Intracellular Distribution and Involvement of GPR30 in the Actions of E2 on C2C12 Cells

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ABSTRACT

G-protein-coupled receptor 30 (GPR30) is an estrogen receptor that initiates several rapid, non-genomic signaling events triggered by E2. GPR30 has recently been identified in C2C12 cells; however, little is known about the intracellular distribution and its role in C2C12 myoblasts and myotubes. By western blotting and immunohistochemistry, we evidenced expression of GPR30. While in C2C12 myoblasts, the receptor was present in nucleus, mitochondria, and endoplasmic reticulum, in C2C12 myotubes, it was additionally found in cytoplasm. Using trypan blue uptake assay to determine cellular death and fluorescent microscopy to evaluate picnotic nuclei and mitochondrial distribution, we demonstrated that treatment of C2C12 myoblasts with G1 (GPR30 agonist) did not protect the cells against apoptosis induced by H2O2 as E2. However, when G15 (GPR30 antagonist) was used, E2 could not prevent the damage caused by the oxidative stress. Further, some of the molecular mechanisms involved were investigated by western blot assays. Thus, E2 was able to induce AKT phosphorylation in apoptotic conditions and ERK phosphorylation in proliferating C2C12 cells but not when the cultures were incubated with G15. Additionally, using G15 antagonist we have found that GPR30 participates in the myogenin expression and creatine kinase activity stimulated by E2 in the first steps of C2C12 differentiation. Altogether these findings provide evidences showing that GPR30 is expressed in diverse intracellular compartments in undifferentiated and differentiated C2C12 cells and mediates E2 actions. J. Cell. Biochem. 9999: 1–13, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: 17β-ESTRA DIOL; GPR30; MYOBLASTS; MYOTUBES

Estrogens in addition to promote differentiation of female secondary sexual characteristics and normal processes of development and/or growth, have gained relevance for its protective action on various cellular types. Regarding skeletal muscle, it has been demonstrated that degenerative diseases of this tissue in menopausal women, is in part due to the absence of estrogens [Greising et al., 2009]. Also, other reports evidence that physiology of skeletal muscle is recovered by 17β-estradiol (E2) administration in ovariectomized mice [Kadi et al., 2002]. Additional studies suggest that this hormone is important in improving muscle mass in cases of atrophy [McClung et al., 2006] and protects skeletal muscle against oxidative damage [Persky et al., 2000]. Therefore, skeletal muscle tissue is a target for E2 actions and it has been established to possess estrogen receptors (ERs) [Lemoine et al., 2002, Wiik et al., 2005]. The most studied ERs in the hormone actions were for a long time ERα, first characterized in 1973 [Jenkins and Desombre, 1973] and ERβ discovered in 1996 [Kuiper et al., 1996]. In our laboratory we have studied the expression and distribution of these receptors in mouse C2C12 myoblasts. We have found that, besides the cytosolic and nuclear localization, ERα was also found in endoplasmic reticulum, perinuclear zone, and to lesser extent in mitochondria, while ERβ localization was mainly observed in mitochondria [Milanesi et al., 2008, 2009].

It is well established that E2 acts through such ERs and activate distinct secondary messenger pathways with different time courses and downstream mechanisms. In the classical mechanism, E2 binds intracellular receptors and then translocate to the nucleus where they function as ligand-dependent transcription factors regulating gene expression [Levin, 2005]. This mode of action is related to long-term responses of the hormone. In contrast, fast non-genomic effects can be also mediated by ERs, regulating different signal transduction pathways, and in many cases, these events appear to be initiated at plasma membrane level [Moriarty et al., 2006]. In our laboratory we have elucidated some of the non-genomic molecular mechanisms involved in the protective action of E2 on skeletal muscle cells. Specifically, we have observed in C2C12 murine skeletal myoblasts...
an antiapoptotic action of the hormone through the classical ERs involving PI3K/Akt/Bad [Vasconsuelo et al., 2008], HSP27 [Vasconsuelo et al., 2010], MAPKs [Ronda et al., 2010a, 2013], and PKC/JNK [La Colla et al., 2015] signalling pathways.

