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Palmitate and Oleate Induction of Acylation Stimulating Protein Resistance in 3T3-L1 Adipocytes and Preadipocytes

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Abstract Acylation stimulating protein (ASP) stimulates triglyceride synthesis and glucose transport via its receptor C5L2. The aims were (i) to evaluate ASP response under insulin-resistant conditions and (ii) to identify mechanisms of ASP resistance using 3T3-L1 adipocytes and preadipocytes. Overnight incubation with palmitate (PAL) or oleate (OLE) induced dose-dependent inhibition of ASP-stimulated glucose transport in adipocytes ($198 \pm 18\%$ +ASP, $100 \pm 4\%$ basal, $131 \pm 14\%$ + ASP + 1 mmol/L PAL) and preadipocytes ($287 \pm 21\%$ + ASP, $100 \pm 4\%$ basal, $109 \pm 13\%$ + ASP + 1 mmol/L PAL). In adipocytes, dose-dependent maximal C5L2 mRNA decreases were $-41 \pm 15\%$ and $-82 \pm 2\%$, with decreased cell-surface C5L2 of $-55 \pm 12\%$ and $-39 \pm 9\%$ (1 mmol/L PAL and OLE, respectively) with no change in preadipocytes. Adipocytes treated with PAL or OLE evidenced inhibition of ASP stimulation of G proteins: Gβ (−50%), Gαq/11 (−50%) and protein kinase C: PKCα-P (−52%), PKCζ-P (−43%). Fatty acid-induced ASP resistance via C5L2 may contribute to altered adipose tissue function and obesity/insulin resistance phenotype in humans. J. Cell. Biochem. 104: 391–401, 2008.

Key words: C5L2; C3adesArg; G protein coupled receptor; glucose transport; intracellular signalling; insulin

The increasing global prevalence of overweight and obese populations is directly related to the increase in associated health problems including increased incidence of coronary heart disease, hypertension, diabetes, and inflammation [Frayn, 2005; Bastard et al., 2006; Poirier et al., 2006]. The obese state results from an imbalance between energy storage and expenditure. Many hormones, including adipokines, contribute to this phenomenon [Fasshauer et al., 2004; Frayn, 2005; Bastard et al., 2006]. One such adipokine, acylation stimulating protein (ASP, also known as C3adesArg), is produced and secreted by white adipose tissue, which is well-recognized as a metabolically active endocrine organ as well as an energy storage tissue [Cianflone et al., 2003; Maslowska et al., 2005].

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 (TG) synthesis (fatty acid esterification), glucose transport and decrease hormone sensitive lipase activity [Cianflone et al., 2003; Kalant et al., 2005; Maslowska et al., 2005]. Through the effects of ASP on intracellular fatty acid re-esterification, ASP increases the lipolytic efficiency of lipoprotein

Abbreviations used: ASP, acylation stimulating protein; 2-DG, 2-deoxy-glucose; FA, fatty acid; GLUT, glucose transport; GPCR, G protein coupled receptor; NEFA, non-esterified fatty acid; NS, not significant; PKC, protein kinase C; PI3kinase, phosphoinositol-3-kinase; TG, triglyceride.

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lipase [Faraj and Cianflone, 2004; Faraj et al., 2004]. Increases in glucose transport are mediated through increased activity and translocation of both Glut4 (in adipocytes) and Glut 1 (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]. ASP activation of C5L2 leads to rapid C5L2 phosphorylation and internalization mediated through βarrestin, followed by activation of phospholipase C, phospholipase A2, phosphoinositol 3 kinase (PI3 kinase), Akt phosphorylation and activation and translocation of protein kinase C (PKC) [Baldo et al., 1995; Kalant et al., 2005; Maslowska et al., 2006].

In vivo, ASP appears to be closely related to postprandial TG clearance. In ASP deficient (C3 knockout) mice and C5L2 knockout mice, the absence of a functional ASP-C5L2 pathway leads to delayed postprandial TG after administration of a fat load [Murray et al., 1999a,b; Paglialunga et al., 2007]. This is further enhanced in diet-induced obese mice or genetically obese *ob*/*ob* mice [Murray et al., 1999b; Xia et al., 2002]. Postprandial chylomicrons stimulate ASP production in human adipocytes in vitro [Scantlebury et al., 1998, 2001], and this has been documented in vivo in humans as well [Saleh et al., 1998; Kalant et al., 2000].

