

Tissue-specific transcriptional initiation and activity of steroid sulfatase complementing dehydroepiandrosterone sulfate uptake and intracrine steroid activations in human adipose tissue

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Abstract

Expression analysis by reverse transcriptase (RT)-PCR indicates that human adipose tissue is not likely to perform *de novo* synthesis of steroid hormones from cholesterol because the mRNAs of cytochromes P450scc and P450c17, and of the steroidogenic-related proteins, steroidogenic acute regulatory protein and steroidogenic factor 1, were not detected. Instead, our data support an intracrine role of adipose tissue, in which adrenal dehydroepiandrosterone sulfate (DHEA-S), the most abundant circulating androgen in man, is selectively uptaken, desulfated, and converted into bioactive androgens and estrogens. Three organic anion-transporting polypeptides-B, -D, and -E, presumably involved in DHEA-S transmembrane transport, were demonstrated at the mRNA level. While sulfotransferase expression was not found, the occurrence of steroid sulfatase (STS), converting DHEA-S to DHEA, was established at the mRNA, protein and catalytic activity levels. The 5'-rapid amplification of cDNA ends analysis showed that STS transcription in adipose tissue is regulated by the use of two promoters which differ from the prevalent placental one. The adipose transcripts

contain a distinct untranslated first exon, 0a or 0b, followed by a common partially translated exon 1b, and nine other exons that are also shared by the main placental transcript. The presence of an upstream open reading frame in the new transcript variants could lead to an N-terminal divergence restricted to the cleavable signal peptide and thus not interfering with the catalytic activity of the mature STS protein. The adipose transcripts are also present in the placenta as minor isoforms. Western blotting revealed the characteristic ~64 kDa band of STS in both the placenta and adipose tissue. The specific enzymatic activity of STS in adipocytes was 118 pmol/10⁶ cells per hour, about 50–100 times lower than in the placenta. A similar rate of [³H] DHEA-S uptake plus desulfation was measured in preadipocytes and adipocytes, equivalent to 40–45 pmol/10⁶ cells per hour. Thus, an excessive accumulation of fat may out-compete other peripheral organs that are also dependent on intracrine DHEA-S utilization, especially when the adrenal production is low or declining with aging.

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Introduction

Adipose tissue is no longer viewed merely as the site of diet-induced storage of excess energy, controlled by external lipogenic and lipolytic hormones, but is also recognized as a *bona fide* endocrine organ releasing adipokines and other bioactive peptides influencing appetite, energy homeostasis, and reproductive competence (Frayn *et al.* 2003, Kershaw & Flier 2004).

This tissue also expresses several terminal steroid-transforming enzymes leading to the secretion of active steroid hormones such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD)/ Δ^5 - Δ^4 -isomerase, 17 β -HSD, and 5 α -reductase for androgens, cytochrome P450arom for estrogens, and 11 β -HSD type 1 for glucocorticoids (reviewed by Belanger *et al.* 2002). Given the

proportional mass of fat depots, their secretions may account for up to 100% of circulating estrogens in postmenopausal women and 50% of circulating testosterone in premenopausal women (Kershaw & Flier 2004). Expression of cytochrome P450c17 and conversion of progesterone to 17 α -hydroxyprogesterone has been reported in adipose tissue (Puche *et al.* 2002), but so far, there is no evidence of other key enzymes for progestogen and corticoid synthesis from cholesterol.

In humans and other primates, the adipose tissue may be supplied with preformed steroids by the adrenal glands. These secrete not only corticoids, but also dehydroepiandrosterone (DHEA) and much greater amounts of DHEA sulfate (DHEA-S), which can be converted into potent androgens and/or estrogens by peripheral tissues, a process defined as intracrine (Labrie *et al.* 2001). DHEA-S is negatively charged

at physiological pH and its uptake requires an active transport across the plasma membrane, which is performed by members of two organic anion carrier protein families: the organic anion-transporting polypeptide (OATP) family and the organic anion transporter (OAT) family. These transporters are expressed in several tissues and display wide and overlapping substrate specificities for many organic compounds, including steroid conjugates. In particular, several OATPs can transport sulfate conjugates of steroids, such as OATP-A, OATP-B, OATP-C, OATP-8, and OATP-E (Hagenbuch & Meier 2003), whereas in the OAT family, only OAT-3 and OAT-4 can mediate the cellular uptake of certain steroid conjugates in the kidney, liver, brain, and placenta (Cha *et al.* 2000, 2001).

Once internalized, DHEA-S must undergo hydrolysis of the sulfate group, carried out by the enzyme steroid sulfatase (STS), before being transformed further. STS is a membrane-bound microsomal enzyme, ubiquitously expressed in mammalian tissues (Martel *et al.* 1994), that hydrolyzes various alkyl (e.g. DHEA-S) and aryl steroid sulfates (e.g. estrone sulfate) (Reed *et al.* 2005). In man, the *STS* gene contains ten exons spread over 146 kbp in the short arm of the X chromosome (Yen *et al.* 1988). The deduced amino acid sequence consists of 583 amino acids with an N-terminal signal peptide of 21 or 23 residues, as there are two dipeptides in tandem that equally fulfill the requirements for recognition by the signal peptidase (Stein *et al.* 1989).

In the present study on human adipose tissue, we have examined by reverse transcriptase (RT)-PCR its steroidogenic competence, the expression of organic anion transporters belonging to the OATP and OAT families, the transmembrane transport of DHEA-S, the transcriptional control of the gene encoding STS, and the specific activity of this enzyme.

Materials and Methods

Collection of adipose tissue for RNA and protein analyses

Biopsies of subcutaneous abdominal adipose tissue were obtained from six female (age: 46–61 years) and three male (age: 39–62 years) patients undergoing abdominal surgery for nonmalignant conditions and were kept at -80°C until RNA or protein analyses. Samples of fresh adipose tissue were also collected and transported immediately to the laboratory for STS assays. All tissue samples were used with full patient consent and institutional review board approval.

