The Effects of 17 β -estradiol on Mitochondrial Biogenesis and Function in Breast Cancer Cell Lines are Dependent on the ER α /ER β Ratio

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Key Words

Estrogen receptor alpha and beta ratio • Breast cancer • 17 β -estradiol (E2) • Mitochondrial biogenesis

cancer, as well as new indicators to the disease progression.

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Abstract

Background/Aims: 17 β -estradiol (E2) is a risk factor for the development of breast cancer, and cause tumorigenesis in epithelial breast cells. Moreover, E2 has distinct effects on different tissues that are attributed to the presence of two estrogen receptor isoforms, ER α and ER β . **Methods:** The effect of E2 on mitochondrial biogenesis and function was investigated in two breast cancer cell lines with different estrogen receptor ratios, MCF-7 (high ER α /ER β ratio) and T47D (low ER α /ER β ratio) cell lines treated with physiological concentrations of E2 (1 nM). **Results:** Mitochondria of the MCF-7 cell line showed an increase in proliferation but a decrease in functionality, while the T47D cell line, with low ER α /ER β ratio, maintained functionality with fewer mitochondria. **Conclusion:** Our results suggest that ERs endowment and its subtypes relation have an effect on treatment response and could contribute new ideas about mitochondria and ERs in breast

Introduction

Estrogens are hormones that have different roles in a number of physiological processes and pathologic conditions such as cancer [1, 2]. It is well known that estrogens, and particularly 17 β -estradiol (E2), are risk factors for the development of breast cancer, and cause tumorigenesis in epithelial breast cells [3]. In the last decades, the focus on estrogen effects has been on the action of these hormones by their binding to estrogen receptors [4, 5]. There are two estrogen receptors: the classical receptor called estrogen receptor alpha (ER α) and the second receptor estrogen receptor beta (ER β) [6]. ER β may have different biological effects than ER α and display different intracellular and tissue distribution patterns [7-9], whilst ER α mediates the proliferative actions of E2 and these effects can be opposed by ER β [10]. In the last few years, researchers have studied the pathways of these receptors with the aim to understand

the importance of these signals in breast cancer.

Moreover, it is known that in mtDNA there are estrogen response elements (ERE) as well as estrogen receptors that can localize in mitochondria in response to several signals [11-13]. On the other hand, mitochondrial biogenesis and reactive oxygen species (ROS) production are under estrogen influence [14, 15]. Similarly, it is demonstrated that mitochondria can modulate the expression of nuclear cell cycle genes and human breast tumor growth [16-18]. For this reason, some authors have given estrogens a new role in the breast carcinogenesis process through the modulation of mitochondrial function and ROS production [14, 19-21].

Additionally, although it is well known that mitochondrial function is altered in cancer cells [15], the supposed theories on the effects of estrogen on mitochondria are controversial, as several studies have shown an increased mitochondrial biogenesis in response to E2 treatment while others have found a mitochondrial dysfunction under the same conditions [12, 14, 22, 23].

The purpose of this article is to investigate whether the influence of E2 on mitochondrial biogenesis and function is dependent on the estrogen receptor ratio. To tackle this aim, we used two breast cancer cell lines: the MCF-7 cell line with a high ER α /ER β ratio; and T47D cell line with a low ER α /ER β ratio.

Materials and Methods

Materials

17 β -estradiol (E2) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mitotracker Green (MTG) was purchased from Molecular Probes (Eugene, OR, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma-Aldrich (St. Louis, MO, USA), Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and treatments

Breast cancer cell lines MCF-7 and T47D bear a great resemblance to one another as human breast adenocarcinoma cell lines with a great difference in ER α /ER β with MCF-7 possessing the highest ratio. Cell lines were purchased from 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% CO₂ in air at 37°C. To evaluate the effects of 17 β -estradiol, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 hours prior to treatment. Experiments were performed when cell cultures reached confluence by providing fresh media supplemented with 1 μ mol/l E2 (Sigma-Aldrich).

