A High Dose of Vitamin E Inhibits Adrenal Corticosterone Synthesis in Chickens Treated with ACTH

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Summary The present experiment was conducted to study the effects of dietary vitamin E on plasma corticosterone (CTC) concentration and adrenal steroid syntheses in chickens treated with adrenocorticotrophic hormone (ACTH). Chickens were divided into ACTH(–) and ACTH(+) groups, and each group was further divided into three subgroups administered with vitamin E (500 or 5,000 mg/kg diet) and without the vitamin. Vitamin E (DL- α -tocopheryl acetate) was mixed with the basal diet at levels of 500 and 5,000 mg/kg and fed for 6 d. ACTH (20 IU/kg body weight) was given daily by intraperitoneal injection for 5 d. α -Tocopherol levels in the plasma and adrenal gland were markedly elevated by vitamin E feeding, and the level of adrenal free cholesterol (CHOL), which is used for steroid synthesis, was significantly decreased by vitamin E feeding in a dose-dependent manner. However, the level of adrenal CHOL ester was unchanged by any treatment. The elevations of pregnenolone, progesterone and CTC levels in the adrenal gland of chickens with ACTH treatment were decreased by vitamin E administration. The elevation of plasma CTC concentration in the ACTH(+) group was dramatically decreased by vitamin E administration, while that concentration was not influenced by the vitamin administration in the ACTH(–) group. These findings indicate that vitamin E suppresses the elevation of the plasma CTC concentration due to ACTH in chickens, possibly by inhibiting the conversion of CHOL ester to free CHOL in the adrenal gland.

Key Words adrenocorticotrophic hormone, vitamin E, cholesterol, corticosterone

Restraint, fasting, transport and exposure to high or low environmental temperatures are typical stressors for animals. During stress, the hypothalamus-pituitary-adrenal axis is activated and glucocorticoid hormone is released from the adrenal cortex (1). Glucocorticoid is essential for maintaining life and normal growth. However, excessive glucocorticoid causes growth inhibition, muscle proteolysis, fat deposition (2), induction of oxidative stress (3, 4) and immune system suppression (5). Thus, minimizing stress is important not only in humans but also in farm animals.

Several studies have shown that vitamin E reduces plasma glucocorticoid levels under different types of stress in rats (6–8). However, the mechanism accounting for this reduction remains unknown.

The pathway in the adrenal cortex leading from a steroid precursor (cholesterol, CHOL) to glucocorticoids involves several different forms of cytochrome P-450. The rate-limiting reaction in this pathway is the side-chain cleavage of CHOL to form pregnenolone (PREG) (9). This step is catalyzed by cytochrome P-450_{scc} (10) located on the matrix side of the inner membrane (11), and is activated by adrenocorticotrophic hormone (ACTH) (9). Activity of this enzyme depends heavily on the amount of CHOL in adrenal glands (12–14). A de-

creased content of adrenal CHOL is correlated with a reduction in the steroid synthesis and exhaustion of free CHOL occurs rapidly, within a few minutes, by ACTH stimulation (14).

Arai (15) has reported that α -tocopherol (TOC) transport may be linked to the cellular CHOL metabolism and/or transport in the liver cell. There are several clinical studies that reported that the plasma CHOL concentration is inversely proportional to the plasma vitamin E concentration (16–18). Recently, Traber and Packer (19) have reported that vitamin E may have a structure-specific role in addition to its antioxidant function.

The plasma concentration of TOC increases only 2- or 3-fold even when the ingestion is about 100-fold in rats (20). This can be explained by the TOC transfer protein system that plays a key role in the preferential retention and the subsequent distribution of TOC to tissues (20). However, Leonhardt et al. (21) has reported that TOC accumulation in tissues is far greater in chickens than other animals. It is known that, in chickens, the plasma TOC concentration increases linearly in response to dietary intake and there is a high relationship between dietary and plasma TOC concentrations (22). From these findings, it was suggested that a large amount of vitamin E may decrease the adrenal free CHOL content and inhibit the stress-induced elevation of plasma corticosterone (CTC) concentration in chick-

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ens.

