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Estrogen Receptors in Human Preadipocytes

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Estrogen influences regional adipose tissue distribution and the accompanying cardiovascular disease risk. To elucidate the mechanisms of this link further, we assessed whether human preadipocytes (PAs) expressed estrogen receptors (ERs) and whether there were any regional or gender differences in ER complement. Human PAs expressed the ERα gene but not ERβ by reverse transcriptase–polymerase chain reaction, possessed ERα protein on Western blotting, and displayed specific 17β-estradiol (E_2) binding with calculated dissociation constants of 0.78 n*M*, 0.96 n*M*, and 1.19 n*M* and maximal binding capacities of 9.3 fmol/mg, 14.6 fmol/mg, and 18.2 fmol/mg from three whole cell binding assays. There were no regional differences in ERα complement for males or females. There were no gender differences in ERα complement for subcutaneous or visceral samples. We conclude that ERα but not ERβ is present in human PAs. This suggests that the effect of estrogen on adipose tissue deposition has a contribution from the direct effect of estrogen on human PAs via ERα.

Key Words: Preadipocyte; estrogen receptor α; estrogen receptor β; adipose tissue.

Introduction

There is significant evidence that estrogen has an important role in regional fat distribution *(1,2)*. There are obvious gender differences in regional fat deposition that contribute to gender differences in cardiovascular disease risk *(3, 4)*. Postmenopausally, women have an increase in abdominal fat volume *(3)* that is independent of changes in body mass index, and is associated with abnormalities in glucose tolerance *(5,6)*. Furthermore, estrogen hormone replacement therapy in post-menopausal women is associated with a lower cardiovascular risk factor profile and less central fat accumulation *(7)*.

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Estrogen is likely to influence adipose tissue deposition by affecting both adipocyte volume and number. Estrogen modifies adipocyte metabolism in vivo. There is lower lipoprotein lipase activity and lipolytic activity in intra-abdominal adipocytes from premenopausal females, in comparison to those from males or postmenopausal females *(8)*, and estrogen hormone replacement therapy increases femoral adipose tissue lipoprotein lipase activity in postmenopausal women *(9)*. 17β-Estradiol (E_2) increases human preadipocyte (PA) replication without affecting differentiation *(10)*, with at least part of this effect likely due to the production of mitogenic factors by PAs in response to estrogen *(11)*.

Although initial studies did not detect the classical estrogen receptor (ER), ERα, in human adipose tissue *(12,13)*, subsequent studies support the presence of ERα in human adipose tissue *(14–16)*. One study reported a regional difference in ERα number, with more ERα in subcutaneous (S) adipose tissue compared with visceral (V) adipose tissue in males *(15)*. Mature adipocytes express the ERβ gene *(17)*. Neither ERα nor ERβ have been identified in human PAs *(15–17)*, despite PAs replicating in vitro in response to estrogens *(10)*, suggesting a direct effect. However, previous studies of ERs in human PAs have been hampered by sample limitations.

We hypothesized that ERs are present in human PAs and that there may be intrinsic differences in the ER complement of S and V PAs, and between males and females. We aimed to assess human PAs for the presence of ERs and for regional and gender differences in ER complement.

We studied S and V PAs from male and female subjects, for ERα and ERβ gene expression using reverse transcription–polymerase chain reaction (RT-PCR), for immunoreactive ERα protein by Western blot analysis, and for specific estrogen binding using a whole cell binding assay and single saturating dose binding. Regional comparisons of S and V PAs from the same individual (paired samples) were made for ERα gene expression using semiquantitative amplification kinetics analysis RT-PCR and for ERα protein by quantitative Western blotting.

Results

Gene Expression

Human PAs expressed the ERα gene as assessed by RT-PCR. A single PCR product band, consistent with the expected 239 basepair product, was visible after 35 PCR

