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Coronary Artery and Cultured Aortic Smooth Muscle Cells Express mRNA for Both the Classical Estrogen Receptor and the Newly Described Estrogen Receptor Beta

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Estrogens exhibit potent anti-atherogenic effects through mechanisms which may involve direct effects on the artery. The existence of the classical estrogen receptor (ERα) in vascular tissues has been established. Recently a new estrogen receptor (ERβ) has been discovered which represents a distinct gene product with homology to the classical ERα. The purpose of the present study was to determine if ERβ mRNA is expressed in vascular tissues of female and male primates. Oligonucleotide primers were developed for the specific RT-PCR amplification of ERα or ERβ mRNA. RT-PCR products of the appropriate size for ERα and for ERβ were observed after amplification of RNA isolated from coronary arteries of both male and female cynomolgus monkeys. Similar results were obtained from cultured aortic smooth muscle cells and from monkey reproductive tissues such as ovary and uterus. The relative expression of ERβ to ERα mRNA was greatest in ovary, on the same order of magnitude in monkey vascular tissues and uterus, while the human breast cancer cell line MCF-7 exhibited a very low level of ERβ relative to ERα. Sequence analysis of isolated RT-PCR products showed >95% similarity between the monkey and the published human sequences for both ERα and ERβ. These findings suggest that estrogen may influence vascular gene expression not only through classical ERα but also through the newly described ERβ. These findings also demonstrate the potential for targeting of these receptors in males for prevention or treatment of heart disease.

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INTRODUCTION

Atherosclerosis is the pathologic process underlying coronary heart disease, the leading cause of death in both men and women. Relative to men, women are protected against atherosclerosis and its clinical complications until after they reach menopause, when cessation of ovarian cyclicity results in reduced circulating estrogen levels. Estrogen replacement therapy reduces the incidence of coronary heart disease in post-menopausal women, and inhibits the progression of diet-induced coronary artery atherosclerosis in ovariectomized cynomolgus monkeys (*Macaca fascicularis*) and other animal models by 50% or more. In both cases, only a small portion of the beneficial effect of estrogen can be explained by effects on lipoproteins and other traditional risk factors [1–5]. These findings and others [6] suggest that estrogen may have direct, and perhaps receptor-dependent, effects on arterial metabolism which are important in this atheroprotection. However, the mechanism by which estrogen acts on the vascular wall to inhibit atherogenesis is not known.

The existence of estrogen receptors (ER) in vascular tissues has been investigated for many years, although their role in vascular metabolism is still poorly understood. Early methods used radio-labeled estrogen to demonstrate hormone binding in arterial tissues [7–12], in cultured aortic endothelial cells [13] and in SMCs [14, 15]. ER mRNA and protein has been detected in cultured rat aortic SMCs [16, 17], in cultured human mammary artery and saphenous vein SMCs [18, 19], and ER mRNA has been found in

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nonhuman primate aorta [20]. In addition, recent studies have demonstrated that ER protein [21] and mRNA [22] are present in human and nonhuman primate coronary arteries, respectively. The ability of native ER to regulate transcription in arterial SMCs has been demonstrated through transient transfection assays with estrogen responsive reporter constructs [17, 18].

Recently, a novel estrogen receptor (ERβ) has been discovered. This new receptor is the product of a distinct gene and is not a splice variant of the traditional ER, which is now being referred to as ERα. The sequences of the transcripts for ERβ have been reported for both rat [23] and human [24]. ERβ is similar to traditional ERα, showing ~95% homology in the DNA binding domain and ~55% homology in the ligand binding domain (within each species). In the rat, *in situ* hybridization studies demonstrated that rat ERβ was expressed in reproductive tissues of both males and females, with highest expression in the prostate epithelial cells and ovarian granulosa cells. The receptor was transcriptionally activated by estradiol, and competition curves demonstrate that the receptor has similar ligand binding characteristics for estradiol as ERα [25]. Studies with the human ERβ also demonstrated that the transcriptional activity could be inhibited with the ERα antagonist ICI 164,384. Northern blot analyses showed highest levels of ERβ in the testis, ovary, and thymus, although expression was also observed in spleen, prostate, intestine, and peripheral blood monocytes [24]. More recent studies have demonstrated that ERβ is expressed in a variety of rat tissues, that the relative expression of ERβ and ERα mRNA varies greatly between different tissues [25], and that in the ovary the expression of ERβ is regulated by gonadotropins [26]. Vascular tissues were not examined in these studies.

