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Hormone and Pharmaceutical Regulation of ASP Production in 3T3-L1 Adipocytes

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ABSTRACT

Several studies have demonstrated increases in acylation stimulating protein (ASP), and precursor protein C3 in obesity, diabetes and dyslipidemia, however the nature of the regulation is unknown. To evaluate chronic hormonal and pharmaceutical mediated changes in ASP and potential mechanisms, 3T3-L1 adipocytes were treated with physiological concentrations of relevant hormones and drugs currently used in treatment of metabolic diseases for 48 h. Medium ASP production and C3 secretion were evaluated in relation to changes in adipocyte lipid metabolism (cellular triglyceride (TG) mass, non-esterified fatty acid (NEFA) release and real-time FA uptake). Chylomicrons increased ASP production (up to $411 \pm 133\%$ $P < 0.05$), while leptin, triiodothyronine, and β-blockers atenolol and propranolol had no effect. Dexamethasone, lovastatin, rosiglitazone and rimonabant decreased ASP production (-53 to -85%, $P < 0.05$), associated with a decrease in the precursor protein C3 (-37% to -65%, $P < 0.01$). By contrast, epinephrine, progesterone, testosterone, angiotensin II and metformin also decreased ASP (-54% to -100%, $P < 0.05$), but without change in precursor protein C3, suggesting a direct effect on convertase activity, possibly mediated by interference (except metformin) due to marked increases in NEFA (5.6–31-fold, increased $P < 0.05$). Both lovastatin and metformin induced decreases in ASP were also associated with decreased TG mass (maximal -60%, $P < 0.05$) and real-time FA uptake (maximum -75%, $P < 0.05$), suggesting a change in adipocyte differentiation status. These in vitro results are consistent with in vivo ASP profiles in subjects, and suggest that ASP may be regulated through precursor C3 availability, convertase activity and differentiation status. J. Cell. Biochem. 109: 896–905, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ADIPOCYTE; FATTY ACID UPTAKE; ADIPOKINE SECRETION

Recently, it has been widely recognized that adipose tissue is not only a storehouse for energy in the form of triglyceride (TG), but it is also a metabolically active organ producing numerous secreted proteins and enzymes such as lipoprotein lipase, cholesteryl ester transfer protein and apolipoprotein E as well as various hormones and cytokines known globally as adipokines, such as leptin, adiponectin, TNFα, to name but a few [Havel, 2004]. Both human and murine white adipose tissue have been shown to synthesize and secrete a number of complement proteins such as C3, factor B and factor D (also known as adipsin), all proteins involved in the alternate complement pathway [Maslowska et al., 1997].

Acylation stimulating protein (ASP) is identical to C3adesArg [Cianflone et al., 2003], and is the product of N-terminal cleavage of C3 through its interaction with factors B and adipsin, generating C3a. As an anaphylatoxin, C3a is rapidly cleaved by carboxypeptidase B or carboxypeptidase N, removing the carboxyl-terminal arginine and generating C3adesArg or ASP [Cianflone et al., 2003]. In plasma, most of the C3a is present in C3adesArg form [Cianflone et al., 2003]. ASP is an adipose tissue-derived hormone that stimulates TG synthesis and glucose transport and also inhibits hormone-sensitive lipase [Cianflone et al., 2003].

ASP levels in vitro and in vivo are modulated by several mechanisms. In vitro, human preadipocytes produce no ASP, but upon differentiation, production increases, simultaneously with an increase in triglyceride synthesis [Cianflone et al., 2003]. Further, in differentiated human adipocytes, insulin and especially dietary lipoproteins chylomicrons markedly increase ASP as well as C3 production, mediated through retinoic acid response (RAR) elements

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[Maslowska et al., 1997; Scantlebury et al., 1998; Scantlebury et al., 2001]. By contrast, NEFA and other lipoproteins had little effect [Scantlebury et al., 1998]. Choy et al. [1992] have demonstrated an increased production of C3, factor B and adipsin during differentiation of 3T3-L1 preadipocytes to adipocytes, resulting in production of ASP and/or C3a. The production of ASP precursors such as C3 or the converting enzyme proteins (factor B and adipsin) have been shown to be regulated by TNF-α, interleukin (IL)-1, IL-4, IL-6, IL-17, aldosterone, estrogen and glucocorticoids in in vitro models in various cells [Kalant et al., 2003].

In vivo, ASP is increased in humans with obesity and associated disorders (cardiovascular disease, diabetes) and, conversely, is decreased with weight loss or exercise [Cianflone et al., 2003]. In mice, plasma ASP also increases with obesity, and is reduced in lean/lipodystrophic mice [Paglialunga et al., 2008]. Further, acute increases of ASP in the postprandial state, which are increased in obesity and diabetes, have been demonstrated in the adipose tissue milieu, as shown in human studies [Saleh et al., 1998; Kalant et al., 2000; Tahiri et al., 2007].

Using 3T3-L1 adipocytes, the aims of the present study were to evaluate the chronic effects of various hormones, including stress hormones and sex steroid hormones, and to investigate the effects of pharmaceutical drugs commonly used in treatment of cardiovascular diseases and metabolic syndrome on ASP production. The factors evaluated were chosen based on the following criteria: (i) target receptors are present in adipocytes, (ii) factors influence an adipose biological process and (iii) in vivo relevance to ASP with (iv) appropriate physiological concentrations used. The potential mechanism for changes was explored by determining the precursor C3, the proportion of C3 converted to ASP and the relationship to lipid metabolism including cell TG mass, lipolysis (NEFA secretion) and TG synthesis measured as real-time fatty acid uptake into adipocytes.

MATERIALS AND METHODS

MATERIALS

Dexamethasone, epinephrine, angiotensin II, triiodothyronine, progesterone, estradiol, testosterone, atenolol, propranolol, lovastatin, rimonabant, metformin were obtained from Sigma (St. Louis). Leptin was purchased from Cedarlane (Burlington, Ontario, Canada). Rosiglitazone was acquired from Sanofi-Aventis (Montpellier, France).

CHYLOMICRON PREPARATION AND FRACTIONATION

Blood was collected from healthy subjects with normal lipid profiles 3 h after a high-fat meal into Vacutainer tubes containing EDTA and placed on ice. Immediately, plasma was isolated by low-speed centrifugation at 4°C, and chylomicrons were isolated by discontinuous preparative ultracentrifugation as described previously. Plasma was layered under 1.006 g/ml salt solution, and chylomicrons isolated by flotation after centrifugation for 30 min at 40,000*g* (11°C) [Scantlebury et al., 2001]. TG concentrations of chylomicrons were measured by enzymatic colorimetric kits from Roche Diagnostics as described below.