Analysis of Cardiolipin content

Cardiolipin content was assayed using Nonyl Acridine Orange (NAO) fluorescence and quantified using a microplate fluorescence reader FLx800 (BIO-TEK Winooski, Vermont, USA) set at 485/20 nm to excitation and 528/20 to emission. The fluorescence was corrected by Hoescht 33342 (Sigma-Aldrich) fluorescence with the same fluorescence reader set at 360/40 nm to excitation and 460/40 to emission.

Citrate synthase, cytochrome c oxidase and ATPase activities

The cell lysates used for enzymatic activities were obtained by scrapping cells in RNAase-free water. CS (citrate synthase; EC 2.3.3.1 [previously 4.1.3.7]) activity was determined by following the increase in absorption of the 5-thio-2-nitrobenzoate ion at 412 nm and 30°C as previously reported [24]. Complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) activity was measured using a spectrophotometric method [25]. Briefly, cell lysate was incubated in 0.1 M NaPO₄H₂, pH 7.0, in the presence of 2 μ g/mL catalase and 5 mM substrate DAB (3, 3' diaminebenzidine-tetrachloride). After 30 s, 100 μ M reduced cytochrome c was added to start the reaction, and the absorbance variation was recorded for 15 min at 450 nm. ATPase (ATP phosphohydrolase, Complex V, EC 3.6.1.3) activity was measured by monitoring the oxidation of NADH at 340 nm and 37°C [26], with an extinction coefficient was 6.22 mM⁻¹ cm⁻¹.

Real time PCR

Total RNA was isolated from cultured cells using TriPure® isolation reagent and quantified using a spectrophotometer set at 260 nm. One μ g of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 μ l volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 2.5 μ M random hexamers, 10 U RNAase inhibitor and 500 μ M each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (-70°C) until the PCR reactions were carried out.

PCR was done for seven target genes: Presenilin 2 (pS2), peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC1 α), nuclear respiratory factors 1 and 2 (NRF1 and NRF2), mitochondrial transcription factor A (TFAM), mitochondrial single strand DNA binding protein (mtSSB) and 18S, using specific primers (see Table 1) SYBR Green technology on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μ L, containing 6.5 μ l Lightcycler® 480 SYBR Green I Master, 0.5 μ M of the sense and antisense specific primers and 2.5 μ l of the cDNA template. The amplification program consisted of a preincubation step for template cDNA denaturation (5 min, 95°C), followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 60°C for PGC1 α , NRF1, NRF2 and mtSSB; and 61°C for 18S, pS2 and TFAM) and an extension step (12s, 72°C). A negative control lacking cDNA template was run in each assay.

Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	T° An. (°C)	Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	T° An. (°C)
			NRF1	CCCgTTACAgggAggTgAg TgTAgCTCCCTgCTgCATCT	60
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61	NRF2	gCgACggAAAgAgTATgAgC gTTggCAGATCCACTggTTT	60
pS2	TTgTggTTTTCTgTgTCA gCAGATCCCTgCAGAAgTgT	61	TFAM	AgATTggggTCgggTCACT CAAgACAATgAAAACCACTC	61
PGC1α	TCAgTCCTCACTggTggACA TgCTTCgTCgTCAAAAACAg	60	mtSSB	TgTgAAAAAggggTCTCgAA TggCCAAAgAATCATCC	60

Table 1. Table and conditions used for RT-PCR. T° An.: Annealing temperature; pS2: presenilin 2; PGC1α: peroxisome proliferator-activated receptor-gamma coactivator-1alpha; NRF1 and NRF2: nuclear respiratory factor 1 and 2; TFAM: mitochondrial transcription factor A; mtSSB: mitochondrial single strand DNA binding protein.

The resulting PCR products were resolved on a 2% agarose gel in 45 mM Tris-borate- EDTA 1 mM buffer (pH 8.0) and visualized by ethidium bromide staining. Bands were analysed with a Chemidoc XRS densitometer (Biorad). The Ct values of the real-time PCR were analysed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (MultiDAnalyses, Sweden).

mtDNA quantification

DNA was isolated from cultured cells using TriPure® isolation reagent following the manufacture protocol and used to quantify mtDNA. 5 ng of the total DNA (quantified using a spectrophotometer set at 260 nm) was amplified using specific primers for 18S and the NADH dehydrogenase subunit 4, with SYBR Green technology on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland).