In the present study, we examined how a high dose of vitamin E affects the adrenal content of free CHOL and precursors of CTC and the plasma concentrations of CHOL and CTC in chickens treated with and without ACTH.

MATERIALS AND METHODS

Animal, diets and schedule. One-d-old male broiler chickens of the Cobb strain were supplied by a local commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). They were housed in an electrically-heated battery brooder and were provided with water and a commercial starter diet (21% CP; Minami Nihon Kumiai Shiryo Co., Kagoshima, Japan) ad libitum for the first 7 d. On day 7, chickens were selected from the two-fold large population for uniform body weight and divided into 6 groups with 6 replications of 2 chickens in each cage. Chickens were housed in wire-bottomed aluminum cages (49 cm height×40 cm width×67 cm depth). The experiment was conducted in temperature-controlled rooms with a 14 h light–10 h dark cycle. The temperature of the room was 25°C, and relative humidity was maintained at 50–70% throughout the experiment. The composition of a basal diet (3,200 kcal ME/kg and 23% crude protein) is shown in Table 1. The basal diet was fed ad libitum during the preconditioning period (from 7 to 9 d of age).

On day 9, chickens were divided into ACTH(–) and ACTH(+) groups, and each group was further divided into E 0, E 500 and E 5,000 subgroups. E 500 and E 5,000 subgroups received the basal diets replaced with equivalent amounts of corn with 500 and 5,000 mg DL- α -tocopheryl acetate/kg, respectively. DL- α -Tocopheryl acetate was purchased from Nacalai Tesque (Kyoto, Japan). Cortosyn Z, a synthetic ACTH, was purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Cortosyn Z contains a tetracosactide that is composed from 24 amino acids. ACTH was given at the level of 20 IU/kg body weight daily by intraperitoneal injection. An equivalent volume of saline was administered in the ACTH(–) group. The birds were treated with vitamin E from 9 to 15 d of age and ACTH was given from 11 to 15 d of age. Body weight and food intake were recorded daily. At the end of the experimental period, all birds were sacrificed by decapitation. Blood was collected into heparinized-test tubes, quickly centrifuged at 4,700×*g* for 10 min at 4°C to separate plasma, and stored at –20°C until analysis. The birds were dissected to remove liver and both adrenal glands. The adrenal glands were stored at –80°C until analysis.

TOC contents in plasma and adrenal gland. TOC contents in the plasma and adrenal gland were assayed using HPLC according to the method of Phoenix (23).

CHOL contents in plasma and adrenal gland. Plasma and adrenal CHOL contents and the adrenal free CHOL content were measured by an enzymatic CHOL assay system using a commercial kit, (Cholesterol E-Test Wako and Free Cholesterol E-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan). After the adre-

Table 1. Composition of basal diet.

Ingredients	%
Ground yellow corn	71.66
Dehydrated alfalfa meal	3.77
Purified soybean protein	19.60
Mineral mixture*	3.31
Vitamin mixture†	0.26
L-Lysine	0.28
DL-Methionine	0.32
Soybean oil	0.80
Analysis	
Crude Protein, %	23
ME	3,200 kcal/kg

* Contained (/kg): CaCO₃ 110 g, CaHPO₄ 660 g, NaCl 113 g, MnSO₄·4-5H₂O 6.600 g, ZnSO₄·7H₂O 4.000 g, FeSO₄ 6.145 g, CuSO₄·7H₂O 233 mg, NaIO₃ 16 mg, and H₂SeO₃ 6 mg.

† Contained (/kg): retinol 1,750,000 IU, cholecalciferol 200,000 IU, riboflavin 2.5 g, thiamine 1 g, pyridoxine 0.5 g, cyanocobalamin 10 mg, pantothenic acid 4 g, nicotinic acid 10 g, menadione 250 mg, folic acid 200 mg, choline chloride 300 mg, biotin 25 mg, DL- α -tocopheryl acetate 2.5 g, and sucrose 978 g.

nal gland was homogenized with saline using a polytron homogenizer, the homogenate was provided for the measurement. The CHOL ester content was calculated by subtracting free CHOL from total CHOL.