CELL CULTURE AND DIFFERENTIATION OF 3T3-L1 CELLS

Murine preadipocytes (3T3-L1) cells were purchased from the American Type Culture Collection (Manassas, VA). All tissue culture media, such as Dulbecco's modified Eagle's medium/F-12 (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS) and tissue culture supplies were from Gibco (Burlington, Ontario, Canada). Preadipocytes (3T3-L1) were cultured using DMEM/F12 supplemented with 10% FBS medium (regular medium), and cells were maintained at low passage number and maintained at 37°C in 5% CO_2 incubator. Preadipocytes were plated on 24-well culture plates at a concentration of 3×10^4 cells/cm^2 and grown to confluence. Two days after, cells were differentiated using a differentiation medium containing 10 μg/ml insulin, 1 μM dexamethasone, and 500 μM isobutylmethylxanthine (IBMX). Two days later, the differentiation medium was removed and supplementation medium containing 10 μg/ml insulin was added. Two days later, the media was changed to regular medium. Differentiated cells (at least 90% of which showed an adipocyte phenotype by accumulation of lipid droplets) were used on days 8–12 after initiation of differentiation.

CELL TREATMENT

Adipocytes were incubated 48 h in serum-free medium with the indicated final concentrations of hormone or drug treatment to evaluate a chronic effect. Cells incubated in serum-free medium alone were used as baseline control. Chylomicrons with or without insulin were incubated both for 18 and 48 h. After the indicated treatment times, the medium was removed and frozen immediately at −80°C for later measurement of ASP, C3, and NEFA levels.

MEDIUM ASP, C3, AND NEFA DETERMINATION

Mouse ASP (C3a) ELISA reagents were from BD Pharmingen (Franklin Lakes, NJ). ASP was measured using a sandwich ELISA. Note the antibody used in this ELISA is specific for a neoepitope exposed in mouse for C3a and the desArginated form ASP and does not cross-react with the native protein C3. Purified rat anti-mouse C3a monoclonal antibody was coated at 2 μg/ml in coating buffer (8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl; pH 7.0) at 100 μl per well in a 96-well plate overnight at 4°C. Plates were blocked with blocking buffer (1% bovine serum albumin in PBS, 250 μl/well, 2 h at room temperature). Following washing (0.05% (v/v) Tween in PBS, three times), purified mouse C3a (0.391–50 ng/ml) and samples (conditioned culture media) were added at 100 μl/well. The sealed plates were incubated for 2 h at 37°C, washed four times, then incubated 1 h at 37°C with 100 μl of 1.0 μg/ml biotin rat anti-mouse C3a in blocking buffer and washed four times. Following incubation (30 min, 37°C) with avidin–horseradish peroxidase conjugate (AV-HRP, diluted 1:1,000 in blocking buffer), plates were washed five times, color reaction was initiated with 100 μl of TMB substrate reagent (reagent A: reagent B, mixed 1:1) and at 5 min, the reaction was stopped with 50 μl TMB stop solution (1.0 M phosphoric acid). Absorbance was read at 450 nm (reading) and 570 nm (blank). ASP concentration was calculated by nonlinear regression using Prism software (Graphpad Software, San Diego, CA). Mouse complement C3 ELISA kit (Kamiya

Biomedical, Seattle, WA) measures total C3, including native and breakdown products, was used according to the manufacturer's instructions and calculated by linear regression. NEFA levels were measured using enzymatic colorimetric kits (Wako Chemical, Richmond, VA) according to the manufacturer's instructions and calculated by linear regression curve.

FATTY ACID UPTAKE IN ADIPOCYTES

QBT™ fluorescent fatty acid uptake assay kit (Molecular Devices, Sunnyvale, CA) was used according to the manufacturer's instructions. Following removal of the culture medium from the cells, 200 μl of SF medium was added followed by 200 μl of BODIPY reagent (diluted in 0.2% BSA in Hanks Balanced salt solution). BODIPY-fatty acid (FA) uptake was measured in real-time every 20 s over 60 min in a bottom-reading fluorescent microplate reader. Area-under-the curve of FA incorporation into adipocytes versus time was calculated by trapezoidal method (Prism, Graphpad Software).

TG MASS AND PROTEIN IN ADIPOCYTES

Following measurement of BODIPY-FA uptake, solutions were removed, cells washed twice gently with ice-cold PBS, and the neutral lipids were extracted with 1 ml of heptane–isopropanol (3:2) overnight at 4°C, and rinsed with an additional 500 μl of the same solvent mix. Lipid extracts were evaporated to dryness in a centrifuge-evaporator (Canberra-Packard Canada) and redissolved in 100 μl heptane–isopropanol (3:2). TG mass was measured using an enzymatic colorimetric assay (Roche Diagnostics, Indianapolis, IN). Soluble protein was solubilized overnight in 1 ml of 0.3 M NaOH and protein levels were determined using the Bradford assay (Bio-Rad, Hercules, CA). TG mass and protein concentration were calculated by linear regression curve.

STATISTICAL ANALYSES

Results are presented as mean ± standard error of the mean (SEM). Each experiment was performed three to four times, with triplicate analyses in each experiment (total n = 9–12). ASP and C3 production were quantified in each experiment, normalized to cell protein (ng/ml media/μg cell protein), and reported relative to the untreated control (CTL = 100%), which was cells incubated in serum-free medium alone. TG cell mass, NEFA medium mass and FA uptake into adipocytes were all normalized to cell protein. Proportion of C3 converted to ASP is calculated as % ASP/C3 (ng/ng). Groups were compared by *t*-tests, one- or two-way ANOVA followed by Dunnett's, repeated measures ANOVA (RM-ANOVA) or linear trend post-hoc test using Prism software (Graphpad Software) for graphs and statistical analysis. Statistical significance was set as $P < 0.05$, where NS indicates not significant.

