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ORIGINAL ARTICLE

Progesterone and estradiol effects on SRC-1 and SRC-3 expression in human astrocytoma cell lines

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Abstract Progesterone (P_4) and estradiol (E_2) regulate many cell functions through their interaction with specific intracellular receptors, which require the participation of coactivators such as SRC-1 and SRC-3 for enhancing their transcriptional activity. Coactivator expression is altered in many cancers and in some of them their expression is regulated by P_4 and E_2. In this study, we determined progesterone and estrogen receptor isoform expression in two human astrocytoma cell lines with different evolution grade (U373, grade III; and D54, grade IV) by Western Blot. We studied the role of P_4 and E_2 on SRC-1 and SRC-3 expression in U373 and D54 cell lines by RT–PCR and Western blot. In U373 cells, P_4 did not modify SRC-1 expression, but in D54 cells it increased SRC-1 mRNA expression after 12 h of treatment without significant changes after 24 h. P_4 also increased SRC-1 protein content after 24 h, but reduced it after 48 h. E_2 did not change SRC-1 expression in any cell line. SRC-3 expression was not regulated by either E_2 or P_4. Our data suggest that SRC-1 and SRC-3 expression is differentially regulated by sex steroid hormones in astrocytomas and that P_4 regulates SRC-1 expression depending on the evolution grade of human astrocytoma cells.

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Introduction

Astrocytomas arise from astrocytes and are the most common primary intracerebral neoplasms in humans [1]. In the Central Nervous System, astrocytes have been implicated in the maturation of neurons, control of the levels of some ions and neurotransmitters in the extracellular space, the development and function of synapses, blood vessel flow, and several diseases [2]. Astrocytomas are mainly found in adults between ages 30 and 50, and possess a high malignancy potential. Astrocytomas are classified according to their histological characteristics in four groups (I–IV), and the survival of patients is inversely related to the tumor's grade [1].

Sex steroid hormones are involved in the regulation of several physiopathological processes in brain, including tumor cell growth [3, 4]. Progesterone (P_4) and estradiol (E_2) regulate several functions through the interaction with their nuclear receptors (PR and ER, respectively) [5–7] which have been detected in several human brain tumors such as astrocytomas, meningiomas, chordomas, and craniopharyngiomas [8–13]. In astrocytomas, a direct relation between the content of PR and the tumor grade has been reported, suggesting that PR-positive tumors possess a high proliferative potential, whereas ER expression is inversely related to astrocytoma grade [8, 9, 14–16]. On the other hand, PR isoforms have been detected in U373 and D54 cell lines, which are derived from grades III and IV human astrocytomas, respectively [17], whereas ER-α isoform expression has been reported for U373 cells [18].

Previous studies in our laboratory have demonstrated that P_4 induces cell proliferation in U373 and D54 human astrocytoma cell lines, which is blocked by its antagonist RU486, suggesting that P_4 effects are mediated by its nuclear PR [17]. In U373 cells, we have recently reported that PR isoforms expression was up-regulated by E_2 and that this effect was blocked by ER antagonist, ICI182780, suggesting that PR regulation by E_2 depends on ER activation [19].

The transcriptional activity of PR and ER has been linked to their interactions with general classes of coregulators that act as coactivators recruited by the activated steroid receptors to target gene promoters. Coactivators enhance the transcriptional activity of steroid hormone receptors through their intrinsic histone acetyltransferase activity, by bridging nuclear receptors with the basal transcription machinery or by interacting with other histone acetyltransferases such as CBP/p300 [20–22].

Several families of coactivators have been characterized. The steroid receptor coactivator family (SRC) of 160 kDa consists of three members: SRC-1 (NcoA-1), SRC-2 (GRIP-1/NcoA-2/TIF-2), and SRC-3 (AIB1/ACTR/pCIP/RAC3/TRAM-1) [20, 23]. Two functionally distinct isoforms of SRC-1 have been identified, SRC-1a and SRC-1e issued from alternative splicing that produce differences in their C-terminal region [24]. SRC-1a and SRC-1e are highly expressed in neurons, but in rat astrocytes SRC-1e was the only isoform detected [25]. It has been reported that SRC-1e enhanced the ability of the ER and glucocorticoid receptor to stimulate transcription to a greater extent than SRC-1a [25, 26].

SRC proteins interact with several ligand-bound receptors including PR and ER [27, 28]. In T47D human breast cancer cell line and in mouse uterus, SRC-1 is preferentially recruited by PR after P_4 administration [29, 30].