RNA extraction and expression analyses

Total RNA was extracted with Trizol reagent (Invitrogen) and kept at -80°C until use. Reverse transcription of 2 μg of total RNA was performed in 20 μl final volume with random hexamers and the ThermoScript RT-PCR System Kit (Invitrogen). After incubation for 10 min at 25°C , RT was carried out for 30 min at 55°C , followed by RT termination

for 5 min at 85°C . Total single-stranded cDNA was amplified by PCR in a 25 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5–2 mM MgCl_2 , 0.2 mM of each dNTP, 0.2 μM of the respective primers and 1.25 U of Biotherm *Taq* DNA polymerase (Società Italiana Chimici, Rome, Italy). Oligonucleotide primers are listed in Table 1 and amplification conditions in Table 2. The extension phase of the last cycle was prolonged by 10 min. An aliquot of the products was run on 1% agarose gel. For each PCR, a negative control was prepared by replacing the cDNA solution with sterile water to check for cross contamination.

Determination of the transcription start site (TSS) of the STS gene expressed in the adipose tissue

The TSS of the *STS* gene was determined with the method of RNA ligase-mediated rapid amplification of cDNA 5'-ends (RLM-5'-RACE) using the FirstChoice RLM-RACE kit (Ambion, Celbio, Milan, Italy). Briefly, 10 μg of adipose tissue total RNA was treated with calf intestinal phosphatase to remove the 5'-phosphate from truncated RNAs, leaving a 5'-OH end. Total RNA was then treated with tobacco acid pyrophosphatase to remove the 5'-cap from full-length mRNAs, leaving a 5'-phosphate to which a 5'-RACE RNA adapter oligonucleotide was ligated with T4 RNA ligase. Ligated mRNAs were then reverse-transcribed with random decamers.

STS transcripts were PCR-amplified using the 5'-RACE outer primer and a specific 3'-reverse primer (STS-4) designed on the exon 5 of the *STS* gene (Stein *et al.* 1989). The amplification procedure consisted of 2 min at 95°C , followed by a touchdown PCR with annealing temperatures decreasing from 68°C to 56°C over 12 cycles and the final 28 cycles at 56°C . The extension phase of the last cycle was prolonged by 10 min. The diluted products were subjected to second and third rounds of amplification using the 5'-RACE inner primer and two specific 3'-reverse primers (STS-6 and STS-10) selected on exon 3 and exon 2, respectively.

The amplicon from the last PCR was purified from the sliced gel band and ligated into a pGEM-T vector using a pGEM-T Vector System I (Promega). Plasmids from positive colonies were purified and four clones were sequenced.

3'-RACE analysis For 3'-RACE analysis, 2 μg of adipose tissue total RNA was incubated in a 20 μl reaction volume with 200 U of SuperScript II RT (Invitrogen), 0.5 mM of dNTPs, 10 mM dithiothreitol (DTT) and 0.5 μM of dT17 primer (5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT-3') for 1 h at 50°C . The first-strand mixture was diluted fivefold and 2 μl were added to 50 μl of the PCR buffer containing 0.2 mM of dNTPs, 0.2 μM of the anchor primer (5'-CTG GTT CGG CCC AGA CTC GAG TCG ACA TCG-3'), 2.5 U of Biotherm *Taq* DNA polymerase, and the gene-specific STS-5 primer. The first-round PCR product was diluted 1:100 and used as a template for the second round of PCR using the STS-7 and anchor

Table 1 Primer sequences used in RT-PCR and 5'- and 3'-RACE analyses

		Sequences	Position ^a	GenBank no.
Primer				
SCC-1	Sense	5'-AAGACTTCACCCCATCTCCGTGAC-3'	1199→1222	M14565
SCC-2	Antisense	5'-ACCCCAGCCAAAGCCCAAGT-3'	1418→1399	M14565
C17-1	Sense	5'-CAATGAGAAGGAGTGGCACCA-3'	1263→1283	NM_000102
C17-2	Antisense	5'-CTTTGAAAGAGTCGATCAGAAAAGAC-3'	1531→1507	NM_000102
AROM-1	Sense	5'-GAATCGGGCTATGTGGACGTGTTG-3'	590→613	Y07508
AROM-2	Antisense	5'-AGATGTCTGGTTTGATGAGGAGAG-3'	749→726	Y07508
SF1-1	Sense	5'-GGTGTCGGGCTACCACTACGG-3'	57→77	U76388
SF1-2	Antisense	5'-TGAAGCCATTGGCCCGAATCTG-3'	355→334	U76388
StAR-1	Sense	5'-CCAGGAGCTGGCCTATCTCCAG-3'	210→231	BC010550
StAR-2	Antisense	5'-CCAGGAGCTGGCCTATCTCCAG-3'	477→456	BC010550
E-SULT-1	Sense	5'-GAATGCAAAGGATGTGGCTGT-3'	493→513	U08098
E-SULT-2	Antisense	5'-TAATCCTGTCCACAAGCTCCTCT-3'	773→751	U08098
DHEA-SULT-1 ^b	Sense	5'-GTGGACAAAGCACAACCTCTG-3'	347→368	NM_003167
DHEA-SULT-2 ^b	Antisense	5'-TCTTACACAATGACCCAGTC-3'	611→591	NM_003167
OATP-A-1	Sense	5'-GGAAGGACTAGAGACTAATGCTGA-3'	1026→1047	NM_005075
OATP-A-2	Antisense	5'-AGCATCAAGGAACAGTCAGGT-3'	1739→1719	NM_005075
OATP-B-1	Sense	5'-AGCCAGCCCAGACCCTCA-3'	274→291	AB026256
OATP-B-2	Antisense	5'-ACATGATCCCCACCACACTCA-3'	755→735	AB026256
OATP-C-1	Sense	5'-GATGGGTTGGAGCTTGGTG-3'	857→875	AB026257
OATP-C-2	Antisense	5'-CCTGCTAGACAGGGTGAGATGT-3'	1562→1541	AB026257
OATP-D-1	Sense	5'-GTGTGTGGGGCAGATGGCA-3'	1646→1664	AB031050
OATP-D-2	Antisense	5'-TTGTCTAGGGTCAGAGTAGAGGCA-3'	2223→2200	AB031050
OATP-E-1	Sense	5'-CGCTGCCTGCAGCTGCCA-3'	1606→1623	AB031051
OATP-E-2	Antisense	5'-AAGTTCCTCGTGTGATTGCATCA-3'	2351→2330	AB031051
OATP-8-1	Sense	5'-GTTGGAGCTTGGTGGCTTGGT-3'	807→827	NM_019844
OATP-8-2	Antisense	5'-TACAGGGGATTGGTAAGGATGCT-3'	1057→1035	NM_019844
OAT-3-1	Sense	5'-TTCCCATCTACATGGTCTTCC-3'	641→662	AB042505
OAT-3-2	Antisense	5'-TTACTTACGCCCATACCTGTTTGC-3'	1473→1450	AB042505
OAT-4-1	Sense	5'-GCAGCGTCTTCACCTCCA-3'	415→431	AB026116
OAT-4-2	Antisense	5'-GTCGAAGACCAGCCCATAGT-3'	1150→1131	AB026116
STS-1	Sense	5'-CTGACTTCTGTCAACCCCT-3'	637→656	J04964
STS-2	Antisense	5'-GTCCATGTTGCTAGTGGGCT-3'	1409→1390	J04964
STS-3	Sense	5'-CAACAGGATCACAAGCTGGA-3'	183→202	J04964
STS-4	Antisense	5'-AGGGTCAGGATTAGGGCTGCT-3'	889→869	J04964
STS-5	Sense	5'-ACCCTCATCTACTTCACAT-3'	1206→1224	J04964
STS-8	Antisense	5'-CACATGCGTCTGTCTGGT-3'	1985→1968	J04964
STS-6	Antisense	5'-AGGTGCTGAGTGAGTTTCACT-3'	409→389	J04964
STS-10	Antisense	5'-CAGTAGGAGGAAAGGGATCT-3'	242→223	J04964
STS-7	Sense	5'-AGGAAGCTGCGGACGACACA-3'	1795→1815	J04964
STS-Pl/1a	Sense	5'-CAGCTGTAGTGAGGTTGCA-3'	23→41	J04964
STS-Ad/0a	Sense	5'-GAGAACCGCTACCATGCAG-3'	34→52	AM072429
STS-Ad/0b	Sense	5'-GAAGAAGTCCGTCCATGTCA-3'	50→69	AM072428