The purpose of the present study was to determine if ERα and ERβ mRNA is expressed in the coronary arteries of female and male cynomolgus macaques, as a prerequisite for examination of ER mediated gene expression by estrogens in these animals.

METHODS

Tissue and cell collection for RNA isolation

Artery and uterus were collected from animals at necropsy. Ovary was collected at the time of ovariectomy. From each tissue, 0.05–0.5 g samples were obtained, cleaned of adherent connective tissues, weighed, placed in a sterile container, and snap-frozen by immersion in liquid nitrogen for future RNA extraction. SMC were obtained from macaque aortas by collagenase/elastase digestion as previously described [27]. Briefly, aortic tissue was aseptically removed from cynomolgus monkeys at necropsy (post mortem interval = 45 min) and placed in Dulbecco's Modified Eagle Medium without phenol red (DMEM). Arteries were carefully cleaned of adherent adventitial tissue using sterile techniques and diced into 0.20 cm^2 sections. The intima-media tissue (0.25 g) was digested with a mixture of 0.225% collagenase (type 2) and 0.05% elastase (Worthington Biochemicals, Freehold, NJ) in phosphate buffered saline (PBS) containing 25 mM HEPES (pH 7.4), 100 μg/ml streptomycin, 10% fetal bovine serum (FBS), and 0.1% glucose until dispersion was apparent (approximately 6 h), and the suspension was filtered through sterile gauze and centrifuged to obtain a cell pellet. Cells were resuspended in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and streptomycin, 1% MEM vitamins, and were seeded and maintained in 150 cm^2 flasks. MCF-7 cells were cultured using conditions previously described [28].

RNA isolation

Tissue sections were pulverized under liquid nitrogen and total RNA and extracted using standardized methods [29, 30]. RNA was extracted from cultured cells directly without pulverization. RNA content was assessed by absorbance at 260 nm, purity was determined using A260/A280 ratios, and intactness was assessed by intensity of staining of 28S and 18S ribosomal RNA bands following agarose gel electrophoresis.

Oligonucleotide primer design

Oligonucleotide primers were designed using current DNA databases which contain sequence information for human ER alpha [31] and human ERβ [24], and 'Oligo' Ver. 5.0 software (National Biosciences, Plymouth, MN). Primers were designed for specific and reproducible amplification of the target mRNA without interference from inadvertent amplification of DNA.

Reverse transcription–polymerase chain reaction

Reverse transcription was carried out at 37°C using MMLV reverse transcriptase along with 5 μg total RNA in a buffer containing 1 mM each mixed NTPs, 5 mM Mg^{2+}, 1 U/μl RNase inhibitor and priming with 2.5 μM random hexamers [32]. Equivalent amounts of the reverse transcribed cDNA products were then aliquoted into separate tubes for the PCR of the individual ER targets. PCR was carried out in a buffer containing 2 mM Mg^{2+}, 0.25 mM each NTPs, 2.5 U/100 μl AmpliTaq^{R} DNA polymerase and 0.15 μM of individual primer pairs for each target. Amplification was carried out using 35 cycles of 2 step PCR as follows: elongation — 30 s at 68°C, denaturation — 30 s at 95°C.

ERα			
(EXON 4)	SENSE	22MER	5'-ATACGAAAAGACCGAAGAGGAG-3'
(EXON 5)	ANTISENSE	20MER	5'-CCAGACGAGACCAATCATCA-3'
ERβ			
(EXON 6*)	SENSE	22MER	5'-GGATGAGGGGAAATGCGTAGAA-3'
(EXON 8*)	ANTISENSE	19MER	5'-CCCGTGATGGAGGACTTGC-3'

*exons for ERβ are extrapolated from information of the ERα gene.

