Endocrine, *vol. 13, no. 3, 369–377, December 2000* 0969–711X/00/13:369–377/$12.25

Stimulation by Interleukin-6 and Inhibition by Tumor Necrosis Factor of Cortisol Release from Bovine Adrenal Zona Fasciculata Cells Through Their Receptors

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Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are synthesized and released from adrenal cells. Therefore, the effects of TNF-α and IL-6 on cortisol release from bovine zona fasciculata (ZF) cells were investigated. IL-6 (10–1000 pg/mL) significantly increased basal and adrenocorticotropic hormone (ACTH)-stimulated cortisol release in a concentration-dependent manner. This stimulatory effect of IL-6 became apparent at intervals as short as 4 h and continued through 24 h. IL-6 also potentiated the cortisol release stimulated by the adenylyl cyclase activator forskolin. By contrast, TNF-α (0.1–10 ng) inhibited basal and ACTH-stimulated cortisol release in a concentration-dependent manner. The inhibitory effects of TNF-α on cortisol release were significant at time intervals as short as 4 h and continued through 24 h. TNF-α inhibited forskolin-stimulated cortisol release. Binding studies demonstrated that ZF cells have IL-6 receptors (100 receptors/cell, K_d of 7.5×10^{-11}) and TNF receptors (200 receptors/cell, K_d of 2.4×10^{-9} *M*). Immunohistochemical analysis provided evidence that the majority of ZF cells have IL-6 receptors, TNF type 1 receptors, and TNF type 2 receptors. Because IL-6 and TNF-α are released from the adrenal cortex and these cytokines modify the release of cortisol from the ZF, IL-6 and TNF-α may play a paracrine or autocrine role in the regulation of adrenal function.

Key Words: Interleukin-6; tumor necrosis factor-α; cytokine receptors; cortisol; adrenal.

Introduction

Cortisol or corticosterone is released from cells of the zona fasciculata (ZF) of the adrenal cortex during stress. During acute stress, the primary control mechanism of glucocorticoid release from the ZF cells is the hypothalamic-pituitary-adrenal axis. Corticotropin-releasing hormone is released from the hypothalamus and increases the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticoids from the ZF cells of the adrenal gland *(1,2)*. However, during chronic stress the relationship between plasma ACTH and plasma glucocorticoid concentration is not always apparent. Therefore, it has been hypothesized that intra-adrenal interactions may also modulate the release of glucocorticoids from the ZF cells of the adrenal gland *(3–6)*. One of the intraadrenal systems that may be involved in the modulation of glucocorticoid release is the cytokine network *(5,7)*.

Cytokines are peptides involved in the regulation of the immune and inflammatory responses. These peptides were originally thought to be produced only by immune cells. However, these proteins are now known to be produced by many different cells and appear to have many functions including an important role in the stress response *(1–3,5,6)*. Rat, human, and bovine adrenocortical cells produce several cytokines including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) *(2,3,5,7)*. Furthermore, the release of IL-6 and TNF-α from rat and bovine adrenocortical cells is regulated by agents that modify adrenal function. Specifically, IL-1 and endotoxin increase the release of IL-6 and TNF from rat and bovine adrenocortical cells. By contrast, ACTH increases the release of IL-6 but inhibits the release of TNF from rat and bovine adrenocortical cells *(7,8)*. Angiotensin II also increases adrenal IL-6 secretion *(7,8)*.

IL-6 and TNF-α modify glucocorticoid release from the adrenal cortex. Therefore, these cytokines may play a role in the regulation of adrenal function. IL-6 increases basal

Received April 28, 2000; Revised July 26, 2000; Accepted August 3, 2000.
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and ACTH-stimulated glucocorticoid release from rat adrenal cells *(9–11)*. Similarly, IL-6 increases basal cortisol release from human and bovine adrenal cells *(12–15)*. Although TNF-α modifies glucocorticoid release from the adrenal gland, the effects of TNF-α on glucocorticoid secretion are not consistent among different model systems. Thus, TNF-α inhibits corticosterone secretion from adrenal cells of normal rats but stimulates corticosterone release from adrenal cells isolated from choleostatic rats *(16,17)*. Similarly, TNF-α inhibits ACTH-stimulated cortisol secretion from fetal human adrenal cells *(18,19)* but has been reported to stimulate cortisol release from adult human adrenal cells *(20)*.

Although it is documented that IL-6 and TNF-α regulate glucocorticoid release from adrenal cells, important questions concerning the effects of these cytokines on glucocorticoid secretion have not been answered. Specifically, the effects of IL-6 on ACTH-stimulated cortisol secretion are not known. Furthermore, the effects of TNF-α on basal and ACTH-stimulated cortisol secretion from the adult adrenal gland are controversial. The time course over which IL-6 and TNF-α modify cortisol secretion is also poorly defined. Similarly, there has been no correlation determined between the effects of IL-6 and TNF-α on adrenal function and the presence of IL-6 and TNF receptors in the adrenal cortex.

In the present study, the effects of IL-6 and TNF-α on cortisol release from adult bovine adrenal ZF cells were determined. Furthermore, the presence of IL-6 and TNF receptors on bovine ZF cells was investigated utilizing binding assays and immunohistochemistry. This model system was utilized because large numbers of adrenal cells for repeated hormone release experiments and cytokine receptor binding assays were required. Bovine adrenal glands, similar to the human adrenal glands release cortisol as the primary glucocorticoid. Unlike human adrenal glands, the bovine adrenal glands are easily available. Furthermore, the bovine adrenal glands are derived from healthy animals whereas the human adrenal glands are generally removed from patients with renal cancer. Therefore, the bovine model system was chosen for these experiments.

