

Glucocorticoid receptors in human preadipocytes: regional and gender differences

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Abstract

Glucocorticoid excess causes visceral obesity and its accompanying insulin resistance, dyslipidemia and hypertension. Glucocorticoids enhance preadipocyte (PA) differentiation and increase their aromatase activity (oestrogen production) and there is regional variability in these PA processes. Therefore, we studied human PAs for the presence of, and any regional or gender differences in, glucocorticoid receptors (GRs).

Confluent subcultured human subcutaneous (Sc) and visceral (Vis) PAs from both genders contained GRs as assessed by GR gene expression and specific glucocorticoid (dexamethasone) binding. The dissociation constant was similar to that of other human cells and there was no difference between Sc and Vis sites or between males and females. There was significantly less GR mRNA in Vis PAs compared with Sc PAs in females ($P=0.008$) but not

in males. There was less glucocorticoid binding in Vis compared with Sc PAs in females, measured by maximal binding capacity ($P=0.035$) or single saturating dose glucocorticoid binding (Bssd) ($P=0.019$). There was no regional difference in specific glucocorticoid binding in males. There was a gender difference with fewer GRs in Vis PAs in females compared with males measured by Bssd ($P=0.006$).

In summary, GRs are present in human PAs. There is a lower GR density in Vis compared with Sc PAs in females, and females have fewer GRs in Vis PAs compared with males. These differences are likely to affect regional aromatase activity and to contribute to the smaller visceral fat mass in females compared with males.

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Introduction

Glucocorticoid excess predisposes to visceral obesity and its accompanying insulin resistance, dyslipidemia and hypertension. Glucocorticoids regulate multiple processes in adipose tissue influencing fat cell size and adipose tissue metabolism (Björntorp 1991, 1996). Moreover, glucocorticoids promote differentiation of human preadipocytes (PAs) into mature adipocytes, increasing fat cell number (Hauner *et al.* 1989). Glucocorticoids also increase the aromatase activity in human PAs (Simpson *et al.* 1981), with this aromatase activity being an important determinant of systemic and local oestrogen concentrations (Folkerd & James 1983, Siiteri 1987).

These findings, that glucocorticoids directly influence important physiological functions of the PA, suggest that human PAs contain glucocorticoid receptors (GRs). This is supported by a single glucocorticoid binding assay in human PAs (Simpson *et al.* 1981), and the presence of GR gene expression in the heterogeneous PA-containing stromovascular fraction of human adipose tissue (Pedersen *et al.* 1996). Human PAs show regional differences in their

susceptibility to differentiate (Adams *et al.* 1997) and in their aromatase activity (Ackerman *et al.* 1981), suggesting that there may be regional differences in GR complement. Studies in whole adipose tissue show that GRs are present in adipose tissue with regional differences in GR complement between visceral (Vis) and subcutaneous (Sc) sites (Rebuffé-Scrive *et al.* 1985, 1990, Miller *et al.* 1987, Pedersen *et al.* 1994).

We hypothesised that GRs were present in human PAs and that there were differences in the GR complement of Vis and Sc PAs. We aimed to assess human PAs for the presence of classical (type 2) GRs and for regional and gender differences in GR density.

Materials and Methods

We studied Sc and Vis PAs from both male and female subjects, for GR gene expression using reverse transcription-polymerase chain reaction (RT-PCR), and for specific glucocorticoid binding using a whole cell binding assay. Regional comparisons of Sc and Vis PAs

Table 1 Clinical characteristics of subjects. Data are expressed as means \pm S.E.M.; ranges in parentheses

	Males (n=10)	Females (n=11)	P
Age (years)	60 \pm 3 (46–75)	50 \pm 4 (35–74)	0.079
Body mass index (kg/m ²)	30.0 \pm 2.1 (22.3–37.4)	31.2 \pm 2.1 (22.9–45.0)	0.686
Waist (cm)	106 \pm 6 (91–114)*	100 \pm 6 (67–144)	0.507
Waist-hip ratio	1.02 \pm 0.02 (0.96–1.09)*	0.89 \pm 0.06 (0.68–1.38)	0.123

*n=7.

from the same individual (paired samples) were made for GR gene expression using semi-quantitative amplification kinetics analysis RT-PCR, and for specific glucocorticoid binding using the whole cell binding assay and single saturating dose glucocorticoid binding.

