Journal of Steroid Biochemistry & Molecular Biology 116 (2009) 93–101

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Expression of estrogen receptor co-regulators SRC-1, RIP140 and NCoR and their interaction with estrogen receptor in rat uterus, under the influence of ormeloxifene

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ARTICLE INFO

Article history:
Received 13 October 2008
Received in revised form 22 April 2009
Accepted 8 May 2009

Keywords:
Ormeloxifene
Estrogen receptor
Co-regulators
Tamoxifen
Uterus

ABSTRACT

Ormeloxifene binds competitively to ERs and antagonizes estrogen-induced gene expression in the uterus. However its detailed molecular mechanisms are not well understood. Present study was aimed to examine the changes in expression pattern of co-regulatory proteins SRC-1 (co-activator), RIP140 and NCoR (co-repressors) and their interaction with ERα in rat uterus under the influence of ormeloxifene (Orm) and tamoxifen (Tam). Adult ovariectomized rats were treated with estradiol (E_2) (5 μg/100 g), or Orm or Tam (200 μg/100 g, s.c.) alone or along with E_2, for 3 days. RT-PCR analysis of uterine RNA and immunoblotting of uterine extracts revealed that expression of SRC-1, RIP140 and NCoR was insensitive to E_2 or Orm or Tam treatment. Direct protein–protein interaction experiments using co-immunoprecipitation revealed that E_2-induced the interaction of ERα with co-activator SRC-1. In rats given Orm alone or along with E_2, there was a significant reduction in E_2-induced effect on ERα–SRC-1 interaction. In case of ERβ and SRC-1, Orm reduced interaction only in the absence of E_2. Interaction of RIP140 or NCoR with ERα was found to be more in rats treated with Orm along with E_2 as compared to that in E_2-treated rats whereas no such recruitment was found in Tam treated rats. Interaction of RIP140 with ERβ was insensitive to Orm or Tam treatment whereas the interaction of NCoR with ERα and ERβ was increased in Orm treated rats. Ormeloxifene also showed inhibitory effects on uterine ER–ERE binding and estrogen-induced expression of progesterone receptor. Taken together, these findings demonstrate that ormeloxifene antagonizes ERα-mediated transcription by inhibiting the recruitment of SRC-1 and inducing the recruitment of RIP140 and NCoR.

1. Introduction

Estrogen plays crucial role in development of normal functions of female reproductive tract, secondary sex characteristics and in reproductive behavior. The biological actions of estrogen are mediated by two subtypes of ER, i.e. α and β. Both receptors belong to a family of ligand-activated nuclear transcription factors [1,2] and share a high degree of homology in their DNA binding domains [3]. However, they differ considerably in their N-terminal regions, which contain the ligand-independent transcription activation function, AF-1 [3,4]. These differences suggest that ERα and ERβ could have distinct functions in terms of gene regulation and may contribute to the selective action of ER-ligands in different target tissues [5,6].

Ligand binding to ER results in a conformational change in the receptor that allows dimerization, DNA binding, interaction with co-regulators, and, ultimately, the modulation of gene transcription [7]. Thus, the estrogen receptor co-regulators are important molecules intervening between the receptors and target genes and are functionally divided into two subclasses, i.e. co-activators and co-repressors. The former stimulates, and the latter suppresses, the transcription of target genes. Thereby, adding the complexity to understand the mechanism of action of various ER-ligands such as estrogens and selective estrogen receptor modulators (SERMs). It is now clear that the agonist action of E_2 causes ER to recruit co-activators, the p160 family of co-activators, SRC-1/NCoA1, SRC-2/TIF2/GRIP1, and SRC-3/RAC3/ACTR/pCIP/AIB1/TRAM-1, have all been shown to recruit histone acetyltransferases (HAT) which disrupt electrostatic bonds on DNA and increase access of transcription factors to the promoter [8,9]. In contrast, the antagonist action of selective estrogen receptor modulators such as tamoxifen causes ER to recruit co-repressors, which down-regulate the transcriptional activity of the ER [10]. The co-repressors, NCoR and SMRT, repress transcription by recruiting histone deacetylases (HDAC) [11]. There-

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doi:10.1016/j.jsbmb.2009.05.006

fore, changing the level of co-regulators in cells has been shown to alter estrogen and SERMs activity [12] and to directly regulate cell growth [13,14]. In addition, there has been tremendous progress in understanding the biochemical nature of ERα and more recently ERβ, and how they interact with estrogen and antiestrogen ligands and with co-regulator proteins that modulate receptor transcriptional activity. These factors no doubt underlie the cell-specific and promoter-specific activities of SERMs in different target cells and at different gene sites.

Ormeloxifene (Orm) has been developed and marketed as once-a-week oral contraceptive [15]. Ormeloxifene binds to ER in a competitive manner and interferes with the ER–ERE-mediated transcription and uterine events leading to blastocyst implantation [16–18]. It is estrogen agonist in bone and cardiovascular system [19–21] while inhibits proliferation of MCF-7 human breast cancer cells [22]. Ormeloxifene binds to both ER subtypes α and β, has been shown to be a selective estrogen receptor modulator, however, its detailed mechanism of action is not fully understood. Moreover, what set of co-regulators are recruited after binding of Orm to ER remain to be investigated.

The present study was therefore, aimed to understand the molecular mechanism of ormeloxifene by investigating its influence on the expression levels of ERα, ERβ and their co-regulators (SRC-1, NCoR and RIP140) in rat uterus. In addition, to evaluate the functional complex formation between the co-regulators and ERα or ERβ, the interaction of co-regulators with ERs was examined by co-immunoprecipitation. Finally, relevance of interaction of co-regulators with ERα and ERβ was compared with the expression of PR. We proposed that the relative expression of co-activator (SRC-1) or co-repressors (RIP140 and NCoR) and their selective interaction with ERα or ERβ is one possibility, which may explain the target site specificity of Orm in rat uterus.

2. Materials and methods

2.1. Chemicals

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Company, USA. γ-P^{32}-dATP (specific activity, 5000 Ci/mmol) was procured from Bhabha Atomic Research Center, Trombay, India. Antibodies were purchased from Santa Cruz, USA.

Ormeloxifene was obtained from Division of Medicinal Chemistry of CDRI, Lucknow, India.

