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ORIGINAL ARTICLE

Opposite effects of dihydrotestosterone and estradiol on apoptosis in the anterior pituitary gland from male rats

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Abstract Hormones locally synthesized in the anterior pituitary gland are involved in regulation of pituitary cell renewal. In the pituitary, testosterone (T) may exert its actions per se or by conversion to dihydrotestosterone (DHT) or 17β-estradiol (E_2) by 5α-reductase and aromatase activity, which are expressed in this gland. Previous reports from our laboratory showed that estrogens modulate apoptosis of lactotropes and somatotropes from female rats. Now, we examined the in vitro and in vivo effects of gonadal steroids on apoptosis of anterior pituitary cells from adult male rats. T in vitro did not modify apoptosis in anterior pituitary cells from gonadectomized (GNX) male rats. DHT, a non-aromatizable androgen, exerted direct antiapoptotic action on total anterior pituitary cells and folliculo-stellate cells, but not on lactotropes, somatotropes, or gonadotropes. On the contrary, E_2 exerted a rapid apoptotic effect on total cells as well as on lactotropes and somatotropes. Incubation of anterior pituitary cells with T in presence of Finasteride, an inhibitor of 5α-reductase, increased the percentage of TUNEL-positive cells. In vivo administration of DHT to GNX rats reduced apoptosis in the anterior pituitary whereas E_2 exerted proapoptotic action and reduced cells in G2/M-phase of the cell cycle. In summary, our results indicate that DHT and E_2 have opposite effects on apoptosis in the anterior pituitary gland suggesting that local metabolization of T to these steroids could be involved in pituitary cell turnover in males. Changes in expression and/or activity of 5α-reductase and aromatase may play a role in the development of anterior pituitary tumors.

Keywords Pituitary · Apoptosis · Estrogens · Androgens · Aromatase · 5α-reductase

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Introduction

Maintenance of tissue homeostasis in the anterior pituitary gland is regulated mainly by hormones and neurotransmitters released from the hypothalamus and by systemic hormones secreted by target glands. Hormones, growth factors, and cytokines locally synthesized in the pituitary gland are also involved in regulation of pituitary cell renewal, acting in an autocrine and paracrine manner [1–4]. The pituitary gland dynamically adapts its responses to coordinate changes in physiological demands [5]. Under basal conditions, daily cell turnover in the adult male pituitary is approximately 1.5 % [6], whereas pituitary mitotic activity in cycling female rats was reported to be two-fold greater than in males [7]. The distribution of mitotic activity amongst different pituitary cell types is similar in both sexes, with lactotropes accounting for the highest proportion of mitotic activity overall [7].

Androgens, the principal male sex hormones, are essential for physiological processes such as differentiation and maintenance of male phenotype and reproductive functions [8]. Production of testosterone (T) by testicular Leydig cells is regulated by the hypothalamic-pituitary–gonadal axis [9]. T controls its own secretion via negative feedback, inhibiting both hypothalamic release of gonadotropin-releasing hormone (GnRH) and anterior pituitary secretion of luteinizing hormone (LH) [10]. T is effective not only in regulating gonadotropin secretion, but also

decreases prolactin (PRL) production and release [11] and increases growth hormone (GH) secretion in male pituitary [12, 13].

T can exert its actions per se or by its conversion to dihydrotestosterone (DHT) and 17β-estradiol (E_2) by 5α-reductase or aromatase activity, respectively [14]. Both enzymes are expressed in the pituitary [15, 16], where their expression is regulated by gonadal steroids [17–19]. Both T and DHT exert their actions through binding to classical androgen receptors (AR) which induce changes in gene expression of target cells [20]. DHT is a powerful androgen with five-fold higher affinity for AR than T [21], and therefore DHT formation amplifies T action in target tissues [22]. AR was detected in about 50 % of anterior pituitary cells from male rats, and was localized in all gonadotropes and other unidentified secretory cells [23]. Recently, AR was reported to be expressed in all endocrine cell types of the male mouse pituitary [24]. Estradiol exerts its actions through activation of estrogen receptors (ER), ERα and ERβ [25]. Approximately 90 % of anterior pituitary cells from male rats express ERα [26], whereas the expression of ERβ is very low [27]. The presence of membrane-associated ERα was also reported in pituitary from female rats [28].

Death by apoptosis is essential for multiple physiological processes such as development, immune responses and tissue homeostasis. Apoptosis is a highly regulated process whose failure can result in pathological conditions, including tumor development. Apoptosis is executed by proteases called caspases which can be activated by ligand binding to cell-surface death receptors (extrinsic pathway) or changes in mitochondrial outer membrane permeability (intrinsic pathway). The extrinsic pathway depends on ligands such as TNF-α, FasL, and TRAIL which interact with death receptors whereas a balance between actions of proapoptotic and antiapoptotic members of the Bcl-2 family controls the intrinsic pathway [29]. The antiapoptotic Bcl-2 family proteins, including Bcl-2 and Bcl-xL, promote cell survival, whereas proapoptotic proteins such as Bax have the opposite effect. These distinct but eventually converging signaling pathways drive terminal events of apoptosis [30].

Changes in circulating sex hormone levels affect proliferation and death in the anterior pituitary gland in female rats [7]. Previous reports from our laboratory showed that estrogens modulate apoptosis of lactotropes and somatotropes from female rats through both extrinsic and intrinsic apoptotic pathways [3]. However, the role of estrogens and androgens in apoptosis of the adult male pituitary is not fully understood. In the present study, we examined in vitro and in vivo effects of gonadal steroids on apoptosis of anterior pituitary cells from male rats. Our results indicate that DHT, a non-aromatizable androgen, exerts antiapoptotic action on anterior pituitary cells from male rats whereas E_2 is proapoptotic, suggesting that local metabolization of T to these steroids could be involved in pituitary cell turnover in males.

