Female adipocyte androgen synthesis and the effects of insulin

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A B S T R A C T

The metabolic syndrome is a cluster of metabolic disorders characterized by insulin resistance and hyperinsulinaemia, and its presence can increase the risk of cardiovascular disease significantly. The metabolic syndrome is associated with increased circulating androgen levels in women, which may originate from the ovaries and adrenal glands. Adipocytes are also able to synthesise steroid hormones, and this output has been hypothesised to increase with elevated insulin plasma concentrations. However, the contribution of the adipocytes to the circulating androgen levels in women with metabolic syndrome is limited and the effects of insulin are not fully understood. The aim of this study was to investigate the presence of steroid precursors and synthetic enzymes in human adipocyte biopsies as markers of possible adipocyte androgen synthesis. We examined pre and mature adipocytes taken from tissue biopsies of abdominal subcutaneous adipose tissue of participating women from the Department of Obstetrics and Gynaecology, of the Royal Derby Hospital. The results showed the potential for localised adipocyte androgen synthesis through the presence of the androgen precursor progesterone, as well as the steroid-converting enzyme 17α-hydroxylase. Furthermore, we found the controlled secretion of androstenedione in vitro and that insulin treatment caused levels to increase. Continued examination of a localised source of androgen production is therefore of clinical relevance due to its influence on adipocyte metabolism, its negative impact on female steroidogenic homeostasis, and the possible aggravation this may have when associated to obesity and obesity related metabolic abnormalities such as hyperinsulinaemia.

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1. Introduction

Adipose tissue plays an important role in the regulation of levels and bioactivity of sex steroids [2,7,13]. Examination into the adipocyte steroidogenic pathway involved in estrogen conversion has shown androstenedione conversion by aromatase to estrone (E1), and that this is cytochrome P450-dependent. Changes in localised levels of these hormones may also be involved in gender-related fat deposition. Studies have detected the presence of enzymes including aromatase, 3-beta-hydroxysteroid dehydrogenase (HSD), 3-alpha-HSD, 11-beta-HSD, 17-beta-HSD, 7-alpha-hydroxylase, 5-alpha-reductase and UDP-glucuronosyltransferase 2B15, in adipose cells and that a correlation exists towards obesity, central fat accumulation, and the metabolic syndrome [8,9]. With the importance of obesity and the association this has with metabolic abnormalities such as insulin resistance [1], hyperinsulinemia and subsequent disorders of the cardiovascular system, examination into adipocyte function and their response to hormonal messengers is of clinical importance. Furthermore, associations have been made between obesity and androgen regulation with androgens being shown to decrease plasma adiponectin, which may subsequently decrease in insulin sensitivity [32].

It is well recognised that androgens are produced in sex organs [5] and the adrenal glands [37,39,42]. However, de novo synthesis of sex hormones also occurs within subcutaneous adipocytes, which have been shown to be more steroidogenically active than visceral adipocytes. However, limited studies have examined the capabilities of peripheral tissues such as skin and adipose tissue to synthesise weaker circulatory androgens. Currently, 15 steroidogenic enzymes are recognised to exist within adipose tissue including aromatase, 3β-hydroxysteroid dehydrogenase [6] type 1 [8], 11β-hydroxysteroid dehydrogenase types 1 and 2 [9], 5α-reductase [10] and 17β-HSD types 2, 3 and 5. It is also recognised that adipocytes contain components necessary for transport and metabolism of cholesterol which is essential for the initial steps of steroid synthesis. Furthermore, CYP11A1 has the capacity to produce pregnenolone, the precursor to progesterone and the foundation to androgen steroidogenesis. This has been demonstrated through the mitochondrial product 27-hydroxycholesterol (27HC) and by the action of CYP27A1 also now known to be present in adipocytes [28]. Based on these findings, the capacity for peripheral tissue to synthesise and inactivate androgens is feasible.

