Calcitonin Reduces Corticosterone-Induced Muscle Proteolysis

Kouji HIGUCHI, Kunioki HAYASHI, Yusuke SHIMOOZAKI,
Akira OHTSUKA, and Yuichiro TOMITA

*Department of Biochemical Science and Technology, Faculty of
Agriculture, Kagoshima University, 1-21-24, Korimoto,
Kagoshima 890, Japan*

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Summary Two-experiments were conducted to clarify the effect of calcitonin (CT) on growth, muscle protein breakdown, plasma corticosterone (CTC) concentration and urinary calcium excretion in young growing rats treated with CTC. Rats received hormonal treatments for 6 days (Experiment 1) or 24 h (Experiment 2). Dose levels of CTC were 10 mg and 5 mg/100 g body weight/day in experiment 1 and 2, respectively, and that of CT was 100 m unit/100 g body weight/day in both experiments. Muscle protein degradation was evaluated by urinary N^{ϵ} -methylhistidine excretion. CT increased 24-h urinary calcium excretion but not 6-day calcium excretion. CTC markedly inhibited growth, accelerated muscle protein degradation and increased calcium excretion in both experiments. However, very interestingly, CT minimized CTC-induced muscle proteolysis, and normalized the CTC-induced decrease in urinary calcium excretion. Furthermore, CT decreased the CTC-induced increase in the plasma CTC concentration. The present observations indicate that CT reduces CTC-induced muscle protein breakdown by reducing the plasma CTC concentration and that increased urinary calcium excretion due to CT treatment may also play a role in reducing CTC-induced muscle protein breakdown.

Key Words calcitonin, corticosterone, muscle proteolysis, N^{ϵ} -methylhistidine

Catabolic effects of excessive glucocorticoid hormones from endogenous or exogenous sources on skeletal muscle are well documented. Glucocorticoids stimulate muscle protein breakdown and inhibit muscle protein synthesis resulting in growth retardation in animals (1–3). However, the mechanism of the catabolic effects of glucocorticoids is not still clearly understood.

It has been suggested that Ca^{2+} plays a critical role in the control of protein breakdown in skeletal muscle (4, 5). Dayton et al. (6, 7) have reported that calpain, a Ca^{2+} -activated neutral protease which requires Ca^{2+} for activation, was thought

to be involved in muscle protein degradation. It is also known that glucocorticoids increase bone resorption and induce osteoporosis (8), and thus induce hypercalcemia and inhibit bone formation (9).

On the other hand, CT is well known to have a hypocalcemic effect and to play important roles in the regulation of blood calcium levels in animals (10,11). Therefore, glucocorticoid-induced muscle proteolysis is expected to be minimized by CT.

In the present investigation, two experiments were conducted to examine the effect of CT on growth, muscle proteolysis, urinary calcium excretion and plasma glucocorticoid concentration in the rats treated with CTC. In the first experiment, rats received daily injections of CTC and CT for 6 days, and in the second experiment, they received a single injection and were examined for 24 h.

MATERIALS AND METHODS

Animal experiment. Two experiments with the same protocol except experimental period were conducted using male Sprague-Dawley rats at 5 weeks of age obtained from Charles River Breeding Co. (Kanagawa, Japan). Rats were individually kept in wire cages in a temperature-controlled room ($24 \pm 1^\circ\text{C}$) with a 12-h light-dark cycle (lights on 6:00–18:00), and fed *ad libitum* a purified diet (25% casein) (Oriental yeast Co., Ltd., Tokyo, Japan). After a 3-day (experiment 1) or 5-day (experiment 2) adaptation period, the rats were divided into four groups (control, CT, CTC, CTC+CT), and received daily hormonal treatment for 6 days in experiment 1 or a single treatment before starting the experiment in experiment 2.

CTC groups received subcutaneous injection of CTC at 10:00 at levels of 10 mg, and 5 mg/100 g body weight/day in experiment 1 and 2, respectively. CTC was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and it was dissolved in corn oil and injected. The CT group received subcutaneous injection of 100 m unit elcatonin/100 g body weight at 10:00. Elcatonin is a synthetic and stable eel calcitonin, which was purchased from Asahi Chemical Industry Co., Ltd. (Osaka, Japan). Elcatonin was dissolved in physiological saline containing 1% bovine albumin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The control group received vehicle injections.