Materials and methods

Drugs and reagents

All drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except for phenol red-free Dulbecco's modified Eagle's medium and supplements (GIBCO, Invitrogen, Carlsbad, CA), FBS (Natocor, Córdoba, Argentina), all terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), antibodies against anterior pituitary hormones (Dr. A. Parlow, National Hormone and Pituitary Program, Torrance, CA), anti-guinea pig Alexa 555-conjugated antibody (Invitrogen, Carlsbad, CA), anti-rabbit Alexa 594-conjugated antibody (Jackson Laboratories, West Grove, PA), anti-mouse Cy3-conjugated antibody (Chemicon, Temecula, CA), streptavidin horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit antibody (Millipore, Temecula, CA) and the materials indicated below.

Animals

Adult Wistar male rats (200–250 g) were kept in controlled conditions of light (12 h light–dark cycles) and temperature (20–22 °C). Rats were fed standard laboratory chow and water ad libitum and kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal protocols were previously approved by the Ethics Committee of the School of Medicine, University of Buenos Aires (Res. N° 1204/2010). Rats were gonadectomized (GNX) 2 weeks before experiments under ketamine (100 mg/Kg, i.p.) and xylazine (10 mg/Kg, i.p.) anesthesia and ketoprofen (5 mg/kg) for analgesia. In some experiments, GNX rats were s.c. injected for two consecutive days with vehicle (propyleneglycol), 17β-estradiol (E_2, 0.2 mg/kg b.w.) or dihydrotestosterone (DHT, 5 mg/kg b.w.) and killed 24 h after the last injection. Anterior pituitary glands were removed within minutes after decapitation. Anterior pituitary cells were dispersed and processed for the experiments described below.

Cell culture

A pool of anterior pituitary cells from 2–5 intact or GNX rats was used for each culture. Anterior pituitary glands

were washed several times with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 µl/ml MEM amino acids, 2 mM glutamine, 5.6 µg/ml amphotericin B, 100 U/ml penicillin, 100 µg/ml streptomycin (DMEM-S) and 3 mg/ml bovine serum albumin (BSA). Then, glands were cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in DMEM-S-BSA, containing 0.75 % trypsin, 10 % fetal bovine serum (FBS) previously treated with 0.025 % dextran-0.25 % charcoal (FBS-DCC) to remove steroids and 45 U/µl deoxyribonuclease type I (Invitrogen, Carlsbad, CA). Finally, cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca^{2+} or Mg^{2+}. Dispersed cells were washed and resuspended in DMEM-S with 10 % FBS-DCC. Cell viability assessed by trypan blue exclusion was over 90 %. Anterior pituitary cells were seeded onto cover slides in 24-well tissue culture plates at a density of 1.5×10^5 cells ml^{-1} $well^{-1}$ for TUNEL assay and immunocytochemistry or at a density of 1×10^6 cells ml^{-1} $well^{-1}$ for Western Blot. Cells were cultured for 24 h in DMEM-S with 10 % FBS-DCC and then incubated for 2 or 24 h in phenol red-free, serum-free DMEM-S supplemented with 0.1 % BSA containing T (10^{-8} M), DHT (10^{-8} M), E_2 (10^{-9} M), or the corresponding vehicle (ethanol, 1- 10 µl/l). In some experiments, anterior pituitary cells were incubated in the presence of Finasteride (10^{-6} M), a 5α-reductase inhibitor, Letrozole (10^{-7} M), an aromatase inhibitor, or vehicle (ethanol, 100 µl/l) for 30 min and then co-incubated with T (10^{-8} M) for further 2 h.

Immunolocalization of aromatase

The presence of aromatase in cultured anterior pituitary cells from intact male rats was detected by double indirect immunofluorescent staining. Briefly, after the culture period, cells were fixed with 4 % PFA in PBS for 10 min. Cells were permeabilized by microwave irradiation and with 0.2 % Triton X-100 and incubated with 10 % normal goat serum-3 % BSA in PBS. Then, slides were incubated overnight with rabbit anti-aromatase antibody (1:100, Abcam, Cambridge, MA) in 1 % normal goat serum in PBS. After rinsing, slides were incubated with anti-rabbit-FITC (1:200) in the same buffer for 1 h. To detect PRL, GH, βLH or ACTH immunoreactivity, slides were incubated with guinea pig anti-rat PRL (NHPP-IC, 1:2500), anti-rat GH (NHPP-IC, 1:1500), anti-rat βLH (NHPP-IC, 1:500) or anti-rat ACTH (NHPP-IC, 1:1000) with 1 % normal goat serum in PBS - 0.2 % Triton X-100 for 1 h. After rinsing, slides were incubated with anti-guinea pig Alexa 555-conjugated antibody (1:200) in the same buffer for 1 h. Finally, slides were mounted with mounting medium for fluorescence (Vectashield, Vector Laboratories Inc., Burlingame, CA) containing 4́,6 diamidino-2-phenylindoledihydrocloride (DAPI), and visualized in a fluorescence microscope. Control slides were incubated with the corresponding normal serum or IgG subtype instead of primary antibody.

Microscopic detection of DNA fragmentation by TUNEL

After incubation, cells were fixed in 4 % paraformaldehyde (PFA) in PBS and permeabilized by microwave irradiation. DNA strand breaks were labeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase according to the manufacturer's protocol. To identify lactotropes, somatotropes, gonadotropes and folliculo-stellate cells, cells were incubated for 1 h with anti-rat prolactin (PRL) antiserum (1: 2500), anti-rat GH antiserum (1:1500), anti-rat luteinizing hormone (LH) antiserum (1:500), or anti-S100β antibody (1:25). Next, slides were incubated with antidigoxigenin-fluorescein antibody (1:10) to detect incorporation of nucleotides in the 3′-OH end of damaged DNA and with anti-guinea pig Alexa 555-conjugated antibody (1:200), or anti-mouse-Cy3-conjugated antibody (1:400) for 1 h. Some slides, after incubation with antidigoxigenin-fluorescein antibody, were incubated overnight with anti-aro matase antibody and followed by anti-rabbit Alexa 594-conjugated antibody (1:400) as described above. Slides were mounted with mounting medium for fluorescence (Vectashield) containing DAPI for DNA staining and visualized in a fluorescence light microscope (Axiophot, Carl Zeiss, Jena, Germany). Total cell number in each coverslip was evaluated by DAPI nuclear staining. The percentage of apoptotic anterior pituitary cells was calculated as $[(TUNEL^+)/\text{total cells}] \times 100$, the percentage of specific apoptotic subpopulations as $[(TUNEL^+\ Hormone^+)/\text{total}\ Hormone^+\ \text{cells}] \times 100$, and the percentage of apoptotic folliculo-stellate cells as $[(TUNEL^+\ S100^+)/\text{total}\ S100^+\ \text{cells}] \times 100$.

