

Chronic testosterone treatment induces selective insulin resistance in subcutaneous adipocytes of women

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Abstract

Adipose tissue plays a central role in determining whole body insulin sensitivity. Several aspects of adipose cell function are regulated by androgens. Given that high androgen levels and insulin resistance are linked in women, we proposed that androgens may influence insulin-mediated glucose metabolism in adipose cells. Preadipocytes harvested from s.c. adipose tissue of healthy women aged 37 ± 5 years were differentiated *in vitro*, then treated with testosterone (T) and/or androgen receptor (AR) antagonists (cyproterone acetate, flutamide) for 48 h. Maximal insulin-stimulated glucose uptake (insulin 10 nM) and increment following insulin stimulation were significantly impaired in cells treated with T 10 and 100 nmol/l. This defect was abolished by cyproterone acetate and partially reversed by flutamide. The effect of T could not be accounted for by altered differentiation status of the adipocytes. In the glucose

metabolic pathway of insulin signaling, treatment of cells with T 10 nmol/l did not alter insulin-stimulated phosphorylation of insulin receptor substrate-1 or Akt, but insulin-stimulated phosphorylation of protein kinase C (PKC) ζ was impaired. Insulin signaling via the mitogenic/gene regulatory pathway, as assessed by extracellular signal-regulated kinase phosphorylation, was unchanged. We conclude that (1) T, or an androgenic metabolite of T, induces insulin resistance in adipocytes of women, selective for metabolic signaling pathways; (2) this defect is via AR; and (3) the defect in signaling is independent of phosphatidylinositol 3-kinase activation and involves impaired phosphorylation of PKC ζ . These findings are relevant to understanding the pathogenesis of insulin resistance in hyperandrogenic women.

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Introduction

Adipose tissue is now recognized to play a central role in determining whole body insulin sensitivity (Minokoshi *et al.* 2003). In animal models, insulin resistance in adipose tissue secondarily results in insulin resistance in skeletal muscle (Abel *et al.* 2001, Yang *et al.* 2005). In the insulin-resistant states of obesity (Stolic *et al.* 2002), type 2 diabetes mellitus (DM; Smith *et al.* 1999), and polycystic ovary syndrome (PCOS; Ciaraldi *et al.* 1992, Dunaif *et al.* 1992, Marsden *et al.* 1994), insulin-stimulated glucose uptake in isolated adipocytes or adipose tissue explants is impaired due to post-insulin receptor defects in signal transduction. The precise molecular mechanisms of these signaling defects are unknown, but it is likely that both genetic and *in vivo* environmental factors are involved.

Androgens regulate several aspects of adipose cell function including lipid metabolism and differentiation. Androgens bind to the androgen receptor (AR), which mediates most of its physiological functions through transcriptional activation of downstream genes. Functional ARs are present in human adipose tissue, both in stromal cells and mature adipocytes (Pedersen *et al.* 1996) and in preadipocytes which have been

differentiated *in vitro* (Dieudonné *et al.* 1998). Androgens have been shown to regulate lipolysis and lipogenesis in the adipose cells of women. In s.c. preadipocytes which have been differentiated *in vitro*, testosterone (T) inhibited catecholamine-stimulated lipolysis associated with decreased expression of hormone-sensitive lipase (HSL) and β 2-adrenoceptors (Dicker *et al.* 2004). The more biologically potent metabolite of T, dihydrotestosterone (DHT), also modulated expression of HSL and lipoprotein lipase (LPL) in isolated s.c. adipocytes of women (Anderson *et al.* 2002). In animal models, prenatal exposure of females to androgens resulted in increased adiposity (Eisner *et al.* 2003) and alterations in adipose tissue lipolysis (Reiling *et al.* 1997) in adulthood, raising the possibility that androgens have a programming effect on adipose tissue. Androgens do not appear to influence preadipocyte proliferation, at least in the rat (Dieudonné *et al.* 2000), but in both rat adipose cells (Dieudonné *et al.* 2000) and 3T3-L1 adipocytes (Singh *et al.* 2006), androgens inhibited adipogenic differentiation. However, whether androgens play a role in the regulation of a key adipose cell metabolic function, insulin-mediated glucose uptake, has not been investigated.

Insulin-mediated glucose transport is dependent on translocation of the glucose transporter GLUT4 to the cell membrane. In adipocytes, stimulation of glucose uptake via GLUT4 involves at least two pathways: (1) insulin receptor substrate (IRS)-1-dependent activation of phosphatidylinositol (PI) 3-kinase and downstream targets Akt/protein kinase B (PKB) and atypical protein kinase C (aPKC) isoforms ζ/λ and (2) the less understood non-IRS-PI3-kinase-dependent activation of the Cbl/CAP pathway (reviewed by Ishike & Klip 2005). The major aPKC in human *in vitro*-differentiated adipocytes that mediates the effects of insulin on glucose transport is PKC ζ (Bandyopadhyay *et al.* 2002). Insulin action on glucose uptake is initiated by activation of the insulin receptor tyrosine kinase that results in phosphorylation of IRS-1 on tyrosine residues. These phosphotyrosine residues act as docking sites for SH2 domain-containing proteins, including PI3-kinase (White & Yenush 1998). The phosphorylated IRS-1 tyrosine 612 is a key site for PI3-kinase activation (Esposito *et al.* 2001). Insulin also has mitogenic/gene regulatory actions, mediated by activation of the mitogen-activated protein kinase (MAPK) signaling cascade via Ras, Raf, and extracellular signal-regulated kinase (ERK) activation (Cobb 1999).