RESULTS

EFFECT OF CHYLOMICRONS ON ASP AND C3 PRODUCTION, NEFA RELEASE, TG MASS, AND FA UPTAKE

Previous studies using human differentiated adipocytes demonstrated a time and concentration dependent stimulatory effect of chylomicrons and insulin on ASP and C3 production [Scantlebury et al., 1998]. To validate the present model of 3T3-L1 mouse adipocytes, increasing chylomicron concentrations at two incubation times, 18 and 48 h, were tested. As shown in Figure 1A, ASP production significantly increased in a dose-dependent manner

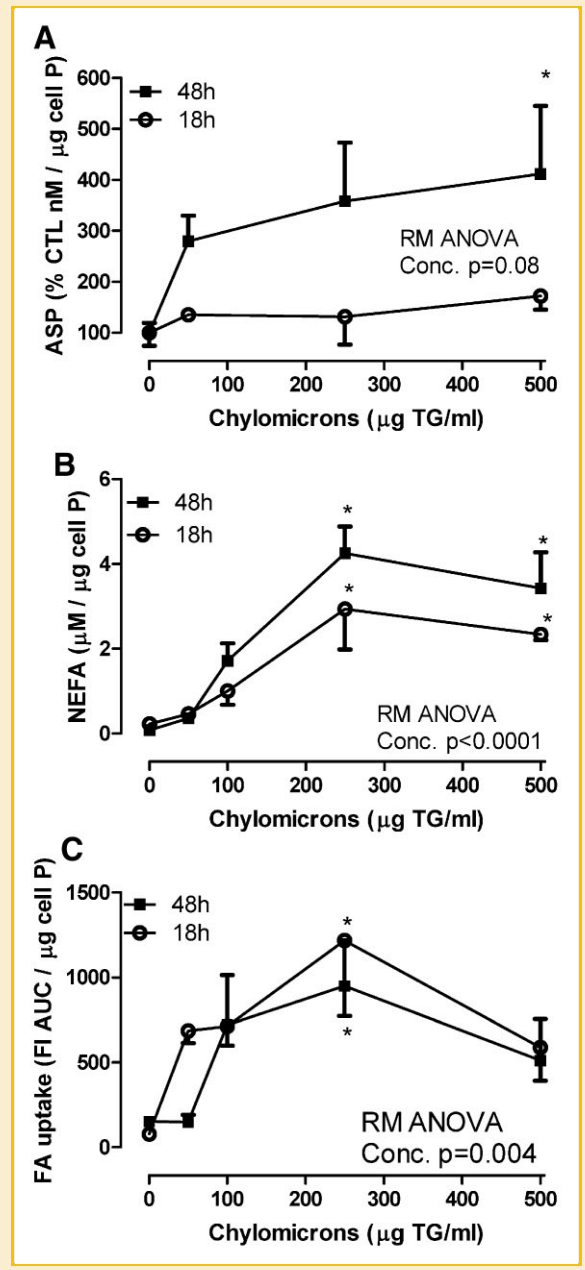

Fig. 1. Chylomicrons stimulate ASP production, NEFA release, and FA uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 18 (circles) or 48 h (squares) with the indicated concentration of chylomicrons. Medium ASP (Panel A), NEFA levels (B) and FA uptake measured as fluorescent fatty acid uptake calculated as area-under-the curve (FI-AUC, panel C) are presented. For ASP (Panel A) results are presented relative to baseline CTL set as 100%. All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) and normalized to cell protein (cell P) where RM (repeated measured) ANOVA results are present in graphs, and $^{*}P < 0.05$ relative to control (CTL).

after 48 h incubation with chylomicrons ($P < 0.05$), with little increase noted at 18 h. At the highest concentration of chylomicrons, there was an increase in C3 conversion to ASP ($100 \pm 21\%$ CTL, $445 \pm 126\%$ chylomicrons, $P < 0.05$) without an effect on C3 secretion (data not shown). In Figure 1B, both 18 and 48 h incubation significantly increased NEFA media concentration (maximum 11-fold at 18 h and 50-fold at 48 h, $P < 0.0001$) as a consequence of both NEFA release from adipocytes and lipoprotein lipase hydrolysis of chylomicrons. No significant change was seen in TG mass after 18 or 48 h incubation with chylomicrons at different concentrations (data not shown). Both 18 and 48 h incubation significantly increased FA uptake up to 14-fold (18 h) and 5-fold (48 h) (Fig. 1C, $P = 0.004$).

On the other hand, the combination of chylomicrons and insulin markedly stimulated C3 secretion at both 18 and 48 h ($290 \pm 98\%$, $P < 0.01$ and $439 \pm 20\%$, respectively, $P < 0.001$). The effect on C3 production further increased ASP production at 48 h (CTL $100 \pm 19.1\%$, [insulin + chylomicrons] $490 \pm 6.4\%$, $P < 0.01$). Note, at baseline (CTL), C3 is far in excess of ASP, where ASP $= 0.23 \pm 0.08$ ng/ml/μg cell protein and C3 $= 39.4 \pm 8.7$ ng/ml/μg cell protein, and on average only $0.76 \pm 0.23\%$ of C3 is converted to ASP (ng/ng). These results on ASP are similar to the results obtained previously in human adipocytes [Maslowska et al., 1997; Scantlebury et al., 1998], and confirm the potential of the 3T3-L1 adipocyte model. Accordingly, in order to evaluate chronic hormone and drug effects, 3T3-L1 adipocytes were incubated for 48 h, and protein secretion and metabolic status evaluated.

EFFECTS OF STRESS HORMONES (DEXAMETHASONE, EPINEPHRINE, AND ANGIOTENSIN II) ON ASP PRODUCTION, C3 SECRETION, AND NEFA RELEASE

3T3-L1 adipocytes were incubated 48 h with increasing concentrations of dexamethasone, epinephrine and angiotensin II. Dexamethasone, a steroid hormone [Sakoda et al., 2000] is also a component of the adipocyte differentiation media (see Materials and Methods Section). Epinephrine acts via adrenergic receptors to stimulate lipolysis in adipocytes [Chernick et al., 1986]; angiotensinogen is both produced by and acts on adipocytes [Giacchetti et al., 2002]. In differentiated adipocytes, dexamethasone treatment decreased ASP production primarily at low concentrations (Fig. 2A; $P < 0.05$). At high dose, dexamethasone reverses the inhibition on ASP. Hypothetically, this phenomenon could be explained by a switch from a glucocorticoid repressor effect (at low dose) to an activator effect at high dose due to mineralocorticoid receptor driven gene expression in 3T3-L1 adipocytes [Caprio et al., 2007]. C3 production is also reduced with dexamethasone treatment at 10^{-7} M (Fig. 2B; $P < 0.01$), resulting in no change in ASP/C3 conversion ratio (Fig. 2C; NS). By contrast, 10^{-6} M epinephrine treatment completely blocked ASP production (Fig. 2D; $P < 0.001$) without affecting C3 secretion level (Fig. 2E; NS) and the conversion ratio of C3 to ASP is then abolished (Fig. 2F; $P < 0.001$). As shown in Figure 2G, only the highest concentration of angiotensin II used (5×10^{-6} M) inhibits ASP production ($P < 0.01$) without affecting C3 level or ASP and C3 ratio (data not shown).

