Estradiol Upregulates c-FLIPlong Expression in Anterior Pituitary Cells

DOI 10.1055/s-0035-1565068
Horm Metab Res

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Rüdigerstraße 14
70469 Stuttgart
ISSN 0018-5043

Thieme

Estradiol Upregulates c-FLIP$_{long}$ Expression in Anterior Pituitary Cells

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Key words
- FasL
- estrogens
- apoptosis
- pituitary
- GH3

Abstract

Anterior pituitary cell turnover depends on a tight balance between proliferation and apoptosis. We have previously shown that estrogens sensitize anterior pituitary cells to pro-apoptotic stimuli. c-FLIP (cellular-FLICE-inhibitory-protein) isoforms are regulatory proteins of apoptosis triggered by death receptors. c-FLIP$_{short}$ isoform competes with procaspase-8 inhibiting its activation. However, c-FLIP$_{long}$ isoform may have a pro- or anti-apoptotic function depending on its expression level. In the present study, we explored whether estrogens modulate c-FLIP expression in anterior pituitary cells from ovariectomized (OVX) rats and in GH3 cells, a somatolactotrope cell line. Acute administration of 17β-estradiol to OVX rats increased c-FLIP$_{long}$ expression in the anterior pituitary gland without changing c-FLIP$_{short}$ expression as assessed by Western blot. Estradiol in vitro also increased c-FLIP$_{long}$ expression in anterior pituitary cells but not in GH3 cells. As determined by flow cytometry, the percentage of anterior pituitary cells expressing c-FLIP was higher than in GH3 cells. However, c-FLIP fluorescence intensity in GH3 cells was higher than in anterior pituitary cells. FasL increased the percentage of TUNEL-positive GH3 cells incubated either with or without estradiol suggesting that the pro-apoptotic action of Fas activation is estrogen-independent. Our results show that unlike what happens in nontumoral pituitary cells, estrogens do not modulate either c-FLIP$_{long}$ expression or FasL-induced apoptosis in GH3 cells. The stimulatory effect of estradiol on c-FLIP$_{long}$ expression could be involved in the sensitizing effect of this steroid to apoptosis in anterior pituitary cells. The absence of this estrogenic action in tumor pituitary cells could be involved in their tumor-like behavior.

received 26.06.2015
accepted 30.09.2015

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DOI http://dx.doi.org/10.1055/s-0035-1565068
Published online: 2015
Horm Metab Res

ISSN 0018-5043

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Introduction

Apoptosis is a highly controlled process involved in the maintenance of homeostasis and elimination of unwanted cells in a tissue. The classical pathways of apoptosis involve the activation of a family of proteolytic enzymes, caspases. The activation of caspases is triggered by both the extrinsic pathway, initiated by death receptor activation such as Fas receptor, and the intrinsic pathway, which involves mitochondrial activity [1]. Upon activation, Fas receptor oligomerizes at the cell surface recruiting FADD (Fas-Associated Death Domain) and procaspase-8 or -10, forming a Death Inducing Signaling Complex (DISC). A key regulatory protein of Fas-initiated death pathway is c-FLIP (cellular FLICE inhibitory protein), recruited to the DISC by binding its own death effector domain (DED) to the DED of FADD. c-FLIP is expressed as 3 different protein isoforms that result from alternative splicing [2,3]: c-FLIP long (c-FLIP$_{long}$, 55 kDa), c-FLIP short (c-FLIP$_{short}$, 28 kDa), and c-FLIP Raji (c-FLIPraji, 25 kDa), the latter found only in some T and B cell lines and in normal human T cells [4]. All 3 isoforms have been shown to modulate the extrinsic death pathway [3,5]. It is widely accepted that c-FLIP$_{short}$ exerts an anti-apoptotic action by competing with procaspase-8 or -10 for their recruitment to the DISC, thus inhibiting procaspase processing and activation [3]. On the other hand, the role of c-FLIP$_{long}$ is controversial since it may act as either anti-apoptotic or pro-apoptotic protein depending on its expression levels [3,5–7]. c-FLIP expression can be regulated at transcriptional and/or translational levels. Some evidence showed that hormones are involved in the regulation of c-FLIP expression in several cell lines and hormone-dependent tissues [8–10]. Androgens were reported to upregulate c-FLIP$_{long}$ gene transcription through the androgen receptor element present in the c-FLIP promoter [10,11].

