CLINICAL STUDY

Aromatase is differentially expressed in peripheral blood leukocytes from children, and adult female and male subjects

A Vottero, V Rochira¹, M Capelletti, I Viani, L Zirilli¹, T M Neri², C Carani¹, S Bernasconi and L Ghizzoni

Department of Pediatrics, University of Parma, Via Gramsci 14, 43100 Parma, Italy, ¹Department of Endocrinology, University of Modena, Modena, Italy and ²Human Genetics Branch, Parma Hospital, Parma, Italy

(Correspondence should be addressed to L Ghizzoni; Email: lucia.ghizzoni@unipr.it)

Abstract

Objective: Aromatase, the key enzyme involved in estrogen synthesis, is expressed in a variety of cells and tissues including human peripheral blood leukocytes (PBLs). The present study was designed to evaluate PBL aromatase gene expression in male and female subjects of different age groups. In addition, differences in gene expression during the follicular and luteal phase of the menstrual cycle in women, and before and after testosterone administration in men, were estimated.

Design: Aromatase mRNA and protein were measured in PBLs obtained from young ($n = 10$) and postmenopausal women ($n = 10$), men ($n = 15$), and prepubertal children ($n = 10$). Aromatase mRNA and protein were also measured during the follicular and luteal phases of the menstrual cycle in women, and before and after the intramuscular administration of 250 mg testosterone enanthate in men.

Methods and Results: Aromatase mRNA measured by real-time PCR in PBLs from women during the follicular phase was significantly higher than during the luteal phase of the menstrual cycle ($P < 0.05$). In men, PBL aromatase mRNA values increased significantly following testosterone administration ($P < 0.05$). PBL mRNA aromatase levels in women during the follicular phase and men after testosterone administration were significantly higher (one-way ANOVA; $P < 0.05$) than in any other group. Children, postmenopausal women, and women during the luteal phase showed the lowest aromatase mRNA expression. The results of the immunoblot analysis confirmed the data obtained by real-time PCR. A positive correlation between PBL aromatase mRNA values and plasma estradiol and estrone levels during the follicular phase of the menstrual cycle was observed in the group of adult women. No other correlations were found.

Conclusions: The aromatase gene is differentially expressed in PBLs from women, men, and prepubertal children, indicating a sexual dimorphism in the enzyme expression and an important role of sex steroids in the modulation of aromatase gene expression.

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Introduction

Aromatase, also called estrogen synthetase or P450arom, is the key enzyme that catalyzes the conversion of testosterone to estradiol, and androstenedione to estrone (1). The P450arom gene (CYP19) spans at least 123 kb and contains nine coding exons (II–X) located on chromosome 15q21.1 in humans (2). Human CYP19 is a member of the cytochrome P450 gene superfamily (1). It is unique among the members of this family because its tissue-specific expression is regulated, at least in part, by the use of different promoter regions, employing alternative splicing mechanisms (I.1, I.2, I.3, I.4, PII) (3, 4). Aromatase is expressed in a number of cells and tissues, such as placenta (5),

gonads (6, 7), various parts of the brain (8), including the amygdala and hypothalamus, fibroblasts (9), adipose tissue (10), normal breast and breast cancer (11–13), and a number of fetal tissues (14), such as liver, brain, and intestine (15). Although aromatase expression with alternative promoter use was reported in human myeloid leukemia cells (16), its presence and regulation in human peripheral blood leukocytes (PBLs) has not yet been fully elucidated. Bernstein *et al.* (17) suggested the presence of 'pseudoaromatase' in PBLs, because of the apparent aromatase activity in the absence of P450arom gene transcripts. More recently, P450arom mRNA and protein, and its activity were detected in PBLs from five normal subjects before and after transformation with the Epstein–Barr virus.

In the latter, the use of proximal promoters I.3 and PII was demonstrated for gene expression (18). These promoters are mainly used in the ovaries (I.3 and PII), and testis and prostate (PII) (3). In addition, a novel exon 1 sequence was recently identified and aberrantly expressed in patients with the aromatase excess syndrome (19). This variant was found both in target tissues, such as mammary gland and fibroblasts, and in lymphocytes, further suggesting a possible correlation between estrogen target tissues and human PBLs.

