Journal of Steroid Biochemistry & Molecular Biology 123 (2011) 133–139

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

17β-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio

A.M. Miró[a,b,1], J. Sastre-Serra[a,b,1], D.G. Pons[a,b], A. Valle[a,b], P. Roca[a,b], J. Oliver[a,b,*]

[a] Grupo Multidisciplinar de Oncología Traslacional, Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Palma de Mallorca, Illes Balears, Spain
[b] Universitat de les Illes Balears, Palma de Mallorca, Illes Balears, Spain

ARTICLE INFO

Article history:
Received 7 May 2010
Received in revised form 3 December 2010
Accepted 10 December 2010

Keywords:
Estrogen receptor
Oxidative stress
Prostate cancer
17β-Estradiol
Uncoupling proteins

ABSTRACT

Estrogen action is mediated by the two receptor isoforms: estrogen receptor alpha and beta. Both receptors are expressed in human prostate tissue and have different action profiles. ERalpha is positively correlated with the malignancy of prostate cancer, while ERbeta may protect against abnormal prostate cell growth. 17β-Estradiol (E2), at least in part, induces cancerous transformations by causing deleterious mutations through the formation of reactive oxygen species (ROS).

The aim was to study the effect of E2 on oxidative stress and the expression of uncoupling proteins (UCPs) and antioxidant enzymes in several prostate cancer cell lines with different ERalpha/ERbeta ratios.

The cell prostate lines with a lower ERalpha/ERbeta ratio had lower oxidative stress, which could be partially explained by the increased expression of antioxidant enzymes and UCPs. Moreover, the action of E2 on the expression of antioxidant enzymes and UCPs was dual and dependent on the ERalpha/ERbeta ratio. Treatments with 0.1 nM E2 in cell lines with high ERalpha/ERbeta ratio produced a decrease in antioxidant enzymes and UCPs levels, with an increase in ROS production. These effects disappeared when the treatment was done in the presence of an ERalpha antagonist (MPP). In the cell lines with greatest levels of ERbeta and the lowest ERalpha/ERbeta ratio, E2 treatment caused the up-regulation of antioxidant enzymes and UCPs with a look-up decrease in ROS production. These effects were reversed when the cells were treated with E2 in the presence of an ERbeta antagonist (R,R-THC).

On the whole, our results suggest a dual E2 effect; increasing or decreasing oxidative stress in part by modulation of UCPs and antioxidant enzymes according to the abundance ERbeta and ERalpha/ERbeta ratio in prostate cancer cell lines.

1. Introduction

Prostate cancer is the most common cancer in men and the second leading cause of cancer death [1]. In industrialized nations, prostate cancer incidence is on the increase, although the number of deaths has declined [2,3]. Epidemiological and experimental studies suggest that estrogens may have cancerogenic and chemopreventative effects on the prostatic epithelium [4,5]. Estrogen action is mediated by specific nuclear receptors that regulate transcription of the target genes by binding to their DNA response elements. Estrogen action is mediated by two receptors, ERalpha and ERbeta, which are often antagonistic to one another [6]. Estrogen via ERalpha stimulates proliferation in the breast, uterus, and developing prostate, while estrogen via ERbeta inhibits proliferation and promotes differentiation in the prostate, mammary gland, colon, lung, and bone marrow stem cells [6].

Both ERalpha and ERbeta receptors are expressed in human prostate tissue [7,8]. The function of ERalpha, the classical receptor, has been thoroughly studied and several groups have reported greater ERalpha expression in cancer specimens than in benign hyperplasia and normal prostate tissue. These data are consistent with other reports in which abundance of ERalpha is positively correlated with the malignancy of prostate cancer [9,10]. Nevertheless, recent reports have shown a frequent loss of ERbeta expression in prostate cancer samples relative to normal prostatic tissue [11,12]. In the primary prostate tumor sites, ERbeta is strongly expressed in low grade prostate carcinoma and is markedly diminished in higher grade tumors [11–13]. These data suggest that ERbeta may protect against abnormal prostate cell growth [11,14]. In fact, even the use of ERbeta agonists has been suggested as a therapy to prevent grade progression in prostate tumors [6].

In the prostate, estrogens have been described to induce cancerous transformation by their genotoxic metabolites, and in part,

* Corresponding author at: Dept. Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Crta. Valldemossa Km 7.5, E07122-Palma de Mallorca, Spain. Tel.: +34 971 259643.
E-mail address: jordi.oliver@uib.es (J. Oliver).
[1] Both these authors equally contributed to the present work.

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.12.004

these metabolites may directly induce genomic damage or via the formation of reactive oxygen species (ROS) [15]. During the onset of cancer, ROS may cause DNA damage and mutagenesis, and they also have a role as secondary messengers, stimulating proliferation while inhibiting apoptosis, which confers a growth advantage to established cancer cells [16,17]. In breast cancer, it has been suggested that 17β-estradiol (E2) induced oxidative stress that resulted from a modulation of antioxidant enzyme status through an estrogen receptor (ER)-dependent mechanism [15,18]. Recently, in our laboratory, we have found that E2, through an ER-dependent mechanism, may increase mitochondrial ROS production by repressing the uncoupling proteins (UCPs) [19]. UCPs are a family of inner mitochondrial membrane proteins whose function is to allow the re-entry of protons into the mitochondrial matrix by dissipating the proton gradient by subsequently decreasing membrane potential and ROS production. In these last years, it has described the importance of these proteins in both cancer development and progression [20].

