Human vascular smooth muscle cells express an estrogen receptor isoform

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Abstract In women, estrogen (E2) exerts a clinically relevant anti-atherogenic effect. The atheroprotective effects of E2 are mediated both by E2-induced changes in systemic factors and by direct effects of E2 on the blood vessel wall. In studies to characterize E2 signaling pathways in vascular smooth muscle cells (VSMC), we recently demonstrated that human VSMC express a functional estrogen receptor [1]. In the present study, we applied a reverse transcription/PCR-based strategy to identify isoforms of the E2 receptor in human VSMC. We now report that in addition to the classical E2 receptor, human VSMC derived from both mammary artery and saphenous vein express an estrogen receptor isoform containing an in-frame deletion of Exon 4 (ERΔ4). RNase protection assays confirm the presence of ERΔ4 message in VSMC and demonstrate it is nearly as abundant as the classical E2 receptor. Transient transfection experiments in VSMC and HeLa cells demonstrate that, in contrast to the classical 67 kDa nuclear-localized E2 receptor, ERΔ4: (a) is a 55 kDa protein that is widely distributed throughout the cell; (b) does not transactivate an E2 response element-driven reporter plasmid in response to E2; and (c) does not modulate transactivation of the ERE-reporter by the classical (wild type) estrogen receptor. Thus, human VSMC express an E2 receptor isoform that does not appear to alter gene transcription. The presence of a novel isoform of the E2 receptor may have important implications for studies of E2-mediated signaling in VSMC.

Key words: Estrogen; Steroid hormone receptor; Signal transduction; Vascular biology

1. Introduction

Ischemic cardiovascular disease is the leading cause of death for adults in the United States. A number of observational epidemiological studies have demonstrated postmenopausal estrogen replacement therapy in women can decrease the incidence of myocardial infarction and cardiac death by 35–50% [2–6]. These epidemiologic studies are supported further by animal studies demonstrating E2-mediated inhibition of experimentally induced atherosclerosis [7–12,46]. The atheroprotective effects of E2 are generally believed to be mediated in part by effects of E2 on systemic factors such as serum lipid levels [2,13,14]. However, systemic effects of estrogens do not account for the majority of the observed atheroprotective effects of E2 [2,3,15,16]. In an effort to reveal additional pathways that may mediate the atheroprotective effects of E2, direct effects of E2 on vascular cells are currently being investigated (reviewed in [17]).

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Direct effects of E2 on vascular cell function were first suggested decades ago [7,18] and have since been shown both in whole animal and *in vitro* studies [10,19–30]. However, the signaling pathways involved in the direct effects of E2 on vascular cells are not yet characterized. In non-vascular cells, most E2-mediated effects are thought to result from ligand-induced activation of the intracellular E2 receptor, a transcription factor capable of regulating expression of genes whose regulatory elements contain a consensus E2 receptor binding sequence (reviewed in [31–34]). Human vascular smooth muscle cells are now known to express an E2 receptor [1,35].

Of interest, earlier studies suggest the presence of multiple E2 binding activities in vascular tissue [18,36,37], but the identity of E2 binding proteins other than the classical E2 receptor remains unknown. Although only the wild type E2 receptor is known to bind E2 and transduce E2-mediated signals in normal human tissues, a number of E2 receptor variant mRNAs now have been identified in breast cancer cells (reviewed in [38–40]). One such mRNA variant, containing an in-frame deletion of Exon 4 (ERΔ4) also recently has been identified in rat brain [41]. These findings suggest some tissues may express variant E2 receptors in addition to the classical E2 receptor. In the present study, we demonstrate the presence of ERΔ4 mRNA in human VSMC. This report represents the first demonstration of an E2 receptor isoform in normal, non-reproductive human tissue.

2. Experimental

2.1. Cell culture

VSMC were cultured, as described [1] from specimens of human saphenous vein and mammary artery obtained from both males and females at the time of cardiothoracic surgery. Cells were maintained in phenol red-free DMEM containing 10% FBS with an E2 content less than 2.6×10^{-11} M (Hyclone Laboratories, Inc., Logan, UT). Cells were identified as VSMC by their characteristic morphology and by immunostaining with anti-smooth muscle specific actin (Sigma, St. Louis, MO), and were harvested for all experiments at passage 2 or less. MCF-7 cells (derived from a human breast carcinoma) known to contain a high level of E2 receptor, and HeLa cells (derived from human cervical carcinoma) that do not express the E2 receptor ([42], and see Results), were maintained in DMEM containing 10% FBS.

