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Effect of collagen I and aortic carboxypeptidase-like protein on 3T3-L1 adipocyte differentiation

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Abstract

Aortic carboxypeptidase-like protein (ACLP) is a secreted protein expressed in preadipocytes and down-regulated during adipogenesis. Results from previous studies on the influence of ACLP overexpression on adipogenesis vary from no effect to complete inhibition. We hypothesized that ACLP may modulate adipogenesis in the presence of collagen I, a protein to which it binds. We compared control (pLXSN) 3T3-L1 preadipocytes with 3T3-L1 preadipocytes stably overexpressing ACLP (pLXSN-ACLP) that were grown in standard vs collagen I–coated dishes. Aortic carboxypeptidase-like protein overexpression, via retroviral transduction, resulted in a 3.2-fold increase in ACLP cellular levels and a 2.1-fold increase in ACLP levels released into medium. Aortic carboxypeptidase-like protein overexpression did not inhibit differentiation in standard dishes. In collagen I–coated dishes compared with standard dishes, control preadipocytes, when induced to differentiate, exhibited the same increase in triacylglycerol accumulation, but showed a significantly higher induction of fatty acid synthase (1.6-fold more), peroxisome proliferator-activated receptor γ (1.4-fold more), and CCAAT/enhancer-binding protein α (1.4-fold more). Aortic carboxypeptidase-like protein overexpression significantly reduced this enhanced induction of fatty acid synthase, peroxisome proliferator-activated receptor γ, and CCAAT/enhancer-binding protein α by 65%, 59%, and 66%, respectively, but had no effect on the accumulation of triacylglycerol during differentiation. Finally, studies on proadipogenic insulin signaling in ACLP-overexpressing preadipocytes demonstrated that insulin-stimulated Akt phosphorylation was significantly decreased by 27% in cells cultured in collagen I–coated dishes vs standard dishes. Our data suggest that ACLP inhibits certain aspects of 3T3-L1 adipogenesis in a collagen I–rich environment.

1. Introduction

Adipose tissue contains stromal preadipocytes that differentiate into mature adipocytes and accumulate lipid [1]. Adipogenesis requires extensive remodeling of the extracellular matrix (ECM), allowing fibroblastic preadipocytes to assume a rounded shape that accommodates lipid accumulation [2]. During 3T3-L1 adipocyte differentiation, the expression of fibrillar collagens I and III and of fibronectin decreases, whereas the opposite occurs for laminar collagen IV, entactin, and laminin [3]. Modulating the ECM in vitro environment can alter adipogenesis. When grown on fibronectin-coated surfaces, lipogenic gene expression and triacylglycerol (TG) accumulation in 3T3-F442A preadipocytes were inhibited [4]. Transforming growth factor–β (TGFβ), which increases expression of fibrillar collagen and fibronectin in 3T3-L1 preadipocytes, inhibits their differentiation [5,6]. Deletion of the membrane-anchored metalloproteinase MT1-MMP disrupts white adipose tissue development in vivo; preadipocytes from these mice do not differentiate in vitro if grown on a 3-dimensional collagen I matrix [7].

Aortic carboxypeptidase-like protein (ACLP), identified in a screen for potential regulators of vascular smooth muscle cell growth and differentiation, is also expressed in preadipocytes [8-10]. Aortic carboxypeptidase-like protein has a signal peptide sequence, a lysine- and proline-rich motif repeated 4 times, a discoidin-like domain, and an inactive carboxypeptidase-like domain [8,11].

Aortic carboxypeptidase-like protein messenger RNA and protein levels in 3T3-L1 and 3T3-F442A preadipocytes

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decrease with differentiation [9,10,12]. We previously reported that overexpression of ACLP in 3T3-L1 and 3T3-F442A preadipocytes does not affect adipogenesis [9,10]. In contrast, another group found that ACLP overexpression in 3T3-F442A preadipocytes inhibited the differentiation response; transdifferentation into smooth muscle-like cells was also noted [12]. The basis for this discrepancy is not clear.

