Effects of Corticosterone *In Vivo* and *In Vitro* on Adrenocorticotropic Hormone Production by Corticotropin Releasing Factor-Stimulated Leukocytes (43911)

GILBERT L. HENDRICKS, III, MAGDI M. MASHALY,[1] AND HERBERT S. SIEGEL
Department of Poultry Science, Pennsylvania State University, University Park, Pennsylvania 16802

Abstract. **Corticotropin releasing factor (CRF) and corticosterone have been shown to affect immune cell function. Previously, we have shown that CRF stimulates immunoreactive adrenocorticotropic hormone (ACTH) production by leukocytes. In this study, splenic leukocytes from corticosterone-injected chickens failed to show a CRF-induced increase in ACTH production. In addition, corticosterone *in vitro* inhibited the production of leukocyte ACTH as well as the stimulatory effect of CRF on splenic leukocyte ACTH production. These findings show that, as with anterior pituitary ACTH production, CRF-stimulated leukocyte ACTH production is inhibited by glucocorticoids.** [P.S.E.B.M. 1995, Vol 209]

The effects of glucocorticoids on the neuroendocrine system—in particular, the hypothalamo-pituitary-adrenocortical axis—are well documented. Corticosterone introduced into the circulation is taken up by different areas of the rat brain, including the hypothalamus (1). In rats, corticosterone has been shown to inhibit corticotropin releasing factor (CRF) production in hypothalamic tissue and adenocorticotropic hormone (ACTH) secretion by pituitary tissue (2). In addition, glucocorticoids inhibit the CRF-mediated secretion of ACTH by cultured rat corticotropic cells (3) and downregulate rat anterior pituitary CRF receptors *in vivo* (4).

Glucocorticoids also affect the immune system; in general, they suppress immune function (5). Hydrocortisone inhibits interleukin-1 (IL-1) secretion *in vitro* (6), and dexamethasone, a synthetic glucocorticoid, inhibits IL-2 (7) and IL-6 (8) secretion *in vitro*. The processing and presentation of antigen to sensitized T cells by human and murine monocytes and macrophages were shown to be inhibited by glucocorticoids *in vitro* (6, 9). Adding corticosterone to feed decreased the size of the chicken spleen, bursa, and thymus (10). Furthermore, antibody titers to SRBC and lymphocyte numbers were inversely correlated with the dose of corticosterone added to the feed.

Glucocorticoids have been shown to cause a redistribution of immune cells; in particular, they inhibit release of murine monocytes into the blood (11) and impede the chemotaxis of neutrophils in guinea pigs (12). Corticosteroids reduce the number of circulating chicken lymphocytes and increase the number of circulating heterophils (13). The decrease in circulating chicken lymphocytes may be due to a sequestering of lymphocytes from the blood into the spleen following an increase in antigen-stimulated plasma corticosterone levels (14).

Glucocorticoids also affect immune cell proliferation; they inhibit Con-A-induced murine (7) and chicken (Trout, personal communication) T-cell proliferation and inhibit pokeweed-induced chicken B-cell proliferation (Trout, personal communication). Although the activities of B cells are inhibited by glucocorticoids, murine lymphocytes that have differenti-

[1] To whom requests for reprints should be addressed at Department of Poultry Science, Pennsylvania State University, 202 Henning Building, University Park, PA 16802.

Received November 18, 1994. [P.S.E.B.M. 1995, Vol 209]
Accepted March 13, 1995.

0037-9727/95/2094-0382$10.50/0

ated into antibody-secreting cells in response to LPS are much more resistant to glucocorticoid inhibition than other mitogen-activated B cells (15).