2.2. Reverse transcription and PCR

Total cellular RNA was harvested by a commercially available guanidinium thiocyanate-based method as described [1]. Reverse transcription was carried out by the random priming method using 16 μg of DNase-treated total RNA (Superscript reverse transcriptase, Gibco-BRL, Grand Island, NY). PCR was performed on an aliquot of the reverse transcription reaction using primers ER3 and ER4 (ER3; 5′-CATAACGACTATATGTGTCCAGCC nucleotides 646–670, and ER4; 5′-AACCGAGATGATGTAGCCAGCCGC nucleotides 1280–1304 of the E2 receptor; cf. Fig. 1) and the following protocol: hot start at 94°C × 2 min, 30 cycles of 94°C × 30 s, 65°C × 30 s, 72°C × 30 s, and a final extension of 72°C × 5 min. An aliquot of this PCR reaction was then reamplified using the same protocol as above with ER3 and the

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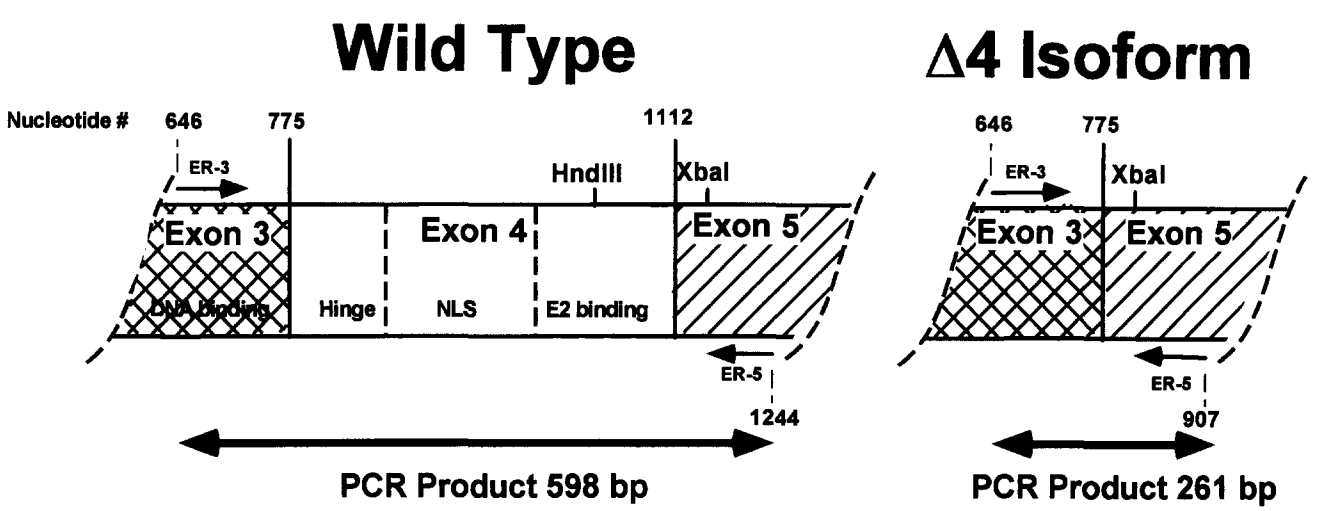

Fig. 1. Summary of the reverse transcription/PCR based strategy to identify E2 receptor isoforms in VSMC. Shown are the intron/exon borders and important functional domains encoded by Exon 4. Using primers ER3 and ER5, PCR amplification of VSMC cDNA encoding the wild-type receptor produces a 598 bp fragment, whereas amplification of cDNA encoding the receptor isoform lacking Exon 4 produces a 261 bp product. Note the position of the *Xba*I site ~ 100 bp distal to the Exon 4/Exon 5 splice junction, and the *Hin*dIII site contained within Exon 4.

nested primer ER5 (ER5; 5′-CCTGGTTCCTGTCCAAGAGCAAGT nucleotides 1220–1244 of the E2 receptor; cf. Fig. 1).

2.3. Southern blotting

The products of the PCR reaction were separated by agarose gel electrophoresis and transferred to nitrocellulose membrane. The membrane was then hybridized at 46°C overnight with a radiolabeled 134 bp probe (methods as described; [1]). The probe was made by restriction digestion of human E2 receptor cDNA (kind gift of Myles Brown) with *Bgl*II and *Xba*I (base pairs 1135–1269 of the E2 receptor cDNA). After washing with 0.1% SSC and 0.1% SDS, the membrane was analyzed by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA).