Subjects/sample preparation

Paired abdominal Sc and omental (visceral) adipose tissue samples approximately 1 cm³ were collected during elective surgery from 10 males and 11 females, with informed written consent. The Princess Alexandra Hospital Research Ethics Committee approved the protocol. None of the subjects was chronically using glucocorticoids or had any endocrine disorder with the exception of one with treated hypothyroidism receiving adequate thyroxine replacement. The male and female subjects were equally obese, with the female subjects tending to be younger with a lower waist-hip ratio, although none of the differences were statistically significant (Table 1).

Samples were transported to the laboratory in Ringers solution and were processed within 15 min by collagenase digestion as previously described (Rodbell 1964). The stromovascular fraction was plated into culture flasks (Corning Inc., Cambridge, UK) in Dulbecco's modified Eagle's medium/Hams F-12 1:1 (DMEM/Hams) (ICN Biomedical Australasia Pty Ltd, Seven Hills, Australia) with 10% fetal bovine serum (Commonwealth Serum Laboratories, Brisbane, Australia) and antibiotics. The cells were grown for 2–3 months and subcultured 3–4 times to obtain a homogeneous cell population of sufficient quantity to study. Paired Sc and Vis PA samples were treated identically, preventing regional differences secondary to *in vitro* effects. The PAs used for the GR gene expression and glucocorticoid binding studies were confluent but undifferentiated, as serum-containing medium is inhibitory to PA differentiation (Entenmann & Hauner 1996). Confluent human forearm skin fibroblasts were used as the GR positive control (Bruning *et al.* 1979).

Confirming the PA phenotype

The subcultured cells were confirmed to be PAs by their ability to differentiate into mature adipocytes under appropriate culture conditions. Confluent subcultured PAs and skin fibroblasts (as a negative control) were incubated in differentiation medium (Adams *et al.* 1997), a serum-free medium containing glucocorticoids and insulin, and an activator of peroxisome-proliferator-activated receptor- γ , BRL 49653 (a kind gift from SmithKline-Beecham, Welwyn, UK). After 14–21 days, the PAs but not the fibroblasts expressed lipoprotein lipase, glycerol-3-phosphate dehydrogenase and leptin, as assessed by Northern blot. Furthermore, the PAs, in contrast to the fibroblasts, accumulated significant quantities of lipid as confirmed by oil-red O staining.

GR gene expression

Confluent PAs were washed with phosphate-buffered saline (PBS) and the RNA was extracted (Chomczynski & Sacchi 1987) and stored at -70°C until use. Total RNA was reverse transcribed using the random hexamer priming option of a commercially available kit (SUPER-SCRIPT Preamplification System for first strand cDNA synthesis, Life Technologies, Gaithersburg, VA, USA). 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 GR and GAPDH primer sequences (Dibattista *et al.* 1993) were obtained commercially (DNA Express, Fort Collins, USA). The PCR was performed in a Corbett Research PC-90 microplate thermal sequencer and the PCR products visualised under ultraviolet illumination after electrophoresis on an ethidium bromide-labelled 2% DNA agarose gel.