It is of interest to investigate whether E2 influenced UCPs expression and consequently oxidative stress in prostate cancer cells with a different ERalpha/ERbeta ratios. To tackle this aim, the effect of E2 on oxidative stress, antioxidant enzymes and uncoupling protein expression was analysed in several prostate cancer cell lines with different endowment levels of ERalpha and ERbeta which were concretely: VCaP cancer cell lines with a high ERalpha/ERbeta ratio, DU145 cell lines that only express ERbeta, and PC3 cell lines with an average of ERalpha/ERbeta ratio.

2. Materials and methods

2.1. Materials and reagents

17β-Estradiol (E2), genistein and testosterone were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) and (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (R,R-THC) were purchased from Tocris (Ellisville, MO, USA). Antisera against uncoupling protein 2 (UCP2) and uncoupling protein 5 (UCP5) were purchased from Alpha Diagnostic International (San Antonio, TX, USA); catalase and glutathione peroxidase (GPx) from Calbiochem (San Diego, CA, USA); ERalpha, ERbeta and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antisera against 4-hydroxy-2-nonenal (HNE) were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell culture and treatments

Prostate cancer cell lines VCaP, PC3 and DU145 were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C. To evaluate the effects of E2, testosterone, genistein, MPP and R,R-THC cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 h before treatment. Experiments were performed when cell cultures reached confluence by providing fresh medium supplemented with 1 nmol/L testosterone or 1 μM genistein or 0.1 nmol/L E2 with or without MPP (1 μM) and R,R-THC (1 μM) for 48 h. Cell lysates were obtained by scraping cells in lysis buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mmol/L NaCl, 0.5%, Nonidet P-40, 10% glycerol, 1 mmol/L EGTA, 1 mmol/L $NaVO_3$, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin; pH 7.4). Protein content was measured with a BCA protein assay kit (Pierce, Bonn, Germany).

2.3. Measurement of carbonyl content

Carbonyl groups were quantified using the Oxyblot protein oxidation detection kit (Chemicon, Chandlers Ford, UK). Derivatization by 2,4-dinitrophenylhydrazine (DNPH) was carried out for 15 min on 5 μg of total cell lysate protein following the manufacturer's instructions. Proteins were transferred onto nitrocellulose membrane by means of a slot-blot system (Bio-Rad). After incubation with anti-DNP antibody, bands were visualized using Immun-Star Western C Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad). To ensure specificity, the oxidized proteins provided by the kit were included as a positive control. Treatment of sample with a control solution served as a negative control for DNPH derivatization.

2.4. Measurement of 4-hydroxy-2-nonenal

For 4-hydroxy-2-nonenal (HNE) analysis, 5 μg of protein from cell lysate was transferred onto nitrocellulose membrane by means of a slot-blot system (Bio-Rad). Membrane was incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against 4-HNE were used as primary antibody. Bands were visualized using the Immun-Star Western C Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad).

2.5. Western blotting

For Western blot analysis, 40 μg of protein from cell lysate was fractioned by SDS-PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against UCP2 (32 kDa, Primary Ab 1:500), UCP5 (36 kDa, Primary Ab 1:500), Catalase (60 kDa, Primary Ab 1:1000), glutathione peroxidase (22.5 kDa, Primary Ab 1:500), ERalpha (66 kDa, Primary Ab 1:1000), ERbeta (54 kDa, Primary Ab 1:200) and actin (45 kDa, Primary Ab 1:1000) (such as housekeeping) were used as primary antibodies. A negative control without primary antibody template was run in each assay. Protein bands were visualized by the Immun-Star Western C kit reagent (Bio-Rad) Western blotting detection system. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad).

2.6. Real-time RT-PCR analysis

Total RNA was isolated from cultured cells using TriPure isolation reagent and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10-μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA was diluted to 1/10 and aliquots were frozen (−70 °C) until the PCRs were carried out.

Real-time PCR was done for seven target genes, UCP1, UCP2, UCP3, UCP4, UCP5, catalase and GPx; and one housekeeping gene: 18S ribosomal RNA (18S).

PCR was carried out using SYBR green technology, purchased by Sigma–Aldrich, on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μl, containing 5 μl SYBR Green JumpStart *Taq* ReadyMix (containing 20 mM Tris–HCl, pH 8.3, 100 mM KCl, 0.4 mM each dATP, dCTP,

dGTP, TTP, glass passivator, stabilizers, 0.05 U/μl *Taq* DNA Polymerase, JumpStart *Taq* antibody, and SYBR Green I dye), 0.5 μM of the sense and antisense primers, 2 mM $MgCl_2$, and 3 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (2 min, 94 °C), followed by 40 cycles consisting of a denaturation step (15 s, 94 °C), and annealing, extension and read fluorescence step (1 min, 60 °C for UCP2, UCP5 and catalase; 61 °C for UCP1, UCP3, UCP4 and 18S; and 66 °C for GPx). A negative control without cDNA template was run in each assay.

The primers used were forward 5′-CTTGGTGTCGGCTCTTATCG-3′ and reverse 5′-CCGTTGGTCCTTCGTTAGTG-3′ for UCP1, forward 5′-GGTGGTCGGAGATACCAAAG-3′ and reverse 5′-CTCGGGC-AATGGTCTTGTAG-3′ for UCP2, forward 5′-GGGATTCTGGTCTT-CACTGC-3′ and reverse 5′-TCCAACCTTCCATTTTGTCC-3′ for UCP3, forward 5′-GCGACAAGGAGTGCGTTATC-3′ and reverse 5′-ATCCAGGGGAAAGGTTGCTA-3′ for UCP4, and forward 5′-CAAGCCGTTGGTCTCCTAAG-3′ and reverse 5′-CGTTTTCAAT-GTCACCCATC-3′ for UCP5, forward 5′-CATCGCCACATGAATGGATA-3′ and reverse 5′-CCAACTGGGATGAGAGGGTA-3′ for CAT, forward 5′-GCGGCGGCCCAGTCGGTGTA-3′and reverse 5′-GAGCTTGGGGT-CGGTCATAA-3′ for GPx, and forward 5′-GGACACGGACAGGATTGA-CA-3′ and reverse 5′-ACCCACGGAATCGAGAAAGA-3′ for 18S.