Discoidin domains can interact with collagen [13,14]; and recently, ACLP was shown to bind to collagen I in a discoidin domain-dependent fashion [15]. Therefore, we investigated whether ACLP overexpression might influence the effect of collagen I on 3T3-L1 adipogenesis.

2. Materials and methods

2.1. Generation of ACLP-overexpressing 3T3-L1 preadipocytes

293T-derived Phoenix-Eco packaging cells (ATCC, Manassas, VA) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin), and 50 μg/mL nystatin (Calbiochem, Gibbstown, NJ). At 80% confluence, the cells were transfected with pLXSN or pLXSN-ACLP constructs (generous gift from Dr M Layne, Harvard Medical School, Boston MA) using calcium phosphate, as described [10]. Viral supernatant was collected 48 hours posttransfection, filtered through 0.45-μm syringe filters, and mixed with hexadimethrine bromide (polybrene; Sigma, Oakville, ON, Canada; 4 μg/mL). It was then added to 50% to 60% confluent 3T3-L1 preadipocytes for 24 hours. Transduced 3T3-L1 preadipocytes were selected with 400 μg/mL Geneticin (Invitrogen, Burlington, ON, Canada) until 80% confluent and then used for the described experiments directly or frozen for later use. Aortic carboxypeptidase-like protein overexpression was performed as described below.

2.2. 3T3-L1 cell culture and differentiation

Stable clones of preadipocytes transduced with retroviral particles generated from pLXSN-ACLP or pLXSN (empty vector) were seeded on regular plastic dishes or on collagen I–coated dishes (BD Biosciences, Mississauga, ON, Canada). They were grown in DMEM supplemented with 10% calf serum (Invitrogen) and antibiotics. Differentiation was induced 2 days postconfluence (day 0) and continued for 8 days. The differentiation medium was DMEM supplemented with 10% calf serum and antibiotics (growth medium). In addition, 0.25 mmol/L IBMX (Sigma) and 0.125 μmol/L dexamethasone (Steraloids, Newport, RI) were added for the first 2 days of differentiation and 100 nmol/L insulin (Roche, Laval, QC, Canada) for the first 4 days of differentiation. Control preadipocytes were maintained in growth medium without adipogenic inducers for 8 days.

2.3. ACLP secretion by 3T3-L1 preadipocytes

pLXSN and pLXSN-ACLP preadipocytes were grown until confluence in 100-mm dishes, and the medium was then replaced with 8 mL of serum-free DMEM supplemented with antibiotics and 1 μmol/L insulin. After 48 hours, medium was collected and centrifuged at 500*g* for 5 minutes; and 50 μL of the supernatant was mixed with 2× concentrated Laemmli buffer [16]. Equal volumes (40 μL) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and processed for immunoblotting as described below.

2.4. Triacylglycerol assay

Confluent pLXSN and pLXSN-ACLP preadipocytes were induced to differentiate or kept in control medium. On day 8, cellular TG was extracted using a solution of 2:3 (vol/vol) isopropanol:heptane. Triacylglycerol accumulation was quantified using a colorimetric assay [9]. Cellular remains were solubilized in Laemmli buffer, and protein was quantified as described below. Triacylglycerol values were normalized to protein content.