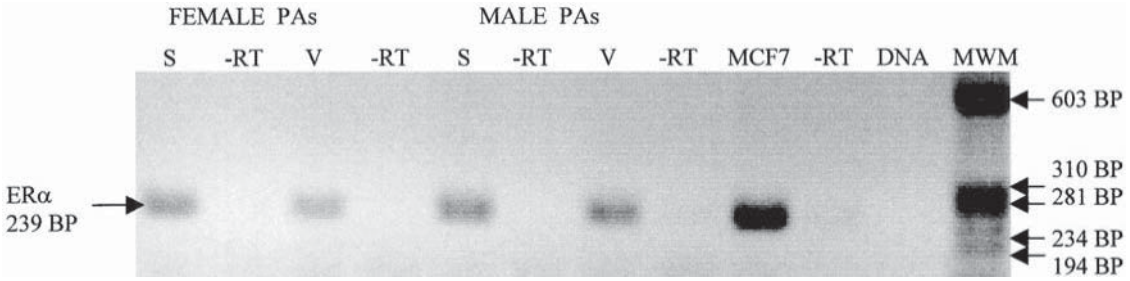

Fig. 1. RT-PCR of ERα showing bands in PA and MCF7 samples but not the corresponding no reverse-transcriptase (-RT) controls or genomic DNA. MWM, molecular weight marker; S, subcutaneous; V, visceral.

cycles for all 16 samples that were successfully reverse transcribed (Fig. 1). The samples included S and V PAs from males and females. The same sized band was present in the MCF7 positive controls, but not in the PA and MCF7 samples not treated with reverse transcriptase (negative controls) or when genomic DNA was used as a template (Fig. 1).

Human PAs did not express ERβ as assessed by RT-PCR. No bands were visible in the PA samples even after 40 PCR cycles, while a single PCR band consistent with the expected 360 basepair product was easily visible after 30 cycles in S and V adipocytes ex vivo, with appropriately negative no reverse transcriptase controls (Fig. 2).

Three male and three female paired S and V PA samples were assessed by dual primer amplification kinetics analysis RT-PCR. While the ERα gene expression was greater in V PAs for all sample pairs, (Fig. 3), there was no significant regional difference for males ($p = 0.25$), females ($p = 0.20$), or the group as a whole ($p = 0.053$). There were no gender differences ERα gene expression in S PAs ($p = 0.42$) or V PAs ($p = 0.44$).

Western Blots

Preadipocyte whole cell lysates ($n = 29$) were assessed for the presence of immunoreactive ERα protein by Western immunoblotting. For all samples, a band was present corresponding to the known molecular weight of ERα, at approximately 65 kDa *(18)* (Fig. 4). The MCF7 positive control had the same band, and also two further faint bands of approx 75 and 80 kDa (Fig. 4). These extra bands present only in the malignant cells are likely to be isoforms of ERα with exon duplications *(19,20)*.

Six male and eight female paired S and V samples have been assessed for regional differences in immunoreactive ERα protein. There was no statistical difference in the immunoreactive ERα protein between S and V PAs for males ($p = 0.71$), females ($p = 0.13$), or the group as a whole ($p = 0.34$). There were no gender differences in ERα protein in S PAs ($p = 0.94$) or V PAs ($p = 0.41$) (Fig. 5).

Estrogen Binding

Nine samples (two males, seven females) were assessed for specific estrogen binding using a whole cell binding assay. Six out of nine PA samples showed saturable specific binding (Fig. 6), with linear Scatchard plots obtained from three assays (one male, two females) allowing calculation of the dissociation constant and maximal binding capacity (Fig. 6). The dissociation constants were 0.78 n*M*, 0.96 n*M*, and 1.19 n*M*. The maximal binding capacities were 9.3 fmol/mg, 14.6 fmol/mg, and 18.2 fmol/mg. The dissociation constants for E_2 binding in human PAs were comparable to other human cell types known to contain ER assayed by similar methods *(21,22)*. The dissociation constant, were also similar to the dissociation constant obtained

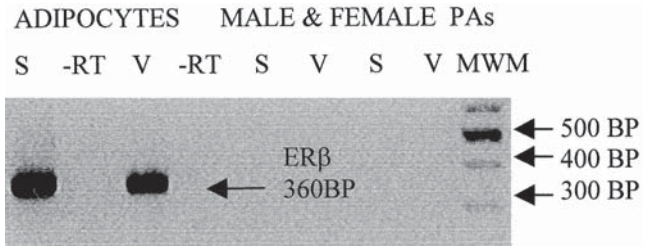

Fig. 2. RT-PCR of ERβ, showing bands in mature adipocyte samples but not the corresponding no reverse transcriptase (-RT) controls or the PA samples. MWM, molecular weight marker; S, subcutaneous; V, visceral.

