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Serotonin Increases Interleukin-6 Release and Decreases Tumor Necrosis Factor Release from Rat Adrenal Zona Glomerulosa Cells In Vitro

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Interleukin-6 (IL-6) and tumor necrosis factor (TNF) are secreted by rat adrenal zona glomerulosa cells. Serotonin increases the release of aldosterone, corticosterone, and cortisol from the adrenal cortex. Therefore, the effects of serotonin on IL-6 and TNF release from rat adrenal zona glomerulosa cells were investigated. Cultures of rat adrenal zona glomerulosa cells were enzymatically prepared and cultured for 4–6 d. The cells were then exposed to serum-free RPMI-1640 medium containing vehicle (RPMI medium alone), serotonin, and/or endotoxin, interleukin-1β, or adrenocorticotrophic hormone (ACTH). Following a 5-h incubation, medium was removed from the cells, and IL-6 and TNF content of this medium determined with bioassays. Serotonin (1–1000 n*M*) increased basal IL-6 release from zona glomerulosa cells, but inhibited basal TNF release from these cells. Endotoxin and interleukin-1β (IL-1β) increased IL-6 and TNF release from zona glomerulosa cells. Serotonin potentiated IL-6 release stimulated by endotoxin and IL-1β, but inhibited TNF release stimulated by these agents. Serotonin potentiated ACTH-stimulated IL-6 release. Serotonin had no effect on IL-6 release from rat anterior pituitary cells. Because IL-6, TNF, and serotonin modify the release of aldosterone and glucocorticoids from adrenal cells, the stimulatory effects of serotonin on aldosterone and glucocorticoid release may be mediated in part by the effects of serotonin on IL-6 and TNF release from adrenal cells.

Key Words: Serotonin; interleukin-6; TNF; ACTH; endotoxin.

Introduction

The complex interactions among the various cells involved in the host response to microbial infection, foreign antigens, stress, and tissue injury are orchestrated, mediated, and controlled by a group of secreted proteins designated as cytokines. These proteins are produced by many different cell types, and act on the cells of the organism in an autocrine, paracrine, and endocrine manner. Cytokines are of central importance in the regulation of immunity, inflammation, tissue remodeling, embryonic development, and endocrine function (Spangelo et al., 1995; Besedovsky and Rey, 1996). Interleukin-6 (IL-6) is a 21–28 kDa glycoprotein and tumor necrosis factor (TNFα) is a 17-kDa polypeptide. These cytokines are produced by many cells, including fibroblasts, endothelial cells, keratinocytes, monocytes, macrophages, T-cell lines, mast cells, and tumor cell lines (Tracey et al., 1989; Hirano et al., 1990; Van Snick, 1990). In addition, IL-6 and TNFα are released from neuroendocrine and endocrine tissues, including the hypothalamus, anterior pituitary, ovary, testes, and adrenal gland (Judd et al., 1990; Spangelo et al., 1990a,b, 1995; Judd and MacLeod, 1992b; Besedovsky and Rey, 1996).

In the rat adrenal gland, in vitro studies have demonstrated that the specific location of IL-6 and TNF release is the zona glomerulosa (Judd et al., 1990; Judd and MacLeod, 1992a, 1995). In the human adrenal, IL-6 and TNF are synthesized by cells in all areas of the adrenal cortex and medulla (Gonzalez-Hernandez et al., 1994, 1996). IL-6 release from the rat adrenal zona glomerulosa cells is stimulated by substances that are known to increase during infection, tissue trauma, or stress. Thus, interleukin-1α (IL-1α), interleukin-1β (IL-1β), TNF, lipopolysaccharide (LPS), dopamine, angiotensin II, and adrenocorticotrophic hormone (ACTH) all increase the release of IL-6 from the adrenal zona glomerulosa (Judd et al., 1990; Judd and MacLeod, 1991, 1992a, 1995; Judd and Ritchie, 1995; Ritchie et al., 1996). Therefore, the adrenal gland may be one of the sources of the plasma IL-6 that is increased during many different types of stress (LeMay et al., 1990;

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Di Padova et al., 1991; Zhou et al., 1993). In addition to IL-6, the adrenal zona glomerulosa also releases TNF. The release of this cytokine from the adrenal gland is stimulated by LPS, IL-1α, and IL-1β, but inhibited by ACTH and dopamine (Judd and MacLeod, 1995; Ritchie et al., 1996).