The Ct values of the real-time PCR were analysed, taking into account the efficiency of the reaction and referring the results to the total DNA amount, using the GenEx Standard Software (MultiDAnalises, Sweden).

2.7. Cell proliferation assay

Cells were plated at 5000 cells per well in 96-well plates in phenol red-free DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C and shifted to phenol red-free DMEM 10% charcoal-FBS 1% antibiotics (penicillin and streptomycin) 24 h before treatment with E2 or testosterone during 48 h. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) was added and plates were incubated for 1 h at 37 °C. The medium was removed and formazan crystals were dissolved by adding 100 μl dimethyl sulfoxide (DMSO). Absorbance of the converted dye was measured at 570 nm with background subtraction at 620 nm using a microplate reader (Power Wave XS, BIO-TEK).

2.8. Measurement of ROS production

Cells were plated at 5000 cells per well in 96-well plates in phenol red-free DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C and shifted to phenol red-free DMEM 10% charcoal-FBS 1% antibiotics (penicillin and streptomycin) 24 h before treatment with E2, genistein or E2 with MPP or R,R-THC during 48 h. After treatment, 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) was added and plates were incubated for 90 min at 37 °C. Assay was performed at 37 °C in a 96-well microplate fluorimeter FLx800 (BIO-TEK instruments, Winooski, VT, USA). Hydrogen peroxide production was assayed by measuring the increase in fluorescence (485 nm excitation, 530 nm emission).

To correct the effect of treatment in the proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) was added and plates were incubated for 1 h at 37 °C. The medium was removed and formazan crystals were dissolved by adding 100 μl dimethyl sulfoxide (DMSO). Absorbance of the converted dye was measured at 570 nm with background subtraction at 620 nm using a microplate reader (Power Wave XS, BIO-TEK).

Table 1
Effect of E2 on VCaP, PC3 and DU145 cell proliferation.

	VCaP	PC3	DU145
Control	100 ± 1.7	100 ± 3.1	100 ± 8.3
E2	112 ± 3.1*	96 ± 1.9	100 ± 2.2
T	113 ± 3.8*		
E2 + T	117 ± 2.5*		

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 100. E2, 17β-estradiol 0,1 nM; T, testosterone 1 nM.

* Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

2.9. Measurement of ROS levels by flow cytometry

Cells were stained with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) for 15 min, washed with PBS containing 10 mM glucose, and analysed immediately using an Epics XL flow cytometer (Beckman–Coulter, Miami, FL, USA). The green fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. 10,000 events were acquired and analysis was performed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL).

2.10. Analysis of mitochondrial membrane potential (MMP) by flow cytometry

Cells were stained with 250 nM tetramethylrhodamine methyl (TMRM) ester for 15 min, washed with PBS containing 10 mM glucose, and analysed by flow cytometry as described above. TMRM is a lipophilic cationic dye that accumulates within mitochondria according to the MMP. The red fluorescence was measured using the FL-2 setting (log mode) after the cell debris was electronically gated out. In each analysis, 10,000 events were recorded and analysed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL).

2.11. Statistical analysis

All data were expressed as means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis was carried out using the Statistical Program package (SPSS 18.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analysed with unpaired Student's *t*-test. A level of $P < 0.05$ was accepted as significant.

3. Results

3.1. Effect of E2 on VCaP PC3 and DU145 cell proliferation

E2 treatment (0.1 M) increased the proliferation of VCaP and had no effect on the proliferation of other prostate cancer cell lines studied (Table 1). Due to the presence of androgen receptor in VCaP cells, the effect of testosterone (1 nM) alone and in combination with E2 also had been evaluated in this cell line with no difference between treatments.

3.2. VCaP, PC3 and DU145 prostate cell lines characterization

The protein levels of estrogen receptor isoforms ERalpha and ERbeta were determined to characterize the prostate cancer cell lines used in this study: VCaP, PC3 and DU145 (Table 2 and Fig. 1). No differences between VCaP and PC3 cancer cell lines were found in ERalpha. In DU145 the levels of ERalpha were undetected (see Fig. 1). ERbeta protein levels were statistically different between all cell lines, with VCaP cells presented the lower levels, PC3 the higher levels (206% considering VCaP as 100%) and DU145 intermediate

Table 2
VCaP, PC3 and DU145 prostate cell lines characterization.

	VCaP	PC3	DU145
ERα	100 ± 9	110 ± 19	UD
ERβ	100 ± 14	206 ± 18*	151 ± 22*,§
Ratio ERα/ERβ	1.00	0.48	0
ROS levels	100 ± 3	88 ± 2*	87 ± 5*
TMRM fluorescence	100 ± 2	124 ± 3*	180 ± 10*,§
Carbonil content	100 ± 7	63 ± 9*	75 ± 13*
4-HNE	100 ± 9	72 ± 14*	65 ± 16*

Data represent the means ± SEM ($n = 6$). Values of VCaP cell line were set at 100. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta, ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; 4-HNE, 4-hydroxy-2-nonenal; UD, undetected.