Furthermore, c-FLIP$_{short}$ expression was shown to decrease during the late secretory phase in normal endometrial tissue [8], suggesting that c-FLIP$_{short}$ expression could also be regulated by gonadal steroids.

We have previously reported that Fas activation induces apoptosis of anterior pituitary cells, especially lactotropes and somatotropes, in an estrogen-dependent manner [12,13]. Since c-FLIP is involved in the regulation of the Fas receptor signaling pathway, we explored whether estrogens modulate c-FLIP expression in anterior pituitary cells from ovariectomized (OVX) rats and tumor pituitary cells. We examined the in vivo effect of 17β-estradiol on the expression of c-FLIP isoforms in the anterior pituitary gland from OVX rats. We also determined the in vitro effect of 17β-estradiol on c-FLIP expression in anterior pituitary cells from OVX rats and GH3 cells, a rat tumor somatolactotrope cell line. In addition, the effect of estrogens on FasL-induced apoptosis of GH3 cells was explored.

Materials and Methods

Drugs

All drugs and reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA except for phenol red free Dulbecco's Modified Eagle Medium (DMEM) and supplements (Gibco, Invitrogen, Carlsbad, CA, USA), fetal bovine serum (Natocor, Córdoba, Argentina), all terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), streptavidin horseradish peroxidase (HRP) conjugated anti-rabbit antibody (Millipore, Temecula, CA, USA), and the materials indicated below.

Animals

Adult female Wistar 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 lab chow and water ad libitum. Rats were ovariectomized (OVX) 2 weeks before the experiments under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia and ketoprofen (5 mg/kg) for analgesia. For the in vivo treatment, OVX rats were injected for 2 consecutive days with vehicle (propylene glycol) or 17β-estradiol (20 µg/100 g b.w.) and killed 24 h after the last injection. Anterior pituitary glands were removed within minutes after decapitation and processed as described below.

All animal work were conducted according to the NIH guidelines and was approved by the Institutional Ethics Committee [Protocol # Res. (CD) 087/2010] at the University of Buenos Aires School of Medicine.

Cell culture

A pool of anterior pituitary cells from 2–4 OVX rats was used for each culture. Anterior pituitary glands were washed several times with DMEM supplemented with 10 µl/ml MEM amino acids, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (DMEM-S) with 5.6 µg/ml amphotericin B, 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, CA, USA). Finally, cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca2+ and Mg2+. Dispersed cells were washed and resuspended in DMEM-S with 10% FBS-DCC. GH3 cells were cultured in flasks containing DMEM-S with 0.56 µg/ml amphotericin B and 10% FBS-DCC. GH3 cells were harvested with 0.025% tripsin-EDTA. Cell viability assessed by trypan blue exclusion was over 90%. Both dispersed anterior pituitary cells and GH3 cells were seeded onto coverslides in 24-well tissue culture plates for TUNEL assay (1×10^5 cells/ml/well), for Western blot (1×10^6 cells/ml/well), or flow cytometry (3×10^5 cells/ml/well) assays. Cells were cultured for 24 h in DMEM-S supplemented with 10% FBS-DCC. After this period, the cells were incubated in red phenol free DMEM-S containing 0.1% BSA and vehicle (ethanol, 100 µl/l) or 17β-estradiol (10^{-9} M) for 24 h. In some experiments, cells were incubated with FasL (1 ng/ml, Upstate, Lake Placid, NY, USA) in the same media for a further 24-h period.

Western blot

Total proteins were extracted from anterior pituitary glands or cultured anterior pituitary cells or GH3 cells with lysis buffer containing 250 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1% Igepal, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, in 50 mM Tris-HCl pH 7.4, and a protease inhibitor cocktail (1:100). Following homogenization and centrifugation at 16 000×g for 30 min, the supernatant was used for immunoblot assay. Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Twenty µg of proteins were size-fractionated in 15% SDS-polyacrylamide gel, then electrotransferred to polyvinyl difluoride (PVDF) membranes. Blots were incubated for 90 min in 5% nonfat dry milk/TBS/0.1% Tween 20 at room temperature and incubated at 4 °C overnight with rabbit c-FLIP antibody (1:500, Cell Signalling Technology, Danvers, MA, USA) in 5% BSA-TBS 0.1% Tween 20 or rabbit c-FLIP$_{long}$ antibody (1:100, Thermo Scientific Pierce Antibodies, Rockford, IL USA). This was followed by 1 h incubation with HRP-conjugated anti-rabbit antibody (1:1 500 for c-FLIP$_{long}$ or 1:500 for c-FLIP$_{short}$ determination). Immunoreactivity was detected by enhanced chemiluminescence (Productos Bio-Lógicos, Buenos Aires, Argentina). Chemiluminescence was detected by chemiluminiscence imaging system (G Box Chemi HR16, Syngene, Cambridge, UK) and bands were quantified using Gene Tools software (Syngene). Intensity data from c-FLIP were normalized with respect to the corresponding β-actin band.