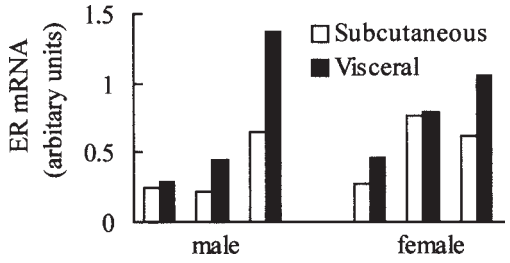

Fig. 3. ERα gene expression in paired subcutaneous and visceral PA samples.

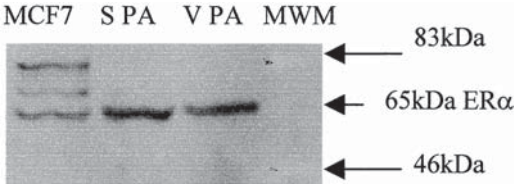

Fig. 4. Western blot for ERα in PAs and MCF7 cells showing native ERα in both and also two higher molecular weight isoforms in the MCF7 cells. MWM, molecular weight marker; S, subcutaneous; V, visceral.

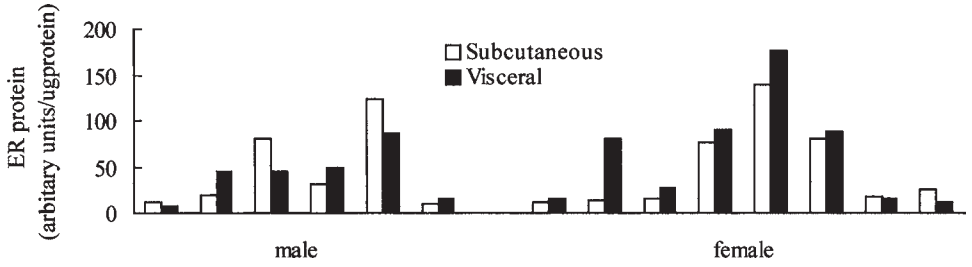

Fig. 5. ERα immunoreactive protein in paired subcutaneous and visceral PA samples.

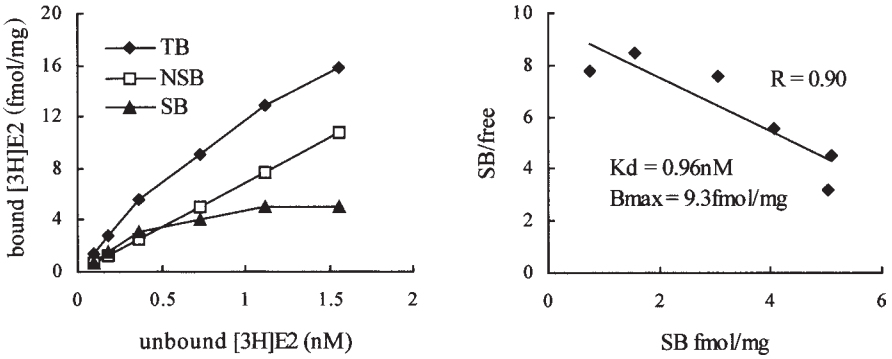

Fig. 6. Example of [^{3}H]E_2 binding in human PAs showing total binding (TB), nonspecific binding (NSB) and saturable specific binding (SB) and corresponding linear Scatchard analysis (right) showing a single binding entity. Bmax, maximal binding capacity; Kd, dissociation constant; R, Pearson correlation coefficient.

for the MCF-7 cell line assayed as a positive control, 0.54 n*M*. The maximal binding capacities for E_2 in human PAs were an order of magnitude lower than the MCF-7 positive control maximal binding capacity of 150 fmol/mg. The Scatchard plots for the other three assays with specific E_2 binding suggested a second low affinity binding site (two assays) or was not interpretable (one assay). For the remaining three samples (one male, two females) specific E_2 binding was low or absent with no obvious technical error to explain this.

Five male and six female paired samples were assessed for specific E_2 binding using the single saturating dose binding method. The results were highly variable. One female V sample showed no specific binding and another had very low specific binding (1.1 fmol/mg). The specific binding for the remaining samples ranged between 7.3 and 53.3 fmol/mg.

