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Non-classical localization of androgen receptor in the C2C12 skeletal muscle cell line

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ABSTRACT

The classical model of testosterone action has been traditionally described as being mediated by the androgen receptor (AR) localized exclusively in the nucleus. However, there is increasing functional evidence for extranuclear localization of AR. We present biochemical and immunological data supporting mitochondrial and microsomal localization of AR in the C2C12 skeletal muscle cell line. As a first approach AR was detected by immunoblotting, using specific antibodies after subcellular fractionation, not only in nucleus and cytosol, but also in mitochondria and microsomes. We then established [^{3}H] testosterone binding characteristics in total homogenates and subcellular fractions. Specific and saturable [^{3}H] testosterone binding sites were detected in mitochondria and microsomes. Immunolocalization of the non-classical AR was also confirmed using confocal microscopy. Sucrose gradient fractionation demonstrated the presence of the AR in lipid *rafts* and caveolae. Besides, the AR interacts physically with Caveolin-1, association that is lost after testosterone treatment. Accordingly, Western blot analysis revealed a decrease of AR expression in the microsomal fraction after testosterone treatment, suggesting translocation of the membrane AR to another subcellular compartment. The non-classical distribution of native pools of AR in skeletal muscle cells suggests an alternative mode of AR localization/function.

Introduction

Many physiological actions of androgens are mediated by the androgen receptor (AR[1]), a member of the nuclear hormone receptor superfamily, which consists of a large group of ligand-regulated transcription factors [1]. The principal physiological androgens, testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT), predominantly mediate their biological effects through binding to the AR. Once AR binds to its ligand, the steroid-receptor complex translocates to the nucleus, where modulation of gene target expression over a period of hours occurs [2–6]. In addition to this steroid hormone transcriptional or genomic mode of action, an increasing body of evidence suggests that androgens can exert rapid (occurring in minutes) non-genomic effects that may also have important biological effects in many tissues [7–10], mediated by the rapid induction of second messenger signal transduction cascades, including the increase in free intracellular calcium, and the activation of protein kinase A (PKA), protein kinase C (PKC), and MAPK. Unlike the classical mechanism of action of the androgens, these events are not abolished by inhibition of transcription and can be activated in some cell and tissue types using macromolecular derivatives of the hormone, not permeable to the plasma membrane, which would indicate the presence of membrane entities which are capable of binding the hormone [11]. The molecular mechanisms underlying these non-genomic actions are poorly understood.

Scientific evidence accumulated in recent years, points to the existence of membrane androgen receptors (mAR), triggering these rapid, non-transcriptional signals. Although, the exact molecular identity of mAR still remains unknown, non-genomic androgen actions manifested within minutes have been reported in various cell types including macrophages and T cells [12,13], LNCaP [14,15], T47D [16], MCF7 [17], DU145 [18–21], C6 [22], PC12 [23] or VSMC cells [24]. The mAR-dependent signaling mechanism was recently characterized in detail in prostate and breast cancer cell lines [21,25–27]. Furthermore, most recent studies have implicated key pro-survival and pro-apoptotic gene products such as Akt, NF-κB, Bad, Fas and caspase-3 in the regulation of the apoptotic response induced by mAR activation in prostate cancer cells [20].

Extranuclear organelles have been described as containing sex steroid receptors. The presence of estrogen and androgen receptors in mitochondria of mammalian cells including skeletal muscle has been documented [28–33].

We have recently demonstrated that testosterone protects against H_2O_2-induced apoptosis in the C2C12 murine skeletal

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[1] *Abbreviations used:* AR, androgen receptor; DHT, 5α-dihydrotestosterone; PKA, protein kinase A; PKC, protein kinase C; mAR, membrane androgen receptors; PAS, protein a sepharose; BSA, bovine serum albumin; BMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sulfate–polyacrylamide gel electrophoresis; MTT, Mitotracker; DMSO, dimethyl sulfoxide.

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muscle cell line, event in which the AR has an active participation [34]. The role of the AR during the anti-apoptotic effect of the androgen might be exerted at different levels: nuclear (in the genomic response), mitochondrial (in the intrinsic pathway) and microsomal (in the response mediated by membrane proteins). Then, the aim of the present work was to identify non-classical localization of the AR in mitochondria and plasma membrane microdomains (caveolae and *rafts*), from where the receptor could mediate the protective effect of testosterone against apoptosis in C2C12 cells.

Materials and methods

Materials

Anti-AR (C-19), anti Lamin B (C-20) and anti-Caveolin-1 (4H312 and N-20) polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Smac/DIABLO monoclonal antibody was obtained from Cell Signaling Technology Inc. (Danvers, MA 01923, USA). MitoTracker (MitoTracker® Red CMXRos) dye, Alexa Fluor 488-conjugated anti-rabbit (A11008) and Alexa Fluor 568-conjugated anti-mouse (A11031) secondary antibodies were from Molecular Probes (Eugene, OR, USA). Testosterone [1,2,6,7-^{3}H(N)] with a specific activity of 70 Ci/mmol was obtained from Perkin Elmer (Boston, MA, USA). Testosterone (T-1500), DHT, 17β-estradiol (E2), progesterone and Protein A Sepharose (PAS) were purchased from Sigma–Aldrich (St Louis, MO, USA). All the other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal muscle cells, from the American Type Culture Collection (ATCC number: CRL-1772™) at Manassas, VA 20108, were cultured in growth medium (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum, 1% nystatin, and 2% streptomycin. Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium [34–36]. Under these conditions, C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate participating in the repair of the tissue when a cellular injury exists [37]. The treatments were performed with 70–80% confluent cultures (120.000 cells/cm^2) in medium without serum containing 10^{-9} M testosterone [34,38,39] or the hormone vehicle, isopropanol, during 60 min. The isopropanol percentage in the culture medium assay, of cells treated with the hormone or the hormone vehicle alone, was less than 0.001%. After treatments, cells were lysed using a buffer composed of 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 20 μg/ml trypsin inhibitor. Total cell lysates were collected by aspiration and centrifuged at 12,000×*g* during 15 min to separate the particulate fraction from the soluble fraction. Protein concentration from the supernatant was estimated by the method of Bradford [40], using bovine serum albumin (BSA) as standard. Cells were cultured in chamber slides for microscopy or in 10 cm plates (Greiner Bio-One, Frickenhausen, Germany) for Western blots.

