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Simvastatin Suppresses Leptin Expression in 3T3-L1 Adipocytes *via* Activation of the Cyclic AMP–PKA Pathway Induced by Inhibition of Protein Prenylation

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Simvastatin inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyses conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis. We demonstrated that simvastatin at 1 μM markedly inhibited adipocyte differentiation measured by Oil Red O staining in preadipocyte cells (3T3-L1), while expression of leptin, a marker of adipocyte differentiation, was suppressed by 1 μM simvastatin for up to 12 days of culture. Next, to elucidate mechanisms underlying the reduction of leptin expression induced by simvastatin, differentiated 3T3-L1 adipocytes were treated with various inhibitors with mevalonate or its metabolite in the presence or absence of simvastatin. Simvastatin time- and dose-dependently suppressed leptin mRNA expression. Heterogeneous nuclear RNA related to leptin mRNA was inhibited by 10 μM simvastatin, while stability of the mRNA was not changed by treatment with simvastatin in transcription-arrested 3T3-L1 cells. Simvastatin inhibition of leptin gene transcription was not abrogated by pre-treatment with cycloheximide, an inhibitor of protein synthesis. Addition of mevalonate or geranylgeranyl pyrophosphate (GGPP), a mevalonate metabolite, abolished simvastatin-induced inhibition of leptin expression in 3T3-L1 cells. Suppression of expression was observed upon addition of GGTI-298, a geranylgeranyl transferase I inhibitor, but not FTI-277, a farnesyl transferase inhibitor. Expression was suppressed by treatment with hydroxyfasudil, a protein prenylation inhibitor. Treatment with phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin, reduced leptin expression in 3T3-L1 cells. Simvastatin dose-dependently increased intra-cellular cyclic AMP (cAMP) concentrations in 3T3-L1 cells, with maximal stimulation obtained at 10 μM. Addition of GGPP abolished simvastatin-induced stimulation of cAMP accumulation and protein kinase A (PKA) activity. H89, an inhibitor of PKA, completely abolished simvastatin-induced suppression of leptin expression. These results suggested that simvastatin reduced geranylgeranylprotein prenylation followed by deactivation of PI3K, leading to cAMP accumulation and subsequent activation of PKA in differentiated 3T3-L1 adipocytes. Finally, PKA inhibited leptin gene transcription without new protein synthesis.

Key words: statins, leptin, adipocyte differentiation, HMG-CoA reductase, prenylation, protein kinase A.

Abbreviations: aP2, adipocyte-specific protein 2; BMP-2, bone morphogenetic protein-2; cAMP, cyclic AMP; C/EBPβ, CCAAT/enhancer-binding protein β; CREB, cAMP responsive element binding protein; DMEM medium, Dulbecco's modified Eagle's medium; DRB, 5,6-dichlorobenzimidazole riboside; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; Glut4, glucose transporter 4; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; hnRNA, heterogeneous nuclear RNA; IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein lipase; MAPK, mitogen activated kinase; ob/ob, leptin deficient; PDE3B, phosphodiesterase 3B; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PPARγ, peroxisome proliferation-activator receptor γ; RT-PCR, reverse transcriptase-polymerase chain reactions; Runx2, Runt-related transcription factor 2; TG, triglycerides.

Adipose tissue, a primary site for energy storage, also acts as an endocrine organ regulating energy homoeostasis *via* secretion of adipokines such as leptin and adiponectin (*1*). Normal adipose tissue development, as well as increases in adipose tissue mass associated with weight gain and obesity, requires formation of mature adipocytes from preadipocytes or stromal progenitor cells (*2*). Factors and processes that mediate conversion of preadipocytes to mature adipocytes have been elucidated in immortalized preadipocyte cell lines such as 3T3-L1, in primary preadipocytes and in stromal progenitor cells (*3*). Adipogenic conversion can be initiated in culture by the addition of glucocorticoids, insulin and agents that elevate intra-cellular cyclic AMP (cAMP) concentrations.

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Thiazolidinediones and prostaglandins, which are ligands of a nuclear hormone receptor—peroxisome proliferation-activator receptor (PPAR) γ—also increase rates of adipogenesis. Exposure to differentiation-inducing agents initiates a well-characterized cascade of gene expression events that lead to the mature adipocyte phenotype. The differentiation cascade begins expression of CCAAT/enhancer-binding protein (C/EBP) β within hours of treatment with inducing agents (*4*). This factor then promotes the expression of PPARγ and C/EBPα (*5*, *6*). Both of these factors are necessary to promote the mature adipocyte phenotype, including leptin and adiponectin secretion as well as insulin-sensitive glucose uptake (*7*). All of these master adipogenic regulators are expressed after initiation of the differentiation programme.

Simvastatin, a prodrug converted to very potent competitive inhibitors of the liver enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, induces blockade of conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis (*8*). Simvastatin, lovastatin and atorvastatin, referred to generally as statins, thus are potent inhibitors of cholesterol biosynthesis that are prescribed widely to lower cholesterol in hyperlipidemic patients at risk for cardiovascular disease (*9*, *10*). Besides lowering serum lipids, statins have a wide range of other actions including inhibition of platelet aggregation and thrombus deposition; promotion of angiogenesis; decreasing β-amyloid peptide, related to Alzheimer's disease; and suppressing T lymphocyte activation (*11*). By inhibiting the initial part of the cholesterol synthesis pathway, statins decrease availability of several important lipid intermediate compounds including isoprenoids such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP); these are attached as posttranslational modifiers to certain proteins, such as small G proteins including Ras and Ras-like proteins (Rho, Rap, Rab and Ral) (*12*).

Pluripotent mesenchymal stem cells including stromal progenitor cells can undergo commitment to several cell lineages including adipocytes, osteoblasts, chondrocytes and myocytes (*13*, *14*); thus, adipocytes originate from a common mesenchymal progenitor. Statins suppress PPARγ and augment Runt-related transcription factor 2 (Runx2), a critical transcription factor for osteoblastic differentiation or osteogenesis in progenitor cells (*15*, *16*). Moreover, statins strongly induce expression of bone morphogenetic protein (BMP)-2 in murine embryonic stem cells, mouse bone marrow stromal cell, and osteoblasts (*15–20*), supporting the concept that BMP-2/Runx2 signalling is crucial for promoting osteoblastic differentiation elicited by statins. Concomitantly, lovastatin and simvastatin inhibited adipocyte differentiation by suppressing expression of fat-cell-specific genes such as PPARγ and adipocyte-specific protein 2 (aP2), impeding subsequent maturation. Thus, statins suppress adipogenic differentiation and instead promoted the osteoblast lineage in pluripotent cells (*15*, *16*).