Plasma CTC concentration. Plasma CTC concentration was measured by a radioimmunoassay using a commercial kit (ICN Biomedicals, Inc., California, USA).

HPLC analyses of steroids. According to the method of Neri et al. (24), steroid assays were carried out with minor modifications. The outline of the procedure is as follows. Adrenal homogenates were mixed with 100 μ L of a dexamethasone (100 ng) solution that was used as an internal standard and a recovery marker. Then steroids were extracted from the homogenate by mixing with 0.6 mL 0.25 M NaOH and 6 mL dichloromethane for 1 min before centrifuging at 4,700×*g* for 10 min. After pipetting out the lipid layer, the extract was washed with 10 mL 0.1 M NaOH and 15 mL distilled water, and then evaporated to dryness under a vacuum and redissolved in 300 μ L 58% methanol. This solution was used for the analyses of PREG, progesterone (PROG), 11-deoxycorticosterone (DOC) and CTC. The steroids were assayed according to the HPLC method of O'Hare et al. (25) using a Shimadzu LC-10AD equipped with a Shim-Pack CLC-ODS column (6.0×150 nm); the reproducibility of the retention time was 1–3% (coefficient of variation). Steroid hormones were monitored by a Tosoh UV-8010 spectrometer. PREG and PROG were detected at 290 nm and DOC and CTC at 248 nm. The compositions of the mobile phases were MeOH: H₂O=80:20 for analyzing PREG and PROG, and 58:42 for DOC and CTC. Steroids were identified by comparison of their retention times with those of the standards. Moreover, for the initial identification, the

same sample was chromatographed alone or with the addition of the standards; this procedure was used during the routine analysis to confirm the findings of some difficult samples. Quantification of steroid hormones was based on peak-area measurement using a Shimadzu C-R6A Chromatopac.

Statistical analyses. Data were analyzed by two-way analysis of variance (ANOVA) using the General Liner Model procedure of the statistical analysis system software package (Release 6.09, SAS Institute Inc., Cary, NC, USA) with Duncan's multiple-range test. A *p* value<0.05 was considered to be statistically significant.

RESULTS

The body weight gain, feed intake and feed efficiency during the period of 4 to 11 d of age, and adrenal gland weight in chickens are shown in Table 2. The body weight gain tended to decrease when the birds were treated with ACTH, and vitamin E administration tended to minimize the ACTH effect. Feed intake was not changed by any treatment and, thus, the feed efficiency tended to be lower in the ACTH group. This effect of ACTH also tended to be minimized by vitamin E administration. Adrenal gland weight was not changed by any treatment.

The effect of dietary vitamin E on TOC and CHOL

Table 2. Effect of vitamin E on body weight gain, feed intake, feed conversion ratio and adrenal gland weight in chickens treated with and without ACTH¹.

Treatment ²	Vitamin E ³ (mg)	Body weight gain (g/4 d/pen)	Feed intake (g/4 d/pen)	Feed efficiency (4 d)	Adrenal weight (mg/100 g BW)
ACTH(−)	0	223±34	321±27	0.69±0.06	9.8±1.4
	500	210±38	297±38	0.68±0.09	10.3±1.9
	5,000	208±32	308±19	0.70±0.05	11.1±1.3
ACTH(+)	0	188±34	307±24	0.63±0.06	10.5±2.6
	500	208±29	298±55	0.68±0.08	9.5±1.9
	5,000	211±39	284±51	0.72±0.07	10.4±2.6
ANOVA ⁴					
ACTH		NS	NS	NS	NS
Vitamin E		NS	NS	NS	NS
ACTH×vitamin E		NS	NS	NS	NS

¹ Values are expressed as means±SD, *n*=6. Means in columns followed by different letters are significantly different, *p*<0.05.