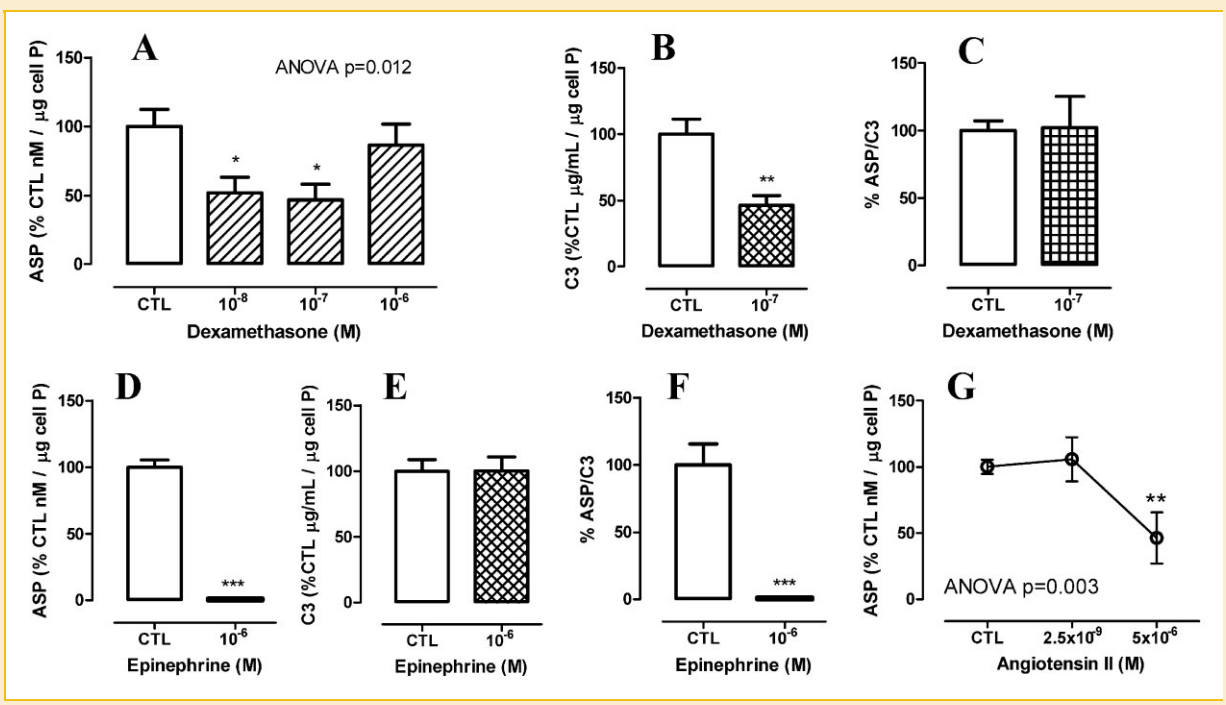

Fig. 2. Dexamethasone, epinephrine and angiotensin II inhibit ASP production in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 48 h with the indicated concentrations of dexamethasone, epinephrine or angiotensin II. Medium ASP (Panels A,D,G) and C3 (Panels B,E) were measured as ng/ml/μg cell protein (cell P) and are presented relative to baseline CTL set as 100%. ASP/C3 ratio (Panels C,F) is calculated based on ASP (ng)/C3 (ng). All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) and normalized to cell protein (cell P) where ANOVA results are presented in graphs, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to control (CTL).

In order to evaluate mechanisms related to ASP production, at the same time we examined cellular TG mass and two key adipocyte functions: lipolysis (measured as NEFA release) and TG storage (measured as real-time fluorescent FA uptake and incorporation into TG). As shown in Figure 3A, there was a concentration dependent effect of dexamethasone on NEFA release: at high concentrations NEFA release was decreased by up to 43% ($P < 0.05$ by linear regression). With epinephrine, there was an inverse relationship between ASP production and NEFA release (Fig. 3B), with a 5.6-fold increase in NEFA at 10^{-6} M treatment ($P < 0.001$), while angiotensin II also increased NEFA release 10-fold at the higher concentration ($P < 0.05$, Fig. 3B). Further, cellular TG mass and fatty acid uptake were also evaluated. None of these three hormones (dexamethasone, epinephrine, and angiotensin II) had any effect (data not shown).

EFFECT OF LEPTIN AND THYROID HORMONE (TRIIODOTHYRONINE, T3) ON ASP PRODUCTION, TG MASS, NEFA RELEASE, AND FATTY ACID UPTAKE

Leptin, an adipocyte specific hormone, influences adipokine expression and secretion [Havel, 2004]. Triiodothyronine is a component of human adipocyte differentiation medium [Jiang et al., 2004]. Leptin had no effect on ASP production over a wide range of concentrations (CTL $100.0 \pm 12.4\%$; 10^{-11} M leptin $86.3 \pm 14.9\%$; 10^{-9} M leptin $98.8 \pm 13.5\%$; 10^{-6} M leptin $88.4 \pm 16.0\%$; pNS), nor did it have an effect on NEFA and FA uptake (data not shown), but decreased TG mass at the highest concentration used ($P < 0.05$ with linear trend analysis, data not shown). With triiodothyronine, there were no significant changes in ASP production (CTL $100 \pm 4.4\%$; 10^{-9} M T3 $88.9 \pm 23\%$; 10^{-7} M T3 $80.1 \pm 26\%$; 10^{-6} M T3 $101.2 \pm 6\%$; pNS), TG mass, NEFA or FA uptake (data not shown).

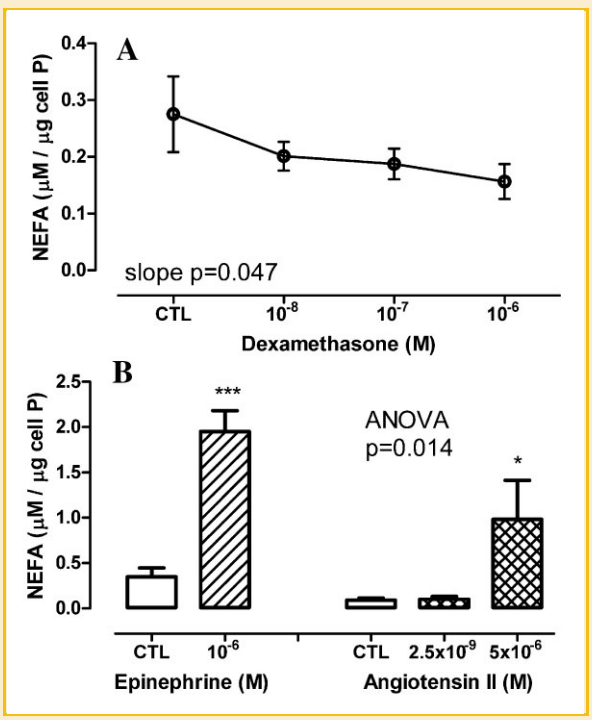

Fig. 3. Dexamethasone, epinephrine and angiotensin II effect on NEFA release in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 48 h with the indicated concentrations of dexamethasone, epinephrine or angiotensin II and NEFA medium content measured as μM/μg cell protein (cell P). All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) and normalized to cell protein (cell P) where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to control (CTL).