To test the hypothesis that a distinct intracrine estrogen synthesis is physiologically present in the different sexes and varies with age and hormonal milieu, we measured aromatase expression and protein in PBLs obtained from adult women and men, and prepubertal children. We elected to study PBLs because they represent a tissue easily obtainable in unlimited amounts. In addition, to assess whether PBL aromatase varies with the menstrual cycle in women and with plasma testosterone concentrations in men, enzyme expression and protein were measured during the follicular and luteal phases of the menstrual cycle in women, and before and after the intramuscular (i.m.) administration of testosterone enanthate in men.

Materials and methods

Subjects

Fifteen adult men (age 27.8 ± 0.8 years) before and 3 days after i.m. administration of testosterone enanthate (Testoviron, Schering, Berlin, Germany) (250 mg), ten young women (age 30.3 ± 1.9 years) during the follicular and luteal phases of the menstrual cycle, ten postmenopausal women (age 56 ± 6.9 years), and ten prepubertal children (five males and five females; age 4.6 ± 1.9 years) were studied. All the premenopausal women had regular menstrual cycles, and the luteal phase was confirmed by progesterone levels. In men, blood samples were drawn 3 days after testosterone injection because peak serum testosterone levels occur between days 2 and 4 after the i.m. administration of testosterone enanthate (20). Twenty milliliters of blood were withdrawn from adults, and 10 ml from children after obtaining informed consent from the subjects studied and the children's parents. This study was approved by the Clinical Research Committee of the Department of Pediatrics at the University of Parma (Parma, Italy).

Generation of an external aromatase RNA calibrator

The aromatase fragment selected for the real-time PCR was amplified by PCR using specific primers (forward: 5'-ATC AGC AAG TCC TCA AGT AT-3'; reverse: 5'-TCT GTG GAA ATC CTG CGT CTT T-3'). The fragment of interest was then run in an agarose gel and

sequenced by the CEQ 2000XL DNA analysis system (Beckman Coulter, Fullerton, CA, USA) to verify its integrity. The 519 bp PCR product was subcloned in the plasmid vector PCR 2.1 TOPO containing a T7 RNA polymerase promoter (TOPO TA Cloning; Invitrogen, Carlsbad, CA, USA), and then the plasmid DNA was isolated and purified by the Qiaprep Spin Miniprep (QIAGEN, Valencia, CA, USA). The aromatase insert was quantified and verified by DNA sequencing on the CEQ 2000XL DNA analysis system using M13 primers. Linearization of the purified plasmid DNA was achieved by restriction with HindIII and RNA synthesized *in vitro* using the RiboMAX large scale RNA production system (Promega, Madison, WI, USA) and T7 polymerase. Purification of the RNA template was performed by DNase treatment followed by phenol–chloroform precipitation and then quantified by a spectrophotometer, and serial dilutions starting from 2500 ng/ μ l to 0.25 pg/ μ l were reverse transcribed. Real-time PCR (ABI PRISM 7700 sequence detector, Applied Biosystems, Foster City, CA, USA) was performed using known quantities of the cDNA obtained after RT-PCR, as described below, and the calibration curve was used for quantification of aromatase from PBLs.

Aromatase expression

Leukocyte separation PBLs were extracted by the Ficoll method. Heparinized human blood was layered over the separation medium and centrifuged at a low speed for 30 min. During centrifugation, differential migration resulted in the formation of several cell layers, leading lymphocytes and other mononuclear cells at the plasma–Ficoll interface.

DNA and RNA extraction The extraction was performed using the single-step phase separation (TRIzol reagent kit; Molecular Research Center, Cincinnati, OH, USA).

