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ORIGINAL PAPER

Comparative effects of prolactin versus ACTH, estradiol, progesterone, testosterone, and dihydrotestosterone on cortisol release and proliferation of the adrenocortical carcinoma cell line H295R

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Abstract In this study, using the H295R cell line as a model system, we investigated the role of prolactin (PRL) and steroid hormones in the growth regulation and cortisol release of adrenocortical cells. H295R cells were treated with increasing doses (10^{-13}–10^{-6} M) of PRL, adrenocorticotropic hormone (ACTH), 17β-estradiol (E_2), progesterone (P_4), testosterone (T), and dihydrotestosterone (DHT). As expected, ACTH raised cortisol secretion and increased the proliferation rate of cultured cells. Incubation with T, DHT, E_2, and P_4 for 24 h did not significantly increase cortisol release. Conversely, PRL concentrations of 10^{-8}–10^{-6} M caused a significant increase in the release of cortisol. Long-term (5 days) stimulation of H295R cells with E_2, P_4, and PRL was a trigger to increased cell proliferation, while T and DHT did not alter H295R cell proliferation. Taken together, these results indicate that steroid hormones exert differential effects on adrenocortical function. Additionally, the present study demonstrates that PRL had biphasic actions in regulating adrenocortical function. PRL may form a novel regulatory system for steroid hormone secretion and cell proliferation in the adrenal cortex.

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Introduction

Prolactin (PRL) is a pleiotropic protein hormone that is secreted from specialized cells of the anterior pituitary gland, the lactotrophs. There is increasing evidence that PRL is involved in the regulation of adrenocortical function. Evidence has been provided that PRL receptors (PRL–R) are expressed in the adrenal cortices of several species [1–5]. Recent findings indicate that PRL stimulates corticosterone secretion from rat adrenocortical cells [6–12]. Other investigators have reported that hyperprolactinemic rats and mice show an increase in absolute and relative adrenal weight [13–16]. Furthermore, PRL has been shown to be involved in the cell proliferation of normal and neoplastic tissues and to activate the mitogen-activated protein kinase cascade in primary cultures of leiomyoma cells, prostate cells, breast cancer cells, and Nb-2 cell lines [17–19]. However, its effects on adrenocortical cells remain to be elucidated.

In this study, the well-characterized H295R cell line was used to compare the effects of PRL with those of adrenocorticotropic hormone (ACTH), 17β-estradiol (E_2), progesterone (P_4), testosterone (T), and dihydrotestosterone (DHT) on cell proliferation and adrenal steroid release.

Results

Effects of PRL, P_4, T, DHT, E_2, and ACTH on H295R cell proliferation

H295R cell cultures were treated with increasing concentrations (10^{-13}–10^{-6} M) of PRL, ACTH, E_2, P_4, T, or DHT

for 5 days to determine comparative dose response among the hormones. Cell proliferation increased significantly in PRL-, ACTH-, E_2-, and P_4-treated cells as compared with control cells (Fig. 1a–d). In contrast, T and DHT did not increase cell growth significantly compared with the control at any concentrations tested (Fig. 1e and f). Cell growth induced by PRL, ACTH, E_2, or P_4 differed from that of controls at concentrations of 10^{-11} M or higher for ACTH, 10^{-12} M or higher for PRL and P_4, and 10^{-13} M or higher for E_2.

Further analysis of results using ANCOVA revealed a significant difference in the regression slopes between PRL and ACTH, E_2 and P_4 ($P < 0.001$). In addition, comparison of the 95% CIs for the slope of each treatment indicated that cell growth induced by PRL was significantly higher than that induced by any other hormones. Indeed, cell growth induced by ACTH was significantly higher than that induced by E_2, but similar to that induced by P_4.

Effects of PRL, P_4, T, DHT, E_2, and ACTH on steroid release in H295R cell

H295R cell cultures were treated with increasing concentrations (10^{-13} –10^{-6} M) of PRL, ACTH, E_2, P_4, T, or DHT for 24 h. Cortisol concentrations were measured from the conditioned medium. Cortisol levels increased in a dose-dependent manner only after administration of ACTH or PRL (Fig. 2a and b). ACTH induced 1.7-fold increases in cortisol levels at 10^{-13} M and promoted a peak at 12.4-fold at 10^{-7} M (Fig. 2a). PRL induced 2.4-fold increases in cortisol levels at 10^{-8} M and promoted a peak at 12-fold at 10^{-6} M (Fig. 2b). Exposure to E_2 (10^{-13}–10^{-8} M), P_4 (10^{-13}–10^{-9} M), T (10^{-13}–10^{-7} M), and DHT (10^{-13}–10^{-7} M) did not affect cortisol secretion in the dose–response exposures (Fig. 2c–f). Most of the steroid hormone treatments decreased cortisol levels at the higher concentrations except for T treatment, which showed an increase in cortisol secretion at 10^{-6} M. ANCOVA revealed a significant difference in the regression slopes between PRL and ACTH ($P < 0.001$). The comparison of the 95% CIs for the slope of each treatment indicated that cortisol secretion induced by PRL was significantly lower than that induced by ACTH.