^aNucleotide position in the reported sequence.^bPrimer taken from Steckelbroeck *et al.* (2004).

primers. The amplification procedure with touchdown PCR was as above. The resultant amplicon was purified from the sliced gel band and directly sequenced.

Nucleotide sequencing

Sequencing was performed on double-stranded DNA directly from PCR products or after cloning using the ABI PRISM dye terminator cycle sequencing core kit (Applied Biosystems, Monza, Italy). Electrophoresis of sequencing reactions was completed on the ABI PRISM model 377, version 2.1.1

automated sequencer. The homology searches were carried out using the Basic Blast program version 2.0 (<http://www.ncbi.nlm.nih.gov/BLAST/>), and alignments were performed using the ClustalW program (<http://www2.ebi.ac.uk/clustalw/>).

Western blot analysis of adipose tissue STS

Samples of adipose tissue were homogenized in an Ultra-Turrax at 4 °C in 100 mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 4 mM MgCl₂, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, and 2 µg/ml each of leupeptin, pepstatin A and aprotin. Cell-free

Table 2 Target gene, PCR primers and conditions used in the RT-PCR analyses

	Primers	[Mg ⁺⁺]	Annealing (°C)	Extension time (s)
Target gene				
P450scc	SCC-1-SCC-2	2 mM	58	15
P450c17	C17-1-C17-2	2 mM	56	20
P450arom	AROM-1-AROM-2	2 mM	58	15
SF-1	SF1-1-SF1-2	2 mM	56	25
StAR	StAR-1-StAR-2	2 mM	56	20
E-SULT	E-SULT-1-E-SULT-2	2 mM	56	20
DHEA-SULT	DHEA-SULT-1-DHEA-SULT-2	1.2 mM	56	20
OATP-A	OATP-A-1 OATP-A-2	1.5 mM	54	40
OATP-B	OATP-B-1 OATP-B-2	1.5 mM	60	30
OATP-C	OATP-C-1 OATP-C-2	1.5 mM	54	40
OATP-D	OATP-D-1 OATP-D-2	1.5 mM	60	35
OATP-E	OATP-E-1 OATP-E-2	1.5 mM	60	40
OATP-8	OATP-8-1 OATP-8-2	1.5 mM	54	20
OAT-3	OAT-3-1 OAT-3-2	1.5 mM	60	40
OAT-4	OAT-4-1 OAT-4-2	1.5 mM	60	40
STS	STS-1-STS-2	1.5 mM	56	40
STS	STS-3-STS-4	1.5 mM	60	40
STS	STS-5-STS-8	1.5 mM	60	40
STS	STS-PI/1a-STS10	2 mM	56	20
STS	STS-Ad/0a-STS10	2 mM	56	15
STS	STS-Ad/0b-STS10	2 mM	60	20

supernatants were obtained after three centrifugations at 1000 *g* for 15 min at 4 °C.

Western immunoblot analysis of STS was done with a specific rabbit antiserum against human STS kindly provided by Dr Bernhard Ugele (Frauenklinik-Innenstadt, University Hospital Munich, Munich, Germany). Human placenta was used as a positive control tissue. Proteins from adipose tissue (50 µg) and placenta (5 and 0.5 µg) were size-fractioned onto 10% SDS-PAGE (Nupage, Invitrogen) and transferred onto a nitrocellulose filter (Roche Applied Science). After a 2 h blocking step at 4 °C with 5% BSA in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.2% Tween-20), the filter was hybridized with 1/10 000 dilution of the antibody against human STS in TBS-T buffer containing 5% BSA overnight at 4 °C. The filter was then washed (3 × 5 min; 1 × 15 min) with TBS-T and further incubated for 1 h at room temperature with 1:5000 dilution of goat horseradish peroxidase (HRP)-conjugated antirabbit IgG antibody (Pierce, Celbio, Milan, Italy) in TBS-T at 25 °C. After washing as above, HRP activity was detected using the SuperSignal West-Dura chemiluminescent kit (Pierce).