Results

Effects of IL-6 on Cortisol

IL-6 increased basal and ACTH-stimulated cortisol release from bovine adrenal ZF cells that had been incubated with this cytokine for 24 h (Fig. 1). Furthermore, the IL-6 augmentation of ACTH-stimulated cortisol secretion was greater than additive. This IL-6 enhancement of basal and ACTH-stimulated cortisol secretion occurred in a concentration-dependent manner with the first significant enhancement of cortisol release occurring at 10 pg/mL of IL-6 and further enhancements of cortisol secretion occurring at IL-6 concentrations up to 1000 pg/mL (Fig. 1). IL-6 (20 pg/mL) augmented ACTH-stimulated cortisol secretion

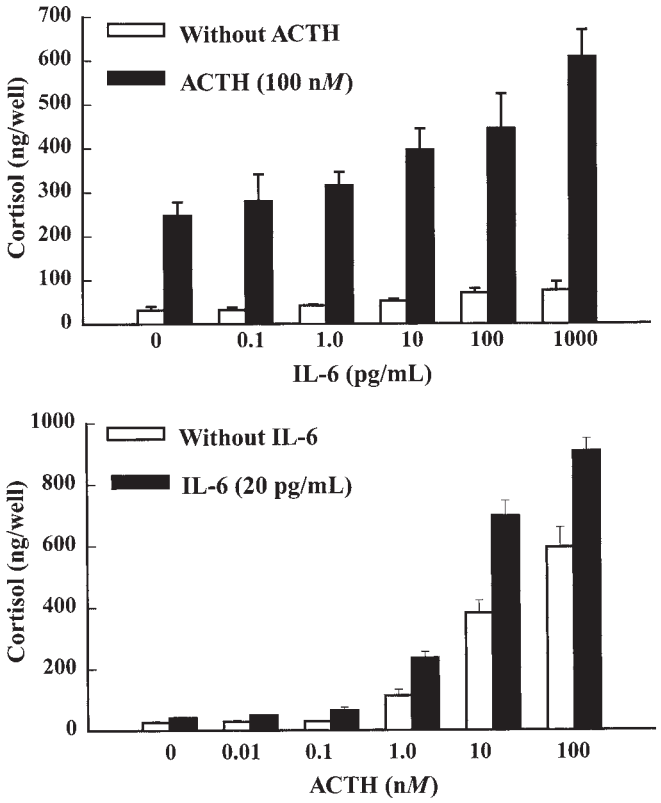

Fig. 1. Effects of IL-6 on basal and ACTH-stimulated cortisol secretion from primary cultures of bovine adrenal ZF cells. (**Top**) IL-6 increased basal cortisol release ($p < 0.05$ for 10–1000 pg of IL-6/mL vs medium alone). ACTH (100 n*M*) increased cortisol release ($p < 0.01$ vs medium alone), and IL-6 increased ACTH-stimulated cortisol secretion ($p < 0.01$ for 10–1000 pg of IL-6/mL vs ACTH alone). (**Bottom**) IL-6 (20 pg/mL) increased basal cortisol secretion ($p < 0.05$ vs medium alone). ACTH increased cortisol release ($p < 0.01$ 1–100 n*M* ACTH vs medium alone), and IL-6 (20 pg/mL) increased ACTH-stimulated cortisol release at all concentrations of ACTH ($p < 0.01$ vs corresponding concentration of ACTH alone). Incubation period was 24 h.

at all concentrations of ACTH tested (0.01–100 n*M*) (Fig. 1). To determine the time course of IL-6 stimulation of cortisol secretion, bovine adrenal ZF cells were exposed to IL-6 in the presence and absence of ACTH for 4, 8, 16, and 24 h. IL-6 (20 pg/mL) significantly increased basal cortisol secretion at 8–24 h but had no significant effect on cortisol secretion at 4 h (Fig. 2). By contrast, ACTH caused a dramatic increase in cortisol concentration after 4 h of exposure, and the cortisol release increased in magnitude throughout the experiment. IL-6 significantly augmented ACTH-stimulated cortisol secretion at the 4-h interval and all subsequent intervals. ACTH increases cortisol secretion through a mechanism involving activation of adenylyl cyclase. Therefore, bovine adrenal ZF cells were exposed to forskolin, a direct activator of the adenylyl cyclase, to determine whether IL-6 could augment cortisol secretion when the ACTH receptor was circumvented. IL-6 significantly increased basal and forskolin-stimulated cortisol

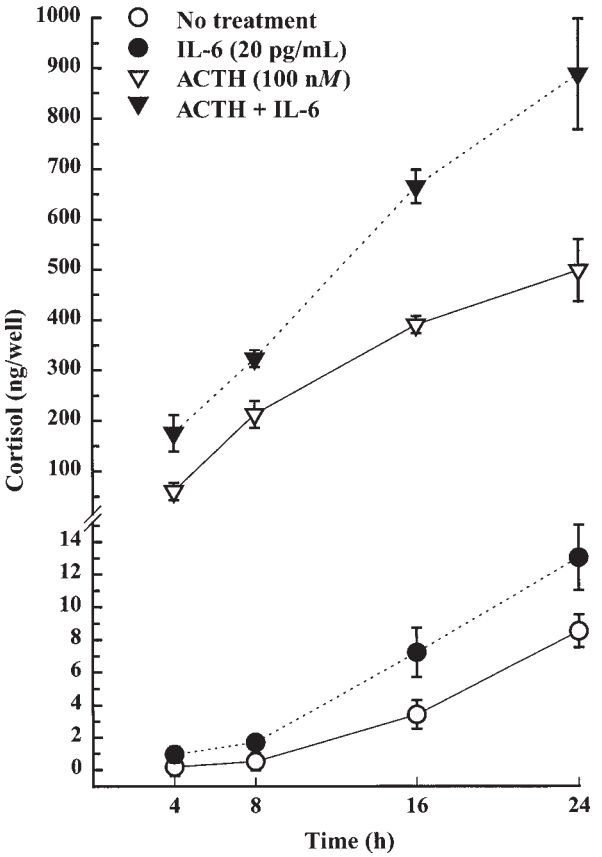

Fig. 2. Time course of the effects of IL-6 on basal and ACTH-stimulated cortisol release from primary cultures of bovine adrenal ZF cells. IL-6 (20 pg/mL) increased basal cortisol release at 8, 16, and 24 h ($p < 0.05$ vs medium alone). ACTH increased cortisol release at 4, 8, 16, and 24 h ($p < 0.01$ vs medium alone). IL-6 increased ACTH-stimulated cortisol release at 4, 8, 16, and 24 h ($p < 0.05$ vs ACTH alone at 4 and 8 h; $p < 0.01$ vs ACTH alone at 16 and 24 h).

