Variant Estrogen and Progesterone Receptor Messages in Human Vascular Smooth Muscle

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Background—Estrogens stimulate growth of breast or uterine cells but have the opposite effect on vascular smooth muscle cells, in which they protect against coronary artery disease with or without concomitant administration of progesterone. A possible cause of differences in hormone action is variable tissue-specific expression of hormone receptor. Therefore, we analyzed the structure of estrogen receptors (ERs) and progesterone receptors (PRs) in human vascular smooth muscle.

Methods and Results—RNA was isolated from human vascular smooth muscle, and the functional domains of ER-α and PR were characterized by reverse transcriptase and polymerase chain reaction. Interestingly, in addition to wild-type ER-α and PR, 5 variant ER-α and 2 variant PR transcripts were found. These variants contained precise deletions of exons encoding regions of the hormone-binding domain. The PR transcripts lacked exon 4 (PRD4) and exon 6 (PRD6). The ER-α transcripts were missing exon 4 (ERD4), exon 5 (ERD5), exon 6 (ERD6), exon 7 (ERD7), and exons 6 and 7. (ERD6,7). ER-β variants were also detected. The PR variants were functionally characterized, and PRD6 was found to be a dominant-negative transcription inhibitor of wild-type receptors. Variant PR was present in premenopausal women but absent in postmenopausal women.

Conclusions—Variant PR and ER transcripts are extensively expressed in human vascular smooth muscle. The complex tissue-specific effects of sex hormones may be mediated by the expression of heterogeneous forms of their cognate receptors. The presence of variant ERs and PRs may be of importance in altering the physiological effects of estrogens or progestins in vascular smooth muscle. (Circulation. 1999;99:2688-2693.)

Key Words: genes ■ muscle, smooth ■ receptors ■ hormones ■ coronary disease

The incidence of coronary artery disease in postmenopausal women is markedly reduced by treatment with exogenous estrogens.1 A possible mechanism of this effect is inhibition by estrogens of vascular smooth muscle cell growth and migration, a process critical to atherogenesis and vascular restenosis.2–4 Estrogen receptors (ERs) are ligand-activated transcription factors. Activated ER binds to estrogen response elements (EREs) in the promoters of target genes, thereby regulating gene expression.5 Among the proteins regulated by estrogens in vascular smooth muscle cells are the progesterone receptors (PRs).6

The effects of estrogens vary markedly in different tissues; for example, estrogens stimulate growth of breast and uterine cells7,8 but inhibit growth of vascular smooth muscle cells.2–4 Progesterone exhibits antagonism to estrogen effects in the uterus9,10 but enhances the effects of estrogens in vascular smooth muscle.11,12 A possible explanation for the heterogeneity of estrogen or progesterone action in different targets is that some tissues express variant forms of ER or PR that have transcriptional effects different from the “wild-type” receptors. There are 2 wild-type ERs, designated ER-α and ER-β,13,14 and 2 wild-type PR isoforms, called PR-A and PR-B.15,16 Additionally, mRNA exon deletion splice variants may arise that encode altered receptor proteins that are missing key functional domains. Variant forms of ER-α arising from exon splicing errors have been observed in breast cancers, meningiomas, and normal human mammary tissue.17–19 Recently, Inoue et al20 reported 3 exon deletion variants of ER in vascular smooth muscle cells isolated from rat aorta. PR splice variants have been reported in breast cancer.21,22

Variant receptors are capable of anomalous transcriptional activities that may dominantly inhibit or enhance the effects of wild-type receptors.23,24 In addition, some variant receptors are constitutively active: they can mimic the transcriptional effects of estrogens or progestins in the absence of hormone. Finally, some variant receptor messages are likely to be incapable of being translated into protein. If these are the most prevalent types of receptor mRNA present, normal receptor-mediated actions of estrogens or progestins may be...
absent. Therefore, expression of variant ER or PR in a particular tissue, such as vascular smooth muscle, could have effects that differ markedly from those of the wild-type receptors.

To analyze the expression patterns of wild-type and variant ER and PR mRNAs, we used reverse transcriptase (RT) and the polymerase chain reaction (PCR) in human vascular smooth muscle. We identified several ER-α and ER-β variants. We also found 2 PR variants that we cloned and sequenced to identify the exon deletions. One of the PR variants, an exon 6 deletion mutant, is a dominant-negative inhibitor.21 We propose that the complex, tissue-specific effects of sex hormones may be mediated in part by the expression, or lack thereof, of heterogeneous types of their cognate receptors.