SRC-1 and SRC-3 are expressed in several tissues including brain where a differential expression and regional distribution have been observed [28]. Ogawa et al. [31] have demonstrated a nuclear distribution of SRC-1 in astrocytes of the rat hippocampus, while SRC-3 is mainly localized in the cytoplasm of astrocytes [25].

SRC-1 is involved in neuronal development and in the sexual differentiation of the brain in rodents [32]. In adult animals SRC-1 is required for the display of female sexual behavior [33], and it participates in tumor progression and survival of several cancer cell lines [34–36]. It has been reported that disruption of SRC-1 gene in mice suppresses breast cancer metastasis [37].

SRC-3 also called AIB1 (amplified in breast cancer-1) is frequently amplified and overexpressed in several cancers such as those of breast, ovary, endometrium, stomach, and prostate [23, 38–40], although a differential SRC-3 expression has been documented in cancer cells. Thus, MCF-7 breast cancer cells present a high SRC-3 expression, whereas T47D cells exhibit a low expression [23]. Overexpression of SRC-3 gene has been correlated with ER and PR positivity in primary breast tumors [41]. Besides, a significant correlation of expression levels between ER and SRC-3 in breast tumors and MCF-7 cells has been reported [42].

It has been observed that acute E_2 administration diminishes SRC-1 mRNA expression in the pituitary of adult male rats without changes in the hypothalamus [43]. In contrast, SRC-1 mRNA expression is induced by the administration of E_2 in the hypothalamic ventromedial nucleus of ovariectomized rats [44]. It has been demonstrated that in MCF-7 cells, E_2 increases SRC-1 expression, but diminishes that of SRC-3 [45, 46]. Thus, SRC-1 and SRC-3 not only participate in gene regulation by E_2 and P_4 but also their expression could be modulated by these hormones. It has been reported that E_2 modulates the expression of astrocyte-specific genes, such as glutamine synthetase and glutaminase in the adult female rat [47].

The regulation of SRC-1 and SRC-3 expression by E_2 and P_4 in human astrocytoma cells is not known. Therefore, we studied the effects of these steroid hormones on SRC-1 and SRC-3 expression in U373 and D54 human astrocytoma cell lines by RT–PCR and Western blot. We also determined the presence of PR and ER isoforms in both cell lines.

Results

Determination of PR and ER isoforms expression in U373 and D54 cell lines

We detected PR-A, PR-B, ER-α, and ER-β expression in human astrocytoma cell lines (U373 and D54) and T47D breast cancer cell line (as a positive control) by Western blot. PR-A and PR-B were detected as bands of 94 and 114 kDa, whereas ER-α and ER-β were determined as bands of 66 and 55 kDa, respectively. In U373 cells, PR-B was the predominant isoform (PR-B:PR-A ratio 3:1), whereas in D54 cells PR-A was the predominant one (PR-B:PR-A ratio 0.66:1) (Fig. 1).

P_4 and E_2 effects on SRC-1 and SRC-3 expression in U373 and D54 human astrocytoma cell lines

We assessed SRC-1 and SRC-3 gene expression both at mRNA and protein levels in U373 and D54 cell lines treated with P_4 and E_2 by RT–PCR and Western blot. In RT–PCR experiments single bands of 320, 338, and 219 bp corresponding to the expected size fragments of SRC-1,

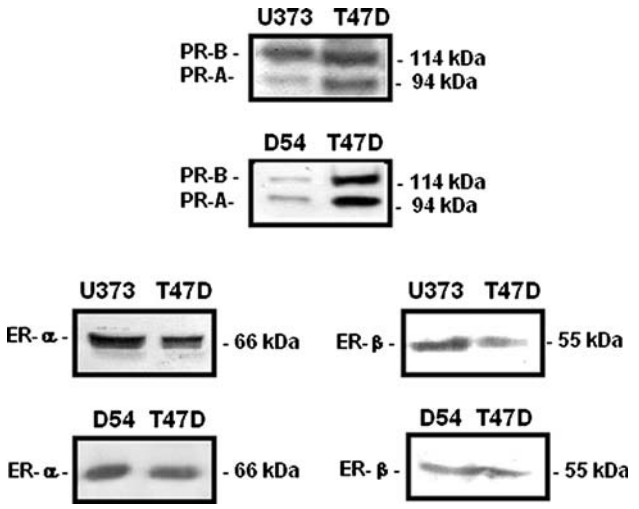

Fig. 1 PR and ER isoforms expression in U373 and D54 human astrocytoma cell lines. D54, U373 and T47D cells were lysed and proteins were separated by electrophoresis on 10% SDS-PAGE. Membranes were incubated with antibodies for PR (*upper panel*), ER-α or ER-*β* (*lower panel*) as described in Materials and Methods