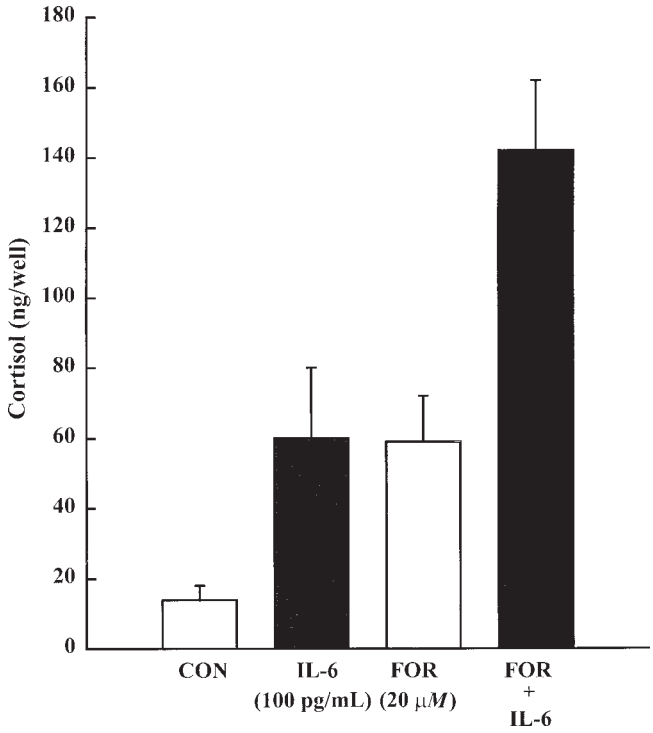

Fig. 3. IL-6 stimulates basal and forskolin-stimulated cortisol secretion from bovine ZF cells. IL-6 (100 pg/mL) increased basal cortisol release ($p < 0.01$ vs medium alone). Forskolin increased cortisol release ($p < 0.01$ vs medium alone) and IL-6 potentiated forskolin-stimulated cortisol release ($p < 0.01$ vs forskolin alone). All incubations were for 24 h FOR, forskolin; CON, control.

release (Fig. 3). Healthy cultured adrenal cells appear flattened and send out many long projections that tightly attach to the cell culture plastic ([*3,13*], unpublished data). If these cells are exposed to toxic agents, are inoculated into the cell culture wells at too high a density, or do not have the cell culture medium changed, the cells take on a rounded appearance, lose the long projections, and become detached from the cell culture plastic (unpublished data). IL-6 had no apparent effect on adrenal cell morphology at any of the concentrations or incubation intervals tested; that is, the adrenal cells retained their flattened appearance with long projections and remained tightly attached to the cell culture plastic (unpublished data).

Effects of TNF-α on Cortisol

In contrast to the stimulatory effects of IL-6, TNF-α (0.001–10 ng/mL) inhibited in a concentration-dependent manner basal and ACTH-stimulated release of cortisol from bovine ZF cells that had been incubated with this cytokine for 24 h (Fig. 4). The first TNF-α concentration to significantly inhibit basal cortisol secretion was 1.0 ng/mL, whereas 0.1 ng/mL of TNF-α significantly inhibited ACTH-stimulated cortisol secretion. ACTH significantly increased cortisol secretion in a concentration-dependent manner at ACTH concentrations of 0.1 n*M* and greater. TNF-α (10 ng/mL) decreased cortisol secretion at all concentrations of ACTH investigated (Fig. 4). To determine the time course of TNF-α inhibition of cortisol secretion, bovine adrenal ZF cells were exposed to TNF-α in the presence or absence of ACTH for 4, 8, 16, and 24 h. ACTH significantly increased cortisol secretion at 4 h, and at subsequent time intervals the amount of cortisol secretion continued to increase (Fig. 5). TNF-α (10 ng/mL) significantly inhibited basal cortisol secretion at 16 and 24 h. By contrast, TNF-α inhibited ACTH-stimulated cortisol release at 4 h and at all subsequent time intervals. TNF-α (10 ng/mL) also inhibited basal and forskolin-stimulated cortisol release (Fig. 6). TNF-α had no apparent effect on adrenal cell morphology at any of the time intervals or TNF-α concentrations tested; that is, the adrenal cells retained their flattened appearance with long projections and remained tightly attached to the cell culture plastic (unpublished data).

IL-6 and TNF Binding to ZF Cells

The presence of the IL-6 and TNF-α receptors in the bovine adrenal ZF was assessed by binding assays. As

