Endocrine Journal 2007, **54** (5), 703–711

Physiological Roles of Prolactin in the Adrenocortical Response to Acute Restraint Stress

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Abstract. The present study characterized the different hormonal responses to stress in the endocrine milieu with different circulating levels of prolactin (PRL) and examined the direct effects of PRL on adrenal steroidogenic responses to adrenocorticotropic hormone (ACTH) using experimentally induced hyperprolactinemia and hypoprolactinemia male rat models. Hyperprolactinemia was induced by transplantation of two adult female rat anterior pituitary glands under the kidney capsule for 2 weeks, and hypoprolactinemia was induced by daily subcutaneous injection of 2-Bromo-alpha-Ergocryptine (CB-154) for 2 weeks. Under stress conditions, the peak levels of ACTH were significantly higher in hypoprolactinemia than normal rats. Meanwhile, the peak levels of corticosterone and progesterone were significantly higher in hyperprolactinemia than in normal and hypoprolactinemia stressed rats. Results of *in vitro* experiments showed that adrenocortical cells in hyperprolactinemia exhibited higher basal levels of corticosterone and progesterone rats than normal and hypoprolactinemia rats. The stimulatory effect of ACTH on corticosterone and progesterone release was higher in hyperprolactinemia than hypoprolactinemia rats. In addition, PRL increased the stimulatory effect of ACTH-induced corticosterone secretion in all rat models. These results suggest that hypoprolactinemia and hyperprolactinemia rats exhibit marked differences in the response of their hypothalamic-pituitary-adrenal (HPA) axis during acute restrain stress. Additionally, these studies emphasize that the adrenal cortex might be more sensitive to ACTH stimulation in endocrine milieu with high levels of PRL resulting in high corticosterone and progesterone release.

Key words: Hyperprolactinemia, Hypoprolactinemia, Stress, HPA axis

(*Endocrine Journal* **54**: 703–711, 2007)

PROLACTIN (PRL) is a pleiotropic protein hormone that is secreted from specialized cells of the anterior pituitary gland, the lactotrophs. Several studies in the literature have examined the role of PRL on adrenal gland function as well as its effects on lactation and reproduction [1–6]. Although adrenocorticotropic hormone (ACTH) is generally considered to play a major role in the regulation of adrenocortical secretion, previous reports showed that PRL could act directly on adrenal gland to drive corticosterone secretion [2, 4, 6, 7–10]. Additionally, PRL receptors (PRL-R) are expressed in the adrenal cortex in several species [1, 7, 11–13].

Disruption of PRL secretion influences the function of HPA axis. It has been reported that hyperprolactinemia activates the function of the hypothalamus, pituitary [14] and adrenal glands [15]. In contrast, hypoprolactinemia seems to inhibit the hypothalamo-hyophyseal-adrenal axis. It had been shown that bro-

Received: January 5, 2007
Accepted: June 27, 2007
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mocriptine suppresses the plasma ACTH concentration in patients with Cushing disease and Nelson's syndrome [16–18]. Additionally, treatment with bromocriptin for 6 days significantly reduced both PRL and cortisol concentrations in castrated pigs [19]. Hyperprolactinemia induced an adrenal hypertrophy and increased the storage of cholesterol esters [6], while hypoprolactinemia resulted in reduced activity of cholesterol ester hydrolase in adrenals [1].

Stress is a fundamental response by the neuroendocrine system that allows the organism to adapt to events in the external milieu [20]. The HPA axis is well known to subserve the body's response to stressors. Under stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), and this provokes the release of ACTH from the pituitary. ACTH triggers the secretion of glucocorticoids from the adrenal cortex. It has been proposed that, in addition to stimulation of the HPA axis, simultaneous production of PRL also occurs during the stress response in rodents [21, 22]. Taken together, these observations lead us to postulate that PRL may regulate the secretion of corticosterone in response to stress.