The objective of this study was to examine the effect of androgens on insulin-mediated glucose transport and insulin signaling in adipose cells of women. We postulated that androgens could be an *in vivo* environmental factor that contributes to insulin resistance in the adipose cells of women, with implications for women with androgen excess, particularly polycystic ovary syndrome (PCOS), a common cause of insulin resistance associated with hyperandrogenemia (Dunaif 1997).

Materials and Methods

Cell culture

Subcutaneous adipose tissue was obtained from healthy women aged less than 50 years (age 37 ± 5 years, mean \pm s.d.) undergoing elective abdominoplasty. The study was approved by the institutional Human Research and Ethics Committee and all patients gave written informed consent. Stromal cells were isolated from fresh tissue by collagenase digestion (Rodbell 1964) and primary cultures were seeded at a density of $\sim 2 \times 10^5$ cells/ml in DMEM (Gibco BRL Life Technologies) containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) plus penicillin and streptomycin (Gibco). At confluence, the cultures were washed in PBS and placed in adipogenic medium Dulbecco's modified Eagle's medium (DMEM) without phenol red, 3% charcoal-stripped FBS (HyClone, Logan, UT, USA), 1 μ mol/l troglitazone (gift of Parke-Davis, Morris Plains, NJ, USA), 25 nmol/l regular insulin (Novo Nordisk, Baulkham Hills, NSW, Australia), 1 μ mol/l dexamethasone (Sigma-Aldrich Corp., St Louis, MO, USA), 200 pmol/l triiodothyronine (Sigma), and 0.5 mmol/l

Isobutyl-1-methylxanthine (IBMX; Sigma). The medium was replaced after 4 days, omitting troglitazone and IBMX, and again after a further 4 days, omitting dexamethasone. After a total of 12 days in adipogenic medium, at which time 70–90% of the cells contained multiple lipid droplets, the cultures were washed with PBS and incubated in DMEM (low glucose, without phenol red) with 0.1% BSA (Cohn modified; ICN, Costa Mesa, CA, USA) for 48 h \pm vehicle (maximum 0.01% ethanol), T (10, 100 nmol/l), and/or AR antagonists (flutamide (1 μ mol/l) or cyproterone acetate (CPA; 1 μ mol/l; Sigma)). The same lots of FBS and BSA were used for all experiments.

Reverse transcription PCR

Total RNA was prepared from cultured adipocytes and AR-positive (LnCaP) and AR-negative (HepG2, L6, PNT1a) cell lines using an RNA Kit (Qiagen). First strand cDNA synthesis was performed using avian myeloblastosis virus reverse transcriptase primed by random hexamers (Promega). PCRs were carried out using the following primer sets (1 μ M final concentration; 5' \rightarrow 3'): AR forward TGTCGTCTTCGAAATGTTATG, reverse GTCGGGC TGGTTGTTGTCGTG and cyclophilin forward CTT GGGCCGCGTCTCCTTC, reverse TGCCGCCAGT GCCATTAT. Thermal cycling conditions were 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 14 s, and on the final cycle, 72 °C for 10 min.

Glucose transport assay

Glucose uptake in three replicate wells for each condition was measured in adipocytes treated as above \pm insulin 0.1, 1, 10 nmol/l for 90 min (Ciaraldi *et al.* 1995). Specific glucose transport over 10 min was calculated in each well by measuring [3 H]-deoxy-D-glucose (Perkin-Elmer, Boston, MA, USA) incorporation and subtracting L-[14 C]-glucose (Perkin-Elmer) incorporation to correct for non-GLUT-mediated glucose uptake. Protein content was measured in each well (bicinchonic acid (BCA) assay; Pierce Biotechnology, Rockford, IL, USA) and glucose uptake expressed as pmol/min per mg protein.

Immunoblotting

Cultured adipocytes were incubated in serum-free medium plus treatments for 48 h, followed by incubation in the same medium \pm insulin (10 nmol/l, 10 min), then scraped on ice in lysis buffer (25 mmol/l Tris-HCl (pH 7.4), 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l Na₃VO₄, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamide, and 0.1 mmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride). Lysates were solubilized by rocking (40 min, 4 °C), then centrifuged for 15 min at 13 000 g, and supernatants stored at -80 °C. Protein content was determined by BCA protein assay. Lysates (25–50 μ g protein) were resolved by SDS-PAGE and immunoblotted with specific antibodies to

insulin receptor β -subunit (IR β ; Transduction Laboratories, San Diego, CA, USA), GLUT1/4 (Chemicon, Temecula, CA, USA, USA), acetyl-CoA carboxylase (ACC; Cell Signaling Technology, Beverly, MA, USA), IRS-1 (Upstate Biotechnology, Lake Placid, NY, USA), phospho-IRS-1 Tyr⁶¹² (Biosource, Camarillo, CA, USA), Akt and phospho-Akt Ser⁴⁷³ (Cell Signaling), PKC ζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-PKC ζ / λ Thr^{410/403} (Cell Signaling), ERK1/2 and phospho-ERK1/2 Thr²⁰²/Tyr²⁰⁴ (Cell Signaling), and appropriate fluorophore-conjugated secondary antibodies (anti-rabbit (Molecular Probes, Eugene, OR, USA) or anti-mouse (Rockland Immunochemicals, Gilbertsville, PA, USA)). Immunoblots were visualized using infrared imaging (Odyssey; Li-Cor, Lincoln, NE, USA) and quantitated using Odyssey software version 1.2. An internal standard was loaded on all immunoblots and results expressed as percentage of standard.

