Original Research

Mevalonate deprivation mediates the impact of lovastatin on the differentiation of murine 3T3-F442A preadipocytes

Manal Elfakhani[1], Sheida Torabi[1], Deema Hussein[1], Nathaniel Mills[2], Guido F Verbeck[3] and Huanbiao Mo[1]

[1]Department of Nutrition and Food Sciences, Texas Woman's University, Denton, TX 76204, USA; [2]Department of Biology, Texas Woman's University, Denton, TX 76204, USA; [3]Department of Chemistry, University of North Texas, Denton, TX 76203, USA
Corresponding author: Huanbiao Mo. Email: hmo@mail.twu.edu

Abstract

The statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity and consequently the synthesis of mevalonate. The use of statins is associated with insulin resistance, presumably due to the impaired differentiation and diminished glucose utilization of adipocytes. We hypothesize that mevalonate is essential to adipocyte differentiation and adipogenic gene expression. Adipo-Red assay and Oil Red O staining showed that an eight-day incubation with 0–2.5 μmol/L lovastatin dose-dependently reduced the intracellular triglyceride content of murine 3T3-F442A adipocytes. Concomitantly, lovastatin downregulated the expression of peroxisome proliferator-activated receptor γ (*Pparγ*), leptin (*Lep*), fatty acid binding protein 4 (*Fabp4*), and adiponectin (*AdipoQ*) as measured by quantitative real-time polymerase chain reaction (real-time qPCR). The expression of sterol regulatory element binding protein 1 (*Srebp-1*), a transcriptional regulator of *Pparγ* and *Lep* genes, was also suppressed by lovastatin. Western-blot showed that lovastatin reduced the level of CCAAT/enhancer binding protein α (C/EBPα) while inducing a compensatory over-expression of HMG CoA reductase. The impact of lovastatin on intracellular triglyceride content and expression of the adipogenic genes was reversed by supplemental mevalonate. Mevalonate-derived metabolites have essential roles in promoting adipogenic gene expression and adipocyte differentiation.

Keywords: Lovastatin, adipocyte, differentiation, HMG CoA reductase, mevalonate, PPARγ, C/EBPα, SREBP-1c, leptin, adiponectin, fatty acid binding protein 4

***Experimental Biology and Medicine* 2014; 239: 293–301. DOI: 10.1177/1535370213517614**

Introduction

Obesity is characterized by, among many other physiological changes, over-accumulation of differentiated adipocytes and is a risk factor for chronic diseases such as type 2 diabetes, hypertension, coronary heart disease, and cancer. At the cellular level, obesity is characterized by hyperplasia and hypertrophy of adipocytes differentiated from fibroblastic preadipocytes. Although adipocyte differentiation starts as a physiological response to energy imbalance, dysregulation of adipogenesis leads to pathological conditions. Suppressing adipocyte differentiation or adipogenesis is one of the potential approaches in obesity prevention.

Adipocyte differentiation is a well-orchestrated multistep process involving several genes. Peroxisome proliferator-activated receptor γ (PPARγ) is one of the central regulators of adipocyte differentiation and is essential to adipose tissue formation *in vivo*.[1] By interacting with CCAAT/enhancer binding protein α (C/EBPα), another adipogenic gene expressed in the early stage of adipocyte differentiation, PPARγ regulates the expression of adipogenic genes including leptin (*Lep*), adipocyte fatty acid-binding protein 4 (*Fabp4 or aP2*), and adiponectin (*AdipoQ*), all playing important roles in fatty acid metabolism and adipocyte functions.[2] PPARγ activity is enhanced by adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element (SRE) binding protein 1 (ADD1/SREBP-1),[1] a promoter of adipocyte differentiation that increases the availability of ligands for PPARγ.[3] SREBP-1c binds to the SRE of adiponectin promoter and stimulates adiponectin expression.[4] PPARγ, C/EBPα, and SREBP-1c are all involved in early stages of adipocyte differentiation.[1]

The 3T3 preadipocytes are a well-established *in vitro* model[5] for studying adipocyte differentiation because their morphology resembles that of fibroblastic preadipocytes from animal fat tissues. Mature 3T3-L1 cells share virtually all characteristics with adipocytes in the adipose tissues.[6] In this study, we used 3T3-F442A preadipocytes, a subclone[7] of 3T3-L1 cells that are able to form fat pads

similar to normal adipose tissues when injected into mice.[8] The formation and appearance of fat droplets in these differentiated cells mimic adipose tissue *in vivo*.

It has been suggested that the signaling of insulin, a promoter of preadipocyte differentiation,[9] requires mevalonate-derived metabolites.[10] Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in the mevalonate pathway to provide mevalonate-derived sterol and non-sterol products.[11] Statins have been shown to inhibit 3T3-L1 preadipocyte differentiation. Nevertheless, their effect on the genes involved in adipocyte differentiation is poorly understood and whether their impact on preadipocyte differentiation is mediated by modulation of HMG CoA reductase and mevalonate deprivation is uncertain; the latter is shown in the contradictory evidence for the ability of supplemental mevalonate to reverse the effect of statins.[12,13] The effect of statins has also not been examined with 3T3-F442A cells.

In this study we evaluated the impact of lovastatin on 3T3-F442A preadipocyte differentiation and the expression of adipogenic genes including *Pparγ*, *Srebp-1c*, *Fabp4*, *AdipoQ*, and *Lep*. We further determined whether the impact of lovastatin is mediated by mevalonate deprivation.