* Significant difference between VCaP cell line and PC3 or DU145 cell lines (Student's *t*-test; $P < 0.05$, $n = 6$).

§ Significant difference between PC3 cell line and DU145 cell line (Student's *t*-test; $P < 0.05$, $n = 6$).

levels (151%). Considering both receptors, ERalpha/ERbeta ratio has also been calculated and VCaP presented the higher range, PC3 the mid-range and DU145 the lower value of this ratio, which was 0, as a result of undetected levels of ERalpha.

Table 2 shows the indicators of mitochondrial function and oxidative stress balance in prostate cancer cell lines: VCaP, PC3 and DU145. Specifically, we measured ROS levels by DCFDA (as an oxidative stress indicator), TMRM fluorescence (as an indicator of mitochondrial membrane potential), and carbonyl and 4-HNE content (as an indicator of protein and lipids oxidative damage, respectively). The highest mitochondrial membrane potential was found in DU145 cells followed by those of PC3, with the VCaP cells presenting the lowest value.

To complete the oxidative stress balance characterization in cancer prostate cell lines, we studied the expression of antioxidant enzymes and UCPs by real time PCR and the protein levels by Western blot. The crossing points (Cp) of catalase, GPx and UCP1–UCP5 of VCaP, PC3 and DU145 cell lines in culture basal conditions are summarized in Table 3, as well as 18S (such as housekeeping). Catalase and GPx were expressed in significant levels in all cell lines studied (Cp < 21), only UCP2 and UCP5 presented a Cp high enough to be considered as a significant gene expression (Cp < 24) in VCaP, PC3 and DU145 cells, while the other UCPs (UCP1, UCP3 and UCP4) presented, in most cases, had a Cp higher than 25, and therefore the expression of these genes may be considered as basal. Only UCP3 in the DU145 cell line presents a Cp value of 22.3.

Table 4 shows protein levels of antioxidant enzymes (GPx and catalase) and main uncoupling proteins (UCP2 and UCP5), in the VCaP, PC3 and DU145 cancer prostate cell lines. Despite of no differences in GPx between the cell lines analysed, the protein catalase levels presented the highest values in PC3 lines and the lowest values of the DU145 cell line, both levels were statistically significant. UCP2 shows no statistically significant differences between lines, although UCP5 protein levels were lower in VCaP with respect to PC3 and DU145.

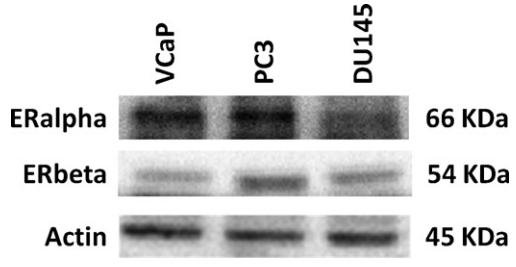

Fig. 1. Representative bands of Western blot are shown. ERalpha, estrogen receptor alpha; ERbeta, estrogen receptor beta.

Table 3
Antioxidant enzymes and UCPs expression in VCaP, PC3 and DU145 cell lines.

	VCaP	PC3	DU145
UCP1	28.3 ± 0.7	28.7 ± 0.2	27.6 ± 0.5
UCP2	20.6 ± 0.1	23.4 ± 0.1	21.7 ± 0.4
UCP3	25.1 ± 0.4	26.2 ± 0.2	22.3 ± 0.4
UCP4	26.5 ± 0.2	26.4 ± 0.3	26.2 ± 0.5
UCP5	21.3 ± 0.1	21.1 ± 0.1	23.3 ± 0.4
Catalase	19.1 ± 0.2	18.6 ± 0.2	21.0 ± 0.4
GPx	17.4 ± 0.2	18.1 ± 0.2	18.7 ± 0.3
18S	8.2 ± 0.1	8.3 ± 0.1	9.4 ± 0.3

Data represent the means of Ct values ± SEM ($n = 6$). UCP, uncoupling protein; GPx, glutathione peroxidase.

Table 4
Protein levels of UCPs and antioxidant enzymes in VCap, PC3 and DU145 cancer prostate cell lines.

	VCaP	PC3	DU145
UCP2	100 ± 10	81 ± 8	92 ± 9
UCP5	100 ± 8	148 ± 11*	175 ± 45*
GPx	100 ± 12	90 ± 13	90 ± 2
CAT	100 ± 11	343 ± 31*	79 ± 4*,§

Data represent the means ± SEM ($n = 6$). Values of VCaP cells were set at 100. UCP, uncoupling protein; GPx, glutathione peroxidase.

* Significant difference between VCaP cell line and PC3 or DU145 cell lines (Student's *t*-test; $P < 0.05$, $n = 6$).

§ Significant difference between PC3 cell line and DU145 cell line (Student's *t*-test; $P < 0.05$, $n = 6$).

3.3. Effect of E2 on antioxidant enzymes and UCPs mRNA levels in VCap PC3 and DU145

The effect of a 48 h E2-treatment on antioxidant enzymes catalase and GPx, and UCP2 and UCP5 mRNA levels was evaluated in order to establish the influence of this hormone on the antioxidant capacity of prostate VCaP, PC3 and DU145 cancer cell lines (Table 5). In Table 5, the fold change for hormone treatment is shown to better elucidate whether E2 treatment has a positive or negative effect on the mRNA expression of these genes. In VCaP cells, E2 treatment had no effect on GPx, but there was a decrease in catalase, UCP2 and UCP5 mRNA levels. Given that an androgen receptor is expressed in the VCaP cell line, the effect of testosterone (1 nM) has also been evaluated and this treatment had no effect on antioxidant enzymes and the UCPs analysed. E2 increased expression of antioxidant enzymes and UCPs, in PC3 cells, was only statisti-

Table 5
Fold-change of UCPs and antioxidant enzymes ARNm in prostate cancer cell lines after hormonal treatment.