Through the presence of common receptors and the production of common hormones, the immune and neuroendocrine systems have the ability to communicate with one another (16). It appears that certain cell populations within the immune system are modulated by the neuropeptide, CRF. Cyclic AMP production by human peripheral blood mononuclear cells is increased by CRF (17), and CRF stimulates human peripheral blood T-cell, but not B-cell, proliferation (18). Human peripheral blood mononuclear cells are stimulated by CRF to secrete IL-1, IL-2 (18), and IL-6 (19). CRF also stimulates human peripheral leukocytes to secrete ACTH (20). The present study was conducted in order to determine if, as with cultured corticotropic cells, glucocorticoids inhibit the CRF-mediated secretion of ACTH by splenic leukocytes.

Materials and Methods

Animals. Single Comb White Leghorn male chickens (Cornell K strain) homozygous at the B15 locus of the major histocompatibility complex (21) were used in both experiments. This strain was chosen in order to reduce variability among individuals. After hatching, birds were housed in a large floor pen and given food and water *ad libitum*. Birds were given 14 hr of light each day (06:00 to 20:00 hr). Two experiments were conducted; in the first experiment corticosterone was administered *in vivo* and in the second experiment it was administered *in vitro*.

Experimental Protocol.

Experiment 1. In order to determine if glucocorticoids regulate production of leukocyte ACTH *in vivo,* eight male chickens, 8 weeks of age, were injected (intramuscularly) with 0.5 mg/100 g body wt of corticosterone (Sigma Chemical Co., St. Louis, MO). Corticosterone was dissolved in ethanol, which was evaporated with nitrogen. Saline (0.9%) was added to the corticosterone to yield a final concentration of 10 mg/ml corticosterone. Each bird was given three injections, 24 hr apart. Eight control birds were injected with an equivalent volume of saline (0.9%) for 3 days. Eighteen hours after the last injection, the birds were sacrificed by cervical dislocation and spleens were removed. Blood samples were taken and smears were prepared in order to determine heterophil/lymphocyte ratios, which have been shown to increase as a result of corticosterone treatment (22). Splenic leukocytes were isolated and cell cultures were prepared and incubated as described previously (23, 24). Briefly, spleens were expressed through 60 and 300 mesh screens, then splenic mononuclear cells were isolated using Histopaque 1077 (Sigma). The leukocyte layer at the interface was removed and the cells were twice washed in RPMI 1640 medium. Cells were kept on ice at all times. Live-dead counts were made using a hemocytometer and trypan blue exclusion and cell viability was determined to be $\geq$95%. After washing twice, leukocytes were counted and three 1-ml suspensions of 1×10^7 cells were made from each spleen. To each of the three sets was added 0, 5, or 1000 ng/well of ovine CRF (Sigma). After 48 hr of incubation at 37°C in an atmosphere of 5% CO_2, cell cultures were centrifuged for 15 min at 650*g*. Supernatants were saved to measure ACTH by radioimmunoassay (RIA) as described previously (24).

Experiment 2. The *in vitro* effects of glucocorticoids on leukocyte ACTH production were also tested. Eight birds, 8 weeks of age, were sacrificed by cervical dislocation, and spleens were removed. Leukocytes were isolated, and cell cultures were prepared as described above. Cell viability was $\geq$95%. From each spleen, six 1-ml suspensions of 1×10^7 cells were prepared; two sets received 50 ng/well corticosterone, two received 5 ng/well, and two received 0 ng/well. Cells were incubated at 37°C in an atmosphere of 5% CO_2 for 2 hr, then half of the cultures received 1000 ng/well of CRF (the amount of CRF shown previously in our lab to elicit a significant dose response [24]). Cells were then incubated and harvested as described above. ACTH was measured in the supernatants by RIA.

Statistical Analysis. Data were analyzed using the SAS (SAS Institute Inc., Cary, NC) general linear models procedure. In Experiment 1, data were analyzed using a two-way analysis of variance model with CRF and corticosterone as the two variables. Average ACTH production by leukocytes from corticosterone- or saline-treated birds at different levels of CRF were analyzed using Duncan's Multiple Range Test. In Experiment 2, a two-way analysis of variance model was used to evaluate the effect of corticosterone on the production of ACTH by CRF-stimulated leukocytes. Average ACTH production by CRF- and non-CRF-treated leukocytes at different levels of corticosterone was analyzed using Duncan's Multiple Range Test.