Materials and methods

Culture and Oil Red O staining of 3T3-F442A cells

Murine 3T3-F442A cells purchased from Dr Howard Green (Harvard Medical School) were cultured in 24 well (1×10^4 cells/0.3 mL medium/well) and 6-well (4×10^4 cells/3 mL medium/well) plates in Dulbecco's modified Eagle's medium (DMEM) adjusted by American Type Culture Collection (ATCC, Manassas, VA) to contain 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, supplemented with 10% bovine calf serum (BCS, Fisher Scientific Company LLC, Houston, TX) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 10% CO_2. Upon reaching ~70% confluency the 3T3-F442A cells were switched from the maintenance medium above to DMEM supplemented with 10% fetal bovine serum (FBS, Fisher Scientific Company), 5 μg/mL insulin (Sigma Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (GIBCO). Lovastatin (gift from Merck Research Laboratories, Rahway, NJ; 1.25 and 2.5 μmol/L) pre-dissolved in dimethyl sulfoxide (DMSO, ATCC) was added to the experimental groups and kept until the end of the study. Control cells were incubated in medium containing DMSO (0.1%, v/v). Following a 2-day incubation the cells were cultured in DMEM supplemented with 10% FBS and 5 μg/mL insulin for additional 2 days. Cells were continued in medium without insulin for additional 5–7 days until the differentiation of adipocytes. Cells were rinsed with phosphate buffer solution (PBS) twice and fixed in 1 mL of 10% formalin per well at room temperature for 1 h. Cells were then rinsed with deionized water and stained with 0.5 mL of 0.3% freshly filtered Oil Red O working solution per well at room temperature for 30 min[14] to visualize cellular neutral lipids. The cells were then washed with 1 mL deionized water per well for three times before photomicrographs of representative fields of monolayer cells were taken with a Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

AdipoRed™ assay for measuring intracellular triglyceride content

Eight days after the induction of differentiation, lipid content was quantified using an AdipoRed™ Assay kit (Lonza, Walkersville, MD) according to manufacturer's instructions. AdipoRed™ contains the hydrophilic Nile Red stain and allows the quantification of intracellular lipid droplets.[15] Differentiated cells were rinsed with 2 mL Hank's Balanced Salt Solution (HBSS) and to each well, 5 mL HBSS and 140 μL of AdipoRed™ reagent were added. After 10 – 15 min. of incubation, the plates were positioned in a Tecan Infinite M200 microplate reader (Tecan Systems Inc., Salzburg, Austria) and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm.

Cell viability assay

To measure the viability of preadipocytes, cultures of 3T3-F442A preadipocytes were seeded (2500 cells/well) in 100 μL maintenance medium in 96-well plates and incubated for 48 h at 37°C in a humidified atmosphere of 10% CO_2. The medium was then aspirated from each well and replaced with 100 μL of fresh medium containing DMSO or escalating concentrations of lovastatin (1.25–10 μmol/L). The cells continued to incubate for an additional 24 or 48 h. Cell viability following a quick rinse with 0.1 mL HBSS was determined by adding 100 μL of serum-free medium and 20 μL of CellTiter 96® Aqueous One Solution (Promega, Madison, WI); plates were held in the dark at 37°C for 2 h and then read at 490 nm with a Tecan infiniteM200 plate reader with Magellan™ software version 6.3 (Tecan Systems Inc.). Absorbances from wells containing cell-free medium were used as baselines and were deducted from absorbances of other cell-containing wells. Cell viability was also measured in mature adipocytes. Cultures of 3T3-F442A preadipocytes were seeded and incubated as described above. Cells were then induced to differentiate as described above and grown to maturation. On day 8–10 of differentiation, the medium was aspirated from each well and replaced with 100 μL of fresh medium containing DMSO or lovastatin (1.25–10 μmol/L). The cells continued to incubate for an additional 24 or 48 h. Cell viability following a quick rinse with 0.1 mL HBSS was determined with CellTiter 96® Aqueous One Solution as described above.

Quantitative real-time polymerase chain reactiont. Murine 3T3-F442A cells cultured in tissue culture dishes (100 mm × 20 mm, Corning Life Sciences, Wilkes Barre, PA) at 2×10^5 cells per dish were incubated in the presence or absence of lovastatin for 8–10 days. Once the cells became mature adipocytes, total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA)

Table 1 Primer sequences (forward and reverse) and GenBank accession numbers used in the quantitative real-time polymerase chain reaction (real-time qPCR)

Gene	Accession#	Primer Sequence	
Pparγ	NM_001127330 & NM_011146	5'-AGAGGGCCAAGGATTCATGACCAGG-3'	5'-TTCAGCTTGAGCTGCAGTTCCAGGG-3'
Srebp-1	NM_011480	5'-TGGCACCCTCTTGCTCTGTAGGCAC-3	5'-GCTGAGGCAGACATCTGCCTACCCA-3'
Lep	NM_008493	5'-TGGAGGTGAGCGGGATCAGGTTTTG-3	5'-TGGCACGTGGGATCTTTCAGAAGCC-3'
Fabp4	NM_024406	5'-GTGTGATGCCTTTGTGGGAACCTGG-3'	5'-TGCGGTGATTTCATCGAATTCCACG-3'
AdipoQ	NM_009605	5'-CGGCAGCACTGGCAAGTTCTACTGC-3'	5'-TTGTGGTCCCCATCCCCATACACCT-3'

Pparγ: peroxisome proliferator-activated receptor γ; *Srebp*-1: sterol regulatory element binding protein-1; *Lep*: leptin; *Fabp4*: fatty acid-binding protein 4; *AdipoQ*: adiponectin.

according to the manufacturer's protocols. RNA concentration was determined using 25 A_{260nm}/mg RNA and purity of the isolated total RNA was determined spectrophotometrically using A_{260nm}/A_{280nm}; a minimal ratio of 1.9 was required for further analysis. The integrity of the purified total RNA was verified by analysis of the 28 S/18 S ribosomal RNA (rRNA) ratio using gel electrophoresis. Samples were run on a 1.5% agarose gel (Tris-acetate (TAE) buffer) at 80 volts for 90 min and visualized by Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA) following the addition of 0.5 μg/mL ethidium bromide.

