Clusterin Decreases Hepatic SREBP-1c Expression and Lipid Accumulation

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Hepatic steatosis is emerging as the most important cause of chronic liver disease and is associated with the increasing incidence of obesity with insulin resistance. Sterol regulatory binding protein-1c (SREBP-1c) is a master regulator of lipogenic gene expression in the liver. Hyperinsulinemia induces SREBP-1c transcription through liver X receptor (LXR), specificity protein 1, and SREBP-1c itself. Clusterin, an 80-kDa disulfide-linked heterodimeric protein, has been functionally implicated in several physiological processes including lipid transport; however, little is known about its effect on hepatic lipogenesis. The present study examined whether clusterin regulates SREBP-1c expression and lipid accumulation in the liver. Adenovirus-mediated overexpression of clusterin inhibited insulin- or LXR agonist-stimulated SREBP-1c expression in cultured liver cells. In reporter assays, clusterin inhibited SREBP-1c promoter activity. Moreover, adenovirus-mediated overexpression of clusterin in the livers of mice fed a high-fat diet inhibited hepatic steatosis through the inhibition of SREBP-1c expression. Reporter and gel shift assays showed that clusterin inhibits SREBP-1c expression via the repression of LXR and specificity protein 1 activity. This study shows that clusterin inhibits hepatic lipid accumulation through the inhibition of SREBP-1c expression and suggests that clusterin is a negative regulator of SREBP-1c expression and hepatic lipogenesis. **(*Endocrinology* 154: 1722–1730, 2013)**

Nonalcoholic fatty liver disease is the most common disease of the liver. Its prevalence has been increasing markedly, and it is considered the hepatic manifestation of metabolic syndrome in obese individuals with insulin resistance (1, 2). Hyperinsulinemia, a hallmark of insulin resistance, potently induces enzymes catalyzing the de novo synthesis of fatty acids in liver (3). A large number of studies point to sterol regulatory element-binding protein-1c (SREBP-1c) as a key transcription factor mediating the stimulating effects of insulin (3–5). SREBP-1c is a membrane-bound transcription factor of the basic helix-loop-helix leucine zipper family and regulates the gene expression of key enzymes implicated in lipid metabolism in liver. Nascent SREBP-1c is embedded in the endoplasmic reticulum and transported to the Golgi in which 2 sequential cleavages generate the active form of mature SREBP-1c (6). The control of hepatic lipogenesis by insulin is mediated by the enhanced transcription of SREBP-1c (7). Several lines of evidence have shown that, within the SREBP-1c promoter sequence, binding sites for liver X receptor (LXR), specificity protein 1 (Sp1), nuclear factor Y, as well as for SREBP-1c itself, are necessary for the full activation of SREBP-1c transcription by insulin (8–10). Therefore, identifying molecules that inhibit these transcription factors may provide effective treatment options for fatty liver disease.

Clusterin, also known as apolipoprotein J, is an 80-kDa heterodimeric disulphide-linked glycoprotein that is widely distributed in various tissues and found in body fluids (11). Clusterin is involved in numerous physiolog-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received October 2, 2012. Accepted March 13, 2013.
First Published Online March 20, 2013

* H.-Y.S. and M.-K.K. contributed equally to this study.

Abbreviations: Clu KO, clusterin knockout; FBS, fetal bovine serum; GFP, green fluorescent protein; HDL, high-density lipoprotein; HFD, high-fat diet; LXR, liver X receptor; Sp1, specificity protein 1; SREBP-1c, sterol regulatory element-binding protein-1c.

ical processes including tissue remodeling, lipid transport, complement inhibition, and the regulation of apoptosis (12–14). Interestingly, clusterin is differentially expressed in many severe physiological disturbances, including cancer, neurodegeneration, diabetes, and atherosclerosis (12). Clusterin has been detected in human and mouse aorta with atherosclerotic lesions but not in normal aorta (15, 16). Clusterin promotes cholesterol efflux from foam cells, indicating that clusterin has a protective and potentially antiatherogenic function (11). Human plasma clusterin exists in high-density lipoproteins (HDL) that contain apolipoprotein A-I and cholesteryl ester transfer protein activity, which play a role in the transport of cholesterol from peripheral tissues to the liver (17, 18). A recent study showed that glucose stimulated clusterin expression in liver and SREPB-1 plays a crucial role in the metabolic regulation of clusterin (19). Taken together, these studies suggest that clusterin may be important for hepatic lipogenesis; however, the effect of clusterin on SREBP-1c expression and hepatic lipogenesis has not been studied yet. The present study demonstrates that clusterin decreases hepatic lipogenesis by inhibiting SREBP-1c expression.