In the present study, we attempted to assess the *in vivo* different hormonal response to stress in three animal models; normal, hypoprolactinemia and hyperprolactinemia rats. In addition, we isolated adrenal cells from normal, hyperprolactinemia and hypoprolactinemia rat models and further investigated the different effect of PRL and/or ACTH on the secretion of corticosterone *in vitro*. In the present study, concentrations of progesterone in plasma and culture media were also measured as the precursor of corticosterone.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), MEM non-essential amino acid solution, penicillin and streptomycin (Invitrogen, Burlington, Ontario, Canada), HEPES (Dojindo, Gaitherburg, MD, USA), collagenase type V, deoxyribonuclease, fetal bovine serum, CB-154 (Sigma-Aldrich Corp., St. Louis, MO, USA), rat adrenocorticotropic hormone (ACTH), rat prolactin (PRL) (NIDDK, NIH, Torrance, CA, USA) were purchased from the sources indicated.

Animals

Adult males Wistar Imamichi rats, age 12 weeks, from the Imamichi Institute for Animals Reproduction (Kasumigaura, Ibaraki, Japan), were used in this study. They were housed 5 animals/cage in a room with controlled lighting (lights on 0500–1900 h), temperature (23 ± 2°C) and humidity (50 ± 10%). They were fed with the rat diet (MR-Breeder, Nosan Corp., Yokohama, Japan) and water *ad libitum*. For hyperprolactinemia rat model, rats were transplanted with two adult female rat anterior pituitary glands (APs) under the kidney capsule for each rat for 2 weeks before experiment according to the method previously described [23]. For hypoprolactinemia rat model, rats were daily subcutaneously injected with 500 μg/0.2 ml/rat of 2-Bromo-alpha-Ergocryptine (CB-154), an inhibitor of prolactin secretion, for 2 weeks [24, 25]. The animals were decapitated 2–3 h after the last CB-154 injection. The experimental protocol was approved by the Animal Ethical Committee in accordance with guide for the care and use of laboratory animals prepared by Tokyo University of Agriculture and Technology.

Response to restraint stress

Normal, hypoprolactinemia and hyperprolactinemia rats were stressed by immobilization in a plastic bag (DecapiCone, Braintree Scientific Inc., Braintree, MA, USA). Blood samples were drawn through the cannula at 0, 15, 30, 60, 120, and 180 min after restraint stress and then after 120 min of resting after 180 min stress. Plasma was harvested from samples following centrifugation and frozen at –20°C until ACTH, PRL, progesterone and corticosterone assay.

In vitro steroid secretion from adrenocortical cells

Adrenal glands were obtained from normal, hypoprolactinemia or hyperprolactinemia rats. The whole glands were used for isolation of adrenal cells, and procedures were performed according to previously described methods [26] with minor modifications. Briefly, the successive steps of cell isolation and cell dissociation were performed in DMEM containing collagenase type V and deoxyribonuclease. After incubation (30 min) at 37°C in collagenase (2 mg/ml, four adrenal fragments per ml) plus deoxyribonuclease (25 μg/ml), cells were disrupted by aspiration with a

sterile 10-ml pipette. After the enzyme treatment, the cell suspension was centrifuged at 200 × g for 5 min and the enzymes were washed out and then filtered through a mesh (pore size, 70 μm). The cell number and viability (over 90%) were assessed by using a hemocytometer and the trypan blue exclusion method. Cells were used in suspension at the concentration of 5×10^4 cells/200 μl, in DMEM medium supplemented with 2% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in 96-well plates for 24 h at 37°C in humidified atmosphere (95% air/5% CO_2) before the start of treatment. After 24 h, media were changed, and cells were incubated in the absence or presence of rat ACTH (10^{-13} to 10^{-7} M) and/or rat PRL (10^{-13} to 10^{-7} M) for 4 h. At the end of the 4-h incubation period, the supernatant was collected and stored at –20°C until assayed for progesterone and corticosterone.

Jugular cannulation

One day before the experiment, rats were anesthetized with ether, and a Silastic cannula (Silastic, Dow Corning, Midland, MI, USA; inner diameter 0.5 mm, outer diameter 1 mm) filled with sterile heparinized saline was inserted through the external jugular vein into the right atrium according to previously described methods [27].