Determination of c-FLIP expression by flow cytometry

Cultured anterior pituitary cells or GH3 cells were harvested with 0.025% trypsin-EDTA and washed in cold PBS. Cells were fixed with 0.1% paraformaldehyde and then permeabilized with 0.1% saponin (MP Biomedicals, Solon, OH, USA) for 10 min in the dark. Next, cells were incubated with rabbit c-FLIP antibody (1:40, Thermo Scientif, Rockford, IL, USA) in PBS/0.05% saponin for 2 h at 4 °C. Then, cells were washed and incubated with an anti-rabbit FITC-conjugated antibody (1:50, Vector Laboratories, Burlingame, CA, USA) in PBS/0.05% saponin for 1 h at 4 °C. Finally, cells were washed, resuspended in PBS and analyzed by flow cytometry (FACS) using a FACScan (Becton Dickinson, NJ, USA). Data were analyzed using WinMDI 98 software. Cells were incubated with isotype control instead of primary antibody to determine the cutoff of c-FLIP fluorescence. c-FLIP fluorescence intensity per cell was expressed as geometric mean of fluorescence (Gmean).

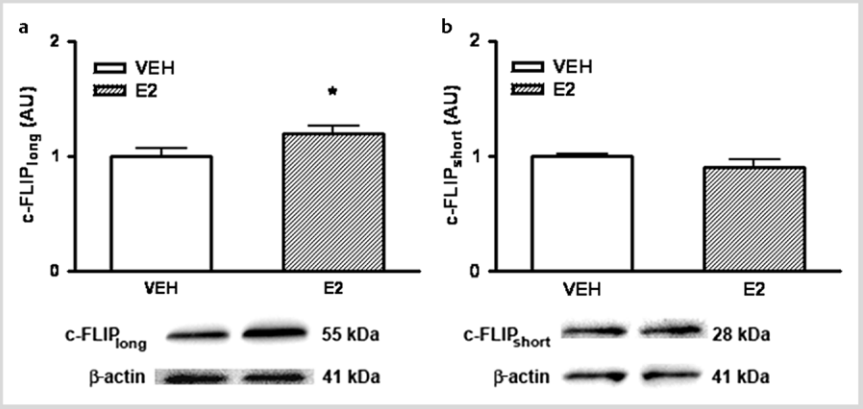

Fig. 1 In vivo effect of estradiol on the expression of c-FLIP isoforms in the anterior pituitary gland: OVX rats were injected for 2 consecutive days with vehicle (VEH, propylene glycol) or 17β-estradiol (E2, 20 µg/100 g b.w.) and euthanized on the third day. Expression of **a** c-FLIP$_{long}$ and **b** c-FLIP$_{short}$ isoforms was evaluated by Western blot. Densitometric data from 5–6 animals per group were normalized by the corresponding β-actin value and analyzed by Student's t-test. Each column represents the mean ± SE of the relative increment respect to vehicle (AU: arbitrary units); * $p < 0.05$. The panels below the graphs show representative blots for each protein using β-actin as loading control.