IRS-1-associated PI3-kinase activity assay

Lysates were subjected to immunoprecipitation with IRS-1 antibodies at 4 °C for 4 h, coupled to protein A sepharose beads, and PI3-kinase activity measured as described (Goodyear *et al.* 1995) with resolution of product using thin layer chromatography and quantitation using a phosphorimager.

Quantification of lipid accumulation in differentiated cells

The percentage of cells accumulating multiple lipid droplets during differentiation was monitored by phase contrast microscopy. Additionally, Oil Red O retention in the cells was measured adapting the method of Singh *et al.* (2006): duplicate wells of differentiated cells which had been incubated in serum-free media with or without T for 48 h were fixed in 2% formaldehyde for 15 min, stained with Oil Red O (0.5%) for 1 h, then extracted with 4% Ipegal in isopropanol for 15 min and absorbance measured by spectrophotometry at 490 nm.

Statistical analysis

Treatments were compared using repeated measures ANOVA with post hoc Dunnett's multiple comparison test. Within-group comparisons were made using two-tailed *t*-tests or Wilcoxon signed rank test when the size of the sample group was ≤ 6 . Data were presented as mean \pm S.E.M. and differences were considered to be significant at $P < 0.05$.

Results

AR expression in cultured adipocytes of women

Consistent with published reports (Pedersen *et al.* 1996, Dieudonné *et al.* 1998), expression of AR was confirmed in cultured adipocytes using RT-PCR (Fig. 1).

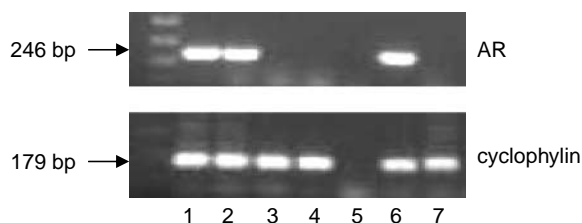


Figure 1 Expression of androgen receptor (AR) in cultured adipocytes of women and various cell lines using RT-PCR. A representative gel (1% agarose, stained with ethidium bromide) is shown. Lane 1, adipocytes patient 1; lane 2, adipocytes patient 2; lane 3, HepG2 cells; lane 4, L6 cells; lane 5, no DNA control; lane 6, LnCaP cells; lane 7, PNT1a cells.

Effect of androgens and androgen receptor antagonists on glucose transport

Maximal insulin-stimulated glucose uptake was significantly reduced by T treatment: T 10 nmol/l reduced maximal glucose transport by 23% and T 100 nmol/l by 30% when compared with vehicle ($n=7$ /treatment group, $P < 0.01$; Fig. 2a). Basal glucose transport was reduced by $\sim 15\%$ in cells treated with T 100 nmol/l when compared with vehicle ($P < 0.05$), but was not altered in cells treated with T 10 nmol/l (Fig. 2a). The increment of glucose uptake after maximal insulin stimulation (i.e. glucose uptake at baseline subtracted from glucose uptake at 10 nmol/l insulin) was reduced by 50–60% in the T-treated cells ($P < 0.01$, T 10 nmol/l versus vehicle; $P < 0.05$, T 100 nmol/l versus vehicle), consistent with the induction of insulin resistance (Fig. 2b).

Concurrent treatment with CPA abolished the effect of T 100 nmol/l on insulin-stimulated glucose uptake (Fig. 2d) and on increment of glucose uptake to maximal insulin stimulation (Fig. 2e; $n=6$ /treatment group). The effects of CPA were similar in the cells treated with T 10 nmol/l, although the changes did not reach statistical significance (Fig. 2c and e). The effect of concurrent treatment with flutamide on glucose uptake in adipocytes treated with T 10 or 100 nmol/l was also assessed ($n=7$ /treatment group). Flutamide alone resulted in lower insulin-stimulated glucose uptake than in vehicle-treated cells, suggestive of a partial agonist effect, although this change did not reach statistical significance (Fig. 2f–h). In adipocytes treated with T 10 nmol/l, flutamide partially corrected the defect in insulin-stimulated glucose uptake (Fig. 2f; P =not significant (NS)). A similar non-significant effect of flutamide was seen in cells treated with T 100 nmol/l, although this effect was attenuated at the maximal insulin concentration (10 nmol/l; Fig. 2g).

Effects of testosterone on adipocyte differentiation

In order to determine whether the impairment of glucose uptake in cultured adipocytes treated with T could be attributed to de-differentiation of the adipocytes, the abundance of differentiation-dependent proteins as well as lipid content of the cells was measured. Incubation of cultured preadipocytes in adipogenic medium for 12 days resulted in a