The mRNA expression levels of *Pparγ*, *Srebp-1c*, *Fabp4*, *Lep*, and *AdipoQ* were analyzed by reverse transcription (RT) followed by quantitative real-time polymerase chain reaction (real-time qPCR). Oligo $(dT)_{20}$ primer and SuperScript® III First-Strand kit (Invitrogen, Grand Island, NY) were used to reverse transcribe 2 μg of total RNA in a 20 μL reaction buffer into cDNA following the manufacturer's instructions. cDNA was diluted by 25-fold with 25 μg/mL acetylated bovine serum albumin solution and 6 μL of diluted cDNA was amplified in a 25 μL PCR solution containing 125 nmol/L of both forward and reverse primers of the gene and iQTM SYBR® Green Supermix (Bio-Rad). Primers (Table 1) were designed using Vector NTI Advance version 11 software (Invitrogen). The cDNA was denatured at 95°C for 3 min followed by 40 cycles of PCR (94°C for 30 s, 60°C for 25 s, 72°C for 25 s, and 78°C for 9 s) by means of an iQTM5 multi-color Real-Time PCR Detection System (Bio-Rad) with Bio-Rad iQ5 Optical System Software (version 2.1). The mRNA levels of all the genes were normalized using ribosomal protein L22 (RPL22) as internal control[16] and the ΔC_T method. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta CT}$ method. To ensure that a single gene was amplified, the melting profile of double stranded DNA product of PCR generated from each primer was analyzed. The specific PCR products were purified with HT ExoSAP-IT® High Throughput PCR Product Clean-Up (USB® Products, Affymetrix Inc., Cleveland, Ohio, USA) and incubated at 37°C for 30 min to degrade remaining primers and nucleotides (dNTPs). The purified double-stranded DNA was warmed at 80°C for 30 min to inactivate the ExoSAP-IT and was then quickly chilled on ice. To confirm specificity, PCR primers were used to sequence both strands of DNA.

Western-blot. Following incubation with 0 and 1.25 μmol/L lovastatin in the presence and absence of 100 μmol/L mevalonolactone for 8–10 days, 3T3-F442A cells were scraped and centrifuged at 1250 r/min for 5 min. The cell pellets were resuspended in 1 mL ice-cold PBS and cell lysate was fractionated as reported.[17] Protein concentration of each sample was determined with BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Membrane and non-membrane extracts (20 μg proteins) were each mixed with equal volume of buffer (62.5 mmol/L Tris-HCl, pH 6.8, 15% [w/v] SDS, 8 mol/L urea, 10% [v/v] glycerol, and 100 mmol/L dithiothreitol) and 1/6 volume of the 4 × SDS loading buffer (150 mmol/L Tris-HCl, pH 6.8, 12% [w/v] SDS, 30% glycerol, 6% β-mercaptoethanol, and bromphenol blue) and incubated at 37°C for 20 min prior to loading to a 10% SDS polyacrylamide gel. Electrophoresis with a Mini PROTEAN® 3 (Bio-Rad) unit and western-blot with antibodies to HMG CoA reductase (gift from Dr. Russell DeBose-Boyd of University of Texas Southwestern Medical Center in Dallas and Howard Hughes Medical Institute) and C/EBPα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were performed as described.[17]

Statistics

One-way analysis of variance (ANOVA) and Tukey's *post hoc* tests were performed to assess the differences between groups using Prism® 4.0 software (GraphPad Software Inc., San Diego, CA). Levels of significance were designated as $P < 0.05$.

Results

We first determined the impact of lovastatin on the differentiation of murine 3T3-F442A cells. Figure 1A (a–c) shows that lovastatin (0–2.5 μmol/L) induced a concentration-dependent reduction in the number of lipid droplets stained by Oil Red O in each cell and the total amount of visible lipids. Supplemental mevalonate (500 μmol/L) reversed the effect of lovastatin by enhancing the lipid content of 3T3-F442A cells (Figure 1A, d–f). Cellular triglyceride content measured by AdipoRed™ Assay was also reduced by lovastatin in a dose-dependent manner (Figure 1B). Consistent with the results of Oil Red O staining, supplemental mevalonate reversed the lovastatin-mediated reduction of triglyceride content.

To evaluate whether the decreased cellular triglyceride was caused by acute cytotoxicity, cell viability was measured with 3T3-F442A preadipocytes prior to (Figure 2A and B) and following (Figure 2C and D)

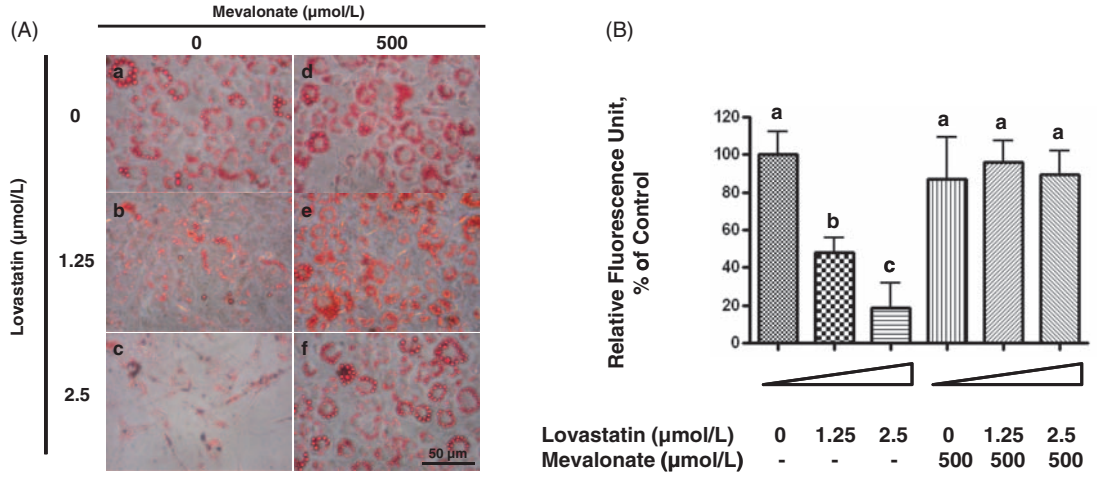

Figure 1 Lovastatin-mediated mevalonate deprivation suppresses the differentiation of murine 3T3-F442A preadipocytes. (A) Phase-contrast microscopy of Oil Red O-stained 3T3-F442A preadipocytes following 8-day differentiation. 3T3-F442A cells were incubated with 0 (a and d), 1.25 (b and e) and 2.5 (c and f) μmol/L lovastatin and 0 (a–c) and 500 (d–f) μmol/L mevalonolactone during differentiation. (B) Triglyceride content of 3T3-F442A cells at the end of 8-day differentiation determined by AdipoRed™ Assay. (A color version of this figure is available in the online journal)