EFFECTS OF SEX HORMONES ON ASP PRODUCTION, C3 SECRETION, AND NEFA RELEASE

Both adipocytes and preadipocytes are responsive to sex hormones [Mayes and Watson, 2004]. 3T3-L1 adipocytes were incubated with increasing concentrations of estradiol, progesterone or testosterone then evaluated for ASP and C3 production. As shown in Figure 4A, estradiol had a slight but not significant effect on ASP production, while treatment with progesterone and testosterone resulted in decreased ASP production at the highest concentration tested (ANOVA $P < 0.05$, at 10^{-6} M, $P < 0.05$), without significantly affecting C3 (Fig. 4B) while the conversion ratio of C3 to ASP decreased with testosterone ($P = 0.03$, Fig. 4C). On the other hand, a

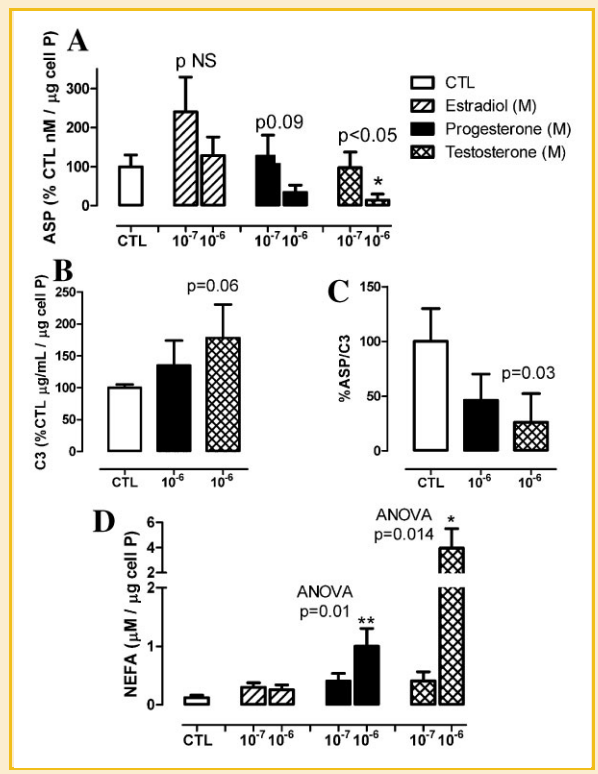

Fig. 4. Sex steroid hormones inhibit ASP production and stimulate NEFA release in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 48 h with the indicated concentrations of estradiol, progesterone or testosterone. Medium ASP (Panel A) and C3 (Panel B) were measured as ng/ml/cell P and are presented relative to baseline CTL set as 100%. ASP/C3 ratio (Panel C) is calculated based on ASP (ng)/C3 (ng). Medium NEFA content (Panel D) was measured as μM/μg cell protein. All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) and normalized to cell protein (cell P) where $^{*}P < 0.05$, $^{**}P < 0.01$ relative to CTL.

TABLE I. β-Blocker Effect on ASP Production, C3 Secretion, ASP/C3 Ratio, NEFA Release, and TG Mass in 3T3-L1 Adipocytes

	ASP (% CTL)	C3 (% CTL)	ASP/C3 ratio	NEFA (μM/μg cell P)	TG (nmol/μg cell P)
CTL	100.0 ± 12.0	100.0 ± 8.8	100.0 ± 15.5	0.35 ± 0.10	0.85 ± 0.09
Atenolol	89.6 ± 17.2	62.9 ± 12.4*	196.5 ± 50.7	0.50 ± 0.10	0.86 ± 0.09
Propranolol	97.4 ± 28.9	64.8 ± 13.8*	164.8 ± 44.5	0.70 ± 0.27	0.65 ± 0.13
Atenolol + propranolol	97.2 ± 23.3	68.9 ± 7.0*	151.0 ± 33.6	0.64 ± 0.10	0.82 ± 0.06

3T3-L1 adipocytes were incubated for 48 h with 10^{-5} M atenolol, propranolol, or both combined (atenolol + propranolol). Medium ASP, C3, and NEFA levels were measured, as well as cell TG and cell protein. For ASP and C3, results are measured as ng/ml/μg cell protein (cell P) and are presented relative to baseline control (CTL) set as 100%. ASP/C3 ratio is calculated based on ASP (ng)/C3 (ng). Results for medium NEFA and cell TG mass are expressed relative to cell protein (cell P). All results are presented as mean ± SEM for 3-4 experiments each in triplicate (n = 9–12 values) where $^{*}P < 0.05$ relative to control (CTL).

significant increase in NEFA release was detected at high concentrations of testosterone ($P = 0.014$) and progesterone ($P = 0.01$, Fig. 3D). None of the sex hormones induced any significant change in TG mass (data not shown).

EFFECTS OF β-BLOCKERS ON ASP PRODUCTION, C3 SECRETION, NEFA RELEASE, AND TG MASS

The β-adrenergic receptor family consists of β_1, β_2, and β_3 subtypes, all three of which are expressed in adipose tissue, where they stimulate lipolysis [Mulder et al., 2005]. Accordingly, we examined the effects of atenolol (β_1 specific [Galletti et al., 1989]), propranolol (adrenergic non-specific [Mulder et al., 2005]), and combination (atenolol + propranolol) on ASP and C3 production, NEFA release and TG mass. As shown in Table I, while there was no effect on ASP production, there was a significant decrease of the precursor protein C3 with the treatments. While the relative proportion of C3 converted to ASP tended to increase, the change was not significant (Table I). Further, there were no significant changes in NEFA release or 3T3-L1 cellular TG mass.