Cell-cycle analysis by flow cytometry (FACS)

Dispersed anterior pituitary cells from GNX rats were fixed in ice-cold 70 % ethanol. DNA was stained with propidium iodide (PI, 50 µg/ml) in PBS containing ribonuclease (10 µg/ml) for 20 min at 37 °C. After centrifugation, cells were washed, resuspended in PBS and analyzed by FACS. Fluorescence intensity of $\geq$10,000 gated cells/tube was analyzed using a FACScan (Becton–Dickinson). Cells with PI staining intensity lower than the G0/G1 peak were considered hypodiploid. Determination of cells in Sub G0/G1 (hypodiploid cells), G0/G1, S, and G2/M-phases of the cell cycle was performed using WinMDI 98 and Cylcherd 1.2 softwares.

Western blot

Total proteins were extracted from cultured anterior pituitary cells in lysis buffer containing 250 mM NaCl, 5 mM $MgCl_2$, 50 mM NaF, 1 mM dithiothreitol (DTT), 1 % Igepal, 0.02 % sodium azide, 0.1 % sodium dodecyl sulpfate (SDS), in 50 mM Tris–HCl pH 7.4 and protease inhibitor cocktail (1:100). Following centrifugation, the supernatant was used for immunoblot assay. Protein concentration of each sample was determined by the Bradford protein assay (BioRad Laboratories, CA). Thirty µg of total proteins were size-fractionated in 15 % SDS–polyacrylamide gel, then electrotransferred to polyvinyl difluoride (PVDF) membranes. Blots were blocked for 90 min in 5 % non-fat dry milk-TBS 0.1 % Tween 20 and incubated overnight with anti-Bax (1: 100, BD Biosciences, CA), anti-Bcl-2 (1:30, Santa Cruz Biotechnology, CA), anti-Bcl-xL (1:500, Stressgen Bioreagents, BC, Canada) and anti-GAPDH (1:5000, Santa Cruz Biotechnology, CA) in the same buffer at 4 °C. This was followed by 1 h incubation with the corresponding HRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:1000). Immunoreactivity was detected by enhanced chemiluminescence (Productos Bio-Lógicos, Buenos Aires, Argentina). Chemiluminescence was detected by chemiluminescence imaging system (G Box Chemi HR16, Syngene, Cambridge, U.K.) and bands were quantified using Gene Tools software (Syngene). Intensity data from Bax, Bcl-2, and Bcl-xL were normalized with respect to the corresponding GAPDH blot. Data were expressed as relative increments compared to respective controls.

Statistical analysis

The percentage of TUNEL-positive cells was analyzed in slides from at least three independent experiments. Results were expressed as percentage of TUNEL-positive cells ±95 % confidence interval (CI) of the total number of cells counted in each specific condition. Confidence intervals for proportions were analyzed by the χ^2 test. Western Blot, hypodiploidy, and cell-cycle data by FACS were expressed as mean ± SE and evaluated by Student's t test. Differences were considered significant when $p < 0.05$. All experiments were performed at least three times.

Results

Expression of aromatase in anterior pituitary cells from male rats

In the male rat pituitary gland, most of the cells expressing either 5α-reductase or aromatase were reported to be gonadotropes [15, 31]. However, the expression of aromatase in other rat pituitary cell types has not been determined previously. Therefore, we first studied the expression of aromatase in different pituitary cell subpopulations from intact male rats. Aromatase was expressed in the cytoplasm of gonadotropes, corticotropes, lactotropes, and somatotropes (Fig. 1a). Although we did not quantify intensity of aromatase immunoreactivity, we observed strong expression of this enzyme in TUNEL-positive cells (Fig. 1b).