In the last decade other receptor was emerging to be an important mediator of non-transcriptional estrogen actions, the novel G protein-coupled receptor 30 (GPR30) or recently named G protein-coupled estrogen receptor 1 (GPER1). The first evidence of this receptor was from Filardo et al. [2000] and later two simultaneous studies indicate that GPR30 is an ER, but distinct from the classic ERα and ERβ [Revankar et al., 2005; Thomas et al., 2005]. GPR30 is a seven transmembrane-domain G protein-coupled receptor (GPCR) and binds E2 with high affinity mediating estrogenic signalling pathways [Revankar et al., 2005; Thomas et al., 2005]. Expression of GPR30 has been identified in multiple tissues, such as uterine endometrium, brain, adrenal, kidney, ovary, endothelium, heart and breast [Haas et al., 2007; Hazell et al., 2009; Wang et al., 2007]. Its intracellular distribution has been demonstrated to localized mainly in the endoplasmic reticulum [Revankar et al., 2005, Prossnitz et al., 2008] but it was also found in the plasma membrane [Thomas et al., 2005; Funakoshi et al., 2006; Cheng et al., 2011], nucleus (Madeo and Maggiolini, 2010; Cheng et al., 2011) and cytoplasm [Chakrabarti and Davidge, 2012; Mo et al., 2013].

GPR30 expression and functions in skeletal muscle cells has been little explored. Thus, studies made in mice, have been shown the presence of GPR30 receptor mRNA [Mårtensson et al., 2009], and its abundance in gene and protein levels has been compared respect to the others ERs, being ERα the most abundant, followed by GPR30 and ERβ [Baltgalvis et al., 2010]. Moreover, it has been reported that GPR30 plays a role in cAMP production induced by E2 in skeletal muscle [Sandén et al., 2011]. Despite these studies, intracellular distribution of the receptor in skeletal muscle cells and its role in the protective action of E2 remain to be elucidated.

The present study was undertaken to determine the subcellular distribution of GPR30 in undifferentiated and differentiated C2C12 cells. Also we investigated the role of the antiapoptotic actions induced by E2 in C2C12 myoblasts and also its role in the hormone effects on C2C12 during differentiation.

MATERIALS AND METHODS

MATERIALS

Anti-GPR30 (1:1000 for Western blot analysis and 1:50 for immunocitochemistry), anti-myogenin (1:1000 for Western blot analysis), and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-KDEL (1:50 for immunocitochemistry) was purchased from Abcam (Cambridge, MA). Anti-pERK (1:1000 for Western blot analysis), anti-pAKT (1:1000 for Western blot analysis) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). MitoTracker Red (MitoTracker Red CMXRos) dye, 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) dye and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:200 for immunocitochemistry) were supplied by Molecular Probes (Eugene, OR). Alexa Fluor 568-conjugated antimouse antibody (1:200 for immunocitochemistry) and anti-b-tubulin (1:1500 for Western blot analysis) was purchased from Thermo Fisher Scientific (Rockford, IL). Creatine Kinase detection kit was supplied by Boehringer-Mannheim (Germany). G1 and G15 were purchase from Cayman Chemical Company (Ann Arbor, MI). All the other reagents used were of analytical grade.

CELL CULTURE AND TREATMENT

C2C12 murine skeletal muscle cells purchased from the American Type Culture Collection (Manassas, VA) were cultured in growth medium DMEM supplemented with 10% inactivated (30 min, 56°C) fetal bovine serum, 1% nystatine, and 2% streptomycin. Cells were incubated at 37°C in a humid atmosphere of 5% CO2 in air. Cultures were passaged every 2 days with fresh medium. For myoblasts studies cultures were performed with 70–80% of confluence (120,000 cells/cm²) for Western blot assays or with 50% of confluence (75,000 cells/cm²) in chamber slides for microscopy. Before treatments, cultures were incubated in medium without serum for 30 min (starvation). During this period of time, cells were exposed to 1 µM G15 when indicated in the experiments. Treatments were carried out by adding vehicle (control: 0.001% isopropanol), 10⁻⁸ M E2 or G1 at the concentrations and times indicated in the experiments. Apoptosis was induced using 0.5 mM H2O2 for 4 h. G1 and G15 were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% (v/v) and was added in control condition in the experiments where G1 or G15 were used. It was verified that concentration of DMSO applied in these experiments did not interfere with the effects of E2 on C2C12 cells [Ronda et al., 2010a]. To induce differentiation from myoblasts to myotubes, C2C12 myoblasts were grown to 90% confluence in the growth medium (GM) and shifted to phenol red free DMEM supplemented with 2% horse serum and the above antibiotics (DM: differentiation medium) during 72 h. DM was replaced at 24 h intervals. Morphological changes of C2C12 cells, Myogenin expression and Creatine Kinase activity at 0 (GM), 24, 48 and 72 h were monitored to evaluate differentiation (Fig. 1). Treatments were carried out by adding vehicle (control: 0.001% isopropanol), 10⁻⁸ M E2, or 1 µM G15 + 10⁻⁸ M E2 during differentiation in the times specified in each assay.

