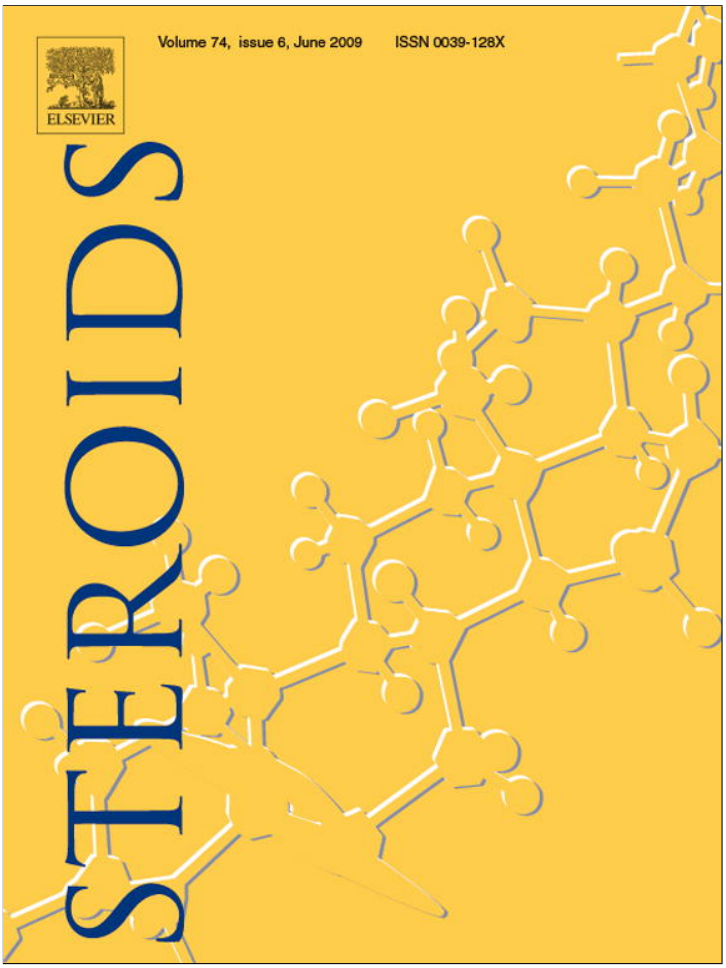

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Expression and subcellular distribution of native estrogen receptor β in murine C2C12 cells and skeletal muscle tissue

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ABSTRACT

We have recently described the expression and intracellular localization of ER α in murine C2C12 cells and skeletal muscle tissue. In separate studies, a protective role of 17β-estradiol against apoptosis exerted mainly at the mitochondrial level was also shown in the C2C12 muscle cell line. However, this functional evidence was in accordance with the participation of ER β. We have then here investigated the expression and subcellular distribution of native ER β in similar skeletal muscle cultured cells and tissue developed *in vivo*. ER β was detected by immunoblotting using specific antibodies and ligand blot analysis after subcellular fractionation. Immunolocalization was confirmed using conventional and confocal microscopy. ER β was found to a great extent in mitochondria and in lower amounts in the cytosolic fraction, differently to ER α which localizes in microsomes, cytosol, mitochondria, and also in the nucleus of muscle tissue. ER β expression was also demonstrated by RT-PCR. Finally, the mitochondrial localization of native ER β in C2C12 muscle cells was corroborated after transient transfection with specific ER β siRNAs. These data raise the possibility that the antiapoptotic action of 17β-estradiol in muscle cells may be related in part to a direct action of the hormone on mitochondria through ER β.

1. Introduction

The estrogen 17β-estradiol (E2) plays an important role in development, cell growth and differentiation, involving genomic and non-genomic mechanisms [1,2]. Usually, the effects of the hormone are mediated via two estrogen receptors (ERs), ER α and ER β, which belong to the nuclear receptor superfamily [3–8]. Briefly, steroid hormones bind to specific receptors which are intracellular transcription factors and exert positive or negative effects on the expression of target genes [5].

Nevertheless, several studies suggest that the ER could be non-classically associated to intracellular membranes [9–13]. Moreover, there is evidence showing that ER α and ER β may be located in the plasma membrane [14–19] and mitochondrial compartments [20–24]. In addition, the non-genomic events triggered by E2 suggest the ability of the hormone to activate extra-nuclear receptors [2]. In agreement with these observations, we have shown that ER α or immunoreactive-related proteins are localized in microsomes/endoplasmic reticulum and mitochondria, in the C2C12 muscle cell line and murine skeletal muscle [25]. We also demonstrated that E2, at physiological concentrations, abrogates H_2O_2 induced-apoptosis in C2C12 cells involving both ER α and ER β and acting at least at two different levels. One of them is inducing PI3K/Akt activation and then BAD phosphorylation, process in which both ER isoforms participate. The other relates to a protective effect on mitochondria integrity and mainly involves ER β [26]. In view of these observations, the aim of this study was to investigate the expression and the subcellular distribution of native ER β in murine C2C12 muscle cells and skeletal muscle tissue. The specific distribution of the two ER isoforms could explain the specific roles of ER α and ER β in the cytoprotective action of E2 against apoptotic stimuli.

2. Experimental

2.1. Materials

Estrogen receptor α mouse monoclonal antibodies clone TE111.5D11 (anti-ER ligand binding domain); clone AER 314 (anti-ER N-terminal transactivation domain) and estrogen receptor β rabbit polyclonal antibody, epitope specific, were purchased from NeoMarkers (Fremont, CA, USA). Estrogen receptor β goat polyclonal antibodies (L-20, Y-19) and rabbit polyclonal antibody (H-150), Y-19 blocking peptide and anti-lamin B goat polyclonal antibody (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MitoTracker (MitoTracker® Red CMXRos) and Alexa 488-conjugated goat anti-rabbit were from Molecular

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Probes (Eugene, OR, USA). Cy™-3 conjugated donkey anti-goat was from Jackson Immunoresearch Inc. (West Grove, PA, USA). Purified recombinant ER α protein was from PanVera Corporation (Madison, WI, USA). [(2,4,6,7-^{3}H(N)] 17β-estradiol with a specific activity of 80-115 Ci/mmol was obtained from New England Nuclear (Chicago, IL, USA). Cytochrome *c* Oxidase Assay Kit, anti-actin polyclonal antibody (A-506), 17β-estradiol, 17α-estradiol, 17β-estradiol-peroxidase, tamoxifen and diethylstilbestrol (DES) were purchased from Sigma–Aldrich (St Louis, MO, USA). ICI_{182780} was obtained from TOCRIS (Ellisville, MO, USA). TRIzol® Reagent, PCR Reagent System and primers were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Reverse Transcription System was from Promega Corporation (Madison, WI, USA). Other chemicals used were of analytical grade. Estrogen receptor β (ER β) ShortCut®siRNA Mix, Estrogen receptor α (ER α) ShortCut®siRNA Mix, Fluorescein-siRNA Transfection Control and TransPass™ R2 Transfection Reagent were purchased from New England BioLabs Inc. (Beverly, MA, USA). Molecular weight colored markers and chemiluminescence blot detection kit were obtained from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Cell culture