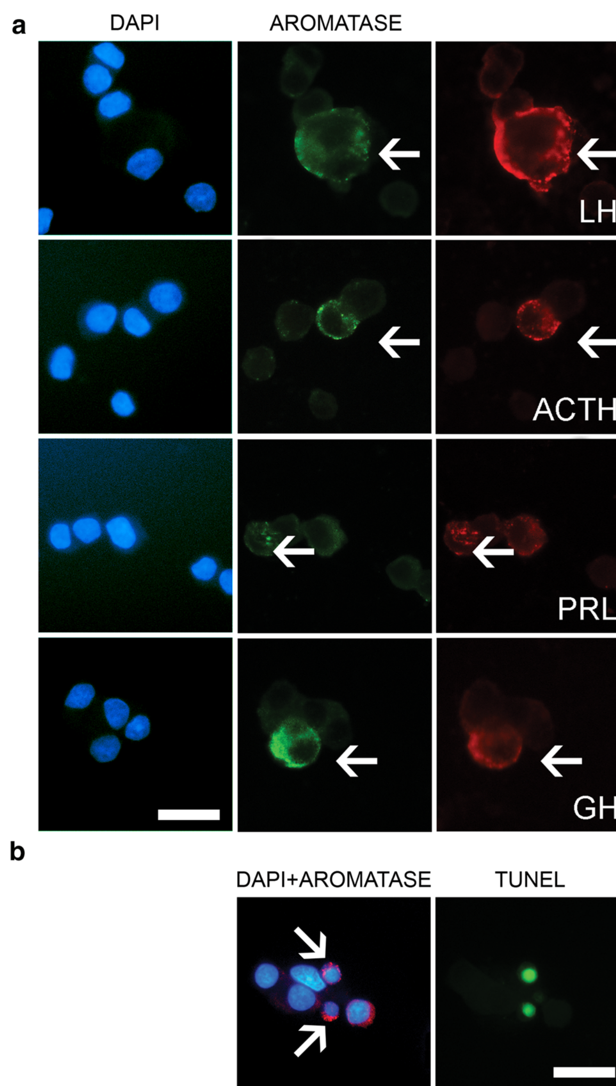

Fig. 1 Expression of aromatase in anterior pituitary cells from male rats. **a** Cultured anterior pituitary cells from intact male rats were processed for detection of aromatase and pituitary hormones by double immunofluorescence. *Left panels* nuclear staining with DAPI; *middle panels* immunocytochemistry for aromatase; *right panels* immunocytochemistry for luteinizing hormone (*LH*), adrenocorticotropic hormone (*ACTH*), prolactin (*PRL*), or growth hormone (*GH*). *Arrows* show a gonadotrope, corticotrope, lactotrope and somatotrope expressing aromatase. **b** *Arrows* indicate aromatase expression in TUNEL-positive cells. *Scale bar* 10 µm

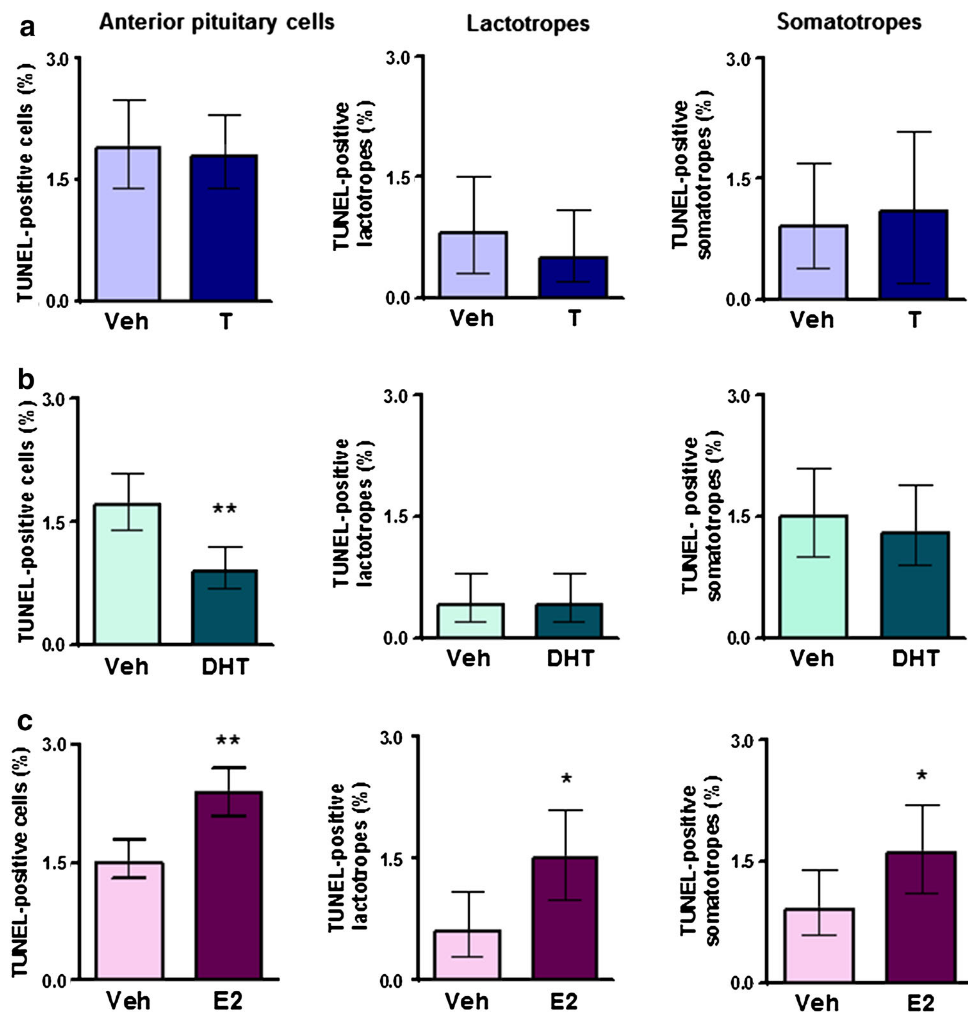

Fig. 2 Effect of gonadal steroids on apoptosis of total anterior pituitary cells, lactotropes, and somatotropes from GNX male rats. Cultured anterior pituitary cells from GNX rats were incubated with **a** testosterone (T, 10^{-8}M), **b** dihydrotestosterone (DHT, 10^{-8}M), **c** 17β-estradiol (E_2, 10^{-9} M) or vehicle (Veh, ethanol 1–10 µl/l) for 2 h. Apoptosis was assessed by the TUNEL method and lactotropes and somatotropes were identified by immunofluorescence. Each column represents the percentage ±CI of TUNEL-positive anterior pituitary cells ($n \geq 2500$ cells/group), lactotropes ($n \geq 600$ cells/group), or somatotropes ($n \geq 800$ cells/group) from 3–4 independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ versus vehicle. χ^2 test

Rapid action of T, DHT, and E_2 on apoptosis of anterior pituitary cells

To investigate the effect of gonadal steroids on apoptosis in the anterior pituitary, cultured cells from GNX rats were incubated in the presence of T (10^{-8} M), DHT (10^{-8} M) and E_2 (10^{-9} M) for 2 h. Apoptosis was determined by TUNEL, and pituitary cell types were identified by indirect immunofluorescence. T did not modify the percentage of TUNEL-positive cells (Fig. 2a). However, DHT decreased the percentage of apoptotic anterior pituitary cells, but not of apoptotic lactotropes or somatotropes (Fig. 2b). On the contrary, E_2 increased the percentage of TUNEL-positive cells as well as the apoptotic rate of both lactotropes and somatotropes (Fig. 2c). Since lactotropes and somatotropes, the pituitary subpopulations with higher turnover [7], were irresponsive to DHT, we explored whether DHT affected apoptosis of gonadotropes and folliculo-stellate cells. DHT did not modify the percentage of apoptotic gonadotropes but decreased apoptosis of folliculo-stellate cells (Fig. 3).