Materials and Methods

Cell culture

The H4IIE rat hepatoma cell line and the HepG2 human hepatoma cell line were cultured in 5% CO_2-95% air at 37°C in MEM (Gibco-BRL, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah) and antibiotics. AML12 cells were cultured in 5% CO_2-95% air at 37°C in DMEM/F12 medium (Gibco-BRL) supplemented with 10% FBS, insulin-transferrin-selenium (Sigma, St Louis, Missouri), 40 ng/mL dexamethasone (Sigma), and antibiotics. After reaching approximately 80% confluence, cells were serum starved for 24 hours in medium containing 0.5% FBS and then treated as indicated in the text.

Generation of recombinant adenovirus

The cDNA encoding rat clusterin was inserted into the *Bgl*II/*Xho*I sites of the pAd-Track-CMV shuttle vector. To produce the recombinant adenoviral plasmid, the resultant shuttle vector was electroporated into BJ5138 cells containing the AdEasy adenoviral vector. The recombinant adenoviral plasmids were transfected, and adenoviruses expressing clusterin (Ad-Clu) were amplified in human embryonic kidney-293 cells and purified using CsCl density centrifugation (Sigma). The viruses were collected and desalted, and the titers were determined using Adeno-X Rapid titer (BD Bioscience, San Jose, California). Control adenoviruses (Ad-GFP) were prepared by the same method. The efficiency of adenoviral infection was assessed using a recombinant adenovirus encoding clusterin fused to green fluorescent protein (GFP; data not shown).

Animal experiments

In vivo experiments were conducted using 8- to 9-week-old male C57BL/6 mice (Samtako, Osan, Korea). To generate clusterin knockout (Clu KO) mice on the C57BL/6 genetic background, clusterin-deficient mice originally generated using a Swiss black genetic background (20) were backcrossed onto the C57BL/6 strain for at least 7 generations. Clu KO mice were kind gifts from Dr I.-S.P. Park (Inha University School of Medicine, Incheon, Korea). All animal procedures were carried out in accordance with institutional guidelines for animal research.

Experimental procedure 1

Mice were divided into 2 groups, fasting and refeeding. Mice were fasted for 48 hours or fasted for 36 hours and refed during the following 16 hours.

Experimental procedure 2

Mice were divided into 3 groups: 1) animals fed a control chow diet (Hyochang, Daegu, Korea); 2) animals fed a high-fat diet (HFD) diet that provides 60% of the calories as fat (D12492; Research Diets, New Brunswick, New Jersey) with an injection of Ad-GFP; and 3) animals fed a HFD diet with an injection of Ad-Clu. Exogenous overexpression of GFP or clusterin was induced in the livers of HFD-fed mice via an injection of adenovirus (1×10^9 pfu per 200 μL) into the tail vein at the indicated time points. After 4 weeks, animals were killed for the collection of liver samples. Liver tissue was fixed with 4% paraformaldehyde and embedded in paraffin. Serial 4-μm sections were subjected to hematoxylin and eosin staining according to standard procedures. Intrahepatic lipid was visualized using the oil red O method and hepatic triglycerides.

Primary cultures of hepatocytes

C57BL/6 or Clu KO mouse hepatocytes were isolated by perfusing the liver via the portal vein. The liver was first perfused with resuspension buffer (140 mmol/L NaCl; 5.4 mmol/L KCl; 0.44 mmol/L KH_2PO_4; 0.34 mmol/L Na_2HPO_4; 0.5 mmol/L EGTA; 25 mmol/L Tricine, pH 7.2) at 4 mL/min for 10 minutes and then perfused with collagenase solution [Hanks' balanced salt solution (9.5 g/L); 0.8 mmol/L $NaHCO_3$; 0.002% deoxyribonuclease I; 0.7 g/L collagenase type I (Worthington Biochemical Corp, Freehold, New Jersey), pH 7.2] at 4 mL/min for 10 minutes. After perfusion, the liver was shaken for 15 minutes at 37°C and filtered through a mesh (85 μm nylon mesh). Hepatocytes were collected by centrifugation at 500 rpm for 5 minutes at 4°C, resuspended in William's E medium (Sigma), and seeded onto collagen type I-coated 60-mm dishes (IWAKI Scitech Kiv, Tokyo, Japan) at a density of 5×10^5 cells/mL. The viability of hepatocytes, as measured by trypan blue dye exclusion, was always greater than 85%. After a 2- to 3-hour incubation, the medium was exchanged with DMEM. Hepatocytes were used subsequently for quantitative real-time RT-PCR.