The C2C12 murine skeletal muscle cell line was gently donated by Dr. Enrique Jaimovich (Universidad de Chile, Santiago, Chile). The cells were routinely cultured in serum-supplemented medium composed of Dulbecco's Modified Eagles's Medium (DMEM) without Phenol Red, 10% heat-inactivated (30 min, 56 °C) fetal bovine serum (FBS), 1% nistatine and 2% streptomycin. Unless otherwise noted, cells were cultured in chamber-slides for microscopy (Nunc Inc, IL, USA) at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium.

2.3. Subcellular fractionation

C2C12 cell confluent monolayers were scrapped and homogenized in ice-cold TES buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml trypsin inhibitor) using a teflon-glass hand homogenizer. A nuclear pellet was obtained by low speed centrifugation ($800 \times g$, 20 min) of the lysed cell preparation. The supernatant was further centrifuged at $10{,}000 \times g$ for 15 min to pellet mitochondria. The remaining supernatant was centrifuged at $120{,}000 \times g$ for 90 min, to yield a soluble supernatant (cytosol) and a plasma membrane/endoplasmic reticulum-containing particulate pellet (microsomes).

To isolate subcellular fractions from mature skeletal muscle, female mice were killed by cervical dislocation, after random selection without regard for the stage of estrous cycle. Skeletal muscle tissue was isolated from legs and placed in ice-cold saline. Connective and adipose tissue were removed. The muscle tissue was homogenized in TES buffer with an Ultraturrax homogenizer using 5 ml buffer/g tissue under ice. The homogenate was filtered through two layers of nylon mesh and subsequently centrifuged as described above for the isolation of subcellular fractions from C2C12 cells.

Protein concentration from each fraction was estimated by the method of Bradford [27], using bovine serum albumin (BSA) as standard.

Contamination of nuclear, microsomal and cytosolic fractions with mitochondrial components was assessed by measuring the activity of the mitochondrial marker enzyme cytochrome *c* oxidase employing the Cytochrome *c* Oxidase Assay Kit (Sigma) according to manufacturer's instructions. Anti-lamin B antibody was employed for the immunodetection of the nuclear protein marker lamin B in the different fractions. Coomassie Blue protein stain was used to ensure the equivalence of protein amounts loaded for Western and ligand blots (data not shown).

2.4. [^{3}H]17β-estradiol binding assays

E2 binding site concentration was determined by means of whole-cell binding assays [28]. C2C12 cells were cultured until confluence in 6-well plates in serum-supplemented medium. Cells were washed with serum-free phenol red-free DMEM and preincubated in the same medium (serum and phenol red-free DMEM (SPRF-DMEM) for 30 min at 37 °C. The medium was replaced with 2 ml of SPRDFMEM containing 5 nM [^{3}H]17β-estradiol. After 90 min of incubation at 37 °C, the cells were exhaustively rinsed with SPRDFMEM to remove the unbound isotope. Trapped radioactivity was extracted with lysis buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 1% Triton X-100). Aliquots from each well were taken in triplicate for both scintillation counting and protein quantitation by the method of Bradford [27]. For determination of non-displaceable binding, a 200-fold molar excess of unlabelled E2 was included in the incubation mixture. For displacement studies, an equivalent excess of cold competitors were also included together with the tritiated hormone.

Blocking experiments of ER β involved incubation of the cells (1 h, 37 °C) in presence of 1:100 dilution of the Y19 polyclonal antibody in DMEM buffer prior addition of the radioactive ligand. In order to allow blocking of intracellular estrogen binding sites in whole cell experiments, membrane permeabilization with saponin (50 μg/ml), 1 min at 37 °C, was performed prior incubation with the antibody.

In all experiments final isopropanol concentration did not exceed 0.01%. Appropriate vehicle controls were used and each condition was assayed in triplicate.

2.5. Western blots

Protein aliquots were combined with sample buffer (400 mM Tris/HCl pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT and 2 μg/ml bromophenol blue), boiled for 5 min and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractionated proteins were then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore), using a semi-dry system. Non-specific sites were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated for 1 h with the appropriate dilution of the primary antibodies. The membranes were repeatedly washed with PBS-T prior incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence blot detection kit was used as described by the manufacturer to visualize reactive products. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers.

2.6. Ligand blots

Subcellular fractions were subjected to 10% SDS-PAGE and blotted to PVDF membranes as described above. The membranes were exhaustively washed (at least 24 h at room temperature) with PBS-T followed by PBS, to remove SDS and allow transferred proteins to renaturalize. After blocking with 5% BSA in PBS (1 h), the membranes were incubated for 1 h at room temperature with 10^{-6} M E_2-peroxidase conjugate. Reactive bands were detected by the enhanced chemiluminescence blot detection kit according to manufacturer's instructions.

Images were digitalized using a Hewlett Packard 3200C scanner at a resolution of 300 dpi and exported to Adobe PhotoShop for digital processing.

2.7. Immunocytochemistry and microscopy

Semiconfluent (60–70%) monolayers were washed with serum-free phenol red-free DMEM, incubated 30 min in the same medium and then fixed 20 min at 20 °C with cold methanol. After fixation, cells were rinsed three times with PBS. Non-specific sites were blocked for 30 min in PBS that contained 5% BSA. Cells were then incubated for 60 min in the presence or absence (negative control) of primary antibodies (TE11.5D11; ER β epitope specific; H-150; Y19 or L20; 1:50 dilution). Purified recombinant ER α protein and Y19 blocking peptide were employed to determine the specificity of anti-ER α and β antibodies immunoreactivity, respectively. To that end, the purified recombinant ER α protein or the blocking peptide were incubated in PBS 1× for 1 h at room temperature with the primary antibody. Then, the incubation medium containing the antibody was added to the fixed cells.

After a PBS wash, the cells were incubated for another 60 min with Alexa 488-conjugated anti-rabbit secondary antibody for H-150, TRITC-conjugated anti-rabbit for ER β epitope specific, Cy™ 3-conjugated anti-goat for Y19 and L20 or FITC-conjugated anti-mouse for TE11.5D11 antibody. The antibodies were prepared in PBS containing 2% BSA and all steps were carried out at room temperature.