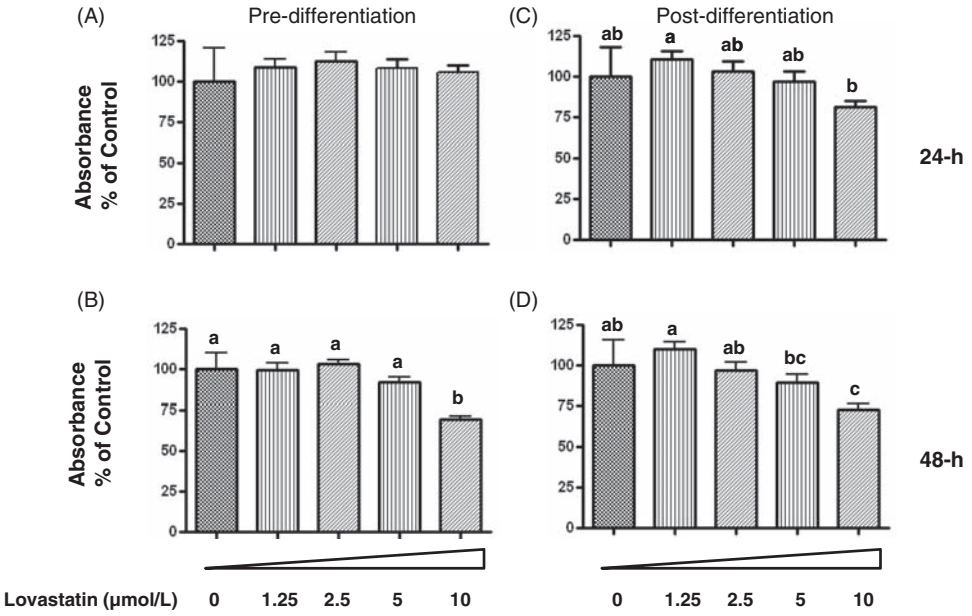

Figure 2 The concentration- and time-dependent impact of lovastatin on viability of murine 3T3-F442A preadipocytes prior to (A and B) and following (C and D) 8-day differentiation. The pre-differentiation (A and B) and differentiated 3T3-F442A cells were incubated with lovastatin (0–10 μmol/L) for 24 - (A and C) and 48 - (B and D) hours before cell viability was determined by CellTiter 96® Aqueous One Assay

differentiation process. When 3T3-F442A preadipocytes were incubated with 1.25–10 μmol/L of lovastatin for 24 (Figure 2A) or 48 h (Figure 2B) prior to differentiation, only 10 μmol/L of lovastatin reduced cell viability following a 48-h incubation. The same concentration of lovastatin (10 μmol/L) was required to decrease the viability of mature, fully-differentiated 3T3-F442A cells (Figure 2C and D), suggesting that the lower lipid content shown in Figure 1A was not due to lovastatin-induced cytotoxicity.

The effect of lovastatin on the expression of *Pparγ* and *Srebp-1c*, two genes that are critical in adipocyte differentiation, was then examined. The PPARγ mRNA level in differentiated 3T3-F442A adipocytes had a near-significance ($P = 0.11$) increase over that in preadipocytes without adipogenic inducers (IDI-) (Figure 3A), an observation likely in accordance with the role of PPARγ in adipocyte differentiation. Consistent with lovastatin-mediated reduction in lipid content (Figure 1), lovastatin at 2.5 μmol/L suppressed the PPARγ mRNA level by >95%. Supplemental mevalonate restored PPARγ mRNA to the control level, suggesting that mevalonate or mevalonate-derived metabolite(s) are required for *Pparγ* expression and potentially adipocyte differentiation. The expression pattern of *Srebp-1c* in preadipocytes prior to and following differentiation

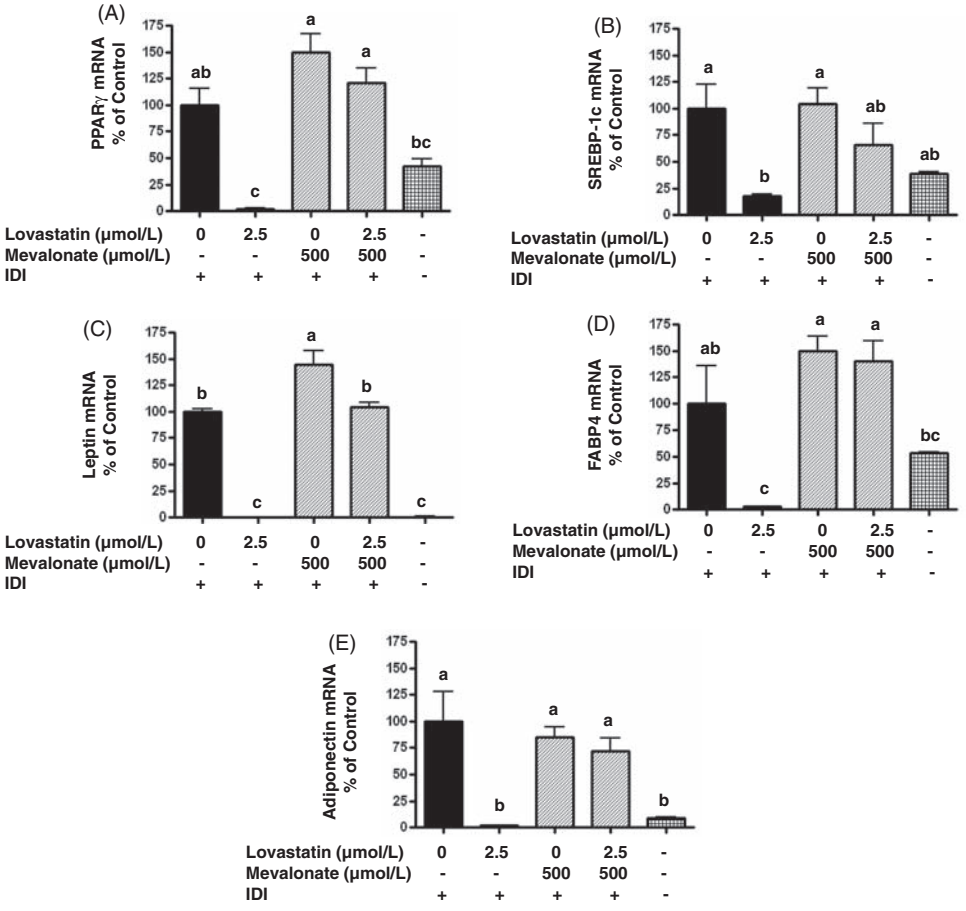

Figure 3 Mevalonate attenuates the impact of lovastatin on the expression of adipogenic genes. 3T3-F442A preadipocytes were incubated in the absence (-) or presence (+) of IDI, lovastatin, and mevalonate as indicated for 8 days. At the end of incubation cells were lysed and total RNA extracted. Cellular mRNA levels of *Pparγ* (A), *Srebp-1c* (B), *Lep* (C), *Fabp4* (D), and *AdipoQ* (E) were measured by RT-PCR

corresponds to that of *Pparγ*, and the impact of lovastatin and supplemental mevalonate on the expression of *Srebp-1c* (Figure 3B) mimics that on *Pparγ* as well.

