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Effect of Corticosterone on Hypothalamic Corticotropin-releasing Hormone Expression in Broiler Chicks (*Gallus gallus domesticus*) Fed a High Energy Diet

Zhigang Song, Lei Yuan, Hongchao Jiao and Hai Lin*

College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong 271018, China

ABSTRACT : This paper reports the peripheral and central effect of corticosterone on feed intake and hypothalamic corticotropin-releasing hormone (CRH) gene expression in chicks fed a high energy diet. Three experiments were conducted: corticosterone was supplemented to the feed (30 mg/kg diet), injected subcutaneously (s.c., 4 mg/kg body weight) or intracerebroventricularly (i.c.v., 4 ng). The results showed that dietary corticosterone significantly increased feed intake. The s.c. corticosterone administration increased feed intake within 1 to 3 h and at 1 to 5 h after the injection. The i.c.v. corticosterone administration increased feed intake within 1 h after the injection, but not at 1 to 3 h. Dietary supplementation and s.c. injection of corticosterone decreased the CRH gene expression in the hypothalamus. Therefore, peripheral corticosterone exerted a decreased effect on hypothalamic CRH mRNA levels, and corticosterone had a stimulating effect on feed intake in broiler chicks fed a high energy diet. (**Key Words :** Broiler Chicks, Corticosterone, Corticotropin-releasing Hormone, Feed Intake)

INTRODUCTION

Stressful situations cause an increase in the activity of the hypothalamo-pituitary-adrenal (HPA) axis (Vandenborne et al., 2005). Glucocorticoids (corticosterone in rats and poultry) are the end product of HPA axis activity and exert feedback effects at the pituitary level and in the hypothalamus (Sato et al., 1975). Corticotropin-releasing hormone (CRH) is the major feedback target of glucocorticoids at the hypothalamic level (Kretz et al., 1999).

Glucocorticoids have shown a "permissive" role in many rodent obesity syndromes (Sainsbury et al., 2001). However, contradictory observations exist regarding the effects of corticosterone on feed intake (Malheiros et al., 2003). Differences in dietary energy content might partially account for these discrepancies (Tannenbaum et al., 1997; Yuan et al., 2008). Chronic stress enhances the preference of rats for palatable or "comfort food" (lard+sucrose) (Bell et al., 2000; Pecoraro et al., 2004). Psychologically stressed women tend to consume more sweet food (sugar, which means high energy) (Epel et al., 2004). Chickens may be able to detect the metabolic changes caused by corticosterone and attempt to modify their food patterns (Covasa and Forbes, 1995). Stressed chickens prefer to consume a higher-energy (lipid) diet (Yuan et al., 2008).

Two key components regulate feed intake: one involves the short-term control of feeding, and the other involves the long-term energy balance regulation by the central nervous system (Richards, 2003). In avian, feed intake may be affected by the dynamics of the hypothalamic-adrenal axis (Denbow et al., 1999). Antoni (1986) described the paraventricular nucleus (PVN) CRH containing neurons as the primary initiators of the stress response organisms. CRH has been considered as the possible anorexigenic factor in mammals and birds (Richard, 1993; Richards, 2003).

Three experiments utilising a high-energy diet (HED) were conducted in the current research. Corticosterone was diet-supplemented or injected to mimic chronic or acute stress. The objective of this research was to investigate the effect of corticosterone on gene expression of CRH in broiler chicken's hypothalamus.

MATERIAL AND METHODS

Animals and diets

One-day old male broiler chicks (Arbor Acres) were

* Corresponding Author : Hai Lin. Tel: +86-538-8249203, Fax: +86-538-8241419, E-mail: hailin@sdau.edu.cn
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obtained from a local hatchery and reared in an environmentally controlled room. The brooding temperature was maintained at 35°C (50% relative humidity) for the first 2 d, and then decreased gradually to 30°C (50% relative humidity) and maintained until the chicks were 10 days old. HED (3,610 kcal ME/kg, 20% CP) was provided. In our previous research (Yuan et al., 2008), corticosterone significantly increased the feed intake of broiler chickens fed with the same HED, but not those with a normal energy feed.

