates cyclic adenosine 3′,5′-monophosphate-dependent protein kinase A activity

cAMP is a direct activator of PKA, so we next investigated whether intracellular cAMP accumulation by DHEA is sufficient to activate PKA in chicken hepatocytes. As shown in Fig. 4, cells incubated with 0·1 μM-DHEA for 20 min or 30 min exhibited a pronounced increase in PKA activity as compared with control cells.

Sterol regulatory element-binding protein-1 protein analysis

To investigate whether the activation of PKA by DHEA induces a change in the level of SREBP-1 protein, we performed a Western blot analysis. As shown in Fig. 5(a), incubation of 0·1 μM-DHEA or 20 μM-forskolin for 1 h caused a significant decrease in the level of SREBP-1 protein. In contrast, no significant difference was found in cells pretreated with PKA inhibitors as compared with cells from the control group (Fig. 5(b)). The level of SREBP-1 protein detected in the DHEA + H89 and the DHEA + PKA inhibitor peptide (PKI) groups was not changed in the present study, indicating that the DHEA-decreased SREBP-1 protein level is mediated by PKA.

Discussion

DHEA has various biological functions in broiler chickens, including beneficial effects on lipid metabolism[16]. However, the cellular and molecular mechanisms of these effects are still unclear. In the present study, we demonstrated that DHEA

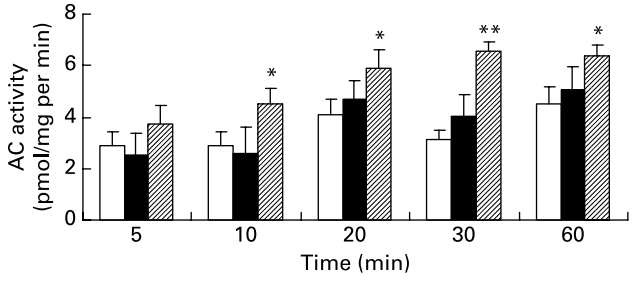

Fig. 2. Effect of dehydroepiandrosterone (DHEA) on adenylate cyclase (AC) activity. Chicken hepatocytes were pre-incubated with 0·2 mM-3-isobutyl-1-methylxanthine (IBMX) or vehicle for 5 min, then treated with 0·1 μM-DHEA (■), forskolin (▨; 20 μM) or vehicle (□) for various periods of time at 37°C. Cyclic adenosine 3′,5′-monophosphate (cAMP) was measured. Values are means (*n* 6), with standard errors represented by vertical bars. Mean value was significantly different from that of the vehicle-treated group: *$P<0·05$, **$P<0·01$.

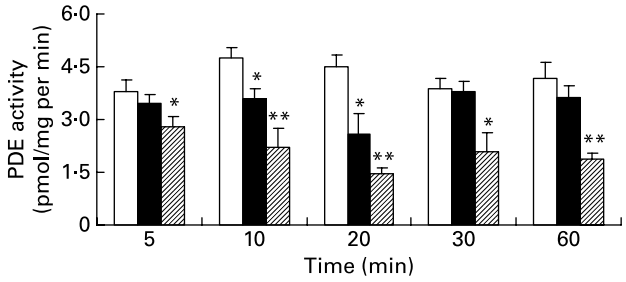

Fig. 3. Effect of dehydroepiandrosterone (DHEA) on cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase (PDE) activity. Cell extracts of chicken hepatocytes were exposed to 0·1 μM-DHEA (■), 0·2 mM-3-isobutyl-1-methylxanthine (IBMX; ▨) or vehicle (□) at 37°C for various periods of time. cAMP in cell extracts was determined. PDE activity was expressed as the rate of cAMP hydrolysis. Values are means (*n* 6), with standard errors represented by vertical bars. Mean value was significantly different from that of the vehicle-treated group: *$P<0·05$, **$P<0·01$.

targets the cAMP signalling pathway in chicken hepatocytes. DHEA directly inhibits the activity of PDE and leads to a marked increase in intracellular cAMP levels. Then, the accumulation of cAMP stimulates PKA activity, which subsequently decreases the level of SREBP-1 protein. Our findings demonstrate a novel signal pathway of DHEA action in chicken hepatocytes and provide a possible explanation for the regulation of lipid metabolism by DHEA in broiler chickens.