Examination of a localised source of androgen production may therefore be of clinical relevance due to its general influence on adipocyte metabolism, its negative impact in female steroidogenic homeostasis and the possible aggravation this may have when associated to obesity and obesity related metabolic abnormalities [11]. In the present study, we hypothesised that the precursors and enzymes involved in the aromatisation and production of steroids within adipose tissue were suggestive of localised androgen synthesis. More specifically, we attempted to support the controversial presence of CYP17 as described by Puche et al. [35] and MacKenzie et al. [29] by showing the expression of the steroid-converting enzyme 17α-hydroxylase [6,29,35], the progesterone precursor, and the controlled secretion of androstenedione in vitro. Our rationale was based on the androgen synthetic pathway found within ovarian steroidogenesis [17], whereby the presence of known precursors and enzymes necessary for thecal steroid metabolism in adipocytes may indicate the presence of androgen synthesis. Subcutaneous adipocyte samples were chosen based on reports of increased expression of steroidogenic enzymes compared to visceral adipocyte samples, which may suggest higher hormonal output.

2. Materials and methods

2.1. Tissue collection

Adipose tissue biopsies (~5 g) were taken from subcutaneous adipose tissue of the abdominal wall of participating women either during a planned surgical procedure or under local anaesthesia in the outpatient clinic [4] in the Department of Obstetrics and Gynaecology, of the Royal Derby Hospital. Inclusion criteria for the study consisted of women with regular menstrual cycles (28 day cycle), normal serum levels of androstenedione (0.2–2.9 nmol/l) and fasting insulin (17.8–173 pmol/l). All women were of childbearing age, ranging from 20 to 45 years with BMI < 35 kg/m². Exclusion criteria consisted of any metabolic or endocrine disease, such as diabetes mellitus and thyroid disease and concurrent treatment with: hormonal therapy such as hormonal contraception, progestogen therapy, thyroxin hormone or...
corticosteroids, metformin treatment or cholesterol lowering agents. The study was approved by the Derbyshire Ethics Committee with all patients providing written, informed consent prior to the surgical procedure. Biopsies were collected in Hanks Balanced 1% penicillin (100 U/ml), streptomycin (100 U/ml), HEPES (15 mM) and transported to the lab. Adipose tissue was minced then digested in DMEM containing 1 mg/ml collagenase (Sigma Aldrich, UK) for 1 h at 37 °C. The digested material was filtered through a double-layered cotton mesh and seeded into T25 flasks for culture incubation at 37 °C 5% O2/95% CO2. Tissue not intended for primary culture processing was snap frozen in nitrogen and stored at −80 °C for later use in Western blotting.

2.2. Primary human subcutaneous adipocyte culture

Isolated cells were centrifuged at 250 \times g for 10 min and the resulting pellet was washed in HBSS prior to re-suspension in high glucose (4500 mg/ml) DMEM (Sigma Aldrich) supplemented with 10% fetal bovine serum.

Briefly, minced adipose tissue was digested in DMEM containing collagenase (1 mg/ml) for 1 h at 37 °C. The digested material was filtered through a double-layered cotton mesh. The isolated cells were then centrifuged at 250 \times g for 10 min and the resulting pellet was washed with HBSS and re-suspended in DMEM including 10% FBS and the cells were seeded at a density of 5 × 10^3 cells/ml in a T25 flask. Cells were maintained in a humidified incubator at 37 °C 5% O2/95% CO2, and grown in DMEM and 10% FBS. Cultures were used at passage 2 when preadipocytes were clearly distinguishable, and cultured until 80% confluent for differentiation.

2.3. Differentiation of pre adipocyte cultures

Preadipocytes were cultured in maintenance medium that included DMEM high glucose + 10% FBS to 80–90% confluence, allowing for cell stability [43]. At day 14, media were removed and exchanged for differentiation medium containing 50 nM insulin, 100 nM dexamethasone, and 0.25 mM 3-isobutyl-1-methylxanthine for culturing cells for the first 3 days. The medium was then replaced with DMEM and 10% fetal bovine serum containing 50 nM insulin and 100 nM dexamethasone and changed every 2 days until accumulation of lipid droplets (d14–21) as described [24] was visible. Oil red O was used to determine droplet formation as stated by Chen et al. [15].