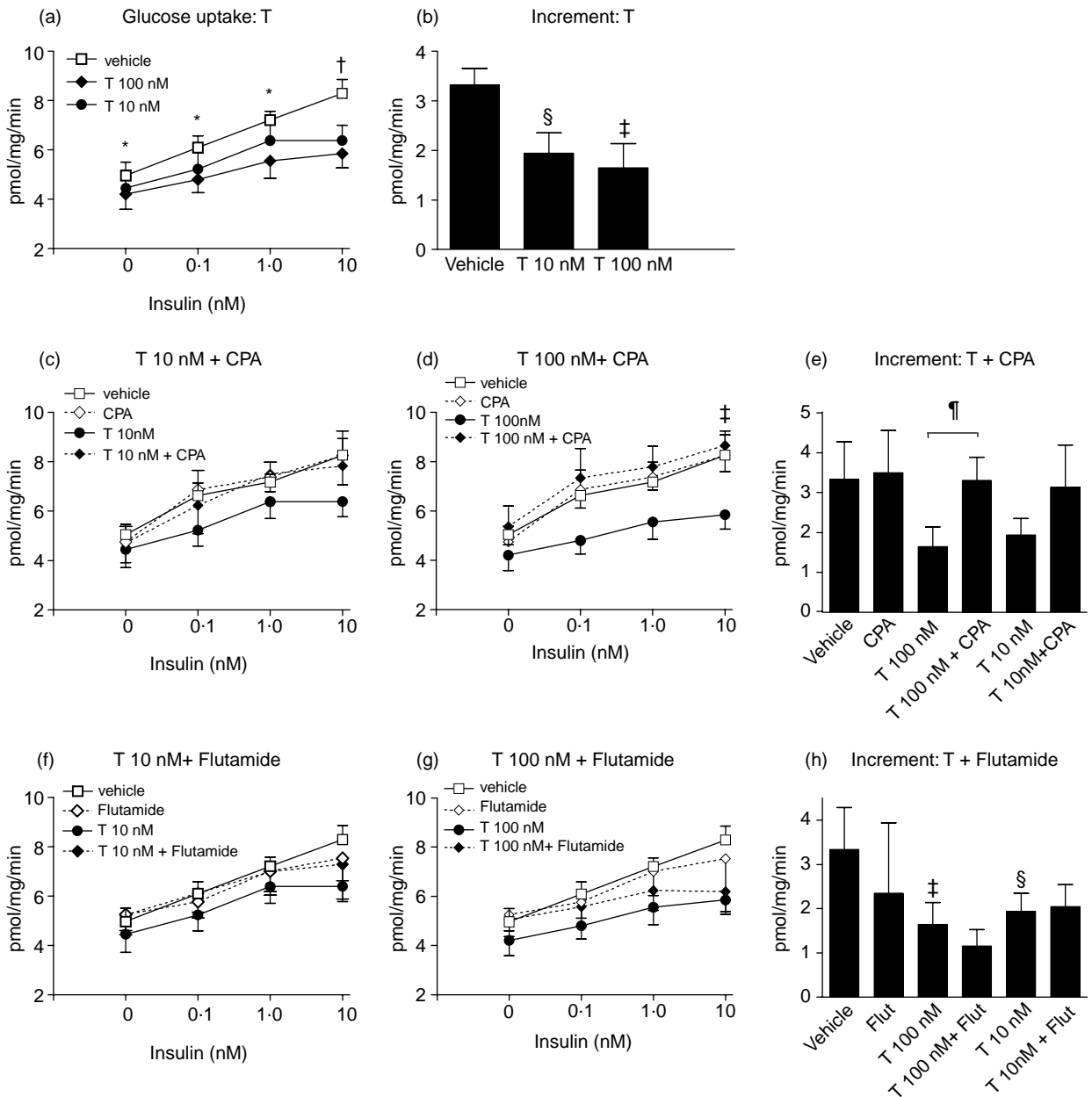


Figure 2 Effect of testosterone (T) and androgen receptor antagonists (cyproterone acetate, CPA; flutamide, Flut) on insulin-stimulated glucose uptake in cultured adipocytes of women. Subcutaneous abdominal preadipocytes were incubated in adipogenic medium for 12 days, followed by treatment for 48 h with or without T and CPA or flutamide. Glucose uptake (insulin 0–10 nmol/l) was measured in cells treated with testosterone (T; 10 nmol/l, 100 nmol/l; $n=7$ /treatment group) (a). The increment (glucose uptake at baseline subtracted from glucose uptake at 10 nmol/l insulin) of the response to insulin was calculated (b). The effect of CPA on glucose uptake was measured in adipocytes ($n=6$ /treatment group) treated with or without CPA (1 μ mol/l) and T 10 nmol/l (c and e) or T 100 nmol/l (d and e) for 48 h. The effect of flutamide (Flut) on glucose uptake was measured in adipocytes ($n=7$ /treatment group) treated with or without flutamide (1 μ mol/l) and T 10 nmol/l (f and h) or T 100 nmol/l (g and h) for 48 h. Data are expressed as means \pm s.e.m. * $P<0.05$, T 100 nmol/l versus vehicle; $^{\dagger}P<0.01$, T 10 nmol/l and T 100 nmol/l versus vehicle. $^{\ddagger}P<0.05$, T versus vehicle; $^{\S}P<0.01$, T versus vehicle. $^{\parallel}P<0.05$, T 100 nmol/l versus T 100 nmol/l + CPA.

marked increase in abundance of IR β , GLUT4, and ACC (Fig. 3a). The abundance of these proteins was not altered when cultured adipocytes ($n=5$ /treatment group) were incubated with T 10 or 100 nmol/l for 48 h (Fig. 3b).

GLUT1 abundance was also unchanged in T-treated adipocytes (Fig. 3c). Similarly, the lipid content of the cells was not affected by incubation with T for 48 h ($n=5$ /treatment group; Fig. 3d).