To determine the effect of T on apoptosis of anterior pituitary cells while avoiding the metabolism of this steroid to either DHT or E_2, we incubated anterior pituitary cells with T for 2 h in presence of an inhibitor of 5α-reductase (Finasteride) which reduces metabolism to DHT, or an inhibitor of aromatase (Letrozole), which blocks aromatization to E_2. Neither Finasteride (10^{-6} M) nor Letrozole (10^{-7} M) per se modified pituitary cell apoptosis (data not shown). However, incubation of anterior pituitary cells with T in presence of Finasteride increased the percentage of TUNEL-positive cells (Fig. 4a) suggesting that this effect could result from increased aromatization of T to E_2. On the other hand, incubation with T in presence of Letrozole did not modify the percentage of TUNEL-positive cells (Fig. 4b).

In vivo effect of DHT and E_2 on apoptosis in the anterior pituitary gland

To explore the in vivo effect of DHT and E2 administration on proliferation and apoptosis in the anterior pituitary,

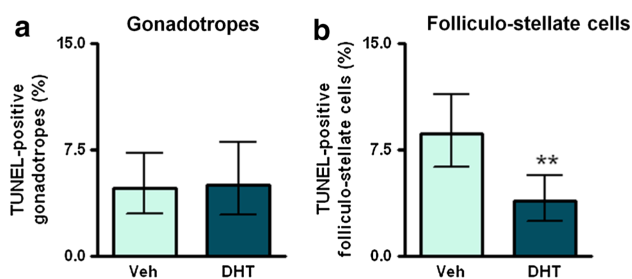

Fig. 3 Effect of dihydrotestosterone on apoptosis of gonadotropes and folliculo-stellate cells from GNX male rats. Cultured anterior pituitary cells from GNX rats were incubated with dihydrotestosterone (DHT, 10^{-8}M) or vehicle (Veh, ethanol 10 µl/l) for 2 h. Apoptosis was assessed by the TUNEL method and gonadotropes and folliculo-stellate cells were identified by immunofluorescence. Each column represents the percentage ±CI of TUNEL-positive gonadotropes ($n \geq 350$ cells/group) or folliculo-stellate cells ($n \geq 500$ cells/group) from 3 independent experiments. **$p < 0.01$. χ^2 test

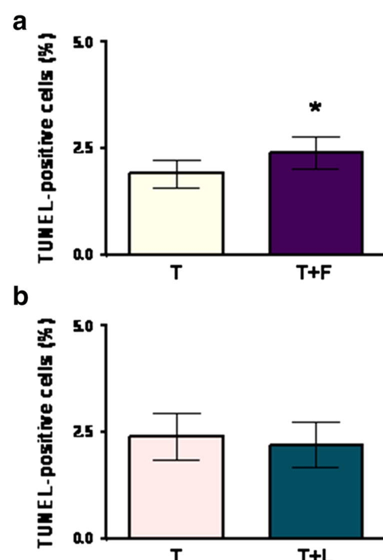

Fig. 4 Effect of testosterone on apoptosis of anterior pituitary cells incubated in presence of Finasteride or Letrozole. Cultured anterior pituitary cells from GNX rats were incubated with testosterone (T, 10^{-8}M) in presence of **a** 5α-reductase inhibitor Finasteride (F, 10^{-6}M) or **b** aromatase inhibitor Letrozole (L, 10^{-7}M) for 2 h. Apoptosis was assessed by the TUNEL method. Each column represents the percentage ±CI of TUNEL-positive anterior pituitary cells ($n \geq 3000$ cells/group) from 3 independent experiments. *$p < 0.05$. χ^2 test

GNX rats were injected for two consecutive days with DHT (5 mg/kg b.w.) or E_2 (0.2 mg/kg b.w.) and killed 24 h after the last injection. Acute DHT treatment decreased the percentage of hypodiploid cells (Fig. 5a) without any change in the percentage of cells in G0/G1-phase, S-phase or G2/M-phase (Fig. 5b–d). On the contrary, E_2 administration increased the percentage of hypodiploid cells (Fig. 6a). Cell-cycle analysis showed that E_2 significantly decreased the percentage of cells in G2/M-phase, without changing the percentage of cells in other phases of the cell-cycle (Fig. 6b–d).

Effect of DHT and E_2 on the expression of pro- and antiapoptotic proteins of the Bcl-2 family in the anterior pituitary gland

To explore whether modulation of expression of proteins of the Bcl-2 family is involved in action of gonadal steroids on pituitary cell apoptosis, we determined the effects of DHT and E_2 on the expression of proapoptotic Bax and antiapoptotic Bcl-2 and Bcl-xL proteins. Cultured anterior pituitary cells from GNX rats were incubated with DHT (10^{-8} M) or E_2 (10^{-9} M) and expression of Bax, Bcl-2, and Bcl-xL was determined by Western blot. Neither DHT (Fig. 7a) nor E_2 (Fig. 7b) modified expression of Bcl-2, Bcl-xL, and Bax or the Bax/Bcl-2 ratio (data not shown).

Discussion

Local synthesis of hormones, growth factors and cytokines participates in regulation of pituitary function and cell turnover [3]. Maintenance of tissue homeostasis in the anterior pituitary gland results from a balance between cell proliferation and death by mechanisms that are tightly regulated. Our present results show that E_2 and DHT exert opposite effects on apoptosis of anterior pituitary cells from male rats. In vitro and in vivo experiments indicated that E_2 is proapoptotic whereas DHT has antiapoptotic action in anterior pituitary cells, suggesting that metabolization of T to these steroids could be involved in tissue homeostasis in the pituitary gland of male rats. Since T can be locally metabolized in the pituitary either to DHT by 5α-reductase activity or to E_2 by aromatase activity, the opposite direct effects of DHT and E_2 on anterior pituitary cell apoptosis may explain the lack of a direct effect of T per se on this apoptotic activity. In fact, T increased pituitary cell apoptosis only when 5α-reductase activity was inhibited with Finasteride.