² ACTH(−) and ACTH(+) groups were intraperitoneally injected with saline and 20 IU ACTH/kg body weight everyday for 5 d, respectively.

³ Chickens were fed a diet containing 0, 500 or 5,000 mg DL-α-tocopheryl acetate/kg diet.

⁴ Two-way analysis of variance.

Table 3. Effect of vitamin E on TOC and CHOL levels in plasma and adrenal gland and adrenal free CHOL level in chickens treated with and without ACTH¹.

Treatment ²	Vitamin E ³ (mg)	Plasma		Adrenal		
		TOC (μg/mL)	CHOL (mg/mL)	TOC	CHOL ester (μg/adrenal)	free CHOL
ACTH(−)	0	0.5±0.2 ^c	1.73±0.31 ^{ab}	0.30±0.14 ^c	83.9±33.6	101.1±22.1 ^a
	500	9.1±4.2 ^b	1.80±0.26 ^{ab}	5.53±2.06 ^b	89.7±14.1	68.2±18.3 ^b
	5,000	22.6±9.1 ^a	1.50±0.14 ^b	13.47±4.32 ^a	105.5±68.4	32.5±5.9 ^{cd}
ACTH(+)	0	0.5±0.2 ^c	1.78±0.20 ^{ab}	0.37±0.08 ^c	83.2±37.3	55.8±22.2 ^{bc}
	500	9.9±5.7 ^b	2.01±0.32 ^a	5.76±3.97 ^b	63.3±28.2	29.4±10.0 ^d
	5,000	28.5±7.5 ^a	1.54±0.18 ^b	15.17±3.52 ^a	69.2±23.7	20.4±6.6 ^d
ANOVA ⁴						
ACTH		NS	NS	NS	NS	0.0001
Vitamin E		0.0001	0.003	0.0001	NS	0.0001
ACTH×vitamin E		NS	NS	NS	NS	NS

¹ Values are expressed as means±SD, *n*=6. Means in columns followed by different letters are significantly different, *p*<0.05.

² ACTH(−) and ACTH(+) groups were intraperitoneally injected with saline and 20 IU ACTH/kg body weight everyday for 5 d, respectively.

³ Chickens were fed a diet containing 0, 500 or 5,000 mg DL-α-tocopheryl acetate/kg diet.

⁴ Two-way analysis of variance.

Fig. 1. Effect of vitamin E on adrenal contents of PREG (A), PROG (B), DOC (C) and CTC (D) levels in chickens treated with and without ACTH. ACTH(-) and ACTH(+) groups were intraperitoneally injected with saline and 20 IU ACTH/kg body weight everyday for 5 d, respectively. Chickens were fed a diet containing 0, 500 or 5,000 mg DL- α -tocopheryl acetate/kg. Values are expressed as means $\pm$ SD. Means bearing different letters are significantly different ($p < 0.05$).

ANOVA	PREG	PROG	DOC	CTC
ACTH	0.05	0.05	NS	0.05
Vitamin E	NS	NS	NS	NS
ACTH $\times$ Vitamin E	0.05	NS	NS	NS

Fig. 2. Effect of vitamin E on plasma CTC level in chickens treated with and without ACTH. ACTH(-) and ACTH(+) groups were intraperitoneally injected with saline and 20 IU ACTH/kg body weight everyday for 5 d, respectively. Chickens were fed a diet containing 0, 500 or 5,000 mg DL- α -tocopheryl acetate/kg. Values are expressed as means $\pm$ SD. Means bearing different letters are significantly different ($p < 0.05$).