In healthy men and women, fasting ASP predicts postprandial TG clearance: the higher the fasting ASP, the greater the delay in postprandial TG clearance [Cianflone et al., 2004]. Further, fasting plasma ASP is increased in obesity, insulin resistance, coronary artery disease and diabetes, even in the absence of obesity [Cianflone et al., 2003; Yang et al., 2006], while weight loss, and exercise decrease ASP [Cianflone et al., 2003; Schrauwen et al., 2005]. Altogether, these studies suggest that increased fasting levels of ASP, especially in the face of delayed TG clearance, are indicative of "ASP resistance," in the same way that increased circulating insulin, even with normal or near normal glucose, is indicative of an "insulin resistant" state. This "ASP resistance" due to a lack of a fully functional ASP-C5L2 pathway has been demonstrated recently in a family with a C5L2 mutation, characterized by increased ASP and dyslipidemia [Marcil et al., 2006].

Insulin resistance is closely associated with obesity and dyslipidemia, elevated fatty acids have been implicated as a contributing factor [Bastard et al., 2006]. 3T3-L1 preadipocyte/adipocyte cell models have commonly been used to examine insulin resistance, usually using insulin stimulation of glucose transport as a marker of insulin response [Van Epps-Fung et al., 1997; Gao et al., 2004; Nguyen et al., 2005]. Treatment of cells with both saturated [palmitate (PAL), stearate], unsaturated [oleate (OLE), linoleate], and mixtures of fatty acids have been shown to inhibit insulin-mediated stimulation of glucose transport [Van Epps-Fung et al., 1997; Gao et al., 2004; Nguyen et al., 2005]. The mechanism of insulin resistance is linked to changes in signaling pathways, including IRS-1, PI3kinase, Akt, and MAP/ERK [Gao et al., 2004; Xie et al., 2006]. Accordingly, the aims of the present study were (i) to evaluate potential ASP resistance in both adipocytes and preadipocytes under conditions which produce insulin resistance, since both types of cells are responsive to ASP as well as insulin and (ii) to identify the mechanism of ASP resistance.

MATERIALS AND METHODS

Materials

3T3-L1 cells were purchased from ATCC USA. 1-Methyl-3-isobutylmethylxanthine, dexamethasone, insulin, oleic acid, palmitic acid, and bovine serum albumin were purchased from Sigma (St. Louis). ^{3}H 2-deoxy-D-glucose (2-DG) was purchased from ICN Biochemicals Canada (Canada). TRIzol reagent was purchased from MRC (Molecular Research Center). 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 μg/ml insulin, 1 μM 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. The medium was changed every 2 days thereafter and adipocytes were used on day 7–8. The cells used for experimentation were over 80% differentiated (as determined by microscopic evaluation).

Fatty Acid Treatment

Adipocytes and preadipocytes were incubated overnight (16–18 h) with OLE-BSA and PAL-BSA (molar ratio 5:1) in (DMEM) supplemented with 10% (v/v) fetal bovine serum. The final concentrations of fatty acids ranged from 0 to 1.0 mmol/L fatty acid, which is within the physiological range of fatty acids in fasting and fed states (0.3–1.4 mmol/L) [Cianflone et al., 2004].

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 [Baldo et al., 1993]. The activity of each ASP preparation was checked by its ability to stimulate TG 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 treatment with the indicated concentrations of fatty acids overnight, cells (adipocytes and preadipocytes) were preincubated in serum free medium for 2 h (containing the same concentration of fatty acids). Cells were then stimulated with ASP (1 μmol/L for 2 h) or insulin (0.1 μmol/L for 1 h) in serum free medium, then assayed for glucose transport. In preliminary experiments we determined that insulin stimulation was maximal after 1–2 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-deoxyglucose (50 μmol/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-deoxyglucose. 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-deoxyglucose uptake per mg soluble cell protein, and expressed relative to the basal untreated cells set as 100%.