SUBCELLULAR FRACTIONATION

We used a well established protocol in our laboratory to obtain the enriched subcellular fractions of C2C12 [Pronsato et al., 2013; Ronda et al., 2013]. Briefly, 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 phenyl-methylsulfonyl fluoride (PMSF), 20 lg/ml leupeptin, 20 lg/ml aprotinin, 20 lg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. The debris was separated by centrifugation (75,000 cells/cm²) in chamber slides for microscopy. The remaining supernatant was centrifuged at 10,000 g for 20 min to pellet mitochondria. Pellets were re-suspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na2VO4, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the
fractions was estimated by the method of Bradford [Bradford, 1976], using BSA as standard and Western blot analysis were performed as described below.

WESTERN BLOT ANALYSIS

Cell cultures were scrapped and resuspended using a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na₂VO₄, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Lysates were collected by aspiration and centrifuged at 12,000g for 15 min. The protein content of the supernatant was quantified by the Bradford procedure [Bradford, 1976] using BSA as standard. Then, lysate proteins dissolved in Laemmli [Laemmli, 1970] sample buffer were separated on 10–12% SDS–polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham, Piscataway, NJ). Membranes were blocked 1 h at room temperature in PBS–T buffer (PBS 0.1% Tween-20) containing 5% dry milk. Membranes were incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the stained cells were visualized using an enhanced chemiluminescent technique according to the manufacturer’s instructions. For reprobing with other antibodies, membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 50 mM mercaptoethanol) for 30 min at 55°C, washed for 10 min in PBS–T, and then blocked and blotted as described above.

IMMUNOCYTOCHEMISTRY

Semi-confluent (50% confluence) monolayers were fixed with methanol at −20°C for 30 min. After fixation, non-specific sites were blocked at room temperature for 1 h in 5% BSA. Cells were incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the stained cells were analyzed with a conventional fluorescence microscope (NIKON Eclipse Ti-S equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera) or confocal scanning laser microscopy (Leica TCS SP2 AOBS). The specificity of the labeling techniques was proven by the absence of fluorescence when the primary or the secondary antibodies were omitted.

MITOTRACKER RED AND DAPI (4',6-DIAMIDINE-2'-PHENYLINDOLE DIHYDROCHLORIDE) STAINING

Coverslips with adherent cells were stained with MitoTracker Red, which was prepared in DMSO and then added to the cell culture medium at a final concentration of 1 µmol/L during starvation. Subsequently, cells were washed with PBS (pH 7.4, 8 g/L NaCl, 0.2 g/L KCl, 0.24 g/L KH₂PO₄, and 1.44 g/L Na₂HPO₄) and fixed with methanol at −20°C for 30 min. Then immunocytochemistry assays were performed.

For DAPI staining, fixed cells were incubated for 30 min at room temperature in darkness with 1:2,000 of a stock solution of DAPI (5 mg/ml) and washed with PBS. At least, two hundred nuclei were counted for each condition and apoptotic nuclei were identified as
those with reduction in its size (“picnosis”) or its fragmentation. Percentage of apoptotic nuclei are shown in graphs.

CELL VIABILITY

C2C12 cells were grown to 70% confluence in six-well plates and treated as indicated. Then, cultures were harvested with trypsin and centrifuged at 8,000g during 6 min. Cells were stained with trypan blue solution and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate.

CREATINE KINASE ASSAY

Creatine kinase (CK) activity in whole cell lysates was measured by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions (kit monotest CK NAC from Boehringer-Mannheim). One unit of activity represents 1 mmol of NADPH generated per min per mg protein.

STATISTICAL ANALYSIS

One-way ANOVAs followed by a multiple comparison post hoc test (Duncan’s test) were used to find statistical differences among group means. Data are expressed as significant at $P < 0.05$. Results are shown as means ± standard deviation (SD) from the indicated set of experiments.

RESULTS

GPR30 EXPRESSION IN C2C12 MYOBLASTS

First, we studied the subcellular localization of the novel estrogen receptor GPR30 in C2C12 cells in proliferative stage. For this end, cell homogenates were subjected to differential centrifugation to obtain the distinct subcellular fractions. Then, organelles were lysate and analysed by Western blot assays using a specific antibody that recognizes an internal region of the receptor. Equal amount of proteins for each fraction were used. Blot in Figure 2A shows the presence of the band corresponding to the predicted molecular weight for GPR30 (~43 KDa), but also the presence of other higher and lower bands. Also, our results showed GPR30 presence in nuclei, mitochondria, and microsomes being more significant in nuclei and microsomes while in the cytosolic fraction GPR30 expression was poorly immunodetected.