EFFECTS OF LOVASTATIN ON ASP AND C3 PRODUCTION, NEFA RELEASE, TG MASS, AND FA UPTAKE

Statin compounds, including lovastatin, inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase activity, the rate-limiting enzyme in cholesterol and isoprenoid synthesis. Adipose tissue contains a large reserve of free cholesterol [Prattes et al., 2000], and adipocytes are influenced by statin treatment [Yin et al., 2007]. As shown in Figure 5A, lovastatin treatment induced a concentration dependent decrease in ASP production ($P = 0.0043$ ANOVA). A lower C3 production (Fig. 5B) contributed to this decrease in ASP. It was further mediated by an effect on the conversion rate of C3 to ASP, shown by a decreased ASP/C3 ratio (Fig. 5C). NEFA release did not change significantly (data not shown). Lovastatin induced significant decreases in both cellular TG

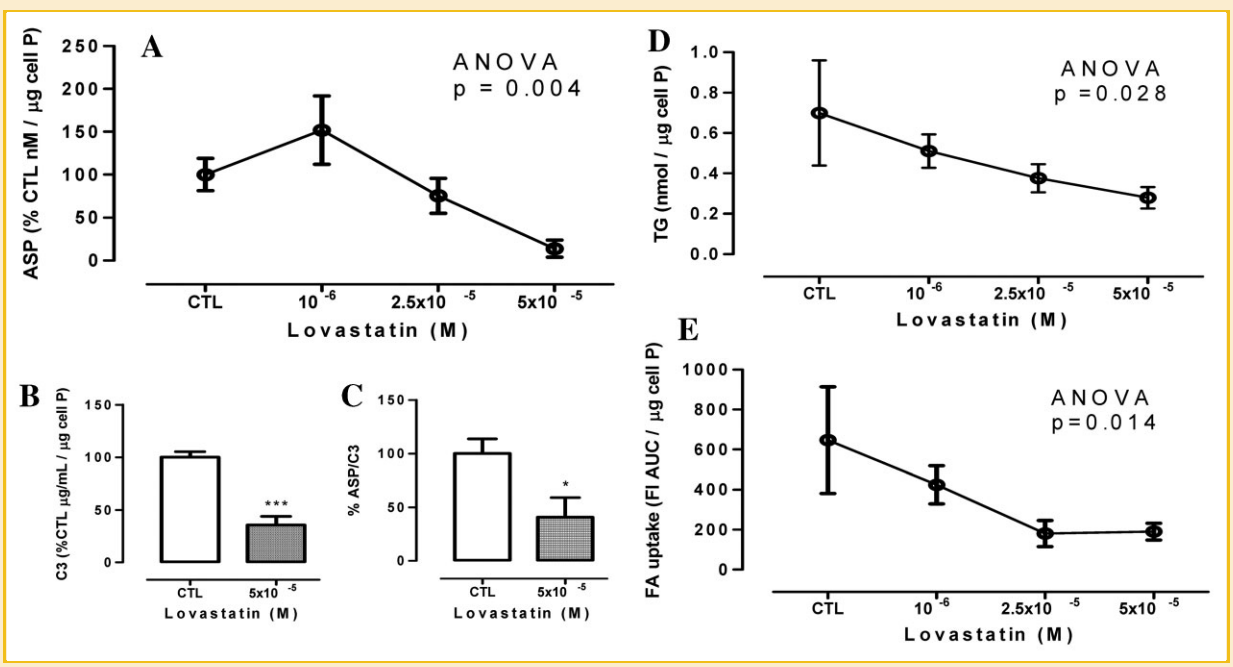

Fig. 5. Lovastatin decreases ASP production, C3 secretion, cell TG mass and FA uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 48 h with the indicated lovastatin concentrations. Medium ASP, C3, and NEFA levels were measured, as well as FA uptake, TG mass and cell protein. For ASP (Panel A) and C3 (Panel B), results are measured as ng/ml/μg cell protein (cell P) and are presented relative to baseline control (CTL) set as 100%. ASP/C3 ratio (Panel C) is calculated based on ASP (ng)/C3 (ng). Results for cell TG mass (Panel D) and FA uptake (Panel E) are all expressed relative to cell protein (cell P). All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) where $^{*}P < 0.05$, $^{***}P < 0.001$ relative to control (CTL).

TABLE II. Rosiglitazone Effect on ASP Production, C3 Secretion, ASP/C3 Ratio, NEFA Release, and TG Mass in 3T3-L1 Adipocytes

	ASP (% CTL)	C3 (% CTL)	ASP/C3 ratio	NEFA (μM/μg cell P)	TG (nmol/μg cell P)	FA uptake (Fl-AUC/μg cell P)
CTL	100.0 ± 4.0	100.0 ± 5.16	100.0 ± 2.55	0.168 ± 0.048	0.45 ± 0.06	300.6 ± 83.8
10^{-9} M Rosi	22.1 ± 8.6***	ND	ND	0.111 ± 0.038	0.40 ± 0.05	848.9 ± 274.7*
10^{-7} M Rosi	24.8 ± 6.2***	53.6 ± 2.9***	62.1 ± 13.2**	0.170 ± 0.043	0.37 ± 0.05	260.6 ± 47.1
10^{-6} M Rosi	39.9 ± 11.7***	ND	ND	0.258 ± 0.081	0.35 ± 0.03	303.5 ± 70.9

3T3-L1 adipocytes were incubated for 48 h with the indicated concentrations of rosiglitazone. Medium ASP, C3, and NEFA levels were measured, as well as FA uptake, cell TG, and cell protein. For ASP and C3, results are measured as ng/ml/μg cell protein (cell P) and are presented relative to baseline control (CTL) set as 100%. ASP/C3 ratio is calculated based on ASP (ng)/C3 (ng). Results for medium NEFA, cell TG mass and fluorescent FA uptake are expressed relative to cell protein (cell P). All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) where $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to control (CTL) and ND indicates not determined.

mass (Fig. 5D, $P = 0.028$ ANOVA) and FA uptake (Fig. 5E, $P = 0.014$ ANOVA), suggesting an effect on de-differentiation of adipocytes [Nishio et al., 1996].

EFFECTS OF ROSIGLITAZONE ON ASP AND C3 PRODUCTION, NEFA RELEASE, TG MASS, AND FA UPTAKE

Rosiglitazone, a thiazolidinedione (TZD), is a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist that induces preadipocyte differentiation to adipocytes [Fu et al., 2005], but by contrast, enhances lipid catabolism in adipocytes [Wang et al., 2007]. As shown in Table II, all concentrations of rosiglitazone decreased ASP production ($P < 0.001$) and this decrease was associated with both decreased C3 secretion ($P < 0.001$) and a lower conversion rate of C3 to ASP ($P < 0.01$) after treatment with rosiglitazone 10^{-7} M. However, there were no differences in adipocyte TG mass or NEFA release, while only the lowest concentration of rosiglitazone (10^{-9} M) significantly increased FA uptake into adipocytes ($P < 0.05$).

EFFECTS OF RIMONABANT ON ASP AND C3 PRODUCTION, NEFA SECRETION, TG MASS, AND FA UPTAKE

Rimonabant acts via the cannabinoid-1 (CB1) receptor, present in adipocytes, inhibiting preadipocyte cell proliferation and increasing adiponectin mRNA expression in adipose tissue of obese fa/fa rats [Gary-Bobo et al., 2006]. Rimonabant decreased ASP production (CTL 100.0 ± 4.4% 10^{-8} M 36.0 ± 9.2%; 10^{-7} M 63.6 ± 9.9% and 10^{-6} M 83.6 ± 18.7%, $P < 0.05$). Both C3 production (CTL 100.0 ± 7.2%; 10^{-8} M rimonabant 63.5 ± 9.5%; $P < 0.01$) and C3 conversion to ASP (CTL 100.0 ± 5.2%; 10^{-8} M rimonabant 61.6 ± 12.8%, $P < 0.01$) are decreased by rimonabant treatment. However, this effect was selective as there was no effect on adipocyte TG mass, NEFA release or FA uptake (data not shown).