After castration of adult male rats, the population of mitotic gonadotropes was reported to increase whereas the mitotic activity of lactotropes was reduced [32]. Also, a rapid increase in mitotic activity was observed in the anterior pituitary gland of adult male rats following gonadectomy, activity that returned to baseline levels about 4 weeks after surgery [33]. This increase in mitotic activity was further raised by T and E_2 but reduced by DHT or T plus Letrozole suggesting that the stimulatory effect of T was likely the result of its local aromatization to estrogens [33]. In this report, changes in apoptotic activity as assessed only by cell morphology were not observed after

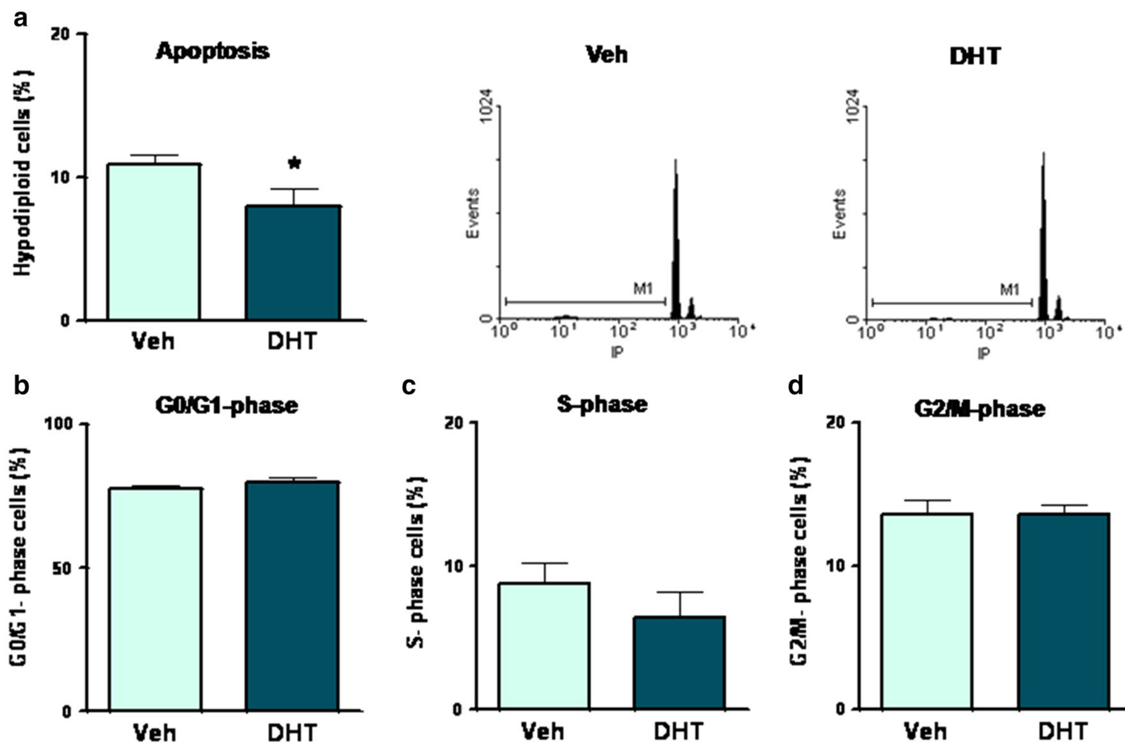

Fig. 5 Effect of in vivo administration of dihydrotestosterone on apoptosis and cell-cycle progression of anterior pituitary cells. GNX rats were injected for two consecutive days with dihydrotestosterone (DHT, 5 mg/kg b.w.) or vehicle (propyleneglycol). 24 h after the last injection, anterior pituitary cells were dispersed and processed for cell-cycle analysis by flow cytometry using propidium iodide. **a** Percentage of hypodiploid cells (*left*) and representative histograms of DNA content (*right*), **b** Percentage of cells in G0/G1-phase, **c** S-phase and **d** G2/M-phase of the cell cycle. Each column represents the mean ± SE of the percentage of cells in Sub G0/G1 (hypodiploid), G0/G1-phase, S-phase, and G2/M-phase ($n = 6$ animals per group from 3 independent experiments). *$p < 0.05$, Studenťs t test

castration or by administration of either estrogens or androgens during the second week after gonadectomy [33]. We now determined apoptosis by more accurate and sensitive methods such as TUNEL or hypodiploidy by FACS. In our in vivo study, performed 2 weeks after castration, apoptosis of anterior pituitary cells was decreased by acute administration of DHT whereas it was increased with E_2 treatment, indicating that regulation of apoptotic processes by gonadal steroids could be involved in maintenance of cell number in this gland.

The in vitro effect of DHT and E_2 on pituitary cell apoptosis suggests that the action of gonadal steroids on apoptotic activity in male rats could result, at least in part, from their direct impact at the pituitary level. The biological activity of androgens occurs predominantly through binding to intracellular AR that interacts with DNA regulatory sequences to alter gene expression. Also, rapid, non-classical actions of androgens result from activation of G-protein coupled membrane-associated AR, stimulation of different protein kinases, or direct modulation of voltage- and ligand gated ion-channels and transporters [34, 35]. A rapid effect of T on prolactin secretion by a subset of lactotropes was reported in the male rat pituitary and it was suggested that this androgenic action was exerted at the cell surface [36]. Similarly, rapid actions of estrogens such as stimulation of prolactin secretion and inhibition of cell proliferation are exerted in the pituitary, probably mediated by membrane-associated ER [37, 38]. Also, we previously showed that E_2 exerts rapid proapoptotic action on anterior pituitary cells from female rats through activation of membrane-associated ERα, especially in lactotrope and somatotrope populations [25, 28]. Now, we observed that both DHT and E_2 exert rapid actions on apoptosis of anterior pituitary cells from male rats suggesting that death signaling cascade initiated very quickly by these steroids could be mediated by membrane-associated ER and AR, although the nature of membrane-associated AR remains elusive [39].