2.4. Subcloning and sequencing of PCR products

PCR products were directly ligated into the pCR II cloning vector (Invitrogen, San Diego, CA). The resulting colonies were screened first by restriction digestion, and subsequently by Southern blotting for the presence of E2 receptor-related sequences. Positive clones were confirmed by sequencing using the dideoxy chain termination method, according to the manufacturer's instructions (Sequenase; United States Biochemical, Cleveland, OH).

2.5. RNase protection assays

A radiolabeled RNA probe, complementary to 261 bp of the ERΔ4 sequence was produced by in vitro transcription (methods as described, [1] of the PCR product subcloned into the pCR II vector (Invitrogen, San Diego, CA). This probe was designed to flank symmetrically the Exon3/Exon5 splice site and thus allow discrimination between wild type ER and ERΔ4 (see Fig. 2). RNase protection assays were carried out as described [1]. The relative proportion of ERΔ4 and wild-type receptor message was determined by quantitative analysis of PhosphorImages independently for both VSMC and MCF-7 cells.

2.6. Transient transfections

The expression plasmid pSV40-ERΔ4 was constructed by cloning a restriction fragment encoding the ERΔ4 cDNA into an appropriately digested expression plasmid for the wild type E2 receptor (pHEGO; kind gift of Myles Brown) and used to express full length ERΔ4. In brief, a portion of the ERΔ4 coding sequence extending from nucleotide 8 to 1244 was amplified by PCR, digested with *Sma*I and *Xba*I and ligated into a similarly digested pHEGO vector. To identify subclones that represented the correct ERΔ4 plasmid, colonies were screened by restriction digestion and Southern blotting, and subsequently confirmed by sequencing.

VSMC or HeLa cells were transfected by electroporation, essentially as described [1]. For protein expression studies, cells were electroporated with one of the following expression plasmids: (a) pHEGO (wild-type E2 receptor cDNA driven by an SV40 promotor); (b) pSV40-ERΔ4 (described above); or (c) pHEGO(−), (control plasmid derived from pHEGO by excision of the E2 receptor cDNA). Cells were harvested 36 h after transfection and assayed either by Western blotting or by immunofluorescent staining, (methods as described [1]), using two different anti-E2 receptor antibodies (D75 and D547 [43]; kind gift of Geoffrey Greene). To examine the potential transcriptional transactivation potential of the E2 receptors, cells were electroporated with the reporter plasmid ERE-*Luc* (containing an E2 response element (ERE) driving expression of the luciferase gene (originally V19TK3 [44]; kind gift of C. Glass), and one or both of the expression plasmids pHEGO or pSV40-ERΔ4, as described [1]. Following transfection, cells were treated with hormone-containing media 36 h prior to harvest for deter-

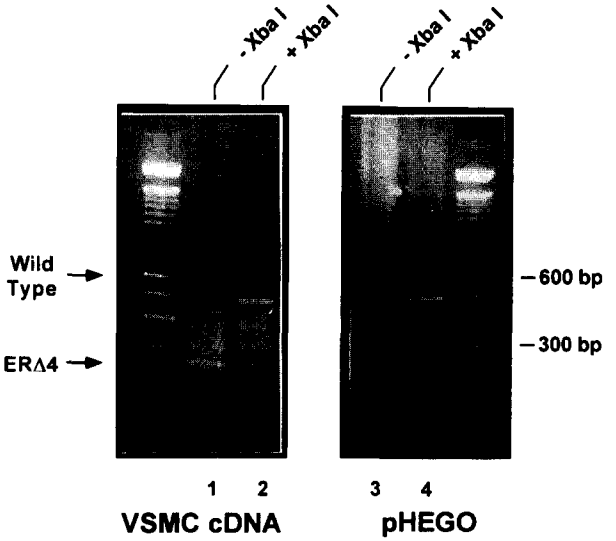

Fig. 2. Reverse transcription/PCR detects two distinct estrogen receptor-related messages in human VSMC. RT/PCR products were amplified using primers that span exon 4 and resolved by agarose gel electrophoresis. Amplification of cDNA derived from human VSMC produced two dominant bands, one of ~600 bp and one of ~260 bp (lane 1). Digestion of the PCR products with *Xba*I reduced the size of the bands by ~100 bp (lane 2). The position of the RT/PCR amplified 598 bp fragment from the classical E2 receptor (pHEGO) is shown in lanes 3 and 4 as a positive control (without and with digestion by *Xba*I, respectively).