2.2. Experimental animals and treatment schedule

All animal studies were performed according to guidelines governed by institutional animal ethics committee. Adult (60-day-old) female rats (Sprague–Dawley strain) of our institute's colony maintained under uniform animal husbandry conditions (temperature 24 ± 1 °C) with free access to pelleted food and water were used in this study. Rats were ovariectomized under light ether anesthesia and given rest period of 2 weeks before giving treatment. These rats were given 17β-estradiol (5 μg/100 g body weight, s.c.), or Orm or Tam (200 μg/100 g body weight, s.c.) or estradiol plus-Orm or -Tam for 3 days. Animals of control group were given vehicle (olive oil) alone. Rats were euthanized on fourth day by giving ether anesthesia. Uteri were removed, cleaned, weighed and stored at −70 °C until analyzed.

2.3. Electrophoretic mobility shift assay (EMSA)

2.3.1. Labeled probe (ERE) preparation

Synthetic oligonucleotides (30mer, Bangalore Genei, Bangalore, India) containing 13 bp perfect palindromic vitellogeninA2-ERE were annealed and 5′-labeled with γP^{32}-dATP using T4 polynucleotide kinase (NewEngland Biolabs, UK). Labeled probes were separated from unlabeled oligonucleotides by spinning through G-50 micro-spin columns (Amersham Biosciences, UK) for 2 min at 4 °C and stored at −20 °C until used.

2.3.2. Nuclear fraction extraction

Nuclear extracts from uterine tissue were prepared as described previously by Navarro et al. [23]. Briefly, tissue was homogenized in TGD buffer (20 mM Tris–HCl, pH 7.5 containing 20% (v/v) glycerol, 2 mM dithiothreitol, 2 mM phenylmethyl sulfonyl fluoride and 2 μg/ml leupeptin) and nuclei were isolated by centrifugation at 800 × *g* for 20 min at 4 °C. The isolated pellet was re-homogenized in TGD buffer containing 400 mM KCl and protease inhibitors, incubated for 3 h at 4 °C and centrifuged at 35,000 × *g* for 30 min at 4 °C. The supernatants (nuclear protein extracts) were collected and stored at −70 °C until used. Protein concentrations were determined by using Bradford method.

2.3.3. Electrophoretic mobility shift assay of uterine ER–ERE complex

Nuclear extract of rat uteri of different groups were diluted 2-fold with TGD buffer and further 2-fold with water in order to bring the 400 mM KCl to final concentration of 100 mM KCl. About 8 μg of protein was pre-incubated with 2 μg of poly-deoxyinosinic-deoxycytidylic acid (poly dI.dC.) for 15 min at 0 °C. The binding reaction was initiated by adding [^{32}P] 5′-end labeled ERE element (∼1 pmol) in 30 μl reaction volume in a final concentration of buffer component 10 mM Tris–HCl pH 7.5, 1 mM DTT, 100 mM KCl and 10% (v/v) glycerol and incubated for 15 min at 20 °C. For the electromobility shift competition assay, 50-fold molar excess of unlabeled competitor oligonucleotide was mixed with the labeled probe before adding them to the binding reaction. For supershift assay, 2 μg antibodies against ERα (MC-20) or ERβ (H-150) were added to reaction mixture before adding oligonucleotide probe. The samples were separated on 5% non-denaturing polyacrylamide gel in 6.7 mM Tris–HCl, pH 6.8, 3.3 mM sodium acetate and 1 mM EDTA at 30 mA with circulating buffer at 4 °C. After electrophoresis, gels were dried and autoradiographed, and shifted bands were quantified on gel documentation system (Gel Doc 2000, Biorad) using Quantity One® software.

2.4. Real-time PCR

2.4.1. RNA isolation and DNase treatment

The uterus was homogenized in Trizol reagent (Invitrogen) and total RNA was extracted from each group as given in manufacturer's instructions. Total RNA concentration was assessed by A_{260} and checked for DNA contamination on an ethidium bromide-stained 2.5% agarose gel. RNA was stored at −80 °C until needed.

2.4.2. PCR

Experiments were performed as per manufacturer's instructions (Qiagen) to determine the optimal primer and RNA concentration for each gene. The primers used for this study were, forward: 5′-GTG TGA GGA TTC TGC CTT TC-3′ and reverse: 5′-CGC TCT CAG GTC TTC TTA CG-3′ for PR; forward: 5′-ATC GTG GGG CCG CCC CTA GGC-3′ and reverse: 5′-TGG CCT TAG GGT TCA GAG G-3′ for β-actin. Finally, 2× Quantitect RT-PCR Master mix was added to 10 ng RNA and 0.5 μM of primers (forward and reverse) in a 25 μl reaction. Reactions were carried out in Mx3000p™ (Stratagene) for 35 cycles (50 °C for 30 min, 95 °C for 15 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s after the initial cycle for SyberGreen at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s). The fold change in expression of gene was calculated using relative expression ratio (*R*) method, with the

β-actin mRNA as an internal control [24]. The *R* of target gene is calculated based on Ct deviation of an unknown sample *vs.* a control, and expressed in comparison to a reference gene.

2.5. Western blot analysis for ERα, ERβ and PR

Uterine tissue of different groups was homogenized (500 mg/ml) in an ice-cold RIPA buffer (i.e. 10 mM Tris, 10 mM EDTA, 0.15 M NaCl, 1% NP-50, 0.5% SDS and protease inhibitors) and incubated on ice for 1 h. The whole cell lysate was obtained by centrifugation at 16,000 × *g* for 15 min at 4 °C. Protein was estimated using Bradford's reagent. Equal amounts of protein were run on 8% SDS-PAGE gels and transferred onto PVDF membrane (0.45 μM, Millipore) in 48 mM Tris–buffer pH 8.3 containing 39 mM Glycine, 0.0375% SDS and 20% methanol for 1 h at 100 V. Non-specific sites were blocked with 5% (w/v) blocking reagent (Amersham Biosciences) in PBS-T (phosphate buffer saline with 0.1% Tween-20) for 1 h at room temperature and then incubated overnight with the primary antibody, anti-ERα (1:10,000), anti-ERβ (1:8000), anti-PR (1:10,000) and anti-β-actin (1:16,000) diluted in PBS-T, at 4 °C. After washes with PBS-T, the membranes were incubated for 1 h with the appropriate secondary antibodies conjugated to horseradish peroxidase. Bands were detected using ECL-detection system (Amersham Biosciences). Densitometric analyses were performed using gel documentation system (Gel Doc 2000, Biorad) using Quantity One® software.