In parallel experiments C2C12 cells were incubated in the presence of 1 nM of E2 during 15 and 30 min at 37 °C prior fixation and immunostaining.

MitoTracker® Red CMXRos was employed for selective stain of active mitochondria, in the presence of H-150 antibody, according to manufacturer's instructions.

Slides were mounted and images were acquired on a *NIKON* conventional microscope (*NIKON Eclipse E 600*) equipped with standard filter sets to capture fluorescent signals.

Images were collected using a digital camera (*NIKON COOLPIX*) and exported to Adobe PhotoShop for digital processing.

2.8. Confocal microscopy

Images were acquired on a Leica TCS SP2 AOBS confocal laser-scanning microscope in an epifluorescence mode. The 488 nm line of an argon ion laser and the 543 nm line of a helium-neon laser were used to excite the samples. A DD 488/543 filter was used to separate red/green fluorescence signals. Cells were imaged through a 63×, 1.3 numerical aperture water immersion objective. Images were collected and saved using the Software LCS (Leica Confocal Software) version 2.61 and exported to Adobe PhotoShop for digital processing.

2.9. Transient transfections with estrogen receptor β shortcut siRNA mix

Cells were plated at an appropriate density to reach 40–60% confluence the day of transfection. For transfection experiments, TransPass™ R2 Transfection Reagent was mixed with ER β ShortCut®siRNA. The mix was incubated for 20 min at room temperature and diluted with complete culture medium. The culture medium of the cells was aspirated and replaced with the diluted transfection complex mixture.

To estimate the transfection efficiency of siRNA, 20 pmol of fluorescein-siRNA were used according to manufacturer's instructions. Cells were then visualized, 24 h post-transfection, in a conventional microscope employing an adequate filter for green fluorescence.

To evaluate the effective silencing of ER β, total protein from cells transiently transfected with ER β ShortCut®siRNA or with TransPass™ R2 Transfection Reagent alone (negative control) were extracted 24 and 48 h post-transfection and ER β expression was tested by Western blot using H-150 rabbit polyclonal antibody. For actin loading control, membranes were stripped with stripping buffer (62.5 mM Tris–HCl pH 6.7, 2% SDS, 50 mM β-mercaptoethanol) and then blocked for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were then incubated 1 h with a 1:10,000 dilution of anti-actin polyclonal antibody (A-5060) as primary antibody. After several washings with PBS-T, membranes were incubated with anti-rabbit conjugated to horseradish peroxidase (1:10,000). Immunoreactive proteins were developed by means of enhanced chemiluminescence (ECL).

For immunocytochemistry and competitive binding assays cells were incubated 24 h with 20 pmol of ER β siRNA or Transfection Reagent without ER β ShortCut®siRNA (negative control).

In competitive binding assays, 5 nM [^{3}H]17β-estradiol was incorporated into the medium to determine the total binding. For determination of non-displaceable binding, a 200-fold molar excess of unlabelled E2, 17α-estradiol, DES, tamoxifen or ICI_{182720} were included in the incubation mixture. For immunocytochemistry, H-150 rabbit polyclonal antibody was employed as primary antibody and Alexa 488-conjugated goat anti-rabbit as secondary antibody.

2.10. Reverse transcription-polymerase chain reaction

Semiconfluent cells in 75 cm^2 culture flasks were rinsed once with 1× PBS (pH 7 4) and then harvested by scraping using TRizol Reagent, for total RNA isolation, according to manufacturer's instructions. The RNA pellet was air-dried for 5 min and subsequently dissolved in 50 μl of deionized water. The concentration and purity of the RNA preparation were determined by measuring the absorbance of RNA at 260 and 280 nm. The RNA integrity was analyzed on a 1% native agarose gel in 1× TBE buffer.

cDNA was prepared with AMV reverse transcriptase using random primers hexamers. The reaction was performed in a total volume of 20 μl containing 5 μg of total RNA, according to manufacturer's instructions. The reaction mixture was incubated at 42 °C for 1 h.

For the PCR reaction, the total volume (20 μl) from the reverse transcription reaction was amplified in a final volume of 100 μl containing 0.5 μM of each primer (forward and reverse). Amplification was performed on an Eppendorf Mastercycler® personal 5332 for 35 cycles with denaturation at 94 °C (30 s), annealing at 58 °C (30 s) and extension at 72 °C (30 s).

PCR primers were specifically designed to amplify mouse ER β employing the Mouse BLASTN program of the *Gen Bank* Sequence database (Table 1). The program was used to analyze the specificity of primer annealing. No matching was obtained with the mouse ER α gene sequence. RT-PCR reaction employing water, instead of C2C12 RNA, was used as a negative control.

2.11. Statistical evaluation

Statistical significance of data was evaluated using Student "*t*" test [29].

3. Results

In previous work we identified and characterized specific intracellular E2 binding sites in the C2C12 skeletal muscle cell line, by competitive binding assays in whole cells in culture employing E2 as a competitor [25]. Displacement assays using the stereoisomer 17α-estradiol, the non-steroidal synthetic estrogenic ligand DES, the antiestrogenic compound ICI_{182780} and the agonist/antagonist tamoxifen corroborated the expression of ER binding sites. By means of immunoblocking experiments in competitive binding assays, Western blot analysis, immunocytochemistry, siRNA knock-down and RT-PCR, we demonstrated the

Table 1
Mouse ER β oligonucleotide primer pairs.

Transcript (Gene bank accession number)	Primers	Location	PCR product (bp)
NM_207707	PF 5′ GAGGAGGTGGGGAGCTGGCCTG 3′ PR 5′ CAGCAGGACTGTAGAATGTCA 3′	121–142 561–541	440

presence of ER α in non-classical intracellular compartments, like microsomes/endoplasmic reticulum and mitochondria [25]. In this work we investigated the presence and subcellular location of ER β in C2C12 cells. We have also compared the subcellular distribution of both the β and α isoforms of ER in the C2C12 cell line and murine skeletal muscle tissue.

We first evaluated the effects of immunoblocking with Y19 specific polyclonal antibody against ER β on muscle cell ligand binding following an *in vivo* classical binding assay protocol performed before with several cell types including C2C12 cells [12,18,22,25].

