ARTICLE

Journal of Cellular Biochemistry 112:1622–1629 (2011)

Journal of Cellular Biochemistry

The Molecular Mechanism of Acylation Stimulating Protein Regulation of Adipophilin and Perilipin Expression: Involvement of Phosphoinositide 3-Kinase and Phospholipase C

Jing WU,[1] Zhou-Yang JIAO,[3] Hui-Ling LU,[2*] Jing Zhang,[1] Han-Hua Lin,[2] and Katherine Cianflone[4]

[1] *Department of Pediatrics, First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China*

[2] *Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China*

[3] *Department of Cardiovascular Surgery, Xiehe Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China*

[4] *Centre de Recherche Institut Universitaire de Cardiologie et Pneumologie de Quebec, Université Laval, Québec, Québec, Canada*

ABSTRACT

The novel adipokine acylation stimulating protein (ASP) is involved in lipid metabolism and obesity-related disorders. Adipophilin and perilipin, two members of the lipid droplet protein family, participate not only in fat storage within adipocytes, but also in ectopic lipid deposition in the form of cytoplasmic triglyceride (TG) droplets within many types of mammalian cells. During differentiation to mature adipocytes, mechanisms controlling the synthesis and turnover of these lipid droplet proteins are only partially understood, the mechanisms regulating gene/protein expression as yet unidentified. In our previous study, ASP has been shown to regulate adipophilin and perilipin expression to facilitate TG synthesis during 3T3-L1 cell differentiation. Our aim in this study was to provide insight into the physiological importance of phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC) in ASP-triggered alteration of adipophilin and perilipin expression. We found that acute (2.5 h) inhibition of PLC or PI3K results in a decrease in mRNA and protein of perilipin and adipophilin at any time during differentiation. The fact that there is such a rapid change even with mRNA levels suggests a rapid turnover of both mRNA and protein independent of a direct ASP effect. Also, the presence of these inhibitors blocked the ASP stimulatory effects with a maximal decrease in gene and protein expression of adipophilin (−45% and −60%, respectively, $P < 0.01$) and perilipin (−96% and −63%, respectively, $P < 0.01$ and $P < 0.05$). These findings provide further understanding of the adipogenic properties of ASP in adipocytes. J. Cell. Biochem. 112: 1622–1629, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: ACYLATION STIMULATING PROTEIN; 3T3-L1 CELLS; TRIGLYCERIDE SYNTHESIS; LIPID DROPLET PROTEIN; PHOSPHOLIPASE C; PHOSPHOINOSITIDE-3 KINASE

In the course of evolutionary history, triglyceride (TG) is the most effective way to meet energy requirements over diverse living organisms from prokaryotes to vertebrates [Williams, 2008]. With energy balance at the cellular and whole-organism levels disturbed due to modern urban lifestyles exemplified by excess acquisition and decreased consumption, over accumulation of lipid droplets in adipocytes and ectopic deposition of TG and free fatty acid (FFA) in non-adipose tissue such as liver and muscle are associated with lipotoxity, which results in glucose and lipid disorder-associated syndromes mainly manifested as insulin resistance (IR) [Goossens, 2008]. In adipocytes, TG synthesis reflects the degree of adipogenesis.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30600213.

*Correspondence to: Hui-Ling LU, Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China. E-mail: huilinglu@yahoo.cn

Received 10 September 2010; Accepted 8 February 2011 • DOI 10.1002/jcb.23076 • © 2011 Wiley-Liss, Inc.

Published online 22 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

Lipid droplets in adipocytes are the primary organelles for efficient storage and timely release of fat [Kadereit et al., 2008]. Lipid droplet-specific proteins were originally identified as perilipin, adipophilin, and the tail-interacting protein of 47 kDa (TIP47) proteins (abbreviated as PAT), but now include additional members with various tissue and subcellular distributions, lipid binding properties and development features [Granneman and Moore, 2008]. It is now accepted that these lipid droplet-specific proteins actively participate in controlling the storage and release of triacylglycerols in lipid droplets [Ducharme and Bickel, 2008]. However, as one cellular indicator of the amount of stored lipids, PAT protein expression is a complex and sequentially highly ordered process regulated at multiple levels, by hormones, cytokines and other molecules [Chen et al., 2008]. Thus a potential approach to regulate lipid accumulation in adipocytes may be to seek a possible key hormonal factor regulating synthesis and expression of lipid droplet-coated proteins.

Acylation stimulating protein (ASP) is generated by adipose tissue through the interaction of Factor B and adipsin with complement C3 and is identical to C3adesArg [Cianflone et al., 2003]. Accumulating in vitro [Murray et al., 1999] and in vivo [Cui et al., 2007] evidence suggests that ASP, a novel adipocyte-derived hormone known to stimulate TGS, is involved in the pathophysiology of obesity [Xia et al., 2002] and other associated complications, such as type 2 diabetes mellitus and cardiovascular diseases [Koistinen et al., 2001; Faraj et al., 2003]. Although insulin has long been recognized as a potentiator, which could physiologically influence TG synthesis (TGS) in adipocytes, its effects have been focused on inhibition of lipolysis [Van Harmelen et al., 1999] rather than directly on FFA esterification which is influenced by ASP [Cianflone, 1997; Van Harmelen et al., 1999].

ASP plays a key role in the regulation of lipid storage in that it stimulates the esterification of fatty acids onto a glycerol backbone, resulting in the augmentation of intracellular TG depots in human preadipocytes, adipocytes, and skin fibroblasts [Cianflone et al., 2003]. C5L2, an orphan receptor, recently was identified as an ASP receptor, providing a strong rationale for identification of the signaling pathway for ASP [Kalant et al., 2005]. ASP activation of C5L2 leads to rapid C5L2 phosphorylation and internalization mediated through β-arrestin, followed by activation of phospholipase C (PLC), phosphoinositol 3 kinase, Akt phosphorylation, activation and translocation of PKC, and phospholipase A2 [Baldo et al., 1995; Kalant et al., 2005; Maslowska et al., 2006]. Activation of both phosphoinositide 3-kinase (PI3K) and PLC were confirmed to be necessary for ASP-stimulated TGS [Maslowska et al., 2006].