2.6. Co-immunoprecipitation (CoIP) of SRC-1, RIP140 and NCoR and immunoblotting

Complex formation between co-regulators and ERα or ERβ was examined in uterine whole cell lysates prepared as described above. Protein A-Sepharose beads were used and desired protein was immunoprecipitated according to manufacturer's instructions. Briefly, 2 μg of either anti-SRC-1 (M-20) or anti-RIP140 (H-300) or anti-NCoR (N-19) was added to 500 μg of cell lysate and samples were incubated for overnight at 4 °C with constant rocking. In negative control, cell lysate was incubated with corresponding non-immune serum instead of anti-SRC-1 or anti-RIP140 or anti-NCoR. Following incubation, 100 μl of beads suspension was added and samples were incubated for 1 h at 4 °C. Immunoprecipitated complexes were collected by centrifugation at 3000 × *g* for 2 min at 4 °C and washed three times with RIPA buffer. Immunoprecipitates were resuspended in Laemmli sample buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 20% Glycerol, 10% β-mercaptoethanol and 0.004% bromophenol blue) to a final concentration of 1 × sample buffer and heated for 5 min at 95 °C. The supernatants were collected by centrifugation at 12,000 × *g* for 30 s at room temperature. Equal volume of immunoprecipitated proteins were run on 8% SDS-PAGE and transferred on PVDF membrane. The proteins were probed with anti-ERα (1:8000) or anti-ERβ (1:6000), followed by the corresponding peroxidase-conjugated secondary antibody. Bands were detected and analyzed as described above. Same membrane was stripped and reprobed with either anti-SRC-1 (1:3000), anti-RIP140 (1:2000) or anti-NCoR (1:2000) depending on their corresponding immunoprecipitated samples. For reprobing, the membrane was first deprobed by incubating it in deprobing buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 98% βME) for 45 min at 50 °C with constant shaking. Membrane was then washed with PBS-T three times and blocked with blocking agent. Rest procedure was same as described before.

2.7. Statistical analysis

Data were compared with their corresponding control groups and expressed as percentage of control. The statistical analysis was performed by one-way ANOVA and significance between treatment groups was determined by Newman–Keul's test. p values >0.05 were considered insignificant.

3. Results

3.1. Effect of Orm on the ER–ERE interaction and the expression of E_2-responsive genes in ovariectomized rats

3.1.1. ER–ERE interaction

Orm modulates transcription by affecting the ability of the estrogen receptor (ER) to bind to DNA elements. Thus, the ability of ER to bind vitellogeninA2-ERE in uterine nuclear extract from E_2 and SERM treated rats were studied by electrophoretic mobility shift assay (EMSA). Fig. 1 represents the results of estrogen receptor–DNA interaction analyzed by electrophoretic mobility shift assay. The amount of the shifted probes was normalized with the ERα expression in the corresponding groups. The value in total binding (control) was taken as 100 and rests of the values were normalized with it. The specificity of ER–ERE binding activity was evaluated by competition assay, where the addition of excess unlabelled ERE resulted in suppression of the specific ER–ERE complex. When specific antibodies to ERα or ERβ were incubated, the ER–ERE complex formation was not observed. This confirmed the presence of both receptors in ER–ERE complexes.

Results revealed that in E_2-treated rats, binding was found to be more ($p < 0.001$) as compared to that in vehicle control group. Likewise, binding was more when rats were treated with Orm or Tam alone or along with E_2 ($p < 0.05$). However, Orm or Tam in the

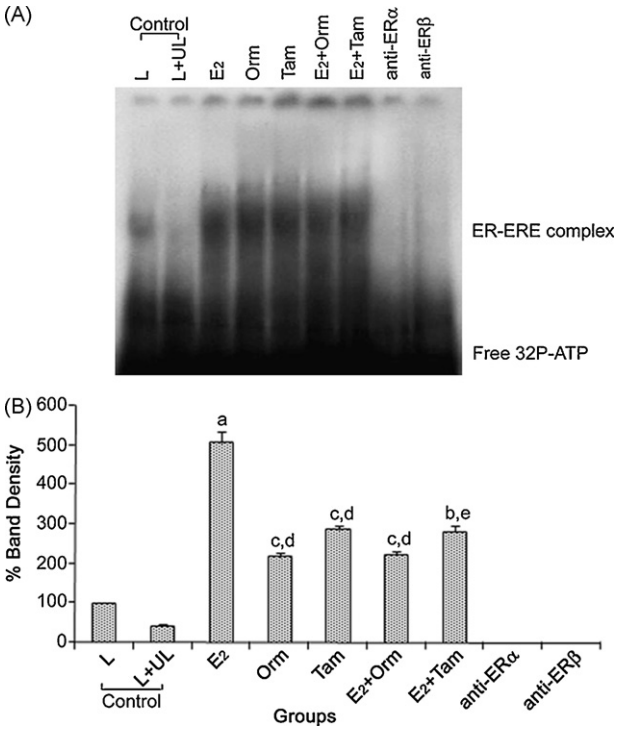

Fig. 1. The effect of Orm on the ER–ERE binding activity of ovx rat uterine nuclear extracts analyzed by EMSA using a ^{32}P-labeled ERE probe. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. (A) Representative autoradiogram showing ER–ERE complex in uterine nuclear extract. ER–ERE binding activity was specific as binding was suppressed in the presence of unlabeled excess oligonucleotide (UL), L-labeled oligonucleotide. (B) Densitometric analysis of the bands of ER–ERE complex. The amount of the shifted probes was normalized with the expression of ERα. The value of total binding (from control group) was taken as 100 and rest samples were normalized with respect to it. Results are presented as mean ± SEM of three separate experiments. [a] $p < 0.001$ *vs.* L (control); [b] $p < 0.01$ *vs.* L; [c] $p < 0.05$ *vs.* L; [d] $p < 0.001$ *vs.* E_2; [e] $p < 0.01$ *vs.* E_2.