As shown in Fig. 1, preincubation of C2C12 cell cultures with Y19 antibody in the presence of saponin reduced specific [^{3}H]17β-estradiol binding by whole cells (−36%; $p < .0.01$). These results are in keeping with an intracellular localization of ER β, as demonstrated before for ER α [25]. The antibody did not significantly displace tritiated estradiol binding when the cells were not subjected to saponin permeabilization (<19%; $p > 0.5$, N.S.). Appreciable differences between the S.D. of control and S.D. of antibody (±saponin) treated cells were observed. This may be attributed to variations in anti-ER β antibody blocking efficiency among independent experiments and/or, in the case of saponin treated cells, to the action of the detergent which can detach cells from the monolayer or induce stress conditions that affect binding capacity.

The immunological constituents of ER β were then analyzed at the subcellular level. C2C12 cell fractions isolated by differential centrifugation were subjected to SDS-PAGE followed by Western blot assays employing an anti-ER β rabbit, epitope specific, polyclonal antibody. As shown in Fig. 2A, high expression levels of a single ∼50 kDa band for ER β were detected in mitochondria, and in lower amounts in the cytosolic fraction. In comparison, the ∼67 kDa band for ER α was predominantly found in microsomes and cytosol and to a lesser extent in mitochondria. The subcellular distribution profile of ER isoforms was also analyzed in murine skeletal muscle tissue. Western blots of these subcellular fractions showed non-classical localization for both ER β and α isoforms (Fig. 2B). Thus, ER β was mainly detected in mitochondria and cytosol and ER α in nucleus and microsomes. The secondary antibody used did not recognize proteins ranging within the expected molecular size (data not shown). Western blot analysis employing an antibody against the nuclear marker lamin B revealed no nuclear contamination in other subfractions (Fig. 3A). Contamination with mitochondria of the nuclear, microsome and cytosolic fractions was also excluded by evaluation of cytochrome *c* oxidase activity in each fraction (Fig. 3B).

Next, ligand blot and Western blot immunoreactivity patterns for ER β were compared. Fig. 4 (left) shows the subcellular localization profile for the E2 binding proteins of ∼67 and ∼50 kDa in C2C12 cells using E_2-peroxidase as ligand, which corresponded with the immunological detection of ER α and ER β, respectively (Fig. 4, right). Also, ligand blot assays showed a significant proportion of estradiol binding proteins of low molecular weight apart from that of ∼67 kDa matching the immunoreactive band patterns obtained with the monoclonal anti-ER α antibody [25].

The extra-nuclear subcellular localization of the ER β isoform was confirmed by indirect immunofluorescence microscopy (Fig. 5). Three different polyclonal antibodies were employed to label methanol fixed C2C12 cells. By means of conventional microscopy we could detect ER β localized in mitochondria. This result was also confirmed by confocal microscopy (Fig. 6), which, employing the H-150 antibody and MitoTracker® Red for ER β- and mitochondria-selective staining, respectively, showed the colocalization of the green and red signals (Fig. 6D). No ER signal was seen in the cells in which the primary antibody was omitted (data not shown).

Fig. 7 shows the immunocytochemical characterization of C2C12 cells subjected to E2 treatments (1 nM, 15 or 30 min) followed by fixation and immunoassaying with anti-ERα or ER β antibodies

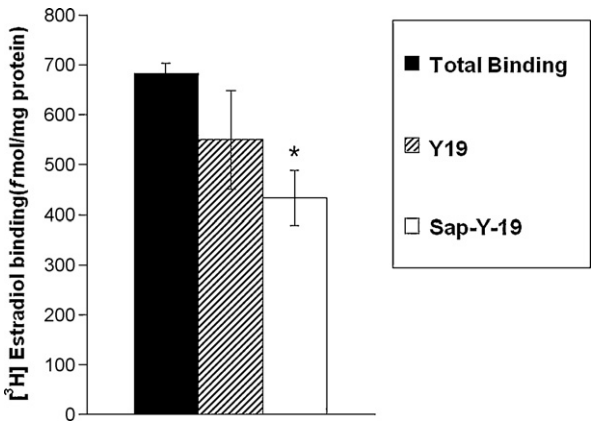

Fig. 1. Inhibition of [^{3}H]17β-estradiol binding by polyclonal antibody Y19 in permeabilized C2C12 cells. Whole cells were incubated for 1 h at 37 °C in DMEM in the absence or presence of Y19 polyclonal antibody against ER β with or without saponin treatment (50 μg/ml; 1 min at 37 °C). Cells were then incubated with [^{3}H]17β-estradiol for 90 min at 37 °C followed by determination of ligand specific binding as described in Section 2. Results are expressed in fmol/mg protein and represent the mean of samples analyzed in triplicate ± S.D. Sap-Y19: cells permeabilized with saponin in presence of Y19 antibody. Y19: non-permeabilized cells incubated with Y19 antibody. $^*p < 0.01$.

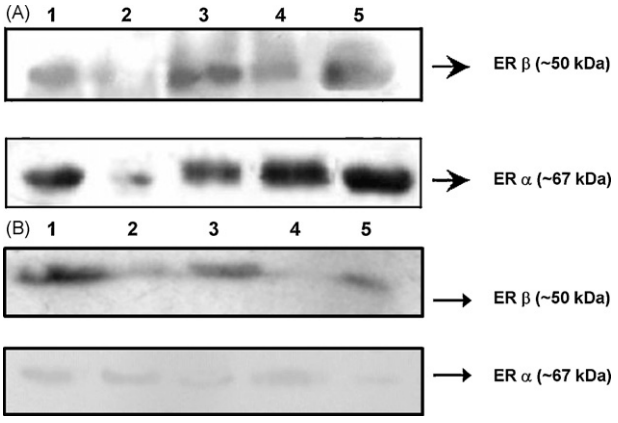

Fig. 2. ER β immunochemical reactivity at the subcellular level in C2C12 cells and murine skeletal muscle tissue. Samples from cell fractions containing equivalent protein amounts (20 μg) were subjected to SDS-PAGE and transferred to PVDF membranes as described in Section 2. Probing was done with: (A) anti-ER β rabbit, epitope specific, polyclonal antibody (upper panel) or an anti-ER α mouse monoclonal antibody, clone: TE111.5D11 (lower panel); (B) anti-ER β rabbit, H-150, polyclonal antibody (upper panel) or an anti-ER α mouse monoclonal antibody, clone: AER 314 (lower panel). The images were focused to show the ∼67 and ∼50 kDa protein bands expected for the α and β isoforms, respectively. Lane 1: total homogenate; lane 2: nuclei; lane 3: mitochondria; lane 4: microsomes; lane 5: cytosol. The subcellular distribution of immunoreactivity for ER β and its comparison with ER α localization in C2C12 muscle cells (A) and murine skeletal muscle tissue (B) is shown.