Body weight and food intake were recorded daily, and 24-h urine was collected to measure $N^{\pm}$ -methylhistidine and calcium excretions. The urine was made up to 100 ml with water after addition of 1 ml 2N HCl, filtered through filter paper and frozen at -20°C until analysis. At the end of the experimental period, rats were killed by decapitation and blood was collected in a heparinized test tube, quickly centrifuged at $5,900 \times g$ for 10 min at 25°C to separate the plasma, and stored at -20°C until analysis.

Chemical analysis. Urinary $N^{\pm}$ -methylhistidine was measured by high performance liquid chromatography (HPLC) as previously described (12). Rat urine

was hydrolyzed in 6N HCl at 110°C for 2 h. The hydrolysates were cooled and made up to 50 ml with water. Fifteen ml of this was evaporated to remove HCl. The residue was dissolved in 5 ml of 0.2 M pyridine and 3 ml of this was applied to a cation-exchange resin column (7×150 mm, Dowex 50W×8, 200–400 mesh, pyridine form). After eluting most of acid and neutral amino acids with 20 ml of 0.2 M pyridine, *N*^ε-methylhistidine was eluted with 20 ml of 1 M pyridine and collected. The eluant was then evaporated and the residue was dissolved in 1 ml of mobile phase (15 mM sodium octane sulfonate in 20 mM KH₂PO₄) and 20 μl of this was used for HPLC analysis. The analysis of *N*^ε-methylhistidine was carried out using a Shimadzu LC6A equipped with a Zorbax ODS column (4.6×150 mm). The column was attached to an oven at 45°C. A Shimadzu fluoromonitor (RF-535) with an excitation wavelength of 348 nm and emission wavelength of 460 nm was used to monitor the fluorescent product.

The plasma CTC concentration was determined by HPLC by the method of Scott and Dixon (13). One ml of plasma, 0.1 ml of 0.25 M NaOH and 7 ml of dichloromethane were mixed and gently shaken for 1 min in a stoppered tube. After centrifugation at 1,300×*g* for 10 min at 25°C, the supernatant was removed and 5 ml of the organic layer was evaporated under reduced pressure. The residue was dissolved in 100 μl of mobile phase (45% w/v methanol) and 80 μl of this was used for HPLC analysis using a Shimadzu LC6A chromatography system equipped with a Shim-pack CLC-ODS column (6×150 mm) with UV detection at 248 nm. The column was attached to an oven at 30°C.

Urinary calcium concentration was measured by atomic-absorption spectrophotometry. A hundred μl of urine was wet-ashed by heating at 240°C for 24 h in 0.5 ml HNO₃ and HClO₄ (1:1) in 5-ml glass tubes. The residues were dissolved in 4 ml solution containing 0.54 M HCl, 20% (v/v) ethanol and 64 mM SrCl₂, and calcium concentrations were determined in a Hitachi 170-30 atomic absorption spectrophotometer.

Statistical analysis. Data were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System.

RESULTS

Experiment 1

Effects of CTC and CT administrations on body weight, food intake, urinary *N*^ε-methylhistidine excretion and urinary calcium excretion in experiment 1 are shown in Table 1.

Growth was inhibited when the animals were treated with CTC or CTC plus CT treatment, and a significant effect of CTC was shown by ANOVA. However, the effect of CT was not significant. Food intakes were decreased by CTC treatment. CT alone did not affect food intake, but concomitant administration of CT tended to normalize the CTC-induced decrease in food intake. Urinary *N*^ε-methylhistidine excretion was markedly increased to 2.4-fold the control by

Table 1. Effect of calcitonin administration for 6 days on body weight gain, food intake, urinary N^{τ} -methylhistidine excretion and urinary calcium excretion in rats treated with corticosterone (experiment 1).