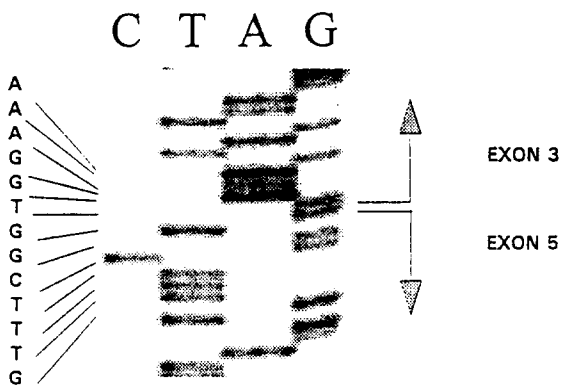

Fig. 3. Sequence analysis confirms the detected estrogen receptor isoform in VSMC contains an in-frame deletion of Exon 4. The PCR products shown in Fig. 2 were subcloned and sequenced. Shown is the portion of the 260 bp PCR product sequence that demonstrates the direct transition from exon 3 to exon 5 coding sequence.

mination of luciferase activity [1]. Equivalent levels of receptor protein expression were documented by Western blotting. Equivalent transfection efficiency between treatment groups was ensured by aliquoting cells from a single electroporation to all treatment groups equally. This was confirmed in a subset of transfection experiments in which the plasmid CMV-CAT (containing the chloramphenical acetyl transferase (CAT) gene driven by the CMV promoter; kind gift of RS Williams) was co-transfected and the luciferase values were normalized for CAT activity. Control cells were treated with vehicle alone (0.02% ETOH). The results of luciferase assays were normalized by comparison with control cells for each individual experiment. Results were compared by Student's t-test with a $P \leq 0.05$ considered significant.

3. Results and discussion

To attempt to identify E2 receptor isoforms in human

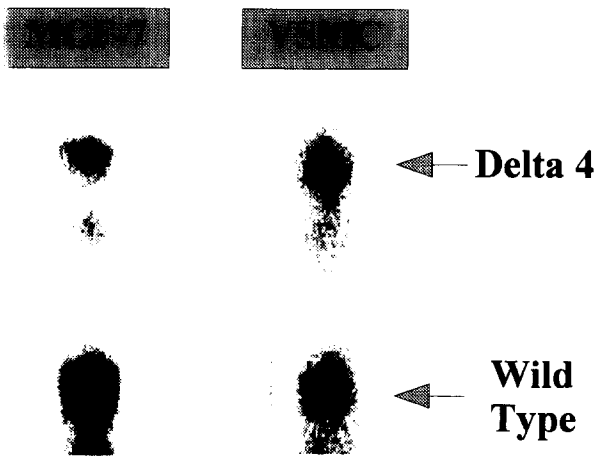

Fig. 4. RNase protection assay detects ERΔ4 message in human VSMC. RNase protection analysis was performed with a radiolabeled probe complementary to 261 bp of the ERΔ4 sequence symmetrically flanking the exon 3/exon 5 splice junction. ERΔ4 message protects the full length probe, whereas the wild-type E2 receptor message protects only smaller fragments (see details in text). Shown is a representative PhosphorImage demonstrating the presence of ERΔ4 mRNA in human VSMC (100 μg total RNA). MCF-7 breast cancer cells (20 μg total RNA) were included as a positive control. An equivalent amount of yeast RNA which does not contain E2 receptor message served as a negative control (data not shown).

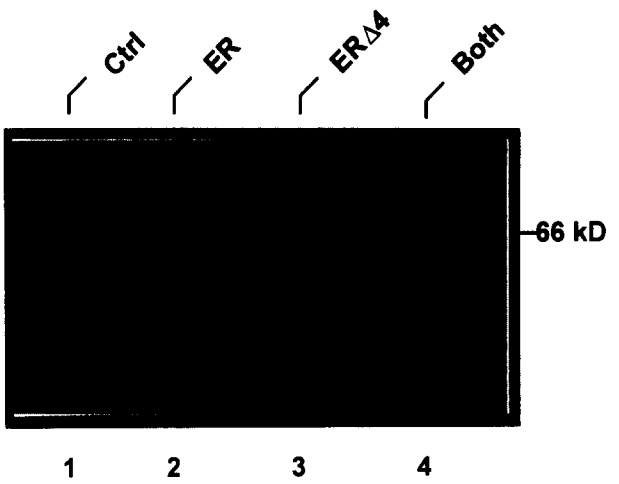

Fig. 5. Immunoblot analysis of the ERΔ4 protein. HeLa cells (which lack endogenous E2 receptor; lane 1) were transfected with expression plasmids encoding either the wild-type E2 receptor (lane 2), ERΔ4 (lane 3) or both receptors (lane 4). Identical quantities of cell lysates were analyzed by immunoblotting with anti-E2 receptor antibody D75. Wild type ER has an estimated M_r of 67 kDa, while the ERΔ4 protein has an apparent molecular weight of 54 kDa, (predicted M_r from primary sequence = 55 kDa).