2.5. Immunoblot analysis

Cells were lysed in 1× Laemmli buffer. Solubilized proteins were quantified using the modified Lowry assay (Bio-Rad Protein Assay Kit, Mississauga, Canada) with bovine serum albumin (BSA) as a standard. Equal amounts of solubilized protein (10-50 μg depending on the experiment) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Nonspecific antigenic sites were blocked, and membranes were incubated with the indicated primary antibodies. For differentiation experiments, the following antibodies were used: mouse anti–peroxisome proliferator-activated receptor γ (anti-PPARγ) (catalog no. sc-7273), rabbit anti-C/EBPα (catalog no. sc-61; both from Santa Cruz, Santa Cruz, CA), rabbit anti-FAS (catalog no. 610962; BD Biosciences), rabbit anti-ERK1/2 (catalog no. 06-182; Millipore, Billerica, MA), and rabbit anti-ACLP (gift from Dr M Layne). For insulin signaling experiments, the following antibodies were used: mouse antiphosphotyrosine (pTyr100; catalog no. 9411), rabbit anti-pAkt (Ser473; catalog no. 9271), rabbit anti-pERK1/2 (Thr202/Tyr204; catalog no. 4370), rabbit anti-Akt (catalog no. 9272; all from Cell Signaling, Danvers, MA), rabbit anti-ERK1/2 (catalog no. 06-182), rabbit anti-IRS1 (catalog no. 06-248; both from Millipore), and rabbit anti-ACLP. Membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA), and protein expression was visualized by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Millipore). Immunoreactivity was quantified using the AlphaEaseFC Software from Alpha Innotech (Santa Clara, CA) and expressed as integrated optical density (IOD) units.

2.6. Insulin signaling

pLXSN and pLXSN-ACLP preadipocytes were seeded on regular or collagen I–coated dishes and grown until confluence. Cells were placed in DMEM supplemented with 1% calf serum and antibiotics for 16 to 18 hours before stimulation for 5 minutes with vehicle, which was Krebs-Ringer-Hepes (KRH) BSA at 2 mg/mL), or with 10 nmol/L insulin in BSA/KRH. Cells were then lysed with 1× Laemmli buffer. Solubilized proteins were quantified, and equal amounts were used for immunoblotting.

2.7. Statistical analysis

Differences between multiple means were analyzed using analysis of variance followed by the post hoc Newman-Keuls test. Differences between 2 means were analyzed using a paired *t* test (GraphPad InStat software version 3.05, La Jolla, CA). $P < .05$ was considered significant.

3. Results

Retroviral infection led to a 3.2-fold increase in ACLP protein expression in pLXSN-ACLP preadipocytes compared with pLXSN preadipocytes (Fig. 1). Elevated ACLP release into the medium was confirmed by detecting the presence of ACLP protein in 3T3-L1 preadipocyte medium. Aortic carboxypeptidase-like protein was 2.1-fold higher in medium conditioned for 48 hours by pLXSN-ACLP preadipocytes vs pLXSN preadipocytes.

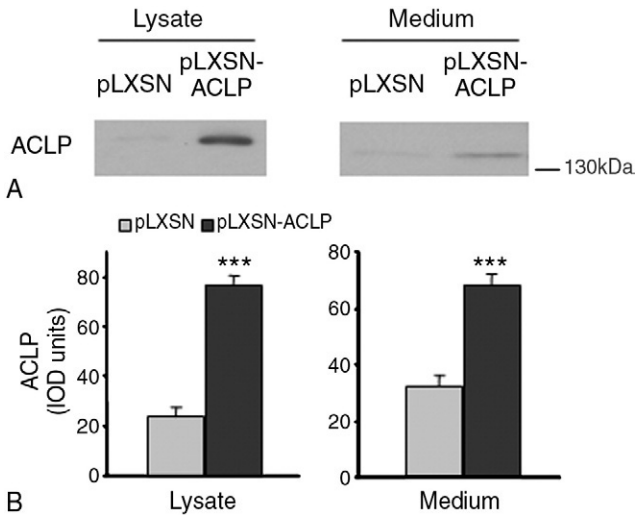

Fig. 1. Aortic carboxypeptidase-like protein overexpression increases 3T3-L1 preadipocyte ACLP release into medium. 3T3-L1 preadipocytes overexpressing ACLP (pLXSN-ACLP) or controls (pLXSN) were grown until confluence. Medium was changed to serum-free DMEM and antibiotics supplemented with 1 μmol/L insulin. After 48 hours, medium was collected, cells were lysed, and each was further processed for immunoblot analysis as described. A, An immunoblot from one experiment, representative of 3 separate experiments, is shown. B, Densitometric analysis of ACLP expression from 3 separate experiments is expressed as mean±SE. ***$P < .001$ compared with pLXSN values.

