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Exogenous administration of chronic corticosterone affects hepatic cholesterol metabolism in broiler chickens showing long or short tonic immobility

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A B S T R A C T

Tonic immobility (TI) is an innate characteristic of animals related to fear or stress response. Animals can be classified into long TI (LTI) and short TI (STI) phenotypes based on TI test duration. In this study, effect of TI phenotype, chronic corticosterone administration (CORT), and their interaction on cholesterol metabolism in liver was evaluated in broilers. LTI broilers showed higher level of cholesterol in liver compared to STI chickens ($p < 0.05$), and CORT significantly increased hepatic cholesterol content ($p < 0.01$). Real-time PCR results showed that both TI and CORT potentially altered ABCA1 and CYP7A1 gene expressions ($0.05 < p < 0.1$), while there was no significant interaction of CORT and TI on both gene expressions. CORT treatment significantly increased the level of SREBP2 ($p = 0.00$), LDLR ($p < 0.05$), GR ($p < 0.05$) and 11β-HSD2 ($p < 0.05$) protein abundance in liver. However, TI phenotype only affected hepatic HMGCR protein expression, and LTI broilers showed higher level of HMGCR protein expression in liver than STI ($p < 0.05$). These results indicate that chronic CORT administration causes hepatic cholesterol accumulation in broiler chickens mainly by enhancing cholesterol synthesis and uptake into liver. LTI chickens had higher amount of total cholesterol in liver, which might be associated with an increase of hepatic HMGCR protein expression. However, there is no interaction between TI and CORT on cholesterol metabolism in liver of broilers.

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1. Introduction

Tonic immobility (TI), an index to assess fearfulness and stress, is an innate response of animals. TI response in chicken is strong and can be clearly divided into long TI (LTI) and short TI (STI) phenotypes based on TI duration. It's generally accepted that LTI means higher sensitivity to fearfulness than STI phenotype (Reese et al., 1984), and that LTI duration and chronic stress negatively affect growth in vertebrates. The BW of LTI line birds is lower than that of STI duration birds (Minvielle et al., 2002). It is well known that broiler chickens are usually confront a multitude of stressors, including human handling disturbance and adverse housing environment (e.g., heat, cold, noise, crowding and wet litter). The responses against these stressors are associated with several serious welfare problems (Hocking et al., 2005; Meluzzi et al., 2008).

In birds, hypothalamic–pituitary–adrenal (HPA) axis shown different responsiveness in two TI phenotypes (Hazard et al., 2005; Hazard et al., 2007). It is well documented that HPA axis controls stress response under endogenous or exogenous stimuli. In birds, fright and anxiety can stimulate HPA axis, then induce the secretion of stress hormone corticosterone (CORT) from the adrenal cortex. CORT is one of the key factors controlling body homeostasis mainly through its specific receptor, glucocorticoid receptor (GR) (Goodwin et al., 2013). As a nuclear hormone receptor, GR is widely distributed in most of cells and organs, which plays key roles in both embryonic development and adult homeostasis. Metabolic enzymes such as 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) control the balance of active CORT concentrations in the circulating system and different tissues in vivo. It's well known that 11β-HSD1 activates, while 11β-HSD2 inactivates glucocorticoids (GCs) (Tomlinson et al., 2004; Holmes and Seckl, 2006). It's reported that CORT effects on TI duration, and microinjection of CORT into brain or blood can increase TI duration in rats (Zamudio et al., 2009; Sandoval-Herrera et al., 2011). Our previous results showed that TI phenotype markedly affects the basal serum CORT concentration in broilers, and STI broilers showed higher level than LTI counterparts (Wang et al., 2013).

In humans, there is a significant association of cortisol excretion rate with HDL cholesterol (Fraser et al., 1999). And it was reported that chronic hydrocortisone administration in Wistar rats significantly

Abbreviations: 11β-HSD, 11β-hydroxyste-roid dehydrogenase; ABCA1, ATP-binding cassette sub-family A member 1; CORT, corticosterone; CYP7A1, cholesterol-7-alpha hydroxylase; CYP27A1, sterol 27-hydroxylase; DEX, dexamethasone; GC, glucocorticoid; GR, glucocorticoid receptor; HMGCR, 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase; HDL, high-density lipoprotein; LDLC, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LXRs, liver X receptors; SREBP, sterol regulatory element binding protein; TCH, total cholesterol; TG, total triglycerides; TI, tonic immobility.

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upgraded cholesterol content in liver (Petrovic et al., 1993). Similarly, Syrian hamster exposures to chronic glucocorticoid cause an increase of cholesterol in plasma (Solomon et al., 2011). In our previous study, we also found that chronic CORT administration causes cholesterol accumulation in pectoralis major of broiler chickens by increasing cholesterol synthesis and uptake (Duan et al., 2014). With respect to cholesterol metabolism, liver highly coordinates and strictly regulates the biological processes, including cholesterol biosynthesis, transformation and transportation, and keeps the maintenance of cholesterol homeostasis (Woollett, 2005; Faust and Kovacs, 2014). Sterol regulatory element binding protein 1 and 2 (SREBP1 and 2) are the key transcriptional factors to activate cholesterol biosynthetic gene expression such as the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (Khesht and Hassanabadi, 2012; Sharpe and Brown, 2013). On the other hand, cholesterol-7alpha-hydroxylase (CYP7A1) and cholesterol-27alpha-hydroxylase (CYP27A1) can decompound cholesterol into excretable bile acids (Monte et al., 2009), and CYP7A1 is the key factor involved in this acidic pathway. Moreover, ATP-binding cassette sub-family A member 1 (ABCA1) plays an important role in high-density lipoprotein (HDL) biogenesis and reverses cholesterol transport to liver for disposal (Mulligan et al., 2003; Levinson and Wagner, 2015). Low-density lipoprotein receptor (LDLR) is a kind of mosaic protein, anchoring on the cell surface and mediating lipoprotein to enter into hepatic cells for metabolism. However, liver X receptors (LXRs) are ligand activated transcription factors involved in promoting reverse cholesterol transport in vivo (Naik et al., 2006).