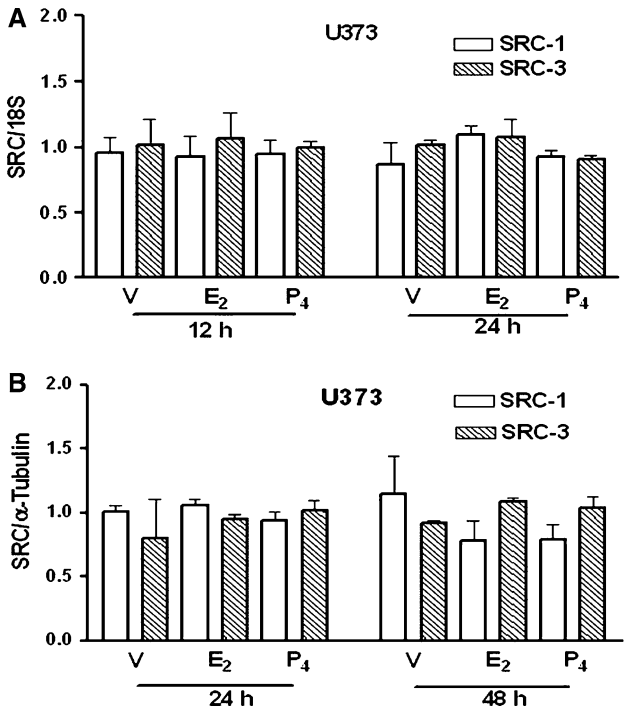

Fig. 2 SRC-1 and SRC-3 mRNA and protein expression in U373 human astrocytoma cell line. **a** Densitometric analysis of SRC-1 and SRC-3 mRNA expression. U373 cells were treated with vehicle (V; 0.02% cyclodextrin in sterile water), estradiol (E_2; 10 nM) or progesterone (P_4; 10 nM) for 12 and 24 h. RNA was obtained, reverse transcribed and PCR conducted using primers for SRC-1, SRC-3 or 18S (used for normalization). **b** Densitometric analysis of SRC-1 and SRC-3 protein content. U373 cells were treated with V, E_2 (10 nM) or P_4 (10 nM) for 24 and 48 h. U373 cells were lysed and proteins (100 μg) were separated by electrophoresis on 6% SDS-PAGE gel. Gels were transferred to polyvinylidene difluoride membranes and then incubated with antibodies for SRC-1, SRC-3 or α-tubulin. The α-tubulin signal was used for normalization. Results are expressed as mean ± S.E.M. of three independent experiments

SRC-3, and 18S were observed. No bands were observed in the negative controls (without RNA and with non-retrotranscribed RNA). In Western blot experiments, SRC-1 and SRC-3 were detected as bands of 160 kDa while α-tubulin was detected as a band of 55 kDa.

In U373 cells, neither P_4 nor E_2 significantly modified SRC-1 or SRC-3 expression after 12, 24, or 48 h of treatment (Fig. 2). In contrast, in D54 cells P_4 increased SRC-1 mRNA expression after 12 h of treatment without significant changes after 24 h (Fig. 3). P_4 also increased SRC-1 protein content after 24 h, but reduced it after 48 h (Fig. 4). SRC-3 expression was not regulated by P_4 in D54 cells (Figs. 3, 4). As in the case of U373 cells, E_2 did not change SRC-1 or SRC-3 expression in D54 cells (Figs. 3, 4). SRC-1 and SRC-3 expression at mRNA and protein levels was similar both in U373 and D54 cells (Figs. 2, 3, 4).

Discussion

In this study, we detected PR-A, PR-B, ER-α, and ER-*β* expression in two cell lines derived from human astrocytoma grade III (U373) and grade IV (D54). The expression of PR and ER isoforms suggests that P_4 and E_2 effects should be mediated by their receptor.

We determined the regulation of SRC-1 and SRC-3 expression by P_4 and E_2 in U373 and D54 cells. We found that only P_4 regulated SRC-1 expression in D54 cells, but not in U373 cells, and that SRC-3 expression was not regulated by either P_4 or E_2.