pLXSN and pLXSN-ACLP preadipocytes were grown in standard or collagen I–coated dishes and kept in either control medium or differentiation medium for 8 days. On day 8, the differentiation of pLXSN and pLXSN-ACLP adipocytes was assessed by morphology, TG accumulation, and the protein expression of genes indicative of the mature phenotype.

pLXSN preadipocytes differentiated in standard or collagen I–coated dishes became rounded and lipid laden (Fig. 2A), indicating that collagen I coating did not affect the morphologic changes of adipogenesis. In standard dishes or collagen I–coated dishes, the TG level was 2.5-fold higher in pLXSN adipocytes than in pLXSN preadipocytes (Fig. 2B), showing that collagen I coating does not affect the TG accumulation of 3T3-L1 adipocytes. However, the induction of FAS was increased 1.6-fold and that of C/EBPα and PPARγ was increased 1.4-fold for pLXSN preadipocytes grown in collagen I–coated dishes vs standard dishes.

In standard dishes, ACLP overexpression in 3T3-L1 preadipocytes did not affect morphology, TG accumulation, or the increase in adipogenic markers during differentiation (Fig. 2). However, when ACLP overexpressors were induced to differentiate in collagen I–coated dishes, the levels of FAS, C/EBPα, and PPARγ were decreased by 65%, 66%, and 59%, respectively, compared with controls (empty vector; Fig. 2C, D).

Insulin increases the phosphorylation of a number of signaling proteins, including IRS-1, Akt, and ERK1/2 [17]. The phosphocontent of these proteins was measured in confluent pLXSN and pLXSN-ACLP preadipocytes seeded on collagen I–coated dishes upon insulin stimulation. In pLXSN preadipocytes, the levels of the phosphorylated forms increased approximately 5-fold for IRS-1, approximately 13-fold for Akt, and approximately 6-fold for ERK1/2 (Fig. 3). In pLXSN-ACLP preadipocytes, insulin-stimulated Akt phosphorylation was 27% lower. There was no significant difference in the insulin-stimulated phosphorylation of IRS-1 or ERK1/2 between pLXSN vs pLXSN-ACLP preadipocytes.

4. Discussion

We have previously reported that ACLP overexpression does not inhibit 3T3-L1 adipocyte differentiation in standard culture conditions [9,10]. Recent studies using murine lung fibroblasts demonstrated that ACLP binds with collagen I [15]. Therefore, we examined whether the ability of ACLP overexpression to modulate the adipogenic response of 3T3-L1 preadipocytes would be different in collagen I–coated dishes.

The differentiation of pLXSN 3T3-L1 preadipocytes into adipocytes on collagen I–coated dishes compared with standard dishes shows a significant up-regulation of differentiation markers but no difference with respect to morphology or TG accumulation. This disconnection