Next we evaluated the genes under the regulation of PPARγ and SREBP-1c. Differentiated 3T3-F442A cells had much higher leptin mRNA level than preadipocytes (IDI-) (Figure 3C), an observation consistent with the role of leptin as a marker of adipocyte differentiation and the expression pattern of *Pparγ* and *Srebp-1c*. In accord with lovastatin-mediated reduction in lipid content (Figure 1) and the expression of *Pparγ* and *Srebp-1c* (Figure 3A and B), lovastatin at 2.5 μmol/L suppressed the leptin mRNA level by >95%. Supplemental mevalonate restored leptin mRNA to the control level, confirming the requirement of mevalonate-derived metabolites in adipocyte differentiation. The expression of *Fabp4* (Figure 3D) and *AdipoQ* (Figure 3E) in adipocytes and preadipocytes and the impact of lovastatin and supplemental mevalonate follow the same pattern as those of leptin.

Consistent with the effect of lovastatin on the adipogenic genes, a lower concentration of lovastatin (1.25 μmol/L) sufficient to reduce cellular triglyceride content (Figure 1) inhibited the expression of C/EBPα protein; supplemental mevalonate (100 μmol/L) attenuated the lovastatin effect (Figure 4). The lovastatin-mediated reduction in C/EBPα

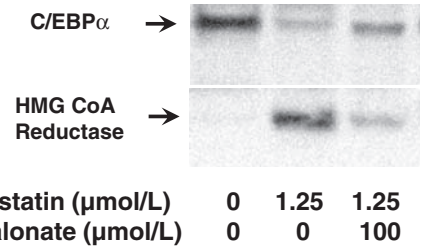

Figure 4 Mevalonate attenuates the impact of lovastatin on the expression of C/EBPα and HMG CoA reductase in 3T3-F442A adipocytes. 3T3-F442A preadipocytes were incubated in the absence or presence of lovastatin (1.25 μmol/L) and mevalonate (100 μmol/L) as indicated for 8 days. At the end of incubation cells were lysed. Cell fractions were subject to western-blot with antibodies to HMG CoA reductase (membrane fraction) and C/EBPα (non-membrane fraction)

level was accompanied by a compensatory increase in the level of HMG CoA reductase protein; supplemental mevalonate attenuated the lovastatin effect.

Discussion

Previous studies have shown that lovastatin,[12,18] atorvastatin,[19] simvastatin,[13,20] rosuvastatin,[13] and pitavastatin[13] inhibit 3T3-L1 preadipocyte differentiation. The anti-adipogenic effect of statins was also shown in animal

studies. Lovastatin inhibited adipogenesis in rabbits.[21] When administered to high-fat-fed pregnant animals, there were decreases in obesity, hypertension, and inflammation in offspring.[22]

Our present study shows that 3T3-F442A cells are more sensitive than 3T3-L1 cells ($IC_{50} = 10\,\mu mol/L$)[12] to lovastatin-mediated suppression of differentiation, suggesting that 3T3-F442A cells are a viable alternative model to study the impact of mevalonate suppressors on adipogenesis. Interestingly, while lovastatin-containing red yeast rice[23] reduced triglyceride content of 3T3-L1 cells, the authors indicated that monocolin K, or lovastatin, did not have such an effect.[24] The level of lovastatin evaluated was not clear.

Our finding that lovastatin downregulates genes critical in adipocyte differentiation, *Pparγ* and *Srebp-1c*, and biomarkers of mature adipocytes, *Lep*, *Fabp4* and *AdipoQ*, is consistent with previous observations that statins inhibited 3T3-L1 preadipocyte differentiation by downregulating *AdipoQ*,[18] *Pparγ*,[19] and *Fabp4*.[13,18] Furthermore, we showed lovastatin-induced downregulation of C/EBPα, an early regulator of adipogenesis, and annihilation of the lovastatin effect by supplemental mevalonate, consistent with a previous finding that atorvastatin-mediated suppression of C/EBPα protein in 3T3-L1 cells was reversed by mevalonate-derived geranylgeranyl pyrophosphate.[19] Chinese red yeast rice also reduced the levels of *Pparγ*, *Fabp4*, and *Lep* in 3T3-L1 cells.[24] Downregulating *Fabp4* may have particular application in controlling metabolic syndrome and diabetes because *Fabp4* gene plays a central role in linking obesity and insulin resistance.[25]

SREBP-1c regulates genes such as fatty acid synthase, glycerol-3-phosphate acyltransferase, and stearoyl CoA desaturase 2 that are important in adipocyte differentiation.[26] Upregulation of ADD1/SREBP provides PPARγ agonists that further stimulate the differentiation process.[27] *Srebp-1a* and *-1c* differ in 5′ exon. In mouse adipose tissues SREBP-1c mRNA dominates, with a 1c:1a ratio of 3 : 1. However, in cultured 3T3-L1 cells, SREBP-1c mRNA was virtually undetectable. Since our primer matches the sequence of exon 19 of *Srebp-1*, it does not differentiate *1a* and *1c*.

Our observation that supplemental mevalonate reversed the impact of lovastatin on cellular lipid content (Figure 1) and the expression of adipogenic genes (Figure 3) and proteins (Figure 4) in 3T3-F442A cells are consistent with previous reports that mevalonate (50–500 μmol/L) reversed statin-mediated inhibition of 3T3-L1 preadipocyte differentiation.[12,19] At a much lower concentration (10 μmol/L), mevalonate was shown to be ineffective in reversing the effect of pitavastatin,[13] consistent with our observation that 5 μmol/L mevalonate did not reverse the effect of lovastatin on intracellular triglyceride content (data not shown).