Subcellular Fractionation

C2C12 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. The *debris* was separated by centrifugation at 100×*g* for 5 min. The upper fraction was collected and a nuclear pellet was obtained by low speed centrifugation (300×*g*, 20 min). The supernatant was further centrifuged at 10,000×*g* for 20 min to pellet mitochondria. The remaining supernatant was centrifuged at 120,000×*g* for 60 min, to yield the cytosolic fraction and a plasma membrane-containing particulate pellet (microsomes). Aliquots of the different fractions were frozen in liquid nitrogen and stored in TES buffer at −70 °C until measurement of testosterone-binding activity and Western blot analysis. Protein concentration from each fraction was estimated by the Bradford method as above. Contamination of nuclear, microsomal and cytosolic fractions with mitochondrial components was assessed by immunodetection of the specific mitochondria marker Smac/DIABLO. Anti-Lamin B antibody was employed for immunodetection of the nuclear marker Lamin B in the different fractions.

Lipid raft fractionation

The purification of lipid *rafts* and caveolae was carried out by sucrose gradient analysis of C2C12 lysates performed according to a modification of previously published protocols [41]. Briefly, cells were grown into confluency in 10 cm dishes and eight dishes were used for each gradient. Monolayers were then rinsed in PBS and lysed with buffer Hepes without glycerol (50 mM Hepes, 300 mM KCl Tris, 0.1 mM EDTA, 1 mM PMSF, 0.1% NP-40, 1 mM leupeptin, 0.5/ml aprotinin). Lysates were scraped from the dishes (2 ml), brought to 80% sucrose in Hepes buffer (2 ml), and placed at the bottom of a 12 ml centrifuge tube. A step sucrose gradient (5–30% in Hepes, 4 ml each step) was layered on the top of the lysates, and the samples were centrifuged at 120,000×*g* for 18–20 h at 4 °C in a Beckman (Fullerton, CA, USA) SW41 rotor. 1 ml fractions were collected from the top of the gradient and centrifuged at 120,000x*g* for 1 h to remove sucrose content and concentrate the sample. Finally, the pellets obtained were solubilized in 30 μl of lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 20 μg/ml leupeptin and 20 μg/ml aprotinin) before subjecting to Western blot analysis with specific antibodies against either AR or Caveolin-1.

Western blot analysis

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 membranes (PVDF, 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 overnight 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 (Amersham, Buckinghamshire, England) 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 (Amersham). Relative quantification of Western blot signals was performed using ImageJ software (NIH, USA).

Immunocytochemistry

Semi-confluent (60–70%) monolayers were washed with serum-free phenol red-free DMEM, incubated 1 h in the same medium and then fixed and permeabilized during 20 min at −20 °C

with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 min in PBS that contained 5% bovine serum albumin. Cells were then incubated overnight at 4 °C, in the presence or absence (negative control) of primary antibodies (anti-AR and anti-Caveolin-1; 1:50 dilution). The primary antibodies were recognized by fluorophore-conjugated secondary antibodies.

Mitotracker (MTT) was employed for selective stain of active mitochondria. Coverslips with adherent cells were stained with MTT, which was prepared in dimethyl sulfoxide (DMSO) and then added to the cell culture medium at a final concentration of 1 μmol/l. After 15 to 30 min of incubation at 37 °C, the cells were washed with PBS and fixed with methanol at −20 °C for 30 min.

Finally, the coverslips were analyzed by confocal microscopy.

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. A 50 mm pinhole was generally used. Cells were imaged through a 63X, 1.3 numerical aperture water immersion objective. Images were collected and saved using the software Meridian and exported to Adobe PhotoShop for digital processing.

[^{3}H] Testosterone binding assays

For whole-cell binding assays, C2C12 cells were cultured until confluence in serum-supplemented media. Cells were washed with serum-free phenol red-free DMEM and pre-incubated in the same media for 30 min at 37 °C. For cell-free binding assays, the total specific androgen binding capacity (empty plus occupied AR site content) of the subcellular fractions was determined by incubating 0.2 mg protein samples in 0.1 ml of TES buffer. The reactions were begun by the addition of 10 nM [^{3}H] testosterone alone (total binding). 10 nM [^{3}H] testosterone and a 1000-fold molar excess of radioinert testosterone were used for determination of non-specific binding. After 16 h of incubation at 4 °C, free [^{3}H] testosterone was separated by carbon/dextran technique [42]. Briefly, 100 μl of carbon/dextran 0.5% in TE buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 2 mM DTT, 0.3 mM PMSF) were added to each tube and the suspension was incubated for 10 min at 4 °C. Then, the mixture was centrifuged for 3 min at 800xg to pellet the charcoal and the supernatant was collected and subjected again to the carbon/dextran technique twice. Trapped radioactivity of the last supernatant was quantified in toluene-based fluid by liquid scintillation spectrometry. Specific testosterone binding sites were determined by subtracting the radioactivity bound in presence of [^{3}H] testosterone alone (total binding) and that retained by the non-specific tubes (non-specific binding).

For saturation analysis, samples of the mitochondrial and microsomal fractions (140 μg) were exposed to a series of [^{3}H] testosterone concentrations ranging from 2 to 80 nM, in a final volume of 0.1 ml. The steroid affinity constant (K_d) and the maximum number of binding sites (B_{max}) were estimated according to the Scatchard equation using the nonlinear curve fitting LIGAND program [43].

For displacement binding experiments, total homogenates at a final protein concentration of 120 μg/0.1 ml were incubated with 10 nM [^{3}H] testosterone in the absence or in the presence of different concentrations of unlabeled steroids (testosterone, DHT, E2 and progesterone), ranging from 10^{-12} to 10^{-6} M. After 16 h of incubation at 4 °C, [^{3}H] testosterone was separated by carbon/dextran technique as described previously, and radioactivity was quantified in toluene-based fluid by liquid scintillation spectrometry.