In the present study, cultured chicken hepatocytes were treated with a serial dilution of DHEA (0·01 μM to 100 μM) for 20 min. A rapid increase in cAMP accumulation was seen in the groups that ranged in concentration from 0·1 μM to 100 μM, and maximal cAMP accumulation was evident in the 0·1 μM-DHEA-treated group. Based on these data, we used 0·1 μM-DHEA to culture cells for various time periods at 37°C. We also found that DHEA-stimulated cAMP is significantly increased from 5 min to 30 min. Therefore, it is important to note that DHEA enhanced cAMP accumulation in a time-dependent manner in chicken hepatocytes.

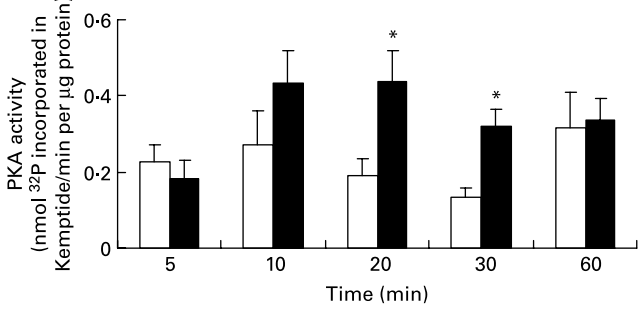

Fig. 4. Effect of dehydroepiandrosterone (DHEA) on protein kinase A (PKA) activity. Chicken hepatocytes incubated for 24 h in phenol-red and fetal bovine serum-free M199 medium were exposed for various periods of time with 0·1 μM-DHEA (■) or vehicle (□) at 37°C. Cell lysates (200 μg) were used to assess the activity of PKA, based on its ability to phosphorylate its specific substrate, Kemptide, using the PKA assay kit (Upstate Biotechnology, Lake Placid, NY, USA). PKA activity was expressed as nmol ^{32}P incorporated in Kemptide/min per μg protein. Values are means (*n* 6), with standard errors represented by vertical bars. * Mean value was significantly different from that of the vehicle-treated group ($P<0·05$).

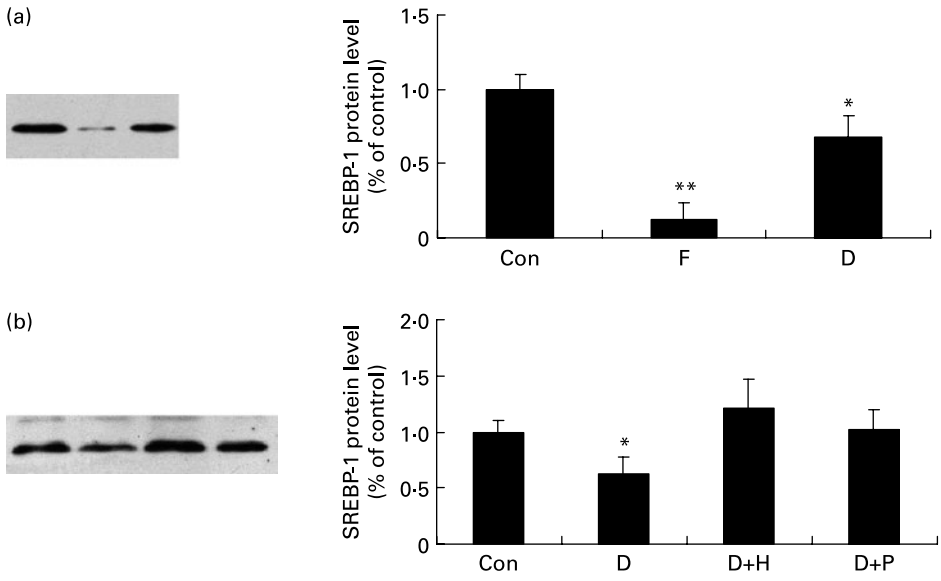

Fig. 5. Effect of dehydroepiandrosterone (DHEA) on protein kinase A (PKA)-mediated sterol regulatory element-binding protein-1 (SREBP-1) protein levels. (a) Chicken hepatocytes were treated with DHEA (D; 0·1 μM), forskolin (F; 20 μM) or vehicle (Con) for 1 h at 37°C. (b) Hepatocytes were pre-incubated with H89 (H; 10 μM), PKA inhibitor peptide (PKI) (P; 2 μM) or vehicle (Con) for 30 min before the addition of 0·1 μM-DHEA for 1 h. SREBP-1 protein levels were detected by Western blot analysis using a SREBP-1 antibody. Results are expressed as a ratio of the level measured in the control group. Representative Western blots are shown. Results are the mean of at least three separate Western blots. Values are means (*n* 4), with standard errors represented by vertical bars. Mean value was significantly different from that of the vehicle-treated group: $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$.