Sulfatase assay

Tissues were homogenized with an Ultra-Turrax in a buffered medium (100 mM KCl, 16 mM K₂HPO₄, 4 mM KH₂PO₄, 1 mM DTT, 1 mM EDTA, 4 mM nicotinamide) and homogenates were centrifuged four times at 1000 *g* for 15 min at 4 °C. In one experiment, adipocytes were homogenized by flushing through a syringe needle. Supernatants were preincubated for 20 min at 37 °C and then

incubated in 1 ml final volume with 74 kBq of [1,2,6,7-³H] DHEA-S (25 µM), previously dissolved in 10 µl of propylene glycol, for 15–120 min at 37 °C. Boiled homogenates were incubated as controls. All incubations were performed in duplicate or triplicate.

Enzyme activity was terminated by adding water-saturated ethyl acetate. [¹⁴C]DHEA was then added as a tracer for recovery control and radiochemical identification. Free steroids were extracted four times with 2 vol of ethyl acetate and separated by thin layer chromatography (plastic sheets precoated with silica gel 60 F₂₅₄; Merck, Milan, Italy) with the solvent systems cyclohexane/ethyl acetate (95:5, v/v; 5–7 runs) for defatting, and cyclohexane/ethyl acetate (1:1, v/v; 2 runs). Chromatoplates were autoradiographed for 5 days and the spots corresponding to unconjugated DHEA were removed, eluted in 20 ml of acetone and counted in a Packard Tri-Carb 1500 Liquid Scintillation Analyzer (Perkin Elmer, Monza, Italy), with a dual-label program.

For each incubate, conclusive identification of labeled metabolites with tracer was based on derivatization by acetylation and rechromatography. Percent conversions were calculated from the last [³H/¹⁴C] ratio in the purification procedure and were thus corrected for losses.

Isolation, culture, and differentiation of human preadipocytes

For preadipocyte culture, adipose tissue samples were collected by liposuction from five healthy female patients (age: 25–40 years) undergoing plastic reduction procedure. After trimming of the surrounding fibrous tissue, dissected adipose lobules were rinsed four times with PBS containing

1% antibiotic and antimycotic solution (AF; Sigma), minced and digested with type IA collagenase (1 mg/ml) (Sigma) for 45 min at 37 °C. Disaggregated cells were passed through a 80 µm nylon mesh to remove undigested tissue and centrifuged at 400 g for 5 min. They were then cultured at the density of 2×10^5 cells/cm² in DMEM–LG medium (Sigma) containing 10% fetal calf serum (FCS, Biochrom-Seromed, Berlin, Germany), 0.1% AF solution, and 0.1 µg/ml bovine fibroblast growth factor. Two days after reaching maximum confluence, cells were induced to differentiate into mature adipocytes by treatment for 10 days with DMEM–LG medium containing 10% FCS, 10 µg/ml insulin, 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX), 6×10^{-5} M indomethacin, and 10^{-6} M dexamethasone (DEX) (Sigma).

[³H]DHEA-S transport studies along with the sulfatase assay in adipose cells

Cultured preadipocytes and adipocytes were centrifuged and resuspended to $1\text{--}2 \times 10^6$ cells/ml in ice-cold transport buffer containing 142 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 1 mM KH₂PO₄, 5 mM glucose, 12.5 mM HEPES, and pH 7.4. For transport studies, aliquots of 0.9 ml of cell suspensions were preincubated for 20 min at 37 °C. The experiment was started by adding 100 µl of transport buffer containing 74 kBq of [1,2,6,7-³H]DHEA-S (50 µM). At fixed times, the reaction was terminated by adding water-saturated ethyl acetate. Since [¹⁴C]DHEA-S to be used as an internal tracer is not commercially available, we have measured conversion to DHEA as an estimate of both transmembrane transport and sulfatase activity. Hence, [¹⁴C]DHEA was added as a tracer for recovery control and identification and free DHEA was measured as above. [¹⁴C]-labeled androstenedione, testosterone, and androstene-3β,17β-diol were also added as tracers in the 6 h incubates.

Results

Expression analysis of steroidogenic enzymes and related proteins in adipose tissue

To establish the steroidogenic competence of human adipose tissue, we analyzed by RT-PCR with specific primers (Table 1) the expression of cytochromes P450_{sc}, P450_{c17}, and P450_{arom}, along with the expression of two steroidogenesis-related proteins, such as steroidogenic factor 1 (SF-1) and steroidogenic acute regulatory protein (StAR). RNAs extracted from human adrenal cortex and placenta were used as controls. Cytochrome P450_{sc} mRNA was expressed in both the adrenal cortex and placenta, P450_{c17} mRNA only in the adrenal and that of P450_{arom} only in the placenta. In the adipose tissue, P450_{sc} and P450_{c17} mRNAs were not detected, whereas a positive signal was obtained for P450_{arom} mRNA, though at a lower level than in the placenta (Fig. 1).

As expected, StAR and SF-1 mRNAs were observed in the adrenal cortex, but not in the placenta, which lacks both proteins (Sugawara *et al.* 1995, Strauss *et al.* 1996). Adipose tissue was also negative for both transcripts (Fig. 1), confirming a previous report on the absence of SF-1 in mammary fat (Clyne *et al.* 2002).