Coimmunoprecipitation

Microsomal fractions from the C2C12 cell line containing 60 μg of protein were immunoprecipitated with 10 μl of a 50% suspension of protein A-agarose after incubating the extracts with 2 μl of the primary antibody indicated in each experiment. The immunoprecipitates were washed three times with buffer Hepes (50 mM Hepes, 10% glicerol, 300 mM KCl Tris, 0.1 mM EDTA, 1 mM PMSF, 0.1% NP-40, 1 mM leupeptin, 0.5/ml aprotinin and 10 μg/ml of trypsin inhibitor). The final pellets were obtained by centrifugation for 3 min at 10,000×*g*, resuspended then in electrophoresis sample buffer without dithiothreitol. Fractionated proteins were electrotransferred to PVDF membranes and then blocked for 1 h with 5% non-fat dry milk in PBS-T. The blots were incubated overnight at 4 °C with primary antibody against the protein of interest. After several washings with PBS-T, the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were developed by means of enhanced chemiluminescence. The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

Statistical analysis

Results are shown as means ± S.E.M. Statistical differences among groups were determined by ANOVA followed by the Duncan test, a multiple comparison *post hoc* test. Data were considered significant at $p < 0.05$.

Results

Androgen receptor expression in the different subcellular fractions of C2C12 muscle cells

As a first approach to the analysis of the subcellular localization of the AR in muscle cells, total homogenates and subcellular fractions of the C2C12 cell line were subjected to Western blot analysis employing the specific antibody against the AR (Fig. 1(A)). We detected not only the well-studied 110-kDa AR protein but also some lower molecular-weight bands. We predicted that these lower molecular-weight bands could be AR splicing products (indicated by arrows in Fig. 1(A)), as the same banding pattern was always detected in independent assays. Besides, the different splicing variants of the classical steroid receptor are not only expressed in nucleus and cytosol, but also located in mitochondrial and microsomal fractions.

In order to evaluate the functional properties of these non classical ARs and to check if they actually are entities capable of binding androgens, we performed competitive binding assays in C2C12 subcellular fractions, employing 10 nM [^{3}H] testosterone and a 1000-fold molar excess of the cold steroid as competitor. As shown in Fig. 1(B), the AR-like androgen binding sites were predominantly detected in nucleus but appreciable specific binding sites were also noticed in mitochondrial and microsomal fractions. These results show not only an AR-like immunoreactive entity with non classical location in mitochondria and microsomes, but also endowed with the functional property of binding testosterone.

Western blot analysis employing the nuclear marker Lamin B revealed no nuclear contamination in the other subcellular fractions. We also excluded mitochondrial contamination in nuclear, microsomal and cytosolic fractions by measuring Smac/DIABLO expression (Fig. 1(C)).

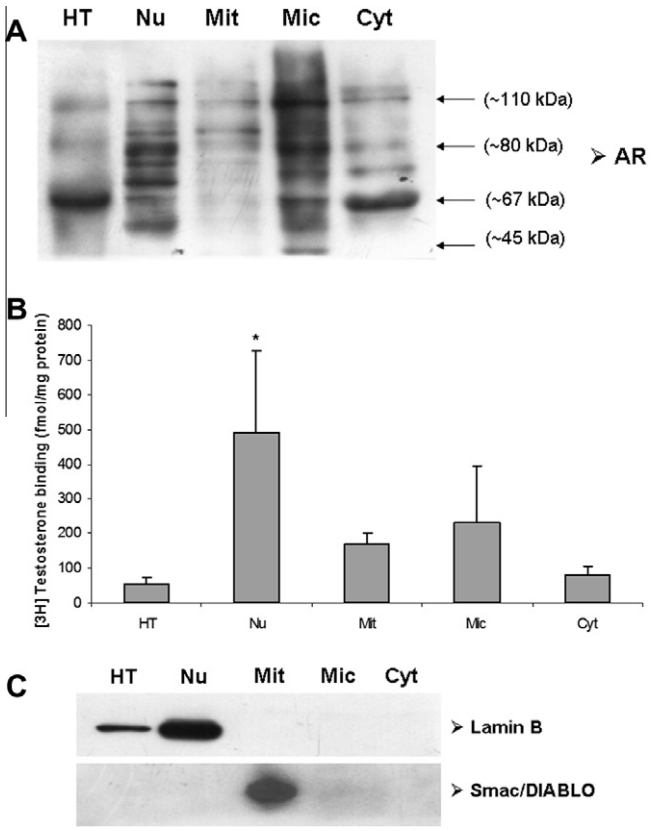

Fig. 1. Subcellular distribution of functional and immunoreactive AR proteins in C2C12 muscle cells. (A) Detection and distribution of the ~110 kDa wild type AR and low molecular weight splicing variants of the AR. Equal protein samples (25 μg) of total C2C12 homogenates (HT), nuclear (Nu), mitochondrial (Mit), microsomal (Mic), and cytosolic (Cyt) subfractions obtained as described in Materials and Methods, were subjected to Western blot analysis using anti-AR antibody. The blot is representative of three independent experiments with comparable results. (B) [^{3}H] testosterone binding capacity of subcellular fractions from C2C12 muscle cells. Equal protein samples (200 μg) of total C2C12 homogenates (HT), nuclear (Nu), mitochondrial (Mit), microsomal (Mic), and cytosolic (Cyt) subfractions were incubated during 16 h in presence of 10 nM [^{3}H] testosterone alone or in combination with a 1000-fold molar excess of testosterone. Total specific binding was calculated as described in Methods. Averages ±S.E.M. are given; $^*p < 0.05$ respect to HT. It may be observed that AR localizes in mitochondria and microsomes keeping its capability of binding testosterone. C) Assessment of purity of subcellular fractions isolated from C2C12 muscle cells. Equal protein samples (20 μg) of total C2C12 homogenates (HT), nuclear (Nu), mitochondrial (Mit), microsomal (Mic), and cytosolic (Cyt) subfractions obtained as described in Materials and Methods, were subjected to Western blot analysis using anti-Lamin B as nuclear marker and anti-Smac/DIABLO as mitochondrial marker.