Discussion

This study was designed to evaluate the effects of PRL on human adrenocortical cells. Well-characterized, hormonally responsive adrenocortical H295R cells were used to

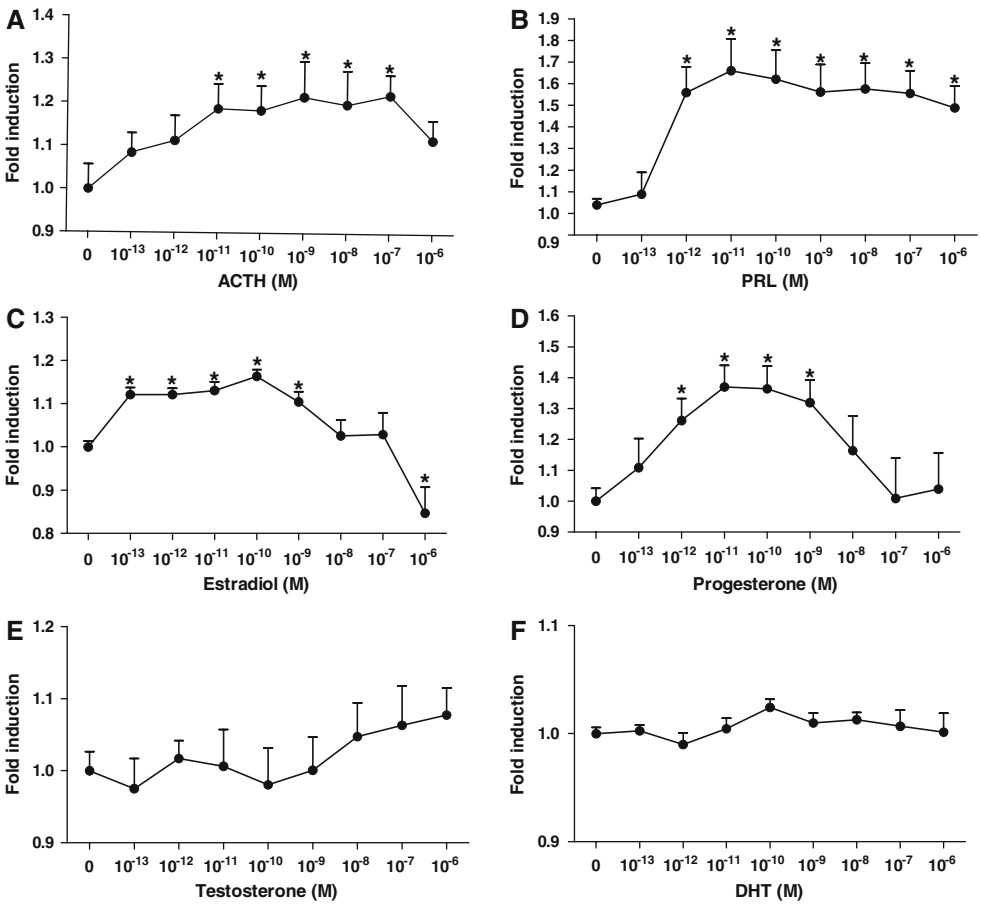

Fig. 1 H295R cell proliferation after treatment with ACTH (**a**), PRL (**b**), E2 (**c**), P4 (**d**), T (**e**), or DHT (**f**) for 5 days. Cell proliferation was measured by CellTiter-GloTM Luminescent Cell Viability Assay. Results are means ± SEM of three different experiments. *, $P < 0.05$ compared with basal proliferation in each treatment