Our recent study showed that treatment of differentiating adipocytes with ASP led to augmented TGS [Wu et al., 2009] and adipophilin/perilipin expression [Wu et al., 2009]. By contrast, insulin affected only glucose transport. We also found that ASP could alter neither temporal profiles of these proteins during adipocyte differentiation nor changes in distribution from adipophilin to perilipin. ASP is thus considered more as a synergist related to lipid metabolism than an initiator for adipophilin and perilipin expression [Wu et al., 2009]. Such findings further raise the possibility that ASP effects on regulation of adipophilin and perilipin gene/protein expression are one key element in promoting TGS. Therefore, the mechanisms involved in perilipin and adipophilin gene/protein expression in response to ASP in 3T3-L1 cells merit further elucidation. We specifically focused our investigation on two possibly related signaling pathway components: PLC and PI3K, which were previously reported to play important roles in ASP stimulated TGS [Maslowska et al., 2006].

MATERIALS AND METHODS

MATERIALS

3T3-L1 preadipocytes were purchased form ATCC (Manassas, VA). Isobutylmethylxanthine (IBMX), dexamethasone (DEX) and insulin (INS) were purchased from Sigma–Aldrich (St. Louis, MO). Mini-Protein II electrophoresis system and Mini trans-blot system were obtained commercially from Bio-Rad Laboratories, Inc (Bio-Rad). Primary antibodies and dilutions used were as follows: anti-adipophilin 1:2,000 (chicken polyclonal, Abcam, Cambridge), anti-perilipin A 1:5,000 (rabbit polyclonal, Chemicon, Billerica), anti-β-actin 1:5,000 (rabbit polyclonal, Cell Signaling Technology, Beverly). ASP was produced as described previously [Maslowska et al., 2006]. U73122 was obtained from Merck (Darmstadt, Germany). Wortmannin was from Sigma (St Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibody, BCA Protein Assay Kit and super-signal reagents were purchased from Pierce (Rockford, IL). PageRular™ Prestained Protein Ladder was ordered from Fermentas (Burlington, Canada). TRIzol® Reagents were obtained from Invitrogen (Life Technologies, Portland).

CELL CULTURE

3T3-L1 preadipocytes were grown at 37°C with 5% CO_2 in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) and Pen-Strep (100 U/ml penicillin and 100 μM streptomycin) in 75 cm^2 flasks to complete confluence for differentiation. Post-confluent cells, cultured in the presence of DMEM/F12 media with 10% FBS, were hormonally stimulated to differentiate with 10 μg/ml INS, 0.5 mmol/L IBMX and 1 μmol/L DEX for 2 days, subsequently with insulin only. By continuous treatment and every 2 days' replenishment, at least 80% cells exhibited adipocyte morphology on day 8–9.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

3T3-L1 cells were washed twice with serum-free DMEM and incubated for 30 min in the presence of U73122 (5 μM) or wortmannin (100 nM) before incubating for another 2 h with 1 μM ASP in the continued presence of this inhibitor. Total RNA was extracted from treated 3T3-L1 cells using TRIzol reagent (Invitrogen Life Technologies). RNA concentration and quality were assessed based on 260 nm/280 nm absorbance ratios. The single strand cDNA synthesis was performed by using TaKaRa RNA PCR kit (Takara, Japan) according to the manufacturer's directions. PCR primers used are listed in Table I. The gene expression levels were normalized to the murine housekeeping gene β-actin. The PCR products present in 25 μl of the reaction mixture were then separated according to size on 2.5% agarose gels and visualized by ethidium bromide staining. The resultant fluorescent bands were digitized and quantified using Bio-Rad Gel Doc XR System (Bio-Rad).

TABLE I. Primers Used for Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

	Gene		Length (bp)	Cycle	Tm (°C)
Mouse	Adipophilin	Forward: 5′-CTCTACCTACGACCTTGTG-3′ Reverse: 5′-GTCTTTCCTCCATCCTGT-3′	212	35	52
	Perilipin	Forward: 5′-AACCCTGCTGGATGGAGAC-3′ Reverse: 5′-GAACCTTGTCAGAGGTGCTTG-3′	407	35	54
	β-actin	Forward: 5′-TCCTCCCTGGAGAAGAGCTA-3′ Reverse: 5′-TCAGGAGGAGCAATGATCTTG-3′	302	35	55

PROTEIN SAMPLE PREPARATIONS AND CONCENTRATION ASSAY

Whole-cell extracts of 3T3-L1 cells were prepared by lysing cells in RIPA lysis buffer (50 mmol/L Tris–Cl, pH 8.0, 150 mmol/L NaCl, 0.2 g/LNaN_3, 1 g/L SDS, 10 g/L NP-40, 100 μg/ml PMSF, 5 g/L sodium deoxycholate, 1 μg/ml Aprotinin), followed by centrifugation at 4°C 12,000 × g for 15 min to pellet insoluble material. Protein content was determined by BCA Protein Assay Kit with BSA as standard. Proteins were denatured by adding 5 × concentrated SDS sample buffer containing a reducing agent and boiling for 5 min.

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS–PAGE) AND IMMUNOBLOT ANALYSIS

Each lane of the gels was loaded with 40 μg protein with 5 μl prestained molecular weight markers used as standards. Lysate proteins were separated by SDS/PAGE using a 12.5% polyacrylamide gel. Following transfer to nitrocellulose at 100 V for 120 min the membranes were blocked and probed with specified primary antibodies diluted in Tris buffered saline containing 5% skim milk at an appropriate proportion for each protein while shaking overnight at 4°C, followed by washing three times in Tris buffered saline with 0.05% Tween-20. The blots were then incubated with HRP-conjugated secondary antibody diluted in buffered saline containing 5% skim milk at 1:5,000 for 90 min while shaking at 37°C, followed by enhanced chemiluminescence (ECL) system detection (Pierce, Rockford).