Leptin, the 16-kDa product of the ob gene, acts as an adipokine, that influences body weight homoeostasis by altering food intake and energy expenditure (*21*, *22*). Lack of a functional leptin protein was shown to be the basis of obesity in mutant (ob/ob) mice. Expression of leptin increases during adipocyte differentiation, and peaks as mature adipocytes fill with lipids (*23*). Animal experiments using hypercholesteromic rabbits showed that atorvastatin reduced serum leptin concentrations, and inhibited leptin release and mRNA expression in the adipose tissue (*24*). The present study was undertaken to better understand how simvastatin inhibits leptin expression in 3T3-L1 adipocytes. We used specific inhibitors of phosphatidylinositol 3-kinase (PI3K), and mitogen-activated kinase (MAPK) signalling cascades regarding leptin expression, because statins have been reported to function at these signalling pathways (*18*, *25*, *26*).

MATERIALS AND METHODS

Cell Culture and Differentiation—Mouse 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained in high-glucose (25 mM) Dulbecco's modified Eagle's medium (DMEM; ICN Pharmaceuticals, Aurora, OH, USA) supplemented with 10% fetal bovine serum (FBS). Media were changed every 2 days, and preadipocytes were maintained at < 50% confluence. All experiments were performed after reaching confluence (Day 0). Preadipocytes (3T3-L1) then were treated with medium to induce differentiation [MDI; DMEM, 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX: Sigma, St. Louis, MO, USA), 1 μM dexamethasone (Sigma), 5 μg/ml insulin (Sigma) and 25 mM D-glucose] for 3 days. At Day 3 following induction, cells were cultured in the high-glucose DMEM containing 10% FBS. Subsequently, medium was changed every second day. Immediately after confluence some cells were exposed to simvastatin (Calbiochem, San Diego, CA, USA) at 1 μM with or without mevalonate (Sigma) at 1 mM for the time periods indicated (*5*, *6*, *23*). In studies of acute effects of simvastatin, 3T3-L1 cells were placed in DMEM with 2% FBS at Day 3, and treated with 1 mM mevalonate (Sigma); 10 μM GGPP (Sigma); and 10 μM FPP (Sigma) in the absence or presence of 10 μM simvastatin for 24 h. At this time point, 3T3-L1 cells were differentiated to mature adipocytes, and expressed adipocyte-specific genes. Inhibitors including 10 μM GGTI-298 (Sigma; a geranylgeranyl transferase I inhibitor); 10 μM FTI-277 (Sigma; a farnesyl transferase inhibitor); 30 μM hydroxyfasudil (Sigma; a protein prenylation inhibitor); 20 μM LY294002 or 100 nM wortmannin (Sigma; both PI3K inhibitors); 10 μM U0126 or 20 μM PD98059 (Sigma; both ERK1/2 MAPK pathway inhibitors); or 10 μM SB203580 (Sigma; p38MAPK pathway inhibitor) were used to treat 3T3-L1 cells for 24 h. In some experiments, 35 μM cycloheximide (Sigma; a protein synthesis inhibitor) or 5 μM H89 (Sigma; a PKA inhibitor) was added to cells for 1 h before the indicated duration of incubation with vehicle or 10 μM simvastatin. Cells were exposed to 75 μM 5,6-dichlorobenzimidazole riboside (DRB, Sigma; a RNA polymerase II inhibitor, dissolved in dimethylsulphoxide) 8 h after exposure to 10 μM simvastatin or vehicle. Cell viability was measured by the WST-1 test (Cell Counting Kit, Dojindo Laboratories, Tokyo, Japan),

after 3T3-L1 cells were treated with vehicle or graded concentrations of simvastatin (up to 10 μM) for 24 h.

Leptin Assay—For the periods indicated, 3T3-L1 cells were treated with vehicle or test substances such as simvastatin, mevalonate and GGPP. Conditioned media from the final 24 h of culture were collected. Leptin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA; Mouse Leptin Assay Kit, Immuno-Biological Laboratories Co., Tokyo, Japan).

Measurements of cAMP Accumulation and PKA Activity—3T3-L1 cells were treated with simvastatin (1 nM–10 μM), GGPP (10 μM) or LY294002 (20 μM) for 3 h. Cellular cAMP was extracted, and measured using a cAMP Biotrak Enzymeimmunoassay (Amersham Biosciences, Piscataway, NJ. USA). PKA activity in 3T3-L1 cells was determined as previously described (*27*). Briefly, after treatment with test agents such as simvastatin (10 μM), mevalonate (1 mM) and GGPP (10 μM), PKA activity was measured using [γ-^{32}P]ATP (ICN Pharmaceuticals) and 5 μg kemptide (Sigma) as substrate.

PI3K Assay—Cells were lysed in lysis buffer, and cleared cell lysates were prepared by centrifugation. The lysates were immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were used in the PI3K assay using PI as substrate in the presence of [γ-^{32}P]ATP (ICN Pharmaceuticals), and the products were separated by thin-layer chromatopraphy followed by autoradiopraphy (*26*).

Oil Red O Staining for Intra-Cellular Triglycerides (TGs)—To stain for TG, cells in the monolayer first were washed three times with PBS and then fixed with 10% formalin in neutral buffer solution for 10 min without shaking. Next, the formalin solution was washed away with PBS followed by 60% isopropanol. Fixed cells devoid of formalin were stained with the freshly prepared Oil-red O solution for 10 min at 23°C, followed by extensive washes with 60% isopropanol (three times). Stained cells were photographed using a digital camera. For quantitative determinations of accumulated TG in cells, Oil Red O staining was eluted with dimethylsulfoxide. The amount of dye eluted was quantified by spectrophotometry at 531 nm (*23*).

Determination of Leptin mRNA—Abundance of leptin mRNA was determined by reverse transcriptase-polymerase chain reactions (RT-PCR). Total RNA was extracted from 3T3L1 cells using guanidine thiocyanate. RT-PCR was performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Total RNA (1 μg) was reverse-transcribed at 42°C for 1 h to synthesize cDNA. Amplification reactions (25 cycles) were performed using the following primers: leptin, forward, 5′-CCCCATTCTGAGTTTGTCCA-3′; reverse, 5′-GCTGAA GAACTAGGTGAGAG -3′ (537-bp product); and cyclophilin, forward, 5′-GTTCCATCGTGTCATCAAGG-3′; reverse, 5′-TTGTGACTGGCTACCTTCGT-3′ (459-bp product). Amounts of leptin transcript were calculated relative to amounts of cyclophilin mRNA present. Signal intensity was quantified with a Molecular Imager FX (BIORAD, Hercules, CA, USA), and the resulting images were analysed using Quantity One 4.1.1 (BIORAD) image analysis software (*18*, *19*).