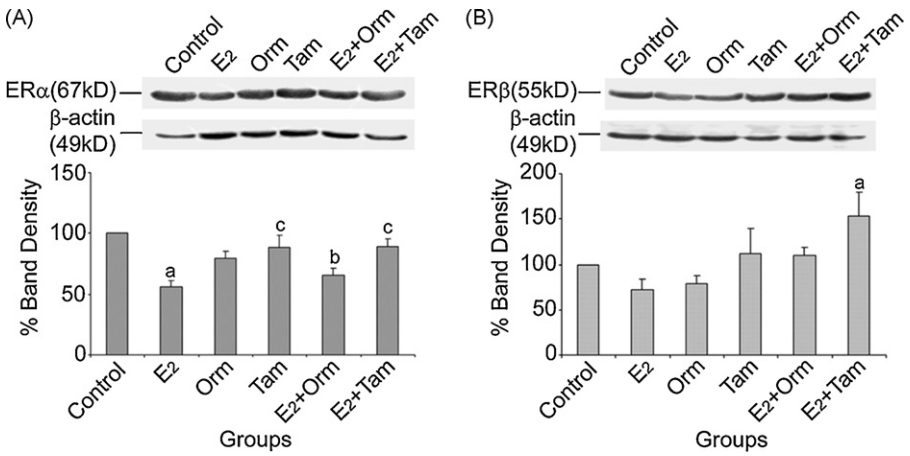

Fig. 2. Expression of ERα and ERβ in rat uterus. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. β-Actin was used as internal control. The values were first normalized to corresponding β-actin levels and then expressed as relative to control group taken as 100. The results are presented as mean ± SEM of 3 independent experiments. (A) Upper panel shows the representative blots of ERα and β-actin and lower panel shows the densitometric analysis of bands. [a] $p < 0.01$ *vs.* control; [b] $p < 0.05$ *vs.* control; [c] $p < 0.05$ *vs.* E_2. (B) Upper panel shows the representative blots of ERβ and β-actin and lower panel shows the densitometric analysis of bands. [a] $p < 0.05$ *vs.* control, *vs.* E_2.

presence or absence of E_2 was able to reduce binding significantly ($p < 0.001$) as compared to that of E_2-treated rats.

3.1.2. *Expression of ERα, ERβ and PR*

The regulation of E_2-responsive genes by Orm was investigated and results were compared with Tam. Since E_2 directly regulates the expression of ERα, ERβ and progesterone receptor (PR), expression of these proteins was examined by Western blotting. Expression of β-actin was used as internal control. The value of control group was taken as 100 and rest of the values were normalized with respect to it.

3.1.2.1. ERα. Fig. 2A shows the results of Western blot analysis of ERα expression in ovx rats. Densitometric analysis of bands revealed that uterine levels of ERα protein decreased ($p < 0.01$) to about 50% of vehicle treated group after E_2-treatment. In addition, no change was observed in Orm or Tam treated rats as compared to that in vehicle treated controls, however, Orm along with E_2 showed decreased ($p < 0.05$) expression levels.

In relation to E_2, both SERMs behaved differently. Tam alone and along with E_2 was able to increase ($p < 0.05$) ERα expression levels. On the other hand, no significant change ($p > 0.05$) was observed in Orm treated rats as compared to E_2-treated rats.

3.1.2.2. ERβ. Expression level of ERβ protein was not significantly ($p > 0.05$) altered in E_2 or Orm or Tam treated rats as compared to vehicle treated control rats. When Orm is co-administered with E_2, the expression level was not changed ($p > 0.05$) significantly, however in rats receiving Tam co-administered with E_2, the expression was increased ($p < 0.05$) significantly to about 53% of that of control group (Fig. 2B).

3.1.2.3. PR-B. PR expression is considered ER-dependent in rat uterus. PR normally exists as two isoforms, PR-A and PR-B originating from two promoters. PR-B is full-length and functional isoform. Therefore, its mRNA expression was measured by real-time PCR and further protein levels were examined by Western blot analysis.

Fig. 3 shows the relative mRNA levels obtained in different treated groups. It was observed that PR mRNA expression levels were induced by 8-fold after E_2-treatment and nearly by 4-fold after Orm or Tam treatment as compared to vehicle treated control rats. When E_2 was co-administered with Orm or Tam, mRNA expression level was found to be 4-fold higher as compared to that of control. Orm and Tam were able to down-regulate the E_2-induced up-regulation of PR mRNA expression.

Fig. 4 shows the results of a Western blot analysis of PR-B. The specificity of band was confirmed by using blocking peptide specific to antibody against PR-B. Blocking peptide was added to antibody prior to the incubation. The blot in which incubation was given with blocking peptide, the specific band disappeared, which confirmed the specificity of band (figure not shown). Densitometric analysis (Fig. 4B) shows that the expression of PR-B was up-regulated 2.5-fold ($p < 0.01$) after E_2-treatment as compared to control group. However, no change in expression ($p > 0.05$) was observed in rats receiving Orm or Tam as compared to control group whereas when Tam was co-administered with E_2, expression of PR-B was up-regulated nearly 1.8-fold ($p < 0.05$) of control group.

3.2. *Effect of Orm on expression of SRC-1, RIP140 and NCoR in ovariectomized rat*

In order to examine the effect of Orm on the expression levels of different co-regulators in rat uterus, expression of co-activator SRC-1 and co-repressors RIP140 and NCoR were investigated by semi-quantitative RT-PCR and Western blotting.

RT-PCR analysis revealed that SRC-1, RIP140 and NCoR expression at mRNA level remained unchanged after E_2 or Orm or Tam treatment of ovariectomized adult rats. No significant change in expression was observed in groups where E_2 was co-administered

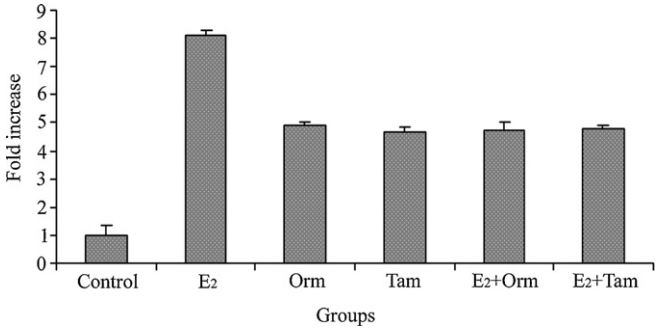

Fig. 3. Changes in PR-B mRNA expression as determined by real-time PCR in rat uterus. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. β-Actin was used as internal control. Bar diagram showing fold increase relative to vehicle control.

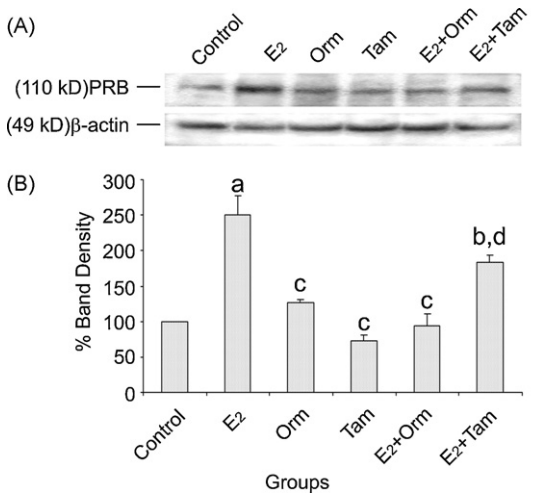

Fig. 4. Expression of PR-B as determined by Western blot in rat uterus. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. β-Actin was used as internal control. (A) Representative blot of PR-B and β-actin. (B) Densitometric analysis of bands. The values were first normalized to corresponding β-actin levels and then expressed as relative to control group taken as 100. The results are presented as mean ± SEM of three independent experiments. $^{a}p < 0.01$ *vs.* control; $^{b}p < 0.01$ *vs.* E_2; $^{c}p < 0.05$ *vs.* E_2.