VSMC, we employed a PCR-based scheme to screen cDNA reverse transcribed from total cellular RNA using primer pairs that span intron/exon junctions. PCR using a primer pair spanning Exon 4 and its 5′ and 3′ splice sites (ER3/ER5; see Fig. 1), produced two predominant bands, one ~ 600 bp and one ~260 bp long (Fig. 2, Lane 1). The 600 bp product corresponds to the predicted size of the fragment from the wild-type gene, and the 260 bp product is the predicted size of a PCR product from which Exon 4 is deleted (cf. Fig. 1). Similar results were obtained when either oligo-dT- or E2 receptor-specific primers were used to prime the reverse transcription reaction during first strand cDNA synthesis (data not shown).

The two predominant PCR products were also characterized by restriction digestion analysis. Digestion with *Xba*I decreased the size of both bands by 100 bp as expected (Fig. 2, Lane 2; cf. Fig. 1). In addition, digestion with HndIII, whose binding site is contained within Exon 4 of the wild-type receptor, reduced the size of the 600 bp product but not that of the 260 bp product (data not shown). The presence of E2 receptor sequences within the PCR products shown in Fig. 2 was also confirmed by Southern blotting with radiolabeled probes derived from wild-type E2 receptor sequences (data not shown).

Based on these preliminary screening experiments, the 600 bp and 260 bp bands shown in Fig. 2 were tentatively identified as portions of the native E2 receptor and an E2 receptor variant containing an Exon 4 deletion. To confirm this hypothesis, the PCR reaction products were directly subcloned and sequenced. Sequence analysis of the plasmid containing the 600 bp insert confirmed it as wild-type receptor (data not shown). Sequence analysis of the plasmid containing the smaller fragment (pER-260) identified it as ERΔ4, an isoform of the wild-type receptor in which the coding sequence contained within Exon 4 is precisely spliced out (Fig. 3).

To quantify the abundance of ERΔ4 message in RNA derived from native VSMC, RNase protection analysis was undertaken using a radiolabeled probe complementary to 260 bp