According to the literature, dehydroepiandrosterone sulfotransferase mRNA is expressed in the adrenal cortex, but not in the placenta (Luu-The *et al.* 1995), while estrogen sulfotransferase mRNA is found in both tissues (Miki *et al.* 2002). None of these enzyme mRNAs showed a detectable expression in the human adipose tissue (Fig. 1), whereas STS mRNA was expressed in both control and adipose tissues. When five samples of cultured preadipocytes, two samples of cultured adipocytes, and nine samples of bioptic adipose tissue were similarly analyzed by RT-PCR, all were found to be positive for STS mRNA.

Expression analysis of known members of the OATP and OAT gene superfamilies in adipose tissue

The expression of the known members of the OATP (subtypes A, B, C, D, E, and 8) and OAT (subtypes 3 and 4) gene superfamilies that might transport sulfate steroids across membranes was assessed by RT-PCR with specific sets of primers in adipose tissue. As shown in three representative tissue samples in Fig. 2, OATP-B, OATP-D, and OATP-E mRNAs were constitutively expressed, whereas the mRNAs of OATP-A, OATP-C, OATP-8, OAT-3, and OAT-4 were not detected in any sample. The identity of the amplicates was verified by sequencing.

As OATP-B's ability to transport DHEA-S is well established in the literature, its expression was confirmed by RT-PCR in all samples of adipose tissue and in cultured preadipocytes and adipocytes.

RLM-5'-RACE and 3'-RACE of STS expressed in adipose tissue

The expression of STS mRNA was initially analyzed by RT-PCR using three sets of primers covering the whole coding region (STS-3–STS-4; STS-1–STS-2; STS-5–STS-8). Using the first set, in which the forward primer is located immediately upstream of the initiation codon of the placental STS cDNA sequence (Stein *et al.* 1989), we could not amplify the first fragment of the coding region. However, two single fragments of the expected sizes were consistently detected on agarose gel with the other two sets of primers covering the remaining coding region. Sequencing in both directions of these fragments showed complete identity between the adipose amplicates and the placental cDNA sequence. These results confirmed the STS expression in adipocytes, but hinted at a sequence divergence at the 5' terminus.

The possible occurrence of alternative splicing in adipose tissue was probed by RLM-5'-RACE, using the placenta as a control. Unlike conventional RACE, which amplifies also

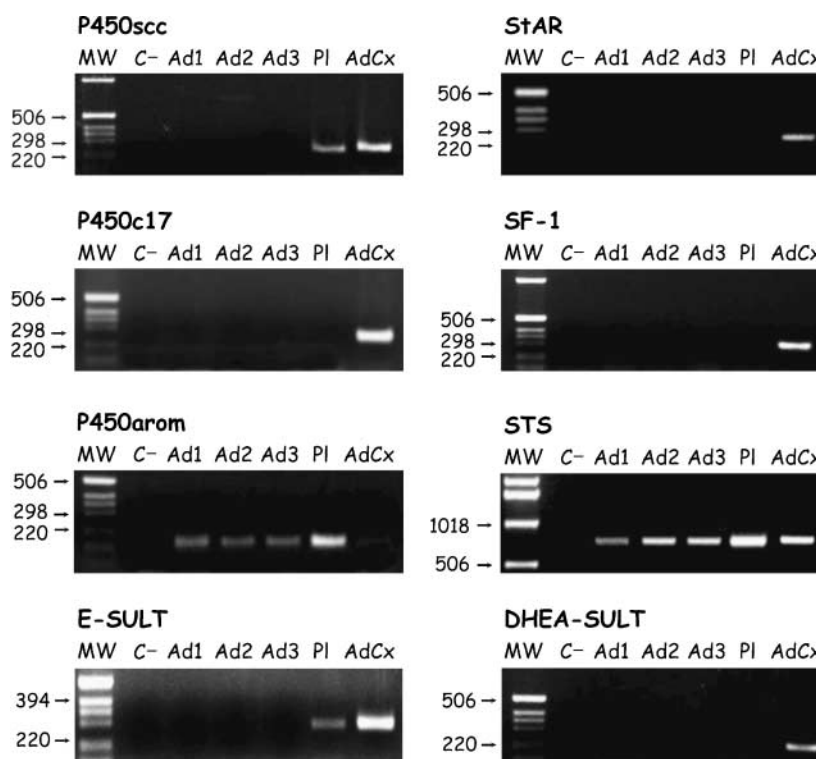


Figure 1 Representative expression analyses by RT-PCR of cytochromes P450scc, P450c17, P450arom, StAR, SF-1, STS, E-SULT, and DHEA-SULT mRNAs in human adipose tissues (Ad1, Ad2, and Ad3), placenta (PI), and adrenal cortex (AdCx), as described in Materials and Methods. MW, molecular weights; C-, no-template negative control (water).

truncated RNAs, RLM-5'-RACE amplifies only capped full-length RNAs. Two transcripts of the STS gene were found in adipose tissue, as compared to a single transcript in the placenta. The sequencing of the placental transcript confirmed the presence of a first exon (here called exon 1a) that is partially translated into the first 3 aa of the placental protein. The TSS was found 205 nt upstream of the first ATG. The ATG context matches only partially the proposed

consensus sequence for the initiation of translation (Kozak 1996), because it lacks a G base at position +4.

As shown in Fig. 3, the two adipose transcripts contain different untranslated first exons (here named 0a and 0b) that are spliced to a common exon 1b. This is then spliced to exon 2, which is identical to that found in the placenta. Exon 0a is 100 nt long and lies 43·28 kb upstream of exon 1b, whereas exon 0b is 77 nt long and lies 42·8 kb upstream of the same

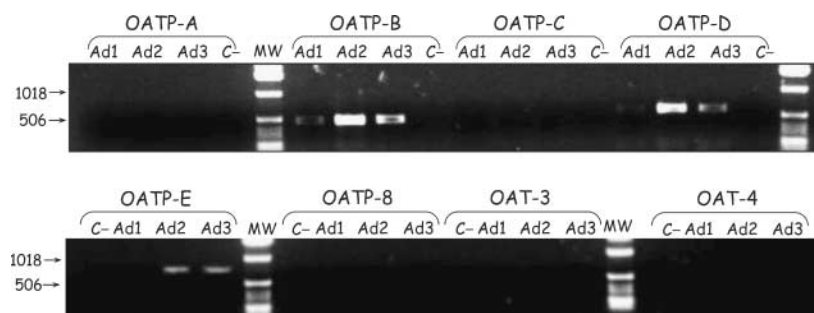


Figure 2 Representative expression analyses by RT-PCR of the mRNAs of organic anion transporting polypeptides (OATP-A, -B, -C, -D, -E, OATP-8, OAT-3, and OAT-4) presumably involved in transmembrane transfer of DHEA-S in human adipose tissues (Ad1, Ad2, and Ad3), as described in Materials and Methods. MW, molecular weights; C-, no-template negative control (water).