Radioimmunoassay (RIA)

Plasma PRL concentrations were measured using National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) kits for rat PRL. Hormones for iodination were rat prolactin-I-5. The antiserum used was antiprolactin-S-9. Results were expressed in terms of NIDDK rat prolactin-RP-2. The intra- and inter-assay coefficients of variation for prolactin were 3.4 and 5.2% respectively.

Concentrations of ACTH [28], corticosterone [29] and progesterone [30] were measured by double-antibody RIAs using ^{125}I-labeled radioligands as described previously. The intra- and inter-assay coefficients of variation were respectively: 11.3 and 11.9% for ACTH, 9.8 and 17.5% for corticosterone and 9.5 and 16.4% for progesterone.

Statistical analysis

The results were expressed as means ± standard error of the means (SEM). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program. Differences among times of sampling were evaluated by two-way analyses of variance (ANOVA) for factorial or repeated measure design with *post-hoc* testing by Fisher's protected least significant difference (PLSD) test. P values of less than 0.05 were considered to be statistically significant.

Results

Hypothalamic-pituitary-adrenal (HPA) axis response to restraint stress

Immobilization stress induced a sharp increase in plasma concentration of ACTH in normal, hypoprolactinemia and hyperprolactinemia rats (Fig. 1, B). In normal and hyperprolactinemia rats, the peak levels of ACTH were observed on 30 min from beginning of stress stimuli. Although the peak levels of ACTH in hypoprolactinemia rats were observed on 60 min from beginning of stress stimuli, the levels of ACTH were greater than normal and hyperprolactinemia. A significant difference was observed 60, 120 and 180 min after stress as compared with normal, and 180 min after stress as compared with hyperprolactinemia. In normal rats, the peak levels of PRL were observed 15 min after stress, followed by a rapid decline to baseline (Fig. 1, A). As expected, plasma PRL level in hyperprolactinemia rats was 40-fold greater than in hypoprolactinemia rats, but there was no significant change of PRL level by stress response in either hyperprolactinemia or hypoprolactinemia rats. Under restraint stress, plasma concentration of corticosterone and progesterone increased in all rat models (Fig. 1, C and D). The levels of corticosterone and progesterone were significantly higher in hyperprolactinemia rats. Plasma ACTH and corticosterone concentrations showed a high positive correlation ($r = 0.82$) in hyperprolactinemia rats (Fig. 2B). A similar correlation was also found in normal and hypoprolactinemia rats ($r = 0.72$ and 0.73, respectively) (Fig. 2A and C). It is also true for the relationship between ACTH and progesterone (data not shown). However, the results showed that there was a clear difference between hypoprolactinemia and hyper-

