Factors Other than ACTH for Manifestation of Circadian Rhythm in Blood Corticosterone Levels in the Rat

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ABSTRACT. In order to examine further the existence of factors contributing to the circadian adrenocortical rhythm, blood corticosterone response to ACTH was determined in the morning and in the evening, using rats pretreated with dexamethasone and Nembutal. By the treatment with dexamethasone at 1300 h for 2 days, plasma ACTH was decreased to nondetectable levels, and the daily variations of blood corticosterone levels were abolished. Adrenal corticosterone content was also minimized. In these rats, following a single injection of 2 or 20 mIU ACTH, a significant increase of blood corticosterone level was observed and this response was greater in the evening than in the morning. An addition of the serum obtained from the rat in the evening and freed both ACTH and corticosterone augmented the responsiveness of cultured adrenal cells to ACTH. This ACTH potentiating effect was not detectable in the serum obtained in the morning. In intact rats, the evening levels of plasma ACTH showed a large individual variation. And the ACTH mean level was higher in the evening than in the morning. The following conclusion was drawn; 1) adrenocortical response to ACTH is augmented by humoral factors other than ACTH. 2) however, peak of circadian adrenocortical rhythm may be brought about mainly by pulsatile secretion of ACTH in the evening.—Key words: ACTH, circadian rhythm, cortisone, rat.


INTRODUCTION

There have been some recent observations which seem to indicate that any factors other than adrenocorticotropic (ACTH) are involved in the fine control of the circadian adrenocortical rhythm in the rat [1, 3, 8]. The amplitude of circadian rhythm in plasma ACTH levels is much smaller than that of plasma corticosterone, and the difference of ACTH levels between the morning and the evening does not reach at the statistical significance [1, 10]. The rhythm of adrenal response to ACTH is found to persist in rats pretreated with dexamethasone-Nembutal [1, 3]. The injection of fixed amount of ACTH maintains the corticosterone rhythm in hypophysectomized rat [8]. Ottenweller et al. [8] reported that the autotransplanted adrenal gland without neural input secreted corticosterone at a normal intermediate level, but failed to manifest the circadian periodicity. Contrarily, we observed the circadian rhythm of blood corticosterone levels in similar autotransplanted rats, and suggested that some humoral factors other than ACTH might be involved in controlling of circadian adrenocortical rhythm [5]. Further, we reported that repeated injections of small amount of ACTH brought about a dramatic increase in blood corticosterone levels, and assumed that clustering of episodical secretion of ACTH produced the peak point of circadian adrenocortical rhythm [6].

Therefore, in the present study, in order to confirm the existence of humoral factors other than ACTH increasing the blood corticosterone levels, it was decided to show that serum free from ACTH augments the adrenocortical response to ACTH in cultured cells. In addi-
tion, a large individual variation of ACTH levels was observed in the evening as compared with that in the morning. This was considered to reflect a pulsatile nature of ACTH secretion in the evening.

MATERIALS AND METHODS

Wister albino rats were housed 5 per plastic cage under an artificial lighting schedule of 12:12 (L:D) with illumination between 0700 h and 1900 h. They were allowed to feed and drink ad libitum. Three months old female virgin rats were used.

In the first experiment, dexamethasone (Fujita pharm. Co) was given, sc, in 100 µl/100 g body weight, at 1300 h for 2 consecutive days. To examine the inhibitory effect of dexamethasone on the daily variation of ACTH, blood corticosterone levels were determined individually at 4 hour intervals starting at 1800 h on the first day, and adrenal corticosterone content and plasma ACTH levels were determined at 1800 h and 0900 h for 2 days. Porcine ACTH (Sigma; 71 IU/mg) was dissolved in 0.005 N HCl: 0.9% NaCl solution. Two or 20 mIU (0.2/ml) ACTH were injected, iv, to 48 rats at 1800 h on the second day or 0900 h on the third day of dexamethasone treatment. In 24 control rats, 0.005 N HCl: 0.9% NaCl solution was injected with the same schedule. All rats were anesthetized with Nembutal (pentobarbital sodium; 4 mg/100 g body weight) 20 min before the ACTH or vehicle injection. Blood samples were obtained individually 0 (just before the injection), 15, 30 and 60 min after the ACTH injection, from the tail tip incision.