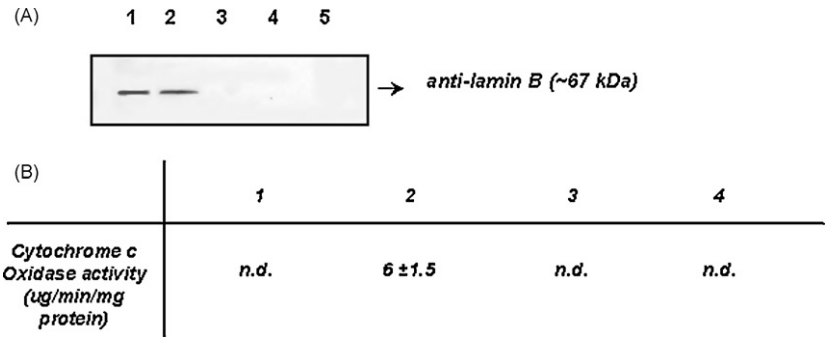

Fig. 3. Assessment of purity of subcellular fractions isolated from C2C12 muscle cells. (A) Lamin B. This protein was immunodetected using a specific antibody (C-20, 1:300 dilution). Lane 1: total homogenate; lane 2: nuclear fraction; lane 3: mitochondria; lane 4: microsomes; lane 5: cytosol. (B) Cytochrome *c* oxidase. Its activity was assayed employing a commercial kit as indicated in Section 2. Lane 1: nuclear fraction; lane 2: mitochondria; lane 3: microsomes; lane 4: cytosol. *n.d.*: not detected.

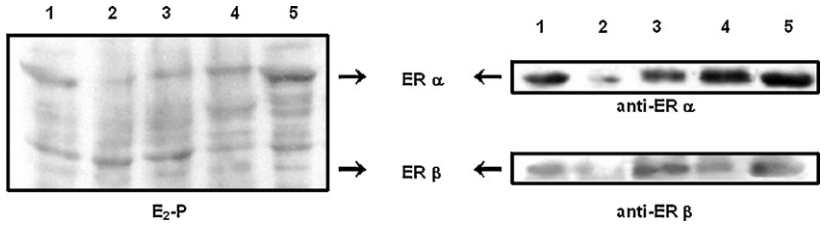

Fig. 4. Subcellular distribution of 17β-estradiol binding proteins in C2C12 muscle cells detected by ligand blot assays and comparison with ER-immunoreactivity. *Left*. Proteins from C2C12 subcellular fractions were subjected to SDS-PAGE, transferred to PVDF membranes and renatured as described in Section 2. E2 binding proteins were labeled with estradiol-peroxidase conjugates (E_2-P). *Right*. *Upper panel*: Western analysis with anti-ER α (TE111.5D11). *Lower panel*: Western analysis with anti-ER β (polyclonal antibody, epitope specific). Lane 1: total homogenate; lane 2: nuclei; lane 3: mitochondria; lane 4: microsomes; lane 5: cytosol.

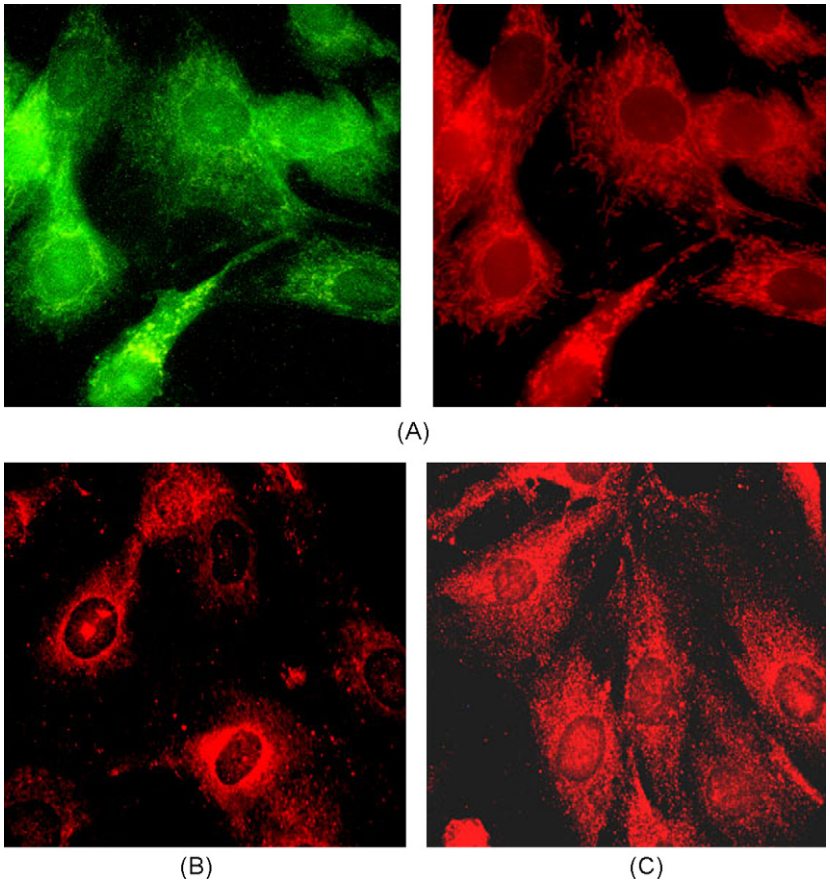

Fig. 5. Immunofluorescence micrographs of C2C12 muscle cells after staining for ER β antigens: conventional microscopy. ER β was detected in methanol fixed and permeabilized cells using H-150 (A), Y19 (B) and L20 (C) antibodies (Section 2). Mitochondria localization with the mitochondrial marker MitoTracker Red is shown (A). Immunocytochemical assays with primary Y19 antibody previously incubated with the respective blocking peptide and negative controls using only fluorescent secondary antibodies rendered a negligible cellular background (data not shown). Original magnification: 600×.