In experiment 1 (Exp. 1), 36 of 72 3-day-old chicks (60.14±1.56 g; 6 pens, 12 per pen) were randomly assigned to corticosterone administration (30 mg/kg diet) (Malheiros et al., 2003; Lin et al., 2004; Lin et al., 2006), and the remainder were fed with the control HED. During the 7 d experimental period, feed intake was recorded daily. At the end of the experiment (10 d of age), chicks with similar body weights were selected in order to avoid the effect of body weight on plasma parameters and hypothalamus gene expression. Blood samples were obtained from the wing vein using heparinized syringes. Plasma was collected after centrifugation at 400×g for 10 min at 4°C and was stored at -20°C for further analysis. The hypothalamus was harvested and preserved in liquid nitrogen until molecular analysis.

In experiment 2 (Exp. 2), 24 of 48 3-day-old chicks (62.95±1.78 g; 12 cages, 4 per cage) were subcutaneously (s.c.) injected with corticosterone (4 mg/kg body weight) (Lin et al., 2004) and the remainder with saline. Feed was deprived for 1.5 h before the injection to synchronize the feed intake behaviour. Feed intake was recorded at 1, 3, and 5 h and plasma was collected at 3 and 5 h. One chicken from each cage (n = 6 for each group) was selected at random at 3 h after the injection and their hypothalami were harvested.

In experiment 3 (Exp. 3), 24 of 48 8-day-old broiler chicks (124.76±4.81 g) were intracerebroventricular (i.c.v.) injected (Davis et al., 1979) with corticosterone (2 μl, 2 ng/μl) and the other half with saline; feed was deprived for 1.5 h before the injection to synchronize the feed intake behaviour. Two chickens from each cage were selected at random at 1 h after the injection and their hypothalami were obtained. Proper injection of the corticosterone was verified by the administered Evans Blue dye in the injecta. Six verified hypothalamus for each group were carried through gene expression determination. The feed intake was recorded and plasma was collected from different chicks at 1 and 3 h after the injection.

Measurements

The concentrations of plasma glucose (No. F006), uric acid (No. C012), triglyceride (No. F001), and free fatty acids (FFA, No. A042) were measured spectrophotometrically with commercial colorimetric diagnostic kits (Jiancheng Bioengineering Institute, Nanjing, China).

Plasma insulin was measured by RIA with guinea pig anti-porcine insulin serum (3V Bio-engineering group Co., Weifang, People's Republic of China). In this measurement, ^{125}I-labelled porcine insulin competes with chicken insulin for sites on insulin-porcine antibodies immobilised to the wall of a polypropylene tube. A high level of cross-reaction has been observed between chicken insulin and guinea pig anti-porcine sera (Simon et al., 1974). The insulin in this study is referred to as immunoreactive insulin. The sensitivity of the assay was 1 m IU/ml, and all samples were included in the same assay to avoid interassay variability. The intra-assay coefficient of variation (CV) was 6.9%.

Gene expression was measured using real-time reverse transcription (RT)-PCR. Briefly, total RNA from hypothalamus was extracted using Trizol (Invitrogen). The quantity and quality of the isolated RNA were determined by biophotometer (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis, respectively, and RT was carried out using RT reactions (10 ml) consisting of 500 ng of total RNA, 5 mmol/L $MgCl_2$, 1 ml RT buffer, 1 mmol/L dNTP, 2.5 U avian myeloblastosis virus reverse transcriptase, 0.7 nmol/L oligo d(T), and 10 U ribonuclease inhibitor (TaKaRa Biotechnology, Co., Ltd, Dalian, People's Republic of China). cDNA was amplified in a 20 ml PCR reaction system containing 0.2 mmol/L of each specific primer (Sangon Biological Engineering Technology & Service Co., Ltd, Shanghai, People's Republic of China) and SYBR green master mix (TaKaRa Biotechnology, Co., Ltd, Dalian, People's Republic of China). Each cycle consisted of a denaturation step at 95°C for 10 s, an annealing step at 95°C for 5 s, and an extension step at 60°C for 34 s. The primer sequences for chicken CRH, β-actin and GAPDH are listed in Table 1. The PCR