		Control	E2	T
VCaP	UCP2	0.00 ± 0.09	−0.44 ± 0.09*	0.05 ± 0.08
	UCP5	0.00 ± 0.08	−0,15 ± 0.02*	0.12 ± 0.08
	Catalase	0.00 ± 0.08	−0.29 ± 0.12*	0.05 ± 0.1
	GPx	0.00 ± 0.09	−0.08 ± 0.15	0.08 ± 0.03
PC3	UCP2	0.00 ± 0.10	0,17 ± 0.18	
	UCP5	0.00 ± 0.09	0.41 ± 0.06*	
	Catalase	0.00 ± 0.12	0.41 ± 0.21	
	GPx	0.00 ± 0.13	0.42 ± 0.06*	
DU145	UCP2	0.00 ± 0.11	0,15 ± 0.12	
	UCP5	0.00 ± 0.18	0.23 ± 0.17	
	Catalase	0.00 ± 0.12	0.20 ± 0.15	
	GPx	0.00 ± 0.06	0.22 ± 0.09*	

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 0. E2, 17β-estradiol 0,1 nM; T, testosterone 1 nM; UCP, uncoupling protein; GPx, Glutathione peroxidase.

* Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

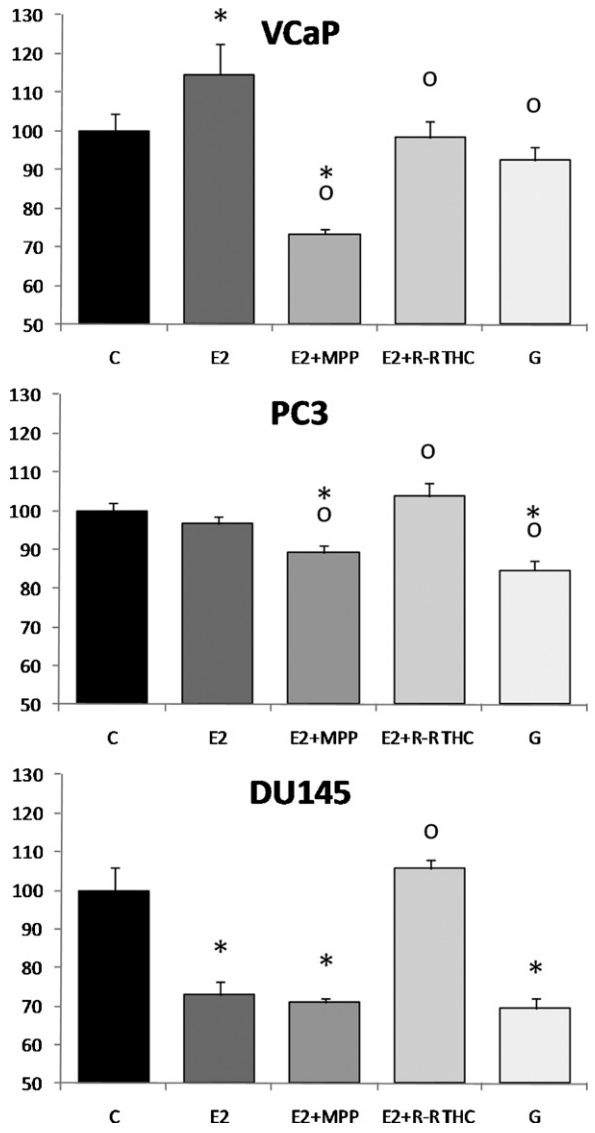

Fig. 2. Effects of 17β-estradiol, genistein and antagonists from estrogen receptors on ROS production. E2, 17β-estradiol 1 nM; MPP1 μM, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy)phenol]-1H-pyrazole dihydrochloride; R,R-THC 1 μM, (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; G, genistein. *Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$). ○Significant difference between antagonists or genistein-treated cells versus E2-treated. cells (Student's *t*-test; $P < 0.05$, $n = 6$).

cally significant for GPx and UCP5 and showed a high tendency in catalase levels ($p = 0.083$). In DU145 cells, the E2 treatment only increases the expression of GPx and had no effect on the catalase, UCP2 and UCP5 mRNA levels.

3.4. Effect of ERalpha and ERbeta antagonists on E2 stimulation of ROS production in VCaP, PC3 and DU145

ROS production of vehicle-treated cells was used as a reference (100%). The effect of estrogen with and without estrogen receptor antagonists (MPP and R,R-THC), and genistein was also determined in all prostate cancer cell lines studied (Fig. 2). Treatment effects were more evident in the VCaP cell line, which showed a higher ERalpha/ERbeta ratio with the statistically significant increase in the ROS production of E2-treated cells (+15% with respect to vehicle-treated cells), while experiencing a decrease in cell ROS production with the ERalpha antagonist (MPP −41% with respect to E2-treated cells) or the genistein (−7% with respect to vehicle-treated cells) treatment. In PC3 cells with a lower ERalpha/ERbeta ratio, ERalpha antagonist and genistein also statistically decreased ROS production, although this inhibition was lower than in VCaP (−7% MPP with respect to E2-treated cells). Moreover, the ERbeta antagonist increased ROS production (R,R-THC, 8% with respect to vehicle-treated cells). ROS production in DU145 cells, which only expresses ERbeta, was affected by E2 (−27% with respect to vehicle-treated cells) and was not affected by ERalpha antagonist or genistein (with respect to vehicle-treated cells), while the ERbeta antagonist statistically significant increased oxidative stress (+33% with respect to E2-treated cells).