STATISTICAL ANALYSIS

Results are presented as the percentage of control values (mean ± SEM) and represent data collected from at least three experiments, with three replicates in each experiment. Data in each figure were analyzed by one-way repeated-measures ANOVA, followed by post-hoc Bonferoni test for individual statistical changes between control groups.

RESULTS

PARTICIPATION OF THE PLC PATHWAY IN ASP STIMULATED ADIPOPHILIN GENE AND PROTEIN EXPRESSION

As shown in Figure 1A, adipophilin mRNA levels at 0, 3, 6, and 9 days were all significantly reduced when PLC was inhibited in cells by U73122 (5 μM, 2.5 h) by 52.8% (day 0, $P < 0.01$), 49.0% (3rd day $P < 0.01$), 35.1% (6th day $P < 0.001$) and 20.6% (9th day $P < 0.05$), respectively (Fig. 1A). Further, U73122 also decreased adipophilin protein levels by 54.4% ($P < 0.05$), 54.1% ($P < 0.05$), 41.9% ($P < 0.05$), 20.5% ($P < 0.05$) at day 0, 1st day, 3rd day, and 4th day (Fig. 1B). This effect was no longer detectable at the 6th day ($P > 0.05$). Pre-exposure to U73122 for 30 min followed by treatment with ASP for an additional 2 h partly counteracted the inhibition by U73122 on adipophilin mRNA, with ASP-induced increases of 1.29-fold ($P < 0.05$) at day 0, 1.27-fold ($P < 0.001$) at 3rd day, 1.15-fold ($P < 0.05$) at 6th day and 1.07-fold ($P < 0.05$) at 9th day (Fig. 1A). Simultaneously, adipophilin protein level was increased 2.08-fold ($P < 0.05$) at day 0, 1.77-fold ($P < 0.05$) at 1st day and 1.51-fold ($P < 0.05$) at 3rd day in ASP + U73122 versus U73122 treatment alone (Fig. 1B). From the 4th day onwards, there was no obvious difference in adipophilin protein levels between U73122 + ASP and U73122 alone (Fig. 1B). Nevertheless, adipophilin mRNA level in U73122 + ASP group was substantially decreased versus ASP alone by 59.3% ($P < 0.01$), 49.4% ($P < 0.05$), 30.7% ($P < 0.05$), and 20.1% ($P < 0.05$) on day 0, 3, 6, and 9, respectively (Fig. 1A). Similarly, adipophilin protein levels in U73122 + ASP group versus ASP alone were decreased by 54.4% ($P < 0.05$) at day 0, 59.0% ($P < 0.05$) at 1st day, 42.8% ($P < 0.05$) at 3rd day, 34.9% ($P < 0.05$) at 4th day and 38.8% ($P < 0.05$) on the 6th day (Fig. 1B).

INVOLVEMENT OF THE PLC PATHWAY IN ASP STIMULATED PERILIPIN GENE AND PROTEIN EXPRESSION

Results showed that the effect of ASP stimulation on perilipin can be partly reversed by U73122 pretreatment: perilipin mRNA level in U73122 + ASP group was 73.1% ($P < 0.01$) less than that in the ASP group on the 3rd day, 54.4% ($P < 0.05$) at 6th day and 21.4% ($P < 0.05$) at 9th day (Fig. 1C). As well, perilipin protein level in U73122 + ASP group was reduced by 53.2%, 66.4%, 46.2%, and 40.6% at the 3rd, 4th, 6th, and 9th days, although significant only for 4th and 9th days ($P < 0.05$, Fig. 1D). Our results also revealed that perilipin levels in U73122 + ASP group were higher than that in U73122 group by 1.28-fold ($P < 0.05$) at day 0 (mRNA) and 1.48-fold ($P < 0.01$) and 1.15-fold ($P < 0.05$) at 3rd day (mRNA and protein, respectively), 1.80-fold ($P < 0.01$) at 4th day (protein), and 1.43-($P < 0.001$) and 1.80-fold ($P < 0.05$) at 6th day (mRNA and protein levels, respectively), although there was no significant difference between the groups at the 9th day (Fig. 1C and D).

REQUIREMENT OF THE PI3K PATHWAY IN ASP STIMULATED ADIPOPHILIN GENE AND PROTEIN EXPRESSION

As Figure 2A and B demonstrate, compared with corresponding normal differentiation group, incubation with wortmannin for 30 min gave rise to a 56.8% ($P < 0.001$) decrease in adipophilin mRNA level at day 0, 48.1% ($P < 0.01$) at 3rd day, 29.1% ($P < 0.01$) at 6th day and 11.7% ($P < 0.01$) at 9th day. Adipophilin protein expression was also significantly suppressed by 60.4% ($P < 0.05$) at