Dual primer amplification kinetics analysis RT-PCR was used to compare GR gene expression in paired Sc and Vis PAs. The GR gene expression was adjusted for any

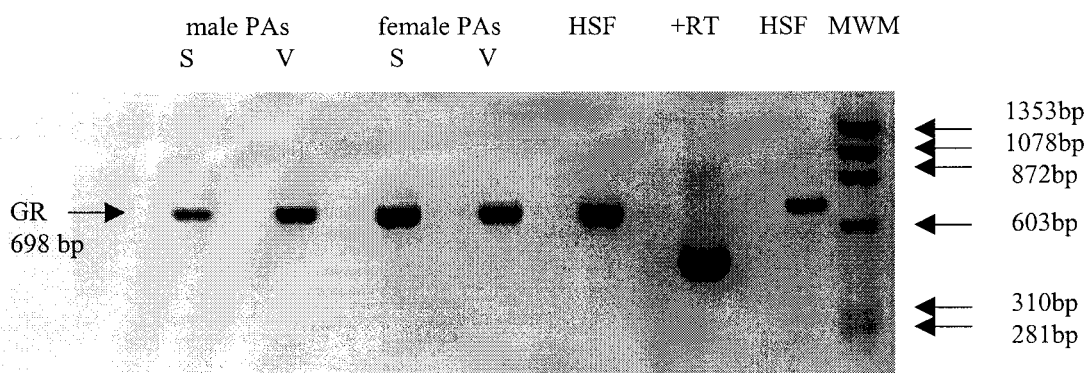


Figure 1 Ethidium bromide-labelled gel of glucocorticoid receptor PCR products, each sample accompanied (on its right) by its minus reverse transcriptase control. bp, base pairs; HSF, human skin fibroblasts; MWM, molecular weight marker; +RT, kit positive reverse transcriptase control; V, visceral; S, subcutaneous.

differences in amount of starting total RNA by adjusting for the expression of GAPDH, giving a result in arbitrary units. Briefly, a 698 base pair sequence of GR cDNA, and a 318 base pair sequence of GAPDH, were co-amplified after an initial 4 PCR cycles with the GR primers only. Aliquots (10 μ l) were removed at GR cycles 23–36 (GAPDH cycles 19–32). Negatives (Polaroid 665 film) of the electrophoresed PCR products were used for densitometry in a Molecular Dynamics personal densitometer. The GR 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 Sc and Vis samples were always assayed together allowing direct comparison of Sc and Vis paired samples' GR gene expression.

Glucocorticoid binding

Flasks (25 cm²) of confluent cells were washed with PBS and preincubated in DMEM/Hams for 30 min at 37 °C. This was followed by a 60-min incubation with one of six serial dilutions of 0.78–25 nM tritiated dexamethasone (³H]Dex) (70–110 Ci/mmol, Amersham Australia Pty Ltd, Sydney, Australia) or 0.78–25 nM [³H]Dex with 250 times excess unlabelled dexamethasone (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) in DMEM/Hams. All incubations were carried out in duplicate or triplicate. An aliquot of the prepared 25 nM [³H]Dex media was added to scintillation fluid (Instagel Plus, Packard, Meriden, USA) and counted in a Minaxi-Tricarb 4000 series scintillation counter, to allow conversion from disintegrations/min to moles. An aliquot of the media was removed near the end of the incubation to obtain an accurate measure of unbound [³H]Dex in each flask. After washing the cells with ice-cold PBS, the cells were lysed and an aliquot was taken from each flask to count bound [³H]Dex, and for protein determination (Bradford 1976). A Scatchard plot provided the binding characteristics for each sample.

Glucocorticoid binding was also assessed using a single 25 nM concentration of [³H]Dex with and without 250 times excess unlabelled Dex to obtain specific glucocorticoid binding at a single saturating dose of [³H]Dex. The purity of the [³H]Dex 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

Vis and Sc PA GR gene expression was compared using the Wilcoxon signed rank test. All other regional comparisons used the Student's paired *t*-test (two-tailed). All gender comparisons used the two-sample *t*-test for means (two-tailed). The Scatchard plots, the two glucocorticoid binding measures comparison, and the comparison of the Vis/Sc PA GR gene expression and the corresponding glucocorticoid binding ratio, 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.