Next, we studied in detail the subcellular distribution of GPR30 in C2C12 myoblasts by immunocytochemistry assays, using different subcellular organelle markers. Figure 2B shows images obtained from fluorescence microscopy where an antibody that recognizes GPR30 together with an Alexa Fluor 488-conjugated secondary antibody (green fluorescence) and the specific nuclear dye DAPI (blue fluorescence) were used. Images revealed that the receptor was present within and around the nucleus being in this last site more evident. Analysis of fluorescence intensity profiles monitored along one cell (white arrow in merge image) confirmed the internal localization of GPR30 in the nucleus and their abundance around it (yellow arrows).

Then, we analysed localization of the receptor in mitochondria using MitoTracker red staining (red fluorescence) as specific marker of this organelle. Images showed in Figure 2C obtained by confocal microscopy demonstrated mitochondrial disposition around the nucleus (red fluorescence) and GPR30 distribution (green fluorescence). The exact superposition of both images (merge) made evident some points of yellow florescence confirming localization of GPR30 in mitochondria. Analysis of fluorescence intensity profiles monitored along one cell (white arrow in merge image) proved different coincidence points in the superposition of both fluorescences (yellow arrows).

Since we have immunodetected by Western blot the presence of GPR30 in microsomes, and it has been widely reported the presence of GPR30 in endoplasmic reticulum in various cell types [Revankar et al., 2005; Prossnitz et al., 2008], then we studied localization of the receptor in such organelle. To this end we used and antibody that recognizes the KDEL sequence of proteins present in endoplasmic reticulum together with an Alexa Fluor 568-conjugated secondary antibody (red fluorescence) and GPR30 was immunodetected as described (green fluorescence). Images obtained by confocal microscopy in Figure 2D show the perinuclear localization of endoplasmic reticulum (red fluorescence) and the GPR30 distribution (green fluorescence). The exact superposition of both images suggested presence of the receptor in such organelle since some points of yellow fluorescence were found, resulting from colocalization between anti-GPR30 and anti-KDEL antibodies. Analysis of fluorescence intensity profiles monitored along one cell (white arrow in merge image) indicated different coincidence sites in the superposition of both fluorescences (yellow arrows).

GPR30 EXPRESSION IN C2C12 MYOTUBES

Similarly as myoblasts, we performed experiments to study expression and localization of GPR30 in C2C12 myotubes. For this end C2C12 cells were differentiated as described under Materials and Methods and subcellular localization of GPR30 receptor was analysed as before using subcellular fractionation and immunocytochemistry assays.

Blot in Figure 3A shows the presence of a band corresponding to the predicted molecular weight of GPR30 (~43 KDa), and similarly to the observation made in myoblasts, different higher and lower bands related with the GPR30 receptor in all fractions were detected. GPR30 was present in all membrane fractions being higher in microsomes and in nucleus. Unlike myoblasts, we have also found high levels of the receptor in the cytosol of myotubes.

Since different bands were immunodetected by Western Blot for myoblasts and myotubes, we compared the molecular weights of the bands detected from total homogenates by Western blot analyses. We found differences in the protein content of bands corresponding to GPR30 (Fig. 3B), while bands of ~38, ~43, and ~52 kDa were common for both culture conditions, two more bands of approximately ~36 and ~46 kDa were also observed in myoblasts and another band of about ~64 kDa was observed in myotubes. The quantifications of total bands corresponding to GPR30 showed that its protein expression in differentiated cells was higher than in undifferentiated cells (Fig. 3C).

Figure 3D, shows the subcellular distribution of GPR30 in myotubes. Images obtained indicate the fusion of cells forming the myotube since various nuclei were observed inside only one cell...
(blue fluorescence). As in myoblasts, GPR30 distribution was observed along the cell (green fluorescence) and inside nuclei being more significant around them, according to the analysed fluorescence intensity profiles (white arrow in merge image).

Figure 3E shows GPR30 distribution (green fluorescence) and mitochondrial disposition using Mitotracker red staining in myotubes (red fluorescence). When both images were superposed (merge) different yellow fluorescence points were evident showing localization of the receptor in mitochondria. Analysis of fluorescence intensity profiles monitored along one cell (white arrow in merge image) proved some coincidence superposition places for both fluorescences (yellow arrows).