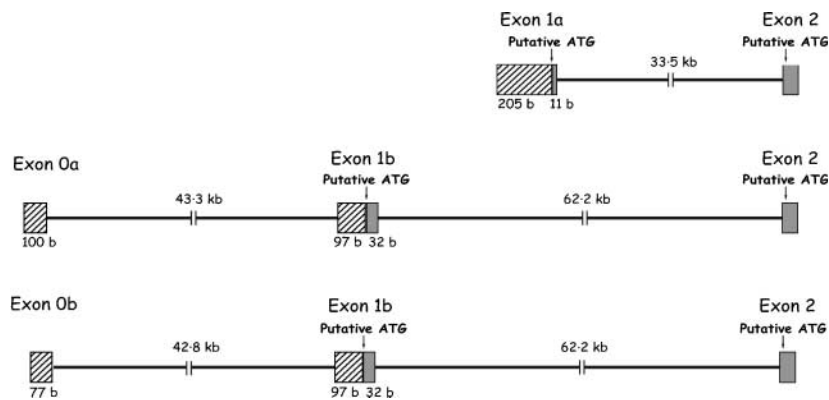


Figure 3 Schematic diagram of the 5'-region variants of STS mRNAs primarily transcribed from alternative promoters in human placenta and adipose tissue. In the main placental transcript, the first ATG is located in the first exon 1a. Two transcripts are expressed in adipose tissue with two different untranslated first exons 0a and 0b and a common exon 1b bearing the first ATG. A second putative ATG is found in exon 2, common to all transcripts. Exons are depicted as boxes; introns are shown as horizontal lines.

exon. The cDNA sequences of the two adipose transcripts have been submitted to the EMBL Nucleotide Sequence Database under the accession numbers AM072429 and AM072428, respectively. The introns display splice signals consistent with the GT/AG rule, except the first intron after exon 0a, in which the 5' splice donor is GC instead of GT. However, this represents the major splice variant (Thanaraj & Clark 2001).

The common exon 1b is 129 nt long and lies 62.19 kb upstream of exon 2. After a segment of 97 nt downstream of its 5' end, exon 1b contains an ATG with a context matching the proposed consensus sequence (Kozak 1996). As this ATG is in frame with the coding sequence of exon 2, the protein encoded in adipose tissue will be 7 aa longer than the placental protein, whose first four aa differs from aa 8–11 of adipose STS (Fig. 4).

To confirm these results, RT-PCR analyses were performed with three different sets of primers, in which a common reverse primer was designed on exon 2 and the sense primers on adipose exons 0a and 0b and placental exon 1a, respectively. As shown in Fig. 5, the adipose tissue does not use the placental promoter, whereas low levels of adipose-type transcripts were found in the placenta. Although analyses were not quantitative, the signals with the set of primers specific for exon 0b were consistently more intense than those for exon 0a.

With regard to the 3'-UTR, 3'-RACE analysis provided a single amplification fragment whose sequence and polyadenylation signal, located 428 bp after the stop codon, were found to be identical to those reported by Stein and co-workers (1989).

Western blotting analysis

The presence of the STS protein was assessed in the samples of adipose tissue and placenta as a control by immunoblot analysis with a polyclonal antibody against human STS. As illustrated in Fig. 6, a protein band was observed in both

placenta and adipose tissue samples. In the latter, the intensity of the immunoreaction was much lower than in the placentas, considering that only 0.5 (P11) or 1 (P12) µg of placental proteins were loaded instead of 50 µg, as in the adipose tissue preparations. This result is in agreement with the biochemical analysis showing that STS activity in adipose tissue is about 1/50 to 1/100 of that measured in placenta (see below). In addition to the characteristic ~64 kDa band of STS, a supplementary band of ~100 kDa was detected in both adipose and placental samples, which is likely due to nonspecific binding.

An attempt to localize STS in adipose tissue by immunohistochemistry with the same antibody was inconclusive, probably because the low level of expression of this enzyme in adipocytes is not detectable in their scanty amount of cytoplasm. Conversely, a strong cytoplasmic labeling was observed in placental syncytiotrophoblast (data not shown).

Measurement of STS activity

STS activity was determined by measuring the conversion of [³H]DHEA-S to DHEA. Homogenates of two adipose tissue samples as well as one sample of homogenized cultured adipocytes were incubated with 25 µM [³H]DHEA-S, a saturating concentration since the *K_m* values reported for human STS were 9.59 µM for the purified protein (Hernandez-Guzman *et al.* 2001), 7.8 µM (Suzuki *et al.* 1992) and 16 µM (Gibb & Lavoie 1984). All incubations were carried out with 1 or 2 mg total protein for 30, 60 and 90 min, because an incubation time of 15 min was preliminarily found sufficient to get a significant signal (0.45% of conversion to DHEA) with 1 mg protein. Under these conditions, the rate of DHEA-S hydrolysis was linear with the incubation time (*r* > 0.94). A homogenate of placental tissue was incubated as a positive control, but for

