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Sex Steroid Hormones Induce Acylation Stimulating Protein Resistance in 3T3-L1 Adipocytes

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ABSTRACT

Acylation stimulating protein (ASP) stimulates triglyceride synthesis and glucose transport via its receptor C5L2. In human studies, ASP is increased in insulin resistant states such as obesity, diabetes, polycystic ovary syndrome and late pregnancy (the latter two associated with altered sex hormones). The aims were (i) to evaluate ASP response and C5L2 expression following treatment with sex steroid hormones and (ii) to identify mechanisms of ASP resistance using 3T3-L1 adipocytes and preadipocytes. Overnight incubation with physiological progesterone (PROG) concentrations induced dose-dependent inhibition of ASP-stimulated glucose transport in adipocytes ($188 \pm 11\%$ +ASP, $100 \pm 4\%$ control, $129 \pm 18\%$ to $85 \pm 7\%$ [ASP + PROG 10^{-8} to 10^{-6} M] and preadipocytes ($263 \pm 18\%$ +ASP, $100 \pm 3\%$ control, $170 \pm 11\%$ to $167 \pm 4\%$ [ASP + PROG 10^{-8} to 10^{-6} M]), while estradiol and testosterone (TEST) were effective only at the highest concentration (10^{-6} M). In adipocytes, dose-dependent maximal C5L2 mRNA decreases were 39–75% ($P = 0.003$), with decreased cell-surface C5L2 of -22% and -27% (10^{-6} M PROG and TEST, respectively) with no change in preadipocytes. Adipocytes treated with PROG displayed decreases in G proteins: Gβ (-55%), Gαq/11 (-56%) as well as complete inhibition of ASP stimulation. PROG significantly decreased basal levels of phosphorylated PKCα (p-PKCα) while there was no change in p- PKCζ. ASP increased p-PKCα and PKCζ to 161% ($P < 0.0.001$) and 160% ($P < 0.01$), a stimulation effectively blocked by PROG (10^{-8} and 10^{-6} M) and TEST (10^{-6} M). Sex steroid hormone-induced ASP resistance via C5L2 may contribute to altered adipose tissue function and insulin resistance phenotype in humans. J. Cell. Biochem. 105: 404–413, 2008.

KEY WORDS: C5L2; C3adesArg; PROGESTERONE; GLUCOSE TRANSPORT; INTRACELLULAR SIGNALLING; ESTRADIOL AND TESTOSTERONE

There is extensive clinical and experimental evidence demonstrating that sex steroids (estrogen, testosterone and progesterone) have a profound influence on distribution and function of adipose tissue. The distribution of fat is different between males and females [Vague, 1947] and sex steroid hormones play a pronounced role in this, as demonstrated by, transsexual treatments [Polderman et al., 1994].

These effects of sex steroid hormones are likely mediated through the presence of their respective receptors which include ERα and ERβ (estrogen receptors), PR-A and PR-B (progesterone receptors) and AR (androgen testosterone receptor). All of these are present in visceral and subcutaneous adipose tissue of both males and females, although there are site, gender and species differences [review Mayes et al., 2004]. It has been suggested that the effects are mediated through both genomic (transcriptional) as well as non-genomic mechanisms. The latter pathway is associated with G protein signalling, cAMP, phosphoinositol-3 kinase and protein kinase C (PKC) cascades [review Boonyaratanakornkit et al., 2007].

Abbreviation used: ASP, acylation stimulating protein; 2-DG, 2-deoxy-glucose; GLUT, glucose transport; GPCR, G protein coupled receptor; NS, not significant; PKC, protein kinase C; PROG, progesterone; TEST, testosterone.

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Further, data supports the presence of additional novel G protein coupled receptors for estrogen, progesterone and testosterone [Boonyaratanakornkit et al., 2007].

The effects of sex steroid hormones in adipose tissue include effects in preadipocytes on altering receptor expression, cell proliferation and preadipocyte differentiation [Mayes et al., 2004; Monjo et al., 2005; Rodriguez-Cuenca et al., 2005]. Effects in adipocytes include effects on receptor expression, lipid metabolism (lipolysis and lipogenesis) as well as adipokine secretion (leptin, adiponectin and resistin) and signalling proteins [Combs et al., 2003; Lappas et al., 2005; Mayes et al., 2004; Monjo et al., 2005].

Regulation of lipid metabolism in adipose tissue is controlled by a number of factors, including insulin and acylation stimulating protein (ASP), both of which increase glucose transport, triglyceride synthesis, lipoprotein lipase activity and decrease lipolysis [Cianflone et al., 2003]. Sex steroid hormones have been shown to diminish insulin action, preventing the stimulation of glucose transport by interfering with insulin receptor signalling [Collison et al., 2000; Muraki et al., 2006]. However the potential effects on ASP and ASP signalling are unknown.

ASP is produced through the interaction of complement C3, factor B and adipsin (all derived from adipose tissue) to generate ASP [Cianflone et al., 2003; Maslowska et al., 2005]. ASP binds to its cell surface receptor C5L2 to increase triglyceride synthesis (fatty acid esterification), glucose transport and decrease hormone sensitive lipase activity [Cianflone et al., 2003; Kalant et al., 2005; Maslowska et al., 2005]. Increases in glucose transport are mediated through increased activity and translocation of both glucose transporter Glut4 (in adipocytes) and Glut1 (in preadipocytes) [Germinario et al., 1993; Maslowska et al., 1997]. The demonstration of C5L2, a seven transmembrane G protein coupled receptor, as an ASP receptor was shown through gain-of-function studies (stable transfection) as well as loss-of-function (antisense and siRNA blocking) [Kalant et al., 2003, 2005]. Previous studies have demonstrated that C5L2 increases with adipocyte differentiation, insulin and thiazolidinedione insulin sensitizer treatment, while TNFα treatment decreases C5L2 [MacLaren et al., 2007]. Treatment with the fatty acids, palmitate and oleate, downregulates C5L2 and decreases ASP stimulation of glucose transport, suggesting an ASP resistant state [Wen et al., 2007].

ASP activation of C5L2 leads to rapid C5L2 phosphorylation and internalization mediated through β-arrestin, followed by activation of phospholipase C, phospholipase A2, phosphoinositol 3 kinase, Akt phosphorylation and activation and translocation of PKC [Baldo et al., 1995; Kalant et al., 2005; Maslowska et al., 2006]. Recently, we demonstrated that ASP activation also leads to increases in the G proteins Gβ and Gαq, as well as increases in phosphorylated PKCα and PKCζ [Wen et al., 2007].