Presence of GPR30 in the reticular network in myotubes was also studied using the anti-KDEL antibody as myoblasts. Images from Figure 3F reveals distribution of the sarcoplastic reticulum (red fluorescence) and the GPR30 along the C2C12 differentiated cell (green fluorescence). The exact superposition of both images obtained by confocal microscopy showed some points of yellow fluorescence resulting from colocalization between both antibodies and suggesting the presence of GPR30 in such organelle. Analysis of fluorescence intensity profiles monitored along one cell (white arrow in merge image) confirmed different coincidence sites in the superposition of both fluorescences (yellow arrows).

ROLE OF GPR30 IN C2C12 MYOBLASTS

To evaluate a possible role of the receptor GPR30 in the protective action of E2 on the C2C12 cells that we previously described [Vasconsuelo et al., 2008; Ronda et al., 2010a, 2013; La Colla et al., 2015]. Since it has been reported that the G1 specific receptor agonist activate the receptor without mediation of signal transduction through ERα or –β [Bologa et al., 2006], we first, investigated whether the activation of GPR30 is sufficient to protect C2C12 cells from death by apoptosis induced by H2O2. To this end, after being deprived of serum for 30 min, cells were preincubated with 0.001% isopropanol (C), 10^{-8} M E2 or 1 μM G1 [Sandén et al., 2011] for 1 h, then apoptosis was induced with 0.5 mM H2O2 in the absence or presence of 10^{-8} M E2 or 1 μM G1 for 4 h. Afterward, existence of pyknotic/fragmented nuclei using DAPI, and subcellular distribution of mitochondria using Mitotracker red staining were analysed by fluorescence microscopy. Images obtained in Figure 4A show that exposure of cells to H2O2 altered the nuclei morphological characteristics and the mitochondrial distribution in C2C12 cells.
respect to control or cells treated with E2. When cells were preincubated with E2 before inducing apoptosis, the effect of H$_2$O$_2$ was prevented (E2 + H$_2$O$_2$). However, when cells were preincubated with G1 before treatment with H$_2$O$_2$ the characteristics for nuclei and mitochondria in apoptotic cells were observed yet (G1 + H$_2$O$_2$). To support this observation, cell death was quantified using trypan blue exclusion assay as we described under Materials and Methods. Figure 4C also evidenced that preincubation of cells with G1 prior to induction of apoptosis with H$_2$O$_2$ was not able to prevent cellular death like E2. These results suggest that the only activation of GPR30 was not sufficient to prevent the effects caused by oxidative stress, but does not exclude a possible involvement of the receptor in the protective action of E2.

Therefore we use the G15 compound, a receptor specific antagonist that has no effect on the other estrogen receptors. This pharmacological agent is currently the most frequently tool used for investigating the role of GPR30 in various cellular systems [Dennis et al., 2011]. For this purpose, cells were serum deprived during 30 min. Then, cells were treated with 0.001% isopropanol (C: control) or 10$^{-8}$M E2 for 1 h followed by induction of apoptosis with
0.5 mM H$_2$O$_2$ in the absence or presence of 10$^{-8}$ M E2 or 1 µM G15 + 10$^{-8}$ M E2 during 4 h. Finally, we evaluated nuclear morphology, mitochondrial distribution, and cell death as before. Results in Figure 5 showed the already established protection of E2 against H$_2$O$_2$ effects (E2 + H$_2$O$_2$) on morphological characteristics of nuclei and mitochondria (Fig. 5A), amount of apoptotic nuclei (Fig. 5B) and cell death (Fig. 5C) in C2C12 cells. Of relevance, such actions of the hormone were diminished in presence of G15 (G15 + E2 + H$_2$O$_2$), suggesting a role of GPR30 in the estrogen protection on myoblasts.

In previous investigations from our laboratory, we demonstrated that E2 is able to induce the phosphorylation of ERK2 MAPK [Ronda et al., 2010b] and that this event is involved in the protective action of the hormone, contributing in the activation of the survival PI3K/AKT/Bad pathway [Ronda et al., 2010a]. Since we have showed a possible role of GPR30 in the protective effects of E2, we then studied using the antagonist G15 and Western blot analyses if the receptor has a participation in such event. To this end, we first studied ERK2 phosphorylation induced by the hormone in the absence and presence of G15. Cells were serum deprived for 30 min and then were treated with 0.001% isopropanol (control), 10$^{-8}$ M E2 or 1 µM G15 + 10$^{-8}$ M E2 for 15 min. Results showed that ERK2 phosphorylation induced by the hormone (E2) was diminished in presence of the GPR30 antagonist (G15 + E2), suggesting an involvement of the receptor in this event (Fig. 6).