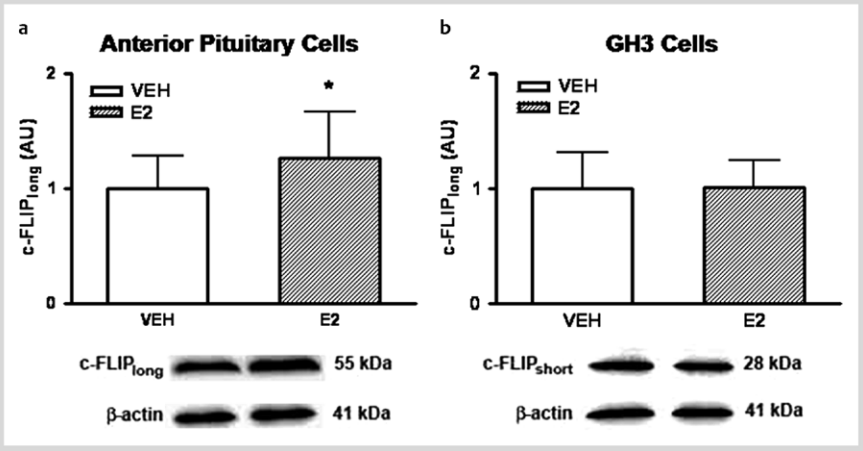

Fig. 2 In vitro effect of estradiol on the expression of c-FLIP$_{long}$ in nontumoral and tumoral pituitary cells: **a** Anterior pituitary cells from OVX rats and **b** GH3 cells were incubated with VEH (ethanol, 100 µl/l) or 17β-estradiol (E2, 10^{-9} M) for 24 h. Expression of c-FLIP$_{long}$ was evaluated by Western blot. Densitometric data from 5–6 independent experiments were normalized by the corresponding β-actin value and analyzed by paired Student's t-test. Each column represents the mean ± SE of the relative increment respect to vehicle (AU: arbitrary units); * $p < 0.05$. The panels below the graphs show representative blots for each protein using β-actin as loading control.

Microscopic determination of DNA fragmentation by TUNEL

After the culture period, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized by microwave irradiation. DNA strand breaks were labeled with digoxigenin-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase (0.18 U/µl) according to the manufacturer's protocol. After incubation in PBS with 10% normal sheep serum for 40 min, cells were incubated for 1 h with antidigoxigenin-fluorescein antibody (1:10) to detect incorporation of nucleotides into the 3′-OH end of damaged DNA. Slides were mounted with mounting medium for fluorescence (Vectashield, Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for DNA staining and visualized in a fluorescence microscope (Axiophot, Carl Zeiss, Jena, Germany). The percentage of apoptotic anterior pituitary cells or GH3 cells was calculated as [(TUNEL+)/total cells] × 100.

Statistical analysis

Normalized Western blot data and c-FLIP expression determined by FACS were expressed as mean ± SE and analyzed by unpaired or paired Student's t-test according to the experimental design. The number of apoptotic cells identified by TUNEL was analyzed in slides from at least 2 independent experiments. Results were expressed as the percentage ± 95% confidence limits (CL) of apoptotic cells of the total number of cells counted in each specific condition. Differences between proportions were analyzed by χ^2 test. Differences were considered significant if $p < 0.05$. All experiments were performed at least twice.

Results

Effect of estrogens on c-FLIP expression in nontumoral and tumoral pituitary cells

In order to explore whether c-FLIP expression is modulated by estrogens, we evaluated the in vivo effect of 17β-estradiol (E2) on the expression of c-FLIP$_{long}$ and c-FLIP$_{short}$ isoforms in the pituitary gland by Western blot. Acute administration of E2 to OVX rats increased c-FLIP$_{long}$ expression in the anterior pituitary gland (**Fig. 1a**) but did not modify c-FLIP$_{short}$ expression (**Fig. 1b**). Then, we determined the direct action of E2 on c-FLIP$_{long}$ expression in cultures of anterior pituitary cells from OVX rats and GH3 cells. E2 increased c-FLIP$_{long}$ expression in anterior pituitary cells (**Fig. 2a**) without changing this expression in GH3 cells (**Fig. 2b**). Considering that constitutive levels of c-FLIP could differ between nontumoral and tumoral pituitary cells, we compared c-FLIP expression in anterior pituitary cells from OVX rats and GH3 cells by FACS. The percentage of cells expressing c-FLIP was lower in GH3 cells than in anterior pituitary cells (**Fig. 3a**). However, c-FLIP fluorescence intensity per cell was higher in GH3 cells than in anterior pituitary cells (**Fig. 3b**).