Accordingly, the aims of the present study were (i) to evaluate the potential effects of sex steroid hormones on ASP function and (ii) to identify potential transcriptional and signalling changes in the ASP–C5L2 pathway. As both preadipocytes and adipocytes express the receptors for ASP and sex steroid hormones, and are responsive to these hormones, both cell types were evaluated.

MATERIALS AND METHODS

MATERIALS

3T3-L1 cells were purchased from ATCC, USA. 1-Methyl-3-isobutylmethylxanthine, dexamethasone, insulin, estradiol, progesterone, testosterone and bovine serum albumin were purchased from Sigma (St. Louis, USA). ^{3}H 2-deoxy-D-glucose (2-DG) was purchased from ICN Biochemicals Canada (Canada). TRIzol reagent was purchased from MRC (Molecular Research Center, USA). The primers were synthesized by AuGCT Biotechnology (China). RNA PCR Kit (AMV) was purchased from TaKaRa Biotechnology (Japan).

CELL CULTURE AND DIFFERENTIATION OF 3T3-L1 CELLS

All cell work was performed using Dulbecco's modified Eagle's medium/F-12 (DMEM) supplemented with 10% (v/v) fetal bovine serum (regular medium). Cells were cultured at 37°C with 5% CO_2. 3T3-L1 preadipocytes were used for experiments at 85–90% confluency. 3T3-L1 cells were differentiated using standard protocols [Maslowska et al., 2005]. Briefly, cells were plated in medium and grown until confluent. Two days post confluency, cell differentiation medium containing 10 ug/mL insulin, 1 uM dexamethasone, and 0.5 mM isobutylmethylxanthine was added to 3T3-L1 cells (day = 0). Two days later the medium was changed to insulin supplementation only; and 2 days later it was changed to regular medium only (DMEM with 10% fetal bovine serum). The medium was changed every 2 days thereafter and adipocytes were used on day 7–9. The cells used for experimentation were over 80% differentiated (as determined by microscopic evaluation).

SEX STEROID HORMONE TREATMENT

Adipocytes and preadipocytes were incubated overnight (18 h) with the indicated concentrations of estradiol, progesterone or testosterone in serum-free medium (DMEM). The final concentrations of hormones ranged from 10^{-8} to 10^{-6} M which ranges from the normal physiological state to the pathological state [Ojeda, 1992].

ASP PREPARATION

ASP was isolated and purified from human plasma as described previously [Baldo et al., 1993; Murray et al., 1997]. Each batch was verified for purity by ion-spray mass spectrometry at McGill University Mass Spectrometry Unit, Montreal, Canada [Baldo et al., 1993]. The activity of each ASP preparation was checked by its ability to stimulate triglyceride synthesis in 3T3-L1 preadipocytes [Maslowska et al., 2006].

GLUCOSE TRANSPORT IN ADIPOCYTES AND PREADIPOCYTES

Glucose transport assays were performed as previously described [Germinario et al., 1993; Maslowska et al., 1997; Tao et al., 1997]. Following overnight treatment with the indicated concentrations of sex steroid hormones, cells (adipocytes and preadipocytes) were preincubated in serum-free medium for 2 h (containing the same concentration of sex steroid hormones). Cells were then stimulated with ASP (1 umol/L for 2 h) or insulin (0.1 umol/L for 1 h) in serum-free medium, then assayed for glucose transport. In preliminary experiments we determined that insulin stimulation was maximal after 1 h incubation, remaining stable for 4 h while ASP stimulation

was maximal by 2 h, remaining stable for 6 h (as described previously [Germinario et al., 1993]). Following stimulation, cells were rinsed with warm (37°C) serum-free, glucose free media, then incubated for 10 min with ^{3}H 2-deoxy-glucose (50 umol/L, final specific activity 60–120 dpm/pmol) in serum-free glucose free media at 37°C. In all experiments, zero-time controls were performed to subtract the background binding of ^{3}H 2-deoxy-glucose. Following incubation, the reaction was stopped with rapid washing of the cells with cold PBS, and cell protein was dissolved in 0.1 N NaOH. Aliquots were taken for scintillation counting. Cell protein was measured by Bradford assay (BioRad, Mississauga, ON). Glucose transport was measured as pmol ^{3}H 2-deoxy-glucose uptake per mg soluble cell protein, and expressed relative to the basal untreated cells set as 100%.

ANALYSIS OF C5L2 AND ADIPONECTIN RECEPTORS R1 AND R2 RNA EXPRESSION IN ADIPOCYTES AND PREADIPOCYTES

Following overnight treatment of the adipocytes and preadipocytes with sex steroid hormones, media was removed and TRIzol reagent was added directly to the tissue culture dishes. Total cellular RNA was isolated according to the manufacturer's instructions, RNA was quantified by spectrophotometry, and all samples had a $A_{260}/A_{280} = 1.8–2.0$. For RT-PCR, 1 μg RNA was reverse-transcribed (final volume = 20 uL). 5 ul of each RT reaction was amplified by PCR (final volume 25 uL). The cDNA was amplified by PCR with the following protocol: 2 min at 94°C; 30 s at 94°C, 1 min at 60°C, 1 min at 72°C for 35 cycles, followed by 7 min extension at 72°C. Amplification was linear over the range used. Primers used were: mouse C5L2 (757 bp): sense 5′-CAG CGG AGA CCT CTT CCT ACT G-3′, antisense 5′-TGA ACG AGC AAG TGC ACA ATG-3′; mouse adiponectin R1 (444 bp): sense 5′-CTG GAC TAT TCA GGG ATT GC-3′, antisense 5′-CCA TAG AAG TGG ACG AAA GC-3′; mouse adiponectin R2 (307 bp): sense 5′-GCA TTG CAG CCA TTA TCG TC-3, antisense 5′-GCA CCA GCA ACC ACA AAG AT-3; mouse β-actin (542 bp): sense 5′-ATG GGT CAG AAG GAC TCC TAT G-3′, antisense 5′-ATC TCC TGC TCG AAG TCT AGA G-3′. Following PCR amplification, aliquots of PCR products were separated on a 2.0% agarose gel by electrophoresis with standard 100-bp DNA markers, stained with ethidium bromide, scanned and analyzed by densitometry.