Similar to mevalonate-mediated reversal of the statin effect, mevalonate-derived non-sterol isoprenoids including farnesol, geraniol,[12] and geranylgeranyl pyrophosphate,[19] but not cholesterol[12,13] or squalene,[12] attenuated statin-mediated inhibition of 3T3-L1 preadipocyte differentiation, suggesting that mevalonate-derived non-sterols rather than sterols are required for adipocyte differentiation. Farnesyl pyrophosphate (FPP), a downstream metabolite of mevalonate, may mediate the reversal effect of mevalonate because FPP is an endogenous PPARγ agonist; this is further supported by the observation that the effect of FPP was counteracted by a PPARγ antagonist on 3T3-L1 differentiation.[18]

The dependency of 3T3-F442A preadipocyte differentiation on mevalonate may also be mediated by protein prenylation. Small GTPases including Ras[28] and Rho[29] are involved in 3T3-L1 differentiation. Farnesylated Ras mediates insulin-induced adipocyte differentiation.[9] Furthermore, insulin stimulated the activities of farnesyl transferase[30,31] and geranylgeranyl transferase II[32] and the prenylation of Ras,[30,31] Rho,[31] Rab-3, and Rab-4[32] in 3T3-L1 adipocytes. Conversely, inhibitors of farnesyl- and geranylgeranyl-transferases block 3T3-L1 differentiation.[9]

The clonal expansion and subsequent differentiation of preadipocytes are also mediated by insulin-like growth factor 1 (IGF-1) receptor[33] that requires mevalonate-derived dolichol for its *N*-linked glycosylation and membrane attachment.[34] This dual impact of statins on the *N*-linked glycosylation and mitogenic activity of IGF-1 receptor and the prenylation of GTPases[35] may explain the higher sensitivity of proliferating and differentiating preadipocytes than that of differentiated adipocytes to statin-mediated impact on cell viability and apoptosis.[36] In this study, we found that the viability of undifferentiating preadipocytes and mature adipocytes was unaffected by up to 48 h of incubation with lovastatin at up to 10 μmol/L, a concentration four-fold higher than that required to inhibit differentiation (Figure 2). The reduced lipid content (Figure 1B) could be a composite effect of diminished cell number and lower level of differentiation following the 8-d incubation; the former may be attributed to lovastatin-mediated inhibition of clonal expansion and the latter, suppression of expression of adipogenic genes. There was also no indication of apoptosis based on ethidium bromide and acridine orange dual staining and fluorescence microscopy in undifferentiating preadipocytes and mature adipocytes following incubation with lovastatin (0–2.5 μmol/L) for up to 48 h (data not shown).

Alternatively, statins block the synthesis of oxysterols that are ligands of LXR, which is a transcriptional activator of ADD-1/SREBP-1 c gene,[37] and reduce SREBP-1c mRNA in rat hepatoma cells[38]; the effect was reversed by mevalonate. It is unknown whether statin-mediated downregulation of *Srebp-1c* in adipocytes is attributed to decreased oxysterol synthesis, or the previously reported failure of cholesterol to attenuate the statin effect is due to poor cellular uptake.

An observation relevant to the role of mevalonate in adipocyte differentiation is that HMG CoA reductase expression is upregulated in differentiating[39] and differentiated[40] 3T3-L1 cells and adipose tissues from obese rodents[41] and humans.[42] HMG CoA reductase is under a multivalent regulation at the transcriptional and posttranscriptional levels.[11] Figure 4 shows that lovastatin induced a compensatory upregulation of HMG CoA reductase – though the reductase would remain inactive in the presence of

lovastatin – due to lovastatin-mediated inhibition of sterol biosynthesis and the consequent lack of sterol-mediated feedback inhibition of the transcription of HMG CoA reductase gene. Supplemental mevalonate reversed the statin-mediated upregulation of HMG CoA reductase gene (Figure 4) and concomitantly, the suppression of adipogenesis (Figures 1 and 3). Though several studies have shown the effect of statins on adipocyte differentiation, we demonstrated herein for the first time that such an effect is accompanied by the modulation of HMG CoA reductase, the direct target of the statins.

In addition, the expression of geranylgeranyl pyrophosphate (GGPP) synthase is upregulated by 20 fold in differentiating 3T3-L1 adipocytes and by 5–20 fold in adipose tissue from ob/ob mice.[43] Upregulation of HMG CoA reductase and GGPP synthase may provide essential intermediates for adipocyte differentiation.

It is controversial whether statins induce insulin resistance.[19,44–46] Our observation that lovastatin downregulates *AdipoQ*, an adipokine produced exclusively in the adipose tissues[47] and a biomarker for insulin sensitivity,[48] may support this possibility. Statin also reduced insulin-induced glucose uptake[36] and lipophilic statins may inhibit glucose-induced calcium signaling and insulin secretion in β-cells.[49] Insulin resistance may also result from statin-induced deficiency of isoprenoids including the coenzyme Q10 with functions in electron transduction chain in mitochondria[50]; mitochondrial dysfunction has been implicated in insulin resistance.[51] On the other hand, reduction in PPARγ activity – and possibly that effected by statins – has been suggested to prevent insulin resistance by potentiating leptin's effect and increasing fatty acid oxidation in heterozygous PPARγ-deficient mice[52]; coincidentally pitavastatin induced GLUT4 mRNA level in 3T3-L1 cells when used during lipid accumulation period (days 8–16)[53] while rosuvastatin also increased insulin sensitivity in an animal model.[54] While statins impair insulin secretion and response in non-obese patients, they may reduce lipotoxicity caused by obesity-associated triglyceride-rich lipoproteins and help maintain glycemic control in obese type 2 diabetes patients.[19]

The essential role of mevalonate in adipocyte differentiation shown herein may provide a molecular target for obesity intervention. Naturally occurring mevalonate suppressors including the vitamin E tocotrienols,[55] terpenoids,[56] genistein,[57] and berberine[40] suppress adipocyte differentiation with concomitant downregulation of PPARγ, C/EBPα, and FABP4 expression. Dietary tocotrienols have also been shown to increase insulin sensitivity.[58] Dietary mevalonate suppressors may have anti-adipogenic benefits without the potential complications such as insulin resistance associated with statins.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. ME, ST, and DH conducted the experiments. HM, NM and GFV contributed to the conceptualization of the studies. HM wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr. Parakat Vijayagopal for technical assistance in the culture of 3T3 cells, Ms. Hoda Yeganehjoo and Ms. Anureet Shah in RNA extraction, and Dr. Brian W. Beck in BLAST search. This study was supported by Texas Department of Agriculture Food and Fiber Research Program and Texas Woman's University Research Enhancement Program.