Liver is known to be the primary site of lipid metabolism in birds (O'Hea and Leveille, 1969). The information about the influence of TI and chronic CORT administration on hepatic cholesterol metabolism in birds is not available. The aim of this study was to investigate TI, chronic CORT exposure and their interaction on hepatic cholesterol metabolism in broilers. Our data will provide a better understanding for the relationship of stress and cholesterol metabolism as well as animal welfare.

2. Materials and methods

2.1. Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University. The sampling procedures followed the “Guidelines on Ethical Treatment of Experimental Animals” (2006) No. 398 set by the Ministry of Science and Technology, China and “the Regulation regarding the Management and Treatment of Experimental Animals” (2008) No. 45 set by the Jiangsu Provincial People's Government.

2.2. Animals and experimental procedure

Animal treatment and experimental procedure have been introduced in detailed in the previous publication (Wang et al., 2013). Simply, a total of 600 broiler breeder eggs (Ross 308), which came from a commercial breeding company, were incubated under standard conditions. 480 newly hatched chickens were wing-banded and housed in an environmentally controlled room following the feeding standard set by the breeding company. The temperature was maintained at 34 ± 3 °C for the first 3 d, and then decreased gradually to 21 ± 3 °C until 28 d of age. Standard commercial broiler starter crumble (12.5 MJ/kg; 21% CP) and finisher pellet (12.8 MJ/kg; 19.5% CP) were provided from 1 d to 20 d and 21 d to 42 d, respectively. Continuous lighting was conducted during the first week and the lighting time decreased by 2 h/week gradually to 18 L: 6D until 21 d and kept constant thereafter to 42 d. Water was available ad libitum.

To establish two segregated groups of STI and LTI phenotypes, all chickens were tested for TI twice on 10 d and 21 d, respectively. On the 41 d the 3rd TI test was performed for confirmation. The protocol followed was described previously (Jones et al., 1991). Briefly, chicken was carried individually to another room devoid of other birds. The chicken was placed on its back on the floor and restrained for 20 s (with one hand on the sternum and one lightly cupping the head of the chicken). The tester remained silent and virtually motionless in the room, out of the bird's sight. If more than 10 s elapsed until the bird righted itself, the duration of TI was recorded. If TI was not attained after 3 attempts, a score of 0 s was given. Conversely, if the bird failed to right itself after 10 min, the test was terminated and a maximum score of 600 s was given for tonic immobility duration. And on the 41 d the 3rd TI test was performed for confirmation. The TI test followed the protocol described in the previous publication (Wang et al., 2013). After 3 times TI test, we selected 80 chickens showing the shortest TI duration (29.6 ± 2.3 s) and 80 scoring the longest duration (246.2 ± 26.8 s), then they were classified into STI and LTI groups.

Chickens of STI and LTI groups were respectively allocated into control (CON) and CORT-treated subgroups to four 2 × 2.7 m^2 pens, chickens in CORT groups of both STI and LTI phenotypes (40 per phenotype) were supplied with water supplemented with 5 mg/L corticosterone (C2505, Sigma, USA) from 27 d to 42 d, whereas those in control groups (40 birds from LTI and 40 birds from STI) were supplied with water supplemented with equivalent volume of the solvent (absolute ethanol). Each broiler chicken consumed approximately 0.2 – 0.3 L water per day in average during the experimental period (27 d to 41 d). On 42 d, all chickens were sacrificed by decapitation, and liver samples were rapidly frozen in liquid nitrogen and then stored at −70 °C until further analysis.

2.3. Cholesterol contents in the liver

Total cholesterol (TCH) in liver was measured by commercial cholesterol assay kits purchased from Applygen Technologies Inc., China (E1005) and Beijing North of Fine Chemicals LLC, China (006301), respectively. In brief, approximately 50 mg of liver sample was homogenized in 1 mL ice-cold buffer RIPA by a Polytron homogenizer (PT1200E, Brinkman Instruments, Littau, Switzerland). 150 μL of homogenates was mixed with 600 μL mixture of chloroform/methanol (2:1, vol/vol), then vigorously shaken for 1 min and stood for 30 min, then centrifuged at 3000 *g* for 10 min. The bottom (chloroform) layer was removed, air-dried and reconstituted in 30 μL mixture of tert-butyl alcohol and methanol (13:2, vol/vol), followed by the TCH determination with an automatic amino acid analyzer (L-8900, Hitachi, Japan).

2.4. RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from 30 mg liver samples using 1 mL of TRIzol reagent (Invitrogen, USA). 2 μg of total RNA was treated with RNase-free DNase and reverse-transcribed to cDNA using the random hexamer primers (Promega, USA). 2 μL of diluted cDNA (1:20, vol/vol) was used for real-time PCR, which was performed with the Mx3000P Real-Time PCR System (Stratagene, USA). The technical variations were normalized using β-actin as an internal control. Primers for real-time PCR (Table 1) were synthesized by Generay Biotech (Shanghai, China). The method of $2^{-\triangle\triangle CT}$ was used to analyze the results and gene mRNA levels were expressed as the fold change relative to the mean value of the control group (Livak and Schmittgen, 2001).

2.5. Protein extracts and western blotting

The protocol extracting total protein has been described in the previous publication. Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). 40 μg of protein extract from each sample was then loaded onto 10% or 12% SDS-PAGE gels and the separated proteins were transferred onto the nitrocellulose membranes (BioTrace, Pall Co, USA). After transfer and blocking,

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