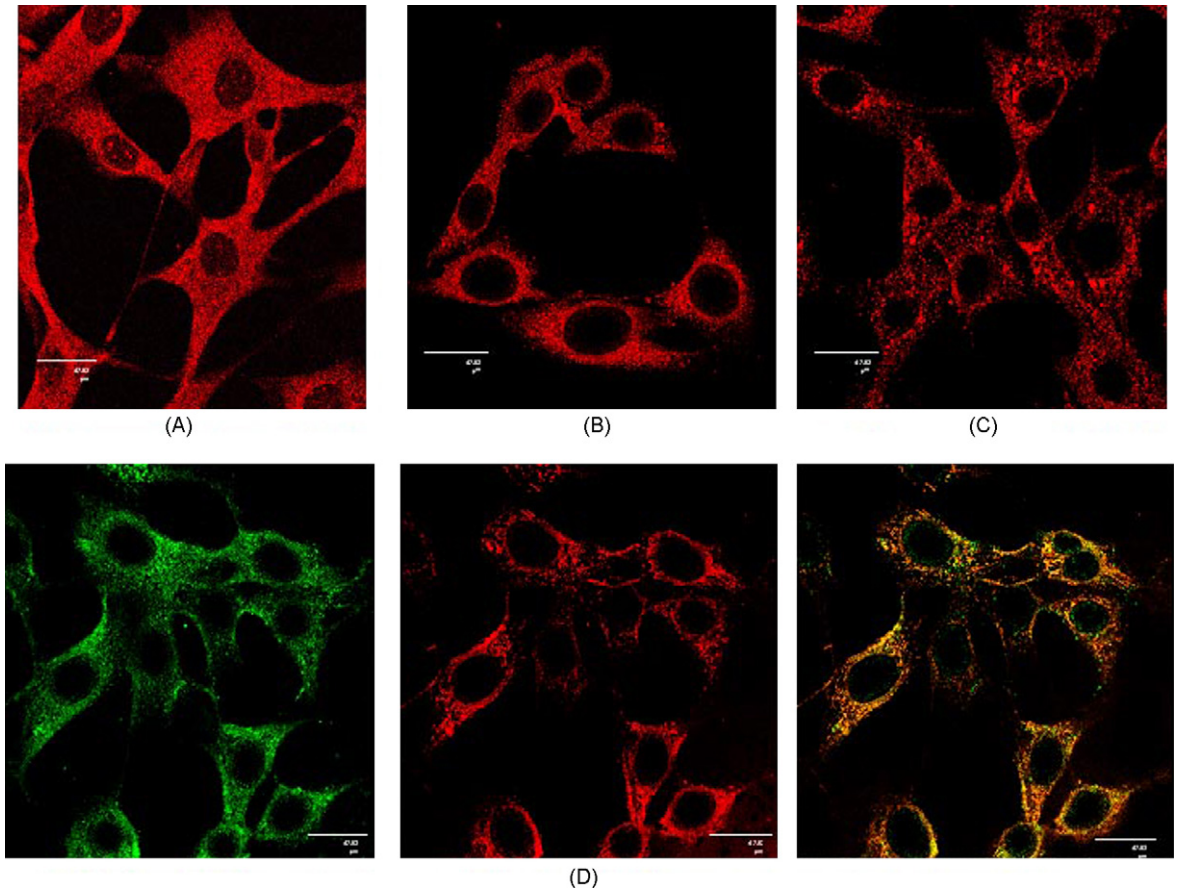

Fig. 6. Immunofluorescence micrographs of C2C12 muscle cells after staining for ER β antigen: confocal microscopy. C2C12 cells were labeled as described in Section 2, using polyclonal antibodies against ER β, epitope specific (A), Y19 (B) L20 (C) and H-150 (D). ER β specific green fluorescence was bright in mitochondrial cell compartments. Staining of mitochondria with the specific marker MitoTracker Red rendered a red signal (D, center) and yellow for the merged image of ER β and mitochondria (D, right). No ER β specific staining was seen when primary antibodies were omitted (data not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

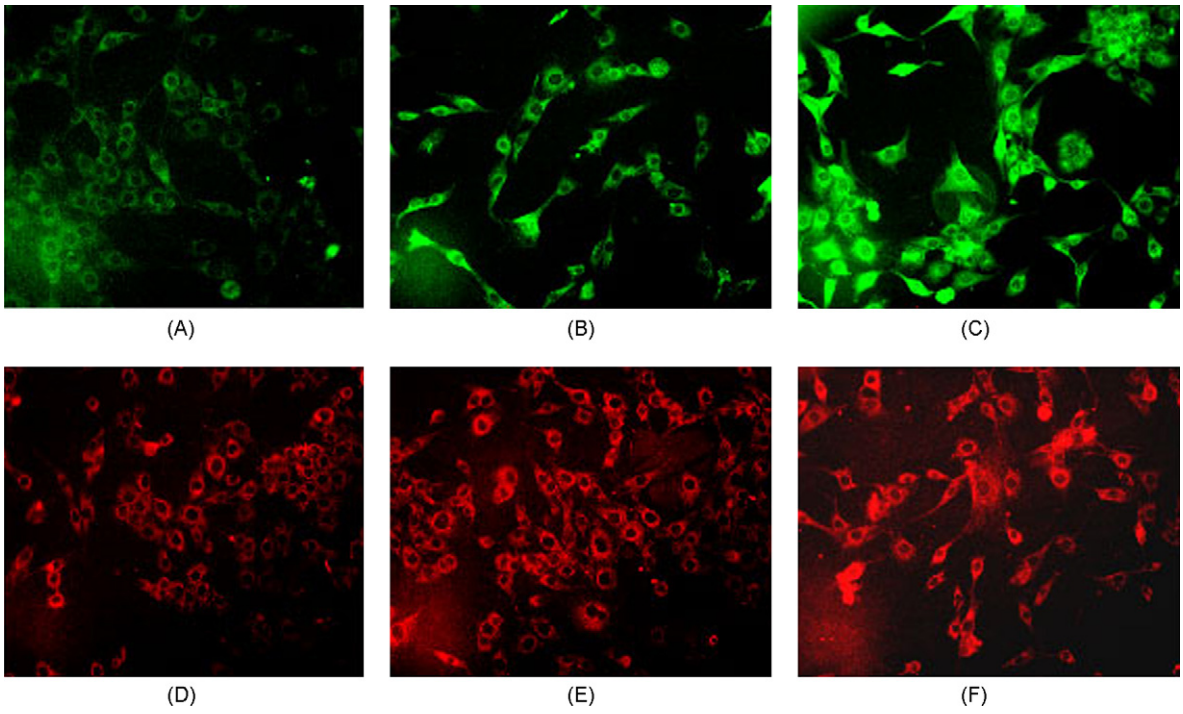

Fig. 7. Localization of ERs after 17β-estradiol treatment of C2C12 muscle cells. C2C12 cells were treated with 1 nM E2 during 15 min (B and E) or 30 min (C and F) or with vehicle for controls (A and D). ER α (*upper panel*) and ER β (*lower panel*) subcellular localization was analyzed by indirect immunofluorescence and conventional microscopy in methanol fixed and permeabilized cells using TE111.5D11 or Y19 antibodies, respectively, as described in Section 2. Original magnification: 200×.