Quantification of Leptin Heterogeneous Nuclear RNA (hnRNA)—Leptin hnRNA was determined by RT-PCR using specific primers designed to amplify hnRNA from an intron to an exon, as described previously (*18*). Briefly, the nucleotide sequences containing exon 1, the interposed intron, and exon 2 of the murine leptin gene were determined using a database (chromosome 6; NT 039340.7, GenBank). A sense primer (5′-TTCTCTTTCCCTTGAAGTACGCAA-3′), spanning nucleotides 29,020,786–800 of exon 3 of the murine leptin gene, and an anti-sense primer (5′-CCAAAG CCACAGGAACCGA -3′), spanning nucleotides 29,021, 137–156 of the intron between exons 2 and 3, were synthesized. Total RNA was extracted from 3T3-L1 cells and treated with ribonuclease-free deoxyribonuclease I (Boeringer, Indianapolis, IN, USA) to remove any contaminating DNA present. RNA (1 μg) was reverse-transcribed to DNA using reverse transcriptase and random hexanucleotide primers. The newly synthesized cDNA was amplified by PCR through 33 cycles. PCR products were loaded onto a 1.2% agarose gel and transferred to a nylon membrane. Southern blotting was performed using a murine leptin genomic DNA probe containing the region of hnRNA and radio-labelled with [α-^{32}P]deoxy-CTP. Amplified RNA from murine leptin gene was corrected for the level of cyclophilin hnRNA by RT-PCR using the same synthesized DNA. PCR was performed, and signal intensity was quantified with the Molecular Imager FX.

Statistical Analysis—All values are expressed as the mean ± SEM of four measurements. Differences between treated and untreated groups were assessed by Student's *t*-test. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's *F*-test. Statistical analysis was performed with the Statview 4.02 software package (Abacus Concepts, Berkeley, CA, USA). A *P*-value below 0.05 was considered to indicate statistical significance.

RESULTS

Simvastatin Inhibits Adipogenic Differentiation—To determine whether simvastatin affected adipogenic differentiation, 3T3-L1 preadipocytes were cultured in high-glucose DMEM medium with or without simvastatin and mevalonate for up to 12 days. Adipogenic differentiation of 3T3-L1 cells were evaluated by Oil Red O staining for lipid (Fig. 1A and B). Oil droplets were clearly seen at Days 8 and 12 of culture. Lipid accumulation in the cells treated with vehicle increased time-dependently for up to 12 days of culture. Simvastatin at 1 μM significantly inhibited lipid accumulation during Days 4 to 12 of culture. To determine whether the inhibitory effect of simvastatin on adipocyte differentiation was dependent on HMG-CoA reductase activity, we added mevalonate (1 mM) to the 3T3-L1 cells in the presence of simvastatin (1 μM). Simvastatin-induced inhibition of lipid droplets formation was completely abrogated by co-treatment with mevalonate, showing that suppression of adipogenic differentiation by simvastatin was mediated by inhibition of HMG-CoA reductase activity (Fig. 1A and B). Gene expression of

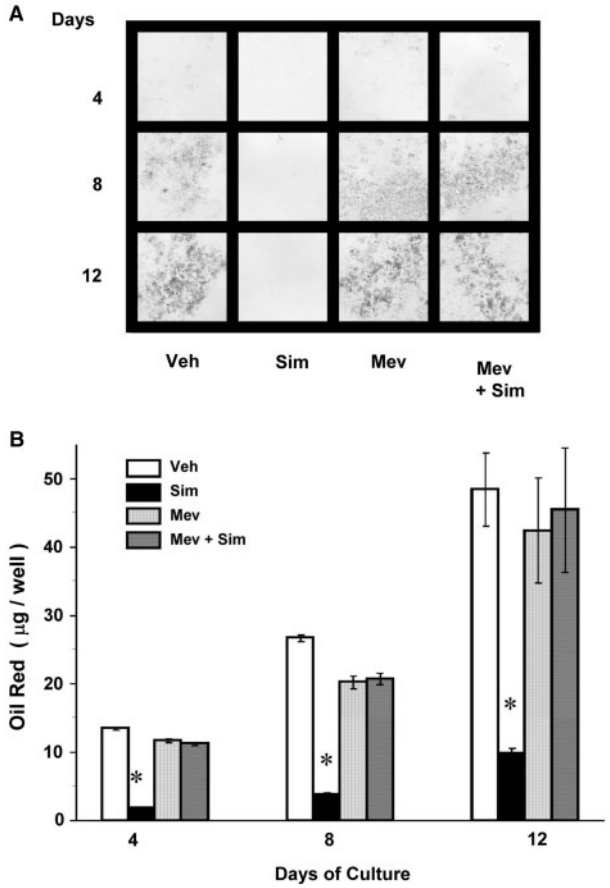

Fig. 1. **Simvastatin inhibits adipogenesis. After confluence, adipocytic 3T3-L1 cells were cultured in MDI medium for 3 days, and then in high-glucose DMEM for 1 to 12 days.** Simvastatin (Sim; 1 μM) and mevalonate (Mev; 1 mM) were added after confluence. Numbers of days represent the period after confluence. (A) Oil Red O staining in 3T3-L1 cells. (B) Quantitation of lipid determined by Oil Red O in 3T3-L1 cells. *$P < 0.01$ compared with vehicle control at each time point.

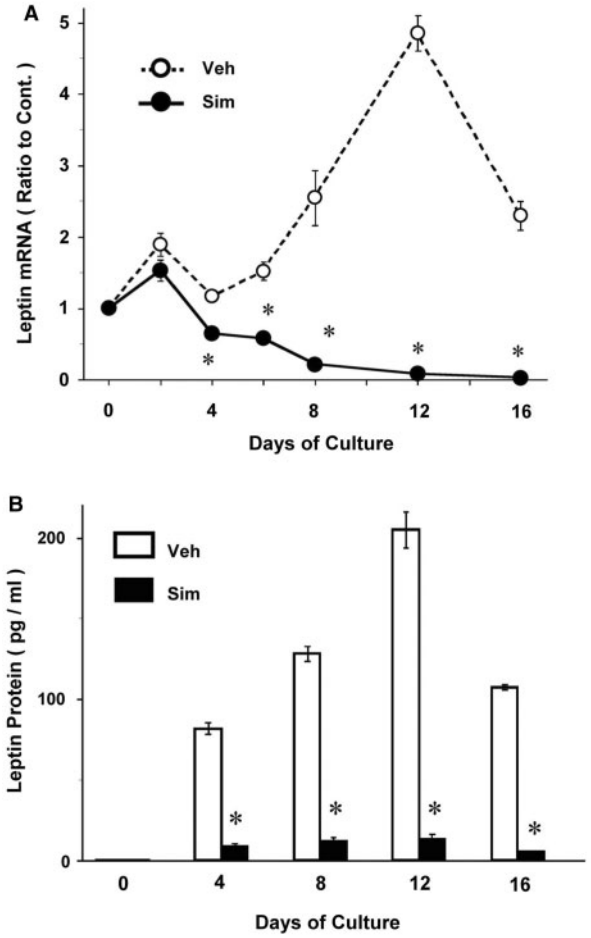

Fig. 2. **Simvastatin suppresses leptin expression in adipogenic 3T3-L1 cells.** (A and B) After confluence, 3T3-L1 cells were cultured in MDI medium for 3 days, and then in high-glucose DMEM. Days of culture represent the period after confluence. Cells were treated with vehicle or 1 μM simvastatin during the indicated time period from Day 0. Changes in expression of leptin mRNA were determined by RT-PCR (A), and changes in secretion of leptin protein for 24 h were measured by ELISA (B). *$P < 0.01$ versus vehicle control at each time point.

adipocyte markers such as C/EBPα, PPARγ and aP2 was monitored by long-term culture of 3T3-L1 cells. Simvastatin at 1 μM inhibited mRNA expression of adipocyte markers other than PPARγ (Supplementary Fig. 1). Thus, simvastatin suppressed adipocyte differentiation in 3T3-L1 cells.