In addition to the adrenal being a source of plasma IL-6, local release of IL-6 and TNF in the adrenal may serve as autocrine/paracrine regulators of adrenal function. TNF and IL-6 modify the release of aldosterone and glucocorticoids from the adrenal zona fasciculata/reticularis and the adrenal zona glomerulosa (Darling et al., 1989; Natarajan et al., 1989; Salas et al., 1990; Jäättelä et al., 1990, 1991). These cytokines also modify the function of the adrenal medulla (Eskay and Eiden, 1992; Nakafuku et al., 1992). Therefore, these peptides may serve an important autocrine/paracrine role in the regulation of adrenal function.

Serotonin increases aldosterone and glucocorticoid secretion from adrenal cells in vitro and in vivo (Muller and Ziegler, 1968; Haning et al., 1970; Bing and Schulster, 1977; Rocco et al., 1990; Aguilera, 1993). Serotonin is stored and synthesized in adrenal medullary tissue (Verhofstad and Jonsson, 1983; Holzwarth and Brownfield, 1985; Delarue et al., 1988, 1992; Kong et al., 1989), but islands of this medullary tissue are scattered throughout the adrenal cortex (Fernandez-Vivero et al., 1993). In addition, serotonin is found in adrenal nerve fibers and in adrenal cortical mast cells (Hinson et al., 1989; Fernandez-Vivero et al., 1993). Serotonin is also released from blood platelets (Gershon and Tamir, 1985), and the concentration of serotonin in the plasma is about 50 n*M*, a concentration that stimulates aldosterone release from adrenal zona glomerulosa cells (Rocco et al., 1990). Because serotonin increases aldosterone and glucocorticoid release from the adrenal cortex, and because the cytokines IL-6 and TNF are released from these tissues, we have determined the effects of serotonin on TNF and IL-6 release from the rat adrenal zona glomerulosa.

Results

Serotonin (10.0 n*M* and greater) stimulated IL-6 secretion in dispersed rat adrenal zona glomerulosa cells in a concentration-dependent manner. The maximum concentration tested (1000 n*M*) resulted in a stimulation of IL-6 release that was about five times basal values. Serotonin simultaneously inhibited TNF release from the zona glomerulosa in a concentration-dependent fashion. The first significant inhibition occurred at 1 n*M* serotonin, and the maximum concentration of serotonin tested (1000 n*M*) resulted in an almost complete inhibition of TNF release (Fig. 1). The adrenal cells cultured from the adrenal zona fasciculata/reticularis and medulla released either undetectable amounts of IL-6 and TNF or marginally detectable amounts of these cytokines. Serotonin did not significantly change the release of IL-6 and TNF from these cells (data not presented). The zona glomerulosa cells utilized in these