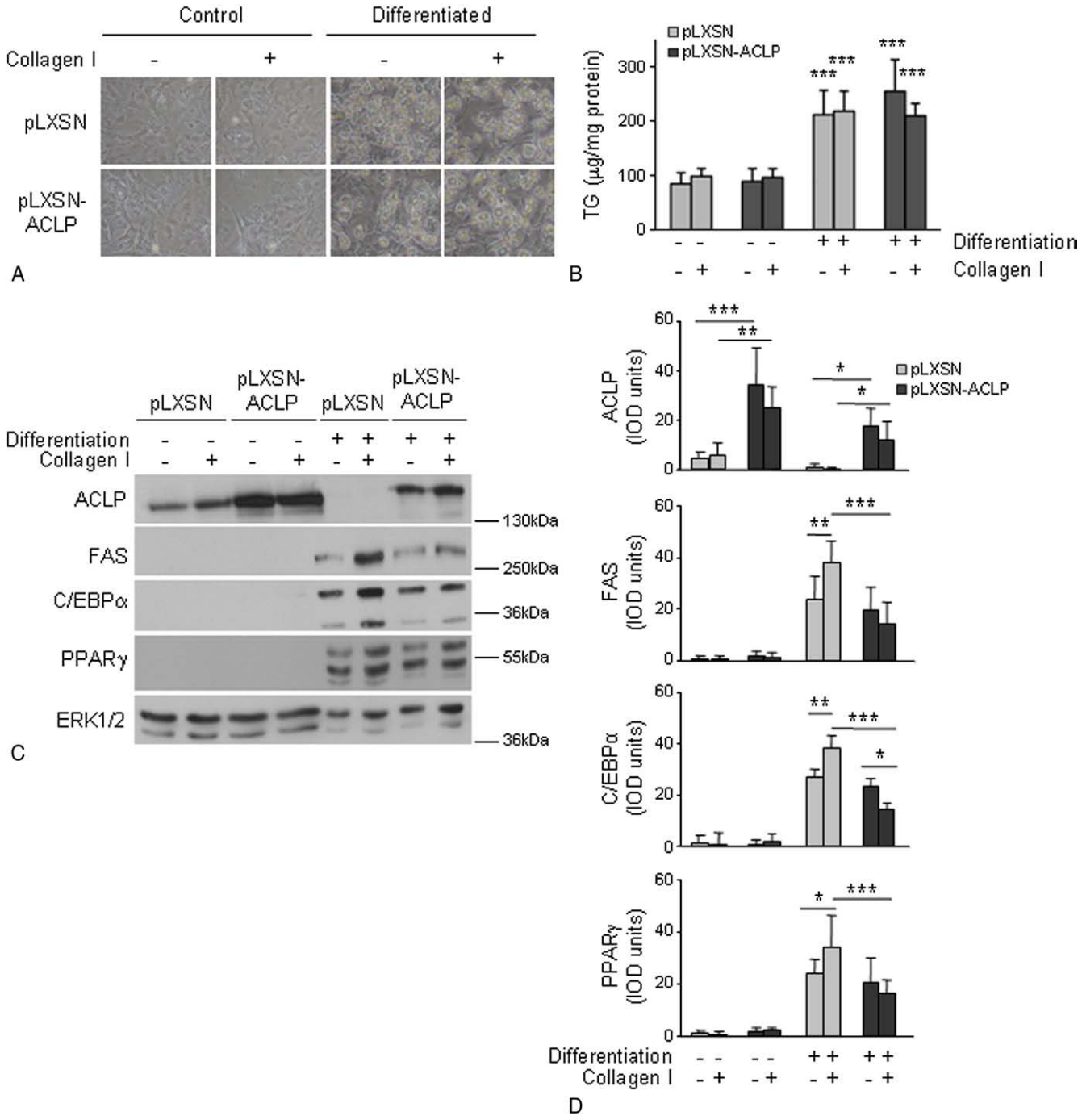

Fig. 2. Adipocyte differentiation on standard vs collagen I–coated dishes and the effect of ACLP overexpression. Confluent 3T3-L1 preadipocytes overexpressing ACLP (pLXSN-ACLP) or controls (pLXSN) were either kept in control medium or induced to differentiate on standard dishes vs collagen I–coated dishes for 8 days. A, Photomicrographs (200×) from one experiment, representative of 5 independent experiments, are shown. B, Triacylglycerol was extracted and quantified. Total TG was normalized to protein content. Results are the mean ± SD of 3 independent experiments. ***$P < .001$ compared with the respective undifferentiated controls. C, Cells were lysed, and equal amounts of solubilized protein were processed for immunoblot analysis using the indicated antibodies. Immunoblots from one experiment, representative of 5 separate experiments, are shown. ERK1/2 serves as a loading control. D, Densitometric results (mean ± SD) from 5 independent experiments are expressed as mean±SE. *$P < .05$, **$P < .01$, and ***$P < .001$ compared with respective undifferentiated controls or between indicated pairs.

between induction of gene associated with the mature phenotype vs TG accumulation has been observed by others. Collagen I enhances the expression of adipogenic genes of primary stromal vascular cells from pigs and humans, as well as 3T3-F442A cells, without altering TG accumulation levels or adipocyte morphology [18-21].