IMMUNOFLUORESCENT FACSCAN ANALYSIS OF CELL SURFACE C5L2 IN ADIPOCYTES AND PREADIPOCYTES

Fluorescence-activated cell scanning (FACS) with anti-mouse C5L2 rabbit polyclonal antibody was used to evaluate cell surface C5L2 expression in adipocytes and preadipocytes. Antibody recognition of human C5L2 was assessed by flow cytometry as described previously [Kalant et al., 2003, 2005]. Rabbit polyclonal anti-mouse C5L2 was prepared through immunization using a peptide representing the N-terminal 23 amino acids of mouse C5L2 [Okinaga et al., 2003]. Following overnight treatment with sex steroid hormones, cells were incubated in serum-free DMEM medium for 2 h containing the indicated sex steroid hormones, then cells were detached using a non-enzymatic cell dissociation solution (Sigma Chemicals, St Louis, MO) for 20–25 min at 37 °C and pelleted by centrifugation. Cells were resuspended in 1 mL PBS with 1.0% BSA containing rabbit anti-C5L2 antibodies (diluted 1:50) and incubated at room temperature for 30 min with gentle rocking. Cells were pelleted by centrifugation, washed twice with PBS and re-suspended in 1 mL PBS with 1.0% BSA containing goat anti-rabbit FITC conjugated secondary antibody (dilution 1:400) (Bethyl, Montgomery, TX) for a 30 min incubation. Cells were washed with PBS and re-suspended in 4% paraformaldehyde in PBS and incubated for 5 min at room temperature. Cells were centrifuged and re-suspended in 0.4% paraformaldehyde and assayed by FACS (FACSort™, Becton Dickinson, USA). Incubation with a non-immune IgG (negative control) was used to evaluate background and set the gating for positive fluorescence. Values are expressed as % positive gated cells. Less than 1% of the negative control cells fell above the gated setting.

WESTERN BLOT ANALYSIS OF SIGNALLING PROTEINS IN ADIPOCYTES AND PREADIPOCYTES

Following overnight sex steroid hormone treatment, adipocytes and preadipocytes were preincubated in serum-free media for 2 h (containing sex steroid hormones) and then stimulated maximally with ASP (5 umol/L) for 4 h as described [Maslowska et al., 2006]. The media was removed and 500 uL of ice cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40; 0.1% SDS, 0.02% NaN_3, 0.5% sodium deoxycholate, 100 ug/mL PMSF, 1 ug/mL aprotinin in PBS) was added to the plates for 10 min at 4°C with gentle shaking. Total cell lysates were collected and centrifuged to remove particulate material (14,000*g*, 10 min, 4°C). Aliquots of the supernatant were stored at −80°C for further analysis. Proteins were measured by Bradford protein assay (BioRad). For Westerns, Laemmli sample buffer was added to aliquots of cell lysate and the samples were boiled for 5 min. Forty ug of cell lysate was loaded per lane and the proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred to PVDF membrane via electroblotting and were blocked in 5% non-fat dried milk (for Gβ, Gαq/11 and actin) or 3% bovine serum albumin (for Thr 410 phosphorylated PKCζ and Ser 657 phosphorylated PKCα) in 0.05% Tween-PBS (60 min) then incubated with the appropriate antibody diluted in the same blocking buffer (overnight). Primary antibodies used were anti-Gβ (1:500, Gβ (T-20): sc-378), anti-Gαq/11 (1:300, Gα q/11 (C-19): sc-392), anti-phosphorylated PKCα (1:300, p-PKCα (Ser 657): sc-12356-R), anti-phosphorylated-PKCζ (1:300, p-PKCζ (Thr 410)-R: sc-12894-R) and anti-actin (1:300, actin (C-11): sc-1615) (all from Santa Cruz Biotechnology, Inc). Negative controls using non-immune IgG or in the absence of primary antibody were used to verify specificity. Antibody incubation was followed by incubation with an enzyme-linked antibody (peroxidase-conjugated goat anti-rabbit immunoglobulin secondary antibody, 1:5000) for 60 min. The immobilized proteins were detected by ECL Plus kit from Amersham Biosciences (Piscataway, NJ, USA) using Kodak film. Immunoblots were quantified by computer assisted photodocumentation system (ChemiImager Ready System, San Leonardo, CA, USA).

STATISTICAL ANALYSES

Results are expressed as means ± standard error of the mean (SEM). Each experiment was conducted multiple times with multiple

replicates (2–4) within each experiment (n value as indicated). For comparison of the differences among groups, one-way or two-way ANOVA was used, followed by Newman–Keuls post-hoc test. Statistical significance was set at P value <0.05, where P NS indicates not significant.

RESULTS

ASP stimulates glucose transport in both adipocytes and preadipocytes [Germinario et al., 1993; Maslowska et al., 1997], and both cell types are responsive to sex steroid hormones [Mayes JS et al., 2004]. Accordingly, both preadipocytes and adipocytes were examined for sex steroid hormone effects on ASP stimulation of glucose transport. 3T3-L1 adipocytes and preadipocytes were incubated overnight with increasing concentrations of estradiol, testosterone or progesterone (as described in methods), then evaluated for ASP-stimulated glucose transport. As shown in Figure 1A, ASP-stimulated glucose transport by $188\% \pm 11\%$, $P < 0.001$ in adipocytes versus control (PBS alone). All concentrations of progesterone (10^{-8} to 10^{-6} M) significantly decreased ASP stimulation of glucose transport ($P < 0.01$ to $P < 0.001$). Testosterone and estradiol were less effective; only the highest concentration of 10^{-6} M significantly inhibited ASP stimulation (both $P < 0.001$). Similarly in preadipocytes, ASP increased glucose transport by $263\% \pm 18\%$, $P < 0.001$ and overnight incubation with progesterone significantly decreased the ASP response (Fig. 1B). As with adipocytes, estradiol was effective only at high concentrations, while testosterone was effective at all concentrations. The effects of sex steroid hormones were also evaluated on insulin-stimulated glucose transport in adipocytes. While higher concentrations of testosterone and estradiol effectively decreased insulin-stimulated glucose transport, consistent with previous reports [Chen et al., 2006a; Collison et al., 2000; Muraki et al., 2006], progesterone had no effect (Fig. 4A). None of the sex steroid hormones had any significant effect on basal glucose transport in adipocytes or preadipocytes (data not shown).