3.5. Effect of E2 with and without ER antagonists on antioxidant enzyme and UCPs protein levels in VCaP and PC3

At this point of the experiment it was of interest to check if the E2 effects in VCaP cell line were caused by ERalpha and in PC3 cell line by ERbeta. For this reason we used MPP (antagonist of ERalpha) in VCaP cell lines, and R,R-THC (antagonist of ERbeta) in PC3 cell line. Fig. 3 shows effect of E2 with and without MPP in the VCaP cell line and the effect of E2 with and without R,R-THC in PC3 cell line on protein levels of antioxidant enzymes, GPx and catalase, and UCP2 and UCP5. E2 treatment in VCaP line decreased the expression of GPx, catalase and UCP5, although it was only statistically significant in GPx and UCP5. These effects were prevented by ERalpha antagonist addition in all the cases. Moreover, in PC3 line E2 treatment increased statistically expression of GPx, catalase and UCP2, effects that were reversed by the ERbeta antagonist reversed.

4. Discussion

Cell lines with a lower ERalpha/ERbeta ratio and higher levels of ERbeta have less oxidative stress than cell lines with a predominance of ERalpha. This state may be explained, at least in part, by the greater levels of antioxidant systems as well as UCP5 protein levels. UCPs are a family of inner mitochondrial membrane proteins whose function is to allow the re-entry of protons into the mitochondrial matrix, dissipate the proton gradient and, subsequently, decrease membrane potential and ROS production [20]. Taking into account the genotoxic effect of ROS, it can be speculated that one of the predictable consequences of UCPs function may be in cancer prevention [21,22] and may offer a clinical benefits for cancer treatment studies [22].

Additionally, the action of E2 on antioxidant enzymes and UCPs expression was dual and dependent on the ERalpha/ERbeta ratio and ERbeta amounts. Thus, treatment with 0.1 nM E2 (physiological concentrations on obese men plasma [23]) in cell lines with a high ERalpha/ERbeta ratio (VCaP) produced a reduction of antioxidant enzymes and UCPs levels, and an increase in ROS production. These effects disappeared when treatment was done in the presence of an ERalpha antagonist. In the cell lines which had high levels of ERbeta and a lower ERalpha/ERbeta ratio (PC3), E2 treatment caused an increase in antioxidant enzymes and UCPs, with a concomitant decrease in ROS production. These effects were reversed when cells were treated with E2 in the presence of an ERbeta antagonist.

These results would agree with those of several authors who have shown in different tissues and cell lines that UCPs and antioxidant enzymes are under the control of sex hormones [24–28]. Moreover, similar results have been described in breast cancer cell lines. In MCF-7 cell lines with a higher expression of ERalpha E2 down-regulates UCPs and antioxidant enzymes, increases oxidative stress, while in MDA-MB-231, which only expresses ERbeta, estrogen cannot down-regulate these proteins and does not increase oxidative stress [19].