### Methods

#### Tissue Culture

As outlined in the Table, normal and diseased coronary artery, saphenous vein, aorta, mammary artery, and iliac artery were used. The sections were placed into DMEM with 1% (vol/vol) antibiotic-antimycotic solution at pH 7.4 (DMEM1AA) (Sigma Chemical Co). The fat and connective tissue were removed, each segment was cut longitudinally, and the endothelial layer was mechanically denuded. After a 10-minute incubation in Ca2⁺- and Mg2⁺-free HBSS (Gibco BRL) containing 73 U/mL collagenase (Worthington), the inner muscular layer was carefully stripped from the outer adventitial layer, and the smooth muscle was incubated in DMEM1AA for 72 hours at 37°C.

#### RNA Isolation

A modified version of a single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction (RNA STAT-60, Tel-Test B Inc) was used to purify the RNA. RNase-free DNase I was used to remove DNA contamination from the RNA.

#### RT-PCR

The GeneAmp RNA PCR kit (Perkin-Elmer) was used with 0.5- to 1.0-μg samples of total RNA and 2.5 μg of murine leukemia virus (MuLV) RT. The RNA was heated to 70°C for 5 minutes before addition of the RT. The RT reaction was performed at 42°C for 60 minutes, then heated to 99°C for 5 minutes to destroy the enzyme. PCR was performed with primers specific for the receptor domains of interest. An initial cycle at 94°C for 1 minute was followed by 35 cycles at 94°C for 30 seconds, 60°C for 40 seconds, 72°C for 90 seconds, and a final extension cycle at 72°C for 7 minutes. [32P]dCTP (DuPont NEN) was incorporated into the PCR reaction. The products were resolved on 1.5% agarose gels.

#### Cloning

The PCR products were inserted into a plasmid vector with a TA cloning kit (Invitrogen) immediately after the reamplified PCR products were obtained. The ligation reaction was performed overnight at 15°C. Cloned amplified PCR underwent automated sequencing at Biotechnology Resource Center, Cornell University.

#### Primers

Oligonucleotide primers were designed for PCR amplification of specific DNA fragments of ER and PR. A primer for β-actin served as an RNA quantification control. The primers amplified specific exons in the DNA binding domain (DBD) or hormone binding domain (HBD) of ER-α (exons 1 to 4) and PR (exons 5 to 8). These primers were able to detect deletions or amplifications in exons 2 to 7 for both ER-α and PR. The primer locations were as follows: primers 1 and 2 (nucleotide [nt] 617 to 637, nt 1082 to 1103) for ER-α DBD (487 bp); primers 3 and 4 (nt 974 to 994, nt 1787 to 1811) for ER-α HBD (840 bp); primers 5 and 6 (nt 2355 to 2375, nt 2675 to 2697) for PR DBD (345 bp); and primers 7 and 8 (nt 2619 to 2642, nt 3393 to 3411) for PR HBD (824 bp).
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Figure 1. Autoradiograph of [32P]-dCTP–labeled RT-PCR of RNA from saphenous vein from premenopausal woman. Amplified DBD for wild-type ER-α and PR (487 and 345 bp, respectively) and HBD for ER-α and PR (840 and 824 bp, respectively) are shown with black arrows. Variant ER-α and PR fragments are marked with white arrows. ER-α and PR variant bands were cut out from the gel, cloned, and sequenced.

The primers used for ER-β amplified both the DBD and HBD (nt 174 to 303, nt 1354 to 1383), generating a fragment of 1212 bp. All the primers were tested on ER and PR cDNA plasmid clones to ensure that they generated the specific products targeted.

Construction of Exon Deletion Mutants
The cDNAs encoding the PR variants were created by PCR with pSG5-hPR1 or -hPR2 (expression vectors for the B or A isoforms of PR) as templates and oligonucleotides designed to selectively eliminate the desired exon. The validity of the constructs was confirmed by sequencing.

Results
Identification of ER-α and PR Transcripts in Human Vascular Smooth Muscle by RT-PCR
To analyze the ER-α and PR transcripts, primers were designed to amplify the encoding mRNA segments of the DBD or HBD of these receptors. RT-PCR was performed with each primer pair, and the labeled products were resolved on an agarose gel. A representative result from smooth muscle tissue isolated from the saphenous vein of a premenopausal woman is shown in Figure 1. The black arrow in each lane marks the predicted wild-type DBD or HBD band for each receptor; all other bands are due to variant transcripts. Note the presence of ≥2 variants in lanes 2 and 3 and 3 variants in lane 4. The variant bands marked by white arrows were isolated, cloned, and sequenced.