The androgen receptor is located in lipid rafts and caveolae of C2C12 muscle cells

To explore the idea of the differential AR partition into membrane fractions within the muscle cell, we carried out discontinuous sucrose density gradient fractionation, as described in Methods, using total C2C12 cell line homogenates. This technique has been used to isolate membrane subdomains that have distinct biochemical composition or properties, such as lipid *rafts* and caveolae. They represent a subcompartment or microdomain of the plasma membrane and are most abundant in certain cell types, including fibroblasts, adipocytes, endothelial cells, type I pneumocytes, epithelial cells, and smooth muscle cells [44,45]. We isolated Caveolin-rich domains by a detergent-free procedure and sucrose gradient flotation centrifugation. An aliquot of each fraction of the gradient was subjected to immunoblot analysis (Fig. 2), using the specific anti-AR and anti-Caveolin-1 antibodies. Interestingly, AR was mostly found in low-density plasma membrane fragments (fractions 3–5). These fractions are composed of lipid *rafts* and caveolae, as confirmed by the presence of Caveolin-1, a 21–24-kDa protein and the principal component of caveolae, which serves as a marker protein for the plasma membrane microdomains (*rafts* and caveolae) [46]. Therefore, the non-classical localization of the AR observed by classical subcellular fractionation in microsomes, is specifically found in caveolae and *rafts*, confirmed by Western blot analysis of the sucrose density gradient fractions.

Immunofluorescence detection of ARs with non-classical localization in C2C12 muscle cells

In order to visualize the non-classical localization of the AR in mitochondria and microdomains detected by Western blot assays, immunocytochemical detection of AR was performed by confocal microscopy.

Mitochondrial AR location was investigated using a polyclonal antibody against the AR (green fluorescence) and Mitotracker (MTT) probe (red fluorescence) for mitochondria-selective staining. The images obtained by confocal microscopy presented in Fig. 3(A) revealed the presence of the AR in mitochondria, demonstrated by the mixture of fluorescences in yellow color (*merge*). Analysis of fluorescence intensity profiles monitored along the cells (white arrow in merge image) showed that the green fluorescence intensity profile matches up with the red intensity profile in many points of the graph, in accordance with the yellow signal observed.

Similar results were obtained when AR (green) and Caveolin-1 (red) antibodies were employed to label methanol fixed C2C12 muscle cells, in order to visualize the membrane AR (Fig. 3(B)). Although the yellow signal obtained from the *merge* of the images was less intense than that gotten for mitochondrial AR and the visualization of color yellow was hard to see, the intensity profiles recoded in green and red channels showed the colocalization of both signals in some points of the graph (indicated with white arrows). Although the intensity profiles do not follow exactly each other and sometimes oscillate in contraphase, we were able to detect some points where green and red signals matched up. The difficulty to see the yellow signal in membranes, was probably due to the huge amounts of Caveolin-1, the principal component of caveolae membranes, respect to AR, so that the green signal is dimmer than the red one and the overlap of colors still being seen as red.

Binding studies of mitochondrial and membrane ARs in C2C12 muscle cells

In an attempt to characterize C2C12 muscle cell mitochondria and membrane androgen binding sites, the association of [^{3}H] testosterone to isolated mitochondrial and microsomal preparations was analyzed in equilibrium binding experiments. As shown in Fig. 4(A) (upper panel), the specific binding of the hormone in mitochondria was a saturable process with respect to the ligand concentration, which ranged from 2 to 80 nM. Saturation data analyzed by linear Scatchard transformation revealed a single high affinity binding component for testosterone (K_d = 64.43 nM) with a maximum binding capacity (B_{max}) of 1052 fmol/mg protein (Fig. 4(A), lower panel). Membrane preparations incubated with [^{3}H] testosterone showed also specific saturable binding. Scatchard analysis of the results revealed a binding affinity for testosterone (K_d = 143.3 nM). The calculated B_{max} was 2496 fmol/mg protein (Fig. 4(B)).

Competitive binding assays were performed in order to confirm the androgen selectivity of the AR detected in C2C12 skeletal muscle cells. They were carried out in C2C12 cell total homogenates, using [^{3}H] testosterone as ligand, and the presence of varying

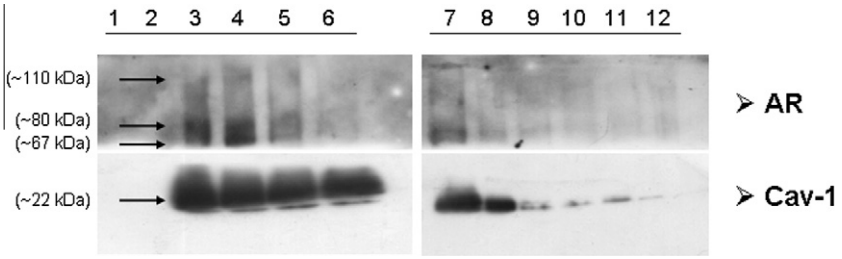

Fig. 2. Co-sedimentation of AR with Caveolin-rich caveolae membrane domain fractions from C2C12 muscle cells. C2C12 cells were lysed and subjected to equilibrium sucrose density gradient centrifugation. Twelve 1-ml fractions were collected from the gradients and processed as described in Methods. Equal protein samples (5 μg of protein) were resolved by SDS–PAGE followed by transblotting onto a PVDF membrane. Western blot analysis was performed with anti-Caveolin-1 and anti-AR antibodies. The blot is representative of three independent experiments with comparable results. The data revealed the presence of the AR in fraction 3–5 corresponding to low-density plasma membrane fragments, implying that the receptor is specifically located in lipid *raft* and caveolae.

concentrations of cold testosterone, DHT, E2 and progesterone (10^{-12}–10^{-6} M) as competitors. Fig. 4(C) revealed a displacement of radiolabeled testosterone by testosterone and DHT, while estradiol and progesterone did not displace the radiolabed hormone, confirming the androgen selectivity of the AR.

