Localized Expression of Aromatase in Human Vascular Tissues

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Abstract—The atheroprotective effects of estrogen are well established and the presence of an estrogen receptor in vascular tissues has recently been reported. Therefore, we investigated the localization of the estrogen-producing enzyme aromatase in vascular tissues to assess the possible contribution of endocrine, paracrine, and autocrine modes of action. Aromatase was found in human vascular smooth muscle cells (SMCs) but not in endothelial cells on in situ hybridization. These observations were further supported by quantitative analysis of aromatase mRNA and the activity in 15 human vascular specimens. Only trace levels of expression were detected in the 3 infants examined, whereas 0.0088 to 0.0806 amol/µg RNA of aromatase mRNA and 12.9 to 122.3 fmol·h⁻¹·mg⁻¹ protein of the activity were detected in 12 of the adult individuals. The switching of tissue-specific exon 1 of the human aromatase gene was also observed in some cases. Aromatase was found to be expressed only in cultured SMCs and not in cultured endothelial cells of human aorta and pulmonary artery and to be regulated through dexamethasone and the signaling pathways of protein kinase A and C. Study results revealed the localized expression of aromatase in vascular SMCs, which indicated a possible direct action of locally produced estrogen in an autocrine or paracrine manner, with possible cross talk between smooth muscle and endothelial cells. (Circ Res. 1999;84:1285-1291.)

Key Words: estrogen • aromatase • in situ hybridization • smooth muscle cell

It is well known that the incidence of cardiovascular disease in premenopausal women is significantly low compared with that in men or postmenopausal women.¹ ² This pathogenic difference is considered to be mainly due to the differences in the estrogen levels supplied to cardiovascular tissues. Epidemiological studies have revealed that estrogen replacement therapy suppresses the development of atherosclerosis after menopause or oophorectomy and reduces cardiovascular mortality.³ An atheroprotective effect of estrogen was also indicated for atherosclerotic progression in experimental animals.⁴ ⁸ This may be explained by an estrogen-induced increase in HDL cholesterol and a decrease in LDL cholesterol⁹ ¹⁰ or by the antioxidant action of estrogen, especially catechol estrogen, toward LDL.¹¹ ¹³ Alternatively, a direct biological action of estrogen on cardiovascular tissues may be an explanation for the atheroprotective effect of estrogen. This action is known to be associated with the suppression of elastin and collagen accumulation in the aorta wall¹⁴ and increases in prostacyclin,¹⁵ ¹⁶ nitric oxide, and endothelium-derived relaxing factor¹⁷ production.

In line with these findings, specific binding proteins for estrogen have been observed in vascular tissues by other investigators.¹₈ ¹⁹ The observation of these specific binding proteins supports the hypothesis that an estrogen-responsive signaling pathway exists in vascular tissues. In fact, functional estrogen receptors have been seen in human vascular smooth muscle cells (SMCs)²⁰ ²² and endothelial cells (ECs).²³ ²⁴ In addition, it has been reported that rat vascular cells have the capacity to synthesize estrogen in an in vitro culture,²² ²⁴ which suggests that a paracrine or autocrine mechanism of estrogen-estrogen receptor signaling may function in vascular cells, as was reported for brain and breast cancer tissues.

The enzyme aromatase, or estrogen synthetase (EC 1.14.14.1), which catalyzes the key step of aromatization of androgen in estrogen synthesis, is widely distributed in gonadal and extraglandular tissues and plays important roles in their physiological functions through tissue-specific regulation of estrogen production. The human aromatase gene was recently shown to comprise multiple exons 1 and promoters that are alternately used in a tissue-specific manner.²⁵ ²⁶ We have found frequent switching of tissue-specific exon 1b (I.4) to exon 1c (I.3) in transcripts of adipose tissue of breast cancer patients,²⁷ which may cause local overproduction of estrogen and the resultant proliferation of estrogen-dependent breast cancer cells. This switching may also cause a local shortage of estrogen and be one of the pathogenic factors in osteoporosis or atherosclerosis. In the present study, we
investigated the localization of aromatase in human vascular tissues and its regulation in cultured vascular cells. The results suggest the possibility of cell-cell interactions and an atheroprotective effect of estrogen locally produced in vascular tissues.