In the second experiment, to examine whether or not the ACTH potentiating factors are present in the blood, blood samples were collected by decapitation at 1800 h (PM serum) or 0800 h (AM serum) from each 15 rats whose adrenals were removed 3 days before. The blood was stored at 4 C for 12 h, and serum was obtained and pooled. Two ml charcoal-dextran T 80 solution (charcoal; 6.25 g, dextran T 80; 0.625 g/100 ml 0.9% NaCl) was added to each 20 ml serum, to remove ACTH. Then serum was incubated for 20 min at 4 C. They were centrifuged at 10000 rpm for 10 min, and supernatants were collected. The same procedure was repeated two times more. Finally, the supernatant was filtrated with Millipore filter (pore size 3.0 µm). ACTH and corticosterone concentrations were determined in 500 µl and 50 µl supernatant fluid, respectively. The remaining fluid (18 ml) was lyophylized.

Forty adrenals were obtained from intact rats of both sexes. The adrenal cortices removed from capsule and medulla were incubated at 37°C for 35 min with 15 ml 137 mM NaCl, 5 mM KCl, 0.7 mM Na2PO4, 0.36 mM CaCl2, 10 mM glucose and 25 mM Hepes containing 0.4% collagenase (Sigma; Type I) and 1% bovine albumin (Sigme; Type V) (PH 7.4) in shaking water bath. Digested tissues were dispersed into cells by flushing through a 5 ml plastic pipet. Then the cell suspension was filtered through a layer of silk screen (mesh 12). The filtrate was collected in a centrifuge tube and spun at 800xg for 5 min at 4°C. The pellets were resuspended in 15 ml DME medium, and flushed to wash the cells. After dissociated cells were washed with DME medium for 4 times, the cells were incubated in 1.5 mm Nunclon plastic dishes at a concentration of 1×10^5 cells/dish. The cells were glown in 0.5 ml DME medium (PH 7.4) containing 5% fetal calf serum (GIBCO, Grandisland, NY), 20% horse serum in humidified atmosphere of 7% CO2, 93% air at 37°C. The cells attached to dish surface were used for experiment after 3 days of culture.

Cells were pre-incubated at 37°C for 30 min with Hepes buffer (PH 7.2). Then medium was removed by aspiration. Thereafter the cells were incubated with or without ACTH at 37°C for 15, 30 or 60 min with 0.5 ml buffer containing AM-serum (50 µl) or PM-serum (50 µl). AM-serum and PM-serum were prepared by adding 18 ml H2O to
lyophylized powder. In this experiment, the dose of ACTH was fixed to 0.05 mIU (about 0.7 ng), since, in a preliminary examination, 0.05 mIU ACTH resulted in a time-dependent increase of corticosterone output between 15 and 60 min in the adrenocortical cell culture. After medium was obtained with aspiration at the end of incubation, corticosterone concentration of medium was measured.

In the third experiment, 36 intact rats were killed by decapitation at 0700 h and 1800 h determining the plasma ACTH level. They were individually caged and handled repeatedly and gently to minimize the stress at the blood sampling by decapitation. About 3 ml blood was collected to a tube containing 3 mg EDTA and 20 μl trasyrol, a protease blocker, and plasma were separated and stored at −40°C. ACTH was extracted from 1 ml plasma following the method of Lesley et al. [4].

Corticosterone levels were determined by the method of Murphy [7] with modification [9]. ACTH levels were determined by the radioimmunoassay reported by Lesley et al. [4]. Iodinated 1–39 ACTH was purchased from New England Nuclear. Anti-ACTH serum was generated using a rabbit immunized with antigen of 1–24 ACTH-BSA conjugated. A comparison of bioassayable ACTH and immunoassayable ACTH [4] in the same pituitary sample yielded B/I ratio of 0.97±0.08 (n=8). The minimum detectable level was 10 pg/tube. The intraassay and interassay variances were 4.9% (n=5) and 7.9% (n=6), respectively.