Table 1. Gene-specific primers used in the analysis of chick CRH gene expression

Gene	GenBank accession no.	Primer sequences (5′-3′)	Orientation	Product size (bp)
CRH	AJ621492	CGCAAATGCTTCTAAACC	Forward	169
		GACTGCTGCTGACACCTT	Reverse	
β-actin	NM_204305	TCTCCCTGGACCTGACTTTC	Forward	211
		GAGGTGACATCAGAGCAGCA	Reverse	
GAPDH	NM_204305	CTACACACGGACACTTCAAG	Forward	244
		ACAAACATGGGGGCATCAG	Reverse	

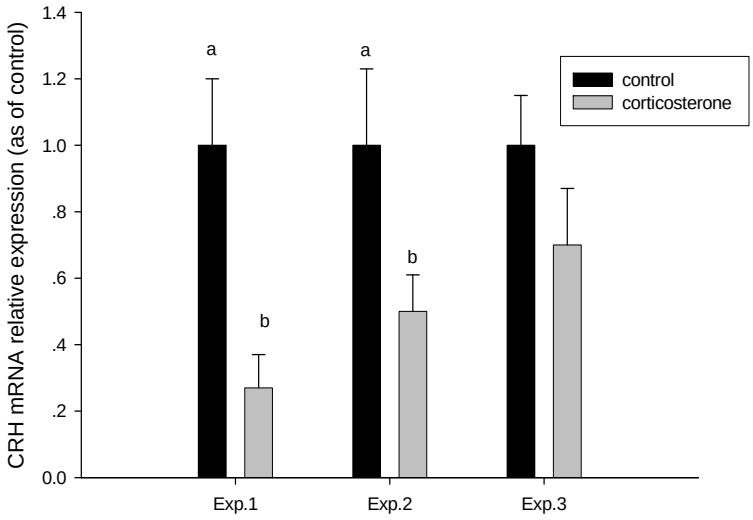

Figure 1. Effect of corticosterone administration on hypothalamic corticotropin-releasing hormone (CRH) mRNA expression (as of control). In Exp. 1, 36 of 72 3-day-old broiler chicks were randomly assigned to corticosterone administration (30 mg/kg diet) and the others to a normal diet; hypothalami (n = 10) were collected after 1 week. In Exp. 2, 24 of 48 3-day-old broiler chicks were subcutaneously injected with corticosterone (4 mg/kg body weight) and the remaining 24 with saline; the hypothalami (n = 6) were collected at 3 h after the injection. In Exp. 3, 24 of 48 8-day-old broiler chicks were intracerebroventricularly injected with corticosterone (2 μl, 2 ng/μl) and the remaining 24 with saline; the hypothalami (n = 6) were collected at 1 h after the injection. Bars in the same experiment with different letters are significantly different ($p<0.05$).

products were verified by electrophoresis on a 0.8% agarose-gel and by DNA sequencing.

The relative amount of CRH gene mRNA was calculated according to the method by Livak and Schmittgen (2001). The CRH mRNA level was normalized to the β-actin level (delta CT) and verified with GAPDH. The delta CT was calibrated against an average CT value of the control chicks. A linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$. The gene transcription result was then reported as the n-fold difference relative to the calibrator.

Statistical analysis

The main effect of the corticosterone treatments on all variables were estimated by one-way ANOVA (SAS Institute, NC). For Exp. 1, n = 10, except for feed intake where n = 3; for Exp. 2 and 3, n = 6. Data are presented as mean±SEM. Means were considered significantly different at $p<0.05$.