Groups	Initial body weight (g)	Body weight gain (g)	Food intake (g)	N^{τ} -methylhistidine excretion ($\mu\text{mol}/100\text{ g BW}/6\text{ days}$)	Urinary calcium excretion ($\mu\text{g}/100\text{ g BW}/6\text{ days}$)
C	197	43 ^a	100.8 ^a	6.0 ^c	5,075 ^a
CT	199	36 ^a	100.4 ^a	6.6 ^c	4,890 ^a
CTC	196	-2 ^b	87.2 ^b	14.2 ^a	4,221 ^b
CTC+CT	197	-1 ^b	94.6 ^{ab}	11.1 ^b	4,917 ^a
Pooled SEM	1.1	4.9	2.26	0.74	81
ANOVA					
CT	NS	NS	NS	$p < 0.01$	$p < 0.05$
CTC	NS	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
CTC $\times$ CT	NS	NS	NS	$p < 0.01$	$p < 0.01$

Values are Ms for five or six rats in each of the following treatment groups: control (C), calcitonin (CT), corticosterone (CTC), corticosterone plus calcitonin (CTC+CT) receiving subcutaneous injection of vehicle, 100 m unit CT, 10 mg CTC, 10 mg CTC plus 100 m unit CT per 100 g body weight (BW)/day, respectively, and pooled SEM. Data were analyzed by analysis of variance and Duncan's multiple range test using Statistical Analysis System. Means in the same column without common letters are significantly different ($p < 0.05$). NS, not significant ($p > 0.05$).

CTC treatment and, very interestingly, the CTC-induced increase in N^{τ} -methylhistidine excretion was significantly reduced by concomitant administration of CT. The effect of CT and the interaction of CTC and CT on urinary N^{τ} -methylhistidine excretion was shown to be significant. Urinary calcium excretion was decreased by CTC treatment and CT normalized the CTC-induced decrease in urinary calcium excretion. ANOVA showed that the effects of CTC and CT and interaction of CTC and CT on urinary calcium excretion were all significant.

Experiment 2

Effects of CTC and CT administrations on body weight, food intake and urinary calcium excretion in experiment 2 are shown in Table 2.

Significant growth inhibition by CTC treatment was also found in the present experiment although the dose level of CTC was decreased to 5 mg/100 g body weight/day. In the present experiment, the dose level was reduced because Tomas et al. (1) have reported that muscle protein breakdown was significantly increased by both 5 mg CTC/100 g body weight and 10 mg CTC/100 g body weight in adrenalectomized rats. CT did not affect body weight gain. The reason why food intakes were not changed significantly by all the treatments might be that the experimental period was short (24 h). Urinary calcium excretion was decreased by CTC, and the decrement was normalized by concomitant administration of CT, as

Table 2. Effect of calcitonin administration for 24 h on body weight gain, food intake and urinary calcium excretion in rats treated with corticosterone (experiment 2).

Groups	Initial body weight (g)	Body weight gain (g)	Food intake (g)	Urinary calcium excretion ($\mu\text{g}/100\text{ g BW}/24\text{ h}$)
C	162	3.6 ^a	15.1	883 ^b
CT	165	4.5 ^a	15.1	1,332 ^a
CTC	163	-0.9 ^b	15.2	603 ^b
CTC+CT	165	1.4 ^{ab}	14.9	933 ^{ab}
Pooled SEM	1.2	0.91	0.24	88
ANOVA				
CT	NS	NS	NS	$p < 0.05$
CTC	NS	$p < 0.05$	NS	$p < 0.05$
CTC $\times$ CT	NS	NS	NS	NS

Values are Ms for five or six rats in each of the following treatment groups: control (C), calcitonin (CT), corticosterone (CTC), corticosterone plus calcitonin (CTC+CT) receiving subcutaneous injection of vehicle, 100m unit CT, 5mg CTC, 5mg CTC plus 100m unit CT per 100g body weight (BW), respectively, and pooled SEM. Data were analyzed by analysis of variance and Duncan's multiple range test using Statistical Analysis System. Means in the same column without common letters are significantly different ($p < 0.05$). NS, not significant ($p > 0.05$).

was observed in experiment 1. However, CT alone significantly increased calcium excretion. This is not consistent with the findings of experiment 1, but maybe due to the difference in the experimental period.

Changes in the plasma CTC concentration in experiment 2 are shown in Table 3. At 6 h after treatment, plasma CTC was markedly increased to 2.9-fold the control by CTC, and it was reduced by concomitant administration of CT. CT alone did not affect the plasma CTC concentration at 6 h after the treatment. At 12 h, no differences were observed between the groups. However, at 24 h, the plasma CTC concentration of the CT group was more than 5-fold that of the control group. This was thought to be due to diurnal variation of the plasma CTC concentration. When all the data were subjected to ANOVA, there were significant effects of CTC and time, and the interactions between CTC and CT and between CTC and time were also significant, but the effect of CT and the interactions between CT and time and among CTC, CT and time were not significant.