To assess cellular capacity to produce adipocyte-derived hormones, we monitored expression of leptin, a well-documented hormone with anti-diabetic properties, in cells of adipocyte lineage (Fig. 2). Cellular leptin mRNA concentrations rose markedly at 8–16 days of culture in 3T3-L1 cells, while 1 μM simvastatin strongly reduced this mRNA expression at 4–16 days of culture (Fig. 2A). Significant suppression of leptin protein secretion was observed in 3T3-L1 cells between 4 and 16 days of culture by the treatment with 1 μM simvastatin (Fig. 2B).

Simvastatin Transcriptionally Suppresses Leptin mRNA Expression—To determine how leptin expression was influenced by simvastatin, the statin was used for short-term treatment of 3T3-L1 cells, showing time-dependent increase in leptin mRNA expression for up to 24 h. Simvastatin treatment at 10 μM significantly inhibited this mRNA expression at the treatment of 18 and 24 h (Fig. 3A). In dose–response experiments, 3T3-L1 cells were treated with graded concentrations of simvastatin for 24 h. A concentration of simvastatin as low as 1 μM significantly decreased leptin mRNA expression and protein secretion. The most prominent reduction was obtained at a statin concentration of 10 μM (Fig. 3B and C), at which 3T3-L1 cells maintained 100% viability as determined by the WST-1 test (Fig. 3D). These results demonstrated that simvastatin at 10 μM for 24 h markedly suppressed leptin expression in 3T3-L1 cells without causing cell toxicity. To determine whether simvastatin transcriptionally altered leptin mRNA synthesis, an hnRNA assay was performed because transcriptional regulation frequently leads to changes in hnRNA

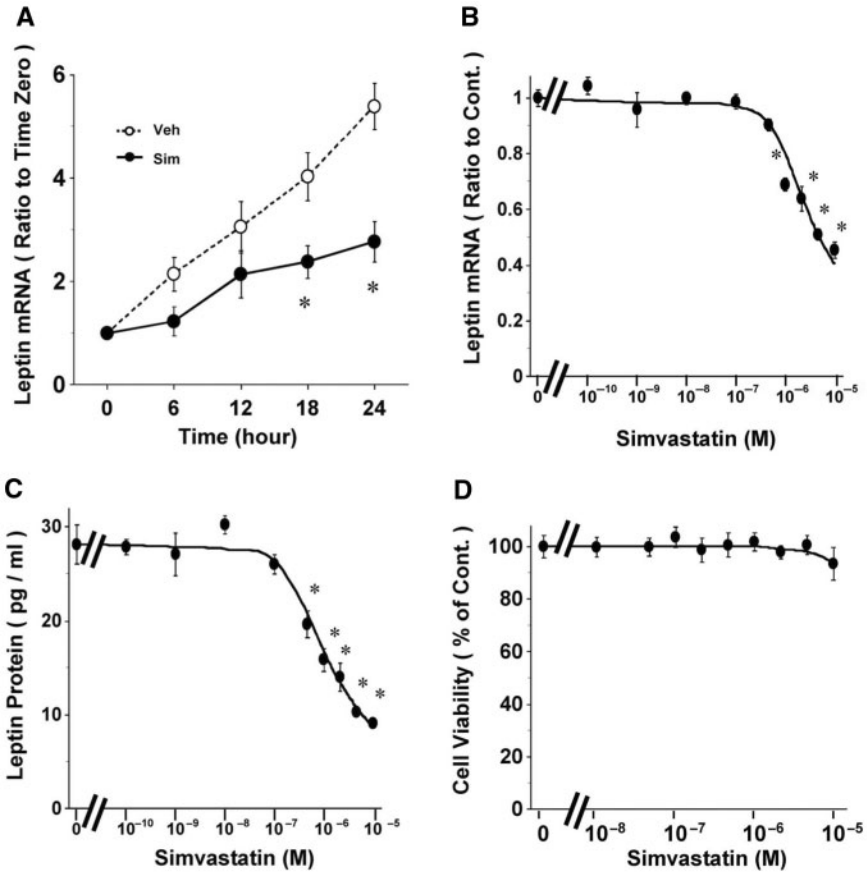

Fig. 3. **Simvastatin inhibits leptin expression without inducing cell toxicity in 3T3-L1 adipocytes.** (A) After 3T3-L1 cells were treated with vehicle (Veh) or 10 μM simvastatin (Sim) for up to 24 h, amounts of leptin mRNA were measured by RT-PCR. (B–D) Cells were treated with vehicle or graded concentrations of simvastatin for 24 h. (B) Leptin mRNA abundance was determined by RT-PCR. (C) Amounts of secreted leptin protein were measured by ELISA. (D) Cell toxicity, assessed as cell viability, was measured using the WST-1 test. *$P < 0.01$ versus vehicle control.

synthesis. Accordingly, concentrations of hnRNA were assessed in the absence or presence of simvastatin. Concentrations of leptin hnRNA decreased significantly in 3T3-L1 cells exposed to 10 μM simvastatin from 3 to 24 h, showing that simvastatin suppressed transcription of the leptin gene (Fig. 4A). Next, we examined the effects of simvastatin on leptin mRNA degradation in 3T3-L1 cells. Cells were exposed to vehicle or 10 μM simvastatin for 18 h, after which 75 μM DRB, a RNA polymerase II inhibitor, was added to cultures. Decay of leptin mRNA occurred at similar rates in both vehicle- and simvastatin-treated cells, indicating that simvastatin did not alter stability of this transcript (Fig 4B). To determine whether the reduction of leptin mRNA by simvastatin required protein synthesis in 3T3-L1 cells, expression was assessed in the presence of cycloheximide, a protein synthesis inhibitor. Cycloheximide did not block the simvastatin-induced reduction of leptin mRNA expression, demonstrating that new protein synthesis was not required to achieve gene suppression (Fig. 4C).