Discussion

In this article, we have shown that cultured undifferentiated human PAs contain the classical ER, ERα, as assessed by the presence of ERα mRNA, immunoreactive ERα protein, and specific estrogen binding. All male and female, S and V PAs tested, displayed ERα gene expression and immunoreactive ERα protein. Most PA samples displayed specific estrogen binding including S and V samples from males and females. ERβ was not expressed in subcultured PAs, but was in mature adipocytes, consistent with previously published findings showing an absence of ERβ gene expression in primary PA cultures and its presence in mature adipocytes *(17)*.

In the estrogen binding assays, linear Scatchard plots were obtained for three out of nine samples, indicating a single binding entity. Two out of nine binding curves and Scatchard plots were consistent with the presence of a second binding site as previously reported for E_2 binding in human whole adipose tissue *(15)*. The low affinity of the second binding site for E_2 makes it unlikely to play a role in mediating estrogen effects in vivo *(23)*, and it does not represent binding to ERβ. ERβ has a high affinity for E_2 similar to ERα *(24)*, and we were unable to detect ERβ gene expression.

The remaining Scatchard plots were uninterpretable despite a degree of specific binding or there was minimal specific binding, despite the presence of ERα gene expression and/or immunoreactive ERα protein in the same samples. Difficulty in obtaining interpretable Scatchard plots for estrogen binding in adipose tissue has been noted previously *(14)*. The lack of concordance in estrogen binding and ERα gene expression is in contrast to human breast tumors where E_2 binding correlated with ER mRNA levels *(25)*, and differed from our previous findings where glucocorticoid binding correlated with glucocorticoid receptor mRNA levels in human PAs *(26)*.

The single saturating dose estrogen binding results were highly variable, including two samples with minimal specific binding. There are a number of technical possibilities for the lack of specific estrogen binding in some samples.

The lowest recorded binding capacities are near the limit of detection by the whole cell binding method and there is relatively high nonspecific binding. Also, fatty acids inhibit ligand binding to ERα *(27)*, and cultured PAs have a small amount of lipid accumulation at confluence, confirming a degree of free fatty acid flux *(28)*. These aspects as well as the presence of a low affinity, high capacity binding site in some samples may have contributed to the variable binding results that have also been noted in the studies of ER in whole adipose tissue *(15)*. Apart from the technical issues, it is likely that there is interindividual variability in ERα expression, as is the case for glucocorticoid and androgen receptor expression in fibroblasts *(29, 30)*. There were no obvious demographic variables associated with a lack of specific estrogen binding.

The confirmation of the presence of ERα in human PAs is in contrast to the studies showing human PAs lack ERα. These studies have used RT-PCR and cytosol binding assays to look for ERα in the stromovascular fraction of whole adipose tissue *(15,16)*. There was an occasional weak ERα PCR product band, but this was felt not to be significant because there was no specific estrogen binding *(16)*. However, the assessment of specific estrogen binding in the stromovascular fraction would be limited by the size of the sample, the free lipid in the sample, and the heterogeneity of the sample.

Having shown ERα was present, we assessed whether there were any intrinsic differences in ERα complement. There were no regional differences in mRNA levels or in immunoreactive ERα protein. We acknowledge that we cannot exclude a small, but potentially biologically important regional difference.

ERα-mediated estrogen effects in human PAs are likely to have a physiological role. The mitotic effect of estrogens in human PAs *(10)* increases PA number, potentially increasing the number of adipocytes. Mitogenic factors produced by breast adipose tissue PAs in response to estrogen *(11)*, may act in a paracrine manner on breast tissue. This hypothesis may be relevant in the pathogenesis of breast carcinoma and may explain why a proportion of ER-negative malignancies respond to anti-estrogen therapy *(31)*.

Although there is no significant regional difference in ERα number in PAs, regional differences in local estrogen production/concentration through paracrine or autocrine mechanisms *(32)* may result in ER-mediated differences in adipose tissue distribution. The PA is the predominant cell responsible for the aromatase activity in adipose tissue, promoting estrogen production from androgenic/adrenal precursors *(33,34)*. There is likely to be a higher local estrogen concentration in peripheral adipose tissue because of its greater aromatase activity *(33,35)*. Several isoforms of 17β-hydroxysteroid dehydrogenase are also present in human adipose tissue, with evidence of regional differences in the level of gene expression that may also influence local estrogen concentrations *(36)*.