Sex steroid hormones are known to mediate their effects through both genomic (transcriptional and protein expression) as well as non-genomic (mediated via signalling pathways) mechanisms. In order to evaluate the mechanism by which sex steroid hormones induce ASP resistance we first examined ASP receptor C5L2 expression (mRNA levels) and cell surface localization. As shown in Figure 2, following overnight treatment with progesterone, C5L2 mRNA expression decreased in adipocytes at all concentrations by 39–75% (Fig. 2C, $P = 0.003$ ANOVA). Estradiol and testosterone treatment resulted in significant decreases in C5L2 mRNA in adipocytes at the higher concentrations (Fig. 2A and B, $P = 0.001$ and $P = 0.002$, respectively by ANOVA). By contrast, no significant change in C5L2 mRNA was detected in preadipocytes (P NS).

Cell surface C5L2 protein expression was evaluated by FACScan analysis, using an antibody specific for C5L2. As shown in Figure 3, treatment with both estradiol and testosterone resulted in significant decreases in cell surface expression of C5L2 in adipocytes only at the highest concentration of 10^{-6} M ($P < 0.05$ and $P < 0.01$, respectively). On the other hand, a significant decrease

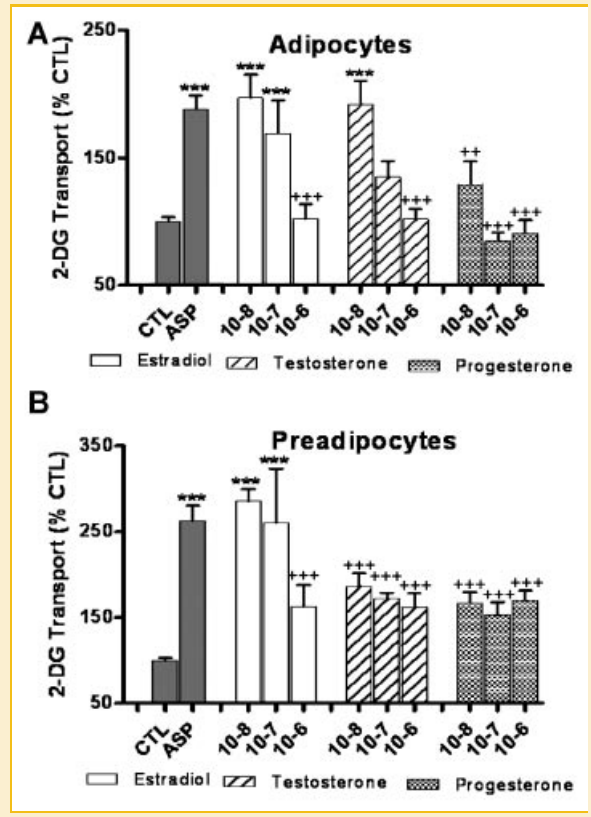

Fig. 1. Effect of sex steroid hormones on ASP-stimulated glucose transport in 3T3-L1 adipocytes and preadipocytes. Adipocytes (A) and preadipocytes (B) were incubated overnight with the indicated concentrations of estradiol, testosterone or progesterone, then stimulated with ASP (as described in methods) and assessed for ^{3}H 2-deoxy-glucose uptake. Results are presented as average ± SEM, where basal glucose transport in the absence of sex steroid hormones (control) was set as 100% for average n = 5 (adipocytes) and n = 9 (preadipocytes) for each data point. Results were analyzed by two-way ANOVA followed by Newman–Keuls post hoc test where $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for stimulation with ASP vs. control (basal glucose transport = 100%) and $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ for sex hormone treated vs. ASP alone.

in C5L2 was detected at all concentrations of progesterone ($P = 0.007$ ANOVA). The decrease in % positive cells was reflected by a decrease in mean fluorescence intensity (MFI), suggesting less C5L2 expression per cell rather than decreased numbers of C5L2-positive cells. As with C5L2 mRNA expression, there was no change in C5L2 cell surface expression in preadipocytes, other than a decrease at the highest concentration of progesterone (Fig. 3B, $P < 0.05$).

In order to evaluate whether these effects were specific for ASP response and C5L2 expression, we also examined insulin response and expression of the adipokine receptor for adiponectin, R1 and R2. As shown in Figure 4A for adipocytes and 4B for preadipocytes, insulin-stimulated glucose transport by two-fold. Overnight treatment with the highest concentrations of estradiol and testosterone decreased the insulin-stimulated glucose transport in adipocytes and preadipocytes, while progesterone had effects in preadipocytes

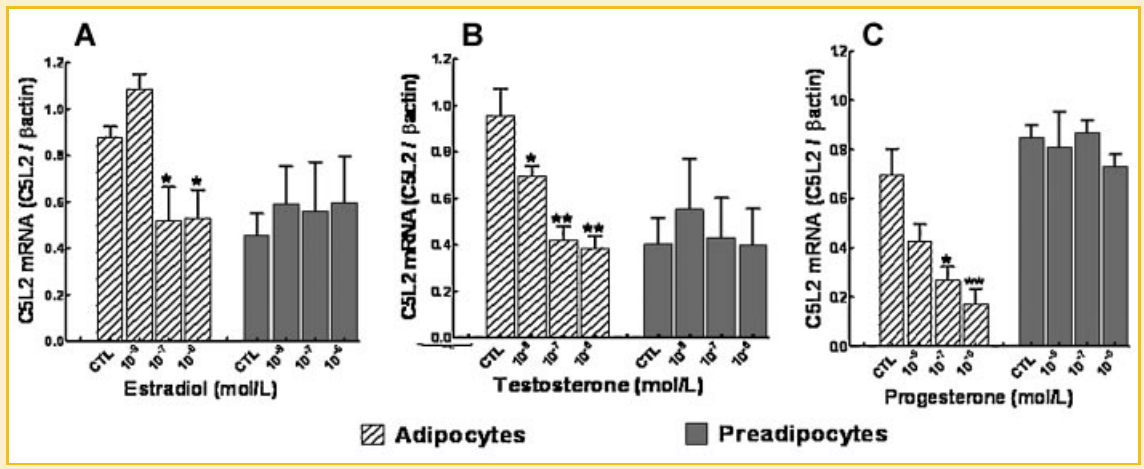

Fig. 2. Sex steroid hormone effect on C5L2 expression in 3T3-L1 adipocytes and preadipocytes. Adipocytes and preadipocytes were incubated overnight with the indicated concentrations of estradiol (A), testosterone (B) or progesterone (C). C5L2 mRNA was then measured by RT-PCR. Results are presented as average ± SEM, for the ratio of C5L2 mRNA/β-actin for n = 4 for each data point. Results were analyzed by ANOVA for adipocytes (all $P < 0.003$) and preadipocytes (all P NS) followed by Newman–Keuls post hoc test where $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. basal expression levels (control) in the absence of sex steroid hormone treatment.

only. Thus results are similar but not identical to the pattern obtained for sex steroid hormone inhibition of ASP action. The effects of sex steroid hormones on adiponectin R1 and R2 are shown in Figures 4C and D. Although expression of adiponectin receptor R1 and R2 clearly increased with differentiation, there was only a slight increase or no change in the adipocytes with either estradiol or testosterone at the highest hormone concentration, in contrast to the downregulation of C5L2 at the same hormone concentrations (Fig. 3A).