Simvastatin Inhibits Leptin Expression in 3T3-L1 Cells via Reduction of Protein Prenylation—To determine the mechanism of leptin mRNA suppression by simvastatin, 3T3-L1 cells were co-incubated with the statin and HMG-CoA reductase-related products, or treated with various inhibitors (Fig. 5). Addition of mevalonate, the immediate product of the reaction catalysed by HMG-CoA reductase, or GGPP, an isoprenoid intermediate, completely abolished the stain-induced inhibition of leptin expression. In contrast, simvastatin still suppressed leptin expression in the presence of FPP, another isoprenoid intermediate (Fig. 5A and B). Inhibition of leptin expression was obtained by treatment of 3T3-L1 cells with the geranylgeranyl transferase-I inhibitor, GGTI-298, but not with a farnesyl transferase inhibitor, FTI-277. Addition of hydroxyfasudil, an inhibitor of protein prenylation, inhibited expression of leptin mRNA and protein (Fig. 5A and B). These results implicated reduced protein prenylation caused by the HMG-CoA reductase inhibitor, simvastatin, in suppression of leptin mRNA expression in adipocytes. We tested three signalling pathways relevant to simvastatin inhibition of leptin expression, using inhibitors of signalling. When we added selective inhibitors of the ERK1/2MAPK pathway such as U0126 and PD98059 or p38MAPK pathway inhibitors such as SB203580 to 3T3-L1 cells, expression of leptin mRNA and protein was not affected

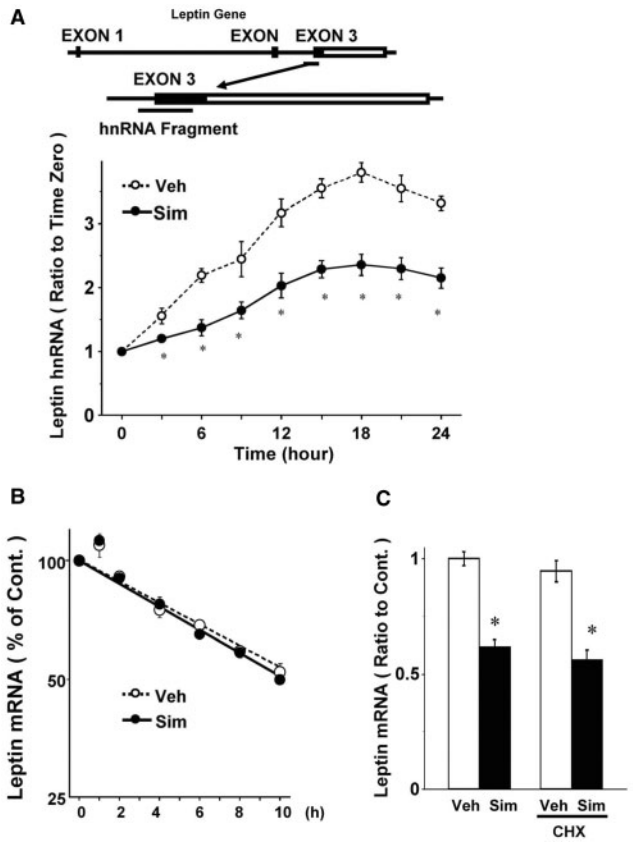

Fig. 4. **Simvastatin directly suppresses leptin mRNA transcription with no requirement for new protein synthesis.** (A) Time course of inhibition of leptin hnRNA by simvastatin in 3T3-L1 adipocytes. Cells were treated with vehicle (Veh) or 10 μM simvastatin (Sim) for the periods indicated. Total RNA was extracted, and amounts of hnRNA fragment (upper panel) region were determined by RT-PCR. (B) Effect of simvastatin on leptin mRNA half-life in 3T3-L1 cells with transcriptional arrest. Cells were exposed to vehicle (Veh) or 10 μM simvastatin (Sim) for 18 h before addition of 75 μM DRB, a transcription inhibitor. At the indicated time points after addition of DRB, total RNA from cells were extracted and leptin mRNA abundance was measured using RT-PCR. (C) Effect of cycloheximide (CHX), a protein synthesis inhibitor, on simvastatin-induced suppression of leptin mRNA expression in 3T3-L1 cells. Cells were treated with 35 μM cycloheximide for 1 h before incubation for 16 h with vehicle (Veh) or 10 μM simvastatin (Sim). Leptin mRNA abundance was measured using RT-PCR. $^*P < 0.01$ versus vehicle control.

(data not shown). However, treatment of cells with two PI3K pathway inhibitors such as LY294002 and wortmannin mimicked the effect of simvastatin (Fig. 5C). Treatment with 10 μM simvastatin or 20 μM LY 294002 suppressed PI3K activities (Fig. 5D). These results suggested that inhibition of the PI3K pathway by simvastatin was involved in inhibition of leptin mRNA synthesis in adipocytes. Suppression of aP2 and C/EBPα mRNA expression by simvastatin appears to be mediated *via* inhibition of prenylation of proteins which regulate signalling pathways other than PI3K pathway, because LY294002 did not affect aP2 and C/EBPα mRNA expression. Simvastatin treatment did not affect PPARγ mRNA expression (Supplementary Fig. 2).

Simvastatin Regulates Leptin mRNA Expression via PKA—We examined involvement of the cAMP-dependent PKA pathway in simvastatin-induced suppression of leptin mRNA expression in 3T3-L1 adipocytes. When the cells were treated with increasing concentrations of simvastatin for 3 h, a dose-dependent increase in cAMP accumulation was observed. Increases in intracellular cAMP concentrations were significant for 1 μM simvastatin, and maximal at 10 μM (Fig. 6A). Simvastatin-induced stimulation of cAMP accumulation was abolished by the treatment with 10 μM GGPP. LY294002, a PI3K inhibitor, at 20 μM significantly augmented cAMP accumulation in 3T3-L1 cells (Fig. 6B). We next tested the involvement in PKA as the downstream effector of cAMP changes induced by simvastatin. A clear increase was found to be induced by 10 μM simvastatin or 20 μM LY294002. Addition of 10 μM GGPP or 1 mM mevalonate abrogated PKA activity of the cells treated with 10 μM simvastatin. Treatment with H89, an inhibitor of PKA, highly inhibited PKA activity in the presence of simvastatin, mevalonate, and GGPP (Fig. 6C). 3T3-L1 cells were incubated with H89, which completely abrogated suppression of leptin expression induced by simvastatin (Fig. 6D and E). These results showed that simvastatin-induced suppression of leptin gene expression was mediated *via* activation of the cAMP–PKA pathway.

DISCUSSION

Many studies document that statins trigger various cellular events through their functions as HMG-CoA reductase inhibitors as well as *via* other signalling pathways (*25*, *26*, *28*, *29*). By modulating the initial part of the cholesterol synthesis pathway, statins decrease concentrations of many important intermediate compounds including isoprenoids that contain GGPP and FPP. Such isoprenoid lipids are attached to certain proteins such as small G-proteins as a post-translational modification (*12*). The present study demonstrated that mevalonate, the direct product of HMG-CoA reductase, as well as GGPP, the metabolite, reversed suppression of leptin gene expression in differentiated 3T3-L1 adipocytes. Furthermore, to assess whether protein modifications participate in suppression by statins of the leptin gene in adipocytes, 3T3-L1 cells were exposed to inhibitors of protein prenylation such as hydroxyfasudil. The inhibitor mimicked the simvastatin-induced effect of leptin gene suppression. However, FPP failed to reverse inhibition of leptin expression induced by simvastatin. Since Ras, a small G protein, is mainly farnesylated by FPP and transduces signals (*29–32*), the Ras protein was unlikely to be involve in the signalling mechanism. In osteosarcoma cells, mevalonate and GGPP preferentially diminished the proapoptotic effect of statins relative to FPP, pointing to a predominant role of protein geranylgeranylation in statin-induced apoptosis (*25*), and in umbilical vein endothelial cells, inhibition of protein geranylgeranylation by statins significantly induced KLF2 transcription factor, whereas inhibition of farnesylation did not (*31*); these are consistent with our findings concerning simvastatin-induced