Preadipocytes were examined following differentiation within in vitro conditions and maintained in a high glucose media (Sigma DMEM). Subcutaneous adipose biopsies were used in primary culture as they were previously determined to be more steroidogenically active than visceral samples [25,36]. Subcutaneous biopsies were also used due to their role in abdominal obesity and their association to metabolic dysfunction [16,20,38].

2.4. Assay formation

Following passage of primary cultured cells at passages of 3–5, the viability of the cells was determined using 1:1 v/v trypan blue and cells suspended in media then observed using a haemocytometer. Approximately 5 × 10^3 cells were plated in 200 μl of adipocyte maintenance media in triplicate 96 well plates and exposed to treatments of either insulin (0, 1, 10, 100 ng/ml), LY292004 (PI3-K inhibitor) + insulin (0, 1, 10, 100 ng/ml), or no-treatment; and incubated for 24, 48 or 96 h. At the relevant time point, cells were harvested, removed from conditioned media, and examined for levels of progesterone and androstenedione.

2.5. Western blot

For Western blotting, cells were carefully washed with HBSS and 100 ml of trypsin/EDTA was added for 2 min before the cell suspension was removed from the well. Cells were counted using a haemocytometer and washed further in HBSS. Cells were then centrifuged at 250 \times g for 10 min and the supernatant was discarded. The cells were then lysed using lysis buffer consisting of Tris base pH 7.5 25 mM, sucrose 300 nM, monothioglycerol 10 mM, EDTA 1 mM, 1% (v/v) igepal, protease inhibitor cocktail 100 l in
100 ml, phosphate inhibitor II (100 l in 100 ml). Lysates were stored at −80 °C for use in Western blotting.

Western blotting: proteins were resolved by 10% PAGE. Following protein quantification using BCA gel electrophoresis was undertaken for examination of CYP17 (Santa Cruz), anti-leptin (antiOB, R&D systems), anti-β-Actin (Sigma). Protein concentration was adjusted to 10 mg/ml using Laemmli buffer (Sigma). The samples of interest were then denatured at 95 °C, before electrophoresis. Each lane was loaded with 100 µg of protein. In addition, the first lane was loaded with 10 µl of Kaleidoscope pre-stained molecular weight markers (Sigma) to facilitate protein size determination. Electrophoresis was run in buffer consisting of Tris (25 mM), Glycine (192 mM) and ddH2O for 1 h at 40 mA [25]. The proteins were then electro-blotted onto nitrocellulose membranes in ice cold transfer buffer (tris base (24 mM), glycine (80 mM), 20% (v/v) methanol) within a BioRad Mini-PROTEAN blotting system consisting of gel transference to nitrocellulose via a charge of 100 V [25] run for ~2 h at 4 °C to transfer.

The nitrocellulose membranes were then blocked by immersion into a bath of 5% Marvel milk and TBS (Tris (12.11 g), NaCl (146.1 g), dH2O) for 1 h. The relevant antibody was then added, and incubated overnight at 4 °C then washed with TBST (×6) for 10 min followed by TBBS washes (×3) for a further 10 min each. The blots were incubated with alkaline phosphatase (AP) conjugated secondary antibodies (BioRad) in 3% milk for 2 h at room temperature with gentle agitation. The membrane was washed again as described above and incubated for 10 min using enhancer and substrate solution (non-isotopic chemiluminescent detection system Immun-Star-Alkaline phosphotase kit (AP:Biorad)). Visualisation was enabled, by using 50 µl of enhancer added to 2.5 ml substrate evenly coating the nitrocellulose blot. Blots were stripped using 15 g glycine, 1 g SDS and 10 ml Tween 20 pH 2.2. The nitrocellulose was then re-probed using Beta-Actin antibody control.

Results were analysed by densitometry and were normalised against the reference protein (β-Actin control) using the Chemi Doc (vers 4.2.1) imaging system [41]. All Western blotting experiments were repeated at least three times for each patient sample.

2.6. Immunofluorescence

Pre-adipocyte cells were seeded at 5 × 10^3 in 24 well plates and grown to required confluence (1–2 days) before careful removal of media and washing in ice cold PBS (×2). The Chinese hamster ovarian (CHO) cell line was cultured for use as a positive control for CYP17. Leucocyte cultures were purified from blood and used as a negative control against all our primary cells.