Total reaction volume was 10 µL, containing 6,5 µL Lightcycler® 480 SYBR Green I Master, 0,5 µM of the sense and antisense specific primers and 2,5 µL of the cDNA template. The amplification program consisted of a preincubation step for denaturation (5 min, 95°C) followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 6°C for 18S and mtDNA), and an extension step (12 s, 72°C). A negative control without cDNA template was run in each assay. The primers used were forward 5'-ggA CAC ggA CAg gAT TgA CA-3' and reverse 5'-ACC CAC ggA ATC gAg AAA gA-3' for 18S and forward 5'-CgT gAC TCC TAC CCC TCA CA-3' and reverse 5'-ATC ggg TgA TgA TAg CCA Ag- 3' for the NADH dehydrogenase subunit 4 (mtDNA).

The resulting PCR products were analysed with the same method before described in section 2.5.

Western blotting

Proteins were isolated from cultured cells using TriPure® isolation reagent following the manufacture protocol. For western blot analysis 50 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 TBS-T (20 mM Tris HCl, 0.13 mM NaCl, and 0.1% Tween 20). Antisera against COXIV (Mitoscience, OR, USA), ATPase, ERα, ERβ TFAM and Tubulin (Santa Cruz Biotechnologies, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Biorad) western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Biorad) and analysed with Quantity One Software (Biorad).

Visualization of mitochondria

Mitochondria were visualized with the mitochondria-specific dye MitoTracker Green as previously described by Rodriguez-Enriquez et al. [27]. The confocal images were acquired on a Leica TCS-SP2 confocal microscope.

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between control and E2-treated cells were analysed by Student's t-test. Statistical significance was set at P < 0.05.

Results

E2 had an effect over estrogen receptor alpha and beta (ERα and ERβ), as shown in Table 2, with a statistically significant decreases in both MCF-7 and T47D cell lines. We used two breast cancer cell lines with different estrogen receptor levels and ratios (Table 2). It can be observed in Table 3 that E2 had a proliferative effect in MCF-7 cell line but not in T47D cell line, with all

	MCF-7		T47D	
	Control	17 β -Estradiol	Control	17 β -Estradiol
ER α (AU)	100 $\pm$ 11	40.1 $\pm$ 9.3*	13.7 $\pm$ 1.8	10.1 $\pm$ 1.6
ER β (AU)	100 $\pm$ 21	35.5 $\pm$ 8.2*	64.4 $\pm$ 8.4	50.3 $\pm$ 8.3
ER α /ER β	1.00	1.13	0.21	0.20

Table 2. Estrogen receptor alpha and beta protein levels in MCF-7 and T47D breast cancer cell lines after 48 hours E2-treatment. ER α : estrogen receptor alpha; ER β : estrogen receptor beta; A.U.: Arbitrary units. Data represent the means $\pm$ SEM (n=6). Values of control (vehicle-treated) cells of MCF-7 were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

	MCF-7		T47D	
	Control	E2	Control	E2
MTT assay (%)	100 $\pm$ 4	111 $\pm$ 3*	100 $\pm$ 7	96.7 $\pm$ 5.0
mtDNA/18S	100 $\pm$ 6	114 $\pm$ 4*	100 $\pm$ 3	75.0 $\pm$ 4.0*
NAO (%)	100 $\pm$ 1	104 $\pm$ 1*	100 $\pm$ 2	100 $\pm$ 2
NAO/mtDNA Ratio	1.00	0.91	1.00	1.33

Table 3. Cellular proliferation and mitochondrial proliferation and differentiation in MCF-7 and T47D breast cancer cell lines. mtDNA: mitochondrial DNA; NAO: 10-N-nonyl-acridine orange. A.U.: Arbitrary units. Data represent the means $\pm$ SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