Together, these data suggest that in adipocytes, one mechanism of induction of ASP resistance is mediated through downregulation of mRNA expression and cell surface localization of C5L2, the ASP receptor. On the other hand, changes in C5L2 level do not appear to be responsible for the ASP resistance evident in the preadipocytes, especially with respect to progesterone. We next looked at the signalling proteins identified in the ASP pathway.

The results for sex steroid hormone treatment on the G proteins, Gβ and Gαq/11, in adipocytes are shown in Figures 5A and B. After overnight incubation of adipocytes, the highest concentration (10^{-6} M) of progesterone significantly decreased Gβ and Gαq/11 by −55% and −56% ($P < 0.01$ and $P < 0.05$), while the highest concentration of testosterone decreased Gαq/11 (−56%, $P < 0.05$). As shown in Table I, progesterone treatment significantly decreased Gαq/11 in preadipocytes. In adipocytes, treatment with ASP significantly increased both Gβ and Gαq/11 by 133% (Fig. 5A, $P = 0.0003$) and 137% (Fig. 5B, $P = 0.008$) in adipocytes, a stimulation that was effectively blocked by the higher concentration of progesterone (both Gβ and Gαq/11) and testosterone (Gαq/11) (P NS for + ASP vs. no ASP), while estradiol treatment had no effect. In preadipocytes, progesterone significantly decreased Gαq/11 (Table I). Representative Western blots are shown in Figure 5C.

The effect of sex steroid hormones on basal and ASP stimulation of PKC isoforms in adipocytes are shown in Figure 6. Progesterone at both concentrations as well as testosterone at the highest concentration significantly decreased basal levels of phosphorylated PKCα (p-PKCα, Figure 6A, $P < 0.001$), while there were no significant changes with PKCζ. In preadipocytes, none of the hormones significantly altered basal levels (Table I). ASP treatment increased p-PKCα and PKCζ by 161% ($P < 0.0.001$) and 160% in adipocytes ($P < 0.01$). This ASP stimulation was effectively blocked by progesterone at both concentrations as well as testosterone at the highest concentration (P NS for + ASP vs. no ASP). In preadipocytes, progesterone significantly decreased the ASP effect (Table I). Representative Western blots are shown in Figure 6C.

DISCUSSION

The results in the present study indicate that physiological concentrations of progesterone as well as high concentrations of testosterone induce ASP resistance in both adipocytes and preadipocytes. In adipocytes the mechanism involves direct downregulation of C5L2 through decreased mRNA expression and decreased cell surface C5L2, as well as interference in the ASP–C5L2 signalling pathway including decreased activation of G protein and PKC isoforms. In preadipocytes, the effects may primarily be mediated through decreased signalling which, although small, may be cumulative and sufficient to induce "ASP resistance" of glucose transport. We have previously demonstrated that ASP stimulation is not Gαs or Gαi mediated (pertussis and cholera toxin insensitive), suggesting association with Gαq/11 [Maslowska et al., 2006]. More recently, a direct increase in Gβ and Gαq/11 was demonstrated upon ASP stimulation [Wen et al., 2007]. We have previously demonstrated the obligatory role of protein kinase C activation and translocation in ASP-mediated stimulation [Baldo et al., 1995] and direct involvement of the protein kinase C isoforms, PKCα and PKCζ [Wen et al., 2007]. Protein kinase C activation is downstream of phosphatidyl inositol 3 kinase (PI3 kinase) and phospholipase C, with subsequent activation of Akt, MAPK/ERK1/2 and cPLA2, all involved in the ASP–C5L2 pathway [Maslowska et al., 2006].

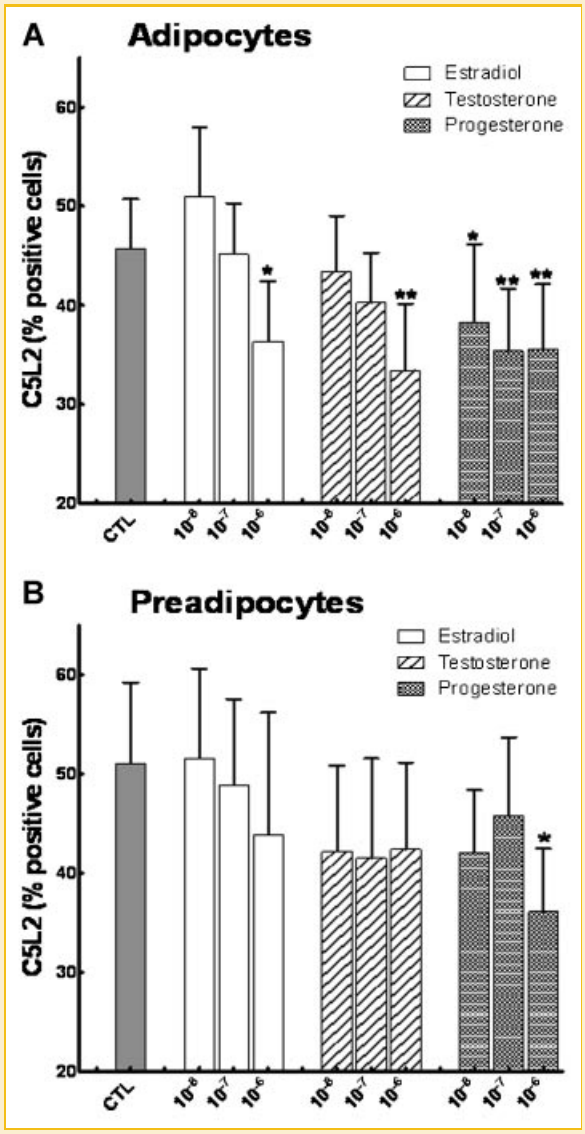

Fig. 3. Sex steroid hormone effect on cell surface C5L2 in adipocytes and preadipocytes. 3T3-L1 adipocytes (A) and preadipocytes (B) were incubated overnight with the indicated concentrations of sex steroid hormones. Cell surface C5L2 was then measured by immunofluorescence FACS analysis using anti-C5L2 antibody. Results are presented as average ± SEM, for the % positive cells (gated based on non-immune antibody set as 0% positive) for n = 6 for each data point. Results were analyzed by ANOVA (for adipocytes, $P < 0.005$ for all sex steroid hormones, for preadipocytes, P NS for estradiol and testosterone, $P = 0.03$ progesterone effect), followed by Newman–Keuls post hoc test where $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. basal expression levels (control) in the absence of sex steroid hormone treatment.