The mRNAs of all steroid receptors, including ER-α and PR, are assembled from 8 exons. Exon 1 encodes the N-terminus; exons 2 and 3 the DBD; exon 4 the nuclear location signal (NLS), hinge, and proximal region of the HBD; and exons 5 to 8 the rest of the HBD. The predicted protein structure of the variant ER-α, determined from mRNA extracted from saphenous vein vascular smooth muscle cells, is shown in Figure 2 for ER-α compared with the wild-type receptors. Variant ER-α mRNAs present in vascular smooth muscle cells include deletions of exon 4 (Δ4), exon 5 (Δ5), exon 6 (Δ6), exon 7 (Δ7), and exons 6 and 7 (Δ6,7).

Figure 2. Wild-type and 5 ER-α variants. Wild-type ER-α mRNA consists of 8 exons that encode the receptor, DBD, HBD, and NLS (small rectangle between exons 3 and 4). Five ER-α variants include deletions of exon 4 (Δ4), exon 5 (Δ5), exon 6 (Δ6), exon 7 (Δ7), and exons 6 and 7 (Δ6,7). mRNA abnormalities and presence of premature stop signals resulting from frameshifts allow deduction of structures of resultant proteins. Three variants (Δ5, Δ6, and Δ7) produce frameshifts that lead to missense amino acids (black boxes) followed by premature truncation.

Prevalence of PR Variants in Human Vascular Smooth Muscle
Results of studies of PR variants in 15 subjects are shown in the Table. The subjects included 5 premenopausal women, 4 postmenopausal women, and 6 men. The men ranged in age from 19 to 69 years. The samples obtained from men contained widely varying percentages of variant or wild-type

Figure 3. Wild-type PR and 2 PR variants showing 8 exons that encompass the mRNA, DBD, HBD, and NLS (small rectangle between exons 3 and 4). Two PR variants that were observed have been cloned and sequenced. One PR variant is an exon 4 (Δ4) deletion. The Δ4 deletion removes the NLS, hinge, and a portion of the HBD. No frameshift occurs, and the remainder of the HBD is translated. The other PR variant, a Δ6 deletion, leads to a frameshift, and the protein is truncated in the middle of the HBD.
PR. PR was present in all the samples from premenopausal women, but a substantial percentage of the PR was of the variant type. The samples from the 4 postmenopausal women contained wild-type but very little or no variant PR. Therefore, in this small sampling of subjects, variant PR appears to be very prevalent in premenopausal women but essentially absent in postmenopausal women.

Discussion

Although premenopausal women and postmenopausal women treated with estrogens have lower incidences of coronary artery disease than men or postmenopausal women not receiving hormone therapy,1 the genomic mechanisms by which this protection is afforded are unknown. Estrogens probably influence atherogenesis and vascular injury through regulation of gene transcription in vascular smooth muscle or endothelium. Estrogens modulate gene transcription by binding to the HBD of ERs.5 The activated DBD of the receptor then binds to the ERE sequences of targeted genes, regulating their transcription. There are 2 wild-type ERs: ER-α, which plays an important role in breast and uterine cells, and ER-β, which has a different tissue expression pattern.13-14 Wild-type PRs are larger proteins than ERs but have the same general structural organization as ERs, with 1 additional complexity: there are 2 natural isoforms, the A and B receptors.15,16

There is functional ER in vascular smooth muscle,6,26 and there is evidence in cell culture and animal models that estrogens inhibit vascular smooth muscle cell growth and migration.2-4 Interestingly, this growth-inhibitory effect of estrogens in vascular smooth muscle differs from the action of estrogens in other tissues, particularly breast and uterus, where estrogens enhance cell proliferation.7,8

Progestins oppose the growth-stimulatory effects of estrogens in uterus and enhance cell proliferation in breast.9,10 In 2 recent studies,11,12 a progestin in high, probably nonphysiological, dosages had growth-inhibitory effects similar to those of estrogens in cultured human and rat vascular smooth muscle. However, in a study in atherosclerotic monkeys,27 a progestin, medroxyprogesterone (MPA), attenuated estrogen-induced coronary vasodilation. In a study in normal monkeys, MPA prevented inhibition of coronary vasospasm by estradiol, but progesterone did not.28 Additional evidence of possible adverse vascular effects of MPA is provided by a recent clinical trial in which use of high-dose MPA in conjunction with estrogen in postmenopausal women with preexisting coronary artery disease increased the risk of adverse cardiac events during the first year.29 Thus, the interactions of progestins and estrogens in vascular smooth muscle are complex and poorly understood. In different tissues, there is marked heterogeneity of estrogen action and, probably, variation in the interactions between estrogens and progestins. One possible explanation for these diverse physiological effects is that the forms of the receptor proteins expressed differ from organ to organ.