AR interacts with Caveolin-1 in microsomes of C2C12 muscle cells

In order to study whether the AR, previously detected in the lipid *rafts*, was interacting with the major component of the caveolae membrane structures, Caveolin-1, coimmunoprecipitation assays using anti-AR with the microsomal fraction from control and testosterone-treated (10^{-9} M, 60 min) C2C12 cells, were performed. The precipitates were then analyzed by Western blot analysis with the anti-Caveolin-1 antibody. As shown in Fig. 5, the anti-AR antibody immunoprecipitated AR associated to Caveolin-1, indicating the interaction between both proteins. When cells were treated with 10^{-9} M testosterone during 60 min, this interaction decreased significantly. The physical interaction between AR and Caveolin-1 was also observed when the coimmunoprecipitation assay was performed using the same antibodies in reverse order (data not shown). However, in this case, given that the much higher amounts of Caveolin-1 immunoprecipitated with the specific antibody, the difference between control and treated condition was undetectable by Western blot assay. Since we have previously demonstrated an active role of the AR in the protective action of testosterone against apoptosis [34], these results lead us to hypothesize on an active participation of a membrane AR. The membrane receptor is probably being induced by testosterone, to translocate to another cellular compartment in order to exert its protective action.

Testosterone treatment induces mAR translocation in C2C12 muscle cells

It has been well described the classical translocation of the inactivated AR. The receptor is localized in the cytoplasmic compartment as a multiprotein complex with heat-shock proteins and immunophilins. Ligand activation is followed by the dissociation from the multiprotein complex, recruitment of coactivators and translocation into the nuclei [47]. However, there is much less information known about the non-classical location of the mAR. Since we demonstrated that the localization of the AR in lipid *rafts* implies a physical interaction with Caveolin-1, and that this association decreases after testosterone treatment, we studied AR localization in the different subcellular fractions after androgen treatment, in order to elucidate where the AR is moving when it dissociates from Caveolin-1. When cells were treated with 10^{-9} M testosterone, a significant decrease in the levels of microsomal AR proteins of ~110 and ~80 kDa was observed respect to untreated cells (Fig. 6(A) and (B)). According to the results of the coimmunoprecipitation experiments, these mARs may lose their interaction with Caveolin-1 leaving the lipid *raft* to another subcellular compartment. In Fig. 6(C), the splicing variants of ~80 and ~45 kDa immunoreactive bands of the AR that decrease their levels in microsomes, after testosterone treatment, account for an increase in their expression observed in the nucleus.

Discussion

Several *in vitro* and *in vivo* reports demonstrate that the androgen-AR signaling pathway is required for skeletal muscle development and for sustaining muscle mass, strength and protein synthesis [48]. We have previously reported that testosterone protects skeletal muscle C2C12 cells against apoptosis through a mechanism involving intermediates of the apoptotic intrinsic pathway and the AR [34]. It is well known that mitochondria play a major role in apoptosis triggered by many stimuli [49]. Of relevance to our work, the presence of steroid hormone receptors in these organelles has been demonstrated in a variety of cells [28–33,50]. Also, recent lines of evidence support the existence of membrane AR involved in triggering rapid non-genomic signals [reviewed in 21].

In view of the above information, we considered of importance to ascertain whether the AR involved in the protective action of testosterone against apoptosis previously observed in C2C12 skeletal muscle cells [34] could exert its antiapoptotic role from different subcellular locations, involving both the intrinsic apoptotic pathway and responses mediated by membrane proteins or genomic actions. Thus, in a first approach to address this issue, in the present work we evaluated the intracellular distribution of the AR in C2C12 cells. Western blot assays of subcellular fractions allowed the immunodetection of a band of ~110 kDa, likely to correspond to the classical AR, in the total muscle cell homogenate and fractions derived therefrom, including mitochondria and microsomes. The AR has been demonstrated to be predominantly a nuclear receptor. However, increasing evidence points to the presence of extranuclear AR entities, structurally and functionally similar to the well-known ARs. Interestingly, additional immunoreactive bands were detected in all the subcellular fractions analyzed. The immunoreactive proteins obtained could be consequence of alternative usage of different in-frame initiations codons or splice variants of the full length AR transcript, as described in previous studies [33,51 and references therein].

Our findings also suggest the presence of functional androgen binding sites in classical and non-classical compartments. The identification and characterization of these androgen binding entities in total homogenate and subcellular fractions of the C2C12 skeletal muscle cell line was made by conventional competitive radioligand binding assays with [^{3}H] testosterone, which