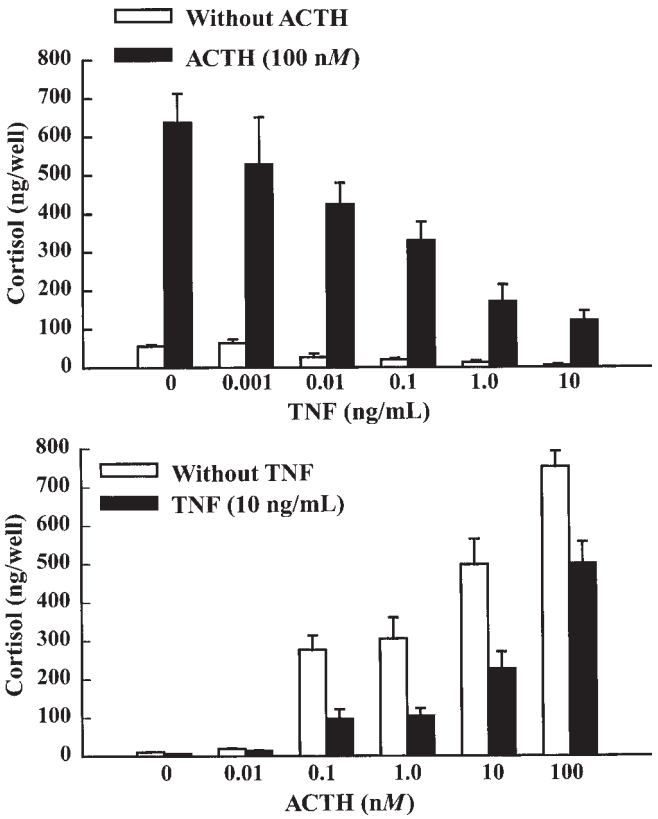

Fig. 4. TNF-α decreases basal and ACTH-stimulated cortisol secretion from primary cultures of bovine adrenal ZF cells. (**Top**) TNF-α decreased basal cortisol release ($p < 0.01$ for 1.0–10 ng of TNF-α/mL vs medium alone) and ACTH-stimulated cortisol release ($p < 0.05$ for 0.1 ng of TNF-α/mL vs control; $p < 0.01$ for 1–10 ng of TNF-α/mL vs ACTH alone). (**Bottom**) TNF-α (10 ng/mL) decreased basal cortisol release ($p < 0.05$ vs medium alone). ACTH (0.1–100 n*M*) increased cortisol release ($p < 0.01$ vs medium alone). TNF-α decreased ACTH-stimulated cortisol secretion ($p < 0.05$ for 0.01 n*M* ACTH vs 0.01 n*M* ACTH and TNF-α; $p < 0.01$ for 0.1–100 n*M* ACTH vs corresponding concentration of ACTH and TNF-α). The adrenal ZF cells were incubated for 24 h.

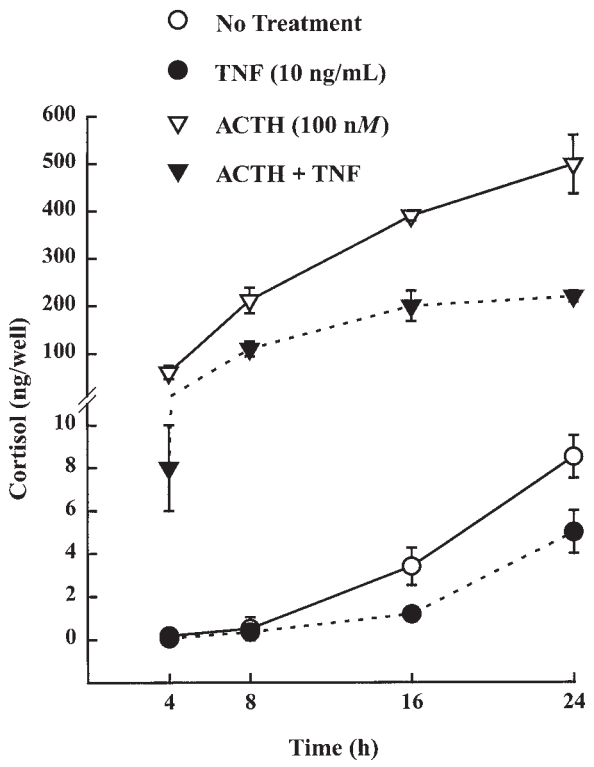

Fig. 5. Time course of the effects of TNF-α on basal and ACTH-stimulated cortisol release from primary cultures of bovine adrenal ZF cells. TNF-α (10 ng/mL) decreased basal cortisol release at 16 and 24 h ($p < 0.05$ vs medium alone). ACTH increased cortisol release at 4, 8, 16, and 24 h ($p < 0.01$ vs medium alone). TNF-α decreased ACTH-stimulated cortisol release at 4, 8, 16 and 24 h ($p < 0.01$ vs ACTH alone).

shown in Fig. 7, the apparent binding of IL-6 and TNF-α to bovine ZF cells was saturable at high concentrations of ligand. The adrenal ZF had 100 IL-6 receptors/cell and the K_d was 7.5×10^{-11} *M*. There was no evidence of cooperativity in the binding of IL-6 to the IL-6 receptor. These cells had 200 TNF receptors/cell and the K_d was 2.4×10^{-9} *M*. As shown in Fig. 7, the binding of TNF-α appeared cooperative with an apparent Hill coefficient of 1.49. The binding of the radioactive IL-6 and TNF-α to the adrenal cells was specific because high concentrations of nonradioactive ligand successfully competed for the entire signal (data not given).

Immunohistochemical Location of IL-6 and TNF Receptors in Bovine ZF Cells

Immunohistochemical analysis of thin sections of bovine adrenal gland demonstrated that the majority of the cells of the ZF stained for IL-6 receptors (Fig. 8). The ZF cells also stained for both TNF type 1 and TNF type 2 receptors. The majority of cells in the ZF had TNF type 2 receptors (Fig. 9). However, there were columns of cells in the ZF that stained intensely for TNF type 1 receptors whereas neighboring columns of cells had diminished staining for these receptors (Fig. 9). The staining for IL-6 receptors, TNF type 1 receptors, and TNF type 2 receptors did not represent nonspecific binding of antibodies to the adrenal glands in that there was negligible staining of the adrenal cells when no first antibody was present (Figs. 8 and 9).

Discussion

IL-6 increased whereas TNF-α decreased basal and ACTH-stimulated cortisol secretion. IL-6 and TNF-α are probably not toxic because they did not modify the morphology of the cells. Furthermore, IL-6 increases cortisol release from bovine adrenal cells and cortisol release is an active process. Therefore, it is unlikely that IL-6 is increasing cortisol secretion through a toxication. As additional evidence that TNF-α is not exerting a toxic effect on the adrenal cells, TNF-α increases adrenal IL-6 release and this