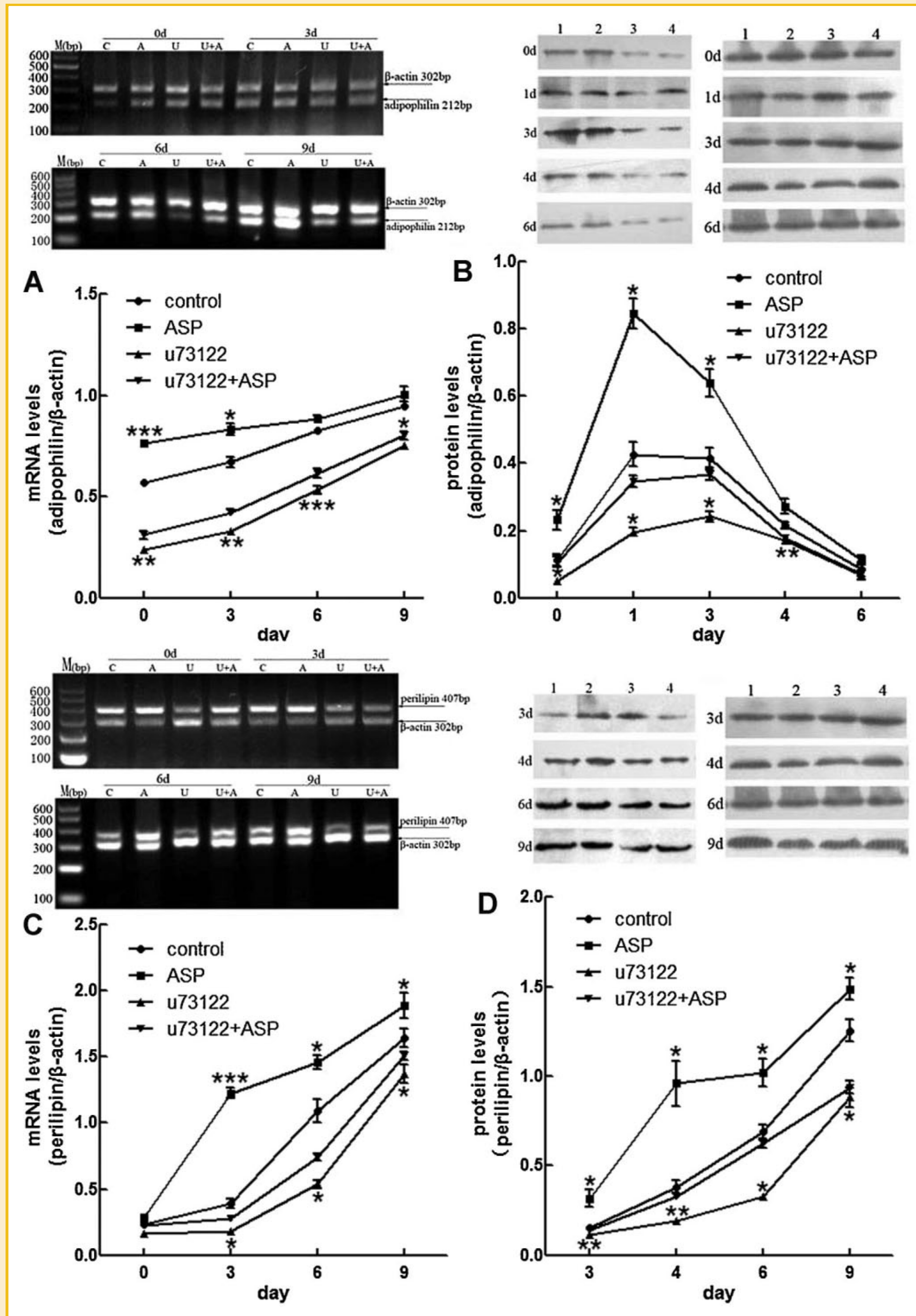

Fig. 1. Effect of PLC inhibition on adipophilin and perilipin mRNA (A,C) and protein (B,D) expression. U73122 was present throughout the experiment with or without additional treatment (ASP or Insulin stimuli) for another 2 h. Results are represented as means ± SEM in three independent experiments, each with triplicate determinations. Asterisks denote statistically significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). M: DNA marker: from 100 to 600 bp; C: control groups; A: ASP treatment groups; U: U73122 treatment groups; U + A: U73122 + ASP treatment groups.

day 0, 82.2% ($P < 0.01$) at 1st day, 72.3% ($P < 0.01$) at 3rd day, 74.7% ($P < 0.01$) at 4th day and 53.8% ($P > 0.05$) on day 6. ASP addition reversed the wortmannin inhibitory effect on adipophilin gene/protein expression; adipophilin mRNA levels in wortmannin + ASP group was 1.45-fold at day 0 ($P < 0.001$), 1.34-fold at 3rd day ($P < 0.001$), 1.17-fold at 6th day ($P < 0.001$) and 1.09-fold at 9th day ($P < 0.001$) when compared to relevant wortmannin groups. Meanwhile, adipophilin protein content of