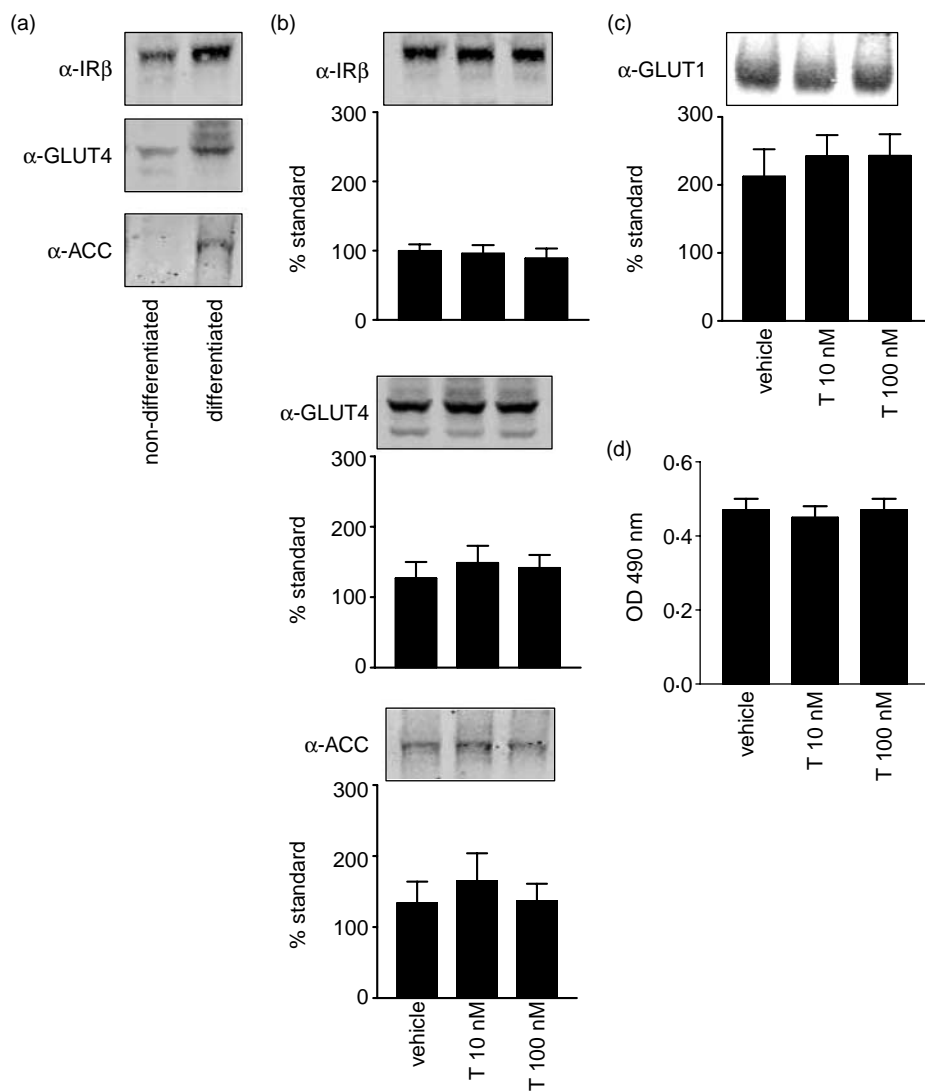


Figure 3 Effect of T on markers of adipocyte differentiation. IR β , GLUT4, and ACC are differentiation-dependent proteins in adipocytes: representative western blots of these proteins in undifferentiated cells versus cells incubated in adipogenic media for 12 days are shown (a). Abundance of IR β , GLUT4, and ACC ($n=5$ /treatment group) (b) and GLUT1 ($n=4$ /treatment group) (c) by western blotting in *in vitro*-differentiated adipocytes treated with T (10 or 100 nmol/l) for 48 h. Data are expressed as means \pm S.E.M. (% of internal standard). Quantitation of lipid content of cultured adipocytes treated with T for 48 h by elution of Oil Red O and measurement of OD 490 nm ($n=5$ /treatment group) (d).

Insulin signaling via IRS-1 and PI3-kinase

Insulin signaling via IRS-1 was assessed only in cultured adipocytes treated with T 10 nmol/l, as the physiological relevance of higher T concentrations is less clear. In cultured adipocytes treated with T 10 nmol/l for 48 h ($n=7$), the abundance of phospho-IRS-1 Tyr⁶¹² following insulin stimulation was unchanged (Fig. 4a). Total IRS-1 abundance did not differ (Fig. 4b) and there was no significant difference in phospho-IRS-1 Tyr⁶¹² when adjusted for total IRS-1

(Fig. 4c). Likewise, IRS-1-associated PI3-kinase activity did not differ in the T-treated cells ($n=9$; Fig. 4d).

Insulin signaling downstream of PI3-kinase

Insulin-stimulated phosphorylation of downstream targets of PI3-kinase, Akt/PKB, and atypical PKC isoform PKC ζ were examined in adipocytes treated with T 10 nmol/l. The abundance of phosphorylated Akt following insulin stimulation and total Akt did not differ in the T-treated cells when