Results

Gene expression

Human PAs expressed the GR gene as assessed by RT-PCR. A single PCR product band, consistent with the expected 698 base pair product was obtained from all 36 PA samples that were successfully reverse transcribed (Fig. 1). The samples included Sc and Vis PAs from males and females. The same sized band was present in the fibroblast positive controls but not in the minus RT negative controls (Fig. 1).

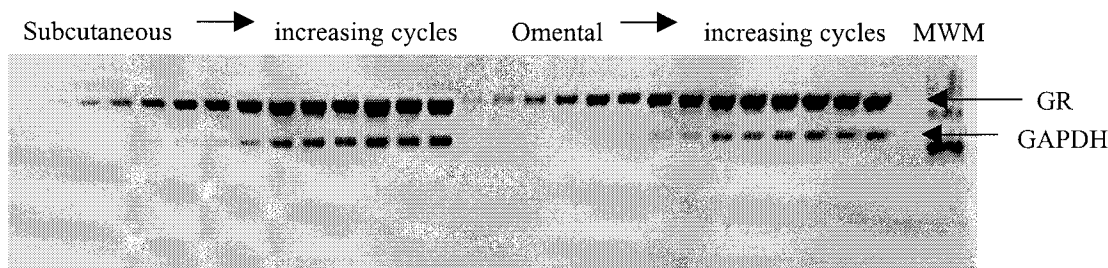


Figure 2 Ethidium bromide-labelled gel of the GR and GAPDH PCR products, over 23–37 and 20–34 cycles respectively, for a paired subcutaneous and visceral (omental) preadipocyte sample. MWM, molecular weight marker.

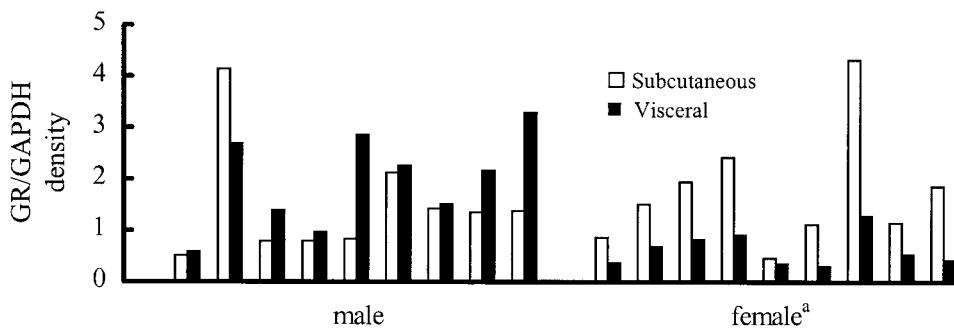


Figure 3 Glucocorticoid receptor gene expression in paired subcutaneous and omental (visceral) preadipocyte samples; ^a $P=0.008$ for subcutaneous versus visceral preadipocytes in females.

Nine male and nine female paired Sc and Vis PA samples were assessed by dual primer amplification kinetics analysis RT-PCR. The GR and GAPDH PCR products showed an increase in density with increasing cycles before reaching a plateau (Fig. 2). For the female subjects, there was significantly less GR gene expression in Vis PAs compared with Sc PAs ($P=0.008$) with all nine Vis samples showing less GR gene expression than the corresponding Sc samples (Fig. 3). For the male subjects there was no significant difference in GR gene expression between Sc and Vis PAs (Fig. 3) ($P=0.066$). There was no statistical difference in GR gene expression in Sc and Vis PAs for the group as a whole ($P=0.327$).

Glucocorticoid binding

All 29 PA samples tested showed saturable specific dexamethasone binding (Fig. 4a). This included both male and female, Sc and Vis samples. Linear Scatchard plots were obtained from 27 samples (Fig. 4b). The dissociation constant (K_d) ranged from 4.67 nM to 24.88 nM. The maximal binding capacity (Bmax) ranged from 152 fmol/mg to 867 fmol/mg. The K_d was comparable to other human cell types known to contain GR assayed by similar methods (Bertovitz *et al.* 1988, Corrigan *et al.* 1991, Dibattista *et al.* 1993). The K_d and Bmax were similar to results for the two forearm fibroblast strains assayed as positive controls (K_d , 6.1 nM and 11.1 nM; Bmax, 367 fmol/mg and 630 fmol/mg).