Materials and Methods

Tissues and Cultured Cells
Infrarenal abdominal aortas were collected at autopsy at Tohoku University Hospital (Sendai, Japan). Various degrees of atherosclerotic changes were detected on histopathological analysis of the specimens. For analysis of aromatase mRNA, the specimens were immediately frozen in liquid nitrogen and stored at −80°C. Human aortic ECs from a 34-year-old white female and SMCs from a 2.5-month-old white male, pulmonary artery ECs from a 34-year-old white female and SMCs from a 45-year-old white female, and umbilical vein ECs from a white female were prepared by Clonetics Corp (San Diego, Calif) and obtained from Kurabo Co (Osaka, Japan). Specimens were checked for morphological features, proliferation rate, and doubling capacity. The ECs were also checked for von Willebrand antigen expression and the uptake of diacetylated LDL. With the use of fluorescent staining, SMCs tested positive for α-smooth muscle actin and negative for von Willebrand antigen. This study was approved by the Ethics Committee on Human Studies of Tohoku University School of Medicine and by the Medical Ethics Committee of Fujita Health University School of Medicine (Aichi, Japan).

In Situ Hybridization
A 27-base aromatase antisense oligonucleotide probe (5′-GCAGTACCAAGTCAGGCTGTGTTAGAGGTGTCCAGCATG-3′) was used for in situ hybridization. The use of this probe in situ hybridization analyses of aromatase mRNA in normal human cycling ovaries and endometrial cancer was previously described. Sense oligonucleotide probes were used as negative controls. The probes were labeled with a 3′-biotinylated tail (Brigati tail). Hybridization was performed with a MicroProbe staining system (Fisher Scientific) by use of manual capillary actions; the method was modiﬁed as previously reported. Tissue sections (3 μm) were placed on Probe ON Plus plus microscopy slides (Fisher Scientific) and were rapidly dewaxed, cleared with alcohol, rehydrated with a Tris-based buffer, pH 7.4 (Universal Buffer, Research Genetics), and then digested with pepsin (2.5 mg/mL; Research Genetics) for 3 minutes at 105°C. The slides were then washed with 105°C for 3 minutes, cooled for ≈1 minute at room temperature, and allowed to hybridize at 45°C for 60 minutes. The sections were then washed twice with 2× SSC buffer (1× SSC contains 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.0) at 45°C (3 minutes per washing) and detected with alkaline phosphatase-conjugated streptavidin (Research Genetics). After the hybridization products were washed once in AP chromogen buffer, pH 9.5, Research Genetics) at room temperature, they were visualized with fast red. The slides were counterstained with hematoxylin, air dried, and then coverslipped for microscopic examination. For every specimen, we used a 20-base poly-T oligonucleotide probe (Research Genetics) to examine the retention of mRNA in human aortic tissues. Intactness of the mRNA in the aortical tissues was further confirmed by 2 distinct bands of 28S and 18S ribosomal RNA on an ethidium bromide–stained agarose gel and then by quantitative analysis of β-actin mRNA in every necropsy specimen. Marked hybridization signals were detected in all cells in the cases examined.

Cell Culture
Vascular SMCs and ECs were plated at 3×10^4 cells per well on 6-well tissue culture dishes and then cultured in modified MCDB131 (Clonetics) medium supplemented with 2% fetal calf serum, 10 ng/mL human epidermal growth factor, 1 μg/mL hydrocortisone, 0.4% bovine brain extract, and 50 μg/mL gentamicin, and in S-BM medium (Clonetics) supplemented with 5% fetal calf serum, 2 ng/mL human fibroblast growth factor, 10 ng/mL human epidermal growth factor, 0.39 μg/mL dexamethasone, and 50 μg/mL gentamicin, respectively. Fetal calf serum was pretreated with activated charcoal to remove steroid hormones. The medium was changed every day. Total RNA was recovered from the cells after being incubated for 24 hours with 20 μmol/L forskolin, 0.2 μmol/L phorbol ester (PMA, phorbol 12-myristate 13-acetate), or 1 mmol/L dibutyryl cGMP. Cells were also cultured in the medium without dexamethasone and used as controls to determine the influence of dexamethasone treatment. After the cells were incubated for 12 hours with 1 μmol/L dexamethasone, aromatase mRNA in the total RNA was quantified.