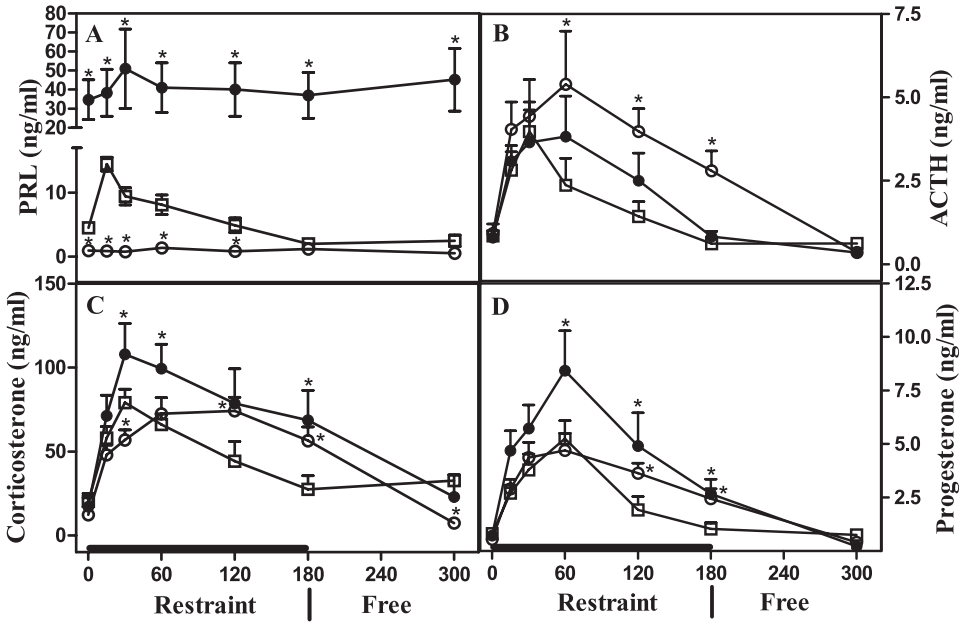

Fig. 1. Effects of restraint stress on plasma concentrations of ACTH (A), PRL (B), corticosterone (C) and progesterone (D) in normal (□), hyperprolactinemia (●) and hypoprolactinemia male rats (○). Each value represents the means ± S.E.M. of five animals. *, $P<0.05$; compared with normal rats.

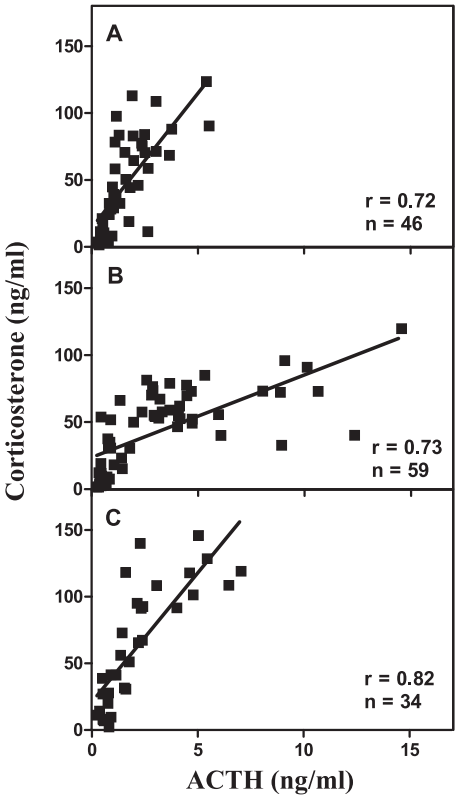

Fig. 2. A positive correlation existed between ACTH and corticosterone levels in normal (A), hypoprolactinemia (B) and hyperprolactinemia male rats (C) during restraint stress.

prolactinemia rats in the stress response of the pituitary and adrenal glands, *i.e.*, secretion of ACTH and corticosterone, respectively. The pituitary stress response of ACTH secretion was higher in hypoprolactinemia rats than hyperprolactinemia rats, whereas the adrenal response of corticosterone secretion was higher in hyperprolactinemia rats than hypoprolactinemia rats.

Direct effects of PRL and ACTH on steroid secretion in vitro

To investigate whether PRL and ACTH could induce corticosterone and progesterone production, primary adrenal cultured cells from normal, hypoprolactinemia and hyperprolactinemia rats were treated with increasing concentrations of PRL (10^{-13} to 10^{-7} M) for 4 h. The difference of basal hormonal level between three rat models was observed. Corticosterone and progesterone production was 3.3- and 1.8-fold, and 4.7- and 1.8-fold in hyperprolactinemia rats than in normal and hypoprolactinemia rats, respectively (Fig. 3, A and B). PRL treatment had no significant effect on the corticosterone and progesterone production in any rat models (Fig. 3). On the other hand, ACTH treatment induced corticosterone and progesterone secretion in a dose-dependent manner in all rat models (Fig. 3). The cor-