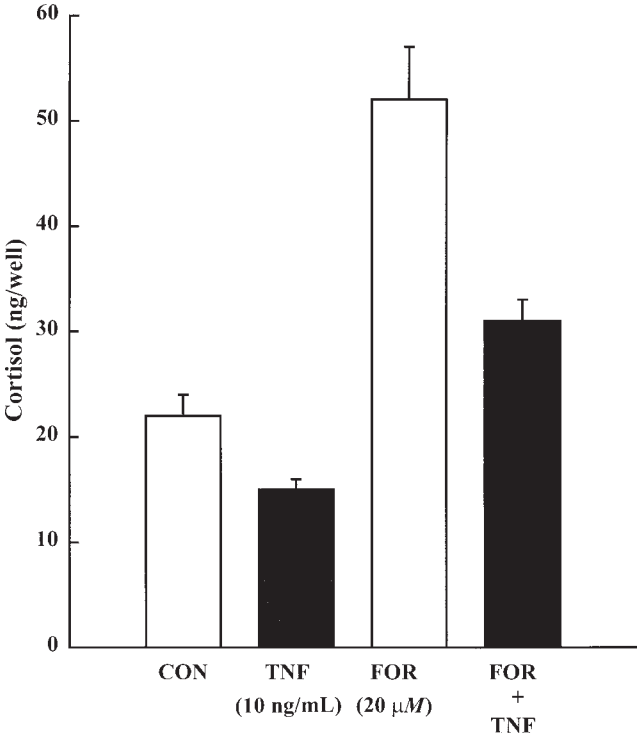

Fig. 6. TNF-α inhibits basal and forskolin-stimulated cortisol secretion from bovine ZF cells. TNF-α (10 ng/mL) decreased basal cortisol release ($p < 0.05$ vs medium alone). Forskolin increased cortisol release ($p < 0.01$ vs medium alone) and TNF-α inhibited forskolin-stimulated cortisol release ($p < 0.01$ vs forskolin alone). All incubations were of 24 h. FOR, forskolin; CON, control.

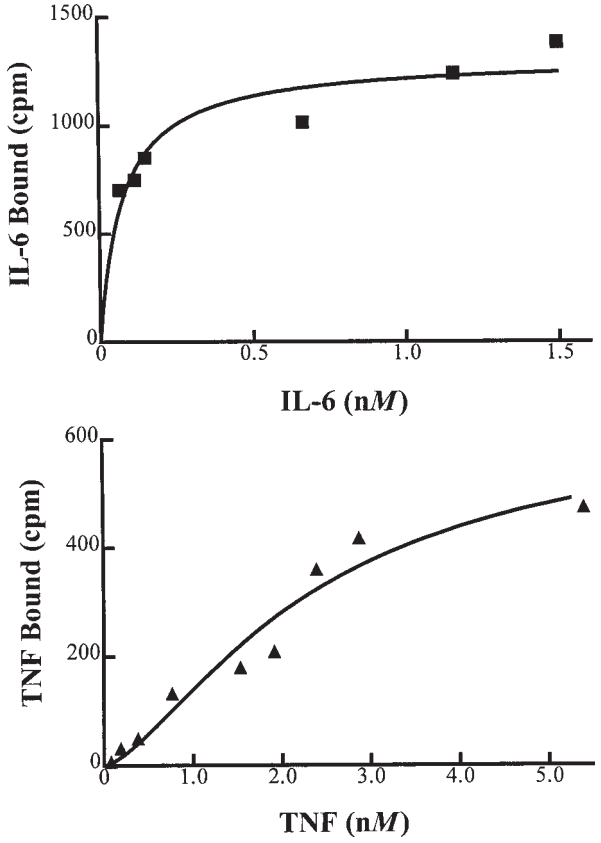

Fig. 7. Binding of radioactive IL-6 and TNF-α to bovine adrenal ZF cells. (**Top**) The K_d of the IL-6 receptor was 7.5×10^{-11} *M* and the cells had 100 IL-6 receptors per cell. (**Bottom**) The K_d of the TNF receptor was 2.4×10^{-9} *M* and the cells had 200 TNF receptors per cell.

release from adrenal cells generally requires IL-6 synthesis *(21)*. Furthermore, in other steroid-secreting tissues, the inhibitory effects of TNF-α on steroid secretion are reversible *(22–24)* and do not involve a decrease in cell number or viability *(22,25)*. Because IL-6 increased cortisol secretion at 10 pg/mL (5×10^{-13} *M*), IL-6-induced cortisol release is not a nonspecific effect of high IL-6 concentrations. The K_d of the IL-6 receptor on bovine ZF cells was 7.4×10^{-11} *M*. The actual K_d of this receptor may be different from this value because human IL-6 was utilized, and human and bovine IL-6 may have different affinities for IL-6 receptors. The IL-6 receptors on ZF cells were high affinity, but the number of IL-6 receptors was low (100 receptors/cell). These results are similar to the data reported for bovine B-cell tumors (250–300 IL-6 receptors/cell, K_d of 1.4×10^{-10} *M*) *(26)*.

In previous studies, IL-6 increased basal and ACTH-stimulated corticosterone release from rat adrenal cells *(9–11)*. In human and bovine cells, IL-6 increases basal cortisol release, but its effect on ACTH-stimulated cortisol release has not been determined *(12–15)*. In some studies, 24 h was required for IL-6 to stimulate corticosterone *(9,10)* or cortisol release *(12,13)*. In other studies, IL-6 increased cortisol release within 12 h, the earliest interval studied *(14,15)*. In the present study, IL-6 increased basal cortisol secretion within 8 h and ACTH-stimulated cortisol release within 4 h. IL-6 concentrations of 5×10^{-13} *M* (*9,10*), 1×10^{-10} *M* (*13*), 3×10^{-10} *M (14,15),* 1.5×10^{-11} *M* (*11*), and 5×10^{-13} *M* (current study) are required to increase cortisol or corticosterone release. These discrepancies may be related to differences in cell culture, the species studied, or the bioactivity of IL-6 preparations.