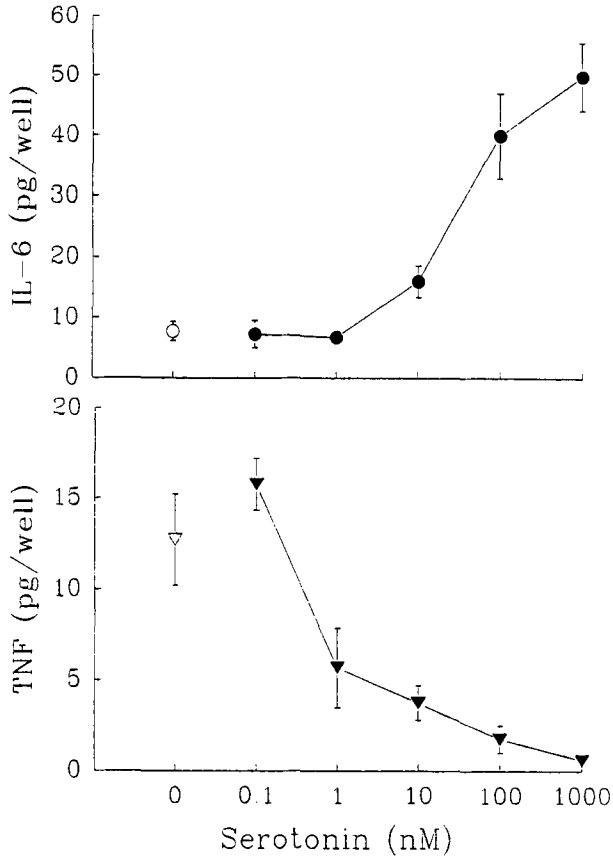

Fig. 1. The effects of serotonin on IL-6 and TNF release from primary cultures of rat adrenal zona glomerulosa cells. Serotonin (10 n*M* and greater) increased ($p < 0.01$) IL-6 release (upper panel). In the lower panel, serotonin (1 n*M* and greater) decreased ($p < 0.01$) TNF release. All incubations were of 5-h duration.

experiments secreted aldosterone (4.8 ± 0.5 ng/well) and corticosterone (105 ± 35 ng/well), whereas the zona fasciculata/reticularis cells secreted corticosterone (250 ± 20 ng/well), but no detectable amounts of aldosterone. Serotonin in a concentration-related manner (1–1000 n*M*) increased aldosterone and corticosterone release from the zona glomerulosa cells (data not presented).

Rat adrenal zona glomerulosa cells were exposed to IL-1β, LPS, and ACTH in the presence or absence of serotonin to determine the effects of serotonin on the cytokine secretion mediated by these regulators of adrenal IL-6 and TNF release. Serotonin (1–1000 n*M*) potentiated LPS-stimulated IL-6 release from zona glomerulosa cells. The effects of LPS and serotonin on IL-6 release were always at least additive, and were generally more than additive at the higher concentrations of serotonin (Fig. 2). In contrast, serotonin inhibited both basal and LPS-stimulated TNF release from the zona glomerulosa cells. At the higher concentrations of serotonin, this inhibition of LPS-stimulated TNF release became complete (i.e., serotonin inhibited LPS-stimulated TNF release to levels similar to that of serotonin alone) (Fig. 2). Similarly, serotonin potentiated IL-1β-stimulated IL-6 release, but inhibited IL-1β-stimulated