DISCUSSION

In the present experiment, the effect of CT administration on growth, muscle protein breakdown, urinary calcium excretion and plasma CTC concentration was examined in rats treated with CTC.

The catabolic effects of CTC on growth and muscle proteolysis were clearly shown in the present study. This has been shown in previous reports as well (2,3).

Table 3. Effect of calcitonin administration for 24 h on plasma corticosterone concentration in rats treated with corticosterone (experiment 2).

Groups	Plasma corticosterone concentration (ng/ml)				
	Time				Pooled SEM
	0 h	6 h	12 h	24 h	
C	307 B	581 ^b , A	341 B	84 ^b , C	60
CT		554 ^b	501	445 ^a	37
CTC		1,682 ^a , A	472 B	331 ^a , B	246
CTC+CT		851 ^{ab} , A	394 B	270 ^a , B	82
Pooled SEM		173	41	38	
ANOVA					
CT			NS		
CTC			$p < 0.05$		
Time			$p < 0.01$		
CTC×CT			$p < 0.05$		
CT×Time			NS		
CTC×Time			$p < 0.05$		
CTC×CT×Time			NS		

Values are Ms for five or six rats in each of the following treatment groups: control (C), calcitonin (CT), corticosterone (CTC), corticosterone plus calcitonin (CTC+CT) receiving subcutaneous injection of vehicle, 100 m unit CT, 5 mg CTC, 5 mg CTC plus 100 m unit CT per 100 g body weight (BW), respectively, and pooled SEM. Data were analyzed by analysis of variance and Duncan's multiple range test using Statistical Analysis System. Means in the same column without common small letters and in the same row without common capital letters are significantly different ($p < 0.05$). NS, not significant ($p > 0.05$).

Although it has been reported that large doses of CT induce body weight loss (14), CT did not affect growth in the present experiment. ANOVA showed that urinary N^{ϵ} -methylhistidine excretion was increased by both CTC and CT administrations, but that the effect of CTC was far greater than that of CT. The present study is thought to be the first one showing the effect of CT on muscle protein breakdown, and the interactive effect of CTC and CT on N^{ϵ} -methylhistidine excretion shows that CT minimizes the CTC-induced increase in the rate of muscle protein breakdown.

Urinary calcium excretion was decreased by CTC, and this was normalized by concomitant administration of CT. CTC-induced hypercalcemia might be caused by the increase in bone resorption and the decrease in urinary calcium excretion. CT might counteract the CTC-induced hypercalcemia by preventing bone resorption and increasing calcium excretion.

The results of experiment 2 were consistent with those of experiment 1 although urinary N^{ϵ} -methylhistidine excretion was not measured in experiment 2. In experiment 2, urinary calcium excretion was decreased by CTC, and this was

normalized by concomitant administration of CT, as was shown in experiment 1. It seems that CTC induces hypercalcemia and results in the increase in muscle protein breakdown. Thus, CT might minimize CTC-induced muscle proteolysis by normalizing the hypercalcemia.

It has been reported that the plasma CTC concentration shows diurnal variation (1), and the value in the control group at 6 h was significantly higher, and that at 24 h was significantly lower than those at 0 and 12 h, respectively. The values at 0 and 24 h should be same because they were from same time of day. However, at present, we can not explain the difference between 0 and 24 h.

It has been reported that CT increases the plasma ACTH concentration (15, 16). In the present experiment, CT tended to increase the plasma CTC concentration at 12 and 24 h after the treatment, as was expected. This might cause a slight increase in muscle proteolysis although N^{ϵ} -methylhistidine excretion was not measured during this period. However, very interestingly, CT decreased the plasma CTC concentration of the CTC-treated rats. The metabolic rate of CTC might be increased by CT administration.

It was clearly shown in the present study that CTC-induced acceleration of muscle protein breakdown is reduced by concomitant administration of CT. Furthermore, it was shown that CT reduces the CTC-induced increase in the plasma CTC concentration. These results indicate that the suppression of the CTC-induced increase in muscle proteolysis by CT might be due to the reducing effect of CT on CTC-induced hypercalcemia. However, further experiments are needed to clarify the effect of CT on calcium concentrations in blood and muscle, and especially on muscle calpain activity in CTC-treated rats.

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