The K_d of the TNF receptor on ZF cells was 2.4×10^{-9} *M* with 200 TNF receptors/cell. This receptor has a K_d of 3.6–5.8×10^{-9} *M* on bovine corpus luteum *(27)* and 2 to 3×10^{-11} *M* on bovine ovarian cells (900 receptors/cell) *(25)*. The differences between the K_d values in these studies may be explained by different types of TNF receptors in different tissues. The human K_d of TNF type 1 receptor (p55) is 2×10^{-11} *M* and of TNF type 2 receptor (p75) is 4×10^{-10} *M (28).* The actual K_d of the bovine TNF receptor may vary from the estimated values because human TNF-α was utilized and human and bovine TNF-α may have different affinities for the TNF receptors. Bovine ZF cells have both TNF type 1 and 2 receptors. Similarly, other cells including

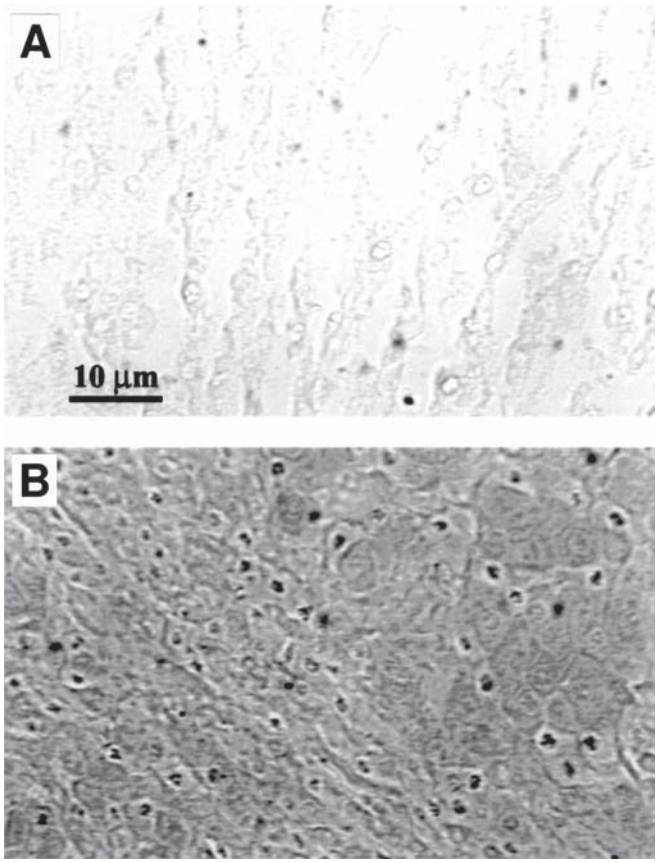

Fig. 8. Micrograph of bovine adrenal ZF cells exposed to antibodies against IL-6 receptors. (**A**) ZF cells exposed to secondary antibody alone (horseradish peroxidase [HRP]-conjugated donkey antirabbit IgG); (**B**) ZF cells exposed to primary antibody against IL-6 (rabbit antimouse IL-6 receptor).

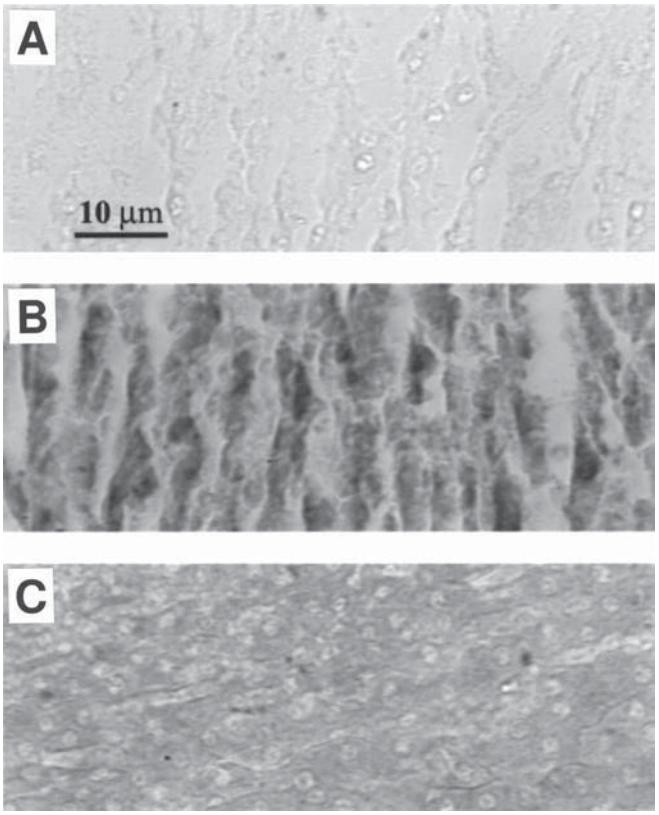

Fig. 9. Micrograph of bovine adrenal ZF cells exposed to antibodies against TNF type 1 and 2 receptors. (**A**) ZF cells exposed to secondary antibody alone (HRP-conjugated donkey antirabbit IgG); (**B**) ZF cells exposed to primary antibody against TNF type 1 receptor (rabbit antimouse TNF type 1 receptor); (**C**) ZF cells exposed to primary antibody against TNF type 2 receptor (rabbit antimouse TNF type 2 receptor).

adipocytes have both types of TNF receptors (*29*). Because TNF-α inhibits cortisol secretion at 0.1 ng/mL (6×10^{-12} *M*), it is probable that these actions are mediated through the higher-affinity type 1 receptors. The type 1 receptor is responsible for TNF-α inhibition of testosterone production by porcine Leydig cells *(30)*. The significance of the type 2 receptors on ZF cells is not known. This receptor is often associated with apoptosis *(31)* and may regulate the growth and differentiation of the adrenal cortex.

TNF-α decreases ACTH-stimulated cortisol release from fetal human adrenal cells and corticosterone release from rat adrenal cells but has no effect on basal glucocorticoid secretion *(16,18,19)*. In other studies, TNF-α increased cortisol release from adult human adrenal cells *(20)* and corticosterone release from adrenal glands of cholestatic rats *(17)*. In the study, TNF-α decreased ACTH-stimulated cortisol secretion from bovine ZF cells over concentration ranges similar to the TNF-α inhibition of fetal human adrenal cells *(18,19)*. TNF-α also inhibited basal cortisol release from ZF cells. These inconsistencies may be related to differences between species, age, physiologic state of the donor animal, or cell culture. The time course of the effects of TNF-α on glucocorticoid release was previously unknown. In the present study, TNF-α inhibited basal cortisol release at 16 h and ACTH-stimulated cortisol release at 4 h.