The successful whole cell binding assays included six paired Sc and Vis samples for each gender (Table 2). There was no significant difference in K_d between Sc and Vis PAs for males ($P=0.90$), females ($P=0.77$), or for the group as a whole ($P=0.78$). Comparing the results for males and females, there were no gender differences in K_d for either Sc or Vis PAs ($P=0.21$ and $P=0.21$ respectively). There was a significant difference between the Sc and Vis Bmax in females ($P=0.035$), with less glucocorticoid binding in Vis PAs, but there was no regional difference in males ($P=0.34$). There was lower Bmax for Vis PAs compared with Sc PAs for the group as a whole ($P=0.023$). Comparing the results for males and females, there were no gender differences in Sc or Vis Bmax ($P=0.89$ and $P=0.45$ respectively).

Ten male and eleven female paired samples were assessed for specific dexamethasone binding using the single saturating dose binding method (Bssd) (Table 3). There was a significantly lower Bssd in Vis compared with Sc PAs in females ($P=0.019$) but not in males ($P=0.78$). There was no significant difference in Bssd between paired Sc and Vis PA samples in the group as a whole ($P=0.19$). Comparing the results for males and females, females had less glucocorticoid binding in Vis PAs ($P=0.006$) with no gender difference for Sc PAs ($P=0.114$).

There was a strong simple linear correlation between the two methods of ascertaining specific glucocorticoid binding, the Bmax and the Bssd ($n=24$, $P<0.001$). This

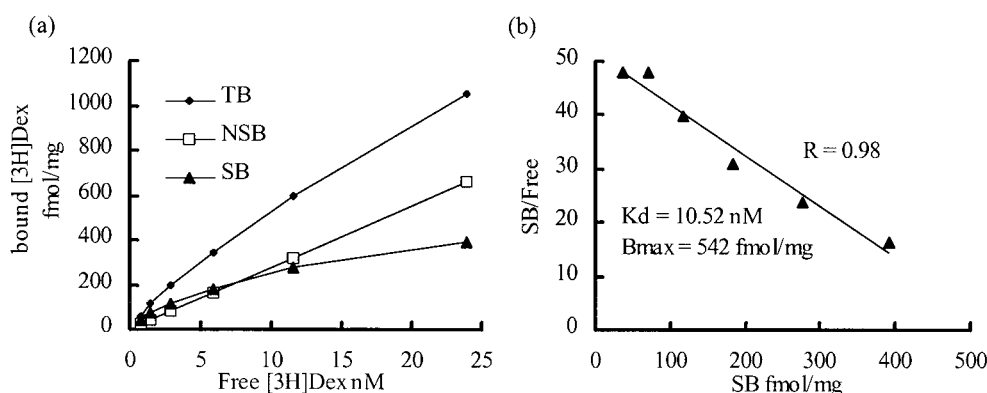


Figure 4 Glucocorticoid binding in human preadipocytes. (a) Graph of [³H]Dex binding in human PAs showing total binding (TB), nonspecific binding (NSB) and saturable specific binding (SB). (b) Corresponding Scatchard analysis. R, Pearson correlation coefficient.

confirmed that the single saturating dose binding method was appropriate for assessing specific glucocorticoid binding in a whole cell system in PAs, as has been shown for androgen binding in cultured fibroblasts using similar

Table 2 Paired subcutaneous and visceral preadipocytes, dexamethasone binding assays. Results are expressed as the mean ± S.E.M.