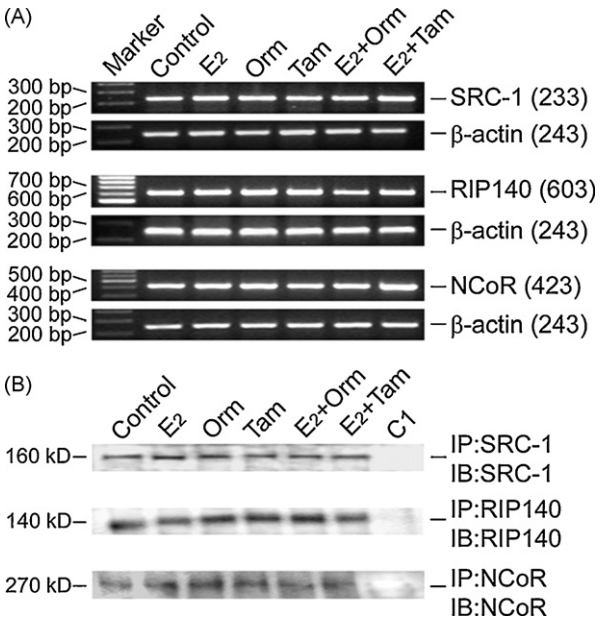

Fig. 5. Expression of SRC-1, RIP140 and NCoR as determined by RT-PCR and immunoprecipitation in rat uterus. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. The results are presented as mean ± SEM of three independent experiments. (A) Representative gel-image of SRC-1, RIP140 and NCoR. β-Actin was used as internal control. (B) Representative blot of SRC-1, RIP140 and NCoR. C1 is the negative control in which cell lysate was incubated with non-immune serum instead of anti-SRC-1 or anti-RIP140 or anti-NCoR.

with Orm or Tam (Fig. 5A). Western blot analysis of co-regulators expression in immunoprecipitated samples of uterine cell extract using specific antibodies revealed no change in expression levels of SRC-1, RIP140 and NCoR under the influence of E_2, Orm or Tam treatment (Fig. 5B).

3.3. *Effect of Orm on interaction of SRC-1, RIP140 and NCoR with ERα and ERβ in ovariectomized rats*

SRC-1, RIP140 and NCoR were immunoprecipitated from uterine cell extract using their antibodies and then immunoblotted with anti-ERα. In negative control, cell lysate was incubated with corresponding non-immune serum instead of anti-co-regulator antibody. As shown in Fig. 2, the expression level of ER was changed in response to different treatments, which might affect its amount in the CoIP complex. So to avoid the masking of the real effect, the CoIP results were first normalized to the corresponding ERα or ERβ levels. Then, the expression level of control group was taken as 100 and rest of the values were normalized with it.

Fig. 6A shows the interaction of SRC-1 with ERα. In E_2-treated rats, interaction with ERα was increased significantly ($p < 0.001$) as compared to that in ovx control rats. In rats where Orm or Tam was given alone or along with E_2, interaction was decreased ($p < 0.01$ or <0.001) significantly as compared to E_2-treated rats. In relation to ERβ (Fig. 6B), interaction was not significantly ($p > 0.05$) affected

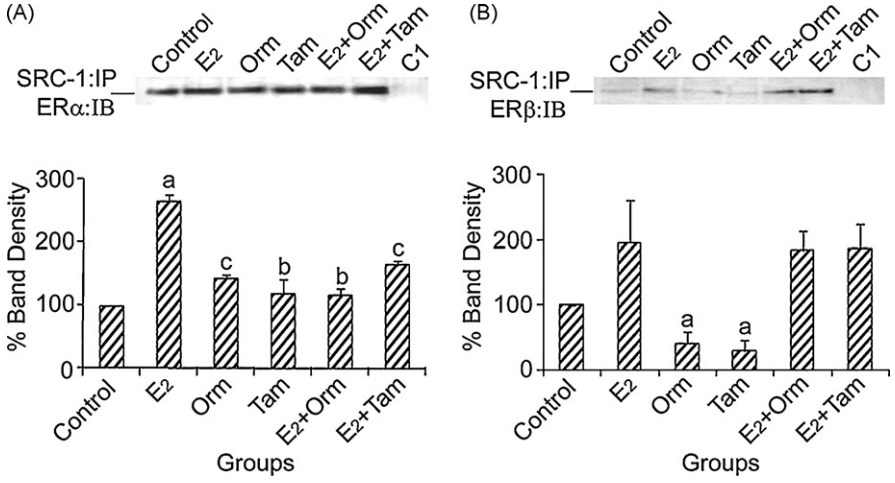

Fig. 6. Interaction of SRC-1 with ERα and ERβ as determined by co-immunoprecipitation in rat uterus. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. Uterine cell lysates were immunoprecipitated with anti-SRC-1 and subsequently immunoblotted with anti-ERα or anti-ERβ. C1 is the negative control in which cell lysate was incubated with non-immune serum instead of anti-SRC-1. The results were presented as mean ± SEM of two independent experiments. The bands were normalized to the expression of ERα or ERβ. The values are expressed as relative to control group taken as 100. (A) Upper panel shows the representative blot of ERα and lower panel shows the densitometric analysis of bands. $^{a}p < 0.001$ *vs.* control; $^{b}p < 0.001$ *vs.* E_2; $^{c}p < 0.01$ *vs.* E_2. (B) Upper panel shows the representative blot of ERβ and lower panel shows the densitometric analysis of bands. $^{a}p < 0.05$ *vs.* control, *vs.* E_2.