Analysis of C5L2 mRNA Expression in Adipocytes and Preadipocytes

Following overnight fatty acid treatment of the adipocytes and preadipocytes, 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, all samples had a $A_{260}/A_{280} = 1.8–2.0$. For RT-PCR, 1 μg RNA was reverse-transcribed (final volume = 20 μL). Five microlitre of each RT reaction was amplified by PCR (final volume 25 μL). 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 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 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 fatty acids, cells were incubated in serum-free DMEM medium for 2 h containing the indicated fatty acids, 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 0–30 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). 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 Signaling Proteins in Adipocytes and Preadipocytes

Following overnight fatty acid treatment, adipocytes and preadipocytes were preincubated in serum free media for 2 h (containing fatty acids) and then stimulated maximally with ASP (5 μmol/L) as described [Maslowska et al., 2006]. The media was removed and 500 μL 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 μg/ml PMSF, 1 μg/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 microgram 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 PKC ζ and Ser 657 phosphorylated PKC α) 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-PKC α (Ser 657): sc-12356-R), anti-phosphorylated-PKCζ (1:300, p-PKC ζ (Thr 410)-R: sc-12894-R) and anti-actin (1:300, Actin (C-11): sc-1615) (all from Santa Cruz Biotechnology, Inc.). Antibody incubation was followed by incubation with an enzyme-linked antibody (peroxidase-conjugated goat anti-rabbit immunoglobulin secondary antibody, 1:5,000) for 60 min. The immobilized proteins were detected by ECL Plus kit from Amersham Biosciences (Piscataway, NJ) using Kodak film. Immunoblots were quantified by computer assisted photodocumentation system (ChemiImager Ready System, San Leonardo, CA).

Statistical Analyses

Results are expressed as means ± standard error of the mean (SEM). Each experiment was conducted multiple times with multiple (2–4) replicates within each experiment (n value as indicated). For comparison of the differences among groups, one-way or two-way ANOVA was used, followed by Bonferroni post hoc test. Statistical significance was set at P-value <0.05, where P NS indicates not significant.

RESULTS

We first verified the cell model of fatty acid induced insulin resistance as published elsewhere [Gao et al., 2004; Nguyen et al., 2005; Van Epps-Fung et al., 1997]. 3T3-L1 adipocytes and preadipocytes were incubated with increasing concentrations of the saturated fatty acid PAL and the mono-unsaturated fatty acid OLE overnight (as described in Materials and Methods), then evaluated for insulin-stimulated

glucose transport. PAL is the major saturated fatty acid in serum non-esterified fatty acids (NEFA) (23–32%) and adipose tissue TG (19.5–27%), while OLE is the major unsaturated fatty acid (25–33% and 41–50%) in serum NEFA and adipose tissue TG, respectively [Cornwell et al., 1962]. The final concentrations ranged from no additional fatty acid up to 1.0 mmol/L fatty acid, concentrations within the fasting-to-fed physiological ranges (0.3–1.4 mmol/L) [Cianflone et al., 2004]. While a concentration of 0.125 μmol/L had no effect on insulin-stimulated glucose transport, 0.5 μmol/L partially inhibited and 1.0 μmol/L of either PAL or OLE completely inhibited insulin-stimulated glucose transport in adipocytes (data not shown), consistent with previous publications [Van Epps-Fung et al., 1997; Gao et al., 2004; Nguyen et al., 2005].

As ASP stimulates glucose transport in both adipocytes and preadipocytes [Germinario et al., 1993; Maslowska et al., 1997], both cell types were examined for fatty acid-induced effects on ASP stimulation of glucose transport in 3T3 cells following overnight incubation with PAL and OLE. As shown in Figure 1A, ASP stimulated glucose transport by $198 \pm 18\%$, $P < 0.001$ versus PBS alone. PAL effectively reduced the ASP response, such that glucose transport returned to basal levels at 1 μmol/L fatty acid. Similar results were obtained with OLE. Similarly in preadipocytes (Fig. 1B), ASP increased glucose transport by $287 \pm 21\%$, $P < 0.001$ versus PBS alone, and overnight fatty acid treatment induced inhibition of the ASP response (Fig. 1). Neither PAL (Fig. 1) nor OLE (data not shown) had any effect on basal glucose transport in either adipocytes or preadipocytes. PAL and OLE were equally effective (*P* NS by two-way ANOVA) in both adipocytes and preadipocytes.