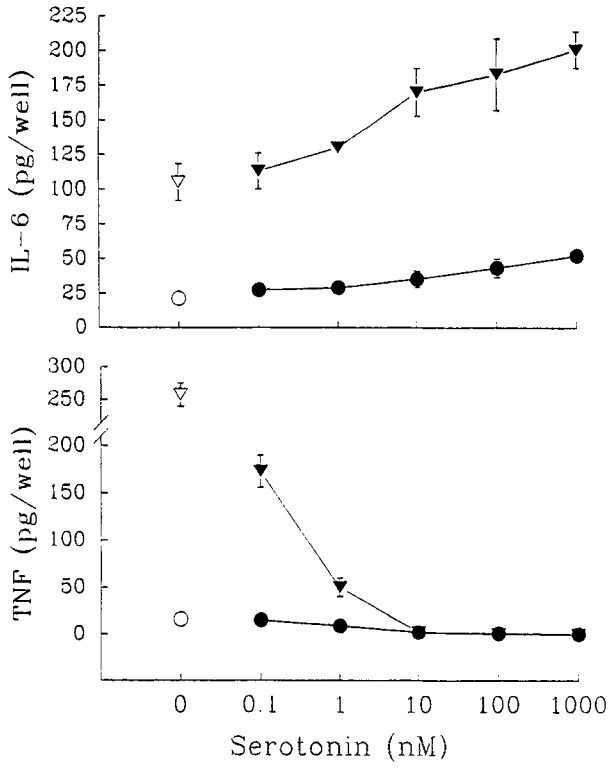

Fig. 2. Serotonin increases basal and LPS-stimulated IL-6 release, but decreases basal and LPS-stimulated TNF release from primary cultures of rat adrenal zona glomerulosa cells. LPS (1 ng/mL) increased ($p < 0.01$) IL-6 and TNF release. Serotonin (10 n*M* and higher concentrations) increased ($p < 0.01$) basal and LPS-stimulated IL-6 release. Serotonin at 1 n*M* and greater concentrations decreased ($p < 0.01$) basal TNF release and decreased ($p < 0.01$) LPS-stimulated TNF release at 0.1 n*M* and higher concentrations. Incubations were 5 h. ●, without LPS; ▼, LPS (1.0 ng/mL).

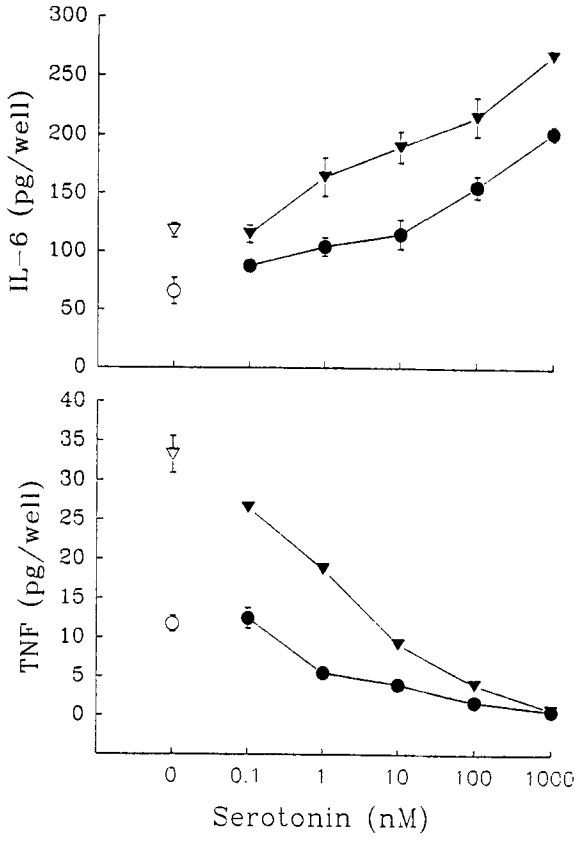

Fig. 3. The effects of serotonin on IL-1β-stimulated IL-6 and TNF release from primary cultures of rat adrenal zona glomerulosa cells. IL-1β (1 ng/mL) increased ($p < 0.01$) IL-6 and TNF release. Serotonin (1 n*M* and higher concentrations) increased ($p < 0.01$) basal and IL-1β-stimulated IL-6 release. Serotonin at 1 n*M* and greater concentrations decreased ($p < 0.01$) basal TNF release. IL-1β-stimulated TNF release was decreased ($p < 0.01$) by serotonin at concentrations of 0.1 n*M* and greater. Incubation period was 4.5 h. ●, without IL-1β; ▼, IL-1β (1.0 ng/mL).

TNF release from adrenal zona glomerulosa cells (Fig. 3). ACTH potently inhibits TNF release from adrenal zona glomerulosa cells (Judd and MacLeod, 1995). Therefore, TNF release from adrenal cells exposed to serotonin and ACTH was not determined. However, serotonin potentiated ACTH-stimulated IL-6 release from adrenal zona glomerulosa cells (Fig. 4).

The effects of serotonin on adrenal IL-6 and TNF release were compared to the effects of serotonin on IL-6 release from primary cultures of rat anterior pituitary cells. The anterior pituitary cells released no detectable amounts of TNF, but released easily detectable quantities of IL-6. Serotonin modified neither basal IL-6 release from pituitary cells nor the IL-6 release stimulated by LPS or IL-1β (data not presented).

