CLINICAL STUDY

Expression and enzymatic activity of the P450c17 gene in human adipose tissue

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Abstract

Objective: P450 aromatase activity increases with age in adipose tissue. Increased oestrogen production has also been observed in obese elderly women, and has been related to the pathogenesis of endometrial cancer. Since peripheral oestrogen production requires the presence of androgenic metabolites, and a recent report from our laboratory showed very low expression levels of P450c17 mRNA in most postmenopausal ovaries analysed, we hypothesised on the existence of an alternative source of androgens. Since steroidogenic enzymes, such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 17 β -HSD have been described in adipose tissue of primates and humans respectively, we aimed to analyse the possible expression of the P450c17 gene in adipose tissue, and its enzymatic capability.

Design: A prospective non-randomised clinical research study.

Methods: Subcutaneous abdominal adipose tissue and random pieces of whole normal ovaries were collected at surgery from nineteen women undergoing bilateral oophorectomy for non-ovarian gynaecological disease. P450c17 mRNA expression levels were measured by RT-PCR/Southern blot analysis, and 17 α -hydroxylase enzymatic activity in dispersed cell homogenates was performed by thin-layer chromatography, using ¹⁴C-progesterone as a substrate.

Results: The study provides the first description of 17 α -hydroxylase activity in adipose tissue and the detection of a new form of the P450c17 cDNA containing a 156 bp in-frame deletion in the first exon. **Conclusions:** The description of 17 α -hydroxylase activity in adipose tissue, together with previously reported enzymatic activities such as 3 β -HSD and 17 β -HSD, might suggest a local production of androgens in this tissue.

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Introduction

A significant decrease in oestradiol constitutes the main hormonal change in the menopause, and causes most of the alterations associated with the climacteric syndrome. Not all postmenopausal women suffer these alterations to the same degree, since they exhibit different circulating levels of oestrogens (1).

Oestrogens, mainly oestrone, are produced by aromatisation of androgens in peripheral tissues by an enzyme known as aromatase cytochrome P450 (P450 arom) (2, 3). In humans, aromatase expression occurs in a number of tissues and cell types, including ovary, brain, skin and adipose tissue, with the latter being the major site of oestrogen biosynthesis in postmenopausal women (4, 5). Hemsell *et al.* were the first to address the significance of human adipose tissue as a major source of oestrogen production and

demonstrated that, in both men and women, there is a progressive increase in the efficiency with which circulating androstenedione is converted into oestrone with advancing age (6). Later, Simpson and colleagues demonstrated that with ageing, there is an increase in the specific activity of the aromatase enzyme in adipose stromal cells and it was concluded that this may result in increased estrone production associated with ageing (7, 8). More recently, the same group determined that this age-related increase in aromatase activity in adipose tissue is the result of increased levels of P450 aromatase transcripts in various body sites of women, including buttocks, thighs and abdomen (9).

Increased oestrogen production in obese elderly women, which has been suggested to play a role in the pathogenesis of endometrial cancer, has been observed in postmenopausal women (10). The higher oestrogen levels found in these women would necessarily

require the presence of androgenic metabolites, which have mainly been attributed to the adrenal gland.

Since José *et al.* (11) reported recently that expression levels of P450c17 mRNA, the key enzyme for steroidogenesis, are in general very low in postmenopausal human ovaries, and because steroidogenic enzymes such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (12, 13) and 17 β -HSD (14) have also been reported in adipose tissue of primates and humans respectively, we focused our attention on investigating the expression and functional capability of the 17 α -hydroxylase (17 α -OHase) enzyme in adipose tissue.

