Estrogens sensitize anterior pituitary gland to apoptosis

D. Pisera, M. Candolfi, S. Navarra, J. Ferraris, V. Zaldivar, G. Jaita, M. G. Castro, and A. Seilicovich. Estrogens sensitize anterior pituitary gland to apoptosis. Am J Physiol Endocrinol Metab 287: E767–E771, 2004. First published June 1, 2004; 10.1152/ajpendo.00552.2004.—Tissue homeostasis results from a balance between cell proliferation and cell death by apoptosis. Estradiol affects proliferation as well as apoptosis in hormone-dependent tissues. In the present study, we investigated the apoptotic response of the anterior pituitary gland to lipopolysaccharide (LPS) in cycling female rats, and the influence of estradiol in this response in ovariectomized (OVX) rats. The OVX rats were chronically estrogenized with implanted Silastic capsules containing 1 mg of 17β-estradiol (E2). Cycling or OVX and E2-treated rats were injected with LPS (250 μg/rat ip). Apoptosis was determined by the terminal deoxynucleotidyl-mediated dUTP nick-end labeling (TUNEL) method in sections of the anterior pituitary gland and spleen. Chronic estrogenization induced apoptosis in the anterior pituitary gland. Acute endotoxemia triggered apoptosis of cells in the anterior pituitary gland of E2-treated rats but not of OVX rats. No differences were observed in the apoptotic response to LPS in spleen between OVX and E2-treated rats. The apoptotic response of the anterior pituitary to LPS was variable along the estrous cycle, being higher at proestrus than at estrus or diestrus I. Approximately 75% of the apoptotic cells were identified as lactotropes by immunofluorescence. In conclusion, our results indicate that estradiol induces apoptosis and enables the proapoptotic action of LPS in the anterior pituitary gland. Also, our study suggests that estrogens may be involved in anterior pituitary cell renewal during the estrous cycle, sensitizing lactotropes to proapoptotic stimuli.

Materials and Methods

LIPOLYPOSACCHARIDE (LPS), an endotoxin of gram-negative bacteria, is commonly used to study neuroendocrine-immune interactions. After systemic administration of LPS, the plasma concentrations of several cytokines, such as TNF-α, IL-1, and IL-6 rise in a temporal-sequential manner (14, 27). Systemic cytokines, as well as those locally released in the central nervous system and pituitary, contribute to the neuroendocrine response to endotoxemia, such as hypothalamic-pituitary-adrenal axis (HPA) activation and hypothalamic-pituitary-gonadal axis inhibition (27, 28, 29). We have reported that systemic LPS and central TNF-α administration exert inhibitory effects on prolactin secretion in male rats by stimulating dopaminergic activity in the hypothalamic-pituitary axis (4), and that LPS (25) and TNF-α (26) reduce in vitro prolactin release from anterior pituitary cells of female rats.

Gonadal steroid hormones modulate the neuroendocrine response to inflammatory stimuli. It has been reported that endotoxin-induced HPA activation is higher in female than in male rats. This sexual difference is abolished by gonadectomy and restored by administration of estradiol to ovariectomized (OVX) rats (28). We have observed that estradiol stimulates basal and LPS-induced TNF-α secretion from anterior pituitary cells of OVX rats (25). Also, TNF-α release from anterior pituitary cells varies along the estrous cycle reaching the highest levels in cells from rats at proestrus (26). The effects of TNF-α in anterior pituitary cells are also modulated by estradiol. We (26) previously reported that TNF-α reduces the proliferation of anterior pituitary cells and inhibits prolactin secretion in an estrogen-dependent manner. TNF-α induces apoptosis of lactotropes and its effect is higher in cells from rats euthanized at proestrus than at other stages of the estrous cycle, suggesting that the steroid environment modulates the proapoptotic action of this cytokine in the anterior pituitary (2). In fact, in anterior pituitary cells from OVX rats, TNF-α significantly increased the percentage of apoptotic lactotropes only when the cells were incubated in the presence of 17β-estradiol (E2) (2).

Considering that LPS induces TNF-α gene expression in the anterior pituitary gland (30), we investigated the effect of the systemic administration of LPS on the apoptotic index in the anterior pituitary of cycling female rats, and the influence of estradiol in the apoptotic response to acute endotoxemia in this gland.

Materials and Methods

Animals. Adult female Wistar rats weighing 200–250 g were used. The animals were kept in controlled conditions of light (lights on from 7 AM to 7 PM) and temperature (20–25°C), with water and food available ad libitum. All the experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In experiments using OVX rats, the animals were castrated under ether anesthesia and implanted under the skin of the back with Silastic capsules (length 20 mm, outer diameter 2 mm) containing 1 mg E2 at the time of ovariectomy. These capsules induce estradiol plasma levels similar to those of rats at proestrus (13). Control animals were implanted with empty capsules. Two weeks later, the animals were injected with LPS (250 μg/rat ip, dissolved in pyrogen-free isotonic saline) or vehicle at 10 AM and euthanized by decapitation 8 h later. In additional experiments, chronically E2-treated rats were injected with LPS (250 μg/rat ip) or vehicle at 10 AM and euthanized 2, 4, 8, or 24 h later.