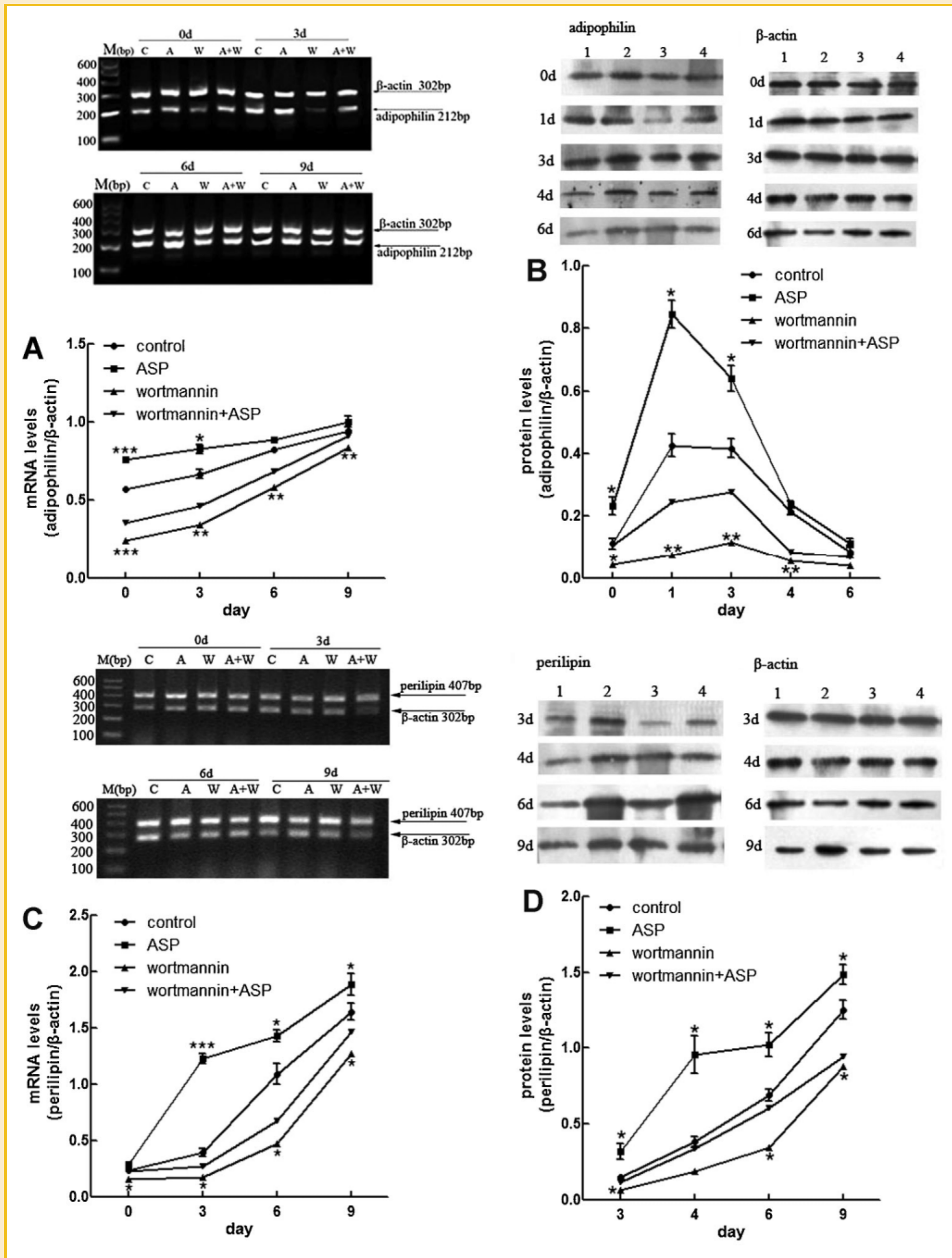

Fig. 2. Effect of PI3K inhibition on adipophilin and perilipin mRNA (A,C) and protein (B,D) expression. Wortmannin was present throughout the experiment with or without additional treatment (ASP or Insulin stimuli) for another 2 h. Results are represented as means ± SEM in three independent experiments, each with triplicate determinations. Asterisks denote statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). M: DNA marker: from 100 to 600 bp; C: control groups; A: ASP treatment groups; W: Wortmannin treatment groups; W + A: Wortmannin + ASP treatment groups.

wortmannin + ASP group was 2.39-fold ($P < 0.01$) at day 0, 3.25-fold ($P < 0.001$) at 1st day, 2.4-fold ($P < 0.001$) at 3rd day, 1.53-fold ($P < 0.01$) at 4th day and 1.76-fold ($P < 0.05$) at 6th day in comparison to that of wortmannin groups, respectively. Adipophilin gene level in wortmannin + ASP group was 53.2% ($P < 0.001$) lower than that in the ASP group at day 0, 44.0% ($P < 0.05$) at 3rd day, 22.5% ($P < 0.05$) at 6th day and 8.9% ($P > 0.05$) at 9th day. Furthermore, adipophilin protein expression in wortmannin + ASP group was markedly down-regulated by 54.4% ($P < 0.05$) at day 0, 70.8% ($P < 0.01$) at 1st day, 56.8% ($P < 0.05$) at 3rd day, 65.0% ($P < 0.01$) at 4th day and 38.8% ($P < 0.05$) at 6th day.

REQUIREMENT OF THE PI3K PATHWAY IN ASP STIMULATED PERILIPIN GENE AND PROTEIN EXPRESSION

PI3K inhibitor wortmannin can obviously attenuate the gene/protein expression of perilipin both during normal differentiation

and following ASP stimulation, although the ASP effect is partly but not completely attenuated, implying that other pathways, such as the PLC pathway, are involved. As shown in Figure 2C and D, when pretreated with wortmannin, perilipin gene level declined by 33.6% ($P < 0.05$) at day 0, 54.9% ($P < 0.05$) at 3rd day, 56.8% ($P < 0.05$) at 6th day and 22.9% ($P < 0.05$) at 9th day during differentiation. Similarly, perilipin protein expression was inhibited by 56.5% ($P < 0.05$) at 3rd day, 50.7% ($P > 0.05$) at 4th day, 49.6% ($P < 0.05$) at 6th day and 29.9% ($P < 0.05$) at 9th day. Perilipin mRNA level in wortmannin + ASP group was 1.44-fold ($P < 0.01$) over that in wortmannin alone at day 0, 1.51-fold ($P < 0.001$) at 3rd day, 1.43-fold ($P < 0.001$) at 6th day and 1.16-fold ($P < 0.001$) at 9th day. Meantime, the amount of perilipin protein in wortmannin + ASP group was 1.75-fold ($P < 0.01$) of that in wortmannin alone at 3rd day, 1.80-fold ($P < 0.001$) at 4th day, 1.74-fold ($P < 0.001$) at 6th day and 1.08-fold ($P < 0.05$) at 9th day. ASP-induced up-regulation of perilipin gene/protein expression could be almost completely blocked by wormannin, with perilipin mRNA decreased by 20.3% ($P > 0.05$) at day 0, 78.3% ($P < 0.01$) at 3rd day, 52.7% ($P < 0.01$) at 6th day and 22.2% ($P < 0.05$) at 9th day. Similarly, perilipin protein content was depressed by 63.7% ($P > 0.05$) at 3rd day, 64.7% ($P < 0.05$) at 4th day, 40.7% ($P < 0.05$) at 6th day and 36.4% ($P < 0.05$) at 9th day.

DISCUSSION

Perilipin was originally identified as an exclusively lipid droplet-associated protein involved in TG accumulation in adipocytes [Brasaemle, 2007; Kimmel et al., 2010]. Its presence in macrophages, smooth muscle cells within the vascular wall and other organs and tissues has been reported recently [Persson et al., 2007; Straub et al., 2008]. Due to its high content in adipocytes and steroid hormone-synthesizing cells coupled with its effects on lipid metabolism, perilipin A has attracted the most attention and has been the focus of much research as compared to other related proteins [Ray et al., 2009; Miyoshi et al., 2010]. Adipophilin is also abundantly distributed in a variety of tissues and cells [Listenberger et al., 2007] in addition to its expression in 3T3-L1 cells at an early stage of differentiation. These two proteins are being studied in great detail with the hope of identifying a key switch for lipid metabolism in adipocytes. They share homologous sequences and similar properties in relation to (i) increased storage of intracellular TG during preadipocyte differentiation and (ii) retention of lipids during macrophage development into foam cells [Brasaemle et al., 2000; Forcheron et al., 2005], and (iii) fatty degeneration in hepatocytes [Subramanian et al., 2004], which suggest that they may be regulated by similar mechanisms or pathways.