Northern blot analysis

H4IIE cells and AML12 cells were treated with TO901317 (Sigma) and insulin (Novo Nordisk, Bagsvaerd, Denmark) and subsequently infected with adenoviral vectors expressing clusterin. Cells were harvested, total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions, and 20 μg of total RNA from

each sample was used. The SREBP-1c and clusterin probes were labeled with [α-^{32}P]dCTP using a random-primer DNA-labeling system (Amersham Biosciences, Little Chalfont, United Kingdom).

Quantitative real-time RT-PCR

Total RNA was isolated from cells and tissue extracts using the Trizol reagent. Reverse transcription was performed using the RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, Maryland). Quantitative real-time RT-PCR was performed using a SYBR Green PCR master mix kit (TOYOBO, Osaka, Japan) and the Light Cycler 480 instrument (Roche Diagnostics, Indianapolis, Indiana). PCR parameters were as follows: 45 cycles of 95°C for 30 seconds, 60°C for 10 seconds, and 72°C for 15 seconds. Primer sequences were as follows: SREBP-1c, forward, 5′-CCATCGACTACATCCGCTTCTT-3′, reverse, 5′-ACTTCGCAGGGTCAGGTTCTC-3′; acetyl-CoA carboxylase, forward, 5′-CGCTCAGGTCACCAAAAAGAAT-3′, reverse, 5′-GGGTCCCGGCCACATAA-3′; stearoyl-CoA desaturase, forward, 5′-CTGCCCCTGCGGATCTT-3′, reverse, 5′-GCCCATTCGTACACGTCATTCT-3′.

Western blot analysis

H4IIE cells and AML12 cells were treated with TO901317 (Sigma), insulin (Novo Nordisk) and subsequently infected with Ad-Clu. Cell lysates were prepared using lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate] containing proteinase inhibitors. The proteins were resolved by SDS-PAGE and then transferred electrophoretically to a polyvinyl difluoride membrane (Millipore, Bedford, Massachusetts). The membrane was blocked by incubation in blocking buffer, incubated with anti-clusterin-α antibody (Santa Cruz Biotechnology, Santa Cruz, California) or anti-SREBP-1c antibody (BD Biosciences) and was then developed using an enhanced chemiluminescence Western blot detection kit (Amersham Biosciences). The membrane was reblotted with antiactin antibody to verify equal loading of protein in each lane. Densitometry was used to quantitate the results using the scientific software program UN-SCAN-IT (Skik Scientific Corp, Orem, Utah).

In vitro reporter assays

HepG2 cells were plated at a density of 7×10^4 cells/well in a 12-well plate and cultured for 1 day in culture medium. Cells