Results

Experiment 1. Combined data from corticosterone injected birds versus saline injected birds show that splenic leukocytes from corticosterone-injected birds produced significantly ($P < 0.0001$) less ACTH (6.3 pg/10^7 cells $\pm$ 0.41 SE, n = 23) than splenic leukocytes from saline-injected birds (9.6 pg ACTH/10^7 cells $\pm$ 0.55 SE, n = 24). Leukocytes treated with 5 and 1000 ng/well CRF and untreated leukocytes from corticosterone injected birds produced significantly ($P < 0.05$) less ACTH than CRF-treated and untreated leukocytes from saline-injected birds (Fig. 1). The heterophil/lymphocyte ratio was significantly ($P < 0.001$)

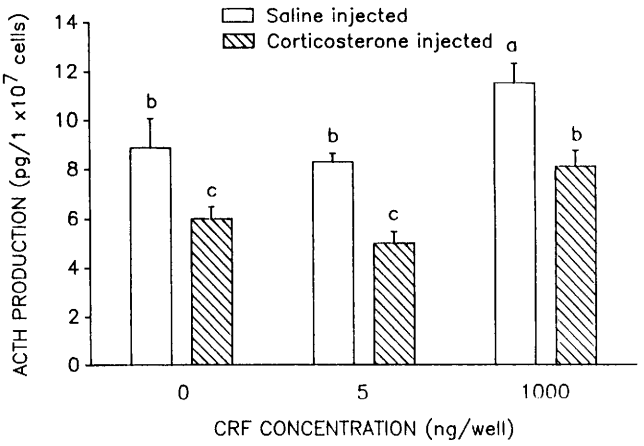

Figure 1. Production of ACTH by 1 × 10^7 chicken splenic leukocytes from corticosterone- and saline-injected birds following 48 hr of incubation with corticotropin-releasing factor (CRF). Values are means ± SE. Values with no common letters are significantly different ($P < 0.05$). $n = 8$.

higher in the blood from corticosterone-injected birds (0.70 ± 0.06) than in the blood from saline-injected birds (0.23 ± 0.03). The corticosterone and CRF main effects were significant, the interaction effect was not significant.

Experiment 2. Corticosterone inhibited splenic leukocyte ACTH production *in vitro* (Fig. 2). Splenic leukocytes incubated in the absence of corticosterone produced 9.7 pg/10^7 cells, while leukocytes incubated with 50 ng/well corticosterone produced significantly ($P < 0.05$) less ACTH (3.0 pg/10^7 cells).

Corticosterone also inhibited the CRF-stimulated increase in splenic leukocyte ACTH production *in vitro* (Fig. 2). Splenic leukocytes incubated with 1000 ng/well CRF and no corticosterone produced an average of 16.8 pg ACTH/10^7 cells. Adrenocorticotropic hormone production declined significantly ($P < 0.05$) to 7.0 pg ACTH/10^7 cells with the addition of 5 ng/well

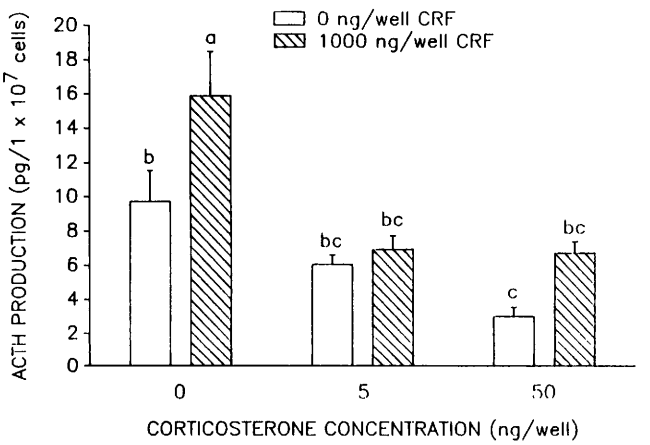

Figure 2. Production of ACTH by 1 × 10^7 chicken splenic leukocytes following 48 hr of incubation with corticosterone and corticotropin-releasing factor (CRF). Values are means ± SE. Values with no common letters are significantly different ($P < 0.05$). $n = 8$

of corticosterone and to 6.5 pg ACTH/10^7 cells following addition of 50 ng/well of corticosterone. Again, corticosterone and CRF main effects were significant, the interaction effect was not significant.