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When cycling female rats were used, animals were monitored by daily vaginal smears. Rats with three or more normal consecutive 4-day estrous cycles were used. Rats at different stages of the estrous cycle were injected with LPS (250 μg/rat ip) or vehicle at 11 AM and euthanized 7 h later.

Drugs. All drugs were obtained from Sigma (St. Louis, MO) including bacterial LPS (Escherichia Coli serotype 011:B8), except terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), primary antibodies against anterior pituitary hormones (kindly donated by Dr. A. Parlow, National Hormone and Pituitary Program, Torrance, CA), and anti-guinea pig rhodamine-conjugated secondary antibody (Chemicon International, Temecula, CA).

Determination of anterior pituitary apoptosis by TUNEL method. Pituitary glands were removed within minutes after decapitation. In some experiments spleen was also removed. The tissues were washed in PBS and fixed in 4% formaldehyde and 2% picric acid in 0.1 M PBS, pH 7.4, for 4 h.

Cryosection sections (16 μm) were permeabilized by microwave irradiation in 10 mM citrate buffer, pH 6, for 5 min, and then in the same buffer with 0.1% Triton X-100 for 4 min at room temperature. DNA strand breaks were labeled with digoxigenin-dUTP using TdT (0.5 U/μl) according to the manufacturer’s protocol (2). The incorporation of nucleotides into the 3’-OH end of damaged DNA was detected with an antidigoxigenin-fluorescein antibody (1:10). Control slices were incubated without TdT. The sections were mounted in anti-fade mounting medium (DABCO) with 4′,6-diamino-2-phenylindole (DAPI) and observed under fluorescence light microscope (Axiopt; Zeiss, Jena, Germany).

Identification of anterior pituitary cells. Anterior pituitary cell populations undergoing apoptosis were identified in paraffin sections (3 μm) by TUNEL staining combined with indirect immunofluorescence for anterior pituitary hormones. After incubation with digoxigenin-dUTP-TdT, the sections were preincubated with 10% donkey serum in PBS for 60 min and then incubated with primary antibodies against anterior pituitary hormones [guinea pig anti-rat PRL (NHPP-IC, 1:2,000), guinea pig anti-rat GH (NHPP-IC, 1:1,000), guinea pig anti-rat βLH (NHPP-IC-2, 1:1,250) and guinea pig anti-rat adrenocorticotropic hormone (NHPP-IC, 1:500)] in 1% donkey serum in PBS for 60 min. Control sections were incubated with dilution buffer. After being rinsed, the sections were incubated for 60 min with donkey anti-guinea pig rhodamine-conjugated secondary antibody (1:200) plus antidigoxigenin-fluorescein antibody (1:10) in the same buffer. The sections were then washed, mounted, and observed under fluorescence light microscope.

Statistical analysis. TUNEL-positive cells were counted on 10–20 fields (×400) in anterior pituitary sections from each rat. The average of TUNEL-positive cells per field for each rat was considered as an individual value. The averages of TUNEL-positive cells per field were analyzed by two-way ANOVA, followed by Tukey’s test. Differences were considered significant when P < 0.05. Results are expressed as means ± SE of TUNEL-positive cells per field (×400).

RESULTS

Effect of estradiol on apoptosis induced by LPS in anterior pituitary gland. OVX and chronically E2-treated rats were injected with LPS (250 μg/rat) or vehicle (control) and euthanized 8 h later. The number of TUNEL-positive cells in the neural and intermediate lobes was very scarce and did not allow statistical comparison between experimental groups. Staining with DAPI allowed observation of the nuclear morphological features of apoptosis in cells of the anterior pituitary lobe, such as chromatin condensation and fragmentation (Fig. 1A). Chronic E2 treatment increased the apoptotic index (the number of TUNEL-positive cells/field) in the anterior pituitary (Fig. 2). LPS administration increased the apoptotic index only in chronically estrogenized rats (Fig. 1B, Fig. 2). Approximately 75% of the apoptotic cells were identified as lactotropes by immunofluorescence (Fig. 1C). No apoptotic somatotropes, gonadotropes, or corticotropes were observed (not shown).

To evaluate whether the effect of estradiol on the apoptotic response to endotoxemia in the anterior pituitary gland was
tissue specific, we explored the effect of LPS on the apoptotic index in spleen of OVX and E2-treated rats. Although the number of TUNEL-positive cells in the spleen was significantly increased by LPS, no differences between OVX and E2-treated rats were observed (Fig. 3).

**Time course of apoptosis induced by endotoxemia in the anterior pituitary gland.** Chronically E2-treated rats were injected with LPS (250 μg/rat) or vehicle at 10:00 AM and killed at different time periods after endotoxin administration. The apoptotic effect of LPS in the anterior pituitary appeared 4 h postinjection and remained evident at 8 h. At 24 h postinjection the apoptotic index of the anterior pituitary gland returned to basal levels (Fig. 4). In vehicle-treated groups (controls) the number of TUNEL-positive cells varied according to the time period of death posttreatment and was significantly reduced at 8 h postinjection (6 PM) (Fig. 4).