Notably, in the presence of collagen I coating, ACLP overexpression blunted some features of adipogenesis,

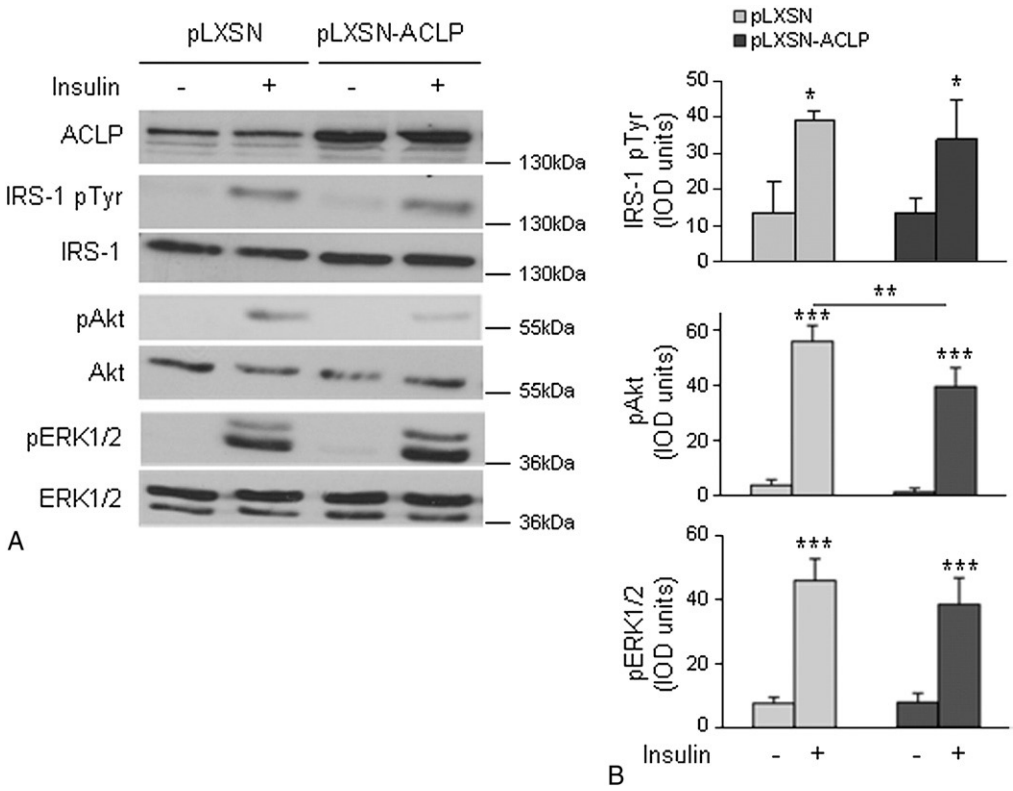

Fig. 3. Aortic carboxypeptidase-like protein overexpression reduces insulin-stimulated Akt phosphorylation in 3T3-L1 preadipocytes grown on collagen I–coated dishes. 3T3-L1 preadipocyte stable clones overexpressing ACLP (pLXSN-ACLP) or controls (pLXSN) were seeded on either standard dishes or collagen I–coated dishes. Two days postconfluence, cells were placed in reduced serum-containing medium for 16 hours, before stimulation with either 10 nmol/L insulin or vehicle for 5 minutes, as described. Cells were lysed, and equal amounts of solubilized protein were processed for immunoblot analysis using the indicated antibodies. A, Immunoblots from one experiment, representative of 3 separate experiments, are shown. B, Densitometric results (mean ± SD) from 3 independent experiments are expressed as IOD units. *$P < .05$, **$P < .01$, and ***$P < .001$ compared with respective vehicle controls or between indicated pair.

characterized by an impaired induction of FAS, C/EBPα, and PPARγ, proteins indicative of the mature adipocyte phenotype. The reason why TG accumulation was unaffected is not known. It might be because, despite the weakened up-regulation of these 3 proteins, the resulting levels were still sufficient for TG synthesis and accumulation to match control levels over the 8 days of the differentiation protocol. A detailed time course would be needed to better examine if the reduced adipogenic gene expression might be associated with a potential delay in the rate of TG accumulation.