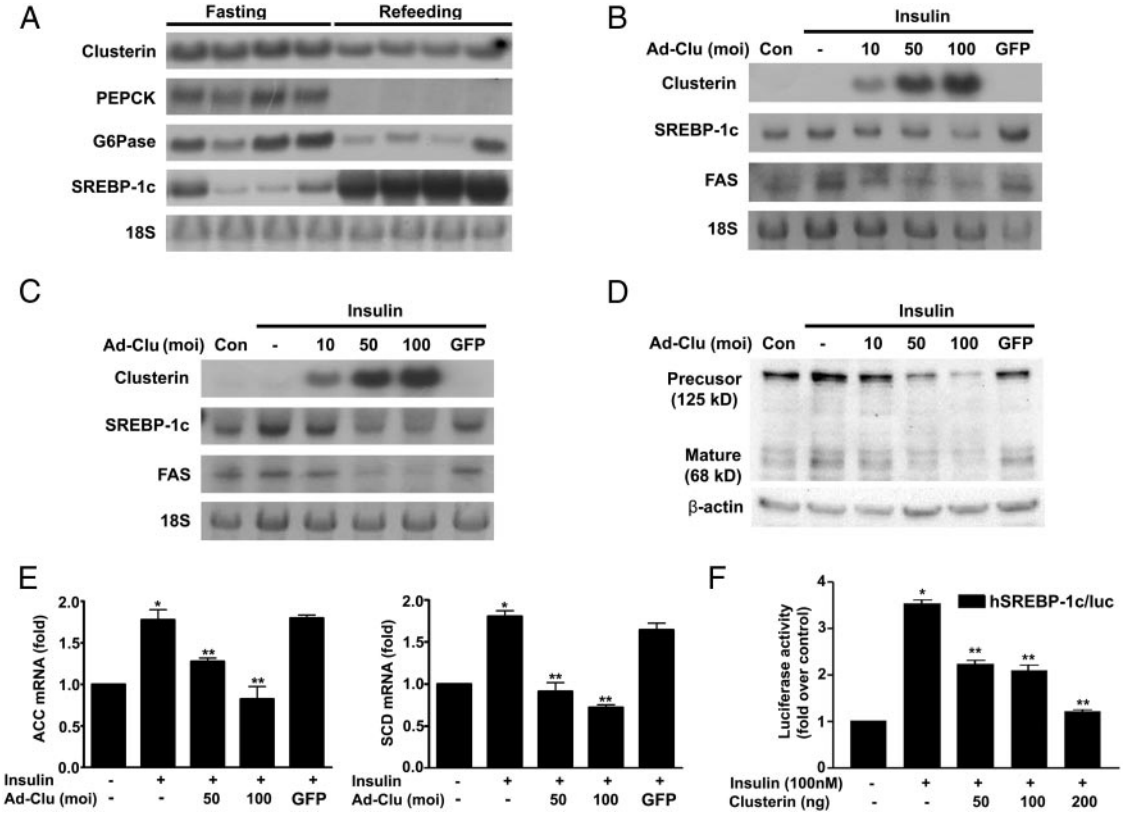

Figure 1. Clusterin inhibits insulin-stimulated SREBP-1c expression in cultured hepatocytes. A, Representative Northern blot analysis of hepatic clusterin and SREBP-1c mRNA expression in the livers of fasting and refeeding mice. B and C, Representative Northern blot analysis showing the effect of clusterin overexpression on insulin-stimulated SREBP-1c and FAS mRNA expression in H4IIE (B) and AML12 (C) cells. FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase. D, Western blot analysis showing the effect of clusterin overexpression on insulin-stimulated SREBP-1c protein expression. AML12 cells were infected with the indicated concentrations [multiplicity of infection (MOI)] of Ad-Clusterin (Ad-Clu) or Ad-GFP (GFP) for 24 hours and treated with insulin (100 nM) for 6 hours. E, Real-time RT-PCR showing the effect of clusterin overexpression on insulin-stimulated acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD) mRNA expression in AML12 cells. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control (Con); $**P < .01$ compared with insulin alone. F, Effect of clusterin on insulin-stimulated SREBP-1c promoter activity. HepG2 cells were cotransfected with a SREBP-1c promoter (−1516/+40) reporter construct (200 ng/well) and the indicated amounts of a clusterin expression construct and stimulated with insulin for 6 hours. Luciferase activity was normalized to β-galactosidase activity. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control; $**P < .01$ compared with insulin alone.