RESULTS

Experiment 1

The hypothalamic CRH mRNA levels of the chicks fed corticosterone decreased to approximately 27% those of the chicks fed the control diet (Figure 1). Data on feed intake, plasma metabolites and hormones are presented in Table 2. Dietary corticosterone significantly increased feed intake, plasma uric acid, glucose, triglyceride, FFA and insulin ($p<0.05$).

Experiment 2

Lin et al. (2004) found that plasma corticosterone reached a peak 1 h after the s.c. injection, and returned to normal at 3 h. In rats, the combined hypothalamic increase in an orexigenic neuropeptide Y, and decrease in CRH, is

Table 2. Effect of dietary corticosterone on feed intake, plasma metabolites, and hormones[1]

	Feed intake (g/d)	Uric acid (mg/L)	Glucose (mmol/L)	Triglyceride (mmol/L)	FFA (μmol/L)	Insulin (μIU/ml)
Corticosterone	24.1±0.2[a]	151.7±19.4[a]	26.2±4.1[a]	1.01±0.21[a]	418±67[a]	27.6±3.2[a]
Control	19.6±0.8[b]	52.6±12.8[b]	11.8±2.9[b]	0.21±0.03[b]	224±48[b]	10.8±0.7[b]

[1] Half of the 72 chicks (3 days old) were randomly assigned to corticosterone administration (30 mg/kg diet), and the other 36 chicks were fed with the control diet. Plasma and hypothalami were obtained after 1 week.

FI = Feed intake; FFA = Free fatty acids.

Data are presented as means±SEM (n = 10, except for feed intake n = 3). Means in a column with superscripts without a common letter differ, $p<0.05$.

the proposed mechanism for the central glucocorticoid-elicited hyperphagia (Zakrzewska et al., 2009). In the current experiment, feed intake showed no significant difference 1 h after s.c. corticosterone injection (Figure 2a). So it was speculated that corticosterone had not performed its effect on gene expression levels. Consequently, the hypothalami were collected 3 h after the treatment. The hypothalamic CRH mRNA levels in the s.c.-injected chicks decreased to approximately 51% those of the chicks in controls (Figure 1). Subcutaneous corticosterone administration increased the feed intake within 1 to 3 h and at 1 to 5 h after the injection, but there was no significant effect on the overall feed intake 5 h after the injection (Figure 2a). Plasma uric acid and FFA were all significantly increased at 3 h and at 5 h after the s.c. injection (Figures 2b and 2e). Plasma glucose and insulin were only significantly increased 5 h after corticosterone administration (Figures 2c and 2f). The s.c. injection of corticosterone had no