Chilled acetone:methanol (1:1) (v/v) was added to each well and left for 10 min before careful removal and further washes in ice cold PBS (×2). Fixed cells were blocked using 20% goat serum in PBS and left for 30 min at RT. Following incubation, blocking solution was removed and the antibody of interest was diluted in PBS as required and added to the wells. Cells were incubated at 4 °C. All procedures were done alongside negative and positive control cells utilising the same protocol. Further negative controls were prepared using fixed cells omitting the primary antibody and stored in PBS overnight at 4 °C. Primary antibodies CYP17 (Santa Cruz), anti-leptin (antiOB, R&D systems), and anti-β-Actin (Sigma) were carefully removed and all cells washed for 5 min in ice cold PBS (×4). A single isomer fluorescein isothiocyanate [26] tag was used bound to the chosen secondary antibody. Following PBS washes, secondary antibody with FITC tag (Sigma SAB3700246/3700265) was added to all cells and incubated at room temperature for 1 h, wrapped in foil to prevent direct light bleaching or fluorophore excitation. Cells then washed in ice cold PBS for 5 min (×7). Cells in PBS were then viewed in a dark room using Carl Zeiss Axiovert 25 Scope with Cell^F imaging software (Build 1131). Images were viewed and edited in a Ziess LSM image browser V4.2.0.121.

2.7. ELISA

Adipocytes were cultured to maturity (see primary and differentiation) and assayed for 3 days before media harvests were examined for progesterone and androstenedione following manufacturers instructions and against calibration curve with ranges (Ridgeway Science, Genway Science). Theca and osteoclast controls were used throughout to support findings, as was the same ELISA kit. Levels of progesterone and androstenedione were determined by ELISA.
Fig. 1. 1A/B–5A/B matched bright fields columns from left to right. 1A/B) Immunofluorescence of leucocytes using CYP17 antibody. Human leucocytes were probed with CYP17 primary antibody acting as a negative control. Results show no CYP17 fluorescence was present. 2A/B) Immunofluorescence of Chinese hamster ovary cells (CHO). CHO cells were used as a positive control allowing the expression of CYP17 to be seen within the cytosol. 3A/B) Fluorescence shows preadipocytes probed with β-actin antibody acted as positive control of IF protocol. 4A/B) Immunofluorescence of human preadipocytes using CYP17 antibody. Fluorescence can be seen within the cytosol. 5A/B) Immunofluorescence of mature human adipocytes using CYP17 antibody. Fluorescence can also be seen within the cytosol.
The progesterone ELISA (Ridgeway) allowed for measurement of samples to a sensitivity of 0.025 ng/ml–0.39 pmol/1000 cells using a standard range of known progesterone samples as supplied. The androstenedione ELISA (GenWay Science) allowed for a sensitivity of 0.019 ng/ml–0.03 pmol/1000 cells and absorbance was measured (570 nm) to create a standard curve. All results were obtained following manufacturers instruction.

2.8. Data and statistical analysis

Normality was tested throughout using Shapiro–Wilk/Kruskal–Wallis normality test using the prism software (vers. 5.0b). Multiple means for unpaired samples were compared using a two way analysis of variance (ANOVA) (non-parametric) with Bonferroni post-hoc test while paired observations were analysed using a two tailed Student’s t-test (parametric). P-values < 0.05 allowed for rejection of the null hypothesis.

3. Results

Negative controls (Fig. 1A) showed no fluorescence in contrast to positive immunofluorescence detected in CHO cells for CYP17 (Fig. 1B). β-actin probing of preadipocytes was used to show fluorescence supporting the protocol used (Fig. 1C). Examination of the presence of CYP17 within pre and mature adipocytes of our human cultures showed that CYP17 was predominantly expressed within the cytoplasm with abundant perinuclear localisation (Figs. 1D/E.). Furthermore westernblot analysis of CYP17 in both pre and mature adipocytes showed significant increased expression in preadipocytes samples (see Fig. 2).