	<i>k_d</i> (nM)	B max (fmol/mg)
Samples		
Male (n=6)		
Subcutaneous	9.3 ± 1.2	465 ± 47
Visceral	9.2 ± 1.7	413 ± 88
Female (n=6)		
Subcutaneous	12.7 ± 2.2	455 ± 43*
Visceral	13.5 ± 2.8	327 ± 63*
Both (n=12)		
Subcutaneous	11.0 ± 1.3	460 ± 31**
Visceral	11.4 ± 1.7	370 ± 53**

*P=0.035; **P=0.023.

Table 3 Paired subcutaneous and visceral preadipocytes, dexamethasone single saturating dose binding results expressed as the mean ± S.E.M.

	B _{ssd} (fmol/mg)
Samples	
Male (n=10)	
Subcutaneous	310 ± 26
Visceral	320 ± 41**
Female (n=11)	
Subcutaneous	240 ± 33*
Visceral	171 ± 28***
Both (n=21)	
Subcutaneous	273 ± 22
Visceral	244 ± 29

*P=0.019; **P=0.006.

methods (Kaufman *et al.* 1983). There was also a strong linear correlation between the relative Vis/Sc GR gene expression and the corresponding Vis/Sc glucocorticoid binding ratio (*n*=16, *P*=0.001).

Discussion

In this paper we have shown that cultured human undifferentiated PAs contain the classical GR. In females, there was significantly less GR gene expression and glucocorticoid binding in Vis compared with Sc PAs. In contrast, in males there was no regional difference in GR complement.

Despite the subculturing and prolonged *in vitro* growth, the cells studied retained their PA phenotype with the ability to differentiate into mature adipocytes, including expressing leptin that is adipocyte-specific, under appropriate culture conditions (Adams *et al.* 1997). This strongly suggests that the regional and gender differences in GR complement are true characteristics of the PA. Moreover, their presence after prolonged *in vitro* growth suggests that these are intrinsic differences, rather than differences due to circulating hormones or differing regional influences. The potential stability of the steroid receptor complement of subcultured cells is exemplified by the consistent regional differences in androgen receptor density and androgen responsiveness of subcultured skin fibroblasts (Brown & Migeon 1981).

The concordance of GR gene expression and specific glucocorticoid binding agrees with previous studies showing that GR number parallels GR mRNA at steady state in a number of systems (Northrop *et al.* 1986, Dibattista *et al.* 1993, Brönnegard *et al.* 1995). It suggests that in confluent PAs, GR density is regulated at the level of gene transcription.

The gender differences in Vis PA GR complement may contribute to the gender differences in regional fat

deposition (Lemieux *et al.* 1993). This is supported by observations that even when circulating sex steroid levels are extremely low, gender differences in adiposity are still apparent. There are gender differences in degree of body fat in prepubertal children, with girls having more fat than boys (Taylor *et al.* 1997). Moreover, there are differences in body fat distribution, with pre-pubertal males having relatively more intra-abdominal fat and less subcutaneous abdominal fat than pre-pubertal females, sufficient to contribute to gender differences in leptin levels (Nagy *et al.* 1997).

The regional differences in GR density found in our study are consistent with, and may explain, the site-specific differences in PA aromatase activity in females. Aromatase activity in PAs is stimulated by glucocorticoids (Mendelson *et al.* 1987), with evidence that this is a direct effect mediated by GRs (Simpson *et al.* 1981). It has been shown that there are regional differences in aromatase expression in confluent cultured PAs from female subjects, with Vis PAs having less aromatase activity than Sc PAs (Ackerman *et al.* 1981). This is likely to be biologically important as PAs are the main cell type in adipose tissue responsible for its aromatase activity (Ackerman *et al.* 1981). Adipose tissue is a major site for the aromatisation of androgens to oestrogens, influencing both systemic oestrogen concentrations and the paracrine effects of steroid hormones in adipose tissue (Folkerd & James 1983, Siiteri 1987, Labrie 1991).