Effect of estrogens on apoptosis in GH3 cells

We previously reported that estrogens exert a sensitizing effect to Fas activation in anterior pituitary cells [12, 13]. To explore whether estrogens sensitize GH3 cells to Fas activation, we determined the effect of FasL (1 ng/ml) on apoptosis of GH3 cells incubated either in the presence or absence of E2 by TUNEL assay. Unlike estrogen sensitizing action in anterior pituitary

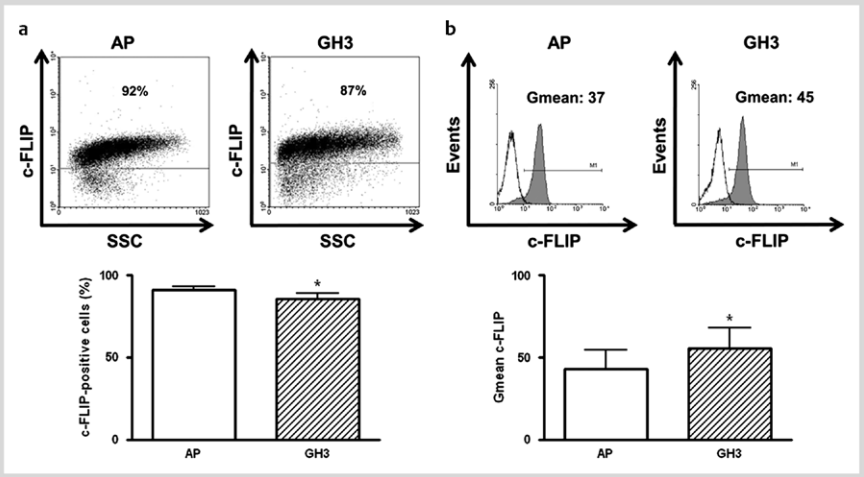

Fig. 3 c-FLIP expression in nontumoral and tumoral pituitary cells: Cultured anterior pituitary cells from OVX rats (AP) or GH3 cells were immunostained for c-FLIP and analyzed by flow cytometry. Each column represents the mean ± SE of **a** the percentage of c-FLIP-positive cells, and **b** the fluorescence intensity of c-FLIP staining (Gmean). Data from 4 independent experiments were analyzed by paired Student's t-test. The upper panels show representative dot plots and histograms of c-FLIP expression in anterior pituitary cells and GH3 cells (solid histogram) and control isotype (open histogram); * $p < 0.05$.

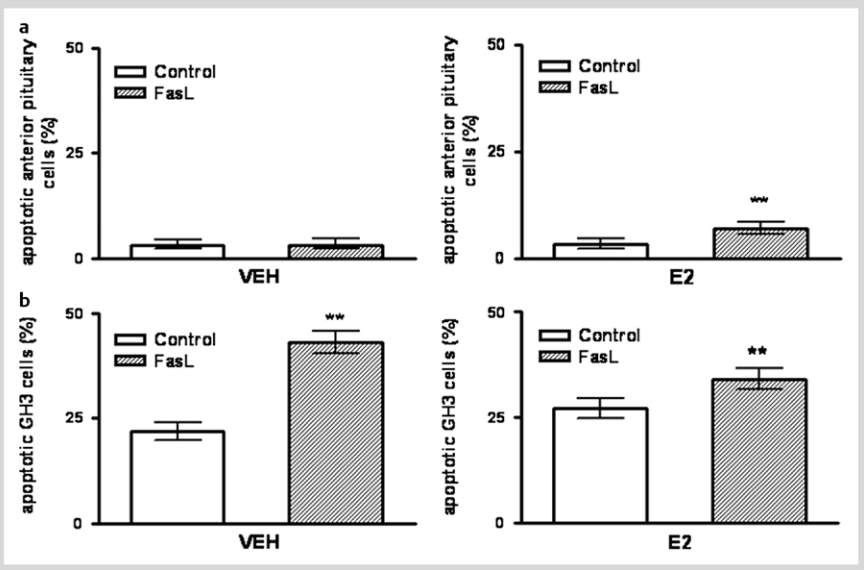

Fig. 4 Effect of FasL on apoptosis of nontumoral and tumoral pituitary cells: **a** Anterior pituitary cells from OVX rats or **b** GH3 cells were incubated with FasL (1 ng/ml) in the presence of VEH (ethanol, 100 µl/l, left panels) or E2 (10^{-9} M, right panels) for 24 h. Each column represents the percentage ± CL of TUNEL-positive anterior pituitary cells (n = 900–1 300 cells/group from 2 independent experiments) or GH3 cells (n = 1 400–1 600 cells/group from 3 independent experiments). ** $p < 0.01$; χ^2 test.

cells from OVX rats (**Fig. 4a**), FasL induced apoptosis of GH3 cells incubated either with or without E2 (**Fig. 4b**).