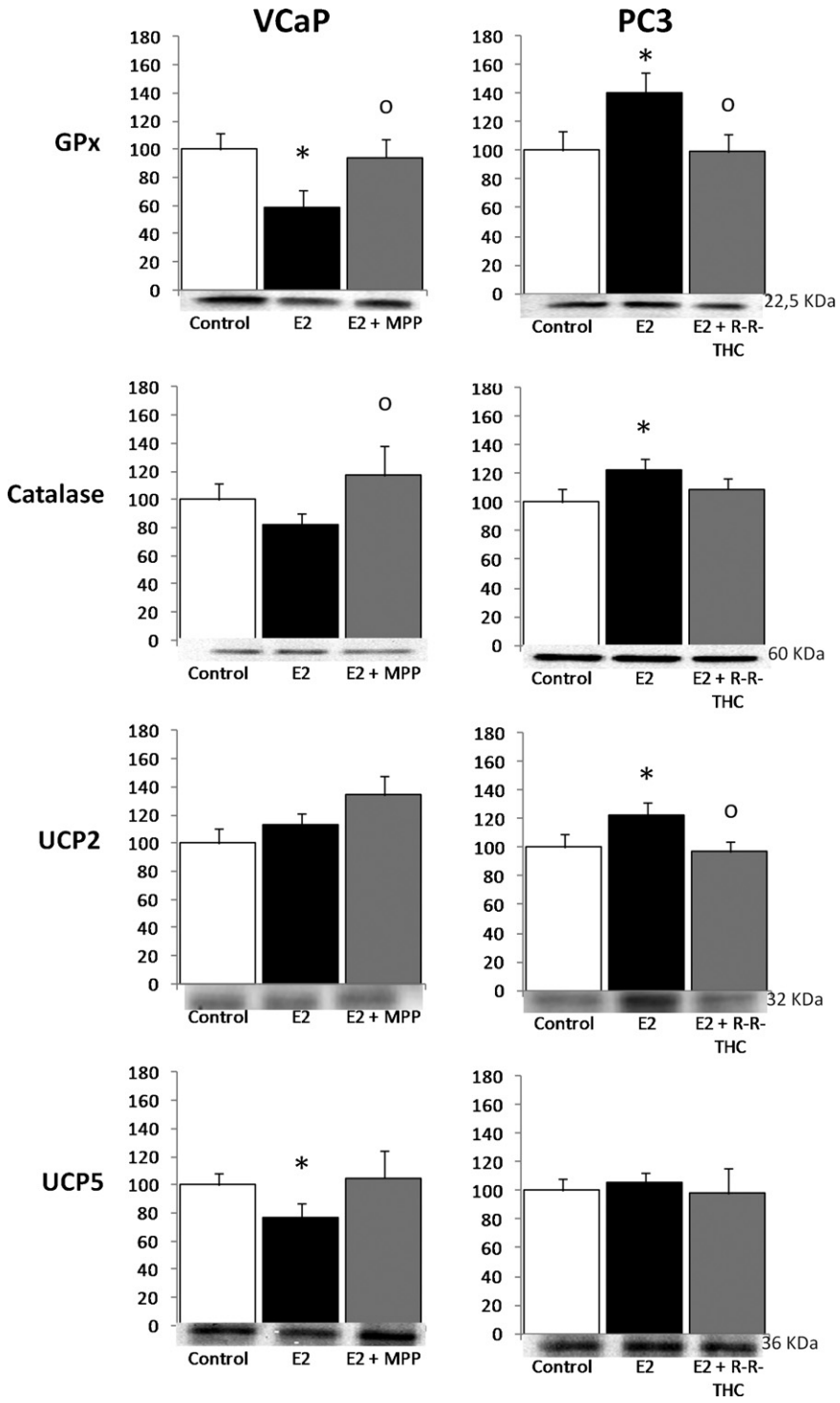

Fig. 3. Effect of 17β-estradiol on GPx, catalase and UCPs protein levels. E2, 17β-estradiol 0.1 nM; MPP 1 μM, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy)phenol]-1H-pyrazole dihydrochloride; R,R-THC 1 μM, (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol. GPx, glutathione peroxidase; UCP, uncoupling protein. Representative bands of Western blot are shown. *Significant difference between E2-treated cells and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$). ○Significant difference between antagonists-treated cells and E2-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

The results of this paper would be consistent with the knowledge that in different cancers the effects that E2 exerts through ERalpha bring about greater oxidative stress and proliferation, whereas the effects through ERbeta have a protective nature when faced with oxidative stress. Thus, in tissues where ERalpha is abundant, E2 is considered a cancer risk, whereas in those tissues in which the predominant isoform receptor is ERbeta, protective effects of E2 when facing oxidative stress have been described [19,29–31]. Moreover, these results agree with the fact that abundance of ERalpha is positively correlated with the malignant prostate cancer and that ERbeta is strongly expressed in low grade prostate carcinoma, while it is markedly diminished in high grade tumors [6]. Several authors have been suggested that ERbeta may protect against abnormal prostate cell growth. Even the use of ERbeta agonists has been suggested as a therapy to prevent grade progression in prostate tumors [6].

Likewise, the antioxidant effects caused by genistein agree with the beneficial effects of phytoestrogens in cancer, as phytoestrogens have reported a 70-fold higher binding affinity for ERbeta than for ERalpha [32], and its difference in affinity for estrogen receptors could explain the protective effects of phytoestrogen.

Another aspect to consider is that some studies have demonstrated that PC3 cancer cells are more aggressive than other prostate cancer cells. These authors believe in the possibility that the aggressive nature of PC3 cells is caused by the lower sensitivity to ROS [33], which could explain why these cells have a greater number of antioxidant systems in response to the higher ERbeta levels.

5. Conclusion

On the whole, our results suggest a dual E2 effect; that an increase or decrease in oxidative stress is regulated by modulating the UCPs and antioxidant enzymes according to the abundance of ERbeta and the ERalpha/ERbeta ratio. Thus, it would be interesting to study these two estrogen receptor isoforms for prostate cancer diagnosis in order to establish possible future treatments. Likewise, the continuation of the study of the role of UCPs in the development of prostate cancer, which could provide valuable information for diagnosis and treatment, since the expression of these proteins has been shown to decrease prostate cancer risk, yet at the same time can have non-desirable effects in neoplastic development, as they confer to these cancer cells a higher resistance to oxidative stress, facilitating their malignancy and chemoresistance.

Acknowledgements

This work was supported by the Spanish Government (PS09/01637). J. Sastre-Serra and A. Valle were funded by grants of Comunidad Autónoma de las Islas Baleares and “Asociación Española contra el cáncer” (AECC), respectively.