EFFECTS OF METFORMIN ON ASP AND C3 PRODUCTION, NEFA SECRETION, TG MASS, AND FA UPTAKE

Metformin, a commonly used drug for treatment of type 2 diabetes mellitus, induces glucose transporter 4 (GLUT4) translocation in adipose tissue of obese Zucker rats and streptozotocin-diabetic rats [Matthaei et al., 1993]. As shown in Figure 6A, all concentrations of metformin effectively decreased ASP production ($P < 0.05$ to $P < 0.001$), however, there was no change in C3 secretion (Fig. 6B) at 10^{-4} M of metformin treatment. Consequently, there was a reduction in C3 conversion to ASP (Fig. 6C, $P < 0.05$). There was no change in NEFA release (data not shown), however there was a concentration dependent decrease in TG mass (Fig. 6D, ANOVA $P = 0.036$) and in FA uptake (Fig. 6E, ANOVA $P = 0.035$).

DISCUSSION

Firstly, we demonstrated that the 3T3-L1 adipocyte cell model secretes both C3 and ASP, consistent with data by Choy et al. [1992], and secondly that both ASP and C3 production are increased by chylomicrons and/or insulin, as shown previously for human differentiated adipocytes [Maslowska et al., 1997; Scantlebury et al., 1998]. Novel results in the present study indicated that various hormones as well as drugs used for obesity, diabetes, coronary heart disease and metabolic syndrome treatment, have varying effects on ASP production in 3T3-L1 adipocytes. Furthermore, in this study, changes in ASP production were variably associated with specific changes in C3 secretion, NEFA release (lipolysis), FA uptake (synthesis) and adipocyte TG mass.

Interestingly, while some factors known to affect adipocyte metabolism (such as leptin and triiodothyronine) had no effect on ASP production, other factors quite potently altered ASP production with or without accompanying changes in lipid markers (TG mass, lipolysis, or TG synthesis measured as FA uptake). As discussed below, the mechanisms for changes in ASP levels could be mediated either as (i) a consequence of changes in the precursor protein C3 secretion (as demonstrated previously in human differentiated adipocytes) [Maslowska et al., 1997; Scantlebury et al., 1998], (ii) as a result of extracellular conversion of C3 to ASP, mediated via convertase action involving factor B and adipsin [Xu et al., 2001], or (iii) related to changes in cellular ASP catabolism.

CHANGES IN C3 SECRETION

A simultaneous change in both C3 and ASP suggests a mechanism related to changes in precursor protein C3 secretion. As all test compounds are known to target adipocyte cellular or nuclear receptors or intracellular enzymes, the inhibition might be mediated directly through changes in C3 gene expression. The effects of chylomicrons with or without insulin on increasing C3 and/or ASP in 3T3-L1 adipocytes are consistent with gene-mediated changes in C3 expression shown previously in other cells (human adipocytes and uterus/placental tissues) [Maslowska et al., 1997; Scantlebury et al., 1998]. Epinephrine, progesterone, testosterone and metformin had no effect on C3 secretion. By contrast, dexamethasone, lovastatin, rosiglitazone, rimonabant,

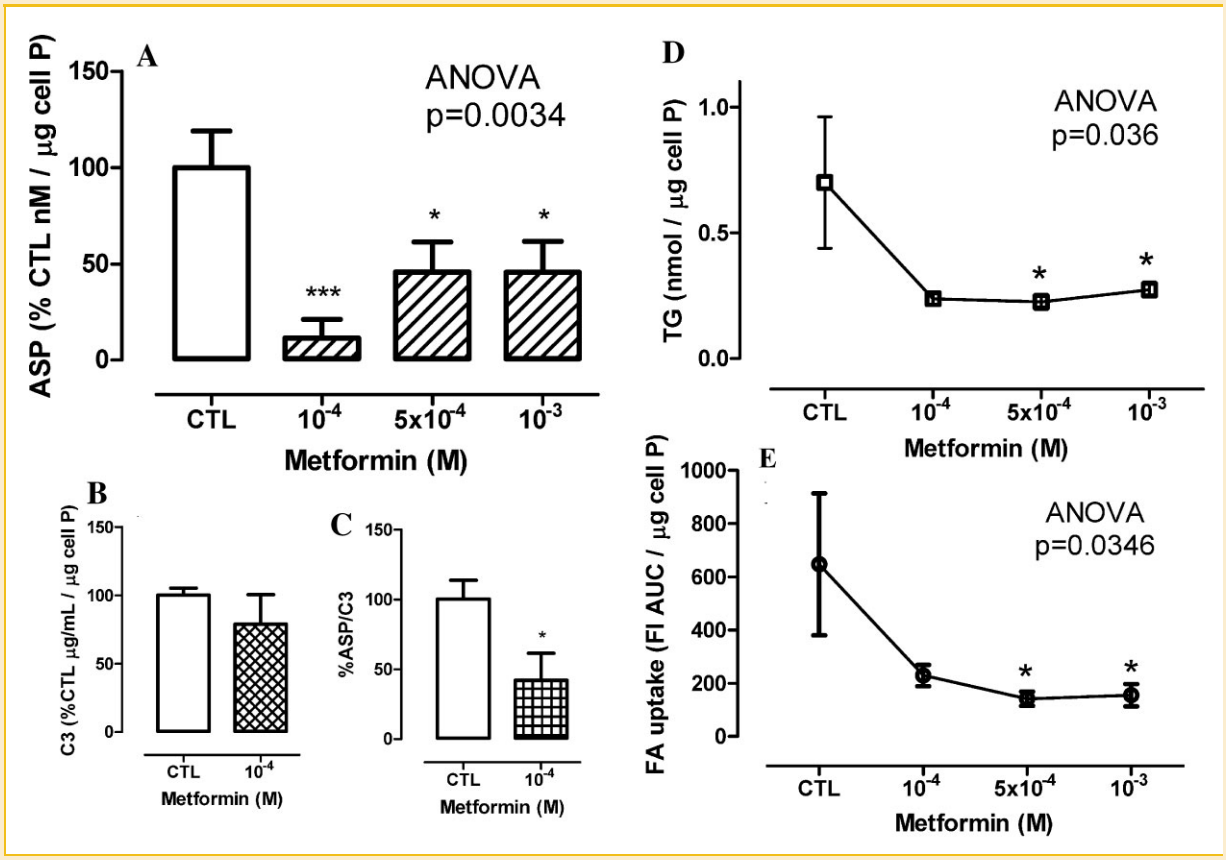

Fig. 6. Metformin inhibits ASP production, TG mass and FA uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 48 h with the indicated metformin concentrations. Medium ASP, C3, and NEFA levels were measured, as well as FA uptake, cell TG and cell protein. For ASP (Panel A) and C3 (Panel B), results are measured as ng/ml/µg cell protein (cell P) and are presented relative to baseline control (CTL) set as 100%. ASP/C3 ratio (Panel C) is calculated based on ASP (ng)/C3 (ng). Results for cell TG mass (Panel D) and FA uptake (Panel E) are all expressed relative to cell protein (cell P). All results are presented as mean ± SEM for three to four experiments each in triplicate (n = 9–12 values) where $^{*}P < 0.05$, $^{***}P < 0.001$ relative to control (CTL).