Materials and methods

Subjects

Subcutaneous abdominal adipose tissue was collected at surgery from 19 women undergoing bilateral oophorectomy for non-ovarian gynaecological disease. Tissues were immediately frozen in liquid nitrogen and kept at -80°C for further RNA extraction, or processed immediately for enzymatic activity assays. Patients, aged from 44 to 73 years, included 10 postmenopausal, 3 perimenopausal and 6 premenopausal women. Mean body mass index was 23–34 kg/m². None of the patients received medication during the three months prior to this study. Random pieces of whole normal ovaries at the ovulatory phase collected at the time of surgery, and peripheral mononuclear cells isolated by density gradient centrifugation from blood donated by healthy volunteers, were used as negative controls for expression and activity of the P450c17 gene.

This study was approved by the Institutional Review Board, and written informed consent was obtained from all patients prior to surgery.

Oligonucleotides

Gene-specific primers 1, 2, 3, 4, 5, 6, shown in Table 1, were designed for the human P450c17 and cyclophilin A (CypA) genes with the Oligo 4.0 software (National Biosciences, Inc., Plymouth, MN, USA). All were synthesised by Boehringer Mannheim GmbH (Mannheim, Germany).

RNA extraction and RT-PCR

Total RNA was extracted from surgically removed tissues and peripheral mononuclear cells using the method described by Chomczynsky and Sacchi (15).

For cDNA synthesis and PCR amplification, 1 μg high-quality intact total RNA was reverse transcribed and amplified in a single step using gene-specific primers for the human P450c17 or cyclophilin A cDNAs, and a mixture of SUPERScript II reverse transcriptase and Taq DNA polymerase. All procedures were performed following the manufacturer's instructions (GibcoBRL, Life Technologies, Rockville, MD, USA) using a DNA Thermal Cycler 2400 (Perkin-Elmer, Foster City, CA, USA).

Conditions for the RT-PCR reaction were as follows: 30 min at 48°C (reverse transcription) and 2 min at 94°C (pre-denaturation), followed by 40 cycles of: 15 s at 94°C (denaturation), 30 s at 55°C (annealing) and 1 min at 68°C (extension) for P450c17 (primers 1 and 2) and cyclophilin A (primers 5 and 6) genes. Extension for 5 min at 68°C was made in the final cycle. Aliquots of 13 μl amplified products were resolved by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Controls consisting of a reaction mixture without RNA were performed to rule out the possibility of contamination by exogenous cDNA. The size of amplified products was consistent with the expected sizes for each cDNA, according to molecular weight markers (EcoRI digested pBR322), indicating that contamination with genomic DNA had not occurred. Finally, template integrity was checked using the cyclophilin A gene as an endogenous marker.

Southern blot analysis

After electrophoresis, the RT-PCR products were transferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Hercules, CA, USA) and hybridised to [γ -³²P]ATP (5000 Ci/mmol) 5' end-labelled internal primers 3 and 4 (Table 1), using T4 polynucleotide kinase (Promega, Madison, WI, USA) to confirm the identity of amplified products and for the detection of low expressed targets. Membranes were incubated overnight with labelled probes using standard methods, with stringency washes of $2 \times \text{SSC}$, 0.5% SDS and

Table 1 Oligonucleotides used in this study.

Gene	Primer sequence		Position	PCR product size (bp)
P450c17	1 sense	5'-ctcttgctgcttcacctag-3'	59–78	527
	2 antisense	5'-tcaaggagatgacattggt-3'	585–566	
	3 internal	5'-tctatggactgtccgttg-3'	538–519	
	4 internal	5'-caggagctcttccatcat-3'	1389–1409	
CypA	5 sense	5'-atggtcaacccaccgtg-3'	15–32	663
	6 antisense	5'-tgcaatccagctaggcatg-3'	678–660	

0.5% sodium pyrophosphate at 50°C. After washing, filters were exposed to autoradiographic films (Hyperfilm; Amersham Pharmacia Biotech, Uppsala, Sweden).