References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, CA Cancer J. Clin. 57 (1) (2007) 43–66.
[2] P.D. Baade, M.D. Coory, J.F. Aitken, International trends in prostate-cancer mortality: the decrease is continuing and spreading, Cancer Causes Control 15 (3) (2004) 237–241.
[3] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics 2002, CA Cancer J. Clin. 55 (2) (2005) 74–108.
[4] W.Y. Chang, G.S. Prins, Estrogen receptor-beta: implications for the prostate gland, Prostate 40 (2) (1999) 115–124.
[5] M.S. Steiner, S. Raghow, B.L. Neubauer, Selective estrogen receptor modulators for the chemoprevention of prostate cancer, Urology 57 (4 Suppl. 1) (2001) 68–72.
[6] M. Warner, J.A. Gustafsson, The role of estrogen receptor beta (ERbeta) in malignant diseases—a new potential target for antiproliferative drugs in prevention and treatment of cancer, Biochem. Biophys. Res. Commun. 396 (1) (2010) 63–66.
[7] G.G. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta, Endocrinology 138 (3) (1997) 863–870.
[8] S. Mosselman, J. Polman, R. Dijkema, ER beta: identification and characterization of a novel human estrogen receptor, FEBS Lett. 392 (1) (1996) 49–53.
[9] H. Bonkhoff, T. Fixemer, I. Hunsicker, K. Remberger, Estrogen receptor expression in prostate cancer and premalignant prostatic lesions, Am. J. Pathol. 155 (2) (1999) 641–647.
[10] B.G. Mobbs, I.E. Johnson, Y. Liu, Quantitation of cytosolic and nuclear estrogen and progesterone receptor in benign, untreated, and treated malignant human prostatic tissue by radioligand binding and enzyme-immunoassays, Prostate 16 (3) (1990) 235–244.
[11] L.G Horvath, S.M. Henshall, C.S. Lee, D.R. Head, D.I. Quinn, S. Makela, W. Delprado, D. Golovsky, P.C. Brenner, G. O'Neill, R. Kooner, P.D. Stricker, J.J. Grygiel, J.A. Gustafsson, R.L. Sutherland, Frequent loss of estrogen receptor-beta expression in prostate cancer, Cancer Res. 61 (14) (2001) 5331–5335.
[12] A. Latil, I. Bieche, D. Vidaud, R. Lidereau, P. Berthon, O. Cussenot, M. Vidaud, Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays, Cancer Res. 61 (5) (2001) 1919–1926.
[13] T Tsurusaki, D. Aoki, H. Kanetake, S. Inoue, M. Muramatsu, Y. Hishikawa, T. Koji, Zone-dependent expression of estrogen receptors alpha and beta in human benign prostatic hyperplasia, J. Clin. Endocrinol. Metab. 88 (3) (2003) 1333–1340.
[14] K.M. Lau, M. LaSpina, J. Long, S.M. Ho, Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation, Cancer Res. 60 (12) (2000) 3175–3182.
[15] M.C.S. Yeh, J. Ni, Y. Yin, E. Chang, M. Zang, X. Wen, Functions of estrogen receptor in prostate and prostate cancer, in: Basic Mechanisms and Therapeutic Approaches, W.S. Publishing, New Jersey, 2005, pp. 293–313.
[16] B. Halliwell, Oxidative stress and cancer: have we moved forward? Biochem. J. 401 (1) (2007) 1–11.
[17] J.L. Martindale, N.J. Holbrook, Cellular response to oxidative stress: signaling for suicide and survival, J. Cell Physiol. 192 (1) (2002) 1–15.
[18] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (10) (1998) 4252–4263.
[19] J. Sastre-Serra, A. Valle, M.M. Company, I. Garau, J. Oliver, P. Roca, Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer, Free Radic Biol Med 48 (4) (2007) 506–512.
[20] A. Valle, J. Oliver, P. Roca, Role of uncoupling proteins in cancer, Cancers 2 (2) (2010) 567–591.
[21] F.M. Santandreu, A.S. Valle, P. Fernandez de Mattos, P. Roca, J. Oliver, Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells, Cell Physiol. Biochem. 24 (5–6) (2009) 379–390.
[22] F.M. Santandreu, P. Roca, J. Oliver, Uncoupling protein-2 knockdown mediates the cytotoxic effects of cisplatin, Free Radic Biol Med 49 (4) (2006) 658–666.
[23] H.K. Kley, T. Deselaers, H. Peerenboom, H.L. Kruskemper, Enhanced conversion of androstenedione to estrogens in obese males, J. Clin. Endocrinol. Metab. 51 (5) (1980) 1128–1132.
[24] R. Guevara, F.M. Santandreu, A. Valle, M. Gianotti, J. Oliver, P. Roca, Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress, Free Radic. Biol. Med. 46 (2) (2009) 169–175.
[25] A.M. Rodriguez, M. Monjo, P. Roca, A. Palou, Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes, Cell Mol. Life Sci. 59 (10) (2002) 1714–1723.
[26] S. Rodriguez-Cuenca, E. Pujol, R. Justo, M. Frontera, J. Oliver, M. Gianotti, P. Roca, Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue, J. Biol. Chem. 277 (45) (2002) 42958–42963.
[27] C Borras, J. Gambini, M.C. Gomez-Cabrera, J. Sastre, F.V. Pallardo, G.E. Mann, J. Vina, 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade, Aging Cell 4 (3) (2005) 113–118.
[28] J. Vina, J. Sastre, F.V. Pallardo, J. Gambini, C. Borras, Modulation of longevity-associated genes by estrogens or phytoestrogens, Biol. Chem. 389 (3) (2008) 273–277.
[29] J. Hartman, A. Strom, J.A. Gustafsson, Estrogen receptor beta in breast cancer—diagnostic and therapeutic implications, Steroids 74 (8) (2009) 635–641.
[30] S. Tamir, S. Izrael, J. Vaya, The effect of oxidative stress on ERalpha and ERbeta expression, J. Steroid Biochem. Mol. Biol. 81 (4–5) (2002) 327–332.
[31] Y. Urata, Y. Ihara, H. Murata, S. Goto, T. Koji, J. Yodoi, S. Inoue, T. Kondo, 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells, J. Biol. Chem. 281 (19) (2006) 13092–13102.
[32] A.M. Sotoca, H. van den Berg, J. Vervoort, P. van der Saag, A. Strom, J.A. Gustafsson, I. Rietjens, A.J. Murk, Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells, Toxicol. Sci. 105 (2) (2008) 303–311.
[33] L. Chaiswing, J.M. Bourdeau-Heller, W. Zhong, T.D. Oberley, Characterization of redox state of two human prostate carcinoma cell lines with different degrees of aggressiveness, Free Radic. Biol. Med. 43 (2) (2007) 202–215.