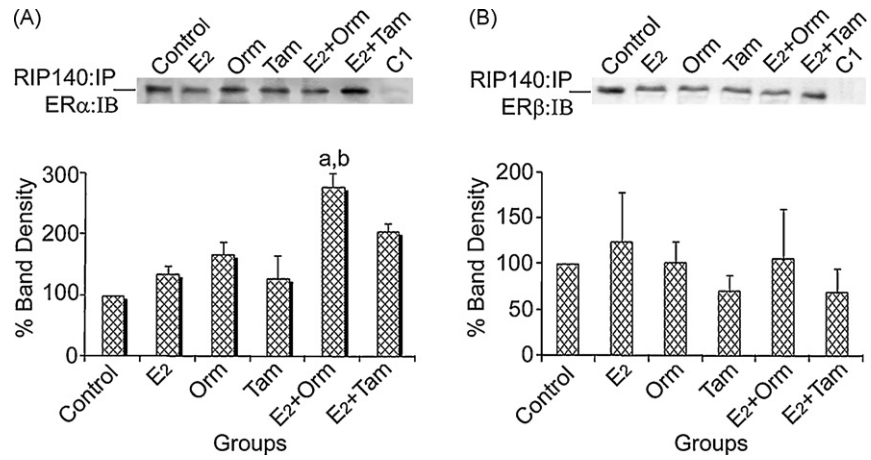

Fig. 7. Interaction of RIP140 with ERα and ERβ as determined by co-immunoprecipitation in rat. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. Uterine cell lysates were immunoprecipitated with anti-RIP140 and subsequently immunoblotted with anti-ERα or anti-ERβ. C1 is the negative control in which cell lysate was incubated with non-immune serum instead of anti-RIP140. The results are presented as mean ± SEM of two independent experiments. The bands were normalized to the expression of ERα or ERβ. The values were expressed as relative to control group taken as 100. (A) Upper panel shows the representative blot of ERα and lower panel shows the densitometric analysis of bands. [a]$p < 0.01$ *vs.* control and [b]$p < 0.05$ *vs.* E_2. (B) Upper panel shows the representative blot of ERβ and lower panel shows the densitometric analysis of bands.

in E_2-treated rats as compared to that in ovx control rats while reduced significantly in Orm or Tam treated rats as compared to ovx controls as well as E_2-treated rats ($p < 0.05$).

Fig. 7A illustrates the interaction of RIP140 with ERα. The RIP140 interaction with ERα was significantly increased when Orm was given along with E_2 as compared to ovx vehicle treated control ($p < 0.01$) as well as E_2-treated rats ($p < 0.05$). Fig. 7B shows the interaction of RIP140 with ERβ. No significant change ($p > 0.05$) was observed in the interaction of RIP140 with ERβ in E_2 or SERMs treated rats as compared to that observed in ovx controls as well as E_2-treated rats. Similarly, when E_2 was co-administered with Orm or Tam, interaction was not significantly ($p > 0.05$) changed.

Fig. 8A shows the interaction of NCoR with ERα. Densitometric analysis revealed that in Orm treated rats, interaction of NCoR with ERα was not changed ($p < 0.05$) but increased significantly ($p < 0.001$) when co-administered with E_2 as compared to ovx vehicle treated control as well as E_2-treated rats ($p < 0.001$ and <0.01 respectively). However, no significant change ($p > 0.05$) in interaction was observed in Tam or E_2 + Tam treated rats. Fig. 8B shows the interaction of NCoR with ERβ. Results showed that in rats treated with E_2 alone or along with SERMs, the interaction remained unaffected as compared to that of vehicle treated controls ($p > 0.05$). Whereas interaction was increased significantly ($p < 0.05$) in Orm treated rats as compared to that observed in control or E_2-treated rats.

3.4. Uterine weight

Estradiol caused a significant increase in uterine wet weight ~2.5-fold of ovx rats when administered at 5 μg dose, s.c. for 3 days. In rat receiving Orm along with E_2 or alone, uterine weight was increased as compared to ovx control rats but it was significantly less than that observed in E_2-treated group ($p < 0.001$). Similarly, tamoxifen also induced the uterine weight gain ($p < 0.001$) in comparison to control but not as efficiently as observed in E_2-treated rats (Fig. 8).

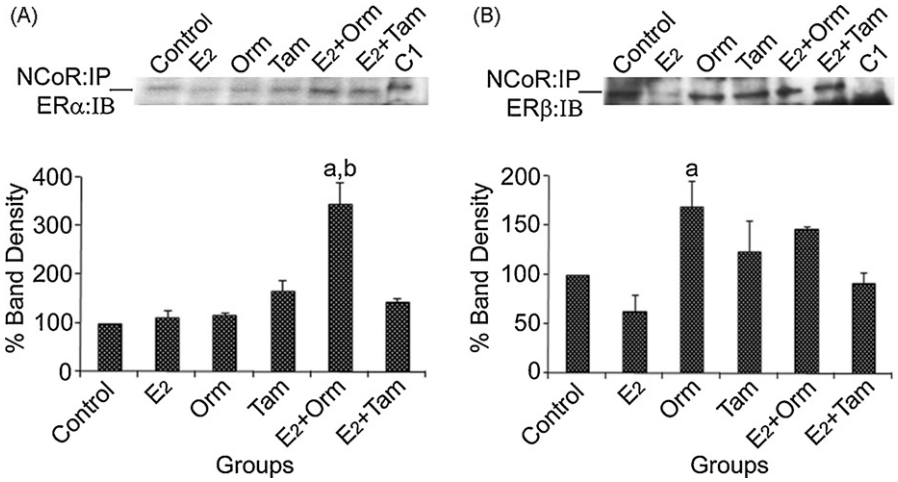

Fig. 8. Interaction of NCoR with ERα and ERβ as determined by co-immunoprecipitation in rat. Ovx rats were treated with E_2 (5 μg/100 g, s.c.) or Orm (200 μg/100 g, s.c.) or Tam (200 μg/100 g, s.c.) or with Orm + E_2 or Tam + E_2 or vehicle (olive oil) for 3 days and euthanized on fourth day. (A) Uterine cell lysates were immunoprecipitated with anti-NCoR and subsequently immunoblotted with anti-ERα or anti-ERβ. C1 is the negative control in which cell lysate was incubated with non-immune serum instead of anti-NCoR. The results are presented as mean ± SEM of two independent experiments. The bands were normalized to the expression of ERα or ERβ. The values were expressed as relative to control group taken as 100. (A) Upper panel shows the representative blot of ERα and lower panel shows the densitometric analysis of bands. [a]$p < 0.001$ *vs.* control and [b]$p < 0.01$ *vs.* E_2. (B) Upper panel shows the representative blot of ERβ and lower panel shows the densitometric analysis of bands. [a]$p < 0.05$ *vs.* E_2.

4. Discussion

The selective action of SERMs in a given cell or tissue is the combined effect of several factors that collectively modulate the ligand-bound ER activity. In the present investigation, modulation of ERE-mediated response, the expression levels of co-regulators, and their interaction with ER under the influence of Orm, a SERM, was studied in adult ovariectomized rat uterus and the results were compared with Tam. Previous evidences are mostly presented on the basis of experiments done in cultured cells, however, little is known of the recruitment of co-regulators in rat uterus that has often been used as an experimental model to study estrogen action.