PCR amplification PCR amplification of the coding region of the P450arom gene was performed with intronic primers reported previously (19). The products of these reactions were run on ethidium bromide-stained 1% agarose gels. Direct PCR sequencing was performed using an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

mRNA quantitation Total RNA was extracted from PBLs, using single-step liquid phase separation (TRIzol reagent kit). cDNA synthesis was accomplished with the random primer using a reverse transcriptase-PCR kit (Boehringer-Mannheim, Mannheim, Germany). real-time quantitative PCR (TaqMan PCR, Applied Biosystems, Foster City, CA, USA) was utilized for mRNA quantitation. Specific primers and a fluorescent probe for aromatase were used (arom forward: 5'-CCC TTC TGC GTC GTG TCA T-3'; arom reverse: 5'-GAT TTT

AAC CAC GAT AGC ACT TTC G-3'; arom probe: 5'-FAM-CTG GAC ACC TCT AAC ACG CTC TTC TTG AGG -3'-TAMRA (FAM, reporter dye; TAMRA, quencher dye)). The TaqMan ribosomal RNA control primers and probe were used to detect the 18S ribosomal RNA levels that provide an endogenous control for PCR quantitative studies (TaqMan ribosomal RNA control reagents, VIC probe, Applied Biosystems, Foster City, CA, USA). Following the TaqMan protocol, the reactions were prepared in triplicate using 250 ng of each cDNA and adding the following reagents at the following concentrations: 1 × TaqMan universal PCR master mix, 900 nM primers, 100 nM probe, and 50 nM 18S ribosomal RNA primers and probe. Reactions were started at 95 °C for 10 min to activate AmpliTaq Gold DNA polymerase (Promega, Madison, WI, USA), and run for 50 cycles at 95 °C for 15 s and 60 °C for 1 min. PCR amplification was performed using the ABI PRISM 7700 sequence detection system (Perkin Elmer Applied Biosystem, Wellesley, MA, USA). Results were normalized using the 18S ribosomal RNA. The relative abundance of aromatase was calculated based on the standard curve and expressed as pg/μg RNA.

Protein extraction Protein content was obtained after lysis of the cells using a sonicator at 4 °C in a buffer containing a cocktail of protease inhibitors, and subsequent centrifugation at 3000 r.p.m. for 10 min at 4 °C. Protein concentration of each sample was determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) using BSA as standard. One hundred micrograms of protein were used for the Western analysis.

Western blot analysis Western blot analysis using a specific anti-aromatase cytochrome P-450 antiserum was performed (Hauptman-Woodward, Buffalo, NY, USA). This antibody recognizes a single band of 55 kDa, the approximate molecular mass of P450arom. One hundred micrograms of protein were applied to 7% precast NuPAGE Tris–acetate gels (Novex, San Diego, CA, USA) and run in parallel with prestained markers (SeeBlue; Novex) to estimate molecular weight. Proteins were then transferred to nitrocellulose membranes and blocked in 5% non-fat milk for 1 h. Immunoblotting was performed at 4 °C overnight, using the specific anti-aromatase polyclonal antibody at 1:6000 dilutions. After washing, the blots were incubated at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin antibody (Dako, Carpinteria, CA, USA) at 1:4000 dilutions. Blots were washed and exposed to chemiluminescence solution for 1 min (ECL kit; Amersham Life Science, Piscataway, NJ, USA), followed by exposure to X-OMAT AR films (Eastman Kodak Co., Rochester, NY, USA). Ovarian granulosa cells were used as controls.

Aromatase activity

Aromatase activity was measured by Δ^4 -androstenedione to estrone conversion in primary cultures of PBLs, and expressed as fmol estrogen/10⁶ cells per h, as previously described (19, 21, 22). Briefly, cell cultures, treated for 19 h with a combination of cAMP (at a final concentration of 500 μM) and phorbol-12,13-diacetate (at a final concentration of 100 nM), were washed with PBS solution and incubated with 1 ml assay medium containing approximately 0.5 μCi 1β-[³H]androstenedione. The culture plates were then placed in an incubator (5% CO₂) at 37 °C for 24 h. The following day, the medium was transferred to a test tube and 300 μl trichloroacetic acid was added, followed by 2 ml chloroform. The unconverted substrate and steroid products were extracted into the organic phase.