Aromatase can locally generate high levels of E_2 that can act through intracrine, autocrine, and paracrine mechanisms [40]. Our findings show that aromatase is expressed not only in gonadotropes, as previously reported [31], but also in lactotropes and somatotropes as recently shown in the human pituitary gland [41]. Recently, aromatase was

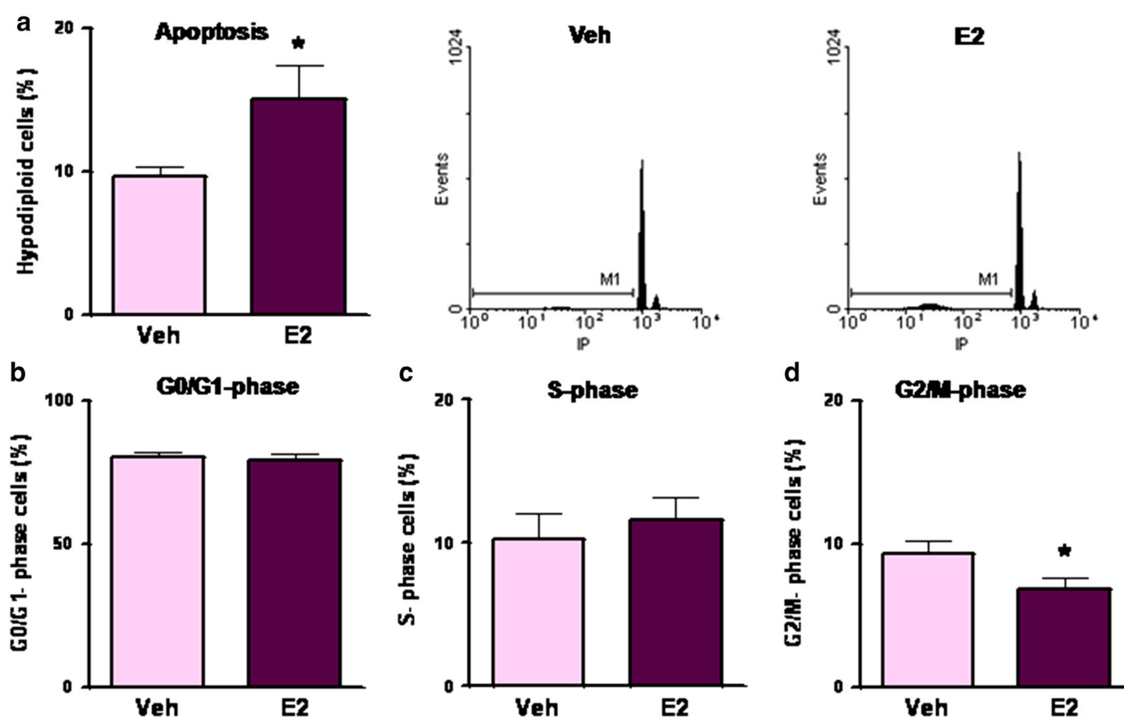

Fig. 6 Effect of in vivo administration of estradiol on apoptosis and cell-cycle progression of anterior pituitary cells. GNX rats were injected for two consecutive days with 17β-estradiol (E_2, 0.2 mg/kg b.w.) or vehicle (propyleneglycol). 24 h after the last injection, anterior pituitary cells were dispersed and processed for cell-cycle analysis by flow cytometry using propidium iodide. **a** Percentage of hypodiploid cells (*left*) and representative histograms of DNA content (*right*), **b** Percentage of cells in G0/G1-phase, **c** S-phase and **d** G2/M-phase of the cell cycle. Each column represents the mean ± SE of the percentage of cells in Sub G0/G1 (hypodiploid), G0/G1-phase, S-phase and G2/M-phase ($n = 7$ animals per group from 3 independent experiments). *$p < 0.05$, Studenťs *t* test

suggested to be expressed in lactotropes from male rats and to be involved in the control of proliferation and prolactin release by transforming T to E_2 [42]. We also observed strong intensity of aromatase expression in apoptotic anterior pituitary cells, suggesting that metabolic conversion of T to E_2 may occur in anterior pituitary cells and that aromatase activity could be involved in regulation of apoptosis of pituitary cells. Also, chronic estrogen treatment was shown to up-regulate ERα and to down-regulate AR expression in the pituitary of male rats, an action associated with a marked rise in cell apoptosis in this gland [43]. As we previously reported in lactotropes and somatotropes from female rats [25], we now show that estrogens exert rapid apoptotic action in these subpopulations of the male pituitary gland. Although in the present study we did not examine the effect of E_2 on apoptosis of other pituitary cell subpopulations, it is possible that this steroid could also exert similar action on them, especially on gonadotropes, which exhibit both ER and AR as well as aromatase activity [23, 31]. Moreover, since steroid hormones can freely and easily cross the cell membrane, it is possible that estrogens produced within a cell type may act on neighboring cells.

In the pituitary gland of male rats, 5α-reductase is localized mainly in gonadotropes [15]. Although in our study we did not observe antiapoptotic effect of DHT on lactotropes, somatotropes or gonadotropes, DHT reduced apoptosis in folliculo-stellate cells. Some functions of folliculo-stellate cells are paracrine regulation of endocrine cells and intercellular communication through gap junctions [44]. The preservation of junctions between folliculo-stellate cells was reported to be dependent on androgens within the male rat anterior pituitary gland [45]. Changes in folliculo-stellate cell turnover could affect functions of secretory pituitary cells.