REFERENCES

1. Tang QQ, Lane MD. Adipogenesis: from stem cell to adipocyte. *Annu Rev Biochem* 2012;**81**:715–36
2. Kawada T, Takahashi N, Fushiki T. Biochemical and physiological characteristics of fat cell. *J Nutr Sci Vitaminol (Tokyo)* 2001;**47**:1–12
3. Kim JB, Wright HM, Wright M, Spiegelman BM. ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand. *Proc Natl Acad Sci U S A* 1998;**95**:4333–7
4. Doran AC, Meller N, Cutchins A, Deliri H, Slayton RP, Oldham SN, Kim JB, Keller SR, McNamara CA. The helix-loop-helix factors Id3 and E47 are novel regulators of adiponectin. *Circ Res* 2008;**103**:624–34
5. Green H, Meuth M. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* 1974;**1**:113–6
6. Cowherd RM, Lyle RE, McGehee RE Jr. Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* 1999;**10**:3–10
7. Green H, Kehinde O. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* 1976;**7**:105–13
8. Green H, Kehinde O. Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J Cell Physiol* 1979;**101**:169–71
9. Klemm DJ, Leitner JW, Watson P, Nesterova A, Reusch JE, Goalstone ML, Draznin B. Insulin-induced adipocyte differentiation. Activation of CREB rescues adipogenesis from the arrest caused by inhibition of prenylation. *J Biol Chem* 2001;**276**:28430–5
10. Chamberlain LH. Inhibition of isoprenoid biosynthesis causes insulin resistance in 3T3-L1 adipocytes. *FEBS Lett* 2001;**507**:357–61
11. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature* 1990;**343**:425–30
12. Nishio E, Tomiyama K, Nakata H, Watanabe Y. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor impairs cell differentiation in cultured adipogenic cells (3T3-L1). *Eur J Pharmacol* 1996;**301**:203–6
13. Nicholson AC, Hajjar DP, Zhou X, He W, Gotto AM Jr, Han J. Anti-adipogenic action of pitavastatin occurs through the coordinate regulation of PPARgamma and Pref-1 expression. *Br J Pharmacol* 2007;**151**:807–15
14. Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 1992;**97**:493–7
15. Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 1985;**100**:965–73
16. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. Evidence based selection of housekeeping genes. *PLoS One* 2007;**2**:e898
17. Fernandes NV, Yeganehjoo H, Katuru R, DeBose-Boyd R, Morris LL, Michon R, Yu ZL, Mo H. Geranylgeraniol suppresses the viability of human DU145 prostate carcinoma cells and the level of HMG CoA reductase. *Exp Biol Med* 2013;**238**:1265–74
18. Goto T, Nagai H, Egawa K, Kim YI, Kato S, Taimatsu A, Sakamoto T, Ebisu S, Hohsaka T, Miyagawa H, Murakami S, Takahashi N, Kawada T. Farnesyl pyrophosphate regulates adipocyte functions as an endogenous PPARgamma agonist. *Biochem J* 2011;**438**:111–9
19. Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control. *Diabetologia* 2006;**49**:1881–92
20. Tomiyama K, Nishio E, Watanabe Y. Both wortmannin and simvastatin inhibit the adipogenesis in 3T3-L1 cells during the late phase of differentiation. *Jpn J Pharmacol* 1999;**80**:375–8