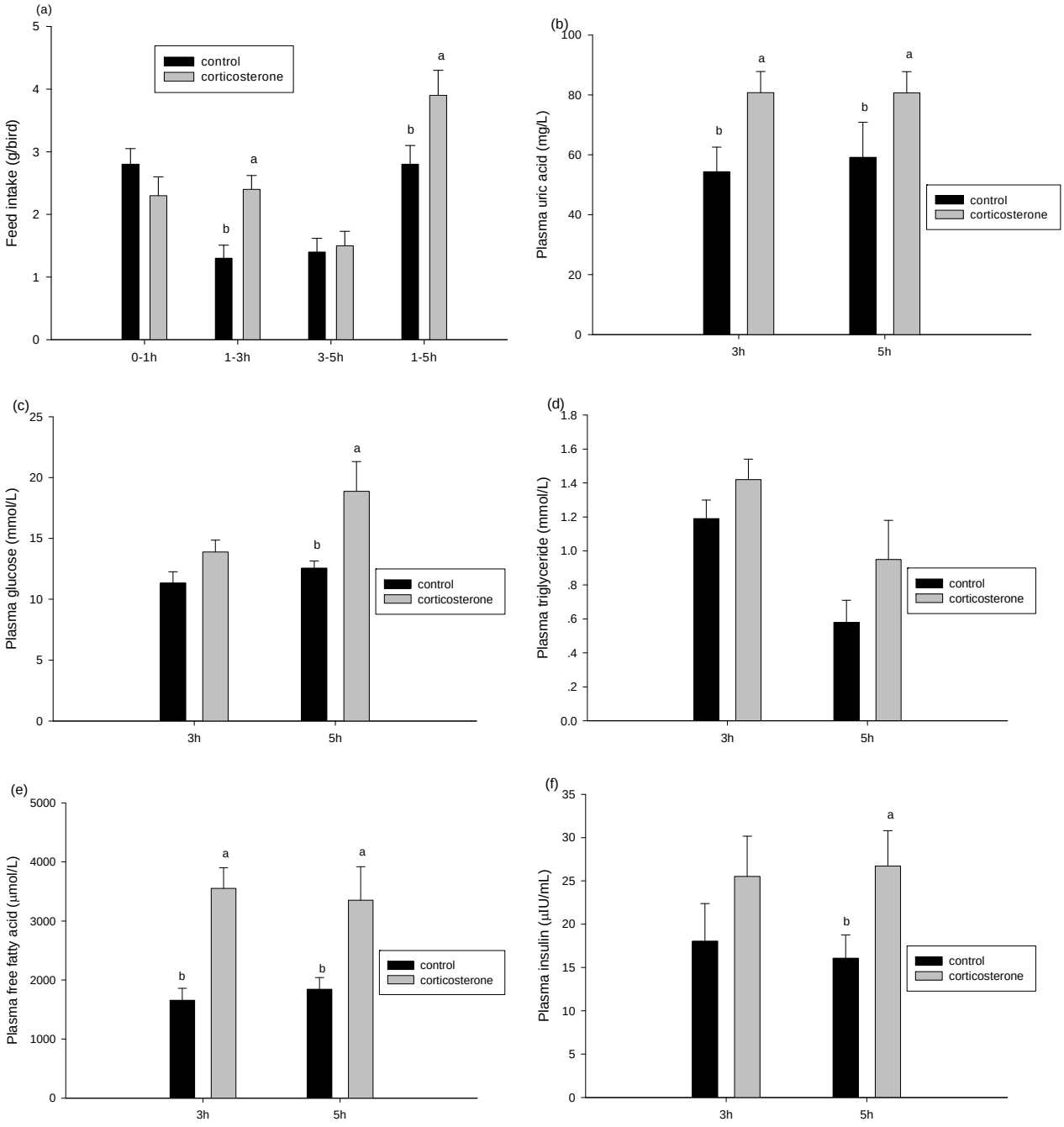

Figure 2. Effect of subcutaneous corticosterone injection on feed intake, plasma metabolites, and hormones. Up to 24 of 48 3-day-old broiler chicks were subcutaneously injected with corticosterone (4 mg/kg body weight) and the remainder with saline. Feed intake was recorded at 1, 3, and 5 h after the injection. Plasma (n = 6) were collected at 3 and 5 h after the injection from different chicks. Bars in the same time point with different letters are significantly different ($p<0.05$).

significant effects on plasma triglyceride (Figure 2d).

Experiment 3

Remage-Healey and Romero (2001) reported that plasma corticosterone abruptly increases after acute stress, starts to decrease after 70 minutes, and returns to normal in 2.5 h. Thus, the CRH mRNA expression at 1 h after the i.c.v. treatment of corticosterone was determined. Although the hypothalamic CRH mRNA levels in the chicks given the i.c.v. corticosterone injection were approximately 70% that of that in controls (Figure 1), no statistical significance was found ($p = 0.15$). The i.c.v. administration of corticosterone increased feed intake within 1 h after the injection, but not from 1 to 3 h after the injection and the overall feed intake for 3 h (Figure 3a). The i.c.v. corticosterone injection had no significant effects on plasma uric acid and FFA (Figures 3b and 3e). Plasma glucose and triglyceride were significantly increased 3 h after the i.c.v. injection (Figures 3c and 3d). In contrast, the plasma insulin was significantly increased 1 h, rather than 3 h, after the i.c.v. corticosterone