Fig. 1. Mitochondrial biogenesis mRNA expression in MCF-7 and T47D breast cancer cell lines after E2-treatment for 48h. mRNA levels were detected by RT-PCR using SYBR Green technology. pS2: presenilin 2; PGC1 α : peroxisome proliferator-activated receptor-gamma coactivator-1alpha; NRF1 and NRF2: nuclear respiratory factor 1 and 2; TFAM: mitochondrial transcription factor A; mtSSB: mitochondrial single strand DNA binding protein. E2: 17 β -estradiol. Bars represent the means $\pm$ SEM (n=6) with the values of control (vehicle-treated) cells of MCF-7 were set at 1. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

results obtained by the MTT assay. The MCF-7 cell line showed significant increases in mtDNA copy

number (14 %) and cardiolipin content (4 %). However, T47D cell line showed a statistically significant decrease

	MCF-7		T47D	
	Control	E2	Control	E2
TFAM A.u. (%)	100 ± 9	65.1 ± 6*	100 ± 16	113 ± 13
COXIV A.u. (%)	100 ± 22	39.9 ± 8.2*	100 ± 45	92.9 ± 29.6
ATPase A.u. (%)	100 ± 35	100 ± 43	100 ± 4	87.5 ± 2.5*
ATPase/COX Ratio	1.00	2.51	1.00	0.94

Table 4. TFAM, COXIV and ATPase protein levels in MCF-7 and T47D breast cancer cell lines. TFAM: mitochondrial transcription factor A; COXIV: cytochrome c oxidase subunit IV. A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

	MCF-7		T47D	
	Control	E2	Control	E2
CS A.U. (%)	100 ± 8	72.4 ± 10*	100 ± 6	127 ± 18
COX A.U. (%)	100 ± 9	56.7 ± 8*	100 ± 7	103 ± 12*
ATPase A.U. (%)	100 ± 4.0	83.8 ± 4.8*	100 ± 5	100 ± 6
ATPase/COX Ratio	1.00	1.48	1.00	0.97

Table 5. Cytrate Synthase, Cytochrome c oxidase and ATPase enzymatic assay in MCF-7 and T47D breast cancer cell lines. CS: Cytrate synthase; COX: cytochrome c oxidase. A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

in mtDNA copy number (25 %) but not in cardiolipin content. For this reason, the NAO/mtDNA ratio was higher in T47D cell line than in MCF-7 cell line (0,91 and 1,33 respect to control as 1,00) after treatment with E2.

In an attempt to localize these differences in mitochondrial biogenesis, we checked the effect of E2 on mRNA expression of different genes involved in this pathway. As shown in Fig. 1, PGC1 α , NRF1, NRF2, TFAM and mtSSB had a time-dependent increase with the E2 treatment. It is worth noting that initial mRNA levels and the amount of the increase observed differed, depending on the cell line. Moreover, these effects were also monitored by the TFAM, COX and ATPase protein levels. Table 4 shows the decrease in TFAM and COX protein levels in MCF-7 cell line (35 % and 60 %, respectively), while in T47D cell line there were no changes after 48 hours of E2-treatment. Furthermore, ATPase protein levels decreased in the T47D cell line (22 %). All of these changes are statistically significant. Consequently, the ATPase/COX ratio showed an increase in MCF-7 cell line and a decrease in T47D cell line.

At this point of the experiment, it was considered of interest to investigate the activities of the mitochondrial-

Fig. 2. Changes in mitochondrial morphology in response to E2. Breast cancer cells were cultured normally and labeled with Mitotracker Green nad imaged, as described in Material and Methods.

related enzymes. As shown in Table 5, citrate synthase activity showed a statistically significant decrease in the

MCF-7 cell line (28 %) while the T47D cell line showed an increase (27 %). Furthermore, COX and ATPase activities were lower in MCF-7 E2-treated cells than in treated controls. Moreover, as shown in Fig. 2, morphology of mitochondria were different in E2-treated cell line, while T47D cell line not showed differences in mitochondrial morphology after treatment.

Discussion

E2 increased mitochondrial biogenesis in MCF-7 and T47D breast cancer cell lines. However, there is a clear difference in the response in the mitochondrial biogenesis of these cell lines when faced this E2 stimulation. MCF-7 cell line (high ER α /ER β ratio) has mitochondria with lower mitochondrial function than T47D cell line (with low ER α /ER β ratio) although this latter line had more functional mitochondria in response to the E2 treatment. These results would be in agreement with the different physiological functions of estrogens displayed once bound to their corresponding receptors [8, 21, 28].