Progesterone was measured from mature adipocyte media following 3 days maintenance (mean 5.5 ± 1.29 pmol/1000 cells). A significant increase was seen in adipocyte media against osteoclast samples (P = 0.018). Ovarian theca cell media was also examined as a positive marker of progesterone secretion as well as an intra assay control throughout all the ELISA techniques performed. Again 3 day untreated media was removed from theca cultures and results were normalised to pmol/1000 cells. Results showed progesterone secretion to be ≈ 3 times that found within the adipocyte media (mean 16.37 ± 0.44 pmol/1000).

Fig. 3 shows ELISA measurements of steroid hormone androstenedione. Examination against positive (theca cells) and negative controls (osteoclasts) showed significant levels of secretion against both 0 treatment and negative control (2.2 pmol/1000 cells).

Androstenedione secretory levels were examined under insulin treatment (0–10 and 10–100 ng/ml) (n = 9) and showed no variation across insulin treatment range (see Fig. 3). CYP17 expression was also examined under the same insulin treatments. CYP17 was seen to increase at around 10 ng/ml treatments with no variation in expression with further increases to 100 ng/ml.

![Fig. 2.](image-url) A) Shows Western blot CYP17 expression of human mature adipocyte (lanes 1–3) and preadipocyte (lanes 4–6) lysates. Exposure at 640 s shows bands at 57 kDa, which were more predominant in preadipocyte samples. The blot was then stripped and re-probed using β-actin to allow for loading control. B) Summarizing the densitometry representative of average expression of CYP17 in pre and mature human samples. Using β-actin normalisation to compare levels of band intensity in Western blot CYP17 expression (see panel A) densitometry graphs were created. A significant increase was seen in expression of CYP17 in pre adipocyte lysates (n = 3,*P < 0.05).
With the possible influence insulin may therefore play in androgen synthesis we examined a possible signalling pathway through inhibition of PI3-K, shown to be involved in ovarian steroidogenesis [31].

**Fig. 4.** Comparison of mature adipocyte androstenedione secretion with and without inhibition of the insulin-signalling pathway. Mature adipocytes (n = 12) were treated with insulin with and without the PI3-K inhibitor LY294004 and androstenedione secretion measured. No significant variation was found between treated and untreated cultures.

4. Discussion

Synthesis and secretion from adipocytes may contribute to the elevated circulating levels of androgens in women with obesity and metabolic syndrome. Androgen secretion from adipocytes may become elevated through the action of insulin in the hyperinsulinaemic state. We have examined the ability of human primary pre and mature adipocytes to independently produce and secrete androstenedione and the effects insulin has on this synthesis. In doing so we may determine a significant contribution to
circulatory androgen levels directly associated to obesity and therefore related to conditions such as insulin resistance, hyperinsulinemia and subsequent disorders of the cardiovascular system, and metabolic syndrome.

Immunofluorescence and comparative phase contrast, showed clear cell morphology and fluorescence representative of CYP17 expression in the cytoplasm of our primary human pre and mature SC adipocyte cultures. Blouin et al. [11] compared human omental and subcutaneous samples for steroidogenic activity at both preadipocyte and mature adipocyte stages [11,12]. They found increased activity in SC cultures with a greater expression of the steroidogenic enzymes AKR1C3, RDH5, AKR1C2, P-450 aromatase, steroid sulfatase, ERα and 17β-HSD-3, following maturation. We however, found perinucleolar staining for CYP17 within our preadipocyte cultures, which pointed to active protein synthesis at this stage of adipocyte development. This coupled with increased expression against our mature adipocyte cultures shown by our Western blot analysis suggests an increase of this particular steroidogenic enzyme during the preadipocyte phase. Interestingly, Blouin also reports CYP19 increases following differentiation contradictory to reports by Mceternan et al., [44] and Diuedonne et al. [45] who both report CYP19 mRNA reduction in mature or differentiated SC cultures [19,30]. A decrease in CYP19 expression following differentiation may occur in order to reduce oestrogen synthesis. With adipocytes unable to proliferate at this phase of development, a reduction in both androgen and oestrogen synthesis at maturity may therefore be associated and also explain the down regulation of CYP17.