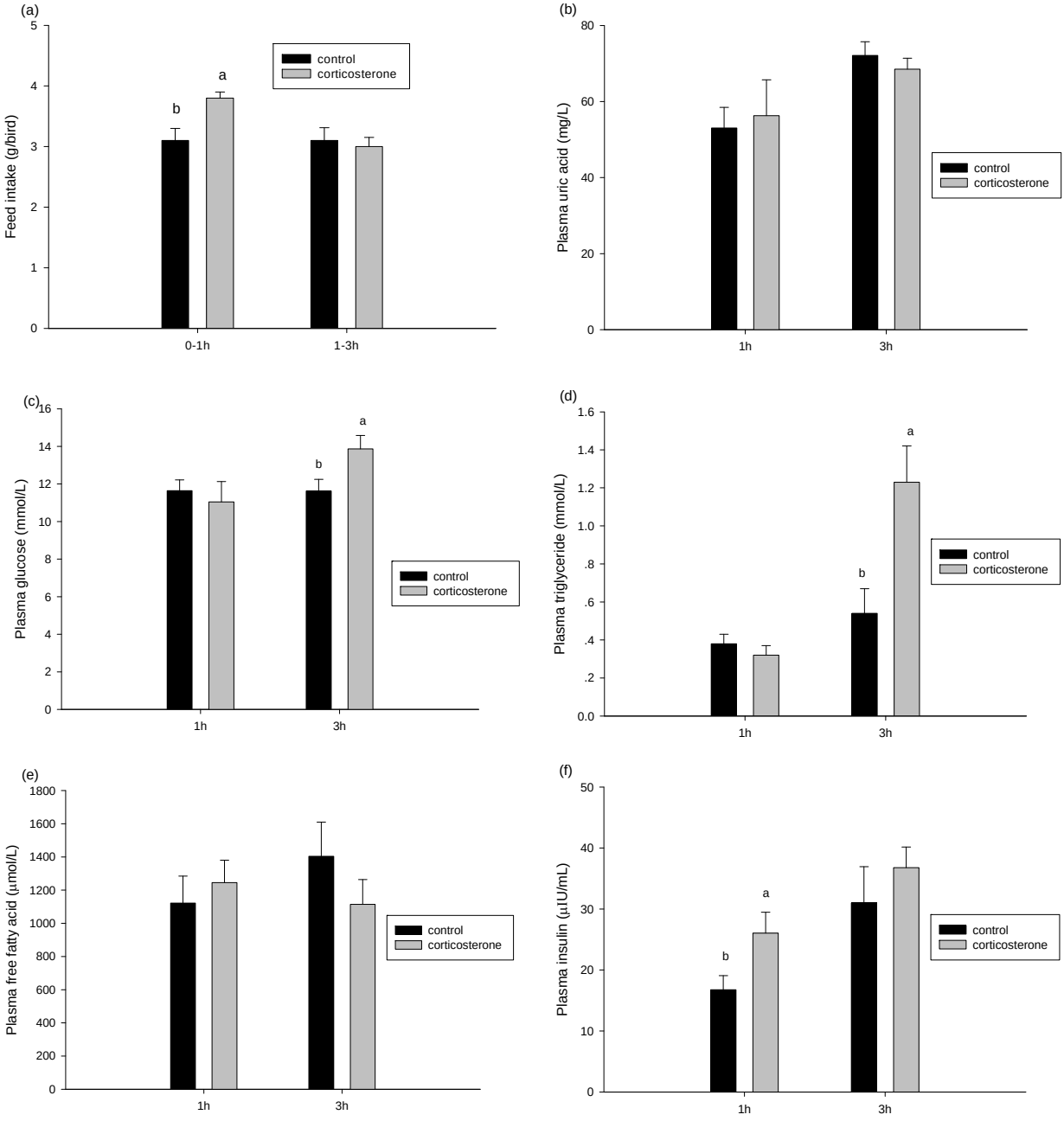

Figure 3. Effect of intracerebroventricular corticosterone injection on feed intake, plasma metabolites, and hormones. Up to 24 of 48 8-day-old broiler chicks were intracerebroventricularly injected with corticosterone (2 μl, 2 ng/μl) and the other half with the same volume of saline. Feed intake was recorded at 1 and 3 h after the injection. Plasma ($n = 6$) were collected at 1 and 3 h after the injection from different chicks. Bars at the same in the time point with different letters are significantly different ($p<0.05$).

administration (Figure 3f).