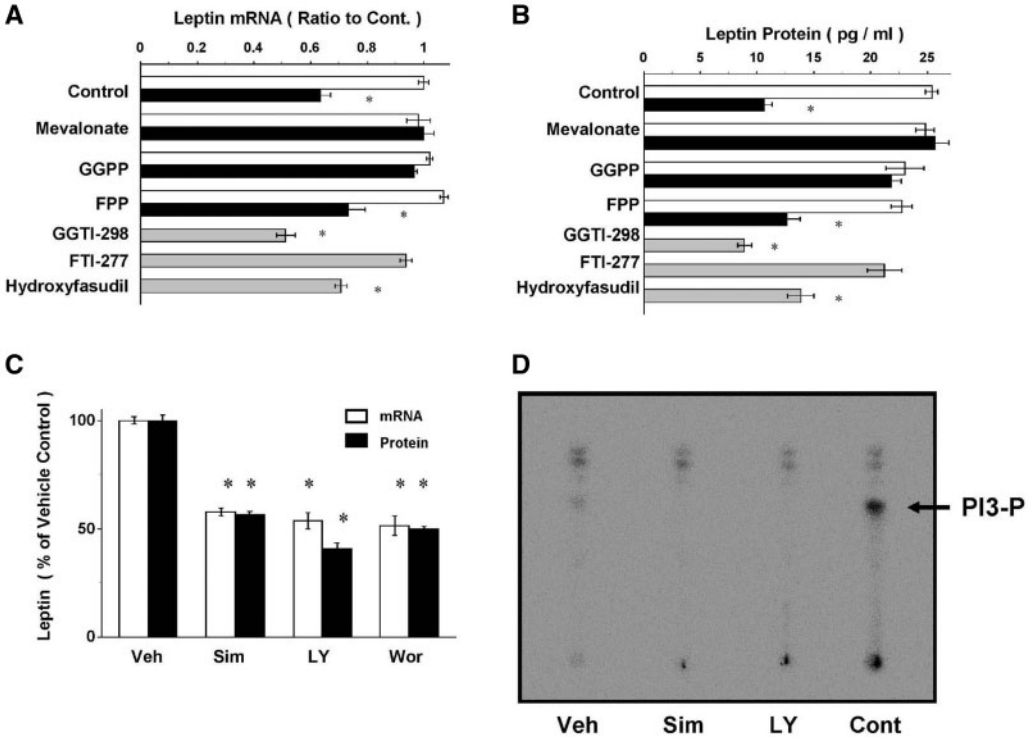

Fig. 5. **Regulation of leptin expression by various inhibitors in simvastatin-treated 3T3-L1 adipocytes.** After cells were treated with various compounds in the presence or absence of 10 μM simvastatin (Sim) for 24 h, leptin mRNA abundance was determined using RT-PCR and secretion of leptin protein over 24 h was measured by ELISA. Effects of HMG-CoA metabolite and transferase inhibitors on leptin mRNA expression are shown in (A), and those on leptin protein secretion are shown in (B). After 3T3-L1 cells were treated with mevalonate (1 mM), GGPP (10 μM), FPP (10 μM), GGTI-298 (10 μM), FTI-277 (10 μM) or hydroxyfasudil (30 μM) in the presence (closed column) or absence (open or grey column) of simvastatin (10 μM) for 24 h, cell extracts and culture media were subjected to measurements. Effects of PI3K inhibitors on leptin mRNA expression are shown in (C). 3T3-L1 cells were treated with PI3K inhibitors, LY294002 (LY; 20 μM) and wortmannin (Wor; 100 nM) for 24 h. Some cells were treated with vehicle (Veh) or simvastatin (Sim; 10 μM) for 24 h. $^*P < 0.01$ versus vehicle control. PI3K activities of 3T3-L1 cells are shown in (D). 3T3-L1 cells were treated with vehicle (Veh), simvastatin (Sim; 10 μM) or LY294002 (LY; 20 μM) for 1 h. Cont represents positive control using authentic PI3K, PI as substrate and labelled ATP.

suppression of leptin gene expression in 3T3-L1 adipocytes. Statins induced relocation and inactivation of Rho A, a small GTPase, in association with induction of apoptosis. Since supplementation of media with GGPP restored the amount of membrane bound-Rho A and fully prevented apoptosis, defective isoprenylation of Rho A induced by statins appears to be necessary and sufficient to elicit the effect in observed osteosarcoma cells (*25*). Our results implicated geranylgeranylated proteins in the downregulation of leptin gene expression in 3T3-L1 adipocytes. At present, Rho A is a prominent candidate among geranylgeranylated proteins, although other proteins possibly may be involved in addition. The target proteins for statin actions must be clarified.

We investigated the major signalling pathways that contribute to leptin gene suppression in 3T3-L1 cells exposed to simvastatin, because it has been reported that statins affect the effects of signalling pathways of ERK1/2 MAPK and p38 MAPK (*25*, *32*). When we added selective inhibitors of the ERK1/2 MAPK pathway, U0129 and PD98059, these inhibitors did not mimic the simvastatin-induced effect. In the ERK1/2 MAPK pathway, the signal is transduced through Ras, which is activated by farnesyl prenylation of the protein (*33*). The present results demonstrated that the effect elicited by simvastatin was independent of farnesylation reactions but dependent on geranylgeranylation, suggesting that statins might not regulate Ras activity. A specific inhibitor of p38 MAPK, SB203580, could not suppress leptin gene expression. These results suggest that ERK1/2 and p38 MAPK pathways are not involved in statin-induced suppression of leptin expression in 3T3-L1 adipocytes.