ANOVA

ACTH	NS
Vitamin E	NS
ACTH $\times$ Vitamin E	NS

concentrations in the plasma and adrenal glands of chickens treated with and without ACTH are shown in Table 3. Plasma and adrenal TOC levels in the ACTH(-) and ACTH(+) groups were markedly elevated by vitamin E administration ($p < 0.0001$) in a dose-dependent manner, and a significant effect of dietary vitamin E was shown by ANOVA. The plasma CHOL concentration was decreased by a high dose of vitamin E administration in both ACTH(-) and ACTH(+) groups, and a significant effect of dietary vitamin E was shown by ANOVA. There was no significant difference in adrenal CHOL ester content between groups. However, the adrenal free CHOL content was decreased in the ACTH(+) group and it was further decreased in the ACTH(+) group with vitamin E administration, although that content was decreased in the ACTH(-) group with vitamin E administration. ANOVA showed that the effects of ACTH and vitamin E on the adrenal free CHOL content were significant ($p < 0.0001$). ACTH treatment increased the adrenal PREG and PROG contents and vitamin E administration minimized both increases, although neither ACTH treatment nor vitamin E administration had any significant effect on the adrenal DOC content (Fig. 1). ANOVA showed that the effect of ACTH on the PREG and PROG contents and the effect of a combination of ACTH and vitamin E on the PREG con-

Fig. 3. Effect of vitamin E on ratios of free CHOL/CHOL (A), PREG/free CHOL (B) and PREG/CHOL ester (C) in chickens treated with and without ACTH. ACTH(-) and ACTH(+) groups were intraperitoneally injected with saline and 20 IU ACTH/kg body weight everyday for 5 d, respectively. Chickens were fed a diet containing 0, 500 or 5,000 mg DL- α -tocopheryl acetate/kg. Values are expressed as means $\pm$ SD. Means bearing different letters are significantly different ($p < 0.05$).

ANOVA	Free CHOL/CHOL ester	PREG/Free CHOL	PREG/CHOL ester
ACTH	0.05	0.003	0.05
Vitamin E	0.01	NS	NS
ACTH $\times$ Vitamin E	NS	NS	NS

tent were significant. The adrenal CTC content was significantly increased 2.4-fold by ACTH treatment, and vitamin E administration significantly inhibited this increase in a dose-dependent manner (Fig. 1). When a quantitative comparison of the effect of vitamin E on adrenal steroid contents was performed in the 5,000 mg E group with ACTH treatment as shown in Fig. 1, vitamin E administration similarly inhibited the ACTH-stimulated syntheses of adrenal PREG, PROG, DOC and CTC, i.e., PREG (-55.6%), PROG (-50.6%), DOC (-34.2%) and CTC (-53.0%). As shown in Fig. 2, the plasma CTC concentration was increased 2.6-fold by ACTH treatment, and vitamin E feeding inhibited the ACTH-stimulated elevation of plasma CTC. The ratios of free CHOL/CHOL ester, PREG/free CHOL and PREG/CHOL ester in the adrenal gland are shown in Fig. 3. The ratios were all changed by ACTH treatment, and ANOVA showed that the effects of ACTH on these ratios were significant ($p < 0.05$). On the other hand, vitamin E administration affected only the free CHOL/CHOL ester ratio and ANOVA showed that the effect of vitamin E was significant ($p < 0.05$).

DISCUSSION

When an animal is exposed to noxious or potentially noxious stimuli, there is an increased secretion of ACTH and, consequently, a rise in the circulating glucocorticoid level. ACTH stimulates CTC synthesis via the mediation of adenylate cyclase and protein kinase in adrenal glands. Excessive glucocorticoid causes growth inhibition, muscle proteolysis, fat deposition (5), induction of oxidative stress (6, 7) and immune system suppression (8). Therefore, relief of stress is important not only in humans but also in animals.

Vitamin E is a strong candidate as an anti-stress agent. However, there are few and rather conflicting findings on the effect of vitamin E on glucocorticoid secretions. Ni et al. (26) reported that vitamin E enhanced ACTH-induced glucocorticoids synthesis in rat adrenal gland. However, al-Shabanah et al. (7) and Shaheen et al. (8) reported that an increase in plasma corticosterone concentration by bacterial endotoxin and swimming stress could be diminished by vitamin E in rats. In contrast, Armario et al. (27, 28) reported that vitamin E did not modify the pituitary-adrenal activity in rats with immobilization stress. Nakamura (29) reported that adrenal function is activated by a small amount of vitamin E but is inhibited by a large amount of the vitamin in rabbits.