Student's t test was employed for statistical comparison between experimental groups, while one-way analysis of variance was used to analyze the daily variation of blood corticosterone levels in serial samples.

RESULTS

A clear circadian rhythm was observed in blood corticosterone levels in the control rat treated with saline. On the other hand, treatment with dexamethasone for 2 days completely prevented the increase of basal level of blood corticosterone in the evening (Fig 1). Adrenal corticosterone content was equalized in the AM and PM groups (PM on the second day: 7.2±1.6, AM on the third day: 7.0±1.3 ng/mg tissue). Plasma ACTH decreased to non-detectable level after dexamethasone treatent. Under this condition, a significant increase of blood corticosterone levels following a single injection of 2 or 20 mIU ACTH was observed (Fig 2). A significant AM-PM difference was observed in the blood corticosterone response to ACTH with a greater response in the evening. On the other hand, injection of acid saline (0.005 N HCl: 0.9% NaCl) failed to increase blood corticosterone levels in both groups (AM and PM).

After the rat serum was treated with charcoal-dextran, ACTH and corticosterone concentrations were found to be less than 10 pg (7.1×10⁻⁵ mIU)/0.5 ml aliquot and less than 100 pg/0.05 ml aliquot, respectively, in both AM and PM serum treated with charcoal-dextran. Fig 3 shows the corticosterone output into culture medium after addition of ACTH, AM-serum, PM-serum or their combinations. A 2–3 fold stimulation of corticosterone output into medium was observed 15, 30 and 60 min after the addition of ACTH. Addition of PM-serum plus ACTH resulted in a further increase in corticosterone output. On the other hand, the corticosterone output after the addition of ACTH plus AM-serum was not different from output in the presence of ACTH alone. No stimulatory effect on the corticosterone output was observed of the serum without the presence of ACTH.

ACTH levels in the plasma obtained from intact rats in the morning fell in a relatively small range from 9.8 to 30.2 pg/ml. While, ACTH level in the evening showed a marked individual variation scattering over a wide range from 15.4 to 93.6 pg/ml. Difference in
Fig. 1. 24 hr patterns of blood corticosterone level in rats treated with dexamethasone (solid line) or saline (broken line). The figure shows the mean value (±SEM) of corticosterone of five rats. No significant variation was observed in blood corticosterone levels in the dexamethasone treated rat.

Fig. 2. The AM-PM difference in blood corticosterone response to ACTH in dexamethasone pre-treated rats. 2 mIU (circle), 20 mIU (square) of ACTH or acid saline (triangle) was injected 0900 h (open symbols) or 1800 h (solid symbols). Zero min represents the time immediately before the injection. Each symbol and vertical bar represents the mean ±SEM of 12 rats. A significant AM-PM difference was observed in blood corticosterone level after injection of ACTH (P<0.01).

Fig. 3. ACTH-potentiating effect of the serum in the corticosterone output from adrenocortical monolayer cultured cells. After pre-incubation for 30 min, AM-serum (AM-S) or PM-serum (PM-S) was added together with (solid line) or without (broken line) ACTH. Incubation medium was collected 15, 30 and 60 min after addition. A significant difference was observed between reactions to PM-S-ACTH and ACTH (P<0.01).

the mean as well as the variance of ACTH level between AM and PM plasma were statistically significant.

DISCUSSION
By the injection of dexamethasone for 2 days, the adrenal corticosterone content was equalized between the morning and the evening, and the daily variation in the blood corticosterone levels was erased. The ACTH level stayed at the nondetectable level after the dexamethasone treatment. Under this condition, a single injection of ACTH resulted in a significant increase of blood corticosterone level with a greater response elicited in the evening. This result is agreement with the observation by Dallman et al. [1]. They reported that the rhythm of adrenal response to ACTH was found to persist in rats treated with dexamethasone [1]. Metabolic clearance rate of plasma corticosterone in the evening is not different from that in the morning [3]. Therefore, the present study together with observation by Dallman et al. [1] demonstrates that some factors other than ACTH augment the responsiveness of adrenal gland to ACTH in the evening. In the pre-
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Fig. 4. Plasma ACTH concentrations in intact rats. Each symbol represents the value of an individual rat. The mean levels in the AM and PM groups were 21.2 and 54.6 pg/ml, respectively. The difference was statistically significant (P<0.05).

vious report, we assumed that the humoral factors other than ACTH are involved in the manifestation of the circadian adrenocortical rhythm, since the rat with the autotransplanted adrenal without neural input also showed a circadian rhythm in blood corticosterone levels [5].