An aliquot of 0.7 ml of the aqueous phase was treated with 2.5% activated, dextran-coated charcoal suspension to remove residual steroids. Tritiated water (³H₂O) formed during the aromatization reaction was measured by counting the radioactivity in the supernatant. Granulosa cells and fibroblasts were used as positive controls.

Hormone assays

An aliquot of blood was centrifuged immediately after collection, and plasma was stored at –20 °C until assayed. Estradiol and estrone were measured using third-generation, ultra-sensitive RIA kits (Diagnostic Systems Laboratories, Webster, TX, USA) with a sensitivity of 8.1 pmol/l and 4.4 pmol/l respectively. The intra- and interassay mean coefficients of variation were 7.4% and 9.3% for estradiol, and 6.4% and 9.1% for estrone respectively. The cross-reactivity of the estradiol antiserum was 2.40% with estrone, 2.56% with 17β-estradiol-3-glucuronide, and non-detectable with testosterone. The cross-reactivity of the estrone antiserum was 1.25% with 17β-estradiol, 0.22% with estriol, 0.10% with 17α-hydroxyprogesterone, and non-detectable with testosterone. Testosterone and progesterone were also measured with RIA kits (Diagnostic Corporation, Los Angeles, CA, USA). The intra- and interassay mean coefficients of variation were 5% and 6.7% for testosterone, and 6.3% and 7.5% for progesterone respectively.

The cross-reactivity of the testosterone antiserum was 3.3% with 5α-dihydrotestosterone, 0.02% with estradiol, and non-detectable with progesterone. The cross-reactivity of the progesterone antiserum was 3.4% with 17α-hydroxyprogesterone, 0.1% with testosterone, and non-detectable with estradiol.

Statistical analysis

All experiments were repeated on at least three independent occasions. Values are reported as means ±

S.E.M. unless otherwise stated. A test for normality was performed on all data. Statistical significance within each group was determined by the paired *t*-test or the Mann–Whitney rank sum test, as appropriate. Comparisons among groups were performed using the one-way ANOVA followed by Scheffé's multiple comparisons tests or the Kruskal–Wallis one-way ANOVA on ranks. Linear association between two variables was analyzed by linear regression analysis. $P < 0.05$ was considered significant.

Results

Hormone levels

Table 1 reports the steroid plasma levels in women, men, and children. As expected, plasma levels of estradiol in women were significantly lower during the follicular than the luteal phase of the menstrual cycle. Conversely, estrone and progesterone concentrations were higher during the luteal phase in comparison with those detected during the follicular phase of the menstrual cycle.

In men, administration of testosterone enanthate significantly increased testosterone as well as estradiol and estrone plasma concentrations. The latter were significantly lower than those in women during the luteal phase, but similar to those in the follicular phase of the menstrual cycle.

In children, estradiol and estrone plasma levels were both significantly lower than those of the other groups.

In postmenopausal women, estradiol levels were significantly lower than in both young women and men, and similar to those detected in children.

Aromatase expression

PBL aromatase mRNA concentrations in men and women are shown in Fig. 1. Women during the follicular phase showed aromatase mRNA values significantly higher than during the luteal phase of their menstrual cycle (follicular phase: 0.9 ± 0.2 pg/ μ g RNA; luteal phase: 0.28 ± 0.07 pg/ μ g RNA; $P < 0.05$) (Fig. 1A). In men, PBL aromatase mRNA values increased

significantly following testosterone administration (baseline: 0.55 ± 0.06 pg/ μ g RNA; after testosterone: 0.7 ± 0.06 pg/ μ g RNA; $P < 0.05$) (Fig. 1B). A comparison among women, men, and children (one-way ANOVA) showed significantly higher mRNA aromatase levels in women during the follicular phase as well as in men after testosterone treatment than in women during the luteal phase, men before testosterone administration, and children ($P < 0.05$; Fig. 1). PBLs from children also expressed aromatase, but their concentration of aromatase mRNA was less abundant than in women during the follicular phase and men (0.14 ± 0.02 pg/ μ g RNA) and similar to that of women during the luteal phase and of postmenopausal women (0.16 ± 0.05 pg/ μ g RNA) (Fig. 1C). In the latter, aromatase mRNA was significantly lower than that detected in young women in the follicular phase, but similar to the levels found in the luteal phase of the menstrual cycle (Fig. 1).