PI3K participates in a wide variety of cellular process, including intra-cellular trafficking, organization of the cytoskeleton, cell growth and transformation and prevention of apoptosis (*34*, *35*). PI3K has a role in differentiation of several cell lines (*36*, *37*), including adipocytes. Activation of this enzyme increases expression of adipocyte-specific proteins including aP2, lipoprotein lipase (LPL), adipsin, glucose transporter 4 (Glut4), CD36 and adiponectin (*38–40*). In osteoblasts, statins stimulated PI3K to affect BMP-2 (*26*) and vascular endothelial growth factor (*18*). Statins also stimulated PI3K in mesenchymal stem cells, vascular endothelial cells and vascular smooth muscle cell (*41–43*). Implication of PI3K as a modulator of simvastatin action on leptin gene suppression was evaluated by

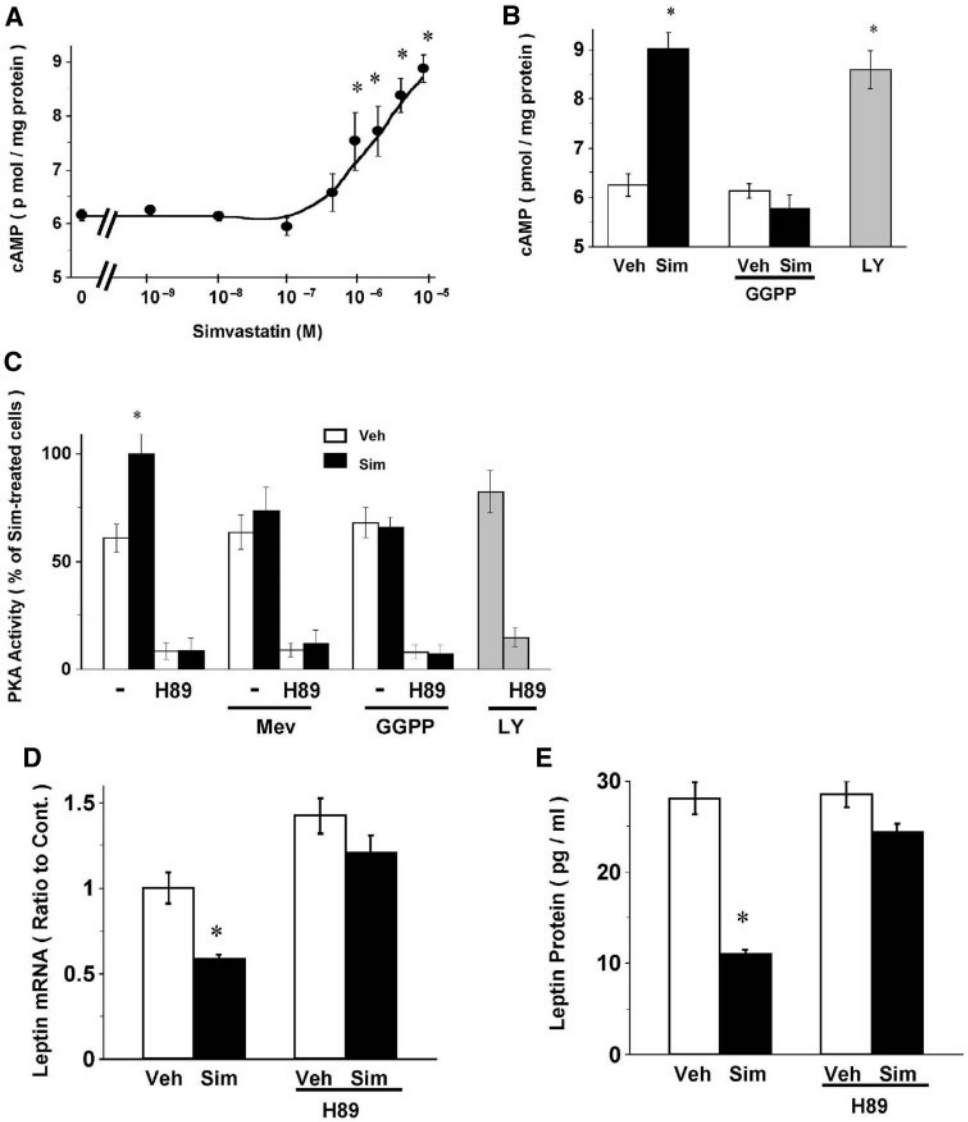

Fig. 6. **Activation of PKA activity is required for simvastatin-induced leptin suppression.** (A) Dose-dependent effect of simvastatin on cAMP accumulation in 3T3-L1 adipocytes. Cells were treated with vehicle or graded doses of simvastatin for 3 h, and then extracted for measurements of cAMP. (B) Effects of GGPP, and LY294002 (LY) on cAMP accumulation. Cells were treated with simvastatin (Sim; 10 μM), GGPP (10 μM) or LY294002 (20 μM) for 3 h. GGPP treatment was performed in the presence or absence of 10 μM simvastatin. (C) PKA activity of 3T3-L1 cells. Cells were incubated with vehicle (Veh), simvastatin (Sim; 10 μM), mevalonate (Mev; 1 mM), or GGPP (10 μM) in the presence or absence of 5 μM H89 for 4 h, and PKA activity was measured. (D, E) Effects of H89, a PKA inhibitor, on leptin mRNA expression (D) and leptin protein secretion (E) were assessed in 3T3-L1 cells. Cells were treated with vehicle (Veh) or simvastatin (Sim; 10 μM) for 24 h. Cells were harvested, and cellular extracts were used for determinations of leptin mRNA concentration. *$P < 0.01$ versus vehicle control.

using two specific inhibitors of the enzyme, LY294002 and wortmannin. Without cell toxicity, both inhibitors at optimal concentrations inhibited leptin gene expression and protein production in differentiated 3T3-L1 cells. Furthermore, we demonstrated that simvastatin directly inhibited PI3K activity in 3T3-L1 cells. These findings strongly suggest that downregulation of leptin gene transcription is mediated *via* the PI3K pathway. These results differ from some previous reports on the effects of increased PI3K by statins. Statins inhibited insulin signalling by disrupted insulin receptor glycosylation (*44*). Insulin increased geranylgeranyl transferase I activity and prenylation of Rho A protein (*45*). Since insulin increases PI3K activity in differentiated adipocytes such as 3T3-L1 cells (*40*), these cells cultured in the presence of FBS containing insulin would retain PI3K activity. Therefore, it is possible that statins inhibit PI3K pathway by interfering in insulin signalling in differentiated adipocytes. Further detailed investigations are required to define the interaction between statin action and intra-cellular insulin signalling on leptin gene expression in adipocytes.

PI3K influences accumulation of intra-cellular cAMP, coupled with the anti-lipolytic action of insulin in adipose

tissue (*46*). A recent study demonstrated that insulin activated phosphodiesterase 3B (PDE3B) *via* PI3K, and, in turn, reduced cAMP concentrations in 3T3-L1 cells (*40*). Reduction of cAMP led to stimulation of leptin and adiponectin expression, consistent with the previous findings that β-adrenergic agonists such as norepinephrine and isoproterenol increased intra-cellular cAMP concentrations followed by a decrease in leptin mRNA expression (*47*, *48*). A recent study showed that leptin secretion by rat adipocytes was profoundly attenuated by cAMP in PKA-dependent manner (*49*). The present study demonstrated that simvastatin clearly increased intra-cellular cAMP concentrations, while simvastatin-induced inhibition of leptin expression was completely abrogated in the presence of H89, a highly selective PKA inhibitor, in 3T3-L1 cells. These findings suggest that elevation of cAMP concentrations as a result of PDE inhibition by PI3K deactivation leads to PKA activation mediating leptin gene suppression in adipocytes (*40*).