In our previous study [Wu et al., 2009], we demonstrated that adipophilin protein reaches a peak at 1 day after initiation of differentiation. While mRNA levels continued to rise during differentiation, protein expression showed the opposite trend. ASP enhanced adipophilin gene and protein expression at early stages of differentiation, whereas ASP could neither induce the re-expression of adipophilin protein nor promote adipophilin gene expression at the terminal differentiation stage. Although perilipin mRNA was present in 3T3-L1 cells at the very beginning of differentiation, perilipin A protein was only detectable at 3 days after differentiation induction, however in general, the temporal profile of perilipin A protein paralleled perilipin mRNA. ASP could not induce perilipin A expression at initiation of differentiation but was able to enhance perilipin gene and protein abundance 3 days after that [Wu et al., 2009].

According to current studies, perilipin A increases lipid accumulation by reducing the basal rate of TG turnover in the absence of lipolytic stimuli [Marcinkiewicz et al., 2006]. Moreover, heterologous expression of perilipin A in 3T3-L1 cells resulted in increased TG storage by reducing the lipolytic rate rather than directly promoting TG synthesis [Brasaemle et al., 2000]. Adipophilin is generally up-regulated in parallel with stored lipid during lipid droplet formation and is present on the surface of lipid droplets from the earliest time of their synthesis [Masuda et al., 2006; Fujii et al., 2009]. Chang et al. [2006] hypothesized that absence of adipophilin may reduce partitioning of TG into lipid droplet at the endoplasmic reticulum, the site of TGS. However, adipophilin and perilipin cannot functionally substitute for each other [Ducharme and Bickel, 2008]. The timely transition from adipophilin to perilipin in adipocytes may be consistent with specialized functions in lipid metabolism in order to facilitate adipogenic processes in 3T3-L1 cells [Ducharme and Bickel, 2008]. Adipophilin and perilipin A gene/protein expression are effectively promoted by ASP at an early stage of differentiation, which ensures a steady increase in adipogenesis. These two proteins were less responsive to ASP at a later stage [Wu et al., 2009], which may represent a self-limiting mechanism for avoiding excess lipid droplet formation at anaphase during adipocyte differentiation.

As previously reported, PLC has been implicated in the modulation of G-protein-mediated adipogenesis [Maslowska et al., 2006; Omatsu-Kanbe et al., 2006], but is not involved in insulin stimulation of TGS or, as shown previously, in glucose transport [Eichhorn et al., 2001; Wright et al., 2003]. Furthermore, although the mTOR pathway is important for insulin-mediated TGS, it is not involved in ASP signaling. These differences in ASP and insulin implicate a divergence of signaling pathways for ASP and insulin. [Maslowska et al., 2006].

PLC activation generates two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which initiate further signal transduction pathways through activation of protein kinase C (PKC) and intracellular calcium release [Sun et al., 2010]. PLC and its downstream effectors Akt, PKC, and ERK were all shown to be implicated in the ASP stimulation of TGS. ASP signaling involves sequential activation of PI3K and PLC, with downstream activation of PKC, Akt, MAPK/ERK1/2, and cPLA2, all of which leads to an effective and prolonged stimulation of TGS. Although there are a number of similarities in the ASP and insulin signaling pathways, there are a number of differences as well. [Maslowska et al., 2006].

In vitro studies have demonstrated that Akt is essential for lipid droplet assembly and controls the expression of perilipin in Drosophila nurse cells lacking Pten (tumor-suppressor protein) [Vereshchagina and Wilson, 2006]. The activity of ERK has also shown to be essential for lipid droplet formation [Andersson et al., 2006]. Some have suggested possible involvement of specific PKC

isozymes in the early phase of lipid accumulation [Chen et al., 2002] and adipophilin expression [Chen et al., 2001]. Given the above experimental evidence, we hypothesized that PLC activity contributed to ASP stimulatory action on perilipin and adipophilin gene and protein expression. We observed the PLC inhibitor U73122 could partly inhibit the ASP-induced up-regulation of adipophilin and perilipin, mainly in the early stage of differentiation, which suggests that the PLC pathway forms only part of the cascade. It is likely that other potential mechanisms together with PLC ultimately mediate regulation of ASP on adipophilin and perilipin.

Previous literature has documented that the PI3K pathway plays an important role in the insulin-signaling cascade leading to glucose transport translocation. Recently, there is considerable interest in the role of PI3K in enhancing adipogenesis. A report [Yu et al., 2008] showed that the PI3K/Akt signaling pathway was essential for adipogenesis of human mesenchymal stem cells (MSCs). Another study [Chuang et al., 2007] further reported that adipogenic induction of lipid accumulation in MSCs enhanced by hyperglycemia also depended on the PI3K signaling pathway. Moreover, a previous study [Maslowska et al., 2006] demonstrated that ASP could stimulate TGS in adipocytes through the PI3K pathway; ASP action was independent of insulin receptor signaling but dependent on $G\beta/\gamma$ subunit activation by ASP interaction with C5L2 receptor. In the present study, PI3K activity inhibition affected, to a certain extent, the ASP-promoted adipophilin and perilipin expression.

PI3K activation and activity changes are programmed events once adipocyte differentiation begin [Sakaue et al., 1998]. During differentiation into mature adipose cells, PI3K signaling could induce the expression of numerous adipogenic markers including peroxisome proliferator-activated receptor gamma 2 (PPARγ2) [Bildirici et al., 2003; Chuang et al., 2007]. PPAR subfamily plays a role in regulating both the promoter region of adipophilin gene [Schmuth et al., 2004; Targett-Adams et al., 2005] and the protein at a post-transcriptional level [Schadinger et al., 2005]. Perilipin and S3-12, another member of the lipid droplet-associating proteins family, both contain the conserved PPAR response elements [Dalen et al., 2004]. It is noteworthy that inhibition of PI3K resulted in decreased expression of adipophilin and perilipin in the basal state in our study. However, more work is now needed to determine the precisely regulatory mechanisms.