Orm binds to ERα with higher affinity than ERβ [18]. In our laboratory, we have previously shown that binding of Orm with uterine ER resulted into the promotion of ER interaction with ERE under transformed conditions [18]. Here, we provided further *in vivo* evidence of modulation of gene transcription by Orm through classical pathway. We demonstrated that in E_2-treated rats, binding of ER to ERE was increased ~5-fold of vehicle control group. In addition, binding was increased in rats treated with Orm or Tam alone or along with E_2 as compared to that of control treated rats but it was less than that of E_2-treated group (Fig. 1). The inhibition of formation of ER–ERE complex was observed in the presence of anti-ERα or anti-ERβ antibody, suggesting the involvement of both receptors in ERE-mediated response in uterus. Therefore, Orm might decrease transcription by decreasing the ability of the receptor to bind to DNA elements specifically. The precise mechanism whereby differences in the kinetics of receptor–DNA interaction are induced by the binding of the ligand and how that relates to the observed behavior of the estrogen receptor *in vivo* is not yet known. Cheskis et al. hypothesized that ligand binding may affect the kinetics of ER interaction with ERE while having minimal effect on its affinity [25]. Also the order of stability of ER–ERE complexes corresponds to the increase in the antagonistic activity of compounds [26]. Therefore, detailed kinetic study is further required to add to our understanding of how transcription may be regulated by Orm and also may give clue to tissue and promoter selective modulation of ER by such molecules.

Besides ER–ERE interaction, ligand binding also induces a conformational change within the ligand binding domain of the ER, and this conformational change allows co-regulator proteins to be recruited and regulate the gene expression. We therefore, determined the effect of Orm on expression levels of ERα, ERβ and PR in ovx rat. We observed that in E_2-treated rats, protein expression of ERα was down-regulated to about 50% of that of control group while expression of ERβ remained unaffected (Fig. 2). This observation is in agreement with a previous study of Nephew et al. who reported that E_2-treatment down-regulates ERα mRNA and receptor protein in a cell type-specific manner in the ovx rat uterus [27]. Varayoud et al. [28] demonstrated that when ovx rats received the high dose of E_2, a significant decrease in both ERα mRNA and protein was observed in the uterus. However, when rats were treated with the low dose of E_2, only the ERα protein was down-regulated and no changes were observed in ERα mRNA expression [28]. Therefore, reduced ERα protein levels in whole uterus could result from its differential regulation within individual cell types and E_2-induced degradation. Several lines of evidence suggest that proteasome pathway is involved in E_2-induced degradation of ERα, which has been implicated in both the overall control of gene transcription and transactivation function of ERα [29,30]. Orm was found to be not affecting ERα expression when given alone whereas when it was co-administered with E_2, the expression was down-regulated to ~34%. Likewise no change in expression was observed with ERβ in Orm treated rats. Therefore, expression of ERα and ERβ in adult ovx rat appears to be insensitive to Orm. However, this is not the case with other SERM, Tam, where the expression levels were up-regulated for both the isoforms of ER. Results indicate that both SERMs regulate the expression of ERα and ERβ in different manner.

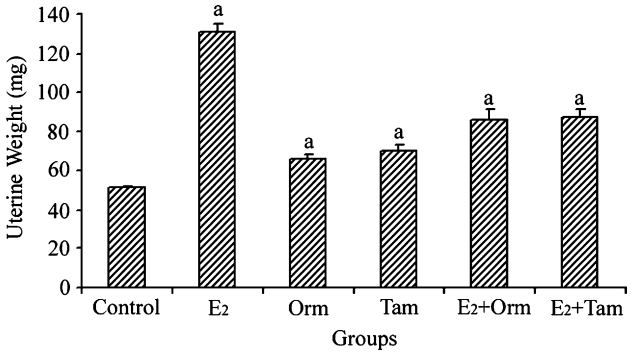

Fig. 9. Effect of Orm on uterine wet weight after 3 days treatment of ovx adult rats with E_2 (5 μg/100 g, s.c.) or Orm/Tam (200 μg/100 g, s.c.) or Orm + E_2 or Tam + E_2. Animals of control group were given vehicle (olive oil) alone. The results are presented as the mean ± SEM of ($n = 6$) independent experiments. [a] $p < 0.001$ *vs.* ovx control; *vs.* E_2.

PR is a downstream target of ERα activation. It is well known that uterine PR can be up- or down-regulated by administration of classical ER agonists depending on cell type [31]. Therefore, its mRNA expression and protein expression were examined in rat uterus. In the present study, we found that after E_2-treatment, PR-B mRNA expression was up-regulated nearly by 8-fold (Fig. 3) and protein level was up-regulated by 2.5-fold (Fig. 4) as compared to that of control group. Our results are in accordance with the data published by Kraus and Katzenellenbogen showing 6-fold increase in PR mRNA and protein expression in immature ovx rat [32]. Here it is evident that Orm and Tam suppress the E_2-induced up regulation of PR expression and thus both SERMs show antiestrogenic action in rat uterus. However, these effects might be cell-specific within the uterine compartments. Parczyk et al. demonstrated that the treatment of ovariectomized rats with estradiol resulted in high PR levels in the uterine myometrium and stromal cells but low PR immunoreactivity in the epithelial cells [33]. These results clearly show that in rat uterus, the activated ER might induce diverging effects on PR expression in different cell types. Our findings indicate that the expression of ERα is insensitive to Orm treatment in adult ovx treated rats. However, it down-regulates the E_2-induced expression of PR and thereby antagonizes the action of E_2. The expression of PR is not directly correlated with expression of ER, however, correlated well with uterine weight data (Fig. 9). Therefore, there must be some other factors, such as growth factors and co-regulators that might influence the regulation of PR by E_2 or Orm or Tam. So, further we determined the expression levels of ER co-regulators to dissect the molecular mechanism of Orm.