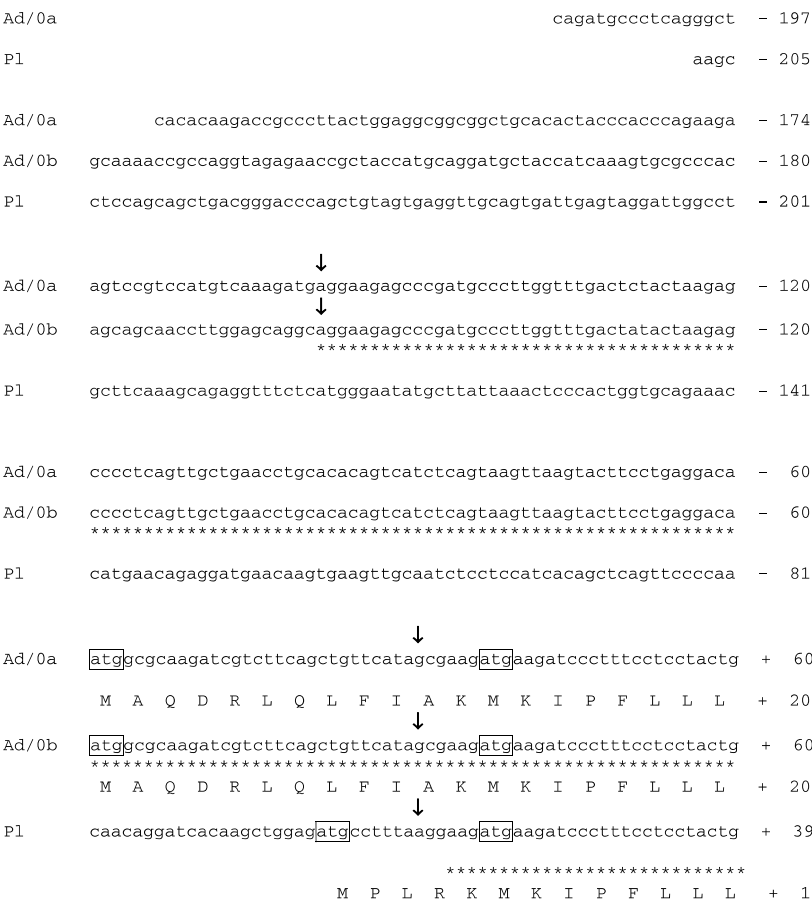


Figure 4 Alignment of the 5' regions found in adipose tissue (Ad/0a and Ad/0b with first exons 0a and 0b respectively) and placenta (Pl) STS transcripts. The putative ATG codons are boxed. Arrows show the intron positions.



Figure 5 RT-PCR analyses of STS mRNAs expressed in seven different human adipose tissues and one placenta with primer pairs specific for a common region in exon 2 and for different 5'-UTR variants (exons 1a, 0a, and 0b). MW, molecular weights; C-, no-template negative control (water).

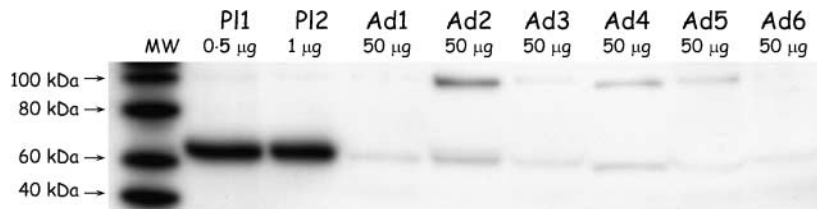


Figure 6 Western immunoblot analyses of STS expression in placenta (PI; 0.5 and 1 µg protein per lane) and six samples of adipose tissue (50 µg protein). Electrophoresis was performed on 10% polyacrylamide gels.

shorter incubation times and with less protein (Table 3). The percent conversions with boiled tissues were always subtracted from experimental values to compensate for the spontaneous hydrolysis of the conjugated substrate.

The specific enzymatic activity of STS in cultured adipocytes was 118 pmol/10⁶ cells per hour, in adipose tissue 1 and 2, 80 and 140 pmol/mg per hour respectively, that is about 50–100 times lower than in the placenta 7 nmol/mg per hour (Table 3).

[³H]DHEA-S transport studies along with the sulfatase assay in adipose cells

To measure [³H]DHEA-S transmembrane transport and intracellular hydrolysis, aliquots of suspensions of preadipocytes and adipocytes were incubated with the conjugate, whose final concentration was adjusted to 50 µM with unlabeled DHEA-S, since a *K_m* of 26 µM for [³⁵S]DHEA-S uptake was reported in isolated mononucleated trophoblastic cells (Ugele *et al.* 2003). After assessing that a minimum incubation time of 1 h was necessary to obtain a significant signal, preadipocytes were incubated for 3 and 6 h, while adipocytes were incubated for 6 h only. Incubations were performed singly, owing to the low numbers of cells available. Free DHEA was measured as above.

Adipocytes produced free DHEA at the rate of 45.83 pmol/10⁶ cells per hour, while preadipocyte rates were 40.8 and 43.3 pmol/10⁶ cells per hour. A search for further metabolites of DHEA, such as androstenedione, testosterone, and androst-5-ene-3β,17β-diol, gave negative results.

Discussion

The present study indicates that *de novo* biosynthesis of progestogens and androgens from cholesterol is unlikely in human adipose tissue, because the mRNAs of key steroidogenic enzymes, such as cytochromes P450scc and P450c17, and of proteins specifically expressed in steroidogenic cells, such as StAR and SF-1, could not be demonstrated in any of the samples. The failure to amplify cytochrome P450c17 cDNA, even with two other sets of primers (data not shown), is at variance with the report by Puche and co-workers (2002), but can be explained by the fact that they used not only RT-PCR, but also Southern blotting analysis to intensify the signal. The level of expression must be minimal anyway.