Analysis of Aromatase Activity
Aromatase activity in vascular tissues was determined according to the published method with a modification. Microsomal fractions were prepared from vascular tissue homogenates by successive centrifugation as described and suspended in 20 mmol/L Tris-HCl (pH 7.5) that contained 1 mmol/L EDTA, 10% glycerol, 5 μg/mL pepstatin, and 5 μg/mL leupeptin. The reaction mixture comprised 0.1 to 1 mg of microsomal fraction, 5 mmol/L MgCl_2, 5 mmol/L glucose-6-phosphate, 5 U of glucose-6-phosphate dehydrogenase, 2 μmol/L rotenone, and 200 pmol of 1β-[H] androstenedione (Dupont NEN) in 0.5 mL of 50 mmol/L Tris-HCl (pH 7.5). After the reaction was preincubated for 3 minutes, the reaction was initiated by the addition of NADPH (0.5 μmol) and continued for 60 minutes at 37°C. Under these conditions, the aromatase activity was linearly correlated with the amount of microsomal protein and the incubation time. The reaction was terminated by adding 3 mL of ice-cold chloroform and applying vigorous shaking and brief centrifugation. The resulting aqueous layer was further extracted with 3 mL of chloroform and treated with 0.5 mL of 5% activated charcoal/0.5% dextran. After centrifugation of the mixture, the radioactivity of the supernatant was counted. Aromatase activity was quantified by measurement of the tritiated water released from 1β-[H] androstenedione. The reaction was also performed in the presence of 10 μmol/L vorozole, a specific aromatase inhibitor, as a specificity control and without NADPH as a background control. Aromatase activity in cultured cells was evaluated as the accumulation of 17β-estradiol aromatized from testosterone during 12 hours culture. Cells treated or untreated with forskolin, PMA, or dibutyryl cGMP for 24 hours were further cultured for 12 hours after the addition of testosterone (1 μmol/L). The amount of 17β-estradiol in the cultured medium was determined by ELISA (Seron Tohoku Research Institute).

Preparation of Total RNA
Total RNA was prepared from autopsy specimens of human arterial tissues. Frozen tissues were homogenized in 5 mol/L guanidine thiocyanate that contained 5 mmol/L sodium citrate and 0.5% sodium sarcosyl, and then total RNA fractions were prepared from the homogenates as described by Chirgwin and colleagues.