Discussion

The adrenal cortex profoundly affects the immune system through the production and release of glucocorticoids. However, recently it has become apparent that the immune system can directly and indirectly influence the activity of the adrenal (Baseman et al., 1989; Spangelo et al., 1995; Besedovsky and Rey, 1996). Several cytokines that have traditionally been associated with the immune system have now been identified in the adrenal gland (e.g., IL-1β, TNF, and IL-6) (Spangelo et al., 1995; Besedovsky and Rey, 1996). The rat adrenal releases IL-6 and TNF from the zona glomerulosa (Judd et al., 1990; Judd and MacLeod, 1991, 1992a, 1995; Judd and Ritchie, 1995; Ritchie et al., 1996) and the mRNA for IL-6 is present in extracts of rat adrenal glands (Schöbitz et al., 1993; Muramami et al., 1993). In the human adrenal gland, the mRNAs for IL-6 and TNF are localized in cells throughout the cortex, and many of the cells that produce IL-6 and TNF are steroid-secreting cells (Gonzalez-Hernandez et al., 1994, 1996). Substances that are physiological regulators of the adrenal cortex (i.e., ACTH, angiotensin II, and dopamine) also regulate the release of TNF and IL-6 from the rat adrenal gland (Judd et al., 1990; Judd and MacLeod, 1991, 1992a, 1995; Judd and Ritchie 1995; Ritchie et al., 1996).

Serotonin stimulates aldosterone and glucocorticoid secretion from adrenal glands in vitro and in vivo (Muller

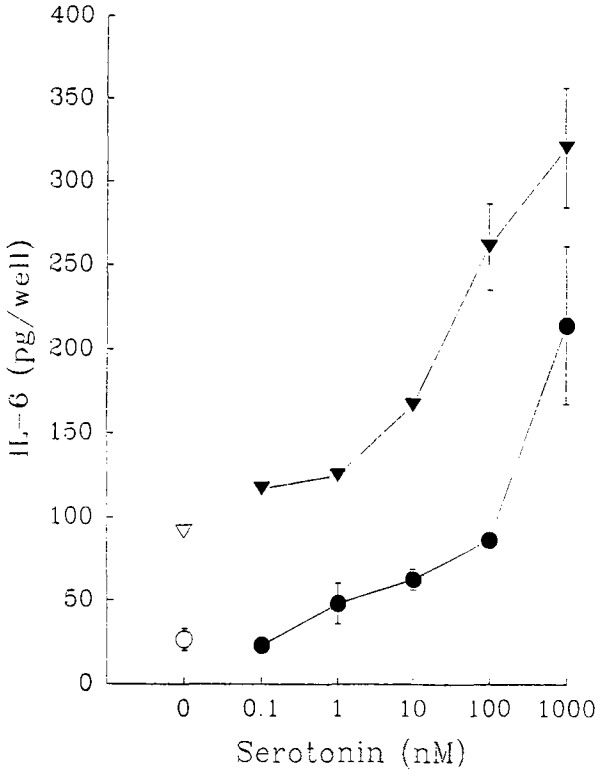

Fig. 4. Serotonin potentiates ACTH-stimulated IL-6 release from primary cultures of rat adrenal zona glomerulosa cells. ACTH (50 n*M*) increased ($p < 0.01$) IL-6 release. Serotonin (10 n*M* and higher concentrations) increased ($p < 0.01$) basal IL-6 release. ACTH-stimulated IL-6 release was increased ($p < 0.01$) by concentrations of serotonin >0.1 n*M*. Incubation duration was 5 h. ●, without ACTH; ▼, ACTH (50 n*M*).