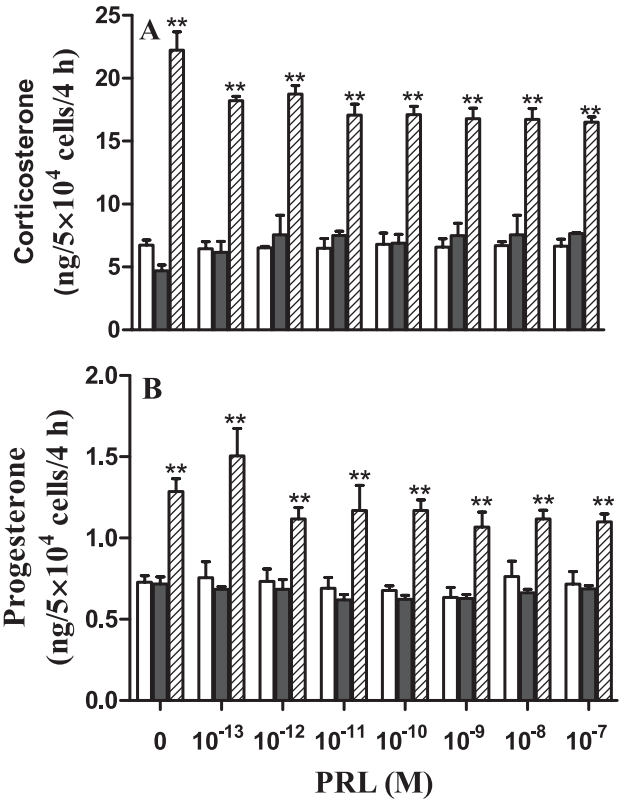

Fig. 3. Effects of PRL (10^{-13} to 10^{-7} M) on release of corticosterone (A) and progesterone (B) in adrenal cells from normal rats (□), hypoprolactinemia rats (■) and hyperprolactinemia rats (▨). Results represent the means ± S.E.M. of two different experiments performed in quadruplicate. *, **, $P<0.05$, $P<0.01$ as compared with the basal levels (0 M), respectively.

ticosterone and progesterone levels in response to ACTH were markedly increased in adrenal cultured cells from normal rats as compared with those from hyperprolactinemia and hypoprolactinemia rats. In addition, the response of ACTH-induced corticosterone release was higher in hyperprolactinemia rats than hypoprolactinemia rats. Although primary hyperprolactinemia and hypoprolactinemia rat adrenal cultured cells treated with ACTH exhibited an increase in their progesterone response, significant differences in progesterone response between hypoprolactinemia and hyperprolactinemia rats were observed at low doses of ACTH stimulation (10^{-13} to 10^{-12} M).

Effects of PRL on ACTH-induced steroid secretion in vitro

To determine whether PRL could co-stimulate the ACTH-induced corticosterone secretion in normal, hypoprolactinemia and hyperprolactinemia rat, the adrenal cells were treated with increasing concentrations of ACTH (10^{-13} to 10^{-7} M) for 4 h, with or without 10^{-13} to 10^{-7} M PRL. PRL (10^{-13} to 10^{-9} M) increased the stimulatory effect of ACTH on corticosterone secretion in all rat models (Fig. 4, A–C). In addition to corticosterone results, PRL increased the stimulatory effect of ACTH on progesterone secretion only in low dose treatment groups (10^{-13} to 10^{-11} M) in normal and hypoprolactinemia (Fig. 4, D–F).

Discussion

The present studies were designed to test the difference of HPA axis response to stress in the endocrine milieu with different circulating levels of PRL. According to the present results, the increased level of plasma PRL in response to transplantation of two adult female rat anterior pituitary glands under the kidney capsule reflects a successfully induced hyperprolactinemia in male rats. In addition, the decreased level of plasma PRL in response to CB-154 injection reflects a successfully induced hypoprolactinemia in male rats. Together with the increased plasma PRL concentration, a high level of plasma corticosterone and progesterone in response to restraint stress was observed. Since hyperprolactinemia has been shown to induce secretion of ACTH [14, 15], the increasing plasma corticosterone during restraint stress may be secondary to an induction in ACTH secretion. However, we have monitored the plasma ACTH during restraint stress and found that hyperprolactinemia did not alter the concentration of ACTH in response to restraint stress as compared with that in normal rats. In the present study, the corticosterone and progesterone release in response to restraint stress were lower in hypoprolactinemia as compared with that in hyperprolactinemia rats. However, no significant difference in ACTH release in response to restraint stress was observed between hypoprolactinemia and hyperprolactinemia. Our observations demonstrated that the adrenal cortex might be more sensitive to ACTH stimulation in endocrine milieu with high levels of PRL resulting in high corticosterone release.