Tissue processing for enzymatic assays

Adipose tissue was minced and incubated with collagenase I (1 mg/ml) (Sigma Chemical Co., St Louis, MO, USA) for 90 min at 37°C in a medium containing 0.5 mmol/l CaCl₂, 5 mmol/l glucose, 3% BSA in Krebs-Henseleit buffer (KHB: 150 mmol/l NaCl, 6.17 mmol/l KCl, 1.54 mmol/l KH₂PO₄, 1.58 mmol/l MgSO₄, 25 mmol/l NaHCO₃ pH 7.4). After incubation, the adipose tissue was filtered through nylon mesh and the adipocytes were separated from the medium and stromal cells. The adipocytes were washed three times with 0.5 mmol/l CaCl₂, 5 mmol/l glucose and 3% BSA in KHB, and stromal cells were recovered by centrifugation at 1000 r.p.m. for 5 min at 4°C. Adipocytes and stromal cells were resuspended in 1 mmol/l CaCl₂, 5 mmol/l glucose and 3% BSA in KHB.

Similarly, ovarian tissues were minced and incubated with collagenase I (4 mg/ml), DNase (10 mg/ml), BSA (10 mg/ml) in medium 199 (Life Technologies) for 90 min at 37°C and centrifuged at 1000 r.p.m. for 5 min at 4°C. Ovarian cells were washed twice with 100 mmol/l KCl, 16 mmol/l K₂HPO₄, 4 mmol/l KH₂PO₄, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, and 2 mg/ml each of leupeptin and pepstatin A (Sigma Chemical Co.) pH 7.4 and resuspended in the same buffer, as described by Vianello *et al.* (16). Protein concentration was determined using the Bradford assay (Bio-Rad) and 15.46 mg or 5.54 mg protein were obtained from 1 g of ovarian or adipose tissues respectively.

Blood peripheral mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation were washed and resuspended with the same buffer used for the ovarian cells (16).

Cell suspensions, corresponding to 5.5 mg protein for adipose tissue, 3 mg protein for ovarian tissue, or 10⁶ peripheral mononuclear cells per tube were mixed with 4 ml buffer containing 100 μ l propylene glycol, 0.77 μ mol/l progesterone and 0.23 μ mol/l ¹⁴C-progesterone (initial specific activity: 55.4000 mCi/mmol;

Dupont-New England Nuclear, Boston, MA, USA) and incubated for 4 h at 37°C in a shaker (17). The reaction was stopped by freezing at -20°C until steroid extraction procedure. Duplicates and negative controls were effected for every sample. The first control contained all reagents without the sample and was incubated under appropriate conditions for 4 h at 37°C. The second control contained all reagents and the sample and was stored immediately at -20°C until processed.

Extraction, chromatography and identification of steroid metabolites

Steroids were extracted four times with ethyl acetate:isooctane (1:1), concentrated by evaporation under N₂, and separated on thin-layer chromatography (TLC) plates (silica gel 60 F254, 20 $\times$ 20 $\times$ 0.2 cm) (Merck/Fisher Chemical, Darmstadt, Germany) with chloroform:ethyl acetate (3:1), as described by Lin *et al.* (18). The chromatoplates were exposed to autoradiographic films (Hyperfilm; Amersham Pharmacia Biotech) for 15 days (adipose), 7 days (ovary) and 30 days (lymphocytes). ¹⁴C-labelled steroids were identified by comigration with UV light-detected authentic steroids. In an attempt to quantify the conversion of progesterone to 17 α -hydroxyprogesterone (17 α -OH-progesterone), the same plates were exposed to phosphor screens and radioactivity in specific spots measured in a Bio-Rad Phosphorimager personal Molecular Imager FX using the Quantity One software (Bio-Rad). The radioactive counts of the final product were divided by the counts of the precursor loaded on each lane and percentage conversions were calculated accordingly.