Discussion

Cells of the immune system and the neuroendocrine system can each produce ACTH in response to CRF stimulation (20, 25). Corticosterone has been shown to inhibit the production of CRF-stimulated ACTH production by rat pituitary tissue *in vitro* (2). The results of these experiments indicate that corticosterone also inhibits production of ACTH by chicken splenic leukocytes and inhibits CRF-stimulated leukocyte ACTH production both *in vitro* and *in vivo*. Our results are in agreement with Smith *et al.* (20), who found that CRF-induced human peripheral blood leukocyte ACTH production was blocked by treatment with dexamethasone *in vitro*.

Glucocorticoids inhibit pituitary ACTH production; corticosterone inhibited ACTH section by rat pituitary tissue *in vitro* (2). Data collected from our experiments suggest that glucocorticoids also inhibits leukocyte ACTH production; splenic leukocytes from corticosterone-injected birds produced less ACTH than splenic leukocytes from saline-injected birds (Fig. 1), and pretreatment of splenic leukocytes with corticosterone *in vitro* results in decreased production of leukocyte ACTH (Fig. 2). The mechanism of glucocorticoid inhibition of leukocyte ACTH production remains unclear. Glucocorticoids inhibit the 8-bromo-cAMP-stimulated, as well as the CRF-stimulated, increase in rat pituitary ACTH, indicating that at least part of the glucocorticoid inhibition of pituitary ACTH production occurs somewhere in the synthesis pathway beyond cAMP formation (3, 26). Glucocorticoids decrease ACTH precursor (pro-opimelanocortin, POMC) mRNA levels in the rat anterior pituitary (27), this result could be due to a decrease in RNA polymerase II complexes transcribing the POMC gene after glucocorticoid treatment (28). If glucocorticoid regulation of ACTH production is similar in leukocytes and pituitary corticotropes, it is possible that glucocorticoids regulate leukocyte ACTH production directly by decreasing POMC mRNA levels.

Not only do glucocorticoids inhibit leukocyte ACTH production directly, they also inhibit the ability of CRF to stimulate leukocyte ACTH production (Fig. 2). The mechanism of glucocorticoid inhibition of CRF induced leukocyte ACTH is also unclear. However, there are similarities between cells of the neuroendocrine and immune systems that suggest CRF-induced leukocyte and pituitary ACTH are inhibited by glucocorticoids through similar mechanisms. As in pituitary corticotropes, murine leukocyte express receptors for CRF (29), and rat leukocytes express receptors form

glucocorticoids (5). Rat anterior pituitary CRF receptors are downregulated by glucocorticoids *in vitro* (4). If glucocorticoids regulate leukocyte function by the same mechanism, glucocorticoids could regulate leukocyte ACTH production by downregulating CRF receptors.

Glucocorticoids and leukocyte ACTH may be involved in coordinating the immune response. During the initiation of an immune response, CRF is produced by the hypothalamus in response to infection (30) and stimulates the production of ACTH by pituitary cells. Corticotropin releasing factor is also produced locally at the site of inflammation in relatively high concentrations (31). This CRF is thought to act in an autocrine or paracrine manner (31) and could stimulate the production of leukocyte ACTH. In birds, both pituitary ACTH (32) and leukocyte ACTH (23, 32) stimulate the adrenal glands, leading to an increase in corticosterone levels. Because an increase in corticosterone level leads to a redistribution of T cells from the blood to the spleen (14), T cells could migrate to the spleen, where they would interface with antigen-presenting cells and B cells in the development of an immune response. Once activated by antigen, B cells may be further influenced by ACTH and glucocorticoids. Adrenocorticotropic hormone (in the presence of IL-2, B-cell growth factors, or B-cell differentiation factors) has been shown to increase antigen-activated human B-cell proliferation, differentiation, and antibody production (34). Although glucocorticoids inhibit the activities of the general B-cell population, B cells that have already differentiated into Ig-secreting cells are much more resistant to glucocorticoid inhibition (15, 35).