It is not clear if regional differences in GR complement cause regional differences in PA differentiation. Glucocorticoids increase PA differentiation (Hauner *et al.* 1989) and there are regional differences in susceptibility to differentiation of human PAs *in vitro*, with Sc PAs differentiating more readily than Vis PAs, but this was true for both genders (Adams *et al.* 1997). However, these studies were performed at supraphysiological glucocorticoid concentrations, possibly obscuring any potential differences dependent on GR complement.

The gender dimorphism in GR distribution was an unexpected but robust finding, occurring both at the level of gene expression and glucocorticoid binding. Gender dimorphism is also seen in adipose tissue lipoprotein lipase levels (Rebuffé-Scrive *et al.* 1989, Fried *et al.* 1993) and leptin expression (Montague *et al.* 1997) with less leptin and lipoprotein lipase in Vis sites compared with Sc sites in females, with a smaller or absent regional difference in males. No direct relationship between the GR complement of PAs and these two products of mature adipocytes, leptin and lipoprotein lipase, can be concluded. However, it is interesting that both leptin and lipoprotein lipase are regulated by glucocorticoids (Fried *et al.* 1993, Russell *et al.* 1998) and show a pattern of expression similar to the GR expression in human PAs. There are other examples of a gender dimorphism in steroid hormone receptor complement. In gonadectomised and adrenalectomised rats, there was

no progesterone receptor expression in adipose tissue in genetic males while it was present in adipose tissue in females (Gray & Wade 1980). It is hypothesised that the *in utero* exposure to sex steroids that results in sexual determination includes this effect on receptor expression.

This is the first study to compare GR numbers in Vis and Sc human PAs. The pattern of GR density found in this study of PAs is different to the results found in whole adipose tissue, where Vis adipose tissue was shown to have a greater number of GR binding sites than Sc adipose tissue with no gender differences (Rebuffé-Scrive *et al.* 1985, 1990, Miller *et al.* 1987, Pedersen *et al.* 1994). A gender difference in regional GR complement between two Sc adipose tissue depots, Sc abdominal and gluteal adipose tissue, has been reported however (Brönnegard *et al.* 1990), and supports the general concept of a gender dimorphism in the regional GR complement of human adipose tissue.

There are a number of possible explanations for the disparity in the regional GR complement in PAs and whole adipose tissue. GR expression may vary between PAs and adipocytes as has been shown in porcine adipose tissue (Chen *et al.* 1995). There are regional differences in the adipocyte size (Rebuffé-Scrive *et al.* 1989) and the cellular composition of whole adipose tissue (Rink *et al.* 1996) that are likely to affect the measured GR density of whole adipose tissue homogenates. In general, Vis adipocytes are smaller than Sc adipocytes from the same individual (Sjöström *et al.* 1972) and a site-specific difference in GR complement in one paper was abolished when the data was expressed per adipocyte (Rebuffé-Scrive *et al.* 1985). In addition, the differing internal milieu of whole adipose tissue from different sites may affect GR expression in tissue explants.

The GR complement of PAs is critical to the response of PAs to glucocorticoids but it is only one aspect of the complex interaction of glucocorticoids with adipose tissue. *In vivo*, glucocorticoids increase Vis adipose tissue at the expense of peripheral Sc tissue (Björntorp 1996). This is likely to be due, in part, to the higher density of GRs in Vis compared with Sc whole adipose tissue, as discussed above. Differences in the local glucocorticoid concentrations are also likely to contribute, with 11 β -hydroxysteroid dehydrogenase type 1, that catalyses inactive cortisone to active cortisol, being present in human PAs with greater activity in Vis compared with Sc PAs (Bujalska *et al.* 1997, 1999).

In conclusion, human PAs contain classical GRs. Females have fewer GRs in Vis PAs compared with Sc PAs, and in comparison with males. This lower GR density in Vis PAs in women is likely to be physiologically relevant, contributing to the regional differences in aromatase activity in females and the smaller intra-abdominal fat mass relative to total body fat in females compared with males.

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