Table 1
Clinical Characteristics of Subjects

	males (n = 9)	females (n = 8)	p
Age in years	64 ± 4 (48–82)	52 ± 6 (29–71)	0.052
Body mass index	25.8 ± 1.7 (20.3–37.3)	25.8 ± 1.4 (22.6–31.2)	0.499
Waist in cm	98 ± 5 (84–118)	90 ± 4 (77–103)	0.107
Waist–hip ratio	0.96 ± 0.02 (0.89–1.09)	0.85 ± 0.02 (0.77–0.95)	0.002

Data expressed as mean ± SEM; ranges in parentheses

Both genders showed the presence of ERα, and there were no gender differences in gene expression or immunoreactive protein. This result supports gender differences in regional adipose tissue deposition being secondary to differences in circulating sex steroid levels, and other gender differences such as the smaller glucocorticoid receptor complement in V PAs in females *(26)*.

In summary, the classical ER, ERα, is present in human PAs with no regional or gender differences in ERα complement. This suggests that estrogens have direct effects on human PAs, but that differences in adipose tissue distribution that are associated with estrogen levels, are not secondary to intrinsic regional differences in ERα complement.

Materials and Methods

Subjects/Sample Preparation

Paired abdominal S and omental (visceral) adipose tissue samples approx 1 cm^3 were collected during elective surgery from nine males and eight females with informed written consent. The Princess Alexandra Hospital Research Ethics Committee approved the protocol. The subjects were all of caucasian origin, undergoing surgery for vascular disease, benign or localized malignant bowel pathology, or benign gynecological conditions. None of the subjects had an endocrine disorder. The male and female subjects were equally obese, with the female subjects tending to be younger, with a statistically significant lower waist–hip ratio (Table 1).

Samples were transported to the laboratory in Ringers solution and were processed within 15 min by collagenase digestion as previously described *(37)*. The stromovascular fraction was plated into culture flasks (Corning, Cambridge, UK) in DMEM/Hams F-12 1:1 (DMEM/Hams) (ICN, California USA) with 10% fetal bovine serum (CSL, Melbourne, Australia) and antibiotics. The cells were grown for 2–3 mo and subcultured 3–4× to obtain a homogeneous cell population of sufficient quantity to study. We have shown that cells cultured in this manner display intrinsic regional and gender differences in glucocorticoid receptor number *(26)*. The cells retain the PA phenotype because when these cells are treated with serum-free differentiation medium (includ-

ing glucocorticoids, insulin, and the thiazolidinedione, rosiglitazone), they differentiate into adipocytes expressing lipoprotein lipase, glycerol-3-phosphate dehydrogenase, and leptin, with visible lipid droplets *(38)* (unpublished observations). Furthermore, the cells show an increase in replication rate in response to E_2, consistent with previous studies *(10)*. The ER studies were performed on confluent undifferentiated PAs grown in serum-containing medium that inhibits differentiation. The cells do not express adipocyte markers, lipoprotein lipase, glycerol-3-phosphate dehydrogenase, or leptin, nor accumulate significant lipid under these conditions. Paired S and V PA samples were treated identically, preventing regional differences secondary to in vitro effects. MCF7 cells, a human breast cancer cell line, were grown in MCF7 medium *(39)* and used as the ERα positive control *(40)*. Isolated mature adipocytes were used as the ERβ positive control *(17)*.

Gene Expression

Confluent PAs were washed with phosphate-buffered saline (PBS) and the RNA extracted *(41)* and stored at −70ºC until use. Total RNA was reverse transcribed using the random hexamer priming option of a commercially available kit (SUPERSCRIPT™, Life Technologies, Gaitherburg, MD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR as a positive RT control, and a minus reverse transcriptase negative control, were included for each RNA sample. Previously published ERα, ERβ, and GAPDH primer sequences *(14,42,43)* were obtained commercially (DNA Express, Fort Collins, CO). The PCR was performed in a Corbett Research PC-90 microplate thermal sequencer and the PCR products were visualized under ultraviolet illumination after electrophoresis on an ethidium bromide-labeled 2% DNA agarose gel.