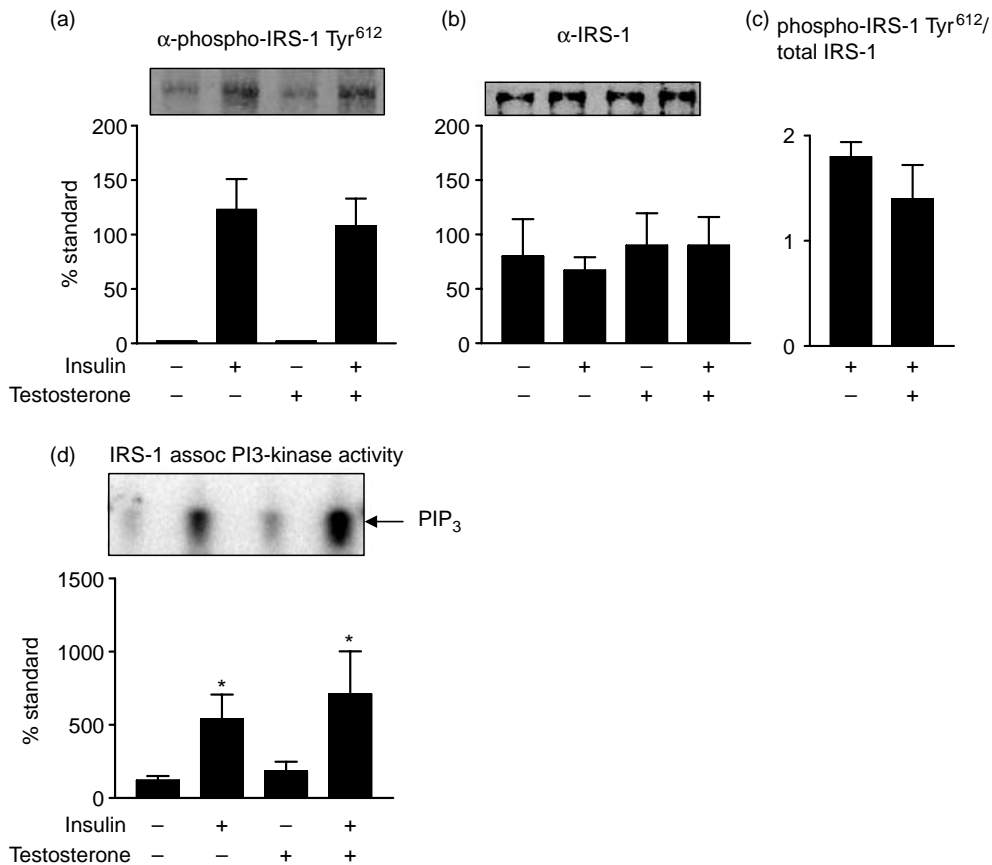


Figure 4 Insulin signaling via IRS-1 in cultured adipocytes treated with T (10 nmol/l) for 48 h. Lysates of cells at baseline or after insulin stimulation (10 nmol/l, 10 min) were resolved by SDS-PAGE and immunoblotted with α -phospho-IRS-1 Tyr⁶¹² or α -IRS-1 ($n=7$) (a–c). PI3-kinase activity was measured in IRS-1 immunoprecipitates, with PIP₃ product resolved by TLC and quantitated using a phosphorimager ($n=9$) (d). Data are expressed as means \pm S.E.M. (% of internal standard). * $P < 0.01$, insulin-stimulated versus baseline under the same conditions.

compared with vehicle ($n=9$; Fig. 5a and b), and when expressed as phospho-Akt adjusted for total Akt, there was a non-significant increase in the T-treated cells (Fig. 5c). Insulin significantly stimulated phosphorylation of PKC ζ in control cells ($P < 0.05$), but not in T-treated cells ($n=7$; Fig. 5d). Total PKC ζ abundance did not differ (Fig. 5e). Phospho-PKC ζ adjusted for total PKC ζ also showed significant stimulation with insulin in vehicle-treated cells only (Fig. 5f). Although the antibodies used to detect PKC ζ also recognize PKC isoforms, λ and τ , cultured human adipocytes do not contain detectable amounts of PKC λ/τ (Bandyopadhyay *et al.* 2002).

Insulin signaling via mitogenic pathway

Insulin regulation of ERK phosphorylation was assessed in order to determine whether the defect in insulin-mediated glucose uptake in adipocytes treated with T was specific for insulin metabolic signaling pathways. Basal and insulin-stimulated phospho-ERK, total ERK, and phospho-ERK

adjusted for total ERK did not differ in the adipocytes treated with T 10 nmol/l when compared with vehicle ($n=12$), consistent with intact insulin mitogenic/gene regulatory signaling in the T-treated cells (Fig. 5g–i).

Discussion

The present study has demonstrated that T induces selective insulin resistance in cultured s.c. adipocytes of women. Chronic T treatment significantly impaired insulin action on glucose metabolism (as assessed by glucose uptake), but did not alter insulin action on the mitogenic/gene regulatory pathway (as assessed by ERK phosphorylation). The T-induced defect in glucose transport was associated with impaired insulin-stimulated phosphorylation of PKC ζ , an atypical PKC isoform downstream of PI3-kinase, which mediates the effects of insulin on glucose transport in human *in vitro*-differentiated adipocytes (Bandyopadhyay *et al.* 2002). The activation of another downstream target of PI3-kinase,