and Ziegler 1968; Haning et al., 1970; Bing and Schulster, 1977; Rocco et al., 1990; Aguilera, 1993). These effects of serotonin on steroid release are mediated via a serotonin type 4 receptor and involve an increase in the intracellular cAMP content of adrenal cells (Rocco et al., 1990; Idres et al., 1991; Lefebvre et al., 1992; Contesse et al., 1994). Adrenal cells are exposed to serotonin that comes from at least four different sources. Serotonin is stored in the cytoplasmic granules of mast cells, and these cells are concentrated in the walls of adrenal arterioles at the point where they penetrate the capsule (Hinson et al., 1989). Stimulation of mast cell degranulation increases aldosterone and corticosterone release from *in situ* perfused rat adrenal glands, whereas ACTH-stimulated corticosterone release is diminished by inhibitors of mast cell degranulation (Hinson et al., 1989). Serotonin is also found in the plasma and probably originates from activated platelets. Plasma concentrations of serotonin can reach 50 n*M*, a concentration sufficiently high to stimulate directly aldosterone and glucocorticoid release from adrenal cells (Rocco et al., 1990) and modify adrenal cytokine release. A third and fourth source of adrenal serotonin is serotonin-containing neurons of the adrenal cortex and medulla and adrenochromaffin cells (Verhofstad and Jonsson, 1983; Holzwarth and Brownfield, 1985; Delarue et al., 1988, 1992; Kong et al., 1989). Islands of chromaffin cells are found scattered throughout the adrenal cortex (Gonzalez-Hernandez et al., 1994) and may explain the islands of serotonin-containing cells scattered in this tissue (Fernandez-Vivero et al., 1993). The serotonergic neurons, mast cells, islands of medullary tissue in the cortex, and plasma serotonin are probably the sources of the serotonin and serotonin metabolites that can be extracted from the adrenal cortex (Lefebvre et al., 1992).

The release of cytokines from adrenal tissue involves a complex interaction in which various substances either potentiate or inhibit the cytokine release stimulated by other substances (Judd et al., 1990; Judd and MacLeod, 1991, 1992a, 1995; Judd and Ritchie, 1995; Ritchie et al., 1996). In this study, serotonin influenced the release of IL-6 and TNF from primary cultures of rat adrenal zona glomerulosa cells. The serotonin-induced increase in intracellular cAMP in adrenal cells (Rocco et al., 1990; Idres et al., 1991) probably explains the effects of serotonin on IL-6 and TNF release from the adrenal zona glomerulosa cells. Serotonin increases basal and stimulated IL-6 release, and decreases basal and stimulated TNF release from adrenal cells. Augmentation of the intracellular cAMP concentration of adrenal cells increases basal IL-6 release and potentiates the IL-6 release stimulated by other secretagogs (Judd and MacLeod, 1992a). In contrast, increasing intracellular cAMP content decreases basal and stimulated TNF release from adrenal cells (Judd and MacLeod, 1995). Therefore, serotonin is probably modifying the release of IL-6 and TNF by increasing adenylate cyclase activity, and thus increases intracellular cAMP concentration. It is noteworthy that serotonin modifies the release of certain cytokines from various tissues (Charles et al., 1991; Palmer et al., 1994; Katz et al., 1994; Wilcox et al., 1994). However, the effects of serotonin on TNF and IL-6 release are unknown. It is apparent that the effects of serotonin on adrenal IL-6 and TNF release are at least in part specific effects, because serotonin does not modify IL-6 release from anterior pituitary cells or adrenal zona fasciculata/reticularis.

Although the rat adrenal zona glomerulosa cells release IL-6 and TNF, the cell type(s) involved in the IL-6 production has not been identified. Furthermore, it has not been determined if a single cell type releases both of these cytokines, or if one cell type releases IL-6, whereas another releases TNF. It is conceivable that these cytokines only represent contamination from circulating macrophages or other blood-borne cell types. Similarly, it is possible that the IL-6 and TNF released from the adrenal zona glomerulosa may simply originate from the fixed macrophages, fibroblasts, or vascular endothelial cells, which could be present in cell preparations. If either of these hypotheses are correct, it would be expected that IL-6 and TNF would be released in similar amounts from both the zona glomerulosa and zona fasciculata/reticularis, since both of these tissues undoubtedly contain fixed macrophages,