PLC activity plays an important role in ATP-induced 3T3-L1 cell differentiation [Omatsu-Kanbe et al., 2006]. Further, 3T3-L1 cells also possess C3a/ASP receptor coupled G protein-activated PLC isozymes, which participate in TGS [Maslowska et al., 2006]. Therefore, it is reasonable to assume that this enzyme might be closely associated with lipid accumulation when preadipocytes differentiate into adipocytes, although the exact control mechanism remains to be further identified. Our data demonstrated that U73122, a potent and widely used inhibitor of PLC, inhibited expression of adipophilin and perilipin during normal differentiation in adipocytes.

Taken together, although the precise intermediate signaling pathway remains to be studied, ASP, adipophilin, and perilipin may play important roles in stimulating TGS in a manner independent to that of insulin.

ACKNOWLEDGMENTS

We are grateful to colleagues at the Centre de Recherche Institut Universitaire de Cardiologie et Pneumologie in Quebec, Canada for experimental assistance and for comments on the manuscript. KC is a Canada Research Chair scholar. This work was supported by grants from National Natural Science Foundation of China (NO. 30600213) to Dr. HuiLing LU.

REFERENCES

Andersson L, Boström P, Ericson J, Rutberg M, Magnusson B, Marchesan D, Ruiz M, Asp L, Huang P, Frohman MA, Borén J, Olofsson SO. 2006. PLD1 and ERK2 regulate cytosolic lipid droplet formation. J Cell Sci 119:2246–2257.

Baldo A, Sniderman AD, St., Luce S, Zhang XJ, Cianflone K. 1995. Signal transduction pathway of acylation stimulating protein: Involvement of protein kinase C. J Lipid Res 36:1415–1426.

Bildirici I, Roh CR, Schaiff WT, Lewkowski BM, Nelson DM, Sadovsky Y. 2003. The lipid droplet-associated protein adipophilin is expressed in human trophoblasts and is regulated by peroxisomal proliferator-activated receptor-gamma/retinoid X receptor. J Clin Endocrinol Metab 88:6056–6062.

Brasaemle DL. 2007. Thematic review series: Adipocyte biology. The perilipin family of structural lipid droplet proteins: Stabilization of lipid droplets and control of lipolysis. J Lipid Res 48:2547–2559.

Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, Londos C. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. J Biol Chem 275:38486–38493.

Chang BH, Li L, Paul A, Taniguchi S, Nannegari V, Heird WC, Chan L. 2006. Protection against fatty liver but normal adipogenesis in mice lacking adipose differentiation-related protein. Mol Cell Biol 26:1063–1076.

Chen JS, Greenberg AS, Tseng YZ, Wang SM. 2001. Possible involvement of protein kinase C in the induction of adipose differentiation-related protein by Sterol ester in RAW 264.7 macrophages. J Cell Biochem 83:187–199.

Chen JS, Greenberg AS, Wang SM. 2002. Oleic acid-induced PKC isozyme translocation in RAW 264.7 macrophages. J Cell Biochem 86:784–791.

Chen CH, Wang PH, Liu BH, Hsu HH, Mersmann HJ, Ding ST. 2008. Serum amyloid A protein regulates the expression of porcine genes related to lipid metabolism. J Nutr 138:674–679.

Chuang CC, Yang RS, Tsai KS, Ho FM, Liu SH. 2007. Hyperglycemia enhances adipogenic induction of lipid accumulation: involvement of extracellular signal-regulated protein kinase 1/2, phosphoinositide 3-kinase/Akt, and peroxisome proliferator-activated receptor gamma signaling. Endocrinology 148:4267–4275.

Cianflone K. 1997. Acylation stimulating protein and the adipocyte. J Endocrinol 155:203–206.

Cianflone K, Xia Z, Chen LY. 2003. Critical review of acylation stimulating protein physiology in humans and rodents. Biochim Biophys Acta 1609:127–143.

Cui W, Paglialunga S, Kalant D, Lu H, Roy C, Laplante M, Deshaies Y, Cianflone K. 2007. Acylation-stimulating protein/C5L2-neutralizing antibodies alter triglyceride metabolism in vitro and in vivo. Am J Physiol Endocrinol Metab 293:E1482–E1491.

Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, Nebb HI. 2004. Adipose tissue expression of the lipid droplet-associating proteins S 3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. Diabetes 53:1243–1252.

Ducharme NA, Bickel PE. 2008. Lipid droplets in lipogenesis and lipolysis. Endocrinology 149:942–949.

Eichhorn J, Kayali AG, Austin DA, Webster NJ. 2001. Insulin activates phospholipase C-gamma1 via a PI-3 kinase dependent mechanism in 3T3-L1 adipocytes. Biochem Biophys Res Commun 282:615–620.

Faraj M, Havel PJ, Phélis S, Blank D, Sniderman AD, Cianflone K. 2003. Plasma acylation-stimulating protein, adiponectin, leptin, and ghrelin before and after weight loss induced by gastric bypass surgery in morbidly obese subjects. J Clin Endocrinol Metab 88:1594–1602.

Forcheron F, Legedz L, Chinetti G, Feugier P, Letexier D, Bricca G, Beylot M. 2005. Genes of cholesterol metabolism in human atheroma: Overexpression of perilipin and genes promoting cholesterol storage and repression of ABCA1 expression. Arterioscler Thromb Vasc Biol 25:1711–1717.

Fujii H, Ikura Y, Arimoto J, Sugioka K, Iezzoni JC, Park SH, Naruko T, Itabe H, Kawada N, Caldwell SH, Ueda M. 2009. Expression of perilipin and adipophilin in nonalcoholic fatty liver disease; relevance to oxidative injury and hepatocyte ballooning. J Atheroscler Thromb 16:893–901.

Goossens GH. 2008. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 94:206–218.

Granneman JG, Moore HP. 2008. Location, location: Protein trafficking and lipolysis in adipocytes. Trends Endocrinol Metab 19:3–9.