Then, GPR30 role in AKT activation was evaluated in apoptotic conditions using the G15 antagonist. As We previously reported, E2 induced an increase in AKT phosphorylation in the absence and presence of H$_2$O$_2$ [Ronda et al., 2010a] (Fig. 7). However, when cells were pre-incubated with G15, the hormone was not able to induce AKT activation under oxidative stress, suggesting a role for GPR30 in the activation of the AKT survival pathway.

ROLE OF GPR30 IN C2C12 DIFFERENTIATION

It has been demonstrated that E2 regulates the first steps of skeletal muscle cell differentiation via ER-α-mediated signals in L6 and...
C2C12 cells [Galluzzo et al., 2009]. Since we have detected GPR30 in differentiated C2C12 cells, we investigated a possible role of this receptor in the actions of E2 on the first steps of myogenesis on C2C12 myoblasts. To this end cultures were grown in growth medium (GM) to confluence and then shifted to differentiation medium (DM) during 0, 24, 48, and 72 h as we described under Materials and Methods. Under these culture conditions, we evaluate the effects of E2 in absence or presence of G15 on different parameters of differentiation. Images obtained by phase contrast microscopy (Fig. 8A) shows the myogenic differentiation of C2C12 cells during 24–72 h. At 72 h of differentiation a high amount of myotubes were observed in all conditions. Images also showed that neither E2 nor G15 + E2, had evident effects on morphological characteristics of cells at the times studied. To evaluate myogenin expression, after treatments, cells were homogenized and subjected to Western blot analyses. Results in Figure 8B show that incubation of cells with E2 statistically increased myogenin protein levels at 24 h of differentiation which was affected by the presence of the G15 antagonist. It was also evident that although there was an increase in protein levels of myogenin by E2 at 48 h of differentiation, this effect was not statically significant. On the other hand, G15 antagonist had no effect on E2 action at 48 h of myogenesis. At 72 h of differentiation, E2 and G15 + E2 had not effects on myogenin expression in C2C12 myotubes respect to control. These results suggest that E2 induced myogenin expression at 24 h of differentiation and that GPR30 receptor was involved in this action at this period of time.

Finally, we evaluated Creatine kinase (CK) activity in whole cell lysates after the indicated treatments. Results revealed an increase of the CK activity under E2 stimulation only at 72 h of differentiation (Fig. 8B) which was abolished by incubation of cells with G15 antagonist (G15 + E2). These results suggest that estrogen induced CK activity at 72 h of differentiation through the involvement of GPR30.

**DISCUSSION**

Results of studies on postmenopausal women and ovariectomized rodents support the contention that E2 is beneficial to muscle strength and that underlying mechanisms involve at least modulation of one of the estrogen receptors [Lowe et al., 2010]. The novel estrogen receptor GPR30 is emerging as an important...
component of the complex system that regulates various organs and tissues. Studies in a mouse model in which the GPR30 gene locus was completely disrupted revealed that this receptor is necessary for normal insulin production, glucose homeostasis, blood pressure and muscle skeletal growth [Mårtensson et al., 2009], demonstrating a role for GPR30 in skeletal muscle tissue performance. Therefore, the knowledge of GPR30 intracellular distribution and the molecular mechanisms that are mediated for this receptor in myoblasts and myotubes will help to understand how E2 modulates the beneficial actions in skeletal muscle tissue. While it is known that ERα and ERβ are present in skeletal muscle cells [Lemoine et al., 2002; Wiik et al., 2005; Milanesi et al., 2008, 2009] and have a role in the protective action of E2 in this tissue [Vasconsuelo et al., 2008], much less is known about GPR30. It has been determined the mRNA expression of this receptor in mice [Mårtensson et al., 2009] and its relative abundance compared to ERα and ERβ amounts [Baltgalvis et al., 2010]. Also GPR30 mRNA levels and its role on cAMP production by E2 have been reported in C2C12 myoblasts [Sandén et al., 2011]. However, there is no data about its intracellular localization, and its role in the estrogen protective action on C2C12 muscle cells. Our results complement such previous studies by reinforcing intracellular distribution of GPR30 and providing evidence that show an involvement of the receptor in the signal transduction triggered during the protective effects of E2 in C2C12 myoblasts. Also, this work contain data that present for the first time the existence and localization of the receptor in C2C12 myotubes, and its role in the earlier steps of C2C12 differentiation.