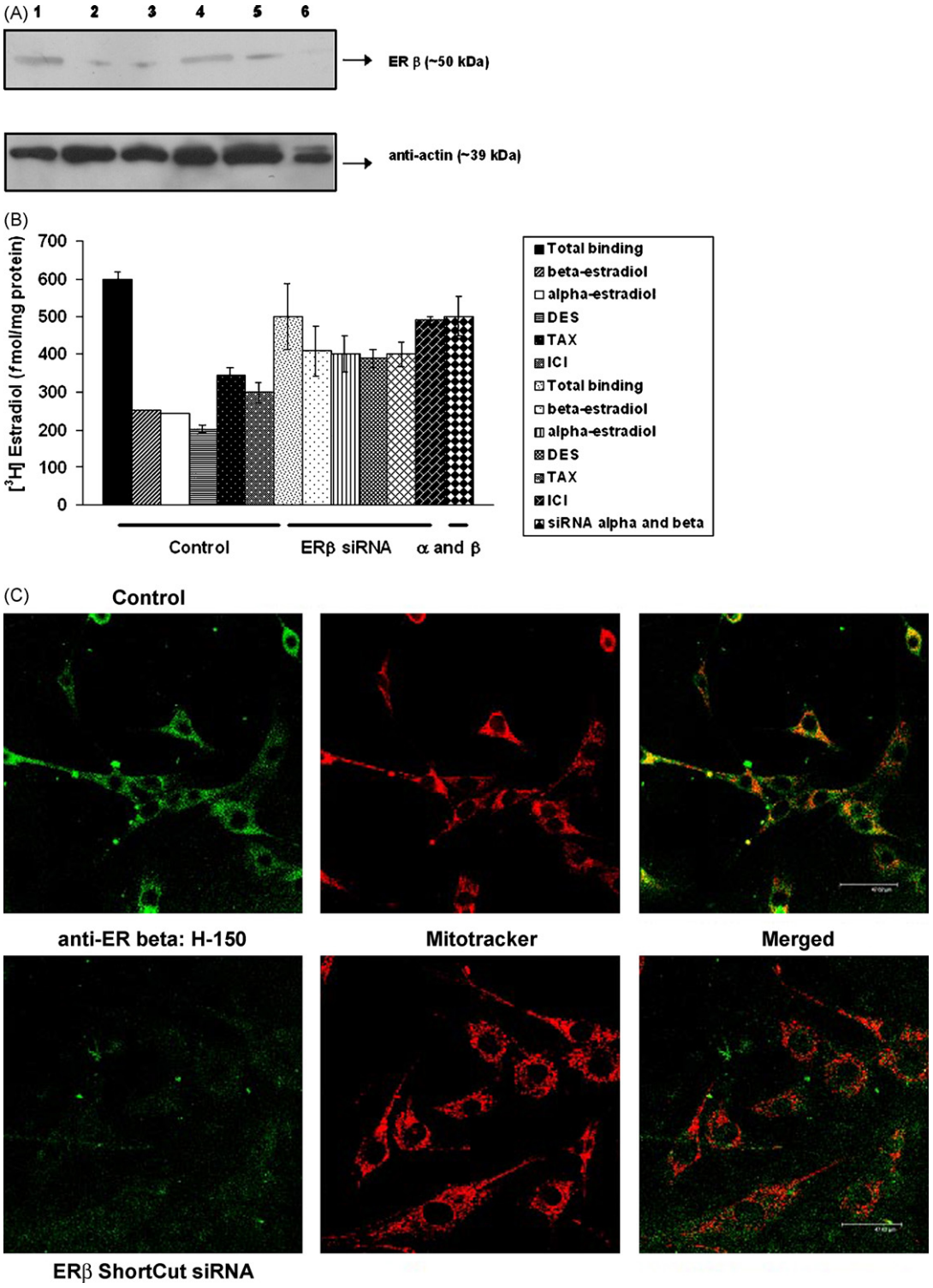

Fig. 8. Effects of ER β silencing by siRNA in C2C12 muscle cells. (A) Blockage of ER β expression. C2C12 cells were transiently transfected with two concentrations of ER β ShortCut® siRNA. Expression of ER β was analyzed 24 h (lanes 1, 2 and 3) and 48 h post-transfection (lanes 4, 5 and 6). *Upper panel*: Western blot of total cell lysates employing ER β rabbit polyclonal antibody, H-150. Lanes 1 and 4: negative controls; lanes 2 and 5: cells transfected with 10 pmol of siRNA; lanes 3 and 6: cells transfected with 20 pmol of siRNA. *Lower panel*: Actin loading control was detected using anti-actin polyclonal antibody. A representative blot from two independent experiments is shown. (B) [^{3}H]17β-estradiol binding properties of whole cells in culture. Living cells were transiently transfected with 20 pmol of ER β siRNA as described in Methods, and then incubated with 5 nM [^{3}H]17β-estradiol alone (total binding) or in combination with a 200-fold molar excess of E2, 17α-estradiol, DES, tamoxifen (TAX) or $ICI_{182,780}$ (non-specific binding). A mix of 20 pmol of ER α and ER β siRNAs was employed to evaluate the effects of double silencing on estradiol binding in competition assays. Control: cells transfected with Transfection Reagent without ER β ShortCut® siRNA. Results are expressed in fmol/mg protein and represent the mean of samples analyzed in duplicate ± S.D. (C) Immunofluorescence confocal microscopy of cells stained for ER β antigen. C2C12 cells were transfected with 20 pmol of ER β siRNA, incubated 24 h post-transfection and then fixed and labeled as described for immunocytochemistry (Section 2). *Upper panel*: negative control; left: shows ER β specific green fluorescence in mitochondria and perinuclear compartments of the cells; center: mitochondrial localization of C2C12 cells with the mitochondrial marker MitoTracker Red rendered a red signal; right: merged image of ER β green fluorescence and mitochondria red fluorescence. *Lower panel*: cells transiently transfected with ER β siRNA; left: weak ER β staining is detected in transfected cells; center: mitochondrial localization with MitoTracker Red; right: merged image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(TE111.5D11 or Y19, respectively). No translocation into the nucleus of green (ER α) or red (ER β) signals were detected (Fig. 7C and F).