Interestingly, IL-6 and TNF-α modify both basal (unstimulated) and stimulated cortisol release from bovine adrenal cells. The mechanism of basal release of cortisol from cultures of bovine adrenal cells is unclear. However, this basal release may represent the release of autocrine or paracrine factors from the primary cultures of adrenal cells *(3)*. These autocrine or paracrine factors may in turn be stimulating the release of cortisol from the bovine adrenal cells. By contrast, the basal release of cortisol may represent an intrinsic steroid-secreting activity of the cultured bovine adrenal cells. In mouse Leydig cells, TNF-α decreases basal testosterone by a mechanism that may involve inhibition of the expression of the enzyme 3 β-hydroxysteroid dehydrogenase δ5 → δ4 isomerase *(23,32)*. However, in several other

steroid-secreting tissues, TNF-α has no effect upon basal steroid hormone release *(22,25)*.

Cytokines may affect adrenal function by modifying macrophage activity in the adrenal gland, and the macrophages in turn modify adrenal function (*3*). However, most ZF cells have IL-6 and TNF receptors and it is improbable that all these cells are macrophages. Therefore, IL-6 and TNF-α modify cortisol secretion by direct effects on the steroid-secreting cells of the bovine adrenal gland. However, an additional indirect effect of IL-6 and TNF-α on adrenal function cannot be excluded.

IL-6 and TNF-α may modify adrenal function through several mechanisms. IL-6 stimulates cortisol release through cyclooxygenase metabolism of arachidonic acid *(14,15)*. In other cell types, IL-6 activates JAK pathways and signal transduction and activators of transcription *(33)*. Adenylyl cyclase is not involved in IL-6 stimulation of cortisol secretion *(15)*. Furthermore, the ACTH receptor is not requisite for IL-6 enhancement of cortisol secretion in that IL-6 enhances forskolin-stimulated cortisol secretion.

Similarly, some of the effects of TNF-α on cortisol secretion occur after the ACTH receptor in that TNF-α inhibits forskolin-stimulated cortisol secretion. In human fetal adrenal cells, TNF-α reduces ACTH-stimulated cortisol release by decreasing the mRNAs for the enzymes involved in steroid synthesis *(19)*. In rat adrenal glands, TNF-α also inhibits angiotensin II–stimulated aldosterone synthesis by inhibiting the 12-lipoxygenase metabolism of arachidonic acid *(34)*. In other steroid-producing tissue, some of the mechanisms of TNF-α inhibition of steroid production have been determined. TNF-α decreases testosterone secretion from mouse Leydig cells in part by a mechanism that is after cyclic adenosine monophosphate (cAMP) production because TNF-α inhibits 8-bromo-cAMP-stimulated testosterone release and the 8-bromo-cAMP augmentation of the mRNAs for cholesterol side chain cleavage enzyme (P450scc), 7α-hydroxylase/17,20 lyase, and 3 β-hydroxysteroid dehydrogenase δ5 → δ4 isomerase *(23,32,35)*. In rat Leydig cells, TNF-α inhibits the stimulation of testosterone release produced by 8-bromo cAMP and human chorionic gonadotropin (hCG) and decreases the mRNA for P450scc *(36)*. In porcine Leydig cells, TNF-α decreases testosterone release from Leydig cells by decreasing the cellular content of steroidogenic acute regulatory protein (StAR) *(30)*. In these cells, TNF-α also decreases the receptors for hCG/luteinizing hormone (LH) and decreases hCG-stimulated cellular cAMP content *(22)*. TNF-α decreases androstenendione release from rat ovarian theca-interstitial cells by mechanisms that may involve a decrease in hCG/LH receptors, decreased LH-stimulated cAMP production, and decreased protein kinase A (PKA) activity *(24)*. In some cells, TNF-α activates sphingomyelinase and nuclear factor-κB (NF-κB) *(37,38)*. It remains to be determined whether TNF-α inhibition of cortisol release from bovine adrenal cells involves the steroidogenic enzymes, StAR, ACTH receptors, cAMP production, PKA activation, arachidonic acid metabolism, sphingomyelinase, or NF-κB.

Cortisol, the primary bovine glucocorticoid, inhibits immune and inflammatory responses. During acute stress, ACTH is the primary regulator of glucocorticoid release *(2)*. However, during chronic stress other factors regulate glucocorticoid release *(4)*. TNF-α and IL-6 play important roles in the stress, immune, and inflammatory responses *(2)*. ACTH and other hormones modify TNF-α and IL-6 released from adrenal cells *(7)*. Because IL-6 and TNF-α play a role in the stress response, are released from adrenal cells, and modify cortisol secretion, these cytokines may have a role in regulating cortisol secretion during chronic stress.

Materials and Methods

Materials

Serum-free RPMI medium and complete RPMI-1640 medium (Gibco-BRL, Grand Island, NY) were made as previously described *(8)*. ACTH 1-24 (Sigma, St. Louis, MO) was dissolved in sterile water (100 μ*M* ACTH), forskolin (Sigma) was dissolved in ethanol (10 m*M*), and recombinant human TNF-α (Calbiochemical, La Jolla, CA) was reconstituted in sterile serum-free RPMI culture medium (10 μg/mL). ACTH, forskolin, and TNF-α were stored in aliquots at –20°C until diluted with serum-free incubation medium immediately prior to an experiment. Recombinant mouse IL-6 was a gift from J. Van Snick (Ludwig Institute, Brussels, Belgium) and was diluted with complete RPMI medium (1 μg/mL) and stored at 4°C. IL-6 was then diluted with incubation medium to the desired concentration immediately before an experiment. ^{125}I- Labeled human recombinant IL-6 and TNF-α were purchased from DuPont NEN (Boston, MA) and stored at –90°C until use.

Isolation and Preparation of Bovine ZF Cell Cultures

Bovine adrenal glands (mature cows) were collected from a local abattoir (Deseret Meats, Spanish Fork, UT), and adrenal ZF cells were isolated from these adrenal glands as explained previously *(8)*. For the experiment involving the release of cortisol, 100,000 ZF cells were pipeted into each well of a 48-well cell culture plate (Costar, Cambridge, MA) containing 750 μL of complete RPMI medium per well. For the experiments involving the detection of IL-6 and TNF receptor, 1×10^6 ZF cells were transferred to a 75-cm^2 sterile T-flask containing 30 mL of complete RPMI-1640 medium. The cells were then cultured for 4–6 d at 37°C in a 5% CO_2:95% air atmosphere. Utilizing these

techniques, we produced ZF cells that secrete cortisol, but no detectable dehydroepiandrosterone or aldosterone (data not presented).