Effects of estrogens on pituitary cells are different depending on species, sex, age, and experimental conditions [37]. Although estrogens have been classically recognized as potent mitogens in the anterior pituitary gland [1], long-term high-dose of estrogens was reported to be insufficient to induce persistent pituitary mitotic activity and growth [46]. Moreover, it is now recognized that estrogens also exert antiproliferative and proapoptotic actions in anterior pituitary cells [28, 38, 47]. In line with these results, we observed that E_2 induced a decrease in the percentage of pituitary mitotic cells together with an

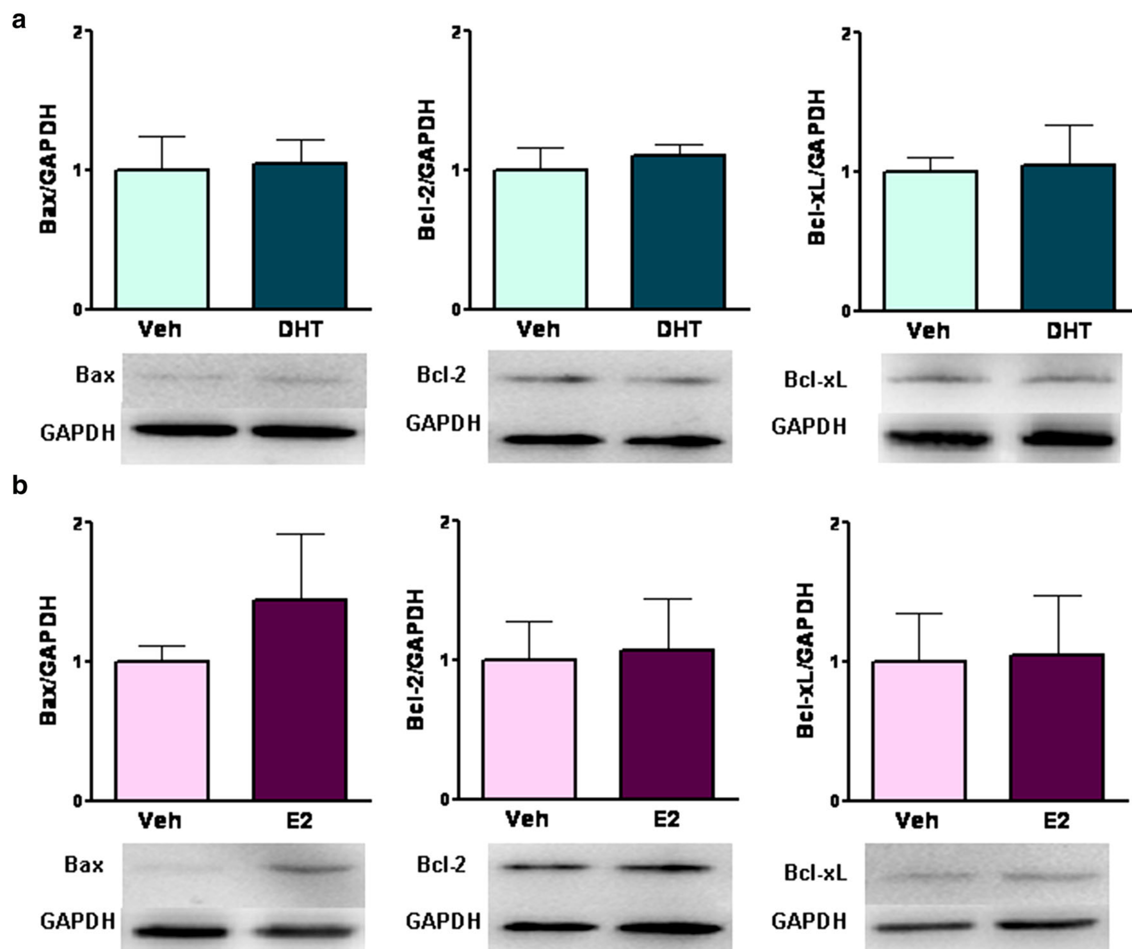

Fig. 7 Effect of dihydrotestosterone and estradiol on expression of Bax, Bcl-2 and Bcl-xL in anterior pituitary cells. Cultured anterior pituitary cells from GNX rats were incubated in presence of **a** dihydrotestosterone (DHT, 10^{-8}M), **b** 17β-estradiol (E_2, 10^{-9} M) or vehicle (Veh, ethanol 1–10 μl/l) for 24 h. Each column represents the mean ± SE of the relative increment respect to vehicle. The panels below the graphs show a representative blot for each protein using GAPDH as loading control. Raw data from 3–4 independent experiments were normalized by the corresponding GAPDH value. Paired Student́s *t* test

increase in the percentage of apoptotic cells. Proapoptotic mechanisms of estrogens in the anterior pituitary of female rats were reported to involve activation of the TNF-α/TNFR1 and Fas/FasL systems, changes in the balance of pro- and antiapoptotic Bcl-2 family proteins, and inhibition of the NFκB pathway [3, 37, 48]. However, we could not detect effects of gonadal steroids on the expression of proteins of the Bcl-2 family in the pituitary of male rats, thus the mechanism of action of both DHT and E_2 on anterior pituitary cell apoptosis in males remains to be determined.

Aromatase expression in the pituitary was reported to be much higher in male than in female rats [16]. Also, aromatase expression in lactotropes is negatively correlated with age [41] and almost completely disappears in the pituitary gland of aged male rats [49]. Since pituitary aromatase expression is downregulated by estrogens [18, 19] and increased by T [19], it is possible that reduced transformation of T to E_2 may be involved in decrease in the apoptotic index in the pituitary with aging.

Microprolactinomas are frequent in women whereas macroprolactinomas are more common in men [50]. However, prolactinomas in postmenopausal women, whose circulating estrogen levels decrease, are large, invasive and usually present very high prolactin levels, opposite to the classical microadenomas diagnosed in younger women [51]. Expression of aromatase was reported to be increased in spontaneous prolactinomas in aged female rats [52] and in human prolactinomas and other functioning and non-functioning pituitary adenomas [53]. Also, aromatase expression was found to be negatively correlated with proliferation in prolactinomas, being higher in patients with complete postoperative remission than in those without remission, suggesting that aromatase expression could be a

good prognosis marker for pituitary tumors [54]. Apoptosis plays an important role in maintaining tissue homeostasis, and therefore alterations in signaling pathways involved in dynamic maintenance of pituitary cell populations have implications in tumor development [55]. Since 5α-reductase and aromatase activities are regulated by gonadal steroids [17–19], changes in expression and/or activity of these enzymes may affect cell turnover, playing a role in the pathogeny of anterior pituitary tumors, especially micro and macroprolactinomas. Further studies are needed to understand the role of androgens and estrogens in basic mechanisms involved in the pathogenesis of pituitary tumors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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