The present results indicate that certain sex hormones, in particular progesterone but also testosterone to a lesser degree, interfere with ASP stimulation of this pathway. It has been suggested that the effects of sex steroid hormones are mediated through both genomic (transcriptional) as well as non-genomic mechanisms (as described in detail in the introduction). In adipocytes, various sex steroid hormones also have effects on additional signalling proteins such as MAP kinase, Akt and CEBPα [Chen et al., 2006b; Mayes et al., 2004; Muraki et al., 2006], pathways that are involved in ASP stimulation. Sex hormones have previously been shown to interfere with specific components of the insulin signalling pathway involved in glucose transport stimulation in both adipocytes and muscle including IRS-1, PI3 kinase and PKCζ [Collison et al., 2000; Corbould, 2007; Muraki et al., 2006]. PKCα is involved in insulin stimulation with interaction with insulin receptor substrate 1 (IRS1) [Oriente et al., 2005]. PKCζ has been well documented as a convergent downstream target of PI3 kinase activation and is directly linked to glucose transporter translocation [Hodgkinson et al., 2005; Kotani et al., 1998; Liu et al., 2006]. As these pathways are also involved in ASP activation, this is likely one mechanism of sex steroid hormone induced ASP resistance.

Sex hormone effects on other receptors have also been examined. While sex steroid hormones have effects, these are not necessarily the same effects as seen for ASP response and C5L2 expression. For example, progesterone has little effect on insulin-stimulated glucose transport (as shown in the present study), whereas this same hormone interferes with ASP effects. Further, studies on adiponectin receptor show that there is a slight increase with estradiol or testosterone, consistent with published data [Tan et al., 2006] yet opposite to the effects on C5L2. Sex steroid hormones estradiol, progesterone and testosterone increase levels of α2 and β3 adrenergic receptors, both G protein coupled receptors [Mayes et al., 2004; Monjo et al., 2005;]. Again the effects on these receptors are distinct from the effects on C5L2, thus sex hormone repression of GPCRs is not a general mechanism.

The implication of sex steroid hormones on ASP resistance is interesting from both a cellular and a physiological point of view. As sex steroid hormones induce a decrease in cellular C5L2 and alterations in signalling pathways, this may possibly interfere with other ASP functions such as triglyceride synthesis. Physiologically, a decrease in ASP function may enhance diversion of available glucose and fatty acids to other tissues (muscle, liver, arterial), leading to lipotoxicity [Bastard et al., 2006] unless disposal mechanisms are upregulated such as increased fatty acid oxidation [Xia et al., 2004]. It should be noted that C5L2 is also expressed in muscle and liver [Kalant et al., 2005; Ohno et al., 2000]. While the function in liver remains to be determined, in muscle, ASP appears to decrease muscle lipoprotein lipase efficiency (as does insulin) [Faraj et al., 2004; Faraj and Cianflone, 2004], and we hypothesize that the presence of ASP resistance in muscle may contribute to muscle lipotoxicity, although this remains to be evaluated directly.

This ''ASP resistant'' state may also be inferred in physiological human studies. Increased plasma ASP and complement C3 (ASP precursor) are associated with both obesity and insulin resistance [Cianflone et al., 2003], and estrogen is a powerful stimulus for C3 production in uterine epithelial cells [Li et al., 2002]. PCOS (polycystic ovary syndrome) in women, which is characterized by abnormal sex steroid hormones, is associated with insulin resistance, as well as increased ASP [Oktenli et al., 2007; Wu et al., 2008], independent of obesity. Similarly, late pregnancy, where estrogen and progesterone levels increase markedly, is associated with insulin resistance, as well as increased plasma ASP levels [Saleh et al., 2007]. Treatment of women with PCOS with metformin, increases insulin sensitivity, and also decreases plasma ASP levels [Oktenli et al., 2007]. While ASP may be increased simply as a