fibroblasts, and endothelial cells. However, the zona glomerulosa releases large amounts of IL-6 and TNF, whereas the zona fasciculata/reticularis releases only small amounts of these cytokines (Judd and MacLeod, 1992a, 1995). Furthermore, serotonin failed to modify significantly IL-6 or TNF release from zona reticularis/fasciculata cells. The zona glomerulosa was successfully isolated from the zona fasciculata/reticularis in the present study, because the zona glomerulosa cells secreted aldosterone, whereas the zona fasciculata/reticularis cells did not. Further, the ratio of aldosterone secretion to corticosterone secretion in the two adrenal preparations in the present study are similar to this ratio in previous studies in which these adrenal zones have been isolated (Haning et al., 1970). Therefore, the cell type(s) in the adrenal that releases IL-6 and TNF appears to be primarily tissue/zone-specific. It is noteworthy that angiotensin II, serotonin, dopamine, TNF, and ACTH all regulated the release of IL-6 and/or TNF from adrenal cells (Judd et al., 1990; Judd and MacLeod, 1991, 1992a, 1995; Judd and Ritchie 1995; Ritchie et al., 1996). However, none of these substances have similar affects on IL-6 release from the pituitary (Spangelo et al., 1990b, 1995). Therefore, the cell type(s) that releases IL-6 from the zona glomerulosa of the adrenal is very different from the IL-6-secreting cells of the anterior pituitary. Similarly, peritoneal macrophages do not release IL-6 when exposed to ACTH (unpublished observation). Although the cell type(s) that produces IL-6 and TNF in the rat adrenal zona glomerulosa is not yet identified, we have determined in preliminary experiments that 80% of the cells in the rat adrenal zona glomerulosa contain IL-6 and that 90% of the cells in these cultures are steroid-secreting cells (unpublished observation). Therefore, it is highly probable that the majority of the IL-6-secreting cells in the zona glomerulosa cultures are steroid-secreting cells. Similarly, a high percentage of cells in the human adrenal that secrete IL-6 or TNF are steroid-secreting cells (Gonzalez-Hernandez et al., 1994, 1996).

Serotonin may not only directly regulate adrenal function, but it also may indirectly affect the adrenal through its influence on IL-6 and TNF release from the zona glomerulosa. Although the effects of IL-6 on the adrenal zona glomerulosa are unknown, IL-6 increases basal and ACTH-stimulated glucocorticoid release from the rat adrenal zona fasciculata/reticularis (Sales et al., 1990). TNF inhibits angiotensin II- and ACTH-stimulated aldosterone release and ACTH-stimulated corticosterone release from rat adrenal cells (Vandermeer et al., 1996). The release of cortisol from the human fetal adrenal zona fasciculata/reticularis is also inhibited by TNF (Natarajan et al., 1989; Jäättelä et al., 1990, 1991), but this cytokine may increase the release of cortisol from the adult human adrenal (Darling et al., 1989). IL-6 and TNF also modify the function of the adrenal medulla (Eskay and Eiden 1992; Nakafuku et al., 1992). Because IL-6 and TNF are present in the adrenal cortex and because these cytokines modify adrenal function, it is probable that IL-6 and TNF are involved in autocrine/paracrine regulation of adrenal function. The anatomy of the adrenal is particularly well suited for paracrine interactions. Although the adrenal zona glomerulosa is the primary site of IL-6 and TNF synthesis in the rat adrenal, blood flow in the adrenal is from the zona glomerulosa to the zona fasciculata/reticularis and medulla (Idelman, 1978; Yeasting, 1985). Therefore, IL-6 and TNF released from the zona glomerulosa may play a paracrine role in the regulation of the zona fasciculata/reticularis and medulla, because substances that are released from the zona glomerulosa are carried by the blood to the zona fasciculata/reticularis and medulla.

In conclusion, the rat adrenal zona glomerulosa produces IL-6 and TNF, and the release of these cytokines is regulated by serotonin. Adrenal tissue serotonin concentration may increase during periods of stress owing to serotonergic nerve stimulation, mast cell degranulation, platelet activation, and release of serotonin from chromaffin cells. This increase in adrenal serotonin concentration may influence the release of IL-6 and TNF from the adrenal cells. The IL-6 and TNF released by adrenal cells in turn may modify adrenal function via paracrine/autocrine interactions. Therefore, serotonin may affect adrenal function directly and/or indirectly through modifying adrenal IL-6 and TNF release.

