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Research paper

Prenatal betaine exposure alleviates corticosterone-induced inhibition of CYP27A1 expression in the liver of juvenile chickens associated with its promoter DNA methylation

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ABSTRACT

Sterol 27-hydroxylase (CYP27A1) plays an important role in cholesterol homeostasis by degrading cholesterol to bile acids. Betaine can alleviate high-fat diet-induced hepatic cholesterol accumulation and maternal betaine treatment programs the hepatic expression of *CYP27A1* in offspring. Excessive corticosterone (CORT) exposure causes hepatic cholesterol deposition in chickens, yet it remains unknown whether prenatal betaine modulates CORT-induced cholesterol accumulation in chicken liver later in life and whether it involves epigenetic gene regulation of *CYP27A1*. In this study, fertilized eggs were injected with saline or betaine at 2.5 mg/egg before incubation, and the hatchlings were raised under the same condition till 56 days of age followed by 7 days of subcutaneous CORT injection. Plasma concentrations of total cholesterol (Tch), HDL- and LDL-cholesterol were significantly increased ($P < 0.05$), after CORT challenge, in both control and betaine groups. However, prenatal betaine exposure prevented CORT-induced increase ($P < 0.05$) in hepatic Tch content. Hepatic expression of cholesterol biosynthesis genes and ACAT1 protein that esterifies cholesterol for storage, were activated in both control and betaine groups upon CORT challenge. However, betaine-treated chickens were protected from CORT-induced repression ($P < 0.05$) in *LXR* and *CYP27A1* expression in the liver. CORT-induced down-regulation of *LXR* and *CYP27A1* coincided with significantly increased ($P < 0.05$) CpG methylation on their promoters, which was significantly ameliorated in betaine-treated chickens. These results suggest that *in ovo* betaine injection alleviates CORT-induced hepatic cholesterol deposition most probably through epigenetic regulation of *CYP27A1* and *LXR* genes in juvenile chickens.

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1. Introduction

Excessive glucocorticoid (GC) exposure is reported to cause metabolic syndrome such as non-alcoholic fatty liver disease (NAFLD) both in mammals and birds (Liu et al., 2016; Morgan et al., 2014; Woods et al., 2015). Cholesterol metabolism disorder is a common feature of NAFLD (Arguello et al., 2015; Ioannou, 2016; Musso et al., 2013). Corticosterone (CORT) is the main active form of GC and also a reliable indicator of stress in the chicken (Rettenbacher et al., 2013). Exogenous CORT administration induces abnormal accumulation of cholesterol in the liver, which drives the progression of liver diseases (Liu et al., 2016).

Cholesterol is an essential biological component of cell membranes that regulates membrane fluidity (Adlakha and Saini, 2016; Nemes et al., 2016). The liver is a key organ involved in the regulation of cholesterol metabolism through highly coordinated and strictly regulated biological processes including cholesterol biosynthesis, transportation and transformation (Faust and Kovacs, 2014; Woollett, 2005). Cholesterol degradation plays a key role in hepatic cholesterol accumulation and pathogenesis of NAFLD (Arguello et al., 2015; Min et al., 2012). Cholesterol-27alpha-hydroxylase (CYP27A1) is required for biosynthesis of bile acid from cholesterol and participates in the degradation of cholesterol in the liver (Duan et al., 2014; Monte et al., 2009). The tran-

Abbreviations: SREBP, sterol regulator element-binding protein; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CYP7A1, cholesterol-7alpha-hydroxylase; CYP27A1, cholesterol-27alpha-hydroxylase; ACAT1, acetyl-CoA cholesterol acyltransferase 1; LDLR, low density lipoprotein receptor; MeDIP, methylated DNA immunoprecipitation; Tch, total cholesterol.

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scription factor liver X receptor (LXR) is a nuclear receptor that can activate the expression of *CYP27A1* (Gilardi et al., 2009). CORT is one of the key factors controlling cholesterol homeostasis primarily through binding and activating its intracellular glucocorticoid receptor (GR) (Duan et al., 2014; Liu et al., 2016). GR, a nuclear transcription factor, can bind to the *CYP27A1* gene promoter and increase its enzyme activity in human HepG2 cells (Tang et al., 2008). However, the mechanism of *CYP27A1* in CORT-induced hepatic cholesterol deposition in chickens is still unclear.

Betaine is necessary for the one-carbon metabolic pathway, which is essential for the epigenetic gene regulation through DNA methylation and histone modifications (Anderson et al., 2012; Lever and Slow, 2010). Also, betaine is used as a feed additive to improve carcass characteristics and meat quality in livestock animals (Eklund et al., 2005) and broiler chickens (Alirezaei et al., 2012; Sayed and Downing, 2011). Recently, we reported that prenatal betaine can program *CYP27A1* expression in neonatal piglets (Cai et al., 2014) and increase hepatic cholesterol accumulation in newly hatched chicks (Hu et al., 2015), through epigenetic modifications. However, it remains unknown whether such changes in newly-hatched chicks may lead to long-term modification of cholesterol metabolism later in life.