In order to evaluate the mechanism by which fatty acids induce ASP resistance we hypothesized that decreased ASP response might be mediated through decreased ASP receptor C5L2 expression (mRNA levels) and cell surface localization or through altered signalling mechanisms. In preliminary experiments, we confirmed that there was a rapid increase in C5L2 expression following induction of differentiation with a (twofold increase at 1 day, reaching a fourfold increase at day 9, (data not shown), consistent with previous data [MacLaren et al., 2007]. As shown in Figure 2A, following overnight treatment with PAL, C5L2 mRNA expression decreased in adipocytes by twofold ($P < 0.01$ ANOVA), although there was no significant change in C5L2 mRNA expression in preadipocytes (*P* NS). OLE treatment also resulted in significant decreases in C5L2 mRNA in adipocytes (Fig. 2B,C, fourfold decrease, $P < 0.01$ ANOVA). Again, there were no changes in C5L2 in preadipocytes (*P* NS).

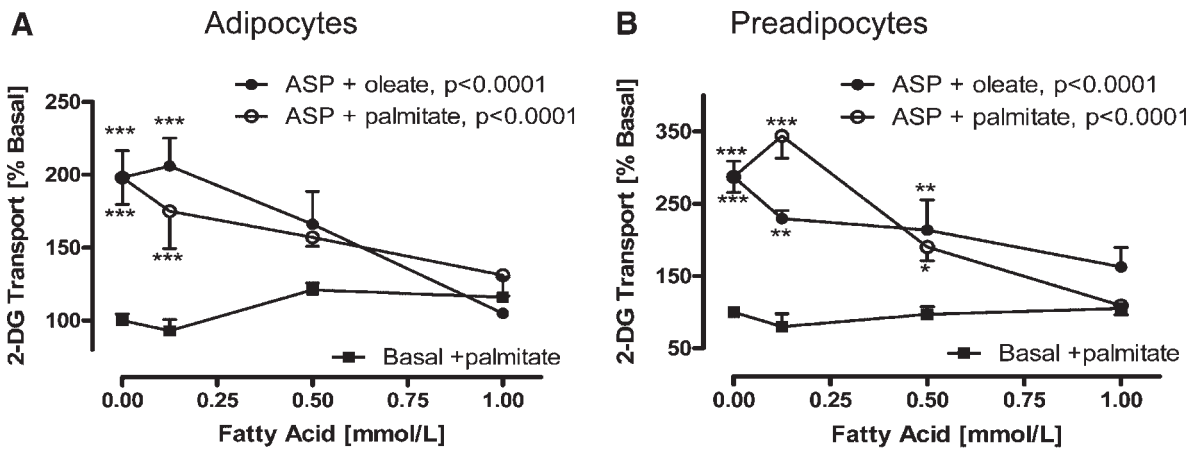

Fig. 1. Palmitate and oleate equally induced ASP resistance in adipocytes and preadipocytes. Adipocytes (**A**) and preadipocytes (**B**) were incubated overnight with the indicated concentrations of palmitate or oleate complexed to BSA, then stimulated with ASP (as described in Materials and Methods Section) and assessed for ^{3}H 2-deoxy-glucose uptake. Results are presented as average ± SEM, where basal glucose transport in the absence of fatty acids was set as 100% for average n = 7 (adipocytes) and n = 11 (preadipocytes) for each data point. Results were analyzed by two-way ANOVA (as indicated on graphs) followed by Bonferroni post-test where $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ for stimulation versus basal glucose transport (100%).