Materials and Methods

Materials

Serotonin (Sigma St. Louis, MO) was dissolved in sterile medium and diluted to the desired concentration immediately preceding an experiment. LPS from *Salmonella typhosa* (Sigma) and recombinant human IL-1β (Biological Modifiers Program, National Cancer Institute, Frederick, MD) were dissolved (10 μg/mL) in sterile serum-free RPMI-1640 growth medium (RPMI) and stored at 4°C. These agents were then diluted with sterile RPMI medium to the required concentration immediately before an experiment. ACTH (Organon, West Orange, NJ) was dissolved in sterile water, diluted to 10 m*M*, and stored in 10-μL aliquots at –20°C until diluted with sterile incubation medium prior to an experiment. Recombinant mouse IL-6 and the 7TD1 cell line were generously provided by J. Van Snick (Ludwig Institute, Brussels, Belgium). The WEHI 164 subclone 13 cells were obtained from D. G. Remick (University of Michigan Medical School, Ann Arbor, MI) with permission of Terje Espevik (University of Trondheim, Trondheim, Norway). TNF standard was obtained from the Biological Modifiers Program, National Cancer Institute (Frederick, MD).

Adrenal Dispersion

Female Sprague-Dawley rats (Sasco, Omaha, NE) weighing 220–250 g were housed in a thermoneutral environment with a 12-h light/12-h dark cycle, and were provided with unlimited food and water. Decapitation took

place in the morning. Adrenal glands were removed rapidly and placed in sterile complete RPMI containing 2.5% FCS, 7.5% horse serum, 19 μg penicillin G/mL, 7.5 μg streptomycin/mL, 15 μg gentamicin/mL, and 0.6 μg fungizone/mL (all from Gibco, Grand Island, NY). The zona glomerulosa of the adrenal glands were separated from the zona reticularis, zona fasciculata, and medulla by previously described techniques (Judd and MacLeod, 1992a). The zona glomerulosa fragments were then enzymatically dispersed according to published technique (Judd and MacLeod, 1992a, 1995). Cells (25–50,000) were added to each well of a 48-well culture plate (Costar, Cambridge, MA) containing 750 μL complete RPMI/well. The cells were cultured for 5 d at 37°C in an atmosphere of 5% CO_2:95% air. In selected experiments, the adrenal fragments containing the zona fasciculata/reticularis and medulla were dispersed, and the resulting cells cultured as explained for the zona glomerulosa.

Anterior Pituitary Dispersion

In select experiments, primary cultures of rat anterior pituitary cells were exposed to serotonin as explained for the adrenal cells. These primary cultures were obtained from female Sprague-Dawley rats as explained previously (Spangelo et al., 1991).

IL-6 and TNF Release and Bioassay

On the day of an experiment, the cell-culture wells containing the pituitary or adrenal cells were examined with an inverted microscope to ensure uniformity of cell number in each well. The complete RPMI was then removed and replaced with 0.5-mL sterile serum-free RPMI, which in turn was replaced with 0.5 mL sterile serum-free RPMI containing the various pharmacological test agents. The adrenal cells were incubated for 4.5–5 h with these agents; the medium then was removed from the cells and stored at 4°C until assayed for IL-6 or TNF content using the either the 7TD1 bioassay for IL-6 or the WEHI 164 bioassay for TNF as previously described (Judd and MacLeod, 1992a, 1995).

Aldosterone and Corticosterone Radioimmunoassays

The aldosterone and corticosterone content of the incubation medium was determined in selected experiments by radioimmunoassays utilizing protocols and reagents from ICN Biomedical Inc. (Costa Mesa, CA).

Statistical Analysis

Data are expressed as pg IL-6/well or pg TNF/well. Each point or bar in the figures represents the mean ± SEM of 4–6 replicates within a single experiment. Data were analyzed by a one-way analysis of variance and the Bonferroni test for multiple comparisons (Wallenstein et al., 1980). Each experiment was repeated at least three times, and each figure illustrates the results from a typical experiment.

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