It is not clear why ACLP overexpression altered the gene markers of adipogenesis only when collagen I was present. Collagen I can interact with cells either by binding to discoidin-domain receptors or integrins [22]. The higher amounts of ACLP released as a result of ACLP overexpression may have bound to collagen I, thereby potentially reducing the ability of collagen I to bind discoidin-domain receptors and redirecting collagen I binding to preadipocyte integrins. Collagen I binding to integrin $\alpha_2\beta_1$ has been reported to inhibit phosphorylation of Akt in osteosarcoma Saos-2 cells [23]. The reduced phosphorylation of Akt that we observed in ACLP-overexpressing preadipocytes in the presence of collagen I is consistent with this model. Akt activation is necessary and sufficient for adipogenesis [24,25]. The inhibition of insulin-stimulated Akt phosphorylation we observed for the ACLP overexpressors may account for the altered adipogenic gene expression. Insulin-stimulated phosphorylation of Akt partly promotes adipogenesis by inactivation of 2 inhibitors of adipogenesis: forkhead box O 1 and GATA binding protein 2 [26-28].

The mechanism by which insulin-stimulated Akt phosphorylation is disrupted is not known. Our data suggest that signaling from the insulin receptor to IRS-1 is intact, based on overall IRS-1 tyrosine phosphorylation levels, and that ERK1/2 phosphorylation is unaffected. Selective changes in specific tyrosine residues may not be detectable by this approach, so that there may be an inhibition of PI 3-kinase docking to IRS-1 or direct inhibition of PI 3-kinase itself. Future studies will be needed to address these possibilities.

Finally, we have verified that ACLP-overexpressing preadipocytes are functionally intact and competent to secrete the higher amounts of ACLP being produced. There was 2.1-fold more ACLP protein present in the medium of overexpressors than of empty vector (pLXSN) preadipocytes. Another group has suggested that ACLP overexpression inhibits 3T3-F442 adipogenesis and promotes smooth muscle transdifferentiation [12]. The ACLP construct used by them possessed a c-myc tag, and perhaps

this may contribute to the differences between their results and ours.

It is relevant that recent investigations have revealed excessive collagen and ECM production as important features of inflamed adipose tissue in rodent and human obesity [29-31]. Studies examining the transgenic overexpression of a constitutively activated version of hypoxia-inducing factor 1α in mice detected excessive cross-linking of collagen I and collagen III leading to collagen fiber formation associated with inflammation in adipose tissue [29]. Human preadipocytes, when exposed to macrophage-secreted factors, overproduce collagen I and fibronectin, forming a fiber network [30]. Collagen VI has also been found to be highly expressed in rodent and human adipose tissue; and mice lacking collagen VI are protected from the metabolic dysfunction associated with obesity, perhaps because of a weakened adipocyte scaffold structure permitting cell expansion without the usual associated cell stress and inflammation [31]. The prominence of collagen within adipose tissue could potentially facilitate ACLP interactions. Aortic carboxypeptidase-like protein can bind collagen I; but whether it can also bind other collagens, such as collagen VI, is not known [15].

Our in vitro studies here have shown that the presence of ACLP affects the ability of collagen I to alter the induction of adipogenic markers. To thoroughly study the potential impact on adipocyte function of the altered differentiation gene program but with normal TG accumulation, a series of responses should be evaluated, including glucose transport, lipolysis, and adipokine production. It will be important for future investigations of ACLP and its cellular effects to include considerations of the role of collagen. It is also possible that more sophisticated collagen-based 3-dimensional matrices may further accentuate the in vitro action of ACLP on specific aspects of adipogenesis.

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