Analysis of Aromatase mRNA
Quantitative analysis of aromatase mRNA in the total RNA fractions was performed by means of reverse transcriptase-polymerase chain reaction (RT-PCR) with the use of a fluorescent dye–labeled primer in the presence of an internal standard mRNA, as previously described. In brief, oligonucleotides of an antisense primer (5′-AACCCAGTACCATCTTGTGTTAGAGGTGTCCAGCATG-3′) for reverse transcription and nested antisense- (5′-TTTTAGGAGTGCAGCATG-3′) and fluorescent dye– (FAM; Perkin-Elmer Co.) labeled sense (5′-TACTACACCGGTTATAGGTTATGCAGT-3′) primers for PCR were synthesized. The coding sequence between the 2 PCR primer sites is 5′ upstream of the reverse transcription primer site in the aromatase transcript and is interrupted by 2 introns in the gene. To prepare an internal standard RNA, modified human aromatase cDNA was constructed by inserting a 21-bp fragment of HoxIII-digested λ-DNA between the 2 PCR primer sites. The internal standard RNA was synthesized in vitro with T7 RNA polymerase with the use of the
modified aromatase cDNA as a template. Both aromatase mRNA in the total RNA and the internal standard RNA were subjected in the same reaction mixture to reverse transcription with Rous-associated virus type-2 reverse transcriptase (Takara Shuzo Co) and a specific primer at 42°C for 40 minutes. The resulting cDNAs were amplified by PCR with the use of fluorescent dye–labeled sense and antisense primers for 24 to 28 cycles. The fluorescent PCR products were analyzed on a 2% agarose gel with a Gene Scanner 362 fluorescent fragment analyzer (Perkin-Elmer Co). The amount of aromatase mRNA in the tissue RNA was calculated from the peak areas of the fluorescent products by the internal standard method. The use of alternating forms of exons 1 of the aromatase gene in vascular tissues was investigated by RT-PCR as previously described.27,34 Oligonucleotide sense primers 1a (5'-CTGGAGGGCTGAACACGTGG-3'), specific for placenta-specific exon 1a (I.1); 1b (5'-GACCAA-CTGGAGCCTGACAG-3'), specific for skin fibroblast/fetal liver-specific exon 1b (I.4); 1c (5'-CCTGTGTTTGGACTTGGAACCA-3'), specific for ovary-specific exon 1c (I.3); and 1d (5'-AACA-GGAGCTATAGATGAAC-3'), specific for ovary/prostate/testis-specific exon 1 d (PII), and a fluorescent dye–labeled antisense primer (5'-CAGAGATCCAGACTCGCATG-3'), specific for exon 3, were synthesized. The cDNAs were obtained from total RNA fractions as described above and amplified by PCR with a fluorescent dye–labeled antisense primer, and primer 1a, 1b, 1c, or 1 d for 24 to 28 cycles. Because the exon 1–specific primers were designed to produce PCR products of different sizes, the fluorescent PCR products were separated by electrophoresis on 2% agarose gels and then analyzed with a Gene Scanner 362. To check the quantity and integrity of RNA samples, β-actin mRNA, which was used as a control, was also analyzed by RT-PCR.27 Aromatase mRNA levels were corrected by comparing them to ubiquitous β-actin mRNA, giving similar results.

Statistical Analysis
Statistical analysis was performed by 1-way ANOVA, followed by use of the Scheffé test. A P value of <0.05 was considered to be significant.

Results

In Situ Hybridization of Aromatase mRNA
The localization of aromatase in human aortic tissues was investigated at the mRNA level by in situ hybridization. As shown in Figure 1, there was heterogeneity in the levels of mRNA hybridization signals. However, SMCs of the tunica media and fibroblasts of the tunica adventitia were predominantly positive (Figure 1-I). Control sections that were hybridized with a sense oligonucleotide probe showed no hybridization signals (Figure 1-II).

Expression of Aromatase in Human Arterial Tissues
The expression of aromatase in human arteries was analyzed by use of autopsy specimens from patients who had died of noncardiac causes with normal coronary arteries and who had never received any treatment involving hormone replacement therapy (Table 1). Significant levels of aromatase activity were detected in all arteries examined. The levels of aromatase mRNA expression in the arterial tissues examined from 3 infants were extremely low or undetectable, whereas they were much higher in adults (0.0088 to 0.0806 amol/μg RNA). Representative RT-PCR profiles of several samples are shown in Figure 2. A significant correlation between the levels of aromatase mRNA and the activity in arterial tissues was observed. Investigation of the use of multiple exons 1 of the human aromatase gene revealed that aromatase mRNA in the arterial tissues examined was transcribed mainly from fetal liver/skin fibroblast–specific exon 1b (I.4) of the gene. Interestingly, the switching between tissue-specific forms of exons 1 was often observed in the aromatase transcripts of adult tissues.

Regulation of Aromatase in Vascular SMCs and ECs
Regulatory factors for aromatase were examined in cultured SMCs and ECs of human aortic, pulmonary, and umbilical vessels. As shown in Table 2, aromatase mRNA was expressed in all the SMCs but not in any ECs, which was consistent with the in situ hybridization results. The level of aromatase mRNA expression in the SMCs was increased by forskolin and PMA, which suggested induction through the protein kinase A (PKA) and C (PKC) signaling pathways. After complete removal of dexamethasone from the SMC culture medium, its effect on the expression was examined. Dexamethasone also caused an increase in expression, whereas dibutyryl cGMP had no effect. Aromatase mRNA in all the SMC was transcribed from exon 1b (I.4) of the gene, regardless of the treatment (data not shown). In agreement with expression of aromatase mRNA, estrogen production
Aortic and pulmonary SMCs aromatized testosterone in the culture medium to 17β-estradiol (109 and 143 pg per 12 h per 1×10^5 cells, respectively). Estrogen synthesis by both types of SMCs was induced by forskolin and PMA but not by dibutyryl cGMP, which is consistent with the induction of mRNA.