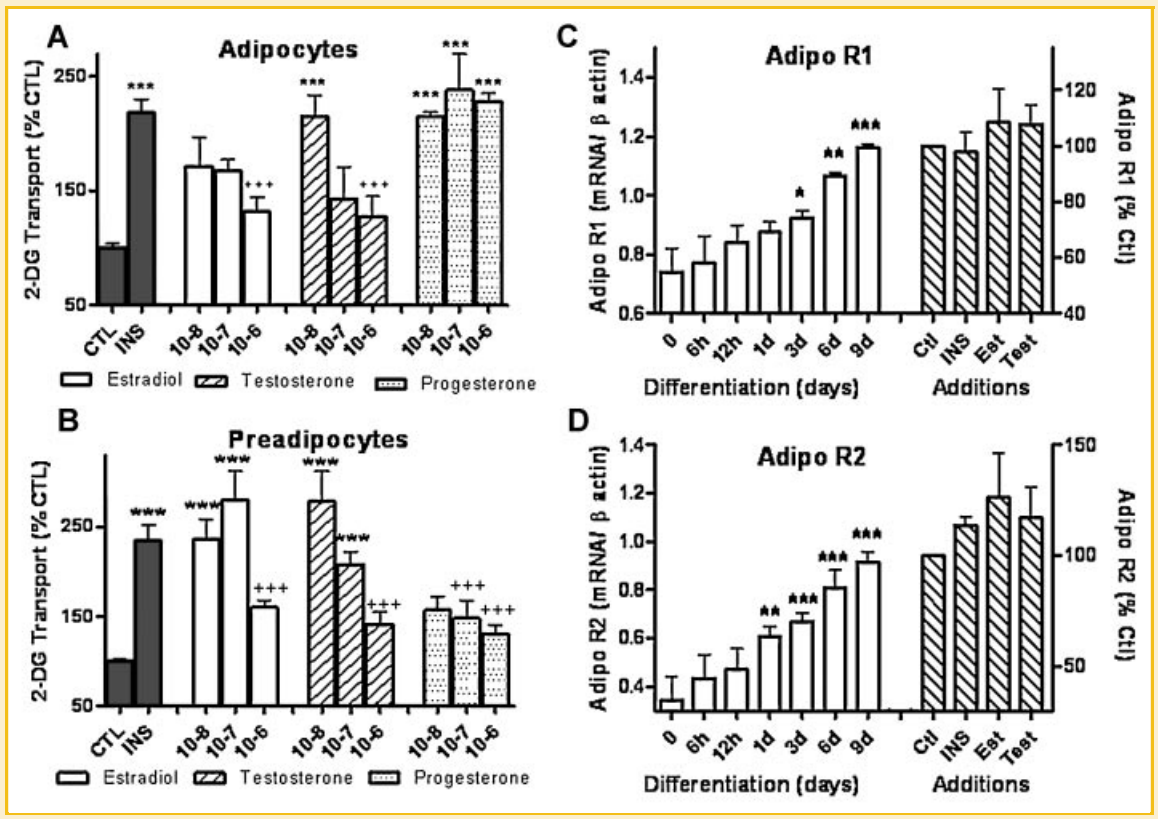

Fig. 4. Effect of sex steroid hormones on insulin-stimulated glucose transport and expression of adiponectin R1 and R2 receptors in 3T3-L1 differentiating adipocytes adipocytes (A) and preadipocytes (B) were incubated overnight with the indicated concentrations of estradiol, testosterone or progesterone, then stimulated with insulin (as described in methods) and assessed for ^{3}H 2-deoxy-glucose uptake. Results are presented as average ± SEM, where basal glucose transport in the absence of sex steroid hormones (control) was set as 100% for average n = 5 (adipocytes) and n = 9 (preadipocytes) for each data point. Results were analyzed by two-way ANOVA followed by Newman–Keuls post hoc test where $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for stimulation with insulin vs. control (basal glucose transport = 100%) and $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ for sex hormone treated vs. insulin alone. For panels C and D, preadipocytes were differentiated for the indicated time in hours (h) or days (d) up to 9 days differentiation. Differentiated adipocytes were then incubated overnight with the indicated addition of insulin (INS, 100 nM), estradiol (Est, 10^{-6} M) or testosterone (Test, 10^{-6} M). Adiponectin receptor R1 (C) and R2 (D) were then measured by RT-PCR. Results for differentiation (left *y*-axis) are presented as average ± SEM, for the ratio of R1 or R2 mRNA/β-actin for n = 4 for each data point. Results for hormone treatment (right *y*-axis) are presented as % control where control = 100% for adipocytes at 9 days differentiation. Results were analyzed by ANOVA for effect of differentiation ($P < 0.001$) followed by Newman–Keuls post hoc test where $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. no differentiation (time = 0).

TABLE I. Sex Steroid Hormone Effects on G Protein Levels and Phosphorylated Protein Kinase C Isoforms in Preadipocytes Stimulated With ASP

		Estradiol		Testosterone		Progesterone		ANOVA
	No hormone	[10^{-8} M]	[10^{-6} M]	[10^{-8} M]	[10^{-6} M]	[10^{-8} M]	[10^{-6} M]	*P*
Gβ protein								
Control	0.95 ± 0.08	0.86 ± 0.12	0.63 ± 0.08	0.98 ± 0.17	0.60 ± 0.02	0.91 ± 0.09	0.62 ± 0.11	NS
+ASP	1.35 ± 0.16	1.30 ± 0.28	1.06 ± 0.09	1.17 ± 0.12	0.89 ± 0.07	0.91 ± 0.03	$0.77 \pm 0.05^{+}$	<0.05
Gαq protein								
Control	0.82 ± 0.06	0.73 ± 0.12	0.67 ± 0.17	0.79 ± 0.14	0.46 ± 0.25	$0.34 \pm 0.09^{*}$	$0.16 \pm 0.02^{**}$	<0.01
+ASP	1.04 ± 0.16	1.17 ± 0.14	0.79 ± 0.06	0.82 ± 0.07	0.80 ± 0.04	$0.53 \pm 0.14^{++}$	$0.42 \pm 0.10^{++}$	<0.001
p-PKCα								
Control	0.59 ± 0.05	0.68 ± 0.13	0.57 ± 0.18	0.65 ± 0.08	0.45 ± 0.10	0.57 ± 0.11	0.47 ± 0.07	NS
+ASP	0.90 ± 0.14	0.89 ± 0.15	0.66 ± 0.18	0.54 ± 0.15	0.67 ± 0.05	0.62 ± 0.02	$0.44 \pm 0.09^{+}$	<0.05
p-PKCζ								
Control	0.22 ± 0.03	0.40 ± 0.09	0.37 ± 0.17	0.18 ± 0.02	0.17 ± 0.04	0.38 ± 0.10	0.26 ± 0.05	NS
+ASP	0.39 ± 0.07	0.51 ± 0.14	0.36 ± 0.08	0.28 ± 0.05	0.39 ± 0.12	$0.24 \pm 0.04^{+}$	$0.27 \pm 0.05^{+}$	<0.05

3T3-L1 preadipocytes were incubated overnight with the indicated concentrations of sex steroid hormones, then stimulated with ASP (as described in methods). Gβ protein, Gαq/11, phosphorylated (p)-PKCα, and p-PKCζ were then measured by Western analysis using specific antibodies. Results are presented as average ± SEM, for the ratio of the indicated proteins/actin for n = 3–4 independent experiments. Results were analyzed by ANOVA for effect of hormone treatments followed by Newman–Keuls post hoc test where $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control and $^{+}P < 0.05$, $^{++}P < 0.01$ for sex hormone treated vs. ASP alone.