Fig. 1. Illustration of PCR primer pairs used for specific amplification of ERα and ERβ.

Analysis of RT-PCR products

RT-PCR products were characterized by electrophoresis on 8% polyacrylamide gels followed by ethidium bromide staining of amplified DNA and photography. Primer specificity was verified by sequencing of the RT-PCR product on a Perkin Elmer/ABI Prism 377 DNA sequencer at the DNA Sequencing and Gene Analysis Facility of the Bowman Gray School of Medicine.

RESULTS AND DISCUSSION

The mRNAs for ERα and ERβ share several regions which are similar in sequence. Using this information, we designed primer pairs for specific amplification of ERα and ERβ mRNA by RT-PCR. These primers were designed for high stringency annealing conditions, and the final selections of primers were based upon regions of high dissimilarity between the two receptor sequences. The primer pair for ERα was derived from sequences in exon 4 and exon 5 of the ERα mRNA (RT-PCR product size 417 bp), while primers for ERβ were derived from sequences in the ERβ mRNA which correspond to the exons 6 and 8 (RT-PCR product size 435 bp) of the ERα gene. The intron and exon structure of ERβ gene has not been reported as yet, so the exon locations were estimated from ERα gene structure (Fig. 1).

RT-PCR amplification of RNA from cynomolgus macaque ovary, uterus and from the cultured human breast cancer cell line MCF-7 resulted in production of DNA products of the predicted size for both ERα and ERβ (Fig. 2). Similar results were obtained for RNA isolated from coronary arteries (left anterior descending) dissected from both female and male macaque hearts, and from cultured aortic smooth

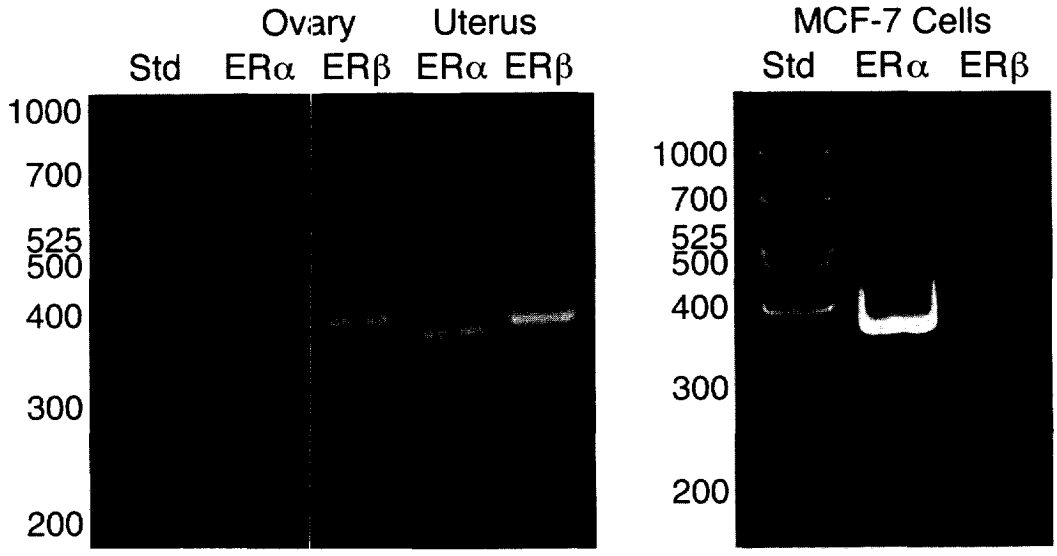

Fig. 2. Demonstration of ERα and ERβ mRNA in macaque ovary, uterus, and the human breast cancer cell line MCF-7 by RT-PCR.