A number of studies have showed that statins directly affect gene expression in many cell lines, including adipocytes and osteoblasts (*15–20*, *22*, *25*, *26*, *50*). The present study demonstrated that cellular signals induced by simvastatin including PKA activation were transmitted to the nucleus to inhibit leptin mRNA expression in 3T3-L1 cells. Simvastatin decreased steady-state amounts of leptin mRNA without modifying the half-life of this mRNA. Although hnRNA may reflect alteration in RNA processing, its abundance correlates with transcription rates and observations with a promoter assay using a luciferase reporter gene (*18*, *51*). Abundance of leptin hnRNA was clearly decreased by simvastatin-induced signals directly influencing the leptin gene to inhibit expression in adipocytes.

The leptin promoter with the TATA box has several sequence motifs recognized by C/EBPα, cAMP-responsive element binding protein (CREB), Sp1 and glucocorticoids (*52*). Other studies and ours using PKA inhibitor, H89, suggest that CREB binds to CRE and suppressed activities of leptin promoter in adipocytes (*49*). C/EBPα is a transcriptional factor important for the transcription of most adipocyte genes including leptin gene to promote adipogenesis (*53*). C/EBPα bound to C/EBP motifs of leptin promoter and stimulated leptin expression (*54*). A highly methylated CpG island within the proximal promoter sequence of the silent leptin gene of preadipocytes is unmethylated in terminally differentiated adipocyte expressing the leptin gene. Direct methylated CpG motifs in this proximal promoter sequence act as specific sites for methyl-CpG binding proteins including C/EBPα that are involved in leptin gene repression (*55*). Therefore, leptin gene transcription is regulated by CREB activated by PKA, C/REBα and glucocorticoid receptors.

Statins including lovastatin and simvastatin have been shown to impair adipocyte differentiation in 3T3-L1 cells and in mouse mesenchymal cells from bone (*15*, *16*, *48*). Both PPARγ and C/EBPα transcription factors are necessary for precursor cells to differentiate into mature adipocytes (*6*, *50*). Statins inhibit PPARγ and C/EBPα expression, suggesting that inhibition of synthesis and activation of these transcription factors is important in inhibition of adipocyte differentiation by statins. A recent study showed that pitavastatin stimulated expression of preadipocyte factor-1, an inhibitory factor for adipocyte differentiation, in 3T3-L1 adipocytes (*50*). Furthermore, statins also reduced expression of adipocyte-specific genes including aP2, LPL, CD36 and Glut4 (*50*, *56*). These studies demonstrated that statins suppressed adipocyte differentiation over the entire cellular life span. The present study showed that simvastatin suppressed gene expression for leptin, an adipocyte hormone or adipokine, which is synthesized and secreted almost exclusively by differentiated adipocytes.

Simvastatin also attenuated mRNA expression of adipocyte markers such as C/EBPα and aP2 by inhibition of protein prenylation, while these downstream pathways might differ from that of leptin gene suppression. We showed that simvastatin attenuated C/EBPα mRNA expression *via* reduction in geranylgeranylated proteins in 3T3-L1 cells. Therefore, simvastatin-induced reduction in leptin mRNA expression is, at least in part, due to suppression of C/EBPα expression. By contrast, PPARγ mRNA expression of 3T3-L1 cells did not change in the presence or absence of simvastatin, suggesting that PPARγ mRNA expression is not implicated in simvastatin-induced suppression of leptin mRNA synthesis. In fact, Nakata *et al*. (*56*) recently showed that atorvastatin did not alter PPARγ expression in differentiated 3T3-L1 adipocytes. Thus, our results raised the possibility that well-differentiated adipocytes were used at the present study.

We propose the model shown in Fig. 7. In which simvastatin, an inhibitor of HMG-CoA reductase, blocks conversion of HMG-CoA to mevalonate. This leads to reduction of downstream products of the mevalonate pathway such as isopentenyl pyrophosphate and GGPP. Decreases in cellular GGPP concentrations result in inhibition of prenylation of crucial signalling proteins including PI3K, inhibiting the activity. Suppression of PI3K occurs in inhibition of PDEs such as microsomal PDE3B in adipocytes, resulting in increases of intracellular cAMP concentrations reflecting reduced cAMP degradation. Activation of PKA by cAMP suppresses leptin gene transcription.

In conclusion, we found for the first time that simvastatin activated the cAMP–PKA pathway to inhibit leptin gene suppression. These findings highlight the importance of PKA activity in statin-induced regulation of adipocyte differentiation. The present study suggests a promising clinical strategy, since inhibition of adipogenesis by statins could alleviate obesity, a well-established risk factor for cardiovascular disease and type 2 diabetes.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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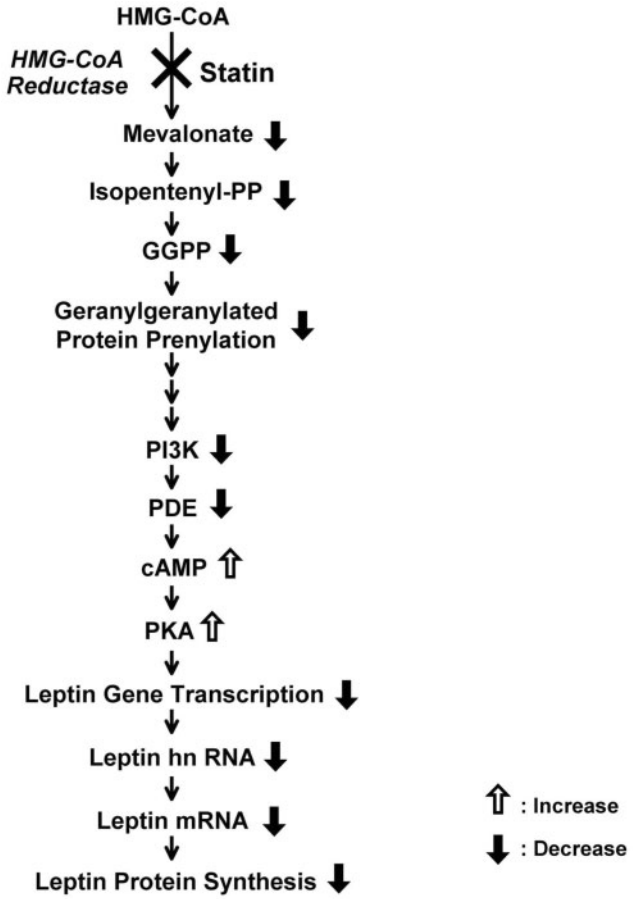

Fig. 7. **Proposed model for mechanisms underlying simvastatin-induced inhibition of leptin expression in 3T3-L1 adipocytes.** Statins such as simvastatin inhibit adipocyte mevalonate synthesis through inhibition of HMG-CoA reductase activity, decreasing GGPP formation. In turn, prenylation of geranylgeranylated proteins is reduced, leading to inhibition of PI3K activity. Suppression of PI3K activity inhibits PDE activity, resulting in increases in intra-cellular cAMP concentrations and ultimately in activation of PKA. PKA suppresses leptin gene transcription without new protein synthesis.

CONFLICT OF INTEREST

None declared.

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