Chickens raised in intensive farming system are exposed to multiple stressors. Stress-induced fatty liver is a common metabolic disease that harms chicken health and causes economic losses in poultry industry. Betaine supplementation can alleviate high-fat diet-induced hepatic cholesterol accumulation in rats (Zhang et al., 2013). Hence questions arise whether prenatal betaine exposure can protect the chicken from stress-induced cholesterol accumulation in the liver later in life, and whether such effect, if any, involves epigenetic gene regulation of *CYP27A1*.

Therefore, here we use CORT treatment, a widely used stress model in birds (Lin et al., 2004; Luo et al., 2013), to investigate whether *in ovo* betaine injection could alleviate CORT-induced hepatic cholesterol accumulation in juvenile chickens, and how such effects, if any, are related to epigenetic regulation of cholesterol degradation gene *CYP27A1*.

2. Materials and methods

2.1. Ethics statement

The experimental protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University, with the project number 31672512. The sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

2.2. Animals and treatment

Fertilized eggs (42.46 ± 0.21 g, ranging from 39.00 g to 44.42 g) laid by Rugao yellow breeder hens were obtained from Poultry Institute of Yangzhou, Jiangsu, China. Before incubation, eggs were randomly divided into control (C) and betaine (B) groups, and were injected with 100 μL of saline or betaine (2.5 mg per egg, B2629, Sigma-Aldrich, USA), respectively, as previously described (Hu et al., 2015). In brief, eggs were injected by advancing a Hamilton syringe into a hole in the middle of the long axis until the yolk membrane was penetrated (approximately 20 mm below the surface). Chicks were hatched inside the incubator and were left to dry completely (up to 12 h) before they were removed. No obvious differences in hatchability or hatching time were observed between two groups. One-day-old chicks were individually weighed, wing labeled and raised according to the standard recommended by the breeder. Chicks were subjected to continuous illumination and the temperature was controlled in the range of 35–37 °C during the first week, and reduced approximately 3 °C per week until 21 °C. The final body weight of the chicken was about 0.6 kg. The breeding density was about 5 kg/m^2. The relative humidity was maintained at 40–60%, and the lighting, ventilation, as well as the feeding and management procedures complied with the Feeding Management Regulations of Ru-gao Chickens. At 56 days of age, 24 male chickens were selected from both C and B groups and chickens in each group were divided to two subgroups, being subjected to either vehicle (CON) or corticosterone (CORT) treatment with daily subcutaneous injection of solvent (15% ethanol) or corticosterone (C2505, Sigma-Aldrich, USA) in a dose of 4.0 mg/kg body mass for 7 days (twice per day, 9:00–10:30 and 21:00–22:30). The dose was determined according to previous publication (Luo et al., 2013). Growth performance was recorded weekly from hatching to 63 days of age. At 64 days of age, all the chickens in 4 groups (12 chickens in each group), C-CON, C-CORT, B-CON and B-CORT, were weighed and killed by rapid decapitation which is considered to be acceptable for euthanasia of birds according to American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition. Blood samples were taken and plasma samples were separated and stored at −20 °C. Liver (without the gall bladder) samples were rapidly frozen in liquid nitrogen and keep at −80 °C for further analysis.

2.3. Determination of cholesterol content in liver and plasma

Cholesterol in plasma and liver was measured by using respective commercial cholesterol assay kit (E1005 and E1015) purchased from Applygen Technologies Inc., China, following the manufacturer's instructions.

2.4. Total RNA isolation and real-time PCR

Total RNA was isolated from 30 mg ground liver samples with TRIzol reagent (Invitrogen, USA) and then treated with RNase-free DNase and reverse-transcribed to cDNA using random hexamer primers (Promega, USA). Two microliters of diluted cDNA (1:25, vol/vol) was used for real-time PCR with a Mx3000P Real-Time PCR System (Stratagene, USA). All primers (Table 1) were synthesized by Generay Biotech (Shanghai, China). Several references

Table 1
Nucleotide sequences of specific primers.

Target genes (accession number)	Primer sequences (5′–3′)	PCR products (bp)
SREBP1 (AY029224)	F: CTACCGCTCATCCATCAACG R: CTGCTTCAGCTTCTGGTTGC	145
SREBP2 (XM_416222)	F: CCCAGAACAGCAAGCAAGG R: GCGAGGACAGGAAAGAGAGTG	108
HMGCR (NM_204485.1)	F: TTGGATAGAGGGAAGAGGGAAG R: CCATAGCAGAACCCACCAGA	137
CYP7A1 (AB109636.1)	F: CATTCTGTTGCCAGGTGATGTT R: GCTCTCTCTGTTTCCCGCTTT	106
CYP27A1 (XM422056.4)	F: AGGACTTTCGTCTGGCTCT R: CTCCGCATCGGGTATTT	185
18S rRNA (DQ018752.1)	F: ATAACGAACGAGACTCTGGCA R: CGGACATCTAAGGGCATCACA	136
CYP27A1 promoter	F: GACACTCGCCCCACCTGC R: CGCTGCTCCTCCGCTCAC	280
LXR promoter	F: GCAAGAGCCCAGCAAT R: CACAAACATAAAGGCATCA	255

SREBP 1 and 2, sterol regulatory element binding protein 1 and 2; *HMGCR*, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; *CYP7A1*, cholesterol-7-alpha hydroxylase; *CYP27A1*, sterol 27-hydroxylase; *LXR*, liver X receptor.

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