The inhibition of steroid receptor function with antagonist is not merely a process of ligand competition but also involves the active recruitment of co-regulatory proteins [34]. Shang and Brown have shown that the molecular basis of unique pharmacology of SERMs is the cell type and promoter-specific differences in co-regulator recruitment that can either enhance or repress transcriptional activity of ER [35]. A few reports have shown that the differential expression or activity of co-regulators in a given cell can modulate the agonist *vs.* antagonist activity of drugs, such as tamoxifen on ER [12]. Present study demonstrated the expression levels of co-regulators and their interaction with ER in ovariectomized rat uterus**.** SRC-1 is a crucial molecule in the first step of the ER-mediated transcription [36], and NCoR and RIP140 are reported to repress the transcription [37,38]. These proteins are the best characterized co-regulators for the nuclear receptor superfamily and are expressed in endometrium [39,40]. Using semi-quantitative RT-PCR

and Western blotting, we have shown that the expression of co-regulators SRC-1, RIP140 and NCoR display no difference in mRNA expression and protein expression in E_2 or Orm or Tam treated rats as compared to control group (Fig. 5). These results are similar to the findings of Nephew et al. who documented that the expression of SRC-1 and RIP140 was insensitive to E_2 and Tam treatment, in ovx immature and adult rats [41]. In human, a previous study showed that protein expression of SRC-1, SRC-2, and SRC-3 did not change during the menstrual cycle except for an increase in glandular SRC-3 during the late secretory phase [39]. Another study showed a decrease in glandular and stromal SRC-1 and N-CoR during the early, mid, and late secretory phase of the cycle [40]. Further, no significant difference in gene and protein expression of NCoR between polycystic ovary syndrome (PCOSE) and normal endometrium was observed [42]. However, Uchikawa et al. have shown an increased expression of NCoR in endometrial hyperplasia as compared with normal and malignant tissues [43]. No change in expression was observed in ishikawa endometrial cells after E_2 or 4-hydroxytamoxifen or pure antiestrogen, ICI164 384 treatment [44].

Further, we studied the interaction of SRC-1, RIP140 and NCoR with ERα in uterine extract of ovx rats using co-immunoprecipitation. Interestingly, Orm or Tam, in the presence or absence of E_2, caused a decrease in the interaction of SRC-1 with ERα as compared to E_2-treated groups (Fig. 6A). On the other hand, Orm or Tam decreased interaction of SRC-1 with ERβ only in the absence of E_2 (Fig. 6B). Our results indirectly support the findings of *in vitro* studies of Wong et al. [45] who used co-activator NR-interaction domain and Cheskis et al. [46] who used surface plasmon resonance to analyze binding of full-length SRC-1 to ERα and ERβ. They showed that interaction of SRC-1 with ERα and ERβ was enhanced in presence of 17β-estradiol whereas inhibited in presence of 4-hydroxytamoxifen, raloxifene and ICI-182,780 [45,46]. The inhibition of E_2-induced interaction of SRC-1 with ERα may be one of the possible mechanisms exerted by Orm, in regulating the ER-mediated transcription to evoke its antagonistic behavior at uterine level under normal physiological condition.

We next determined the interaction of RIP140 with ER. It is an unconventional ER co-regulator which acts mainly as a negative regulator of hormone-dependent nuclear receptor activity, thus counterbalancing the effect of co-activators [47]. We demonstrated that Orm in the presence of E_2 was found to significantly enhance the interaction between RIP140 and ERα with respect to E_2-treated groups. However, Tam did not show such response (Fig. 7A). In relation to other isoform, the interaction of RIP140 with ERβ was not significantly changed in E_2 or SERM treated rats as compared to vehicle treated control rats (Fig. 7B). It is reported that transcriptional repression mediated by RIP140 occurs by competition with co-activators [48]. Evidence was provided that the *in vitro* binding of RIP140 and SRC-1 to nuclear receptors was competitive and might account for the repression of transcription by RIP140 [49]. Therefore, it might be possible that Orm induces the interaction of RIP140 and regulate gene transcription by inhibiting the interaction of ER with SRC-1 and simultaneously by recruiting other co-repressors.

Evidences show that in absence of hormone, NCoR binds to certain nuclear receptors, including retinoic acid and thyroid hormone receptors; and to steroid receptors in the presence of antagonist [38,33]. Binding of NCoR is different from co-activators because it contains an extended LXXLL-like motif that cannot dock into a cleft formed by the activated receptors [50]. Here, we documented that Orm increased the interaction of NCoR with ERα in the presence of E_2 while interaction of NCoR with ERβ was increased in the absence of E_2. However, no change in interaction was observed in Tam treated rats. In E_2-treated rats, interaction of NCoR with ERα and ERβ was not affected and remained comparable to that of vehicle treated control (Fig. 8). This is contradictory to the earlier reports documented by Wu et al. [51] where they have provided an indirect evidence of interaction of NCoR with ERα. They showed that in the presence of E_2, the NCoR was redistributed to form intranuclear incomplete foci together with estrogen receptor and thus suppress its transactivation function [51]. The formation of complete foci suggests the formation of transcriptionally active complex. In another report, it was demonstrated that extracts from HeLa cells transfected with ERα and MCF-7 cells, treated with 4-OH-Tam, ERα was co-immunoprecipitated with NCoR antibody, but not in untreated cells or in cells treated with E_2 [52]. In line with this report, Webb et al. demonstrated that ICI and raloxifene are more efficient than tamoxifen in promoting ERα binding to the NCoR *in vivo* and *in vitro* [53]. From our results, it appears that Orm induces the recruitment NCoR and thereby preventing the formation of co-activator complex to inhibit the E_2-mediated response in rat uterus.

Altogether, Orm in presence of E_2 was found to increase the interactions of ERα–RIP140 and ERα–NCoR while it inhibits the interaction of ERα–SRC-1. The clinical relevance of this recruitment is the critical role of SRC-1 in the agonist activity of tamoxifen in uterus [35]. Moreover, SRC-1 protein is significantly associated with insensitivity to endocrine treatment [54]. However, our data clearly indicates that Orm interacts with ERα and it recruits co-repressors (RIP140 and NCoR) not co-activator (SRC-1) in order to mediate its effect at uterine level as an antagonist. Likewise, in certain tumors where co-activators are over-expressed, Orm might be able to antagonize E_2 action by attenuating the E_2-induced recruitment of co-activator(s) to the transcriptional machinery.

Present study has revealed that under the influence of Orm, ERα preferably recruits co-repressors in uterus. Orm as reported earlier, interacts with both ER subtypes α and β, thus, it may also manifest estrogenic or antiestrogenic response in non-reproductive target tissue, e.g. bone, nervous system and cardiovascular system where ERβ is a dominant subtype. In addition, since the expression of various co-activators and co-repressor varies in different cell types, Orm may be able to show agonistic or antagonistic profile through different co-regulators in different cell types. Future studies on cell-specific ER-co-regulators interaction might unravel full spectrum of tissue selective actions of ormeloxifene.

Acknowledgements

We thank Sh. P.K. Bhattacharya and Sh. N.P. Mishra for technical assistance in animal work. The study was supported by grants from Ministry of Health and Family Welfare, Government of India. AD and RS are recipients of research fellowship from Council of Scientific and Industrial Research, New Delhi, India

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