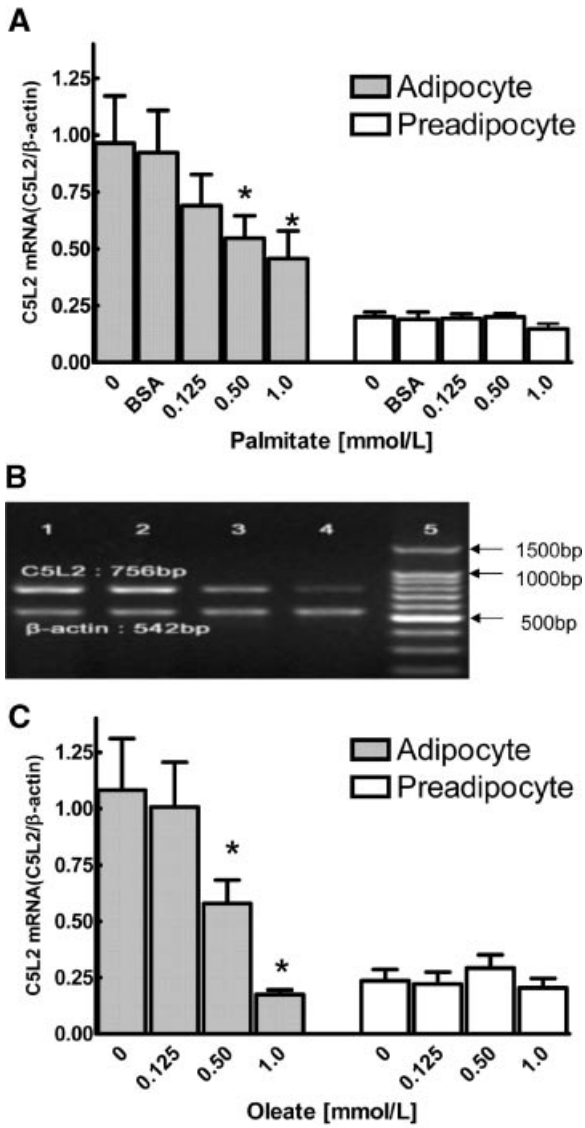

Fig. 2. Palmitate and oleate decreased C5L2 expression in adipocytes but not preadipocytes. Adipocytes and preadipocytes were incubated overnight with the indicated concentrations of palmitate (**A**) or oleate (**C**) complexed to BSA. C5L2 mRNA was then measured by RT-PCR. Results are presented as average ± SEM, for the ratio of C5L2 mRNA/β-actin for n = 4–5 for each data point. Results were analyzed by one-way ANOVA for adipocytes (palmitate $P < 0.01$ and oleate $P < 0.01$) and preadipocytes (palmitate $P <$ NS and oleate P NS) followed by Bonferroni post hoc test where $^*P < 0.05$ versus basal expression levels in the absence of fatty acids. **Panel B**: Representative RT-PCR results for C5L2 and β-actin in adipocytes for 0 (**lane 1**), 0.125 mmol/L (**lane 2**), 0.50 mmol/L (**lane 3**) and 1.0 mmol/L (**lane 4**) oleate, with the DNA bp ladder shown in **lane 5**.

Cell surface expression of C5L2 was evaluated by FACScan analysis, using an antibody specific for C5L2. As shown in Figure 3, treatment with both PAL and OLE resulted in decreases in cell surface expression of C5L2 in adipocytes (by two-way ANOVA, $P < 0.0001$ for both OLE and PAL) with PAL producing a slightly greater decrease ($P = 0.04$ vs. OLE). The decrease in % positive cells was reflected by a decrease in mean fluorescence intensity (MFI) from 42.3 (basal) to 15–17 (OLE treatment) and 18–22 (PAL treatment) over the range of OLE and PAL concentrations, 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 (data not shown).

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 protein, 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. We next looked at the signaling proteins identified in the ASP pathway. Previous studies [Baldo et al., 1995;