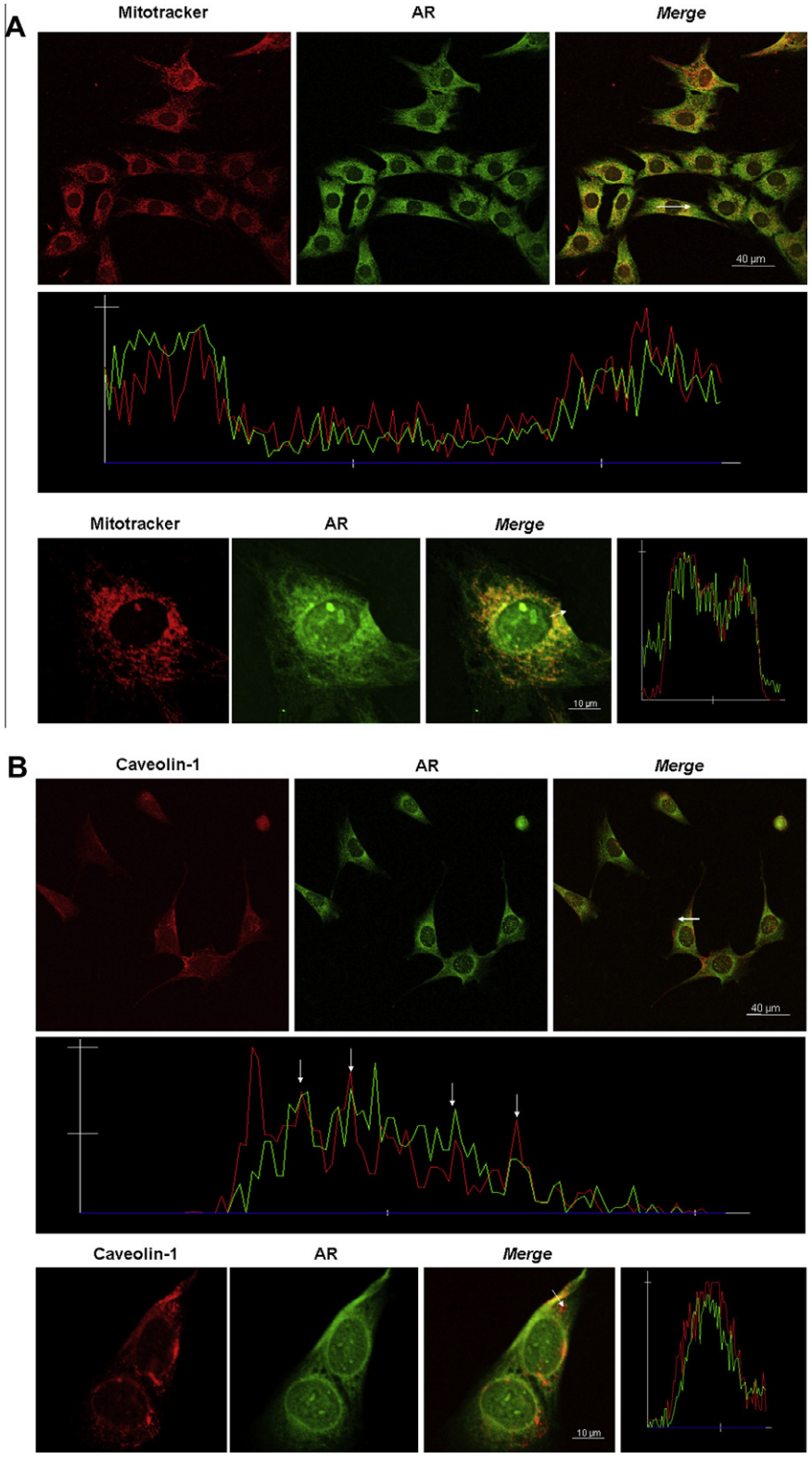

Fig. 3. Immunofluorescence localization of AR in mitochondria and membrane of C2C12 muscle cells. (A) Upper panel: Mitochondria of C2C12 cells were stained with Mitotracker (red fluorescence), and immunocytochemical assays were performed by confocal microscopy using anti-AR antibody (green fluorescence). Fluorescence intensities monitored along the cells (white arrows in merge images) are shown. At least ten fields per slide were examined. Representative photographs are shown. Magnification: 63X. **Lower panel:** Magnified image showing one C2C12 cell labeled with Mitotracker and anti-AR as mentioned above. (B) **Upper panel:** C2C12 cells were double labeled using polyclonal antibodies anti-AR (green fluorescence) and anti-Caveolin-1 (red fluorescence) as described in Materials and Methods. At least ten fields per slide were examined. Representative photographs are shown. Magnification: 63X. **Lower panel:** Magnified image showing two C2C12 cells labeled with anti-Caveolin-1 and anti-AR as mentioned above. Fluorescence intensity profiles are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