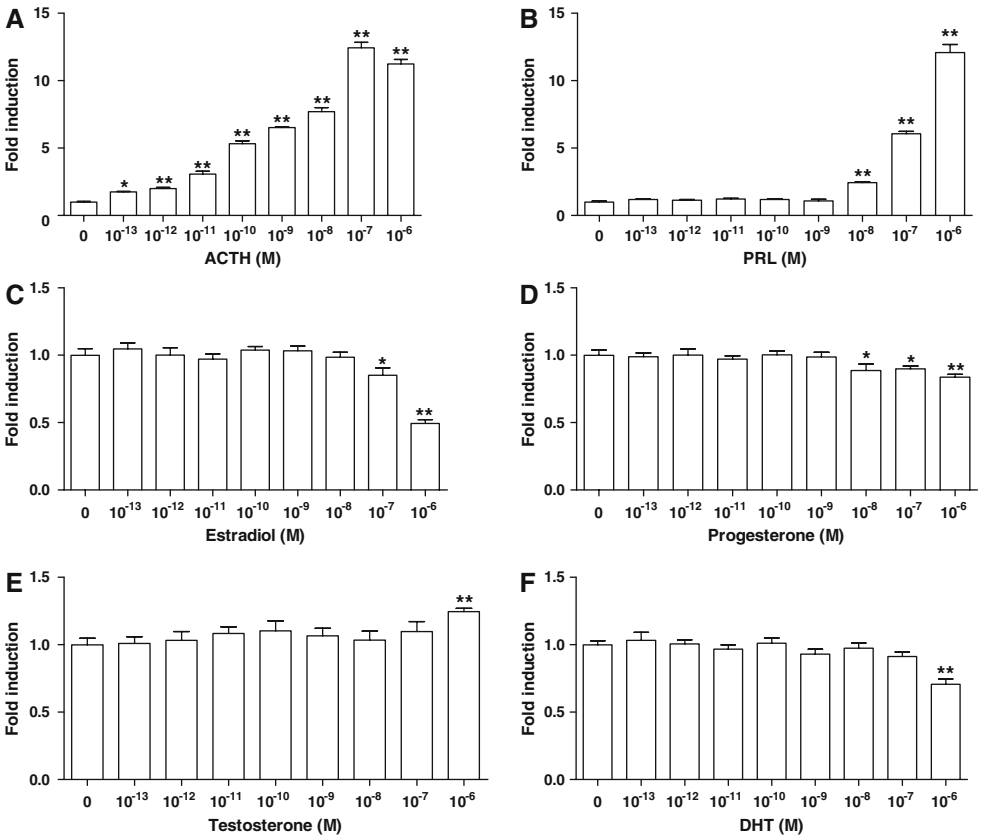

Fig. 2 Dose–response effect of ACTH (**a**), PRL (**b**), E2 (**c**), P4 (**d**), T (**e**), or DHT (**f**) after 24 h incubation on cortisol secretion in H295R cell. Results are means ± SEM of three different experiments. *, **, $P < 0.05$, $P < 0.01$ compared with basal levels in each treatment, respectively

compare the effects of administration of PRL with the effects of ACTH, E_2, P_4, T, and DHT on cell proliferation and cortisol release. In this study, we demonstrated that PRL is implicated in the regulation of cellular proliferation and steroidogenesis in adrenocortical H295R cells. Although ACTH, E_2, and P_4 each stimulated H295R cell proliferation in dose–responsive manner, when compared with PRL treatment the responses were muted. These data highlight the distinctly increased cell-proliferative responses induced by PRL compared with those regulated by ACTH and sex steroid hormones. To date, the mechanism of action of PRL on human adrenocortical growth is not well defined. In agreement with previous studies [13–16] that demonstrated a PRL-induced increase in rat and mouse adrenal weights, our results show direct evidence that PRL is capable of increasing adrenocortical cell proliferation.

This study also clearly demonstrates that PRL is able to induce cortisol release from a human adrenocortical carcinoma cell line. A possible explanation for this phenomenon could be an increase in steroidogenic enzyme activity. We previously found that PRL increases steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage (CYP11A1) gene expression, and corticosterone release from rat adrenal cells [20] However, this study was not designed to address this question, and therefore future studies will be necessary to investigate the specific effects of PRL on steroidogenic enzyme activities. The pattern of response for cortisol observed in H295R cells exposed to PRL differed somewhat from that observed in cells exposed to ACTH. However, the effect of ACTH on cortisol in the H295R assay was greater than for PRL. We hypothesize that PRL is an essential regulator of human adrenocortical cell proliferation.