Using subcellular fractionation and immunocytochemical markers we identify that in C2C12 myoblasts, GPR30 expresses in nucleus, mitochondria, endoplasmic reticulum, and in differentiated C2C12 cells, we have also found its expression in cytoplasm. Regarding mitochondria, even though evidences show that GPR30 participates in mitochondrial events in heart modulated by E2 [Bopassa et al., 2010; Rowlands et al., 2011] its existence and localization in this organelle has not been determined yet in any cellular type. Our results are novel in demonstrating the localization...
of the receptor in mitochondria in undifferentiated and differentiated C2C12 cells. The fact that we observed GPR30 expression around the nucleus in both culture conditions, and that subcellular fractionation showed the existence thereof in the nuclear fraction, leads us to believe that the receptor may be integrated into the nuclear envelope which is contiguous with the endoplasmic reticulum where we have also found GPR30 expression. Interestingly, it has been shown that there is a traffic of the receptor in absence of agonist, via endosomes, from the plasma membrane and its accumulation in the perinuclear region [Cheng et al., 2011]. Furthermore, we observed fluorescence specific points corresponding to the receptor into the nucleus which may be indicating additional mechanisms respect to those already known. Actually, it has been shown a nuclear localization of GPR30 that induces gene expression in breast cancer cells [Madeo and Maggiolini, 2010]. GPR30 is a seven transmembrane receptor [Luttrell, 2006], however we could not confirm whether it is present in the plasma membrane. Although the majority of GPCRs are expressed in this place, it is becoming accepted that some GPCRs may be functionally expressed at intracellular sites [Gobeil et al., 2006]. Since, this is particularly true for GPCRs with lipophilic ligands, we suggest that GPR30 is inserted in nuclear, mitochondrial and endoplasmic reticulum membranes. Interestingly, it has been shown that G protein βγ subunits are initially targeted to the endoplasmic reticulum, where they subsequently associate with G protein α subunits [Smrcka, 2008], providing the requisite machinery for GPR30 to initiate signalling cascades. It is certainly possible that under appropriate conditions, intracellular GPR30 could exist or translocate to the cell surface or vice versa. In fact, it has been demonstrated that GPR30 localized in the plasma membrane of rat hippocampal neurons and in GPR30-transfected HeLa cells where the receptor can translocate to the cytoplasm upon agonist stimulation [Funakoshi et al., 2006]. However since E2 (which is the specific GPR30 ligand) is permeable to the plasma membrane, an intracellular localization of the receptor is certainly consistent with its function. Respect to GPR30 expression in cytoplasm and, in agreement with our results in differentiated C2C12 cells, it has been reported in various cell types the localization of the receptor in this compartment [Chakrabarti and Davidge, 2012; Mo et al., 2013], however the mechanism of action of GPR30 in this place is still unknown. More studies are needed to elucidate the role and mechanism of action of this receptor with seven transmembrane domains in the cytoplasm.

It is evident from our Western blot data that GPR30 appears to have a variety of bands in all organelles suggesting existence of the receptor with different molecular weights. Moreover, the expression of some bands is different depending on culture conditions. GPR30, besides having seven transmembrane domains, has N-amino terminus that links glycoproteins [Luttrell, 2006]. Therefore, we
hypothesized that such bands observed could correspond to different glycosylated forms of the receptor, since the predicted molecular weight detected by the GPR30 antibody used has an apparent molecular mass of ~44 kDa. According with this supposition, it has been demonstrated by using blocking peptides that molecular weights more than 38 kDa corresponds to glycosylated forms of GPR30 [Filardo et al., 2007; Maiti et al., 2011]. Alternatively, different bands observed for GPR30 in our experiments could be products from splicing or proteolysis processes. Homodimerization or interaction with other proteins have been reported before [Sandén et al., 2011] and isolated vascular preparations [Tica et al., 2011]. Therefore, more studies are needed to elucidate possible contribution of GPR30 could homodimerize or interact with other proteins. It would be of interest to determine the nature of these bands by proteomic analysis and gene sequencing to evaluate their biological significance. Additionally, our experimental data show a substantial amount of differences in expression and intracellular distribution of GPR30 between myoblasts and myotubes, suggesting clearly a differential role of the receptor in both culture conditions.