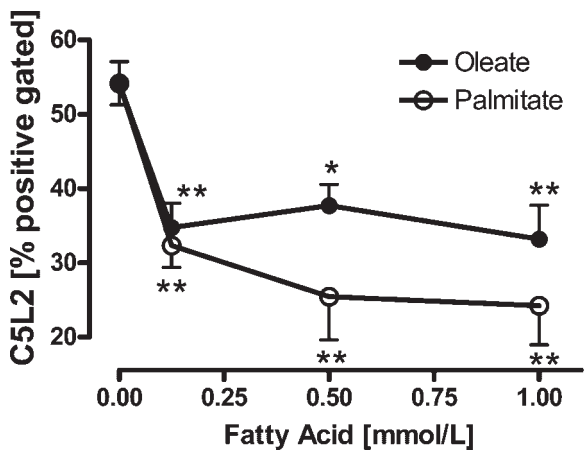

Fig. 3. Palmitate and oleate decreased cell surface C5L2 in adipocytes. Adipocytes were incubated overnight with the indicated concentrations of palmitate or oleate complexed to BSA. Cell surface C5L2 was then measured by immunofluoresence FACS analysis using anti-C5L2 antibody. Results are presented as average ± SEM, for the % positive cells (gated based on non-immune antibody set as 0% positive) for n = 5–6 for each data point. Results were analyzed by two-way ANOVA ($P < 0.0001$ for oleate and palmitate effect, with palmitate versus oleate $P = 0.04$), followed by Bonferroni post hoc test where $^*P < 0.05$ and $^{**}P < 0.01$ versus basal expression levels in the absence of fatty acids.

Maslowska et al., 2006] have demonstrated that ASP interaction with C5L2, a seven transmembrane serpentine receptor, triggers activation of phospholipase C and PI3 kinase as well as phosphorylation of Akt, consistent with a G protein coupled receptor (likely Gq). Further, ASP increased total PKC activity as well as translocation of PKC to the cell membrane, although the specific PKC has not been identified [Baldo et al., 1995]. Accordingly we evaluated G protein expression and levels of phosphorylated PKC isoforms.

As shown in Figure 4A, following ASP treatment of adipocytes, there were significant increases in Gβ protein, Gαq/11 protein, phosphorylated PKCα (p-PKCα) and p-PKCζ relative to actin ($P < 0.05$ to $P < 0.001$). Similar changes were detected in preadipocytes for Gβ, Gαq/11, p-PKCα) and p-PKCζ (data not shown). There was no change in actin levels for any treatment (data not shown).

The results for PAL and OLE treatment on the G proteins, Gβ and Gαq/11, in adipocytes are shown in Figure 5A,B. After overnight incubation of adipocytes with PAL and OLE, both Gβ and Gαq/11 were decreased by −43% to −62% ($P < 0.05$ to $P < 0.01$ as indicated) even in the absence of ASP treatment. Similar results were obtained with preadipocytes (data not shown). Further, both OLE and PAL effectively blocked the ASP stimulation, such that there was no longer any significant ASP stimulation, and values were comparable to basal levels (Fig. 5A,B). The effect of PAL and OLE on basal and ASP stimulation of PKC isoforms are shown in Figure 5C,D. PAL significantly decreased both basal and ASP stimulated p-PKCα and p-PKCζ (Fig. 5C,D, $P < 0.05$–0.01). OLE incubation, however, decreased basal and ASP-stimulated levels of p-PKCα only (Fig. 5C, −32% and −48% respectively, $P < 0.05$). Similar results were obtained with preadipocytes (data not shown).