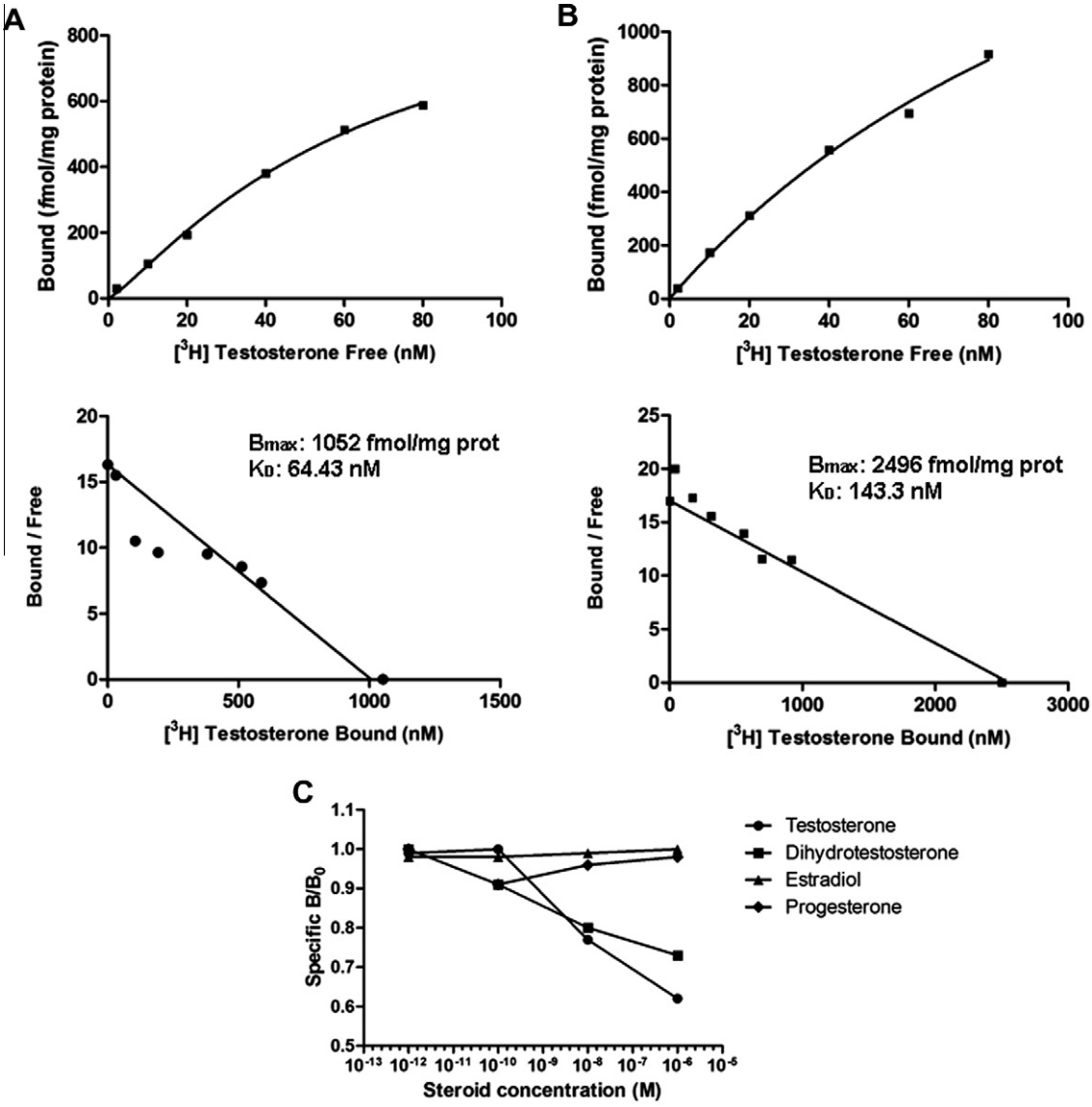

Fig. 4. Saturation analysis of [^{3}H] testosterone binding to mitochondrial and microsomal sites from C2C12 muscle cells. (A) Saturation binding assay in mitochondrial fraction. **Upper panel:** Mitochondrial protein fraction (140 μg) was incubated overnight at 4 °C in the presence of different concentrations of [^{3}H] testosterone, which varied from 2 to 80 nM. **Lower panel:** K_d and B_{max} values for the mitochondrial binding sites were determined from Scatchard plots, based on saturation bindings. Estimated binding parameters at equilibrium are indicated. (B) Saturation binding assay in microsomal fraction. **Upper panel:** Membrane proteins (140 μg) were incubated overnight at 4 °C in the presence of different concentrations of [^{3}H] testosterone, which varied from 2 to 80 nM. **Lower panel:** K_d and B_{max} values for the microsomal binding sites were determined from Scatchard plots, based on saturation bindings. Estimated binding parameters at equilibrium are indicated. (C) Representative curves of steroid competition for androgen binding in C2C12 cells. Total homogenates (120 μg) were incubated with 10 nM [^{3}H] testosterone alone (total binding, Bo) or in the presence of the indicated concentrations of unlabeled steroids (B: testosterone, DHT, estradiol, progesterone) ranging from 10^{-12} to 10^{-6} M.

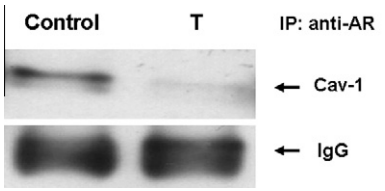

Fig. 5. AR interacts with Caveolin-1 in C2C12 muscle cells. Microsome lysates (60 μg protein) from C2C12 cells treated with vehicle, 0.001% isopropanol (C) or 10^{-9} M testosterone (T) during 60 min, were immunoprecipitated using anti-AR and immunoblotted with anti-Caveolin-1 antibody. The IgG levels are shown as loading control. The blot is representative of three independent experiments.

demonstrated specific binding activity in the different subcellular fractions, predominantly located in the nuclear pool ($^*p < 0.05$ respect to the total homogenate).

Moreover, the specific binding in mitochondrial and microsomal fractions isolated from C2C12 cells was a saturable process with respect to the ligand concentration. Scatchard linearization of the saturation binding data was consistent with a single set of affinity binding sites. Of importance, immunoblots with anti-Lamin B antibody excluded the possibility of nuclear contamination of the particulate preparations. Also Smac/DIABLO immunodetection demonstrated no cross-contamination with mitochondrial components in the other subfractions.

Thus, the AR specific binding sites, detected in total homogenates, were present not only in cytosol and nucleus but also in association with other particulate subfractions. This is in agreement with studies where an appreciable content of ARs was detected in mitochondrial and microsomal preparations in different cell types [12–24,33,52].