Discussion

The anterior pituitary gland undergoes considerable cell turnover in several physiological conditions such as the estrous cycle [14]. During the estrous cycle, the highest apoptosis rate is achieved at proestrus [15,16] when the highest levels of circulating estradiol occur. It is now well documented that estrogens exert antiproliferative and pro-apoptotic actions in anterior pituitary cells [17–20]. The regulatory mechanisms of estrogens in apoptosis of anterior pituitary cells involve modulation of both extrinsic and intrinsic death pathways [12,13,21]. In the present study we show that estradiol, both in vivo and in vitro, increases c-$FLIP_{long}$ expression in anterior pituitary cells, suggesting that regulation of c-FLIP could be involved in the sensitizing effect of estrogens to pro-apoptotic stimuli in these cells.

c-FLIP is one of the key regulatory proteins of Fas-initiated death pathway [22]. c-$FLIP_{short}$ isoform blocks procaspase-8 recruitment, thus acting as an inhibitor of apoptosis triggered by death receptor activation. However, c-$FLIP_{long}$ has a dual function in apoptosis depending on its expression level. Overexpression of c-$FLIP_{long}$ inhibits cell apoptosis, whereas at physiological expression levels, c-$FLIP_{long}$ heterodimerizes with procaspase-8 or -10, facilitating their activation at the DISC [6,22–24]. In fact, a reduction in endogenous c-$FLIP_{long}$ concentration was reported to decrease Fas-induced apoptosis [6].

Even though the c-FLIP promoter lacks an estrogen response element, it has binding sites for transcription factors such as NFκB, AP1, and SP1, all of which can be transactivated by estrogens [25,26]. Since estrogens modulate alternative splicing in pituitary tumors [27,28], it is possible that they can modify c-$FLIP_{long}$ expression through regulating alternative splicing of c-FLIP or expression of splicing factors in the pituitary [29]. Also, c-FLIP expression could be regulated post-transcriptionally [30]. JNK-mediated phosphorylation induces c-$FLIP_{long}$ proteasomal degradation [31]. Since estradiol can inhibit JNK pathway [32], it is possible that it could inhibit c-$FLIP_{long}$ degradation by blocking JNK pathway.

c-$FLIP_{long}$ is upregulated by androgens in the rat prostate and some human prostate cancer cell lines [10,11]. c-$FLIP_{long}$ expression was found to be higher in androgen-unresponsive prostate cancer cell lines than in responsive ones. The failure of androgens to increase c-FLIP expression in those cell lines with higher basal expression was suggested to be associated with dysregulation of c-$FLIP_{long}$ expression during the transition from androgen-dependent to androgen-independent behavior of prostate cancer cells [10]. Even though GH3 cells express functional

membrane and nuclear estrogen receptors [33–35], our results indicate that, unlike what occurs in anterior pituitary cells, estrogens fail to modify c-FLIP$_{long}$ expression in GH3 cells and to sensitize them to Fas activation. The fact that c-FLIP expression per cell in GH3 cells was higher than in anterior pituitary cells could explain why FasL induced apoptosis of GH3 cells in an estrogen-independent manner. Similarly, we recently reported that TNF-α-induced apoptosis in GH3 cells is estrogen-independent [36]. The lack of estrogen modulation of c-FLIP$_{long}$ expression in GH3 cells could be responsible, at least in part, to their tumor-like behavior.

In summary, herein we have presented evidence that c-FLIP$_{long}$ expression can be upregulated by estrogens in the anterior pituitary. The stimulatory effect of estradiol on c-FLIP$_{long}$ expression could be involved in the sensitizing effect of this gonadal steroid to apoptosis in anterior pituitary cells. We also presented evidence that estrogens fail to modulate c-FLIP$_{long}$ expression and FasL-induced apoptosis in tumor pituitary cells suggesting that dysregulation in estrogenic control of anterior pituitary cell turnover could have consequences for tissue homeostasis. Further studies are necessary to ascertain a role of c-FLIP$_{long}$ in anterior pituitary cell apoptosis and to understand the physiological effect of c-FLIP$_{long}$ in the maintenance of cell renewal in this gland.

Acknowledgements

We would like to thank Dr. Marianela Candolfi for the interest, support, and advice, and Miss Mercedes Imsen and Mr. Martín Brahamian for their kind assistance with animal care and handling.

Conflict of Interest

The authors declare no conflict of interest.

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