Results

Amplification conditions of the P450c17 cDNA gene, using primers 1 and 2 (Fig. 1), were previously standardised in our laboratory using RNA from human ovary tissue as a template (11). The same primers and conditions were used to investigate the putative expression of this gene in human adipose tissue obtained from 10

Figure 1 Schematic representation of human P40c17 cDNA. Numbers in boxes indicate the 8 exons of the cDNA, flanked by the 5' and 3' untranslated regions (UTR 5' and UTR 3') respectively. Numbers in brackets correspond to the primers used to amplify two different regions of the cDNA. Primers 1 and 2 were used to amplify the expected 527 bp of the 5' region of the cDNA. Primers 3 and 4 correspond to internal sequences of the cDNA used as probes in the RT-PCR/Southern analysis.

postmenopausal, 3 perimenopausal and 6 premenopausal patients. Human peripheral blood lymphocytes were included as a negative control for the RT-PCR/Southern blot experiments.

Southern blot analysis of amplified P450c17 cDNA products showed that both ovarian and adipose tissues express the P450c17 gene, while expression in lymphocytes is absent (Fig. 2A). Besides detecting the 527 bp product that corresponds to the P450c17 cDNA, our experiments also showed the presence of a 371 bp product (Fig. 2A) which corresponds to a putative new form of the P450c17 mRNA, since it is specifically recognised by the internal primer (primer 3) used as a probe in the Southern experiment (Fig. 2A). As expected, hybridisation with an internal oligonucleotide from the 3' part of the gene (primer 4), not represented in the amplified cDNA fragment, yielded a negative result (Fig. 2B). In general, it appears that the smaller-sized product is more prominent in adipose tissue than in the ovary and also that the relative expression of the amplified products does not correlate with the menopausal status of the patients.

Cloning and sequencing of the amplified 371 bp product demonstrated a deletion of 156 bp in the first exon of the P450c17 which maintains the original open reading frame. The exact sites for the 156 bp deletion found in all adipose tissue samples tested are shown in Fig. 3.

Amplification signals were totally negative in lymphocytes, regardless of the probe used (Fig. 2A and B), thereby demonstrating the tissue-specific expression of P450c17 in adipose tissue and the appearance of a putative new form of this gene in ovary and adipose tissues.

To correlate expression of the P450c17 mRNA with enzymatic activity, dispersed cells were incubated with

^{14}C -progesterone and samples collected at different times for further steroid extraction. Analyses of the steroids, produced during a range of 1 to 18 h of substrate incubation, were analysed by TLC, and production of $17\alpha\text{-OH}$ -progesterone was detected after 2 h (data not shown). Incubation times of 4 h were selected for further experiments, which also included dispersed ovarian cells and lymphocytes as positive and negative controls respectively. Results of these experiments are shown in Fig. 4 where an hydroxylated form of progesterone is observed in ovary and adipose tissue, although not in lymphocytes. Cold metabolites included in each sample, and therefore run simultaneously with labelled products, permitted the identification of the labelled metabolites as progesterone and $17\alpha\text{-OH}$ -progesterone (Fig 4, lane 3). When cells were omitted from the reaction mixture and the reaction was allowed to occur under identical conditions (Fig. 4, lane 1), only progesterone was observed. The same results were observed when, in the presence of cells, the enzymatic reaction was abolished by keeping the mixture at -20°C (Fig 4, lane 2). Both controls indicate the presence of enzymatic $17\alpha\text{-OH}$ ase activity in ovary and adipose tissue, although not in lymphocytes. While no activity was detected in three postmenopausal ovaries with low P450c17 mRNA levels (data not shown), the nine adipose tissue samples studied displayed $17\alpha\text{-OH}$ ase activity, independent of the hormonal status of the patients.