Linear regression analysis between the different hormones and aromatase mRNA values failed to find any significant correlation in men and children. In contrast, a positive correlation between aromatase mRNA values and plasma estradiol ($r = 0.70$, $P < 0.05$) and estrone levels ($r = 0.72$, $P < 0.05$) during the follicular phase of the menstrual cycle was detected in women.

The results of the immunoblot analysis of aromatase expression are depicted in Fig. 2. As for mRNA, the 55 kDa protein was visualized at higher levels in PBLs from women during the follicular than the luteal phase of the menstrual cycle (Fig. 2A). In men, PBL aromatase protein levels were significantly increased by testosterone administration (Fig. 2B). Children presented less abundant PBL protein concentrations than the other groups (Fig. 2C). Analysis of the coding region of the P450arom gene in PBLs from some of the subjects studied showed no abnormalities in the sequence of this gene.

No aromatase activity was detected in PBLs from any of the subjects studied, whereas a marked enzyme activity was found in granulosa cells and fibroblasts, used as positive controls (aromatase activity in granulosa cells: 68.3 ± 21.8 fmol/ 10^6 cells per h; aromatase activity in fibroblasts: 3.7 ± 0.5 fmol/ 10^6 cells per h).

Table 1 Hormonal values in the patient groups studied. Values are means $\pm$ S.D.

	Estradiol (pmol/l)	Estrone (pmol/l)	Progesterone (pmol/l)	Testosterone (nmol/l)
Women				
FP	118.98 ± 49.45	100.61 ± 36.66	2.86 ± 0.63	
LP	$317.98 \pm 169.45^*$	$257.56 \pm 131.72^*$	$28.94 \pm 4.45^*$	
Men				
Baseline	95.92 ± 25.37	96.25 ± 34.73		22.03 ± 4.66
After testosterone	$127.86 \pm 30.62^{**}$	$126.80 \pm 45.13^{**}$		$35.53 \pm 7.38^{**}$
Children	$14.83 \pm 20.30^\dagger$	$6.03 \pm 14.76^\dagger$		
Postmenopausal women	$60.98 \pm 47.36^\dagger$			

FP, follicular phase; LP, luteal phase.

* $P < 0.001$ vs FP; ** $P = 0.001$ vs baseline; $^\dagger P < 0.05$ vs men and women.

Figure 1 (A) PBL aromatase mRNA values in female subjects during the follicular (FP) and luteal phase (LP) of the menstrual cycle. * $P < 0.05$ vs LP. (B) PBL aromatase mRNA values in male subjects before (Pre T) and after (Post T) testosterone administration. § $P < 0.05$ vs Post T. (C) PBL aromatase mRNA values in children and postmenopausal women (PW). ** $P < 0.05$ vs FP, Pre and Post T. Values are means $\pm$ S.E.M.

Discussion

The results of the present study have shown for the first time that the aromatase gene is expressed in PBLs from women, men, and prepubertal children, and that its pattern of expression differs in the various study groups. Specifically, aromatase gene was predominantly expressed in PBLs from women and varied according to the menstrual cycle and estradiol plasma concentrations. Male subjects exhibited lower PBL levels of aromatase mRNA than women in the follicular phase of the menstrual cycle. Moreover, testosterone administration in males led to an increase in PBL aromatase gene expression. Children together with postmenopausal women showed the lowest aromatase gene expression.