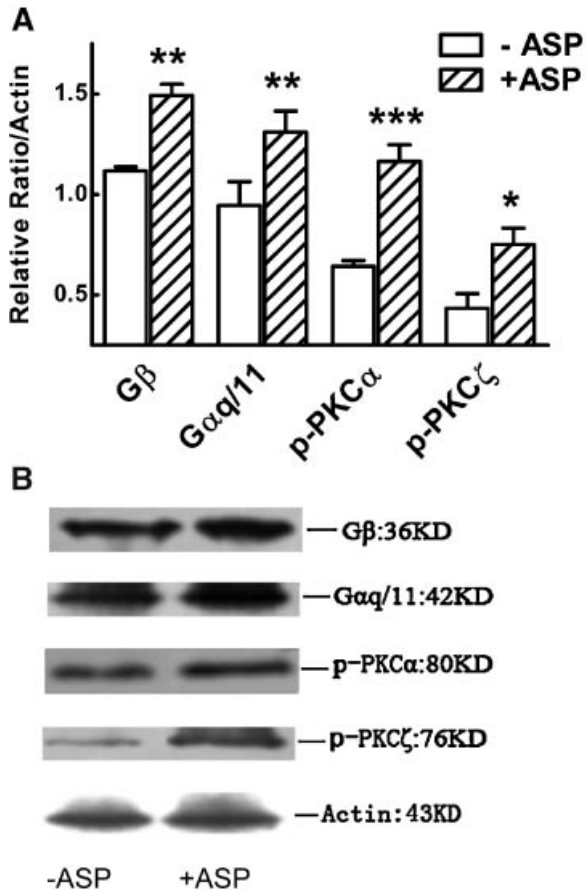

Fig. 4. ASP stimulated protein expression of specific G protein and protein kinase C isoforms in adipocytes. Adipocytes were incubated with ASP. The indicated proteins (Gβ, Gαq/11, phosphorylated (p)-PKCα and phosphorylated (p)-PKCζ were then measured by Western analysis using specific antibodies. **Panel A**: Results are presented as average ± SEM, for the ratio of the indicated protein/actin for n = 7 independent experiments. Results were analyzed by *t*-test for ASP stimulation versus untreated cells where $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. **Panel B**: Representative Western blots for ASP stimulation in adipocytes for untreated (left band) versus ASP stimulation (right band) for each protein, with the molecular weight of the band indicated.

DISCUSSION

The results in the present study indicate that the fatty acids PAL and OLE 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 signaling pathway including decreased activation of G protein and PKC isoforms. This study also provides direct evidence of the involvement of Gαq/11, which complements previous data. 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]. Further the present study demonstrates involvement of the PKC isoforms, PKCα and PKCζ. We have previously demonstrated the obligatory role of PKC activation and translocation in ASP activation [Baldo et al., 1995]. PKC activation is