Cortisol Release Experiments

Following 4–6 d in culture, the complete RPMI was removed from the ZF cells. Sterile serum-free RPMI (0.5 mL/well) was then quickly added to each well. This medium was immediately removed and replaced with 0.2 mL of serum-free RPMI containing the various pharmacologic agents. The adrenal cells were then incubated with these agents for 4–24 h, and the medium was removed from the cells and stored frozen until assayed for cortisol. During the course of each experiment, the morphology of the cells was observed with an inverted microscope (Talaval 31 model; Zeiss, Jana, Germany).

Radioimmunoassay

The cortisol concentration in the samples was determined utilizing a cortisol radioimmunoassay kit from ICN Diagnostics (Costa Mesa, CA). The minimal detectable amount of cortisol was 25 pg, and the maximal concentration of cortisol that was on the linear portion of the cortisol standard curve was 1000 pg. The samples of cell culture medium from the bovine adrenal cells were diluted to fall within this range of cortisol values. Interassay variation was 10% and intraassay variation was < 20%.

Binding Assay for IL-6 and TNF Receptors

Following 4–6 d in culture, the culture medium was removed from the ZF cells in the T-flasks. The adrenal cells were enzymatically detached from the cell culture plastic as explained previously (*39*) and the cells were centrifuged to form a pellet (200*g*, 6 min). One milliliter of binding medium (serum-free RPMI-1640 containing 25 m*M* HEPES, 10 mg/mL of bovine serum albumin, and 0.01% NaN_3, pH 7.2) (Sigma) was added to each cell pellet, and the centrifuge tubes were shaken lightly until the cells were resuspended. Cell viability and number were determined by trypan blue exclusion (Sigma). The cell suspension was then diluted with binding medium to yield 1 million cells/50 μL. Aliquots (50 μL) of this cell suspension were dispensed into a series of 1.5-mL microfuge tubes. Varying concentrations of ^{125}I-labeled human recombinant IL-6 or TNF-α (DuPont NEN) were added to the tubes. Nonradioactive IL-6 (100 n*M*) or TNF-α (500 n*M*) was also added to some tubes to determine nonspecific IL-6 or TNF-α binding. Binding medium was added to each tube so that the total volume in each sample was 200 μL. The tubes were incubated on ice for 180 min with gentle agitation every 10–20 min.

An olive oil:dibutyl phthalate (2:8 [v:v]) (Sigma) solution (700 μL) was pipeted into a second series of 1.5-mL microfuge tubes. At the end of the 180-min incubation, 190 μL of the reaction mixture from each tube was layered on top of the oil solution in the corresponding microfuge tube. The tubes were then centrifuged at (6300*g*) for 3 min in a microfuge. The two liquid phases were then aspirated from the cell pellet and the microfuge tubes cut just above the level of the cell pellets. The cell-associated radioactivity was subsequently measured in a gamma counter. The data were graphed as the concentration of IL-6 or TNF-α vs counts per minute of the cytokine bound to the cells and analyzed by nonlinear regression. The equation utilized to fit these data is as follows:

$$\text{Bound} = (B_{max}\,[L]^n)/(K_D{}^n + [L]^n)$$

in which B_{max} is maximal binding; [*L*] is the concentration of the ligand; and $n = 1$ if there is no cooperativity, $n > 1$ if there is positive cooperativity, and $n < 1$ if there is negative cooperativity.

Immunohistochemical Detection of IL-6 and TNF Receptor in Bovine Adrenal Gland

Bovine adrenal glands were collected, transported to the laboratory, fixed, sectioned into 10 μm sections, and attached to slides as explained previously *(8)*. Immunohistochemistry was performed by covering the adrenal sections with 1 mL of 10 m*M* Tris-buffered saline (pH 7.6) containing antibodies, peroxidase suppressor, or enzyme detection reagents *(8)*. The primary antibodies utilized in this study consisted of the following polyclonal antibodies (1/200 dilution): rabbit antimouse IL-6 receptor, goat antimouse TNF type 1 receptor, and goat antimouse TNF type 2 receptor (Santa Cruz Biotechnology, Santa Cruz, CA). The binding of primary antibody was detected with an HRP-conjugated secondary antibody (1/200 dilution, donkey antirabbit IgG antibody for the IL-6 receptor assays [Pierce, Rockford, IL] or donkey antigoat IgG antibody for the TNF receptor assays [Santa Cruz Biotechnology]). The adrenal slices were then exposed to a metal-enhanced DAB detector (Pierce). As a control for nonspecific antibody binding, selected adrenal gland sections were exposed to the secondary antibody without prior exposure to primary antibody. Slides were visualized under a light microscope (Model 770; Carl Zeiss, Thornwood, NY) and the images digitally captured and processed as explained previously *(8)*.

Statistical Analyses

Each experiment was repeated at least three times. Each experiment was initially analyzed with the chi-square test of variance to determine whether all experimental groups had similar variances. If significant differences among the variances of the various experimental groups were evident (i.e., $p < 0.05$ by the chi-square test), the hormone concentration data were transformed by a log transformation and the transformed data utilized in all subsequent statistical analyses. If no significant differences between the variances of the experimental groups were evident (i.e., $p > 0.05$ by the chi-square test), the actual hormone concentra-

tions were utilized in all subsequent statistical analyses. The transformed or nontransformed data were analyzed by analysis of variance followed by the Bonferroni post tests for multiple comparisons *(40)*. Representative data from these experiments were graphed as a mean the standard ± SEM.

Acknowledgments

We wish to express gratitude for the cooperation of the management and workers of the Deseret Meat Company, Spanish Fork, Utah which provided the adrenal tissue for this research. We thank the Brigham Young University College of Biological and Agricultural Sciences and the Department of Zoology for the Professional Development Grant that funded this research.

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