Conversion rates from progesterone to $17\alpha\text{-OH}$ -progesterone, obtained with the Quantity One Quantitation Software version 4 (Bio-Rad), indicated a wider range for adipose tissues (0.48% to 3.17% per mg protein) than for the ovary (4.6% per mg protein), consistent with the variable mRNA expression levels

Figure 2 Expression of the P450c17 gene in human adipose tissue, ovary, and lymphocytes using the RT-PCR/Southern blot technique. (A) One microgram total intact RNA obtained from adipose tissues of premenopausal (○) and postmenopausal (●) women, human ovaries at the ovulatory stage and lymphocytes (Lymph), was reverse-transcribed into cDNA and amplified by PCR in a single-step procedure, using specific primers for the P450c17 gene (primers 1 and 2 from Table 1). The RT-PCR products were resolved by electrophoresis in 1% agarose gel, transferred to nylon membranes and analysed by the Southern blot technique, using a specific internal primer as a probe (primer 3). Autoradiographs corresponding to adipose tissues were exposed overnight while those corresponding to ovarian samples were exposed for approximately 3 h. (B) The same filters were sequentially hybridised with primer 4, which is not represented in the amplified 5' product (see Fig. 1) and represents a negative control for the signals obtained with primer 3. (C) Amplification signals of the human cyclophilin A cDNA obtained by RT-PCR, detected upon ethidium bromide staining. The cyclophilin A cDNA was used as an internal control for RNA integrity.

42 ...ATG TGG GAG CTC GTG GCT CTC TTG CTG CTT ACC CTA GCT TAT TTG TTT TGG CCC
M W E L V A L L L L T L A Y L F W P
96 AAG AGA AGG TGC CCT GGT GCC AAG TAC CCC AAG AGC CTC CTG TCC CTG CCC CTG
R K R C P G A K Y P K S L L S L P L
150 GTG GGC AGC CTG CCA TTC CTC CCC AGA CAT GGC CAT ATG CAT AAC AAC TTC TTC
V G S L P F L P R H G H M H N N F F
204 AAG CTG CAG AAA AAA TAT GGC CCC ATC TAT TCT GTT CGT ATG GGC ACC AAG ACT
K L Q K K Y G P I Y S V R M G T K T
258 ACA GTG ATT GTC GGC CAC CAC CAG CTG GCC AAG GAG GTG CTT ATT AAG AAG GGC
T V I V G H H Q L A K E V L I K K G
312 AAG GAC TTC TCT GGG CGG CCT CAA ATG GCA ACT CTA GAC ATC GCG TCC AAC AAC
K D F S G R P Q M A T L D I A S N N
366 CGT AAG GGT ATC GCC TTC GCT GAC TCT GGC GCA CAC TGG CAG CTG CAT CGA AGG
R K G I A F A D S G A H W Q L H R R
420 CTG GCG ATG GCC ACC TTT GCC DTG TTC AAG GAT GGC GAT CAG AAG CTG GAG AAG
L A M A T F A L F K D G D Q K L E K
474 ATC ATT TGT CAG GAA ATC AGT ACA TTG TGT GAT ATG CTG GCC ACC CAC AAC GGA
I I C Q E I S T L C D M L A T H N G
528 CAG TCC ATA GAC ATC TCC TTT CCT GTC TTC GTG GCG GTA ACC AAT GTC ATC TCC
Q S I D I S F P V F V A V T N V I S
582 TTG A...
L

99 256
||
T G G C C C A A G A C T A C A G T G A T T

Figure 3 Nucleotide sequence of the 5' region of human P450c17 cDNA. The upper part of the figure represents the nucleotide sequence of human cytochrome P450c17 mRNA (accession # M14564), encompassing exons 1, 2 and part of exon 3. Nucleotide sequences of primers 1 and 2 (see Table 1) used for RT-PCR amplification of the cDNA are underlined. Italicised letters correspond to the deleted 156 bp of exon 1, from position 100 to 255 of the depicted sequence. The lower part of the figure represents the nucleotide sequence of the RT-PCR product corresponding to the deleted form of the P450c17 cDNA. Notice that nucleotides 99 and 256 are now side by side in the new putative form.