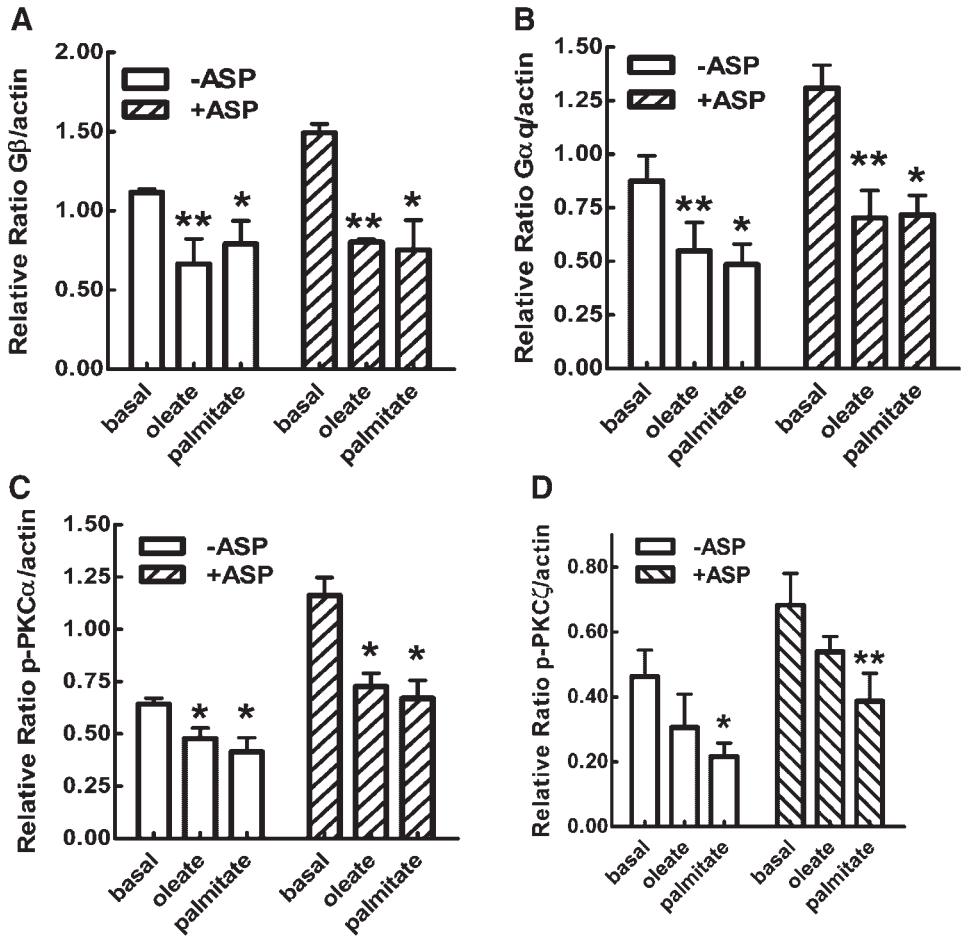

Fig. 5. Palmitate and oleate induced decreased G protein levels and protein kinase C isoforms in adipocytes stimulated with ASP. Adipocytes were incubated overnight with 1 mmol/L palmitate or oleate complexed to BSA then treated with ASP (as described in Materials and Methods Section). Gβ protein (**A**), Gαq/11 (**B**), phosphorylated (p)-PKCα (**C**) and phosphorylated (p)-PKCζ (**D**) 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 1 way ANOVA for all groups (all $P < 0.005$ for fatty acid effect) followed by Bonferroni all-pairwise post-test where $^{*}P < 0.05$ and $^{**}P < 0.01$ for fatty acid treatment versus no fatty acid.

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]. 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 translocation [Kotani et al., 1998; Hodgkinson et al., 2005; Liu et al., 2006].

The implication of ASP resistance is interesting from both a cellular and a physiological point of view. As fatty acids induce a decrease in cellular C5L2 and alterations in signaling pathways, this may possibly interfere with other ASP functions, such as stimulation of TG synthesis, fatty acid re-esterification, lipoprotein lipase efficiency, and inhibition of lipolysis. Unfortunately, one caveat of the present cell model is that the treatment with fatty acids precludes proper evaluation of these assays. Physiologically, a decrease in ASP function may enhance diversion of available 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 [Ohno et al., 2000; Kalant et al., 2005]. While the function in liver remains to be determined, in muscle, ASP appears to decrease muscle lipoprotein lipase efficiency (as

does insulin) [Faraj and Cianflone, 2004; Faraj et al., 2004], and we hypothesize that the presence of ASP resistance in muscle may contribute to muscle lipotoxicity, although this remains to be evaluated directly.

Fatty acids play a central role in inducing insulin resistance in the whole organism [Bastard et al., 2006], and this has been demonstrated experimentally in cell models [Van Epps-Fung et al., 1997; Gao et al., 2004; Nguyen et al., 2005]. Moreover, fatty acids also induce resistance to endothelin-mediated stimulation [Rachdaoui and Nagy, 2003] and EGF-mediated stimulation [Van Epps-Fung et al., 1997] of glucose transport in 3T3-L1 cell models. In the present study, we have demonstrated that fatty acids also interfere with ASP mediated function. Further, fatty acids may mediate their effects on insulin resistance through stimulation of TNFα secretion [Suganami et al., 2005]. TNFα, IL-1β, IL-6 and macrophage secreted factors, all of which are associated with obesity and insulin resistance in vivo [Bastard et al., 2006], have also been shown to directly cause insulin resistance in 3T3-L1 cells models [Tagami et al., 2002; Lagathu et al., 2003, 2006; Permana et al., 2006]. Interestingly, the induction of a resistant state, whether it be resistance to ASP, insulin, endothelin or EGF, appears to be mediated by interference with common signaling molecules including βarrestin, Gαq, PI3 kinase, Akt, and various PKC isoforms [Imamura et al., 2001; Ogihara et al., 2004; Liu et al., 2006; Xie et al., 2006].

This common mechanism in the development of a resistant state has physiological implications. Not only do multiple factors appear to contribute to insulin resistance, as demonstrated both physiologically [Bastard et al., 2006], and in cell models (see above), but leptin resistance, adiponectin resistance and ASP resistance have all been proposed to contribute to (or be present in) an insulin resistant state [Ozata et al., 2002; Munzberg and Myers, 2005; Dyck et al., 2006]. This suggests the presence of a general adipose tissue resistant state.

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 signaling parameters, and provides a basis for understanding and examining ASP resistance in human studies.

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