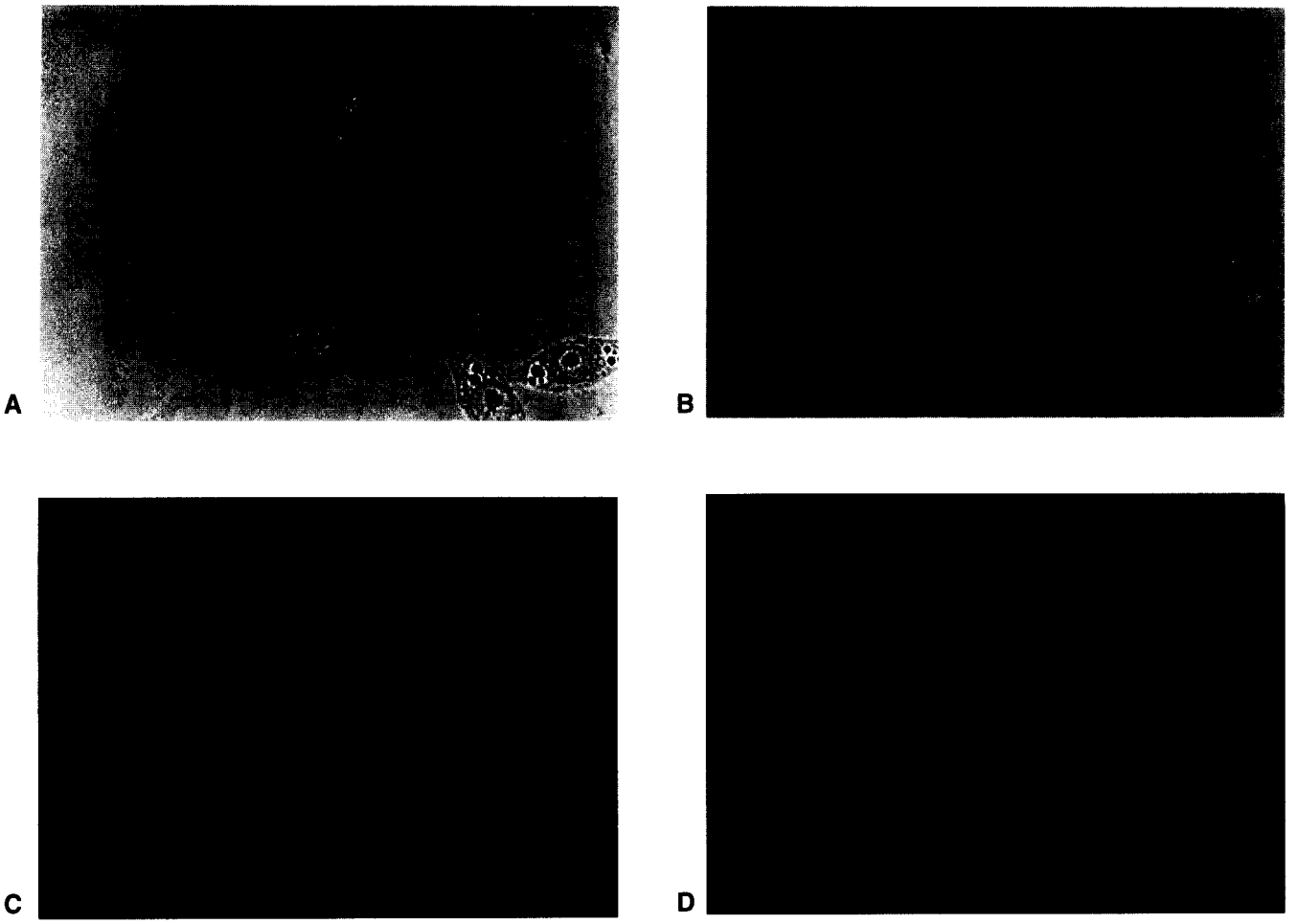

Fig. 6. The ERΔ4 protein is not confined to the nucleus. Shown are HeLa cells transfected with an expression plasmid for either the wild-type (A,C) or ERΔ4 (B,D) receptor and viewed by phase contrast (A,B) or immunofluorescence microscopy (C,D). Receptor protein was identified by immunostaining with the Anti-E2 receptor antibody D75. Note that unlike the wild-type receptor, the ERΔ4 protein is not confined to the nuclear compartment and appears diffusely distributed throughout the cell (Magn. = 40×).

of the ERΔ4 sequence. The probe was designed to flank symmetrically the Exon3/Exon5 splice site in ERΔ4 such that hybridization with the ERΔ4 mRNA would produce a protected fragment larger than those produced by hybridization with the wild type receptor (260 bp vs. two 130 bp fragments, respectively). This strategy prevents any degraded probe from being incorrectly identified as representing a receptor isoform. RNA prepared from low-passage, freshly cultured VSMC derived from either saphenous vein or mammary artery was used in all RNase protection assays. The representative RNase protection assay shown in Fig. 4 demonstrates human VSMC make significant levels of ERΔ4 mRNA. No protected fragment of this size was detected when a comparable amount of yeast RNA was used as a negative control (data not shown). Quantitative analysis of RNase protection assays performed on VSMC obtained from both males and females demonstrates a ratio of ERΔ4/wild type E2 receptor of 0.64 ± 0.11 ($n = 10$). The ratio of ERΔ4 to wild type E2 receptor in MCF-7 cells, for comparison, is 0.51 ± 0.16 ($n = 5$).

To explore the functional role of ERΔ4, the receptor isoform was next subcloned into a suitable expression plasmid, pSV40-ERΔ4. The ability of pSV40-ERΔ4 to induce expression of full length ERΔ4 receptor protein was investigated in transient transfection studies using HeLa cells, which have no endogenous E2 receptor [42]. First, protein lysates of HeLa cells transfected with (a) control plasmid lacking receptor sequences; (b) pHEGO; (c) pSV40-ERΔ4; or (d) both receptor plasmids were analyzed by immunoblotting. As shown in Fig. 5, no receptor was detectable in HeLa cells transfected with the control plasmid (lane 1). However, transfection of HeLa cells with pHEGO or pSV40-ERΔ4 resulted in approximately equivalent levels of expression of proteins with the predicted molecular weights of 67 kDa (native ER) or 55 kDa, (ERΔ4), respectively (Fig. 5, lanes 2–4). Similar results were obtained when a different anti-E2 receptor antibody was used for immunoblotting (D574, [43] not shown).