The present study clearly shows that an increase in plasma CTC level due to ACTH is diminished in chickens by a high dose of vitamin E. Thus, it was suggested that vitamin E might directly reduce CTC synthesis in the adrenal gland. To examine this hypothesis, we measured adrenal steroids in chickens treated with ACTH.

In chickens, the major corticosteroid present in the adrenal gland is CTC because little 17 α -hydroxylase activity exists in the adrenal gland and therefore cortisol cannot be synthesized (30). Steroids produced in the adrenal cortex are derivatives of CHOL. Adrenocortical

cells are almost completely dependent upon the continuous supply of CHOL from serum lipoproteins (31). CHOL is esterified and stored as lipid droplets in adrenocortical cells before synthesizing steroids. CHOL esterase hydrolyzes CHOL ester to free CHOL before conversion of free CHOL to PREG in adrenal mitochondria by P-450_{scc} which is activated by ACTH. In adrenal glands, PREG produced from CHOL is converted to PROG and the PROG is further hydroxylated to give DOC. The DOC is finally hydroxylated to form CTC in adrenal glands. There are studies showing the effect of vitamin E deficiency on steroidogenesis (32–34). However, little attention has been paid to the effect of a high dose of vitamin E on steroidogenesis.

Analyses of steroids in the adrenal gland of chickens suggest that the decrease in CTC synthesis due to vitamin E was caused by depletion of free CHOL in the adrenal gland.

Plasma and adrenal TOC levels in the vitamin E-administered chickens (5,000 mg/kg) with and without ACTH treatment were about 50-fold higher than their control levels. This is consistent with the findings of Leonhardt et al. (21) who showed that TOC accumulation in tissues was greater in chickens than in other animals. Although the plasma CHOL concentration in chickens treated with and without ACTH was decreased by a high dose of vitamin E (5,000 mg/kg), the adrenal CHOL ester content was not changed by the vitamin E administration. However, the adrenal free CHOL content in chickens treated with and without ACTH was significantly decreased by vitamin E administration in a dose-dependent manner (Table. 3). The PREG/free CHOL ratio in the adrenal gland of chickens was clearly increased by ACTH treatment (Fig. 3-B). This suggests that the decrease in free CHOL content due to ACTH in the adrenal gland of chickens may be caused by an increase in PREG formation in the gland. However, vitamin E administration did not change this ratio significantly. Thus, it is obvious that the decrement of adrenal free CHOL content due to vitamin E was caused by the reduction of the formation of free CHOL from CHOL ester. The effect of vitamin E on the free CHOL/CHOL ester ratio in the adrenal gland of chickens was significant, but that on the PREG/free CHOL ratio was not (Fig. 3). The levels of adrenal PREG, PROG, DOC and CTC were all lowered by vitamin E administration in ACTH-treated chickens (Fig. 1). These findings clearly indicate that the inhibition of CTC synthesis by vitamin E occurs at the step of free CHOL formation in the adrenal gland of chickens treated with ACTH. Although vitamin E administration decreased adrenal free CHOL content in ACTH-untreated chickens, an inhibition of adrenal CTC synthesis was not shown, because adrenal PREG synthesis in the chickens was stimulated, but not significantly, by vitamin E administration (Fig. 3-B).

Deficiency of CHOL leads to insufficient synthesis of adrenal steroid hormones (12–14, 35). These observations are consistent with the findings in the present study.

The present investigation showed that a high dose of

vitamin E minimizes the elevation of plasma CTC level due to ACTH in chickens, possibly by inhibiting the conversion of CHOL ester to free CHOL in the adrenal gland. Measurement of activities of enzymes regulating this step in the adrenal gland, e.g., CHOL esterase and P-450_{scc}, is in progress in our laboratory. Furthermore, our previous study has shown that CTC induces oxidative stress and that this effect of CTC can be minimized by a high dose of vitamin E (7). Thus, the present study suggests that vitamin E works as a strong anti-stress agent.

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