DISCUSSION

When the broiler chickens were provided with a high energy diet, corticosterone significantly increased feed intake, plasma metabolites, and insulin (Exp. 1), which is in line with our previous research (Yuan et al., 2008). The same diet was used in Exp. 2. The overall feed intake within 3 or 5 h of the injection showed no significant difference; however, within 1 to 3 h or from 0 to 1 h after injection, the feed intake increased, which indicates that corticosterone induced the chickens to get enough feed earlier than their control counterparts (Figures 2a and 3a) after the s.c. corticosterone injection. The mechanism underlying these phenomena might be related to variations in exogenous corticosterone. When chickens were provided with dietary supplemented-corticosterone, their plasma corticosterone reached the highest level at 7 d (Lin et al., 2004). However, plasma corticosterone reaches its peak 1 h after s.c. injection, and then gradually returns to normal level in chickens (Lin et al., 2004), and even lower than the normal in rats (Dallman et al., 2007). Therefore, the results suggest a stimulatory corticosterone effect on feed intake when the chickens were supplied with a high-energy diet. Furthermore, the higher feed intake at 1 h after the i.c.v. corticosterone administration (Exp. 3) prompted a feed-forward rather than a feedback effect. The results from rats confirmed this hypothesis. Centrally administered glucocorticoids stimulate hyperphagia (Remage-Healey and Romero, 2001), which is different from the classic glucocorticoid feedback mechanism. A similar result was found in rats. Contrary to that observed with the peripheral corticosterone treatment (Bell et al., 2000), the i.c.v. corticosterone treatment of adrenalectomised rats reduces the volume of sucrose solution consumed while minimally affecting food intake (Laugero et al., 2002). These data, together with our results, strongly suggest that central and peripheral corticosterone affect feed intake via different mechanisms.

Given that corticosterone and CRH affect each other, and CRH plays a role in animal feed intake regulation (Richard, 1993; Richards, 2003), the effects of corticosterone on CRH gene expression was further explored. Little information is available on the effects of corticosterone on CRH mRNA expression in birds. In mammals, declining corticosterone levels at the PVN and the median eminence increases CRH mRNA levels (Plotsky and Sawchenko, 1987). In the current experiment, the decreased CRH mRNA caused by the dietary and s.c. corticosterone, and not by the i.c.v. corticosterone, indicates the indirect and/or peripheral effect of corticosterone on CRH gene expression. In contrast, a higher concentration (as much as 110 ng) of i.c.v. corticosterone increased CRH immunoreactivity in the PVN (Laugero et al., 2002). However, Tanimura and Watts (1998) reported high corticosterone concentrations inhibit and prolong CRH gene expression in the PVN, whereas low corticosterone concentrations facilitate it. Nevertheless, only one corticosterone concentration was used in Exp. 3; it will be precarious to conclude that central corticosterone does not affect CRH gene expression in the hypothalamus of broiler chickens. The effect of different concentrations of central corticosterone on CRH mRNA expression, temporally and dimensionally, should be further investigated.