In the MCF-7 cell line, which has highest reported ER α /ER β ratio [29, 30] E2 increased mtDNA copy number, although mitochondrial function was diminished since Citrate Synthase, cytochrome c oxidase and ATPase activities suffered a statistically significant decreases. Moreover, cytochrome c oxidase protein levels were also lower after E2 treatment. However, for the T47D cell line, with lower ER α levels than the MCF-7 cell line, E2, despite a decrease in mtDNA copy number per cell, maintained mitochondrial function, as enzymatic activities did not decrease per cell with E2. This is noteworthy as given that even citrate synthase has a tendency to increase, the enzymatic activities indicate functional mitochondria.

These estrogen receptor isoform dependent differences in mitochondria fit with the dual effect of E2 found in different tissues [31]. Thus, E2 has a beneficial role in some tissues such as: heart, brain, muscle, brown adipose tissue, and liver [32-34], where this hormone increases mitochondrial function. This effect agrees with studies that shown more functional mitochondria in female rats and these findings have been associated with a lower oxidative stress [35, 36]. However, E2 has a role in tissue malignancy in: ovary, mammary gland and uterus; and has been related to lifetime exposure to estradiol and other cancer risk factors [8]. These effects could be attributed to the difference in the predominant estrogen receptor isoform in the tissues [9].

Expression of mitochondrial biogenesis genes was increased in MCF-7 cell line, but to a lesser degree than in T47D cell line. Amongst all genes studied, it should be underscored that TFAM, had a higher expression in the T47D cell line than in the MCF-7 in response to E2 treatment. Moreover, there was a decrease in TFAM protein levels after a 48h E2 treatment respect to its control in the MCF-7 cell line, while in the T47D cell line, there was an increase in this protein. High TFAM levels are known to stimulate differentiation, while low levels of TFAM stimulate mitochondrial proliferation [37-40]. Thus, the resultant TFAM levels would be in agreement with mitochondrial functionality observed in these breast cancer cell lines. Another important mitochondria biogenesis factor is NRF-2, which also increased with E2, although differed in its magnitude of expression according to cell line, showing a higher increase in the T47D cell line than in the MCF-7 cell line. NRF-2 has been related to mitochondrial maturation, owing to the fact that it regulates traffic and assembly related-genes of the mitochondrial proteins [41]. Thus, the effect of E2 on mitochondrial biogenesis genes indicate different responses in the genes involved in mitochondrial biogenesis, as the MCF-7 cell line showed a response to proliferation while the T47D showed a differentiation response.

Lately, mechanisms has been proposed to modulate mitochondrial number and the deleterious effects from signals like ROS or UV light known as mitoptosis [42]. Mitoptosis in normal conditions removes dysfunctional mitochondria [43]. There are studies that show that in some cancers, mitoptosis is inhibited, and for this reason, the damaged mitochondria accumulated in the cells would create mitochondrial dysfunction, affecting mitochondrial biogenesis [44]. Mitoptosis could be explained by the increase in MCF-7 mtDNA copy number caused by an accumulation of dysfunctional mitochondria, or as well by the decrease observed in the T47D cell line, when removing the damaged mitochondria accumulated in the pretreatment with Charcoal, where the beneficial effects of E2 in this cell line there are not present.

In fact, if we observed mitochondrial morphology, we appreciate a several change in morphology of E2-treated MCF-7 mitochondria according to all the results.

In summing up, the results suggest that E2 acts over function, proliferation and mitochondrial differentiation, according to the estrogen receptor ratio (ER α /ER β), with lesser mitochondrial activity present in the cell line with a predominance of ER α , while in contrast, the cell line with a lower ER α /ER β ratio had more functional mitochondria.

Thus, our results reinforce the idea that depending on the estrogen receptor endowment and its subtype relation, breast tumors could have different response to treatment and prognosis, with ER β clinical analyses could provide an indicator to the disease progression and provide new insight of the role of mitochondria and estrogen receptors in breast cancer.

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