Kadereit B, Kumar P, Wang WJ, Miranda D, Snapp EL, Severina N, Torregroza I, Evans T, Silver DL. 2008. Evolutionarily conserved gene family important for fat storage. Proc Natl Acad Sci USA 105:94–99.

Kalant D, Maclaren R, Cui W, Samanta R, Monk PN, Laporte SA, Cianflone K. 2005. C5L2 is a functional receptor for acylation stimulating protein. J Biol Chem 280:23936–23944.

Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, Londos C. 2010. Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. J Lipid Res 51:468–471.

Koistinen HA, Vidal H, Karonen SL, Dusserre E, Vallier P, Koivisto VA, Ebeling P. 2001. Plasma acylation stimulating protein concentration and subcutaneous adipose tissue C3 mRNA expression in nondiabetic and type 2 diabetic men. Arterioscler Thromb Vasc Biol 21:1034–1039.

Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, Brown DA, Brasaemle DL. 2007. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. J Lipid Res 48:2751–2761.

Marcinkiewicz A, Gauthier D, Garcia A, Brasaemle DL. 2006. The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. J Biol Chem 281:11901–11909.

Maslowska M, Legakis H, Assadi F, Cianflone K. 2006. Targeting the signaling pathway of acylation stimulating protein. J Lipid Res 47:643–652.

Masuda Y, Itabe H, Odaki M, Hama K, Fujimoto Y, Mori M, Sasabe N, Aoki J, Arai H, Takano T. 2006. ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. J Lipid Res 47:87–98.

Miyoshi H, Souza SC, Endo M, Sawada T, Perfield JW II, Shimizu C, Stancheva Z, Nagai S, Strissel KJ, Yoshioka N, Obin MS, Koike T, Greenberg AS. 2010. Perilipin overexpression in mice protects against diet-induced obesity. J Lipid Res 51:975–982.

Murray I, Köhl J, Cianflone K. 1999. Acylation-stimulating protein (ASP): Structure-function determinants of cell surface binding and triacylglycerol synthetic activity. Biochem J 342:41–48.

Omatsu-Kanbe M, Inoue K, Fujii Y, Yamamoto T, Isono T, Fujita N, Matsuura H. 2006. Effect of ATP on preadipocyte migration and adipocyte differentiation by activating P2Y receptors in 3T3-L1 cells. Biochem J 393:171–180.

Persson J, Degerman E, Nilsson J, Lindholm MW. 2007. Perilipin and adipophilin expression in lipid loaded macrophages. Biochem Biophys Res Commun 363:1020–1026.

Ray H, Pinteur C, Frering V, Beylot M, Large V. 2009. Depot-specific differences in perilipin and hormone-sensitive lipase expression in lean and obese. Lipids Health Dis 8:58.

Sakaue H, Ogawa W, Matsumoto M, Kuroda S, Takata M, Sugimoto T, Spiegelman BM, Kasuga M. 1998. Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase. J Biol Chem 273:28945–28952.

Schadinger SE, Bucher NL, Schreiber BM, Farmer SR. 2005. PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. Am J Physiol Endocrinol Metab 288:E1195–E1205.

Schmuth M, Haqq CM, Cairns WJ, Holder JC, Dorsam S, Chang S, Lau P, Fowler AJ, Chuang G, Moser AH, Brown BE, Mao-Qiang M, Uchida Y, Schoonjans K, Auwerx J, Chambon P, Willson TM, Elias PM, Feingold KR. 2004. Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes. J Invest Dermatol 122:971–983.

Straub BK, Stoeffel P, Heid H, Zimbelmann R, Schirmacher P. 2008. Differential pattern of lipid droplet-associated proteins and de novo perilipin expression in hepatocyte steatogenesis. Hepatology 47:1936–1946.

Subramanian V, Rothenberg A, Gomez C, Cohen AW, Garcia A, Bhattacharyya S, Shapiro L, Dolios G, Wang R, Lisanti MP, Brasaemle DL. 2004. Perilipin A mediates the reversible binding of CGI-58 to lipid droplets in 3T3-L1 adipocytes. J Biol Chem 279:42062–42071.

Sun Q, Zang WJ, Chen C. 2010. Growth hormone secretagogues reduce transient outward K^+ current via phospholipase C/protein kinase C signaling pathway in rat ventricular myocytes. Endocrinology 151:1228–1235.

Targett-Adams P, McElwee MJ, Ehrenborg E, Gustafsson MC, Palmer CN, McLauchlan J. 2005. A PPAR response element regulates transcription of the gene for human adipose differentiation-related protein. Biochim Biophys Acta 1728:95–104.

Van Harmelen V, Reynisdottir S, Cianflone K, Degerman E, Hoffstedt J, Nilsell K, Sniderman A, Arner P. 1999. Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. J Biol Chem 274:18243–18251.

Vereshchagina N, Wilson C. 2006. Cytoplasmic activated protein kinase Akt regulates lipid droplet accumulation in Drosophila nurse cells. Development 133:4731–4735.

Williams KJ. 2008. Molecular processes that handle – And mishandle – Dietary lipids. J Clin Invest 118:3247–3259.

Wright DC, Fick CA, Olesen JB, Craig BW. 2003. Evidence for the involvement of a phospholipase C-protein kinase C signaling pathway in insulin stimulated glucose transport in skeletal muscle. Life Sci 73: 61–71.

Wu J, Lu HL, Hu XF, Liang XY, Lin HH, Wang HW, Fan XW. 2009. Effect of acylation stimulating protein on the perilipin and adipophilin expression during 3T3-L1 preadipocyte differentiation. Sheng Li Xue Bao 61:56–64 (Chinese).

Xia Z, Sniderman AD, Cianflone K. 2002. Acylation-stimulating protein (ASP) deficiency induces obesity resistance and increased energy expenditure in ob/ob mice. J Biol Chem 277:45874–45879.

Yu W, Chen Z, Zhang J, Zhang L, Ke H, Huang L, Peng Y, Zhang X, Li S, Lahn BT, Xiang AP. 2008. Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells. Mol Cell Biochem 310: 11–18.