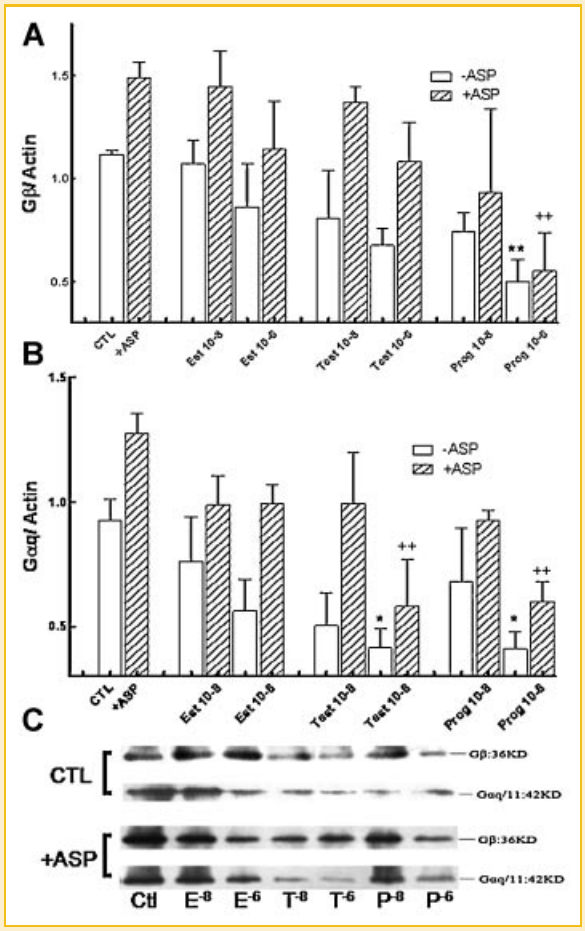

Fig. 5. Sex steroid hormone effects on G protein levels in adipocytes stimulated with ASP. 3T3-L1 adipocytes were incubated overnight with the indicated concentrations of sex steroid hormones, then stimulated with ASP (as described in methods). Gβ protein (A) and Gαq/11 (B) were then measured by Western analysis using specific antibodies. Results are presented as average ± SEM, for the ratio of the indicated proteins/actin for n = 3–4 independent experiments. Results were analyzed by two-way ANOVA ($P < 0.01$ Gβ and $P < 0.02$ for Gαq/11) followed by Newman–Keuls post hoc test where $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control and $^{+}P < 0.05$, $^{++}P < 0.01$ for sex hormone treated vs. ASP alone.

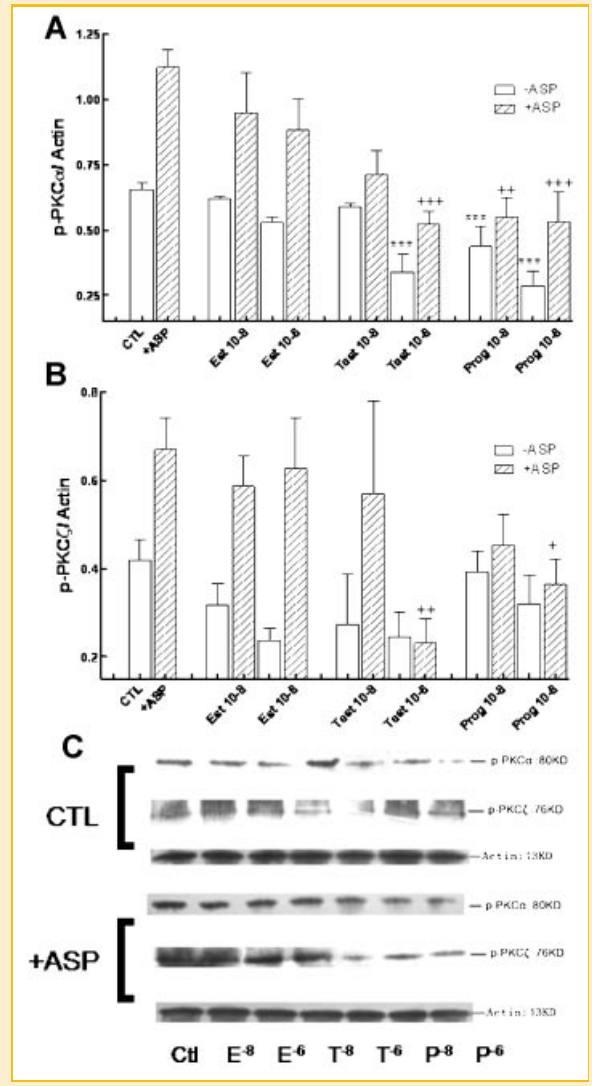

Fig. 6. Sex steroid hormone effects on phosphorylated protein kinase C (p-PKC) isoforms in adipocytes stimulated with ASP. 3T3-L1 adipocytes were incubated overnight with the indicated concentrations of sex steroid hormones, then stimulated with ASP (as described in methods). Phosphorylated (p)-PKCα (A) and phosphorylated (p)-PKCζ (B) were then measured by Western analysis using specific antibodies. Results are presented as average ± SEM, for the ratio of the indicated proteins/actin for n = 3–4 independent experiments. Results were analyzed by two-way ANOVA ($P = 0.0002$ p-PKCα and $P = 0.03$ p-PKCζ) followed by Newman–Keuls post hoc test where $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control and $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ for sex hormone treated vs. ASP alone.

response to the insulin resistance (since ASP is also increased in other insulin resistant states such as obesity, diabetes and cardiovascular disease), the present cellular studies support the interpretation that the alteration in ASP pathway may be a direct response to the effect of the sex steroid hormones on down-regulation of C5L2. Consequently, these physiological increases in sex steroid hormones in PCOS and late pregnancy likely induce decreased C5L2 expression and signalling, leading to ASP resistance. Therefore ASP resistance may be contributing independently to the insulin resistance which is already recognized as characteristic of these conditions. The increased plasma ASP associated with PCOS and late pregnancy may be a marker of an ASP resistant state.

It is suggested that there is an optimal physiological window for sex hormones with extremely low and excessively high concentrations associated with insulin resistance [Mayes et al., 2004]. For example, low levels of estrogen during menopause or a defect in aromatase action are associated with insulin resistance [Mayes et al., 2004]. Similarly increased sex steroid hormones in PCOS women, and in late pregnancy are also associated with insulin resistance [Oktenli et al., 2007; Saleh et al., 2007; Wu et al., 2008]. Further, sex steroid hormones not only interfere with insulin hormone action in adipocytes, but have also been shown to influence adipokine hormone secretion from adipocytes, including leptin, resistin and adiponectin [Lappas et al., 2005; Mayes et al., 2004], and in the present study, ASP function. This common mechanism in the

development of a resistant state has many physiological implications. Multiple factors appear to contribute to insulin resistance, as demonstrated by changes in whole body metabolism [Bastard et al., 2006], and in cell models (see above). Leptin resistance, adiponectin resistance and ASP resistance have all been proposed to contribute to (or be present in) an insulin resistant state [Dyck et al., 2006; Munzberg et al., 2005; Ozata et al., 2002]. This suggests the presence of a general adipose tissue resistant state which can be influenced by sex steroid hormones.

In summary, we provide direct evidence of ASP resistance in a cellular model. Further, we have shown that the mechanism of action involves both changes in expression of C5L2 as well as signalling parameters, and provides a basis for understanding and examining ASP resistance in human studies. This state has direct relevance to specific physiological conditions, such as PCOS and late pregnancy.

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