The intracellular distribution of ERΔ4 protein also was studied by immunofluorescent staining of HeLa cells transfected with either the control plasmid pHEGO(−), pHEGO, or pSV40-ERΔ4. As shown in Fig. 6, expression of the ERΔ4 isoform resulted in a staining pattern strikingly different from that of the wild type receptor. The wild-type E2 receptor protein was more widely distributed and confined to the nucleus (Panel C), whereas the ERΔ4 isoform was not predominantly nuclear (Panel D). HeLa cells transfected with the control vector alone showed no specific staining (data not shown).

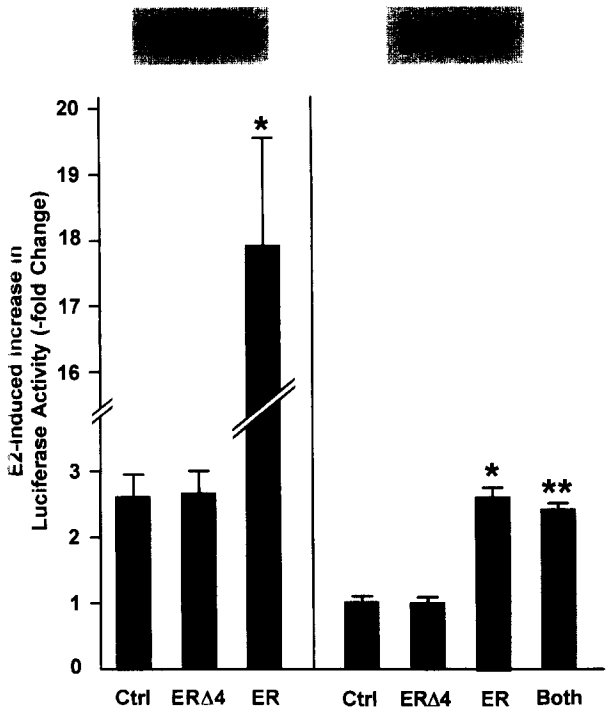

Fig. 7. ERΔ4 does not activate E2 response element-driven gene expression or affect wild type E2 receptor transactivation in either VSMC or HeLa cells. Human VSMC and HeLa cells were co-transfected with an ERE-driven reporter plasmid along with expression plasmids encoding the wild-type receptor, ERΔ4, both receptors, or neither. Approximately equal receptor protein expression was documented by Western blotting (cf. Fig. 5). Shown is the relative increase in luciferase activity in response to 48 h of 10^{-7} M E2 treatment. In VSMC, overexpression of the wild-type receptor markedly increases E2-induced ERE activation (as reported [1]), whereas overexpression of ERΔ4 has no effect on ERE activation by the endogenous receptor. In HeLa cells, the wild-type receptor, but not the ERΔ4 isoform, confers E2-induced activation of the ERE and again, the ERΔ4 does not effect ERE activation by wild-type E2 receptor.

3.'. *ERΔ4 receptor isoform function*

To begin to define the function of ERΔ4, we first investigated the potential transcriptional activity of the ERΔ4 isoform. Several functional domains of the wild-type E2 receptor are intact in the ERΔ4 isoform (e.g. DNA binding domain, transcriptional activation domains, dimerization motif) while others are omitted (e.g. hinge region, nuclear localization signal and 56 of 240 amino acids of the hormone binding domain; cf. Fig. 1). Therefore, ERΔ4 could be transcriptionally silent, or it could act as either a dominant negative or dominant positive E2 receptor. To investigate these possibilities, we used an ERE-*Luc* based reporter system in cotransfection experiments with the receptor isoforms. VSMC or HeLa cells were co-transfected with either (a) control plasmid; (b) pHEGO; (c) pSV40-ERΔ4; or (d) both receptor plasmids. Basal activity and E2-induced transactivation of the ERE were determined. Equivalent protein expression for both the wild type and Δ4 expression plasmids was confirmed by western blotting (cf. Fig. 4 and data not shown).