Dual primer amplification kinetics analysis RT-PCR was used to compare ERα gene expression in paired S and V PAs. The ERα gene expression was adjusted for any differences in amount of starting total RNA by adjusting for the expression of the housekeeping gene, GAPDH, giving a result in arbitrary units. Briefly, A 239 basepair sequence of ERα cDNA, and a 318 basepair sequence of GAPDH, were co-amplified after an initial eight PCR cycles with the ERα primers only. Aliquots (10 µL) were removed at ERα cycles 29–38 (GAPDH cycles 21–30). Negatives (Polaroid 665 film) of the electrophoresed PCR products were used for densitometry in a Molecular Dynamics personal densitometer. The ERα band density at a cycle number chosen in the log linear range for both samples was adjusted by the GAPDH band density chosen similarly. Paired S and V samples were always assayed together, allowing direct comparison of S and V paired samples' ERα gene expression.

Western Blot Analysis

The Western analyses were performed on whole cell lysates prepared as suggested by the antibody suppliers (Santa Cruz, CA). Confluent PAs or MCF7 cells were scraped into lysis buffer with 1% tergitol type NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/mL phenylmethylsulfonyl fluoride, 45 µg/mL aprotinin, 1 n*M* sodium orthovanadate, incubated on ice for 30 min, and centrifuged at 6500*g* for 20 min at 4ºC. The supernatant was subjected to SDS-PAGE with a 5% stacking, 10% resolving gel, and the proteins were electrophoretically transferred to a polyvinylidene difluoride (PDVF) membrane (Biorad). The PDVF membrane was blocked with 5% skim milk powder overnight at 4ºC, and incubated with rabbit polyclonal antibody against human ERα (1:1000) for 45 min at 25ºC, and then with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) for 30 min at 25ºC. Immunodetection was performed using chemiluminescence detection reagents (ECL™, Amersham, Buckinghamshire, UK) as per the supplier's instructions.

ERα protein levels in S and V samples were compared using a modification of the immunoblotting technique that has successfully been used for quantifying other proteins *(44)*. The paired S and V whole cell lysates were assayed together. At least two different concentrations of each sample were loaded and the conditions were optimized to ensure band density was proportional to protein loaded for an individual sample. The ERα protein band density measured in a Molecular Dynamics personal densitometer was corrected for differences in the total protein for each sample, measured by the Bradford method *(45)*.

Estrogen Binding

Twenty-five cubic centimetre flasks of confluent cells were washed with PBS and preincubated in DMEM/Hams for 30 min at 37ºC. This was followed by a 60 min incubation with one of six serial dilutions of 0.125–3 n*M* tritiated E_2 ($[^3H]E_2$) (70–120 Ci/mmol, Amersham) or 0.125–3 n*M* $[^3H]E_2$ with a 250× excess of unlabeled E_2 (Sigma), in DMEM/Hams. Media without phenol red (Life Technologies) were used for both the preincubation and incubation, as phenol red is an estrogen agonist that binds to ERα *(46)*. All incubations were done in duplicate or triplicate. An aliquot of the prepared 3 n*M* $[^3H]E_2$ media was added to scintillation fluid (Instagel Plus, Packard) and counted in a Minaxi-Tricarb 4000 series scintillation counter, to allow conversion from disintegrations/per minute to moles. An aliquot of the media was removed near the end of the incubation to obtain an accurate measure of unbound $[^3H]E_2$ in each flask. After washing the cells with ice-cold PBS, the cells were lysed and an aliquot was taken from each flask for bound $[^3H]E_2$ count and for protein determination *(45)*. A Scatchard plot provided the binding characteristics for each sample.

Estrogen binding was also assessed using a single 3 n*M* concentration of $[^3H]E_2$ with and without a 250× excess of unlabeled E_2 to obtain specific estrogen binding at a single saturating dose of $[^3H]E_2$. The purity of the $[^3H]E_2$ was regularly checked by thin layer chromatography as per the

manufacturer's instructions and was always greater than 95% at the time of assay.

Statistical Analysis

V and S PA ERα gene expression and immunoblotting were compared using the Student's paired *t*-test (two-tailed). All gender comparisons used the two-sample *t*-test for means (two-tailed). The Scatchard plots were tested for simple linear correlation using Pearson's correlation coefficient applying the appropriate degrees of freedom. The statistical analyses were performed using the data analysis function of Microsoft Excel Version 5 and the SPSS data analysis package version 8. A result with a *p* value of less than 0.05 was taken as statistically significant.

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