The difference in aromatase gene expression detected among the different study groups under conditions of distinct sex steroid concentrations suggests an important role of sex steroids in the modulation of aromatase gene expression in human PBLs. Specifically, it seems

that a regulatory loop is operating, so that in the presence of relatively low estrogen levels, such as those found in the follicular phase of the menstrual cycle, an increased expression of the aromatase gene in PBLs occurs. In contrast, high estrogen levels, such as those characteristic of the luteal phase of the menstrual cycle, are associated with a diminished gene expression. The positive correlation between aromatase gene expression and estrogen levels measured during the follicular phase of the menstrual cycle might indicate that peripheral aromatase significantly contributes to serum steroid concentrations during the follicular phase. In contrast, the absence of any relation between aromatase expression and estrogen levels during the luteal phase implies that, during this phase of the menstrual cycle, the gonadal synthesis of estrogen prevails. This distinctive modulation of aromatase gene expression might be instrumental in protecting peripheral target tissues from the variation in estrogen activity caused by the fluctuation of circulating estrogen levels in premenopausal women (23). Specifically, in young

Figure 2 Representative immunoblot analyses of PBL aromatase protein in (A) women ($n = 4$; W1–W4) during the follicular (FP) and luteal phase (LP) of the menstrual cycle, (B) men ($n = 5$; M1–M5) before (B) and after testosterone administration (T), and (C) children ($n = 4$).

women such a regulatory mechanism may preserve peripheral tissues from relative estrogen deficiency during the follicular phase by increasing aromatase gene expression when circulating estrogens fall. Conversely, in postmenopausal women, this regulatory loop does not seem to be active, since low circulating estrogen concentrations are not associated with elevated PBL aromatase mRNA levels, contributing to the maintenance of the hormonal quiescence typical of this age.

The lack of any significant correlation between enzyme gene expression and serum estrogen levels in men might be related to a gender-specific pattern of the regulation of PBL expression, as recently described in human adipose tissue (24). In this case, instead of a negative feedback as postulated in females, a priming effect of estrogen on aromatase gene expression can be hypothesized in men, accounting for the increased aromatase gene expression following the estrogen level augmentation caused by testosterone administration (25, 26). In children, the extremely low levels of both PBL aromatase mRNA and serum estrogen may account for the lack of correlation between enzyme expression and serum estrogen levels.

In humans, a number of tissues have the ability to express the aromatase gene and hence synthesize estrogens (27, 28). Aromatase gene expression in these various sites is under the control of tissue-specific promoters regulated by different cohorts of transcription factors (3, 4). In particular, gonads utilize a promoter localized immediately upstream of the transcriptional start site (promoter PII that binds the transcriptional factors CREB and SF1, and is regulated by cAMP and gonadotropins). PBLs have also been shown to utilize the proximal promoters I.3 and PII for gene expression (16, 18). Since PBLs are an easily accessible tissue they might be used as a model for studying the regulatory mechanisms of the gene expression in tissues sharing the same transcriptional mechanisms.

No aromatase activity has ever been reported in PBLs from normal subjects, whereas a significant enzyme activity was detected in PBLs from patients with the aromatase excess syndrome. In the present study, no aromatase activity was detected in PBLs from the different subjects whereas a significant enzyme activity was quantified in the cells used as positive controls. This suggests that the presently available method is not as sensitive at measuring enzyme activity as is the real-time PCR at detecting small amounts of mRNA. However, the functional significance of the present findings cannot be disregarded, as the enzyme activity that cannot be detected in an assay using a limited amount of PBLs does not necessarily reflect that which can be derived from all circulating PBLs. An alternative hypothesis that might explain the undetectable enzyme activity is the potential presence of a truncated aromatase protein in PBLs with no particular

meaning for human physiology. Indeed, at least two different truncated aromatase mRNA isoforms have been detected in male rat germ cells (29), and an inactive truncated polypeptide was described in rabbit pre-ovulatory granulosa cells (30). However, the presence of a single band of the expected molecular size detected by the Western blot analysis of our samples, and the absence of any sequence abnormality of the P450arom gene in PBLs from some of the subjects studied, rule out this hypothesis.

In conclusion, the aromatase gene is differentially expressed in PBLs from women, men, and prepubertal children, and sex steroids seem to modulate gene expression in a gender-dependent fashion. The physiologic significance of these findings is presently unknown and deserves further investigation, as well as validation in tissues in which aromatase activity is detectable. However, since sex steroids seem to play an important role in regulating the immune process and, given the higher incidence of autoimmune disorders in women, it may be hypothesized that variations in PBL aromatase gene expression might contribute to the distinct gender-specific prevalence of the different autoimmune diseases.

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