Consistent with our previous report [1], treatment of VSMC with 10^{-7} M E2 resulted in a 2.6 ± 0.42-fold increase in Luciferase activity, reflecting activation of the ERE by endogenous VSMC E2 receptor (Fig. 7). Overexpression of the ERΔ4 isoform did not alter the E2-induced activation of the ERE (2.7 ± 0.35-fold increase; $n = 8$, P = n.s vs. control). In contrast, and as shown previously [1], E2-induced activation of the ERE was markedly enhanced by overexpression of the wild type E2 receptor (16.7 ± 2.7-fold activation; $n = 5$; $P < 0.001$ vs. control or ERΔ4). In an additional series of transfections using VSMC, a CAT expression plasmid also was co-transfected, and all luciferase results were normalized to CAT activity. In these experiments, the ratio of E2-induced activation of ERE in ERΔ4 transfected cells compared to sham transfected cells was 1.1 ± 0.1 ($P = 0.75$; $n = 6$), confirming the lack of effect of ERΔ4 on activation of the endogenous E2 receptor. To examine the transcriptional transactivation capacity of ERΔ4 in a cell that does not contain endogenous E2 receptors, HeLa cells were also studied. As shown in Fig. 7 (right panel), E2 does not activate the ERE in sham transfected cells. Overexpression of the ERΔ4 isoform also does not confer E2-mediated activation to these cells. Transfection of HeLa cells with wild type ER (pHEGO) does, however, confer E2-mediated transactivation of the ERE. Again, the magnitude of this response is unchanged when pHEGO and ERΔ4 are co-transfected (2.6 ± 0.4 vs. 2.4 ± 0.1, P = n.s, $n = 3$). Thus, in both VSMC and HeLa cells, ERΔ4 appears to be transcriptionally silent.

We have demonstrated that human VSMC express mRNA for an isoform of the E2 receptor containing an in-frame deletion of Exon 4. Although a number of E2 receptor variants have been identified in tumor cells, they have been considered to be mutations related to the transformed nature of the cells studied. The ERΔ4 variant was originally cloned from malignant tissue [39], and similarly considered to have resulted from a tumor-related mutation, until the recent report of ERΔ4 expression in normal uterine cells [40].

In previous studies of ERΔ4, the presence of ERΔ4 message was detected by reverse transcription/PCR [39–41], but the level of ERΔ4 message expression was not investigated. In the present study RNase protection assays were used to show the relative proportion of wild type to ERΔ4 message is ~3:2. The presence and high relative proportion of ERΔ4 message in VSMC has a number of implications. First, it suggests previous studies of E2 receptor expression should be reassessed, since most methods capable of detecting the wild-type receptor (both antibody-based and oligonucleotide probe-based) also will likely detect ERΔ4. This has specific implications for immunohistochemical studies of the intracellular receptor distribution since unlike the wild-type receptor, ERΔ4 is diffusely distributed. The heterogenous cytoplasmic staining noted previously in VSMC [1,35] thus may in part represent ERΔ4 protein within the cells. Definitive investigation of this possibility will require development of ERΔ4-specific antibodies.

Despite the present demonstration of ERΔ4 expression in VSMC and previous reports identifying ERΔ4 in other cell types, a number of important questions regarding ERΔ4 remain. For example, due to a lack of ERΔ4-specific antibodies, ERΔ4 protein expression has not yet been characterized in any cell type. A second issue that has not yet been adequately explored is what is the function of the ERΔ4 isoform. Although it is possible ERΔ4 serves no function, the relatively high abundance of ERΔ4 message compared to wild-type message makes this less likely. Several functional possibilities are made unlikely or excluded by the present data. Though it was speculated initially that ERΔ4 might be a constitutively active orphan

receptor with a basal level of transcription of E2-responsive genes [41], in the current study overexpression of ERΔ4 did not increase the basal activity of ERE reporters in either HeLa cells or VSMC. Also, ERΔ4 by itself was not capable of transcriptional transactivation of the ERE in response to E2, nor did it affect the ability of the wild-type receptor to do so. These findings are consistent with those of Koehorst et al who studied the ERΔ4 variant from MCF-7 cells [40]. These authors demonstrate further, that overexpressed ERΔ4 protein binds neither the ERE nor E2. Our data do not exclude the possibility that ERΔ4 is an orphan receptor, that (a) recognizes a DNA sequence different than the ERE (though the DNA binding domain of the protein is essentially intact); or (b) binds to a ligand other than 17β-estradiol. It is also possible ERΔ4 might alter transcriptional events in a hormone-independent fashion following activation by an alternative pathway, as has been seen for a hormone-independent E2 receptor activation by dopamine [45].

In summary, the data presented here show that human vascular smooth muscle cells express abundant ERΔ4 message and support the hypothesis that this receptor variant is an E2 receptor isoform. The protein, when expressed, is not confirned to the nucleus, nor does it lead to E2-responsive transcriptional transactivation or influence transactivation by wild type E2 receptor. Further studies will address any relationship between the ERΔ4 protein and the low-affinity E2 binding sites previously reported in vascular cells, as well as the rapid, nongenomic effects of E2 on vascular smooth muscle cell tone [17].

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