Figure 4 Thin-layer chromatography. Autoradiograph of the free steroids extracted from the incubate of human adipose tissue, ovary and lymphocyte dispersed cells incubated with 0.23 μ mol/l 14 C-progesterone and 0.77 μ mol/l unlabelled progesterone. Labelled steroids were identified by comigration with UV light-visualised authentic steroids. Lane 1, reaction mixture incubated under appropriate conditions to permit enzymatic activity, in the absence of cells. Lane 2, samples stored at -20°C to block enzymatic activity until extraction of the steroids. Lane 3, samples incubated under appropriate conditions to permit enzymatic activity. Autoradiographs were exposed for different times depending on the tissue: ovary 1 week, adipose tissue 2 weeks, lymphocytes 4 weeks. Prog, progesterone; 17OHProg, 17 α hydroxyprogesterone.

detected in the adipose tissue. According to these values, and under the experimental conditions used in this study, adipose tissue 17α -OHase activity would represent between 10.5% and 69% of the premenopausal ovarian activity, which is considered as 100%. Because the protein yield per gram of tissue is 3 times higher in the ovary than in the adipose tissue, we can surmise, roughly, that the progesterone to 17α -OH-progesterone conversion efficiency in the adipose tissue ranges from 3% to 23% in relation to that exhibited by the ovary.

Discussion

This paper describes for the first time the expression and enzymatic activity of the P450c17 gene in human adipose tissue.

The demonstration that adipose tissue exhibits 17α -OHase activity, together with the previously reported enzymatic activities such as 3β -HSD and 17β -HSD, might suggest a local production of androgens in this tissue. A recent report from our laboratory showed that P450c17 mRNA expression levels are very low in most postmenopausal human ovaries and similar to those observed in the early follicular phase (11). By performing activity assays with homogenates of three menopausal ovaries, we were not able to observe any hydroxylated product, which indicated that the 17α -OHase activity in low-expressing postmenopausal ovaries was undetectable with the methods used (results not shown). These results indicate that in the ovary, 17α -OHase activity will be precisely controlled by the many regulators that temporarily modulate its expression at mRNA level. In contrast, all adipose tissues tested showed a wide range of 17α -OHase activity, independent of the hormonal status of the patient. These results suggest differential tissue-specific regulation of the P450c17 gene in the ovary and adipose tissue.

During menopause, obese patients at high risk for endometrial adenocarcinoma and cardiovascular disease are on the safe side for osteoporosis (19). Although higher aromatase activity has been the most accepted explanation for hyperoestrogenism associated with obesity (20), the exclusive contribution of the adrenal gland when ovarian function decreases renders the increase in oestrogenic metabolites difficult to understand. Although, on the basis of available evidence, it is premature to conclude that adipose tissue represents an additional source of androgens in postmenopausal women, our findings offer a very reasonable hypothesis in relation to its possible contribution.

Women suffering from polycystic ovary syndrome exhibit hyperandrogenism which, in some cases, is associated with obesity and insulin resistance (21). While different factors have been postulated to contribute to this syndrome, the possibility that adipose tissue

could produce its own androgens and thus contribute to the hyperandrogenic state might better explain the vicious circle described between this syndrome and obesity.

Interestingly, our results demonstrated the existence of a putative new form of the P450c17 mRNA that includes a deletion of 156 bp from exon 1 and maintains the open reading frame. We do not know at present what the significance of this new form is or why it exhibits a relative preferential expression in adipose tissue. Identification of this deleted form and further functional analysis will be required to determine its physiological significance.

The results presented in this study, together with previous descriptions of 3β -HSD, 17β -HSD and aromatase in adipose tissue, might suggest the existence of steroidogenic pathways never previously described in this tissue. Their definition, understanding of the regulatory mechanisms that might drive P450c17 gene expression in a tissue-specific manner, and analysis of the physiological significance of a putative second P450c17 mRNA form are the goals on which our laboratory is currently focused.

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