Variant ERs arising from exon splicing errors have been reported in human breast cancers and normal human mammary tissue.17-19 Recently, Inoue et al20 reported the presence of 3 ER-α variants in smooth muscle isolated from rat aorta. Variant receptors may have anomalous transcriptional activities: some may dominantly inhibit or enhance the effects of wild-type receptors. In addition, some variant receptors are constitutively expressed and may not require binding of their hormone ligand to produce their effects. Therefore, understanding the structural types and functional characteristics of variant ERs and PRs in the vascular bed could lead to valuable insights into the actions of estrogens and progestins in these tissues. We examined human vascular smooth muscle.

Figure 4. Autoradiograph of 32P-labeled RT-PCR (with primers specific for ER-β) of RNA from saphenous vein from premenopausal woman. Black arrow indicates 1212-bp wild-type PCR product. Splice deletion variants of ER-β and an insertion variant are indicated by white arrows.

ER-β Variant Transcripts Are Present in Human Vascular Smooth Muscle

In Figure 4, the vascular smooth muscle in a saphenous vein segment removed from a premenopausal woman expresses variant ER-β transcripts. In addition to the wild-type ER-β bands (black arrows), several variant PCR products (open arrows) are present. We found 1 variant band in the HBD that was larger than the wild type. A similar variant was described recently in rat ovary and prostate cells.25
to determine whether messages encoding ER-α variants were present.

Using RT-PCR, we discovered the presence of 5 HBD variants of ER-α in vascular smooth muscle: Δ4, Δ5, Δ6, Δ7, and Δ6,7 exon deletions. The Δ4 variant is not transcriptionally active alone, nor does it alter the transcriptional activity of wild-type ER-α. The Δ7 variant is not transcriptionally active alone and does not alter wild-type ER-α transcription in HeLa cells, but it exerts a dominant-negative effect on wild-type ER-α transcription in yeast cells. The transcriptional effects of the Δ6 and Δ6,7 ER-α variants are unknown. Three of these transcripts, the Δ5, Δ6, and Δ7 exon deletions, produce frameshifts that encode missense amino acids, followed by premature truncation. The other 2, the Δ4 and Δ6,7, are without frameshifts. The Δ5, Δ6, Δ7, and Δ6,7 variants lack portions of the HBD and would be expected to be constitutively active in the absence of hormone; this has been confirmed for the Δ5 variant.

Of considerable interest is our finding of PR splice variants in human vascular smooth muscle. These included a Δ4 and a Δ6 exon deletion variant. Because this is the first description of PR splice variants in any vascular tissue, we sequenced the mRNA. The Δ4 deletion removes the NLS, the hinge, and a portion of the HBD. Because no frameshift occurs, translation of the remainder of the HBD would be expected. The Δ6 deletion leads to a frameshift, which truncates the protein in the HBD. Richer et al reported the functional characteristics of these PR variants. The Δ4 has no transcriptional activity. The lack of an NLS probably renders this variant unable to enter the nucleus to compete with the activity of the wild-type PR. However, the Δ6 variant, when overexpressed, markedly inhibits the activity of both isoforms of wild-type PR. Therefore, at least 1 PR variant is capable of dominantly inhibiting the effects of wild-type PR. Differences in transcriptional effects, which are dependent on the type of PR expressed, could cause differences in the effects of progestins in vascular tissue. There is also evidence that PR can alter the function of ER.

Samples from postmenopausal women showed little or no PR variant mRNA in the tissue samples (Table). Three of the 4 postmenopausal women were taking estrogen replacement therapy with no progestin. It is possible that exogenous estrogen induces expression of a proportionate wild-type PR level above that found in premenopausal women and men. Alternatively, postmenopausal women may produce only wild-type mRNA regardless of the type of hormonal stimulation.

We discovered by RT-PCR that messages encoding several variants of ER-β were also present in vascular smooth muscle. Because the entire structure of wild-type ER-β is not known, we did not attempt to functionally characterize these variants. It has recently been reported that in ER-α knockout mice, estrogens inhibit vascular smooth muscle growth and migration. Because ER-β was present in the blood vessels of these mice, ER-β is probably transcriptionally active in vascular tissue. The concomitant presence of variants of ER-β could have important effects on the transcriptional activity of wild-type ER-β and ER-α.

We conclude that there are exon deletion variants of ER-α, ER-β, and PR in human vascular smooth muscle. Because these variants may have entirely different transcriptional effects than the wild-type receptors from which they are derived, they are potentially of physiological importance. An attractive concept is that some of the puzzling differences in estrogen and progestin effects in different target tissues could be explained by heterogeneous expression of their cognate receptor variants. To further address the possible importance of variant ERs or PRs, specific antibodies need to be developed for each variant to determine whether and in what quantities the variant mRNAs are translated.

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References


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