The normal circulating concentration of DHEA in human adult plasma has been shown to vary from 0·01 to 0·07 μM[22]. In a recent study from our laboratory, we found that the 10 μM- or 100 μM-DHEA treatment of chicken hepatocytes caused cellular activation of peroxisomal and fatty acid β-oxidation (X Tang, HT Ma, GQ Huang, JF Miao and SX Zou, unpublished results). Thus, these results may be applicable to the human research model, since the concentrations of DHEA used in the present study (0·01–100 μM) are consistent with human adult physiological plasma levels.

The intracellular second-messenger cAMP acts as a mediator of multiple hormonal signals and is synthesised by hormone-activated AC and degraded by PDE[23,24]. In the present study, 0·1 μM-DHEA had no significant effect on AC activity during the experimental period from 5 min to 60 min. In contrast, a marked suppression of PDE activity was observed at 10 min and 20 min. These results suggest that enhanced cAMP accumulation by DHEA may be primarily attributable to inhibited PDE. It is reported that PDE effectively controls the intracellular cAMP level and consequently plays an important role in the regulation of cell metabolism. The ability to regulate PDE activity can therefore be expected to provide a powerful way to manipulate the magnitude and duration of the cAMP response[25]. However, the mechanism of DHEA suppression of PDE activity is unclear. According to previous studies, various hormones, including glucagon[26], growth hormone[27] and the thyroid hormones[28–30] have been shown to regulate, in intact rats, high-affinity cAMP–PDE activity in liver particulate fractions, and the stimulation of PDE seemed to be most probably realised by phosphorylation. Therefore, we speculate that DHEA, a steroid hormone, may affect the inhibition of PDE phosphorylation in chicken hepatocytes.

SREBP genes, the transcription factors of the leucine zipper family, have been described as regulators of biosynthesis of cholesterol and fatty acids in the liver[31]. As in mammals, SREBP-1 is preferentially involved in the activation of genes that control the synthesis of fatty acids in chickens[32]. The previous study reported that glucagon and its signal mediator, cAMP, suppresses SREBP-1 in rat primary hepatocytes[33]. PKA, a cAMP-dependent protein kinase, is classically recognised as a fasting signal to activate β-oxidation and to oppose TAG synthesis, although it regulates cellular function in eukaryotic cells by suppressing SREBP-1[34]. As shown in Fig. 4 and in Fig. 5, DHEA treatment caused a significant up-regulation of PKA activity at 20 min or 30 min, and then subsequently decreased the SREBP-1 protein level in cultured chicken hepatocytes. Our finding indicated that DHEA had a significant negative effect on SREBP-1, mediated by PKA in response to elevated cAMP. This was in accordance with a recent report that found that SREBP-1 was suppressed by PKA in C57BL6/J mice liver after the treatment of PKA activators[35]. Many studies have claimed that liver X receptors (LXR) belong to a nuclear receptor superfamily and are the primary activators of the SREBP-1 promoter[36,37]. The cAMP/PKA signal could at least partially explain fasting suppression of SREBP-1 through repression of LXR activity[35]. Few studies have reported the effects of DHEA on the cAMP/PKA signal pathway in poultry. Therefore, given the present results and those from previous studies, we speculate that DHEA can act via a cAMP cascade to up-regulate PKA activity that directly phosphorylates LXRα protein and inhibits its signalling, resulting in suppression of SREBP-1 *in vitro*. However, further study is needed to more precisely validate this hypothesis.

In summary, using an *in vitro* model for the study of fat deposition reduction in poultry, we found that DHEA reduced the protein level of SREBP-1 induced by the cAMP/PKA signal pathway in chicken hepatocytes. This DHEA effect

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was associated with an elevation of intracellular cAMP concentration and blocked by the inhibition of PKA. This initial demonstration of a novel cAMP/PKA-mediated anti-obesity effect of DHEA on chicken hepatocytes suggests that reduction of the protein level of SREBP-1 might be mechanistically at least in part through PKA phosphorylation in LXR. On the basis of results from the present study, it may be speculated that DHEA is a novel anti-obesity agent for the prevention of fat deposition reduction in poultry; however, this supposition requires further investigation.

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X. T. collected the data and wrote the manuscript. S. Z. and H. M. designed the study. We thank S. Z., H. M., Z. S. and C. L. for interviewing the participants and X. X. for the PKA activity assay when necessary. We are also grateful to Dr William W. Riley, Technical Consultant, International Division, Hinapharm Pharmaceutical Co., Ltd (Foshan, People's Republic of China) for his critical reading of the manuscript. All authors contributed to the manuscript.

None of the authors had conflicts of interest.

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