Glucocorticoids could modulate inappropriate immune cell activation and function, limiting the severity of the immune response (5). The ability of glucocorticoids to limit the immune response appears to be crucial to the proper function of the immune system; in rats, glucocorticoid insufficiency results in autoimmune disease, such as arthritis (36).

The stimulation of leukocyte ACTH production by CRF and the inhibition of CRF-induced leukocyte ACTH production by corticosterone suggests the presence of a negative feedback system similar to that of the hypothalamo-pituitary adrenocortical axis. Corticotropin releasing factor induces the production of avian leukocyte ACTH (24), and CRF-stimulated avian leukocytes produce an ACTH-like substance that increases adrenal steroidogenesis (23). The results presented here show that corticosterone inhibits CRF-induced avian leukocyte ACTH production. The data also suggest that glucocorticoids act directly on avian leukocytes to inhibit ACTH production. This effect parallels the action of glucocorticoids in the neuroendocrine system: glucocorticoids inhibit pituitary ACTH production at the level of the hypothalamus and the pituitary (2).

1. McEwen BS, Weiss JM, Schwartz LS. Uptake of corticosterone by rat brain and its concentration by certain limbic structures. Brain Res **16**:227–241, 1969.
2. Buckingham JC. Effects of adrenocortical and gonadal steroids on the secretion *in vitro* of corticotrophin and its hypothalamic releasing factor. J Endocr **93**:123–132, 1982.
3. Vale W, Vaughan J, Smith M, Yamamoto G, Rivier J, Rivier C. Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. Endocrinology **113**:1121–1131, 1983.
4. Hauger RL, Millan MA, Catt KJ, Aguilera G. Differential regulation of brain and pituitary corticotropin-releasing factor receptors by corticosterone. Endocrinology **120**:1527–1533, 1987.
5. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr Rev **5**:25–44, 1984.
6. Snyder DS, Unanue ER. Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. J Immunol **129**:1803–1805, 1982.
7. Gillis S, Crabtree GR, Smith KA. Glucocorticoid-induced inhibition of T-cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J Immunol **123**:1624–1631, 1979.
8. Woloski BM, Smith EM, Meyer WJ, III, Fuller GM, Blalock JE. Corticotropin-releasing activity of monokines. Science **230**:1035–1037, 1985.
9. Hirschberg H, Hirschberg T, Nousiainen H, Braathen LR, Jaffe E. The effects of corticosteriods on the antigen presenting properties of human monocytes and endothelial cells. Clin Immunol immunopathol **23**:577–585, 1982.
10. Gross WB, Siegel PB, DuBose RT. Some effects of feeding corticosterone to chickens Poult Sci **59**:516–522, 1980.
11. Thompson J, van Furth R. The effect of glucocorticosteroids on the kinetics of mononuclear phagocytes. J Exp Med **131**:429–442, 1970.
12. Ward PA. The chemosuppression of chemotaxis. J Exp Med **124**:209–226, 1966.
13. Siegel HS. Blood cells and chemistry of young chickens during daily ACTH and cortisol administration. Poult Sci **47**:1811–1817, 1968.
14. Trout JM, Mashaly MM. Heat, adrenocorticotrophic hormone and lymphocyte distribution. Poult Sci **73**:1694–1698, 1994.
15. Roess DA, Bellone CJ, Ruh MF, Nadel EM, Ruh TS. The effect of glucocorticoids on mitogen-stimulated B-lymphocytes: Thymidine incorporation and antibody secretion. Endocrinology **110**:169–175, 1982.
16. Blalock JE. A molecular basis for bi-directional communication between the immune and neuroendocrine systems: A review. Physiol Rev **69**:1–32, 1989.
17. Singh VK, Leu SJC. Corticotropin-releasing factor-induced production of cyclic AMP by human peripheral blood immunocytes. Immunol Lett **35**:239–246, 1993.
18. Singh VK, Warren RP, White ED, Leu SJC. Corticotropin-releasing factor-induced stimulation of immune functions. Ann NY Acad Sci **594**:416–419, 1990.
19. Leu SC, Singh VK. Stimulation of interleukin-6 production by corticotropin-releasing factor. Cell Immunol **143**:220–227, 1992.
20. Smith EM, Morrill AC, Meyer WJ, Blalock JE. Corticotropin-releasing factor induction of leukocyte derived immunoreactive ACTH and endorphins. Nature **321**:881–882, 1986.
21. Erf GF, Marsh JA. Triiodothyronine affects mitogen respon-