Discussion

Although the atheroprotective effects of estrogen have been considered to be the result of an indirect action on plasma lipoproteins, a direct action on vascular tissues has recently been suggested on the basis of many lines of evidence. The estrogen receptor or binding proteins in vascular SMCs and ECs have been identified by means of immunocytochemical studies and ligand-binding assays. In addition, the trait of estrogen synthesis was also reported for rat arterial SMCs and bovine aortic ECs in culture. The present study, in demonstrating the localization of aromatase (estrogen synthetase) in human arterial SMCs, indicates the potential for autocrine and/or paracrine activity of estrogens. Estrogen produced in vascular SMCs might regulate SMC functions, such as contractility, vasodilation, and collagen synthesis, and also EC functions, such as prostacyclin and endothelin synthesis, nitric oxide production, and angiogenesis. Conversely, products of vascular EC may influence SMC functions. Recent findings that modulation of endothelium-derived nitric oxide release is dependent on the number of estrogen receptors in the blood vessel wall suggests a local vascular mechanism for estrogen-induced atheroprotection. The results in the present study suggest the possibility that signal transduction pathways involving estrogen/estrogen receptors (binding proteins) could operate in vascular tissues to effect communication between vascular SMCs and ECs.

In this study, we demonstrated the presence of aromatase mRNA in vascular SMCs. In addition, we demonstrated that aromatase in vascular tissues is catalytically active (Table 1) and, in fact, SMCs are able to produce 17β-estradiol after the addition of testosterone as a substrate to the culture medium (Table 2). A significant amount of 17β-estradiol was produced by all cultured SMCs, which was consistent with a recent report, whereas undetectable levels of 17β-estradiol were observed in ECs. The human aortic, pulmonary arterial, and umbilical vein ECs were cultured in the medium with charcoal-stripped serum. Because the suboptimal medium containing charcoal-stripped serum is known to inhibit

<table>
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ND indicates not detectable; ellipses, not determined.

Figure 2. Quantitative analysis of aromatase mRNA in human aortic tissues. The total RNA from aortic tissues of patients 1, 7, 8, and 9 (Table 1) was subjected to RT-PCR in the presence of an internal standard RNA with a fluorescent dye-labeled primer. Fluorescent PCR products (solid lines) showed 2 peaks derived from aromatase mRNA (Ar) and the internal standard RNA (St) at positions of 378 and 399 bp, respectively. The internal size markers (broken lines) of 262, 293, 317, and 439 bp are also shown.
estrogen receptor expression in EC,

38 the EC were also cultured in the medium with untreated serum. Neither aromatase activity nor aromatase mRNA in the EC were detected in both the culture mediums (data not shown). It is feasible that vascular SMCs produce a significant amount of 17β-estradiol in vivo because the substrates, testosterone and androstenedione, are readily available from the blood, and 17β-hydroxysteroid dehydrogenase is also present in vascular SMC.

We recently reported elevated levels of aromatase mRNA and the switching of alternately spliced forms of exons 1 in transcripts in human breast cancer tissues. 27 Similarly, the present study indicated significant levels of aromatase mRNA and frequent switching of alternate forms of exons 1 of the gene in adult arterial tissues. The aromatase mRNA levels in arterial tissues of the adult individuals in the present series were >10-fold higher than those in breast cancer tissues. 27 It is possible that the local concentration of estrogen reached in arteries is considerably higher than that in the circulating plasma, and aromatase in vascular SMCs could play important roles in vascular physiological functions through local production of estrogens. Also, aromatase mRNA is mainly transcribed from exon 1b (1.4), and the switching of alternate forms of exons 1 was often found in both adult arterial and breast cancer tissues.