atenolol and propranolol all decreased C3 secretion; all factors but the last two (atenolol and propranolol) were also associated with accompanying decreases in ASP. Whether these compounds selectively affect C3 gene expression, or are a consequence of global adipocyte changes remains to be determined in future studies.

CHANGES IN ASP PRODUCTION

The factors studied produced variable changes in ASP: increases, decreases or no change. Chylomicrons, with or without insulin, increased ASP production levels. On the other hand, leptin and triiodothyronine did not affect ASP levels, while atenolol and propranolol, in spite of changes in C3 secretion, also did not alter ASP production. Dexamethasone, lovastatin, rosiglitazone and rimonabant all induced decreases in both ASP and C3. While decreases in ASP associated with comparable decreases in C3 are likely a result of a decrease in precursor protein availability (as discussed above), an additional contribution related to decreased convertase activity may also exist, such as with lovastatin, rosiglitazone and rimonabant treatment, based on additional decreases in ASP/C3 ratio. Lastly, there were specific decreases in ASP production that were independent of any changes in C3, suggesting an effect that targets the extracellular convertase step. These included treatment with epinephrine, progesterone, testosterone, and metformin.

POTENTIAL MECHANISMS FOR CHANGES INDUCED

In the present study we specifically used chronic rather than acute treatments with the various hormones and drugs, as these are typically what are experienced in the physiological situation. Consequently, alterations in C3 secretion or ASP production may be a consequence of an overall modification of adipocyte status, since many of these treatments also induced changes in adipocyte TG mass, lipolysis or TG synthetic capacity. Potential mechanisms (as discussed below) might include (i) adipocyte de-differentiation, (ii) modification of convertase (stabilization or NEFA interference), or (iii) transition to more sensitive adipocytes although these interpretations remain to be tested.

As gene expression of C3, adipsin and factor B, as well as ASP production are all adipocyte differentiation dependent [Choy et al., 1992], as is also expression of the ASP receptor C5L2 [MacLaren et al., 2007], de-differentiation of adipocytes may explain a decrease in ASP and/or C3. Chronic incubation with factors such as lovastatin and metformin, which led to decreased cell TG mass and decreased

fatty acid uptake are consistent with this, as lovastatin for example has been shown to inhibit adipogenesis [Nishio et al., 1996].

Increases in local NEFA concentration have been shown to interfere with lipoprotein lipase action by inhibiting catalytic activity and destabilizing the proteoglycan anchoring, releasing lipoprotein lipase from its cell surface location [Amri et al., 1996]. As conversion of C3 to ASP is also dependent on cellular anchoring of the active convertase as well as positive and negative regulators (properdin, factor H and I) [Xu et al., 2001], the presence of high NEFA concentrations, such as induced with epinephrine, progesterone, testosterone and angiotensin II, may lead to destabilization of the convertase, and subsequent reduction in ASP production. Further, high levels of NEFA lead to a decrease in mRNA expression and cell surface levels of C5L2 [Wen et al., 2008a], recognized as an ASP receptor [Kalant et al., 2005], and a decrease in ASP stimulation of glucose transport [Wen et al., 2008a], suggesting ASP resistance. Similarly, incubation with progesterone, testosterone and dexamethasone not only led to changes in ASP production (as shown here), but was also associated with decreased C5L2 mRNA expression and cell surface protein [Wen et al., 2008b] as well as decreased ASP response [MacLaren et al., 2007; Wen et al., 2008b], providing further support for an ASP resistant state. Conversely, chylomicrons, a lipoprotein particle, may act to stabilize the convertase in vivo, in a manner analogous to the effect of lipopolysaccaride (LPS), which is used to enhance C3 convertase activity in vitro, and as shown in vivo [Fujita et al., 2007].

Increased ASP internalization and catabolism could also contribute to decreases in ASP through increased C5L2 receptor expression. However, this is less likely as conditions which lead to decreased ASP (high NEFA, dexamethasone, progesterone and testosterone) also lead to decreased C5L2, as mentioned above [MacLaren et al., 2007; Wen et al., 2008a,b].

Finally, increased adipocyte ''sensitivity'' may also contribute to alterations in ASP production. We have previously demonstrated that rosiglitazone treatment increases mRNA expression and cell surface levels of C5L2 [MacLaren et al., 2007]. The reciprocal response may lead to decreased ASP production (as shown here).

The physiological relevance of these in vitro data should be noted. First, all factors tested were used in physiological/treatment ranges; and all factors have known adipose targets (receptors and enzymes) with demonstrated effects on adipose metabolism (as referenced in results). Further, in vivo studies in various subject groups led to the in vitro testing of the factors. Specifically, plasma ASP and/or C3 are increased in obesity, diabetes and cardiovascular disease, many of which are associated with dyslipidemias [Cianflone et al., 2003]. ASP and C3 associations with lipids and obesity have been examined in a sex hormone related context (polycystic ovary disease, pregnancy and menstrual cycle studies [Oktenli et al., 2007; Saleh et al., 2007, 2009; Wu et al., 2009]) and in an energy metabolism related context (hypothyroid and hyperthyroid subjects [Yu et al., 2006] and exercise [Schrauwen et al., 2005]). Finally, the effects of metabolic treatments such as rosiglitazone and metformin have been evaluated with respect to ASP and/or C3 [Ebeling et al., 1999; Koistinen et al., 2001; Oktenli et al., 2007; Tahiri et al., 2007] and simvastatin has been shown to decrease C3. Overall, in vivo physiological changes in ASP and/or C3 are consistent with the reported in vitro effects of these factors in the present study.

In summary, using the 3T3-L1 adipocyte cell model for evaluation of ASP production and C3 secretion, we have demonstrated changes induced by hormones and drugs. These results provide a basis for understanding the physiologic regulation of ASP and its associated function in lipid storage.

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