**Effect of endotoxemia on apoptosis in anterior pituitary along the estrous cycle.** To evaluate the possible modulatory effects of circulating levels of estradiol on the apoptotic action of LPS, female rats at different stages of the estrous cycle were injected with LPS (250 μg/rat) or vehicle at 11 AM and euthanized 7 h later. The administration of LPS significantly increased the apoptotic index in the anterior pituitary gland of rats at proestrus and diestrus, but not at estrus. The apoptotic response to LPS was significantly higher at proestrus than at diestrus I (Fig. 5).

**DISCUSSION**

The present study shows that, during acute endotoxemia, apoptosis is triggered in the anterior pituitary gland and that this response is modulated by estradiol. Systemic administration of LPS is a useful tool to elucidate the response of the hypothalamic-pituitary axis to inflammatory stimuli (4, 27, 28, 29). Once LPS binds to circulating LPS-binding protein and CD14, this complex interacts with Toll-like receptor type 4, which can trigger apoptosis (11). Because CD14 and Toll-like receptor type 4 are expressed in the anterior pituitary gland (19), LPS could be exerting a direct proapoptotic effect. However, most of the neuroendocrine actions of LPS are mediated by its stimulatory actions on the secretion of various proinflammatory cytokines in peripheral immune cells, brain, and...
pituitary gland (9, 27, 28, 29, 30). TNF-α plays a central role during endotoxemia, initiating a cascade of synthesis of other factors required for an appropriate neuroendocrine response to infection (14). TNF-α is expressed in the pituitary gland after LPS administration (30) and is released from anterior pituitary cells in response to LPS (25). Since the anterior pituitary gland expresses TNF-α receptors (31) and this cytokine induces apoptosis of anterior pituitary cells (2), circulating and/or locally produced TNF-α could be involved in the apoptotic response of the anterior pituitary to endotoxemia.

The immune response presents sexual dimorphism. Autoimmune diseases are more prevalent in females than in males, and the development of some autoimmune diseases is modulated by circulating levels of gonadal steroids (8, 20). In postmenopausal women, estrogens inhibit cytokine release and HPA activation in response to inflammatory stimuli (23). However, in OVX rats, estradiol enhances the adrenocorticotropic hormone secretory response to LPS, without affecting the increase in TNF-α, IL-1, or IL-6 plasma levels (28). In the present study, we observed that the systemic administration of LPS induces apoptosis of anterior pituitary gland in chronically estrogenized rats but not in OVX rats. These results suggest that estradiol increases the responsiveness of anterior pituitary cells to LPS, which by direct action or through auto/paracrine factors may trigger apoptosis in the anterior pituitary gland. The lack of effect of LPS in OVX rats seems to be tissue specific, because no differences were observed in LPS-induced apoptosis in the spleen between OVX and E2-treated rats. Chronic estradiol treatment not only sensitized the anterior pituitary cells to the proapoptotic effect of LPS, but also induced apoptosis per se in the anterior pituitary gland. The variations observed in basal anterior pituitary apoptosis in E2-treated rats killed at different times suggest diurnal changes in the proapoptotic action of estradiol, as was described for the effects of estrogens on lactotrope proliferation (13).

Estrogens have been reported to enhance neuronal survival through genomic and nongenomic mechanisms (10), and to inhibit the apoptosis induced by TNF-α in neurons (16). Also, estrogens have been shown to exert proliferative and antiapoptotic actions in a prolactin secreting pituitary cell line derived from F344 rats, a strain particularly sensitive to estradiol-induced pituitary tumors (3). However, estradiol inhibits IGF-I- and insulin-induced anterior pituitary cell proliferation (15), and induces apoptosis in somatolactotrope GH4C1 cells that overexpress ER-α (17). Evidence shows that lactotrope apoptosis occurring after termination of lactation involves changes in the expression of modulators of cell survival such as Bax and p53 (1). The p53 tumor suppressor protein can induce cell death, and therefore, the inhibition of p53-induced apoptosis can promote tumor progression (7). Estradiol increases p53 gene expression in human breast cancer MCF-7 cells, and it has been suggested that this effect may contribute to regulatory pathways leading to inhibition of estradiol-induced cell proliferation (24). In fact, in Sprague-Dawley rats, anterior pituitary p53 mRNA levels increase in response to estrogen treatment, whereas they remain undetectable in F344 rats (34). There is evidence that cell proliferation and apoptosis are coupled processes. The activation of cell proliferation necessarily primes the cellular apoptotic program that, unless abrogated by appropriate survival signals, automatically removes the affected cell (5). In fact, it was observed that adenalem...

In conclusion, estradiol induces apoptosis in the anterior pituitary gland and increases its sensitivity to proapoptotic stimuli such as LPS, suggesting that estrogens may be involved in anterior pituitary apoptosis during the estrous cycle.

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