It has been reported that P_4 treatment (3 days) increases SRC-1 expression in stroma, but decreased it in luminal epithelium in mice uterus [30]. We found that P_4 only modifies SRC-1 expression in human astrocytoma cells with the highest evolution grade (D54), suggesting that the molecular environment present in these cells is fundamental for SRC-1 regulation by P_4.

PR isoforms are differentially expressed in human astrocytomas, González-Agüero et al. [14] observed that PR-B was the predominant isoform expressed in astrocytoma grade III and IV. We observed that PR isoforms expression pattern was different in U373 and D54 cells. Thus, PR-B was the predominant isoform in U373 cells, whereas in D54 cells PR-A was the predominant one. This suggests that PR isoforms exert a differential role in the regulation of SRC-1 expression by P_4 in human astrocytoma cells. In fact, it has been reported that PR-A and PR-B modify the expression of different genes in the uterus and breast cancer cells [48–50].

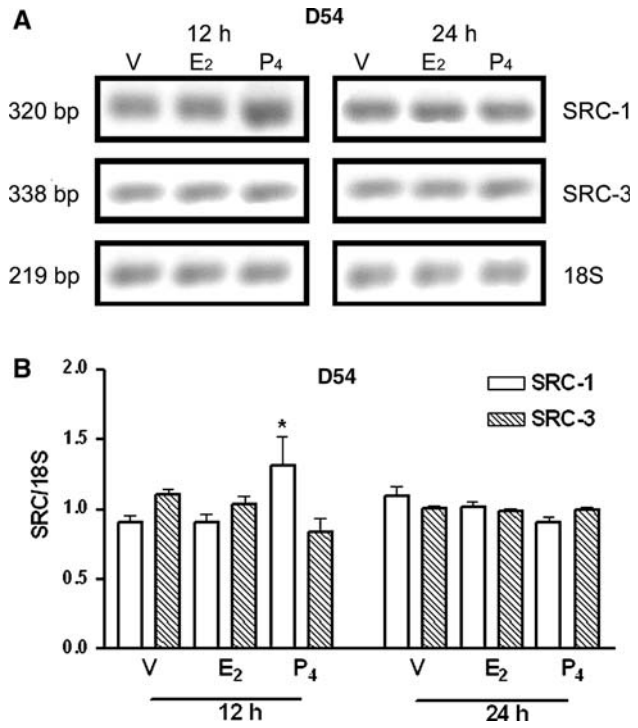

Fig. 3 Regulation of SRC-1 and SRC-3 mRNA expression by E_2 and P_4 in D54 human astrocytoma cell line. **a** RT–PCR analysis of SRC-1 and SRC-3 mRNA in D54 cells treated with V, E_2 (10 nM) or P_4 (10 nM) for 12 and 24 h. Representative assay of four independent experiments. **b** Densitometric analysis of SRC-1 and SRC-3 mRNA expression. RNA was obtained, reverse transcribed and PCR conducted using primers for SRC-1, SRC-3 or 18S. Coactivators levels were corrected by 18S. Results are expressed as mean ± S.E.M. * $P < 0.05$ compared to V