By showing the existence of the steroidogenic enzyme CYP17 and its cellular localisation within both pre and mature adipocytes evidence exists towards localised adipocyte androgen synthesis. Currently adipocytes are known to take part in steroidogenic activity through oestrogen and androgen modification [12,22,40]. This requires circulatory precursors and the existence of an androgen synthetic pathway although theorised, has not been reported [12]. The presence of androgen receptors (AR) as determined by Pedersen et al. [33], in both pre and mature adipocytes may also support the presence of an androgen pathway [3,14,21,33]. Furthermore a feedback mechanism may be occurring with reports of decreases in adipocyte proliferation and adipogenesis when exposed to androstenedione [14]. It has also been shown that ARs are more abundant within preadipocytes more so in human and animal model comparison [18], which supports a greater involvement of androgen at the preadipocyte stage of development. This is suggested by our finding of significantly greater levels of CYP17 expression within human preadipocytes. By showing the existence of CYP17 both pre/post differentiation, we support the important regulatory role androgen may have and therefore require localised synthesis.

Progestosterone plays a vital role as a precursor within ovarian androstenedione synthesis. By determining its presence in adipocyte secretion we have shown the requisites necessary for this steroidogenic pathway. It is understood that progesterone is synthesised by the ovary, testis, adrenal glands and the placenta during pregnancy. Although it is yet to be established, it is also believed that neurons also have the ability to locally produce progesterone. If this were viable it may occur through P450scc/desmolase action on the cholesterol precursor and further converted to pregnenolone for conversion by dehydrogenase [23,34]. With adipocytes shown to have the necessary proteins and related precursors necessary to also locally biosynthesise progesterone the possibility remains feasible and supports our findings of progesterone presence in isolated adipocyte cultures. Although, progesterone can also be stored at any stage of preadipocyte development this was accounted for through passage of our cultures.

With the discovery of the progesterone receptor by Sherman et al. [46] more evidence pointed to progesterone’s involvement in adipocyte metabolism. Secretion from localised production may allow self-regulation and research has shown that progesterone may be involved in the regulation of lipogenesis through stimulation of transcription factors such as ADD1/SREBP1c [27].

Our continued examination of steroid secretion showed androstenedione present within our media harvest. We ran the same protocol under varied insulin treatments to determine if any influence on levels of secretion existed. In doing so we hoped to examine one of the key influences to stimulating maturation and effects at the more biologically activity stage of development. The addition of our insulin treatment range showed an increase in androstenedione secretion at 10–100 ng/ml significant against the negative osteoclast control and with no variation between these treatments. From this we suggest that insulin influences androstenedione metabolism in mature adipocytes at this treatment level causing an increase in secretion over 3 days. We took these results further and examined a possible pathway in which insulin
may affect androstenedione synthesis similar to that seen in ovarian androgen synthesis. The protocol used was similar to that used by Munir et al. [34] and used a PI3-K inhibitor (LY294002) in combination with insulin treatment levels previously shown to increase androstenedione levels [31]. This allowed us to examine the effects insulin had on levels of secretion. The results showed that no variation occurred between inhibited and un-treated samples. This would suggest that if insulin were to be stimulating androstenedione secretion it is not working through the PI3-K pathway. Our further examination into the progesterone precursor required for localised androstenedione synthesis, also showed supportive evidence of a hormonal influence and the pathway involved in androgen synthesis. Although no significant difference in progesterone level was found under hormonal treatments, the results (not shown) showed a possible trend towards decreased progesterone synthesis at 10 ng/ml insulin. This is the same level required to cause androstenedione levels to increase. If a pathway utilising progesterone existed in the conversion to androgen then a possible reduction may be seen at this point due to demand.

If we were to determine that progesterone was active in an adipocyte androgen synthesis pathway and that adipocyte cells are also producing increased levels of androgen at the preadipocyte phase then the increased levels associated to insulin fluctuations required for maturation would support the increase in androgen secretion. From a clinical perspective this may be linked with conditions found in metabolic syndrome and diabetes with symptomatic hyperinsulinaemia acting to increase androstenedione, which is increased levels associated to insulin that adipocyte cells are also producing increased levels of androgen at the preadipocyte phase then the possible reduction may be seen at this point due to demand.

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