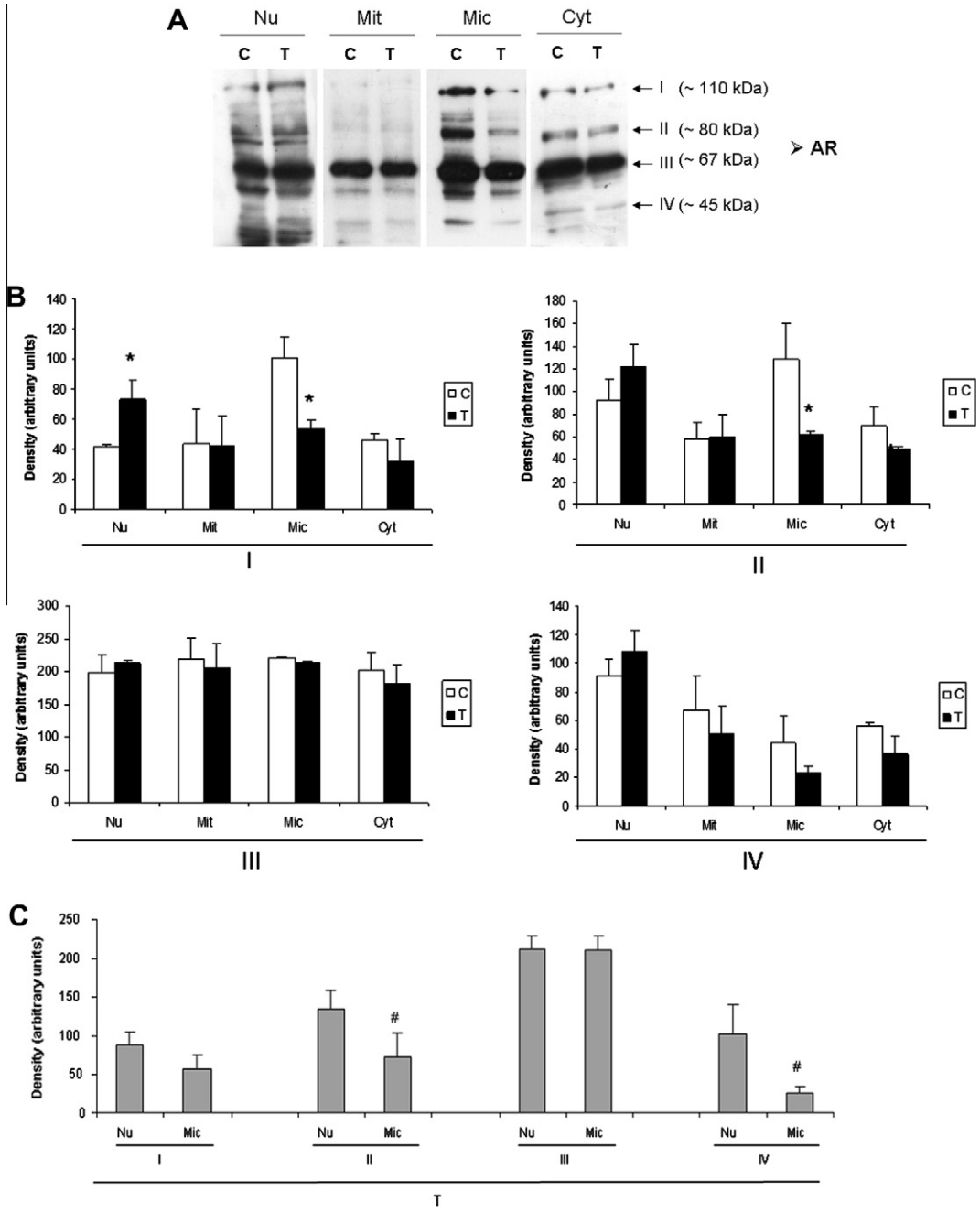

Fig. 6. Subcellular expression of AR after testosterone treatment in C2C12 muscle cells. (A) C2C12 cells were incubated with 0.001% isopropanol (C) or 10^{-9} M testosterone (T) during 60 min. Then, equal protein samples of nuclear (Nu), mitochondrial (Mit), microsomal (Mic), and cytosolic (Cyt) subfractions obtained as described in Methods were subjected to Western blot analysis using anti-AR antibody. The blot is representative of three independent experiments with comparable results. (B) Densitometric quantification of the AR wild type (I) and the splice variants (II, III, IV) in the different subcellular fractions are shown. Averages ±S.E.M. are given; $^{*}p < 0.05$ respect to untreated cells. (C) Densitometric quantification of the AR wild type (I) and the splice variants (II, III, IV) in nucleus and microsomes after testosterone treatment are shown. Averages ±S.E.M. are given; $^{\#}p < 0.05$ respect to the nucleus counterpart band.

Displacement studies using the classical androgen steroids testosterone and DHT and the steroid hormones E2 and progesterone, confirmed the specificity of the muscle intracellular AR binding sites. The androgen binding sites detected in total C2C12 cell lysates showed affinity and specificity for androgen steroid competitors, as indicated by the displacement of radiolabeled testosterone by testosterone and DHT, but not by estradiol and progesterone.

In agreement with the observed non classical subcellular distribution of AR binding entities, immunocytochemical assays with confocal microscopy by staining the cells with anti-AR antibody and Mitotracker red (MTT) or anti-Caveolin-1 antibody, confirmed the presence of immunoreactive AR entities in mitochondria and microsomes, respectively. Although the cell membrane AR signal was more difficult to see in confocal microscopy images, probably due to the huge amount of Caveolin-1 respect to AR, the fluorescence intensity profile showed some points of colocalization of the AR with the integral plasma membrane protein Caveolin-1.

Moreover, the discontinuous sucrose density gradient fractionation showed the presence of the AR in the low-density plasma membrane fragments, corresponding to lipid *rafts* and caveolae. Caveolae represent a subcompartment of the plasma membrane that has been previously implicated in signal transduction,

including G protein coupled signaling events [44,45,53–57]. Caveolin is an integral membrane protein and the principal component of caveolae membranes. At steady-state, greater than 90% of Caveolin is both morphologically and biochemically localized within plasma membrane caveolae [46,58,59]. In this work we have also demonstrated that the non-classical localization of the AR in caveolae imply a physical interaction with Caveolin-1. However, as shown by Western blot analysis, this association is impaired after testosterone treatment as the levels of some splice variants of the AR, in the microsomal fraction, decrease after exposure to the hormone whereas their amounts in the nucleus appear increased. These results lead us to think that testosterone activates the membrane AR inducing their translocation to other subcellular compartments, e.g. nucleus.

This receptor dynamics may participate in the protective effect of testosterone against apoptosis of muscle cells in conjunction with BAD/BAX/PARP inactivation previously observed [34].

Altogether, the results of this study allow us to propose the presence of extranuclear AR in the C2C12 murine myoblast cell line. The biochemical and immunological similarity between the reactive entities detected in mitochondria and membrane compartments with the AR strengthen the general idea of the existence of a subpopulation of AR with a non-classical location.

Regarding the participation of AR in testosterone regulation of skeletal muscle function, we have previously shown that typical changes of apoptosis such as nuclear fragmentation, cytoskeleton disorganization, mitochondrial reorganization/dysfunction, cytochrome c release, PARP cleavage and the loss of mitochondrial membrane potential induced by H_2O_2, are abolished when cells are previously exposed to the hormone in C2C12 muscle cells. The employment of the antagonist of the AR, flutamide, inhibited this protective action [34,39], suggesting a functional role of the AR, detected to a greater extent in mitochondria and lipid *rafts* of skeletal muscle cells in the present work.

Conclusions

There is evidence that skeletal muscle is a target tissue for androgens. The loss of muscle mass and strength with aging, also referred to as sarcopenia, has been associated with a deficit of sex hormones as the levels of testosterone and/or estrogens decline with aging. Although it has been shown that testosterone supplementation increases muscle mass in healthy young and old men [60], the exact mechanism of androgen-dependent sarcopenia remains to be clarified. Characterization of the relative roles of the muscle cell AR proteins with non-classical intracellular distribution detected in the present study may provide basis on androgen modulation of skeletal muscle development.

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