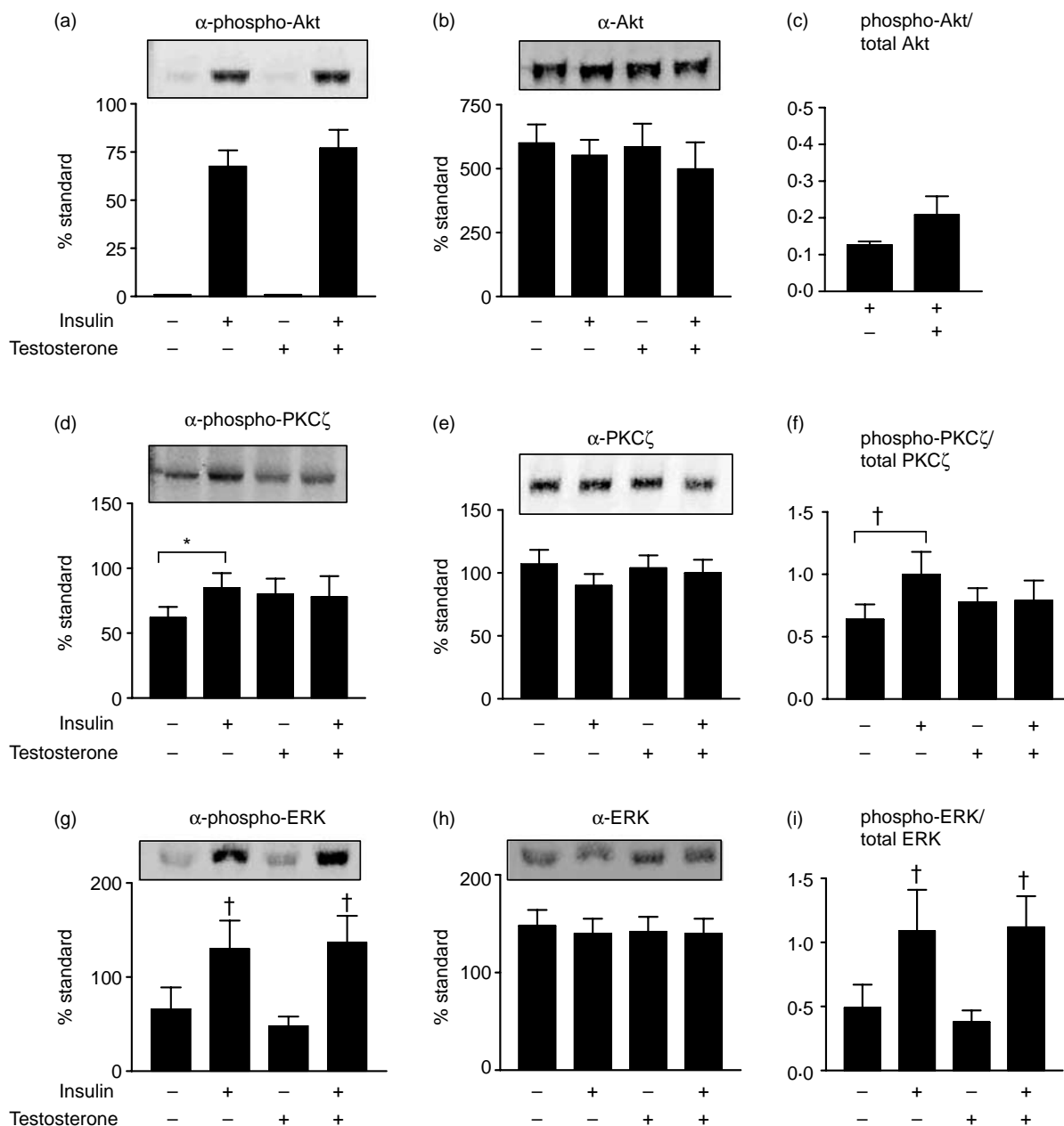


Figure 5 Insulin signaling downstream of PI3-kinase and also signaling via the mitogenic pathway in cultured adipocytes treated with T (10 nmol/l) for 48 h. Lysates of cells at baseline or after insulin stimulation (10 nmol/l, 10 min) were resolved by SDS-PAGE and immunoblotted with α -phospho-Akt and α -total Akt ($n=9$) (a–c), α -phospho-PKC ζ and α -total PKC ζ ($n=7$) (d–f), or α -phospho-ERK and α -total ERK ($n=12$) (g–i). Data are expressed as means \pm S.E.M. (% of internal standard). * $P<0.05$, † $P<0.01$, insulin-stimulated versus baseline under the same conditions.

Akt, was not impaired in the T-treated cells, and no defects in the proximal insulin signaling pathway via IRS-1 were identified. Defects in aPKC activation, due to poor responsiveness of aPKC to the lipid product of PI3-kinase, PIP₃, have been reported in both skeletal muscle and adipocytes in obesity, prediabetes, and type 2 DM (Beeson *et al.* 2003, Farese *et al.* 2005), suggesting that the present

finding of impaired insulin-mediated phosphorylation of PKC ζ induced by T in adipocytes of women is physiologically relevant.

Given that the AR antagonists cyproterone acetate and flutamide attenuated the effect of T on glucose uptake, it can be concluded that the action of T was mediated via the classical AR. Testosterone is metabolized to estradiol in

adipose tissue catalyzed by P₄₅₀ aromatase. However, it is unlikely that the effect of T on glucose uptake was estrogen mediated, given both the response to AR antagonists and the low expression of aromatase in *in vitro*-differentiated human adipocytes (Clyne *et al.* 2002). Testosterone is also metabolized to the significantly more biologically active androgen DHT, catalyzed by 5 α -reductase. This enzyme is expressed in adipose tissue (Martel *et al.* 1994). Thus, it is possible that the effects of T observed in the present studies were at least partially mediated by conversion to DHT.

Androgens have been shown to inhibit adipogenesis in rodent adipose cells (Dieudonné *et al.* 2000, Singh *et al.* 2006). However, supraphysiological T (1 μ mol/l) used for 10 days during *in vitro* differentiation of adipose cells of women did not alter the differentiation status of the cells, as indicated by glycerol-3-phosphate dehydrogenase activity (Dicker *et al.* 2004). In the present study, cells were treated with T for 48 h at the completion of 12-day incubation in adipogenic medium. The expression of proteins IR β (Smith *et al.* 1988) and GLUT4 (Hauner *et al.* 1998), the abundance of which increases markedly during the differentiation of preadipocyte to adipocyte, and ACC, an enzyme with a central role in fatty acid metabolism (Ruderman *et al.* 2003) did not differ in the T-treated and control cells. The lipid content of the T-treated cells was also unchanged. The defect in insulin-mediated glucose uptake observed in the T-treated adipocytes could not therefore be accounted for by an effect on the degree of adipocyte differentiation, suggesting a specific action of T on adipocyte metabolism.