In line with our previous studies (Lin et al., 2004; Lin et al., 2006), s.c. corticosterone injection resulted in increased plasma uric acid and FFA in the present study. Together with the results of Exp. 1, these outcomes are consistent with the roles that peripheral corticosterone plays on nutrient metabolism. Corticosterone promotes the conversion of proteins and lipids into glucose during the stress-induced activation of the HPA axis (Gross and Cidlowski, 2008). The s.c. injection of dexamethasone, a synthetic glucocorticoid, increases hepatic fatty acid *de novo* synthesis (Cai et al., 2009). Thus, the increased plasma FFA might be the result of enhanced peripheral lipolysis and strengthened *de novo* synthesis in the liver. In contrast to the high plasma uric acid and FFA at 3 h and 5 h after the s.c. corticosterone injection, the plasma glucose only increased at 5 h. Stress-induced high corticosterone was accompanied by the mobilisation of proteins and peripheral lipids, stimulation of hepatic gluconeogenesis, and increased central fat deposits (Dong et al., 2007). Considering the similar overall feed intake in the two treatments, we conclude that the higher plasma glucose was the result of a stimulated hepatic gluconeogenesis, which was accomplished with the substrates of proteolysis and peripheral lipolysis. Similarly, plasma insulin only increased 5 h after the s.c. corticosterone administration. Considering that plasma corticosterone returns to normal 3 h after s.c. corticosterone injection (Lin et al., 2004), a possible explanation for this lagged increment of plasma insulin seemed to be related more to the feed-back effects of plasma metabolites. Increased levels of plasma glucose and lipids can stimulate insulin biosynthesis and secretion (Obici and Rossetti, 2003), and stress-induced high plasma FFA and glucose are connected with insulin resistance in broiler chickens (Yuan et al., 2008).

In Exp. 3, we measured the direct effects of corticosterone on the central nervous system and the subsequent metabolic variations. Due to the lack of information on this topic in birds, several preliminary experiments were conducted. Referred to previous reports in Dawley-derived rats (Laugero et al., 2002), four dosages (1 ng, 2 ng, 4 ng and 8 ng) of corticosterone were tested.

The results showed that 4 ng of corticosterone i.c.v. was enough to evoke stress in broiler chicks, as shown by the plasma insulin, glucose, and triglyceride levels. In line with our preliminary experiments, i.c.v. corticosterone administration caused higher plasma glucose and triglyceride levels at 3 h after injection. However, the increased levels of plasma uric acid and FFA were not observed after i.c.v injection of corticosterone. Compared to peripheral effects of corticostereon on these parameters as reported by previous researchers (Lin et al., 2004; Yuan et al., 2008), current results indicate a corticosterone regulation mechanism different from its peripheral mechanism. It has been reported that the same dose of central or peripheral glucocorticoids showed different effect on feed intake and body weight in rats (Zakrzewska et al., 1999). The higher glucose and triglyceride levels were believed to partly come from the postprandial effect of the increased feed intake 1 h after the corticosterone injection. Nevertheless, the effects of corticosterone could not be denied. The impact of glucocorticoids on triglyceride is minimal when the diet composition maintains a low lipid flux, but becomes highly significant when the diet lipid flux increases (Mantha et al., 1999). In contrast to plasma glucose and triglyceride, plasma insulin increased at 1 h rather than 3 h, which indicates a direct central corticosterone feed-forward effect on plasma insulin. Little information about this effect is available in poultry. Central glucocorticoids induce hyperinsulinemia in obese rats, in which a central glucocorticoid-mediated stimulation of the vagal drive to the pancreatic β-cells is believed to be involved (Stubbs and York, 1991). Corticosterone infused into the central nerve system in adrenalectomised rats acts in a stimulatory, feed-forward fashion on subsequent ACTH secretion (Dallman et al., 2004). Central but not peripheral glucocorticoid infusion in adrenalectomised male rats increases basal and substrate-induced insulinemia through a parasympathetic pathway (Sainsbury et al., 2001).

In conclusion, corticosterone stimulated feed intake in broiler chicks fed with a high-energy diet. Peripheral administration of corticosterone decreased hypothalamic CRH mRNA levels. Peripheral corticosterone might have an indirect effect on CRH gene expression and plasma insulin, and might be mediated by substances from proteolysis, peripheral lipolysis, and/or fatty acid synthesized *de novo* in the liver. Central corticosterone had a possible direct feed-forward effect, but the underlying mechanisms need to be further investigated.

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