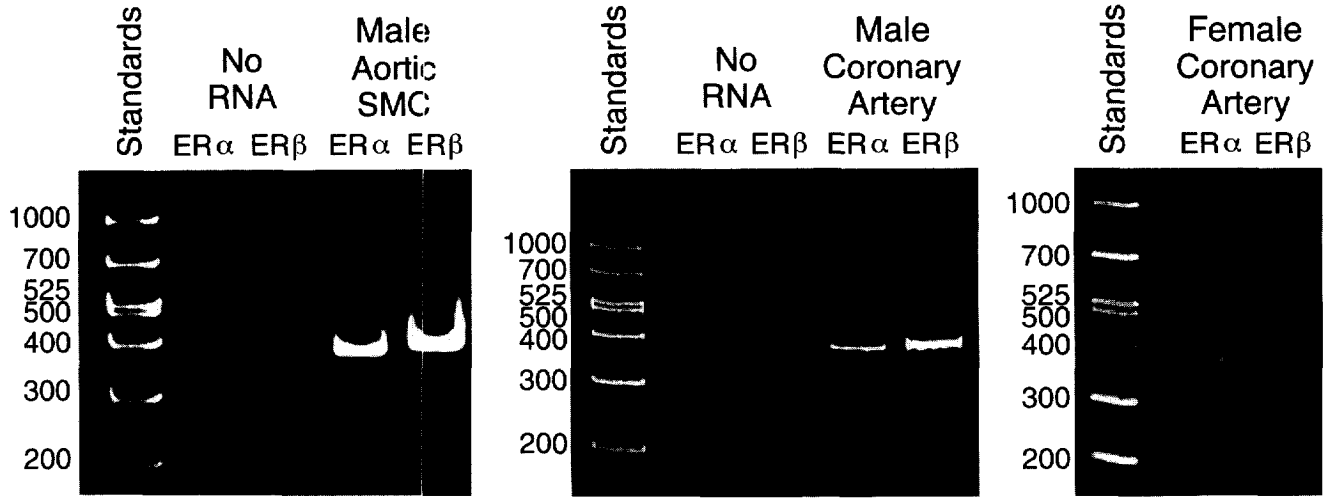

Fig. 3. Demonstration of ERα and ERβ mRNA in coronary arteries of male and female cynomolgus macaques by RT-PCR. Lanes represented by No RNA represent negative control amplifications.

muscle cells (male donor) (Fig. 3). The specificity of the PCR primers was verified by sequence analysis of the PCR products. The sequences of the regions amplified for cynomolgus monkey ERα and ERβ were >95% homologous to the sequences published for their human counterparts.

Comparison of the intensity of staining of the RT-PCR products suggests that the relative expression of ERβ mRNA to ERα mRNA was greatest in the ovary and of the same order of magnitude in the monkey vascular and uterine tissue. In contrast, the human breast cancer cell line MCF-7 exhibited a very low level of ERβ relative to ERα. Previous studies of rat tissue expression of the two receptor messages suggested that the expression of ERα was greater than ERβ in the rat uterus. The apparent discrepancy could be accounted for by a number of mechanisms. Rat uteri were obtained from intact animals, the uterus in the present study was obtained from an intact animal that had been treated with a triphasic oral contraceptive regimen containing ethinyl estradiol and levonorgestrel [33], a treatment likely to have effects on estrogen receptor expression. Further studies are necessary to determine the extent to which the expression of these receptors are regulated by hormonal therapies in both reproductive and non-reproductive tissues.

These results represent the first evidence for ERβ mRNA expression in non-human primate tissues and provide a new dimension for the study of estrogen action and hormone dependence of tissues. In addition, though the role of ER in mediating estrogen-associated effects on cardiovascular risk remain uncertain, the expression of ERα and ERβ mRNA in male coronary arteries suggests the possibility of targeting of these receptors for prevention or treatment of coronary artery disease in males through the use of tissue specific selective estrogen receptor modulators (SERMS). Recently, ERβ has been shown to act in an opposite manner from ERα with respect to ligand-mediated transcription from an AP1 site [34], highlighting the potential influence that alterations in the relative expression of ERα and ERβ might have on tissue responses to mammalian and plant estrogens, antiestrogens, and SERMS. Research addressing these issues will provide new insights into the tissue specific mechanisms of estrogen action.

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