Aromatase in vascular SMCs was induced by cAMP, PMA, and dexamethasone (Table 2), thus indicating participation of PKA, PKC, and glucocorticoid receptor, respectively, in the regulation of vascular aromatase. We also examined the effects of other activators and inhibitors of PKA and PKC on the expression of aromatase in vascular SMCs (data not shown). The expression of aromatase was also induced by dibutyryl cAMP, which acted as a PKA activator, and inhibited by KT5720, which acted as a PKA inhibitor. Similarly, PMA induced aromatase as a PKC activator, whereas 4α-PMA as a negative control of a PKC activator did not cause any increase of aromatase. In addition, H-7, a PKC inhibitor, inhibited an increase of aromatase, although the inhibition by H-7 is not strictly specific for PKC. It has been reported that vascular SMCs express several kinds of receptors, such as the prostaglandin-I2 receptor and endothelin receptor, that participate in cross talk regulation between vascular SMCs and ECs. Because prostaglandin I2 and endothelin transduce intracellular signaling through cAMP/PKA and inositol 1,4,5-triphosphate/diacylglycerol/PKC, respectively, they may be candidates of physiological regulatory factors of vascular aromatase. On the contrary, cGMP, an intracellular second messenger of the atrial natriuretic peptide and nitric oxide, did not have any effect on the expression of vascular aromatase. There were some inconsistent responses to aromatase inducers, especially PMA, between aromatase mRNA and estrogen synthesis activity in cultured SMCs (Table 2). This may reflect the difference in the half-lives of the mRNA and the enzyme protein. Because the half-life of mRNA is usually much shorter than that of a protein, the aromatase mRNA levels determined in this experiment will roughly reflect the transcription rate at the time point of 24 hours after induction, whereas the activity will roughly reflect the accumulated amount of the aromatase protein synthesized during 24-hour culture for induction and 12-hour culture for the assay. In addition, 17β-estradiol synthesized by cultured SMCs may be converted to other metabolites that are undetectable by ELISA 17β-estradiol assay. This may also cause inconsistent results.

The dissociation constant of the high-affinity estrogen receptor in cultured vascular cells was reported to be 8×10⁻²¹ or 5×10⁻⁸ mol/L, 19 or for 17β-estradiol, whereas the concentration of 17β-estradiol in the adult human serum was reported to be usually 3×10⁻¹¹ to 2×10⁻⁹ mol/L, which is the value for the preovulatory peak of estrogen in women. This indicates that the circulating level of plasma 17β-estradiol is far lower than the Kd value of the estrogen receptor in vascular cells for 17β-estradiol. The close juxtaposition of vascular ECs and SMCs in blood vessels suggests that estrogen levels in vascular tissues might be locally sufficient for efficient activation of the estrogen receptor and regulation of the physiological functions of vascular cells. A similar situation has in fact been reported for the estrogen-dependent proliferation of breast cancer cells. 40,41 Many in vivo and in vitro studies have indicated that estrogen has inhibitory effects on the proliferation and DNA synthesis of SMCs, 42,48 and indeed the atheroprotective effect of estrogen has been explained.
on this basis. There have also been reports of contradictory data, such as the (1) potentiation of DNA synthesis by estrogen; (2) suppression of SMC proliferation by tamoxifen; and (3) induction of c-fos (proto-oncogene associated with mitogenesis) in SMCs by estrogen. Recently, Morales and colleagues described the wound healing effects of estrogen on cultured umbilical vein ECs and stimulation of neovascularization in oophorectomized mice. Bone ECs can also be induced to proliferate by estrogen. These observations suggest the possible involvement of estrogen in angiogenesis.

In conclusion, the present demonstration of aromatase in the SMCs of the human aorta raises the possibility that estrogen may play important roles in various physiological functions of the vascular EC-SMC system in a paracrine or autocrine manner. Additional studies are necessary to elucidate how this relates to the atheroprotective and angiogenenic effects of estrogen.

Acknowledgments

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