In the present study, ACTH-potentiating effect on adrenal steroid secretion was observed in the serum obtained in the evening. After charcoal-dextran treatment, ACTH and corticosterone concentrations in the serum depleted by less than $7 \times 10^{-5}$ mIU and 100 pg, respectively. It is not likely that this amount of ACTH or corticosterone affects the adrenal steroid secretion. Therefore, these results suggest that any ACTH-potentiating factors are present in the blood obtained in the evening. The entity of this factor is unknown. However, recently Iida et al. [2] reported that ACTH potentiating activity was observed in the extract from cerebrum, thyroid, lung or liver. The PM-serum may contain more cholesterol (corticosterone precursor) than AM-serum, since free cholesterol may be but lipoprotein-bound cholesterol may not be removed by charcoal-dextran treatment.

Dallman et al. [1] have suggested that the diurnal change of adrenal responsiveness to ACTH rather than change in plasma ACTH level may cause the circadian adrenocortical rhythm, since the daily variation of plasma ACTH level is much smaller than that of plasma corticosterone. In fact, the difference between ACTH in the morning and that in the evening is not always statistically significant [1]. In the present study, however, it is likely that ACTH is predominant in inducing of peak of blood corticosterone rhythm as compared with the putative adrenotropic factors other than ACTH. Because 1) suppression of ACTH secretion by dexamethasone abolished the daily variation of blood corticosterone level; 2) plasma which shows ACTH-potentiation did not stimulate corticosterone secretion from adrenocortical cells without ACTH; 3) the injection of ACTH resulted in the increase of blood corticosterone level in the evening as well as in the morning when the blood corticosterone level was very low in normal rats; 4) the mean level of plasma ACTH was significantly higher in the evening than in the morning (Fig 4). These results suggest that the daily rhythm of blood corticosterone level is brought about mainly by the daily variation of plasma ACTH. We reported previously that repeated injections of small amount of ACTH produced a dramatic increase of blood corticosterone levels, suggesting that ACTH acting on the adrenal gland with pulses markedly stimulates the steroidogenic response of the adrenal [6]. An individual variation in plasma ACTH level in the evening seems to imply that the secretory pattern of ACTH is pulsatile rather than tonic in the evening.

In conclusion, 1) adrenocortical response to ACTH is further augmented by some
humoral factors other than ACTH. 2) However, daily rhythm of blood corticosterone level may be brought about mainly by the daily variation of plasma ACTH. Especially, peak of adrenocortical rhythm may be brought about by pulsatile secretion of ACTH occurring in the evening.

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REFERENCES

要約
ラット副腎皮質ホルモンの日周リズム成立に対するACTH以外の因子について：村上 昇（宮崎大学農学部家畜生理学教室）——ラットにデキサメタゾンを2日連続投与し、血中の副腎皮質刺激ホルモン（ACTH）、副腎皮質ホルモン（CS）、および副腎のCS含量の日内変動を消去した後、ACTHを午前あるいは午後に単一投与すると、血中のCSレベルにおいて、午後投与の方が著明に高い反応性を認めた。この増強因子が液性のものであるか否かを知るために、副腎摘出ラットを午前または午後に屠殺し、得た血清中からACTHを完全に除去した後、副腎細胞の単層培養系に添加した。結果、午後の血清のみ、ACTHのCS分泌能を促進させる効果が認められた。一方、正常ラットの血中ACTHレベルを測定すると、午後の方が有意に高い値を示し、個体間には大きな変異があり、ACTHの律動分泌の存在が示唆された。以上のことから、午後に、副腎のACTHに対する反応性を高める因子がラット血中に存在することが推察され、また、血中のACTHレベルは午後に上昇し、その分泌パターンは律動的である可能性が示唆された。