Competitive binding assays and immunocytochemistry employing cells transiently transfected with a specific ER β siRNA were performed in order to confirm the identity of functional and structural relationships of the classical ER β and the endogenous ER β detected in C2C12 skeletal muscle cells. To estimate the transfection efficiency of siRNA, fluorescein-labeled siRNA was used as a positive control. High transfection values were obtained with this probe (~70% after 24 h). To corroborate the efficacy of ER β silencing with ER β siRNA, two different amounts of siRNA and transfection times were evaluated. Effective silencing of ER β was detected by Western blot analysis after 24 h and 48 h post-transfection with 10 and 20 pmol of siRNA (Fig. 8A). Fig. 8B shows competitive binding assays performed in C2C12 whole cells transfected for 24 h with ER β siRNA (20 pmol), using [^{3}H]-17β-estradiol as ligand and an excess of cold E2, 17α-estradiol, DES, tamoxifen (TAX) or $ICI_{182,780}$ as competitors. Specific binding was significantly reduced by transfection in comparison to non-transfected controls. Also, cells transfected with ER β siRNA alone or ER α and β siRNAs together, conserved some specific binding activity that can be attributed to ER α and ER α-like binding proteins of low molecular weight, respectively. Finally, ER β immunoreactivity was evaluated by confocal microscopy analysis employing H-150 polyclonal antibody, in C2C12 cells transiently transfected with ER β siRNA. Fig. 8C (lower panel) shows representative micrographs of C2C12 cells 24 h post-transfection and their respective negative controls (upper panel). Transfected cells with ER β siRNA revealed a high decrease in ER β immunostaining (lower panel, left), compared with the negative controls (upper panel, left).

Coupled RT-PCR was performed with total RNA extracted from the C2C12 cells. The chosen primer pair (Table 1) amplifies a 440 bp fragment from ER β mRNA. As shown in Fig. 9, a single band of the expected fragment size was obtained. Sequence analysis of the PCR product showed high sequence homology with the mouse ER β on a Blast search of the sequence database (data not shown).

These results confirm the structural relationship of endogenous C2C12 ER β with the classical and well-known ER β, as well as the subcellular localization of the immunoreactive protein in this cell line.

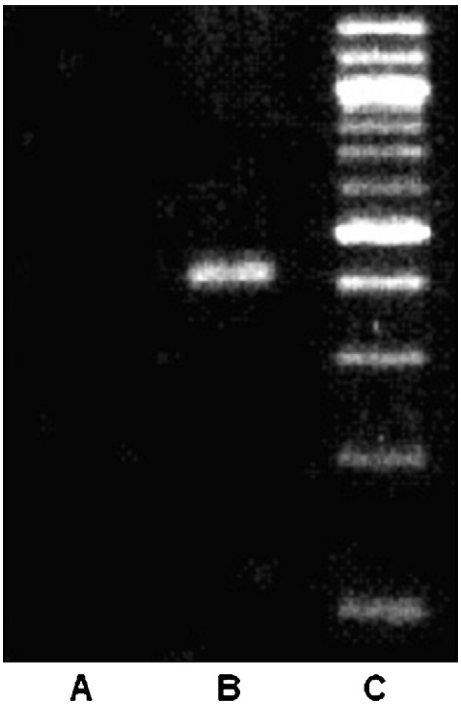

Fig. 9. Expression of ER β in C2C12 muscle cells. Total RNA was extracted from cultured C2C12 cells. RNA samples were subjected to RT and subsequently to PCR using a primer pair specifically designed to the mouse ER β gene (Table 1). The reaction product was analyzed on agarose gel and stained with ethidium bromide. (A) Negative control. (B) PCR product. (C) DNA ladder (100 bp). The size of the ER β PCR product was ~440 bp.

4. Discussion

It is well known that mitochondria play a major role in apoptosis triggered by many stimuli [30]. We have previously reported, using the C2C12 cell line, that E2 protects skeletal muscle cells against apoptosis through a mechanism involving both the α and β isoforms of the estrogen receptor (ER). Interestingly, a marked protection of mitochondria by the hormone which was mediated mainly by ER β was observed [26]. This study provides relevant information on the subcellular localization of ER β which contributes to understand the key role of this estradiol receptor isoform protecting muscle cells at the mitochondrial level.

We have recently shown, by means of conventional radioligand assays, the presence and subcellular localization of specific E2 binding sites in C2C12 murine skeletal muscle cells. Moreover, high affinity mitochondrial binding sites were identified by Scatchard analysis [25]. The use of different estrogen agonists and antagonists allowed us to characterize the estrogen binding entities as ER-like. Various lines of biochemical, immunological and molecular evidence supported the localization of ER α in microsomes/perinuclear region and to a lesser extent in mitochondria of C2C12 muscle cells [25].

In the present work, immunoblocking of [^{3}H]-17β-estradiol binding by C2C12 cells using a highly specific ER β isoform antibody established the presence of estrogen binding ER β entities in this muscle cell line. In keeping with this observation, treatment with the same anti-ER β antibody, as transfecting with ER β siRNA, blocks the inhibition of cytochrome *c* release by E2 [26].

Ligand blot analysis of C2C12 subcellular fractions revealed that the ER β-like specific binding detected in whole cells in culture was mainly localized in mitochondria and to a lesser extent in cytosol. This is in agreement with previous studies in which an important content of ERs was detected in mitochondrial preparations [14–24, 31 and references therein, 32 and references therein].

Determination of specific markers lamin B and cytochrome *c* oxidase activity excluded cross-contamination with nuclei or mitochondria, respectively, of other subfractions, making unlikely the possibility that artifacts due to homogenization or cell fractionation procedures might have altered significantly the native distribution of ER β in C2C12 muscle cells.

Western blot analysis of subcellular fractions derived from C2C12 cells and murine skeletal muscle showed high expression levels of ER β immunoreactivity in mitochondria and lower amounts in the cytosolic compartment, in accordance with the ligand blot data.

In agreement with the above findings, immunocytochemical studies by conventional and confocal microscopy confirmed the mitochondrial localization of ER β immunoreactive entities in the C2C12 cell line. Furthermore, the subcellular location of ER β was not modified by ligand (E2) pretreatment of C2C12 cells, similarly to ER α [25], different to other cell lines, which undergo rapid reorganization of perinuclear ERs, especially ER α into the nucleus, in response to E2 [17]. However, the fact that ER immunoreactivity did not translocate into the nucleus of C2C12 muscle cells during hormonal treatments might be evidence reenforcing the extra-nuclear location established for the ERs.

Competitive binding assays and immunocytochemistry approaches employing C2C12 cells transiently transfected with a

specific ER β siRNA mix strengthened the functional and structural relationships between the classical known ER β and the endogenous ER β detected in C2C12 muscle cells, in agreement with the results of RT-PCR analysis showing high homology with mouse ER β. In addition, we could verify the non-classical localization of the receptor.

Our data establishing ER β localization in mitochondria suggest that the antiapoptotic action of estrogens at the mitochondrial level could be consequence of a direct action of the hormone on the organelle, in accordance with the fact that mitochondrial genes contain ERE-like sequences [33].

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