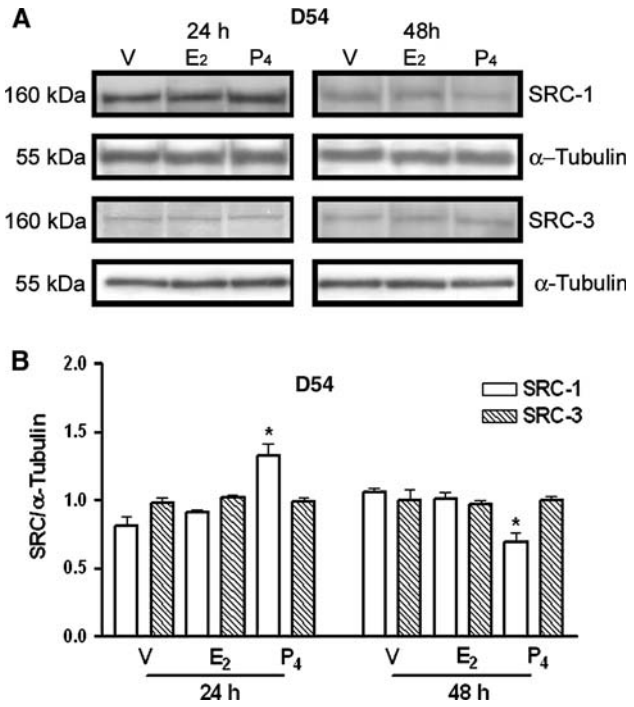

Fig. 4 Regulation of SRC-1 and SRC-3 protein content by E_2 and P_4 in D54 human astrocytoma cell line. **a** Western blot analysis of SRC-1 and SRC-3 content in D54 cells treated with V, E_2 (10 nM) or P_4 (10 nM) for 24 and 48 h. Representative assay of four independent experiments. **b** Densitometric analysis of SRC-1 and SRC-3 protein expression. Proteins were detected by Western blot and corrected by using data of α-tubulin protein expression. Results are expressed as mean ± S.E.M. * $P < 0.05$ compared to V

We found that P_4 increases SRC-1 mRNA expression in D54 cells 12 h after its administration. This increase was reflected at SRC-1 protein content 12 h later. The mechanism involved in this effect is not known, however, it is possible that P_4 regulates SRC-1 expression by PR through an indirect pathway since SRC-1 gene promoter lacks progesterone responsive elements (PRE), but PR can bind other transcription factors, including specificity protein 1 (Sp1), activator protein 1 (AP1), signal transducer, and activator of transcription 5 (STAT5), to regulate gene promoters that lack PRE sequences [51].

Interestingly, we observed that P_4 diminishes SRC-1 protein content 48 h after its application, an effect not previously reported in other cell lines. SRC-1 is degraded through ubiquitin-26S proteasome, since in the presence of 26S proteasome inhibitor MG132, SRC-1 content was at higher steady state level [52], and under physiological conditions the content of SRC-1 was significantly increased after MG132 injection into the rat brain [7], it is possible that P_4 could induce SRC-1 phosphorylation with the subsequent signalling to its degradation by 26S proteasome.

In contrast to SRC-1, the expression of SRC-3 was not modified by P_4 in any astrocytoma cells. This suggests a selective modulation of SRC coactivator family expression by P_4 that should be related to a differential role of SRC-1 and SRC-3 in transactivation function of PR in astrocytomas. In T47D cells, ligand-bound PR preferentially interacts with SRC-1 [29], whereas, SRC-3 is induced by E_2 [53].

Although in MCF-7 breast cancer cells SRC-1 and SRC-3 expression is regulated by E_2, we did not find such regulation in astrocytoma cells. This lack of effect is not due to the lack of ER isoforms expression, as we found that both ER isoforms were present in U373 and D54 cell lines.

Differences in SRC-1 and SRC-3 regulation are important because they can participate in the regulation of different genes by sex steroid hormones in the same cell that in the case of astrocytomas could be related with tumor progression or metastasis. Our data suggest that SRC-1 and SRC-3 expression is differentially regulated by sex steroid hormones in astrocytomas and that P_4 regulates SRC-1 expression depending on the evolution grade of human astrocytoma cells.

Materials and methods

Cell culture and treatments

U373 and D54 human astrocytoma cell lines derived from human astrocytoma grades III (ATCC, Manassas, VA) and IV, generously obtained by Dr. Andres Gutiérrez from Dr. Sontheimer (Bringham, Alabama) laboratory were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, and 0.1 mM non-essential amino acids (GIBCO N.Y.) for 24 h. Medium was changed by DMEM phenol red-free medium supplemented with 10% fetal bovine serum without steroid hormones (Hyclone, Utah), 1 mM pyruvate, 2 mM glutamine, and 0.1 mM non-essential amino acids (GIBCO N.Y.) at 37°C under a 95% air, 5% CO_2 atmosphere during 24 h. Then, the following treatments were applied for 12, 24, and 48 h: (a) vehicle (V; 0.02% cyclodextrin in sterile water); (b) E_2 (10 nM); and (c) P_4 (10 nM).