E_2 and P_4 each stimulated H295R cell proliferation, whereas T and DHT did not alter cell proliferation. Some epidemiologic and experimental studies suggest that sex steroid hormones could be involved in adrenocortical tumorigenesis. In fact, adrenocortical tumors are more frequent in woman than in men, especially in those exposed to estro–progestins [21, 22]. Moreover, a recent study demonstrated that 17β-estradiol enhances proliferation of the human adrenocortical carcinoma cell line NCI-H295R [23], whereas androgens inhibit NCI-H295R cell proliferation [24, 25]. The present study found that E_2 and P_4 increased H295R cell proliferation, with no direct effect on cortisol secretion. This agrees with a previous study showing that these cells are sensitive to low doses of E_2 and P_4 in terms of proliferation [26]. However, the increase in proliferation may be accompanied by increased cortisol secretion during long-term E_2 and P_4 treatment. In the present experiment, T tended to increase cell proliferation at high concentrations (10^{-8}–10^{-6} M) but DHT inhibited

cell growth. Additionally, treatment with T at 10^{-6} M stimulated an increase in cortisol secretion. It has been shown that H295R adrenocortical carcinoma cells are able to convert androgens to estrogens, which then contributes to enhanced H295R cell proliferation [23, 27]. These observations suggest that the increased cell proliferation with T treatment may be due to its conversion to estrogen.

Taken together, the effects of PRL that we observed on the regulatory functions of H295R cell lines indicated responses similar to those elicited by ACTH, but different from those induced by sex steroid hormones. PRL may participate in a novel regulatory system for steroid hormone secretion and cell proliferation in the adrenal cortex.

Materials and methods

Materials

Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12), penicillin–streptomycin solution, and trypsin/EDTA were purchased from Invitrogen-Gibco (Carlsbad, CA, USA). ITS+Premix and NuSerum were purchased from BD Bioscience (San Jose, CA, USA). Recombinant human PRL (AFP 795) was provided by NIDDK (NIH, Torrance, CA, USA). Estradiol-17β (E_2), 4-androstan-17β-ol-3-one (testosterone; T), 5α-androstan-17β-ol-3-one (DHT), and synthetic human ACTH (1–24) were purchased from Sigma-Aldrich Corp. (St. Loui, MO, USA). Progesterone (P_4) was purchased from Wako (Osaka, Japan).

Cell culture

The H295R human adrenocortical carcinoma cell line was purchased from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA), and cells were grown in 100 $\times$ 20 mm Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) with 12 ml of supplemented medium at 37°C in 5% CO_2 in air. Culture medium was 1:1 DMEM/F-12 nutrient mixture supplemented with 1% ITS+Premix and 2.5% NuSerum. The medium was changed every two to three times per week and the cells were detached from flasks by trypsin/EDTA. For exposure, cells were seeded in 96-well plates at initial concentrations of 3×10^4 cells/well in phenol red-free DMEM/F-12 medium with 1% ITS+Premix and 2.5% charcoal–dextran-treated NuSerum, and cells were allowed to attach for 24 h. After the attachment period, the medium was changed and the experiment was initiated. Cells were exposed for 24 h to different concentrations of hormones dissolved in methanol. The diluent for the hormones was added to the controls. The final concentration of methanol was 0.01%.

Hormone measurement

Concentrations of cortisol were measured by double antibody RIAs using ^{125}I-labeled radioligands as described previously [28]. The intra- and inter-assay coefficients of variation were 2.9% and 3.9%, respectively. The lower limit of the assay sensitivity was 5 pg/tube.

Cell proliferation assay

Cell proliferation was determined by viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. Briefly, cloned H295R cells were trypsonized and plated in 96-well plates at initial concentrations of 8×10^3 cells/well in phenol red-free DMEM/F-12 medium with 1% ITS+Premix and 2.5% charcoal–dextran-treated NuSerum. Cells were allowed to attach for 24 h; then, the seeding medium was removed and replaced by fresh medium. A range of concentrations of the test hormones was added to the medium. The diluent for the hormones was added to the controls. The bioassay was terminated on day 5 of culture of H295R cells by using CellTiter-GloTM Luminescent Cell Viability Assay (Promega, Madison, WI, USA) in a luminometer (Luminescencer-JNR AB2100; ATTO, Tokyo, Japan) following manufacturer-suggested protocol.

Statistical analyses

The results were expressed as means $\pm$ standard error of the means (SEM). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program. Differences among doses of each treatment were evaluated by one-way analyses of variance (ANOVA) with the factorial or repeated measure design and post hoc testing by least significant difference (LSD) test. Analysis of covariance (ANCOVA) was performed to test for homogeneity of regression slopes among hormones when the dependent variable was regression over dose. The 95% confidence intervals (CIs) for parameter estimates were calculated for each hormone and compared to identify which regression slopes differed. To further delineate effects of hormones across dose, fixed-effects ANOVA was performed using the LDS adjustment for multiple comparisons to compare least-square means. P values of less than 0.05 were considered to be statistically significant.

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