siveness in sex-linked dwarf and Cornell K strain chickens. Devel Comp Immunol **11**:395–406, 1987.
22. Gross WB, Siegel HS. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Disease **27**:927–979, 1983.
23. Hendricks GL III, Siegel HS, Mashaly MM. Ovine corticotropin-releasing factor increases endocrine and immunologic activity of avian leukocytes *in vitro*. Soc Exp Biol Med **196**:390–395, 1991.
24. Hendricks GL III, Mashaly MM, Siegel HS. Validation of an assay to measure adrenocorticotropin in plasma and from chicken leukocytes. Poult Sci **74**:337–342, 1995.
25. Chan J, Lu C, Seidah NG, Chretien M. Corticotropin releasing factor (CRF): Effects on the release of pro-opiomelanocortin (POMC)-related peptides by human anterior pituitary cells *in vitro*. Endocrinology **111**:1388–1390, 1982.
26. Bilezikjian LM, Vale WW. Glucocorticoids inhibit corticotropin-releasing factor-induced production of adenosine 3′,5′-monophosphate in cultured anterior pituitary cells. Endocrinology **113**:657–662, 1983.
27. Schachter BS, Johnson LK, Baxter JD, Roberts JL. Differential regulation by glucocorticoids of pro-opiomelancortin mRNA levels in the anterior and intermediate lobes of the rat pituitary. Endocrinology **110**:1442–1444, 1982.
28. Eberwine JH, Roberts JL. Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. J Biol Chem **259**:2166–2170, 1984.
29. Webster EL, Tracey DE, Jutila MA, Wolfe SA Jr., DeSouza EB. Corticotropin-releasing factor receptors in mouse spleen: Identification of receptor-bearing cells as resident macrophages. Endocrinology **127**:440–452, 1990.
30. Tepperman J. Metabolic and Endocrine Physiology (4th ed) New York: Year Book Medical, p181, 1980.
31. Karalis K, Sano H, Redwine J, Listwak S, Wilder RL, Chrousos GP. Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone *in vivo*. Science **254**:421–423, 1991.
32. Nagra CL, Baum GJ, Meyer RK. Corticosterone levels in adrenal effluent blood of some gallinaceous birds. Proc Soc Exp Biol Med **105**:66–70, 1962.
33. Siegel HS, Gould NR, Latimer JW. Splenic leukocytes from chickens injected with *Salmonella pullorum* antigen stimulate production of corticosteroids by isolated adrenal cells. Proc Soc Exp Biol Med **178**:523–530, 1985.
34. Alvarez-Mon M, Kehrl JH, Fauci AS. A potential role for adrenocorticotropin in regulating human B-lymphocyte functions. J Immunol **135**:3823–3826, 1985.
35. Claman HN. Corticosteroids and lymphoid cells. N Engl J Med **287**:388–397, 1972.
36. Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW, Wilder RL. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. Proc Natl Acad Sci USA **86**:2374–2378, 1989.