Our data support an intracrine role of adipose tissue as a terminal activator of circulating inactive androgen precursors into potent sex steroids. The detection of P450arom mRNA confirms previous findings about the occurrence of

Table 3 Sulfatase activity measured by production of DHEA from DHEA-S substrate in adipose tissues and adipocytes

	Adipose tissue 1 (pmol/mg per hour)		Adipose tissue 2 (pmol/mg per hour)		Adipocytes (pmol/10 ⁶ cells per hour)		Placental (mol/mg per hour)	
	Mean	S.D.	Mean	S.D.			Mean	S.D.
1 mg×30 min	71.67	5.77	175.00	57.66	–	–	7.05	0.25
1 mg×60 min	75.83	6.29	141.67	18.08	–	–	–	–
1 mg×90 min	60.56	6.75	117.22	17.95	–	–	–	–
2 mg×30 min	87.50*	–	143.75	19.44	–	–	–	–
2 mg×60 min	106.25*	–	121.90	10.85	–	–	–	–
0.5 mg×15 min	–	–	–	–	–	–	6.59	0.89
0.5 mg×30 min	–	–	–	–	–	–	7.85	1.02
0.5 mg×45	–	–	–	–	–	–	6.43	3.22
10 ⁶ cells×60 min	–	–	–	–	127.50*	–	–	–
10 ⁶ cells×120 min	–	–	–	–	108.75*	–	–	–
	80.36	17.39	139.91	22.85	118.13	13.26	6.98	0.64

Each experiment was performed in triplicate except the experiments marked with *, which were performed singly.

aromatizing activity for estrogen synthesis in adipose tissue, as reviewed by Belanger *et al.* (2002). The fact that the enzymes catalyzing the conversions of DHEA to androstenedione, testosterone, and androst-5-ene-3 β ,17 β -diol were not evidenced in the incubates with cultured preadipocytes, contrary to the experiment of Le Bail and collaborators (2002) on MCF-7 breast cancer cells, is probably due to a longer incubation time (20 h) in their case. A marked expression of these enzymes was not expected in the incubates with adipocytes, because 3 β -HSD/ Δ^5 - Δ^4 -isomerase and 17 β -HSDs (types 2, 3, and 5), together with aromatase, appear to occur in adipose stromal cells and preadipocytes rather than in differentiated adipocytes (Belanger *et al.* 2002).

Although adipose tissue can uptake a variety of free and conjugated steroids, the most important precursor appears to be DHEA-S, which circulates at much higher concentrations than free DHEA, gonadal steroids, and corticoids (Labrie *et al.* 2001). We have found that the adipose tissue transcribes the genes encoding three OATPs, namely OATP-B, OATP-D, and OATP-E, but no other members of the OATP and OAT families. The same OATPs plus OAT-4 are also expressed in placental tissue, where OATP-B is the predominant mediator of Na⁺-dependent transport of DHEA-S (Ugele *et al.* 2003). The same transporters are also present in the human mammary gland (Pizzagalli *et al.* 2003) and temporal lobe (Steckelbroeck *et al.* 2004). Interestingly, we have measured similar rates of uptake and hydrolysis of DHEA-S by both preadipocytes and adipocytes, suggesting that fat cell differentiation is not associated with major changes in DHEA-S transport and desulfation. Thus, excessive adipose tissue accumulation may potentially extract substantial amounts of DHEA-S from the circulation to the detriment of other peripheral organs with a rather constant mass that are also dependent on this steroid, a situation that may be suspected when the adrenal production of DHEA-S is inherently low or declining with aging (Labrie *et al.* 2001).

While the expression of sulfotransferase genes was not observed in adipose tissue, STS expression at the mRNA, protein, and catalytic activity levels was instead conclusively established. Notably, fat cells do not utilize the main placental promoter leading to the partially translated first exon 1a, but display two differently spliced transcripts leading to two distinct untranslated first exons 0a and 0b, followed by a common partially translated exon 1b, placed at a greater distance from exon 2 than exon 1a. These two variants, in which the one with exon 0b seems to prevail over that with 0a, were also detected in the placenta as minor isoforms.

The use of different first exons combined with alternative promoters is a mechanism allowing spatial- and/or temporal-specific regulations of transcription, whose selectivity may be further enhanced by different sets of transcriptional regulatory factors in target cells. In many genes, like *CYP19* encoding human cytochrome P450arom (Bulun *et al.* 2003), alternative

splicing does not alter protein translation in different tissues. In the case of the *STS* gene, however, the location of the initiation codon in a different exon for placenta (1a) as compared to the isoforms 0a and 0b (1b) results in proteins with dissimilar N-terminal regions. Nevertheless, this should not interfere with the catalytic activity of the protein, because the N-terminal regions correspond to the signal peptide that is post-translationally cleaved during STS maturation (Stein *et al.* 1989).

A putative second initiation codon in frame with the remaining of the coding region was found in exon 2 of both placenta and adipose tissue cDNAs but, like the ATG in the placental exon 1a, its context matches only partially the Kozac consensus sequence, as it lacks a G base at position +4. If this alternative ATG is actually used, it would eliminate any sequence difference in the N-terminal region of placental and adipose STS proteins. A heterogeneity of human STS mRNA transcripts due to alternative polyadenylation sites has also been described (Ferrante *et al.* 2002), which may indirectly affect catalytic activity by influencing mRNA half-life.

The proximal promoter of placental *STS* gene is peculiar because it lacks a functional TATA box, as do the promoters of many housekeeping genes (Weis & Reinberg, 1992), but unlike them, it has a low GC content and lacks multiple binding sites for Sp1. It also seems to be not tightly regulated because it lacks binding sites for known transcription factors, though several distal regulatory elements have been characterized within the 1.3 kbp region upstream of the TSS (Li *et al.* 1996). A preliminary sequence analysis of the 5'-flanking region of each new first exon of adipose tissue failed to reveal a TATA box or an initiator element. Computer analysis of the proximal promoters with the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>) showed no consensus elements for transcription factors related to steroidogenesis, such as steroid receptors or StAR and SF-1. Notably, the program CpG islands (<http://125.itba.mi.cnr.it/cgi-bin/wwwcpg.pl>) evidenced a 200 bp GC-rich region 50 nt upstream of exon 0b.

These data support the conclusion that the transcriptional regulation of adipose tissue STS differs from that of placental STS and disclose new avenues of investigation in the field of breast tumors, where STS expression is increased and may indirectly support breast tumor growth by boosting a local formation of estrogens.

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