21. Pengde K, Fuxing P, Bin S, Jing Y, Jingqiu C. Lovastatin inhibits adipogenesis and prevents osteonecrosis in steroid-treated rabbits. *Joint Bone Spine* 2008;**75**:696–701
22. Elahi MM, Cagampang FR, Anthony FW, Curzen N, Ohri SK, Hanson MA. Statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular risk factors in their offspring. *Hypertension* 2008;**51**:939–44
23. Endo A, Hasumi K, Negishi S. Monacolins J and L, new inhibitors of cholesterol biosynthesis produced by Monascus ruber. *J Antibiot (Tokyo)* 1985;**38**:420–2
24. Jeon T, Hwang SG, Hirai S, Matsui T, Yano H, Kawada T, Lim BO, Park DK. Red yeast rice extracts suppress adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 cells. *Life Sci* 2004;**75**:3195–203
25. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996;**274**:1377–9
26. Konig B, Koch A, Spielmann J, Hilgenfeld C, Hirche F, Stangl GI, Eder K. Activation of PPARα and PPARγ reduces triacylglycerol synthesis in rat hepatoma cells by reduction of nuclear SREBP-1. *Eur J Pharmacol* 2009;**605**:23–30
27. Fajas L, Fruchart JC, Auwerx J. Transcriptional control of adipogenesis. *Curr Opin Cell Biol* 1998;**10**:165–73
28. Benito M, Porras A, Nebreda AR, Santos E. Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. *Science* 1991;**253**:565–8
29. Hara Y, Wakino S, Tanabe Y, Saito M, Tokuyama H, Washida N, Tatematsu S, Yoshioka K, Homma K, Hasegawa K, Minakuchi H, Fujimura K, Hosoya K, Hayashi K, Nakayama K, Itoh H. Rho and rho-kinase activity in adipocytes contributes to a vicious cycle in obesity that may involve mechanical stretch. *Sci Signal* 2011;**4**:ra3
30. Goalstone ML, Draznin B. Effect of insulin on farnesyltransferase activity in 3T3-L1 adipocytes. *J Biol Chem* 1996;**271**:27585–9
31. Goalstone ML, Leitner JW, Berhanu P, Sharma PM, Olefsky JM, Draznin B. Insulin signals to prenyltransferases via the Shc branch of intracellular signaling. *J Biol Chem* 2001;**276**:12805–12
32. Goalstone ML, Leitner JW, Golovchenko I, Stjernholm MR, Cormont M, Le Marchand-Brustel Y, Draznin B. Insulin promotes phosphorylation and activation of geranylgeranyltransferase II. Studies with geranylgeranylation of rab-3 and rab-4. *J Biol Chem* 1999;**274**:2880–4
33. Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem* 1988;**263**:9402–8
34. Dricu A, Wang M, Hjertman M, Malec M, Blegen H, Wejde J, Carlberg M, Larsson O. Mevalonate-regulated mechanisms in cell growth control: role of dolichyl phosphate in expression of the insulin-like growth factor-1 receptor (IGF-1R) in comparison to Ras prenylation and expression of c-myc. *Glycobiology* 1997;**7**:625–33
35. Siddals KW, Marshman E, Westwood M, Gibson JM. Abrogation of insulin-like growth factor-I (IGF-I) and insulin action by mevalonic acid depletion: synergy between protein prenylation and receptor glycosylation pathways. *J Biol Chem* 2004;**279**:38353–9
36. Mauser W, Perwitz N, Meier B, Fasshauer M, Klein J. Direct adipotropic actions of atorvastatin: differentiation state-dependent induction of apoptosis, modulation of endocrine function, and inhibition of glucose uptake. *Eur J Pharmacol* 2007;**564**:37–46
37. Castrillo A, Tontonoz P. Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. *Annu Rev Cell Dev Biol* 2004;**20**:455–80
38. DeBose-Boyd RA, Ou J, Goldstein JL, Brown MS. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc Natl Acad Sci U S A* 2001;**98**:1477–82
39. Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J Clin Invest* 1997;**99**:838–45
40. Huang C, Zhang Y, Gong Z, Sheng X, Li Z, Zhang W, Qin Y. Berberine inhibits 3T3-L1 adipocyte differentiation through the PPARγ pathway. *Biochem Biophys Res Commun* 2006;**348**:571–8
41. Le Lay S, Krief S, Farnier C, Lefrere I, Le Liepvre X, Bazin R, Ferre P, Dugail I. Cholesterol, a cell size-dependent signal that regulates glucose metabolism and gene expression in adipocytes. *J Biol Chem* 2001;**276**:16904–10
42. Le Lay S, Ferre P, Dugail I. Adipocyte cholesterol balance in obesity. *Biochem Soc Trans* 2004;**32**:103–6
43. Vicent D, Maratos-Flier E, Kahn CR. The branch point enzyme of the mevalonate pathway for protein prenylation is overexpressed in the ob/ob mouse and induced by adipogenesis. *Mol Cell Biol* 2000;**20**:2158–66
44. Freeman DJ, Norrie J, Sattar N, Neely RD, Cobbe SM, Ford I, Isles C, Lorimer AR, Macfarlane PW, McKillop JH, Packard CJ, Shepherd J, Gaw A. Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. *Circulation* 2001;**103**:357–62
45. Sever PS, Dahlof B, Poulter NR, Wedel H, Beevers G, Caulfield M, Collins R, Kjeldsen SE, Kristinsson A, McInnes GT, Mehlsen J, Nieminen M, O'Brien E, Ostergren J. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial–Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* 2003;**361**:1149–58
46. Group TDALIDS. The effect of aggressive versus standard lipid lowering by atorvastatin on diabetic dyslipidemia: the DALI study: a double-blind, randomized, placebo-controlled trial in patients with type 2 diabetes and diabetic dyslipidemia. *Diabetes Care* 2001;**24**:1335–41
47. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995;**270**:26746–9
48. Asterholm IW, Scherer PE. Enhanced metabolic flexibility associated with elevated adiponectin levels. *Am J Pathol* 2010;**176**:1364–76
49. Yada T, Nakata M, Shiraishi T, Kakei M. Inhibition by simvastatin, but not pravastatin, of glucose-induced cytosolic Ca2+ signalling and insulin secretion due to blockade of L-type Ca2+ channels in rat islet beta-cells. *Br J Pharmacol* 1999;**126**:1205–13
50. Bliznakov EG. Diabetes and the role of isoprenoid biosynthesis. *FEBS Lett* 2002;**525**:169–70
51. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science* 2005;**307**:384–7
52. Yamauchi T, Waki H, Kamon J, Murakami K, Motojima K, Komeda K, Miki H, Kubota N, Terauchi Y, Tsuchida A, Tsuboyama-Kasaoka N, Yamauchi N, Ide T, Hori W, Kato S, Fukayama M, Akanuma Y, Ezaki O, Itai A, Nagai R, Kimura S, Tobe K, Kagechika H, Shudo K, Kadowaki T. Inhibition of RXR and PPARgamma ameliorates diet-induced obesity and type 2 diabetes. *J Clin Invest* 2001;**108**:1001–13
53. Ishihara Y, Ohmori K, Mizukawa M, Hasan AU, Noma T, Kohno M. Beneficial direct adipotropic actions of pitavastatin in vitro and their manifestations in obese mice. *Atherosclerosis* 2010;**212**:131–8
54. Naples M, Federico LM, Xu E, Nelken J, Adeli K. Effect of rosuvastatin on insulin sensitivity in an animal model of insulin resistance: evidence for statin-induced hepatic insulin sensitization. *Atherosclerosis* 2008;**198**:94–103
55. Uto-Kondo H, Ohmori R, Kiyose C, Kishimoto Y, Saito H, Igarashi O, Kondo K. Tocotrienol suppresses adipocyte differentiation and Akt phosphorylation in 3T3-L1 preadipocytes. *J Nutr* 2009;**139**:51–7

56. Gong Z, Huang C, Sheng X, Zhang Y, Li Q, Wang MW, Peng L, Zang YQ. The role of tanshinone IIA in the treatment of obesity through peroxisome proliferator-activated receptor gamma antagonism. *Endocrinology* 2009;**150**:104–13
57. Kim MH, Park JS, Seo MS, Jung JW, Lee YS, Kang KS. Genistein and daidzein repress adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells via Wnt/beta-catenin signalling or lipolysis. *Cell Prolif* 2010;**43**:594–605
58. Chen CW, Cheng HH. A rice bran oil diet increases LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity in rats with streptozotocin/nicotinamide-induced Type 2 diabetes. *J Nutr* 2006;**136**:1472–6

(Received July 10, 2013, Accepted October 24, 2013)