The adrenal responsiveness to ACTH for corticosterone release was suppressed, whereas pituitary responsiveness to CRH release was increased in hypo-

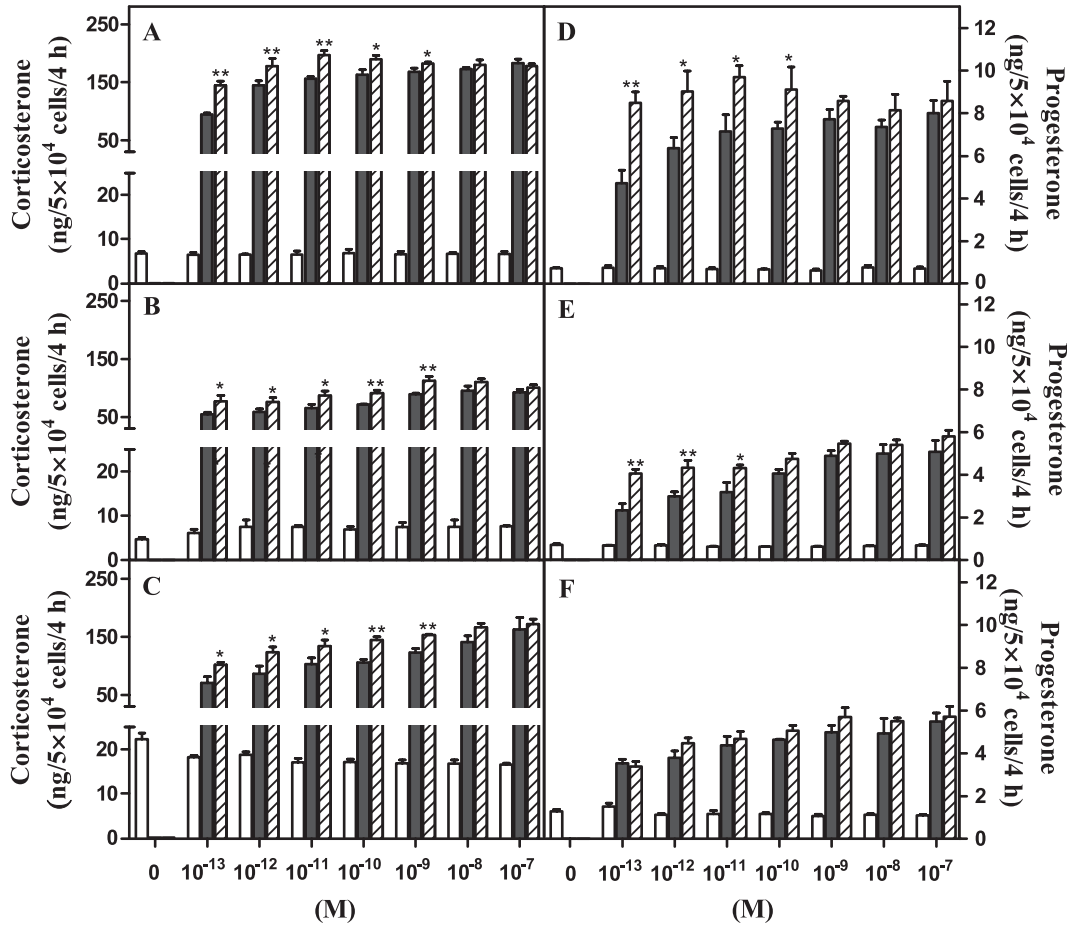

Fig. 4. Effects of PRL (10^{-13} to 10^{-7} M; □), ACTH (10^{-13} to 10^{-7} M; ■) or combination of PRL and ACTH (▨) on release of corticosterone (A, B, C) and progesterone (E, F, G) in adrenal cells from normal (A, D), hypoprolactinemia (B, E) and hyperprolactinemia male rats (C, F). Results are represents the means ± S.E.M. of two different experiments performed in quadruplicate. *, **, $P<0.05$, $P<0.01$ as compared with ACTH-stimulated, respectively.

prolactinemia as compared with hyperprolactinemia. The present study clearly indicated that hypoprolactinemia cause hyposecretion of corticosterone, while results in the hypersecretion of ACTH due to reduction of negative feedback effect by glucocorticoids from the adrenal cortex. These results also suggest that hypersecretion of CRH probably occurs in hypoprolactinemia rats.