The post-AR pathway whereby T caused impairment of insulin-mediated glucose uptake and phosphorylation of PKC ζ in adipocytes of women remains to be elucidated. While the mechanisms for androgen effects in tissues such as prostate have been extensively studied, there is little information on androgen actions in human adipose tissue, although novel pathways are now being identified, for example, interaction of AR with β -catenin and activation of Wnt signaling (Singh *et al.* 2006). Although the present studies have focused on the insulin signaling pathway, androgens could potentially modulate multiple pathways involved in cellular metabolism with consequences for insulin sensitivity, including AMP-activated protein kinase (AMPK), an enzyme with a central role in cellular energy balance and lipid oxidation (Ruderman *et al.* 2003) that has also been shown to activate pPKC ζ (Chen *et al.* 2002, Luna *et al.* 2006).

In the present studies, T 10 nmol/l for 48 h had significant effects on insulin-stimulated glucose uptake in adipocytes. Whether lower T concentrations have a similar effect is unknown. Although T 10 nmol/l is higher than the normal female circulating concentration, the T concentration within the adipose tissue of women approaches this level (Deslypere *et al.* 1985). Thus, T 10 nmol/l is not significantly supraphysiological. The local T concentration in the adipose tissue of women with hyperandrogenemia is not known, but is conceivably even higher than 10 nmol/l. The present studies examined the effects of chronic androgen treatment of

adipose cells because the *in vivo* situation in women with hyperandrogenemia is one of sustained increases in androgen levels rather than rapid fluctuations. Rapid effects of androgens via non-genomic mechanisms have been characterized in various cell types (Simoncini & Genazzani 2003), although not yet in human adipose tissue, and the relevance of these effects to the *in vivo* situation is unclear.

The present studies may be relevant to understanding why hyperandrogenism and insulin resistance are associated in women. High T levels have been linked to multiple cardiovascular disease (CVD) risk factors in women (Oh *et al.* 2002, Korytkowski *et al.* 2005). Low levels of sex-hormone-binding globulin (SHBG), a plasma protein that regulates bioavailability of circulating T, have been independently linked with CVD in women (Reinecke *et al.* 2002). Untreated women with androgen excess due to non-classic congenital adrenal hyperplasia (21-hydroxylase deficiency) have reduced insulin sensitivity (Speiser *et al.* 1992, Saygili *et al.* 2005). Short-term T administration to female adult mice causes impaired insulin-stimulated glucose uptake in skeletal muscle (Holmång *et al.* 1992), although there have been no similar studies in adipose tissue. Taken together, these lines of evidence suggest that androgens play a role in the regulation of insulin sensitivity in women and implicate elevated androgen levels in the development of insulin resistance and associated risk of CVD.

The most common cause of androgen excess in women is PCOS, due to both intrinsic upregulation of steroidogenesis (Nelson *et al.* 1999) and augmentation of androgen production by high circulating insulin levels (Nestler *et al.* 1998). In addition, PCOS is characterized by ovulatory dysfunction and insulin resistance (Dunaif 1997). Insulin resistance in PCOS has been characterized in skeletal muscle where the pathogenesis involves both intrinsic, presumably genetic, post-receptor defects in insulin metabolic signaling, as well as acquired defects due to *in vivo* environmental factors (Dunaif *et al.* 2001, Corbould *et al.* 2005, 2006). Adipocytes from women with PCOS also have post-insulin receptor-binding resistance to insulin effects on glucose uptake (Ciaraldi *et al.* 1992, Dunaif *et al.* 1992, Marsden *et al.* 1994). In women with hyperandrogenemia, insulin resistance, assessed by hyperinsulinemic-euglycemic clamp, is to some extent reversible by treatment with AR antagonists or agents that suppress ovarian steroidogenesis (Moggetti *et al.* 1996, Dahlgren *et al.* 1998). These studies suggest that a high circulating androgen level may be an *in vivo* environmental factor contributing to insulin resistance in PCOS, although other studies have given conflicting results (Dunaif *et al.* 1990, Lasco *et al.* 1995). In a recent study of adolescent girls with PCOS, serum androgen levels were an independent risk factor for metabolic syndrome (Coviello *et al.* 2006). It remains possible that hyperandrogenism in PCOS is part of a vicious cycle whereby hyperinsulinemia promotes increased androgens, which in turn contribute to insulin resistance in adipose tissue. Given that in animal models, insulin resistance in adipose tissue secondarily results in insulin resistance in

skeletal muscle (Abel *et al.* 2001, Yang *et al.* 2005), the present data suggest an indirect mechanism whereby androgens could contribute to skeletal muscle insulin resistance in women with hyperandrogenemia.

In conclusion, this study has shown that chronic T treatment of *in vitro*-differentiated adipose cells in women results in insulin resistance, which is selective for glucose uptake, with intact mitogenic/gene regulatory signaling. Insulin signaling via IRS-1 was not impaired, but a downstream defect in insulin-stimulated phosphorylation of PKC ζ was identified in the T-treated adipocytes. The effects of T on glucose uptake were AR mediated, but the post-AR pathway for these effects remains to be identified. These findings are relevant to understanding the pathogenesis of insulin resistance in women with hyperandrogenism, especially PCOS, and suggest that the contribution of androgens to the metabolic defects in these conditions warrants further study.

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