In the present work we demonstrated a role of GPR30 in the estrogen protective actions on C2C12 myoblasts since the use of the antagonist G15 suppress the survival actions of E2 on apoptotic nuclei quantity, mitochondrial distribution and cellular death induced by oxidative stress. According with this results, it was recently described a participation of GPR30 in the protective effects of E2 on neurons [Chen et al., 2015] and endothelial cells [Ding et al., 2015]. The fact that the use of G1, which has been employed to activate GPR30 while not EREs or –β [Bologa et al., 2006], was not sufficient to protect the oxidative damage caused by H2O2, suggested us that the only activation of GPR30 by E2 is not sufficient to promote myoblasts survival. Actually, we have already shown a contribution of ERα and ERβ in the protective action of E2 on C2C12 cells [Vasconsuelo et al., 2008]. Thus, we propose that the hormone is able to induce a sinergic activation of the three estrogen receptor to promote cell survival in C2C12 myoblasts. Accordingly, it has been reported that the GPR30 receptor may act together with intracellular estrogen receptors to activate cell-signaling pathways which support neuroprotection after global ischemia [Hazzell et al., 2009]. Moreover, functional cross-talk between receptors has been demonstrated in ovarian cancer cells [Albanito et al., 2007], uterine epithelial cells [Gao et al., 2011] and isolated vascular preparations [Tica et al., 2011]. Therefore, more studies are needed to elucidate possible interactions among estrogen receptors in E2 actions on C2C12 myoblasts.

Estrogens initiate multiple intracellular signalling cascades. Although classical ERs have been demonstrated to mediate many of these responses, the signalling capabilities of GPR30 in response to estrogen have just begun to be described. As a GPCR, GPR30 couples to G proteins and modulates second messenger pathways, from which CAMP and Ca2+ have emerged as the messengers most frequently associated with GPR30 signalling [Revankar et al., 2005; Filardo et al., 2007]. In C2C12 cells it has been demonstrated that E2 induces CAMP through GPR30 [Sandén et al., 2011] and it has been reported that stimulation of GPR30 leads to PI3K/AKT activation resulting in antiapoptotic and cardioprotective properties of the receptor [Weil et al., 2010]. In addition, several groups have also reported ERK phosphorylation through GPR30 [Filardo et al., 2000; Revankar et al., 2005]. As we have previously demonstrated that E2 induces ERK activation [Ronda et al., 2010b] and that this event participates in the survival PI3K/AKT pathway modulated by the steroid [Ronda et al., 2010a], in the present work using the G15 compound we found that GPR30 antagonist attenuated ERK and AKT phosphorylation induced by E2, suggesting the involvement of GPR30 receptor in the protective signalling triggered by E2 in C2C12 myoblasts.

Different actions of E2 on myoblast differentiation have been demonstrated. While Ogawa et al. [2011] have found that the hormone inhibits myogenesis at long times of differentiation (8 days), Galluzzo et al., [2009] have shown that E2 positively modulates the first steps of skeletal muscle cell myogenesis via ER-α-mediated in L6 myoblasts. In the present work using C2C12 cells, we reinforce the findings which show that E2 positively modulates the first steps of L6 myoblasts differentiation. Images obtained by phase contrast microscopy did not reveal morphological changes in C2C12 myogenesis in cells treated with E2 respect to control. However, we evidenced changes in biochemical markers of differentiation. Our hypothesis is based on the fact that E2 first increased myogenin expression at 24 h and then CK activity at 48 h of differentiation. According with this findings it has been stablished that myogenesis is a highly ordered process that involves at least four temporally separable events: (1) entry of myoblasts into the differentiation pathway, as indicated by the rapidly and transitory induction of myogenin; (2) irreversible cell cycle withdrawal, as indicated by the expression of p21; (3) expression of terminal differentiation markers such as myosin heavy chain and creatine kinase activity; and finally (4) cell fusion that is evident morphologically [Sabourin and Rudnicki, 2000]. Additionally, our data show an involvement of the novel estrogen receptor GPR30 in E2 modulation of myogenesis. The fact that G15 antagonist was able to abrogate myogenin expression and CK activity induced by the E2 suggest us a contribution of GPR30 in the first steps of C2C12 differentiation regulated by the steroid hormone. To our knowledge this is the first evidence showing a role for GPR30 in myogenesis.

Finally, results presented in this work together with the previously obtained from our laboratory demonstrate the co-existence and involvement of the three estrogen receptors, ERα, ERβ, and GPR30 in E2 actions on C2C12 cells, suggesting a possible interaction between them or between the different signalling pathways triggered by the hormone. Thus, a new door of investigation is open to elucidate how E2 perfectly orchestrates stimulation of these receptors and subsequent signalling cascades and/or transcription factors modulation to give a final beneficial response on skeletal muscle tissue. Moreover, our results reinforce evidences for an important role of E2 in muscle physiology indicating that this steroid hormone acts as a skeletal muscle trophic factor.

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