Previous studies have shown that PRL could act directly on adrenal gland to drive corticosterone secretion [2, 4, 6, 7–10]. In the present study, PRL stimulated the secretion of corticosterone from cultured adrenal cells in synergy with low levels of ACTH in all rat models. The mechanism by which PRL supports the action of ACTH on the adrenal gland has yet to be fully elucidated. One possibility includes a PRL-induced promotion of adrenal steroidogenesis. It is well known that ACTH regulates glucocorticoid secretion through the cyclic AMP (cAMP) pathway [31–37]. It has been shown that the action of PRL might be mediated through G-protein-adenylate cyclase coupling and cAMP production [38, 39], where in chronic hyperprolactinemia enhances receptor-G-protein-adenylate cyclase coupling and cAMP production [2]. On the other hand, hypoprolactinemia inhibited the activity of adenyl cyclase [5]. The present study suggested that PRL might exert a direct action on the adrenal cortex to enhance the ACTH action though increased cAMP production.

In accordance with the present results suggesting that direct adrenal effects of PRL may be important for steroidogenesis in the response to stress, there are observations from animal studies in which glucocorticoid levels can remain elevated during stress, despite de-

clining CRH and/or ACTH concentrations [20, 21, 40–42]. Previous studies have demonstrated that exercise stress induced an increase in circulating PRL in humans [43–48]. However, the direct effects of PRL on adrenal gland during stress remain to be elucidated.

In rat adrenals, biosynthesis of corticosterone involves the parrticipation of cholesterol side-chain cleavage (rate-limiting step), 3β-hydroxysteroid dehydrogenase (3β-HSD), 21β-hydroxylase, and 11β-hydroxylase activities [49]. In this study, PRL induced an increase in both progesterone and corticosterone by adrenal cells. It has been shown that chronic hyperprolactinemia induces adrenocortical cell hypertrophy, and intracytoplasmic cholesterol and cholesterol esters [6]. The protein expression of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage (P450scc) enzyme in zona fasciculata (ZF) cells were inhibited in hypoprolactinemia induced by bromocriptine-treated rats [5]. A previous study has suggested that PRL increases the release of corticosterone *via* cAMP cascades and 3β-HSD activity [2]. Such evidence lends support to the idea that PRL may contribute to a sustained corticosterone response by regulating adrenal steroidogenesis enzyme.

On the other hand, the high levels of PRL may increase adrenal sensitivity to submaximal doses of ACTH. PRL-R mRNA has been detected in both the adrenal cortex and medulla of many species [7, 11–13]. Adrenal sensitivity to ACTH is regulated by adrenal innervation (for example, splanchnic nerve stimulation) with the subsequent release of catecholamines and vasoactive neuropeptides that affect adrenal blood flow and hence, increase the presentation of ACTH to the steroidogenic cells of the adrenal cortex and result in an increase of corticosterone release [50–58]. Indeed, while accumulated evidence shows the effect of PRL on adrenal innervation remains controversial, the physiological role of PRL, whether circulating or intraadrenal, in this interplay requires further investigation.

In summary, the present results suggest that normal, hypoprolactinemia and hyperprolactinemia rats exhibit marked differences in the response of their HPA axis during acute restrain stress. Based on *in vitro* results, this evidence suggested that, due to high levels of PRL, the adrenal cortex could be more sensitive to ACTH stimulation resulting in high corticosterone and progesterone release.

Acknowledgments

We are grateful to Dr. A.F. Parlow and the Rat Pituitary Hormone Distribution Program (NIDDK, NIH, Torrance, CA, USA) for providing rat prolactin RIA materials and rat prolactin for *in vitro* study, and to Dr. G.D. Niswender, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA for providing antisera to progesterone (GDN337). This study was supported in part by a Grant-in-Aid for Scientific Research (B-18310044, The Japan Thailand Joint Research) from the Ministry of Education, Culture, Sport, Science and Technology of Japan and the Japan Society for the Promotion of Sciences.

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