Total RNA extraction and RT–PCR

Total RNA was isolated from U373 and D54 cells 12 and 24 h after treatments with the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction according to the TRIzol Reagent manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1% denaturing agarose gels in the presence of 2.2-M formaldehyde. The first-strand cDNA was synthesized from 5 μg of total RNA by using SuperScript II reverse transcription (Invitrogen) and oligo $(dT)_{18}$ primers according to its protocol. Two μl of RT reaction were subjected to PCR to simultaneously amplify a gene fragment of SRC-1, SRC-3, and 18S ribosomal RNA. This was used as an internal control. The sequences of the specific primers for SRC-1 amplification fragment (from +1,015 to +1,333) were 5′- CCTCCAGCTATTACGGGTGTAG-3′ in the sense primer and 5′- ATGATGAAAGGTTGCAT GTCTG-3′ in the antisense. The primers used for SRC-3 amplification region (from +2,103 to +2,440) were 5′-GTCATTCCTCCTTGACCAACTC-3′ in the sense and 5′-ATCCCTGTCCAGCAGGTATCTA-3′ in the antisense. The 25-μl PCR reaction included: 2 μl of previously synthesized cDNA, 2.5 μl 10× buffer PCR II, 1.25 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μM of each primer, and 2.5 units of Taq DNA polymerase. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. After the initial denaturation step at 94°C for 5 min, PCR reaction was performed for, 28 (SRC-1), 30 (SRC-3), and 25 (18S) cycles. The cycle profile for SRC-1, SRC-3, and 18S amplification was: 1 min at 94°C, 1 min at 55°C (SRC-1), 62°C (SRC-3), or 60°C (18S) and 1 min at 72°C. A final extension cycle was performed at 72°C for 5 min. The number of performed cycles was within the exponential phase of the amplification process. All PCR products were always studied and analyzed together throughout the experiments. 25 μl of PCR products were separated on 2% agarose gel and stained with ethidium bromide. The image was captured under a UV transilluminator. The intensity of SRC-1, SRC-3, or 18S bands was quantified by densitometry using the Scion Image software (Scion Corp., Maryland). Coactivators expression level was normalized to that of 18S.

Protein extraction and western blotting

After 24 and 48 h of treatment, cells were homogenized in TDG lysis buffer with protease inhibitors (10 mM Tris–HCl, 1 mM dithiothreitol, 30% glycerol, 1% Triton X-100, 15 mM sodium azide, 1 mM EDTA, 4 μg/ml leupeptin, 22 μg/ml aprotinin, 1 mM PMSF, and 1 mM sodium ortovanadate). Proteins were obtained by centrifugation at 20,000×g, at 4°C for 15 min, and quantified by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Proteins (100 μg) were separated by electrophoresis on 6% (coactivators) or 10% (PR and ER isoforms) SDS-PAGE at 60 V. Colored and enhanced chemiluminescence markers (Bio Rad, CA, USA and Gibco-BRL, Maryland) were included for size determination. Gels were transferred 1 h to polyvinylidene difluoride membranes (Millipore) (211 mA, at room temperature in semi dry conditions), which were blocked at room temperature with 5% non-fat dry milk and 0.5% bovine serum albumin for 2 h. Membranes were then incubated with 0.5 μg/ml of antibodies against SRC-1 (Upstate 05-522; mouse monoclonal), SRC-3 (Santa Cruz sc-5305; mouse monoclonal), and PR (NeoMarkers RB-1492-P; mouse polyclonal), which recognizes two PR isoforms (PR-A and PR-B), ER-α (Santa Cruz sc-8002), or ER-β (Santa Cruz sc-6821) at 4°C overnight. Blots were then incubated with a 1:5,000 dilution secondary antibody conjugated to horseradish peroxidase (Santa Cruz sc-2033) for 1 h. Signals were detected by enhanced chemiluminescence (ECL) (Amersham, NJ).

In order to correct for differences in the amount of total protein loaded in each lane, SRC-1, SRC-3, PR, ER-α, and ER-β protein contents were normalized to that of α-tubulin. Blots were stripped with glycine (0.1 M, pH 2.5, 0.5% SDS) at 4°C overnight and at 37°C for 30 min, and reproved with a 1:10,000 dilution mouse anti-α-tubulin monoclonal antibody (Sigma T9026, Saint Louis, MO) at room temperature for 2 h. Blots were incubated with a 1:15,000 dilution goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz sc-2033) for 45 min at room temperature. Signals were detected by ECL. The

intensity of coactivators, PR and ER isoforms, and α-tubulin signals was quantified by densitometry using Scan Primax 600p apparatus (Colorado, Utrecht, the Netherlands) and the Scion Image software (Scion Corp., Maryland).

Statistical analysis

The mRNA and protein expression levels of coactivators were expressed as means ± S.E.M. and analyzed using SPSS 12.0 for Windows (SPSS Inc, Chicago, Il). Kruskal–Wallis groups test was used to determine differences between the effects of hormonal treatments and the control for each time point. Mann–Whitney test was used for comparison between groups. $P < 0.05$ was considered as statistically significant difference.

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