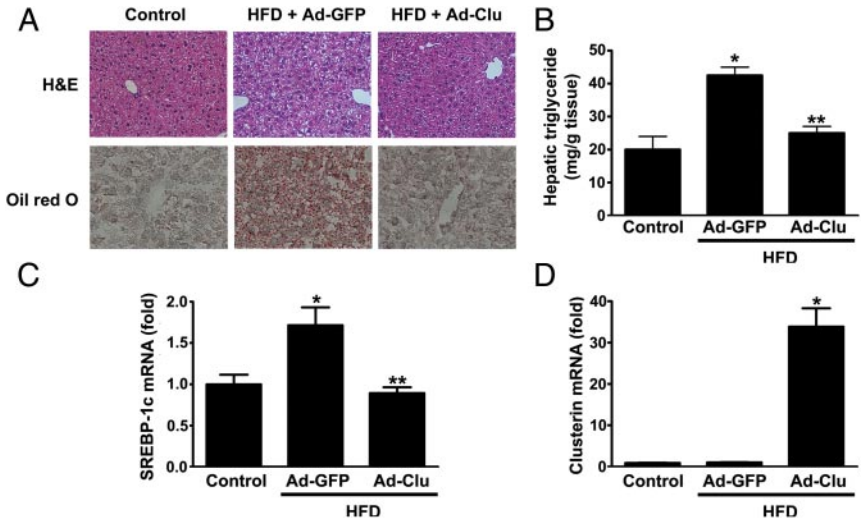

Figure 2. Clusterin reduces lipid accumulation and SREBP-1c expression in the liver of mice fed a HFD. A, Histological analysis of liver sections of HFD-fed mice after a tail vein injection of Ad-Clusterin (Ad-Clu) or Ad-GFP. Upper panel, Hematoxylin and eosin staining; lower panel, oil red O staining. Original magnification, ×100. B, Triglyceride concentration of HFD-fed mice after a tail vein injection of Ad-Clusterin or Ad-GFP. Data in bar graph are the means ± SEM of 3 independent measurements. $*P < .05$ compared with control diet-fed mice; $**P < .05$ compared with HFD-fed mice. C, Representative real-time RT-PCR analysis of SREBP-1c mRNA expression in the livers of HFD-fed mice after a tail vein injection of Ad-Clusterin or Ad-GFP. Data represent the means ± SEM of 3 independent measurements. $*P < .05$ compared with control diet-fed mice; $**P < .01$ compared with HFD-fed mice. D, Representative real-time RT-PCR analysis of clusterin mRNA expression in the livers of HFD-fed mice. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control diet-fed mice or HFD-fed mice.

were transiently transfected with the indicated promoter constructs (200 ng/well) and other cDNAs using the Lipofectamine 2000 transfection reagent (Invitrogen). Cells were cotransfected with a plasmid encoding β-galactosidase as an internal control. Cells were transfected for 5 hours, washed to remove plasmid, and then maintained in conditioned medium. Cells were harvested 24 hours after transfection and assayed for luciferase and β-galactosidase activity. Twenty microliters of cell lysate containing 15 μg of protein were analyzed using the luciferase assay system, according to the manufacturer's instructions (Promega, Madison, Wisconsin). Luciferase activity was measured using a SIRUS luminometer (Berthold, Pforzheim, Germany) and was normalized to β-galactosidase activity. Luciferase reporter constructs containing the human wild-type SREBP-1c promoter [pSREBP(−1516/+40)-luciferase] were kind gifts from Dr E. Tarling (Queen's Medical Center, Nottingham, United Kingdom) (21) and wild-type rat SREBP-1c promoter [pSREBP(−1516/+40)-luciferase] and a mutant SREBP-1c promoter containing 2 LXR-binding site mutations [pSREBP(−1516/+40) mutLXR1/mutLXR2-luciferase] were kind gifts from Dr L. Cagen (University of Tennessee, Memphis, Tennessee) (*8*).

Electrophoretic mobility shift analysis

Nuclear extracts were prepared from HepG2 cells using the NucBuster protein extraction kit (Calbiochem, La Jolla, California) according to the manufacturer's instructions. Nuclear extracts (6 μg) were incubated at room temperature with 60 000 cpm of a ^{32}P-labeled double-stranded oligonucleotide encoding the LXR-binding site oligomer 5′-CAG TGA CCG CCA GTA ACC CCA GC-3′ and the Sp1-binding site oligomer 5′-TAA CGG GGC GGG GCG AAT-3′ as a probe. Competition experiments were performed with unlabeled LXR-or *Sp-1*-binding site oligonucleotide. Supershift experiments were performed by incubating the binding reaction with supershifting antibody. Samples were separated by electrophoresis in a 4% acrylamide gel in 0.5× Tris-borate EDTA [0.045 M Tris, 0.045 M boric acid, 1.0 mM EDTA (pH 8.0)] at 150 V (constant voltage).

Statistical analysis

Data are the means ± SEM. An ANOVA was used to determine the significant differences in multiple comparisons and was performed using the Duncan test. $P < .05$ was considered statistically significant. All experiments were performed at least 3 times.

Results

Clusterin inhibits insulin-stimulated SREBP-1c expression in cultured hepatocytes

First, we determined whether clusterin is regulated by nutritional status and found that clusterin expression was regulated by fasting and refeeding cycles. Refeeding after fasting dramatically decreased hepatic clusterin and gluconeogenic gene expression but increased SREBP-1c expression (Figure 1A). The expression patterns of clusterin

Table 1. Body Weight, Fat Weight, and Food Intake of Mice Fed a HFD With or Without Ad-Clusterin (Ad-Clu)

	Control	HFD+Ad-GFP	HFD+Ad-Clu
Body weight, g	25 ± 0.26	28.7 ± 0.2[a]	27.9 ± 0.2[a]
Epididymal fat weight, g	0.29 ± 0.01	0.61 ± 0.05[a]	0.55 ± 0.15[a]
Food intake, g/d	2.22 ± 0.15	2.19 ± 0.19	2.13 ± 0.16

Values are mean ± SEM.

[a] $P < .05$ compared with control mice.

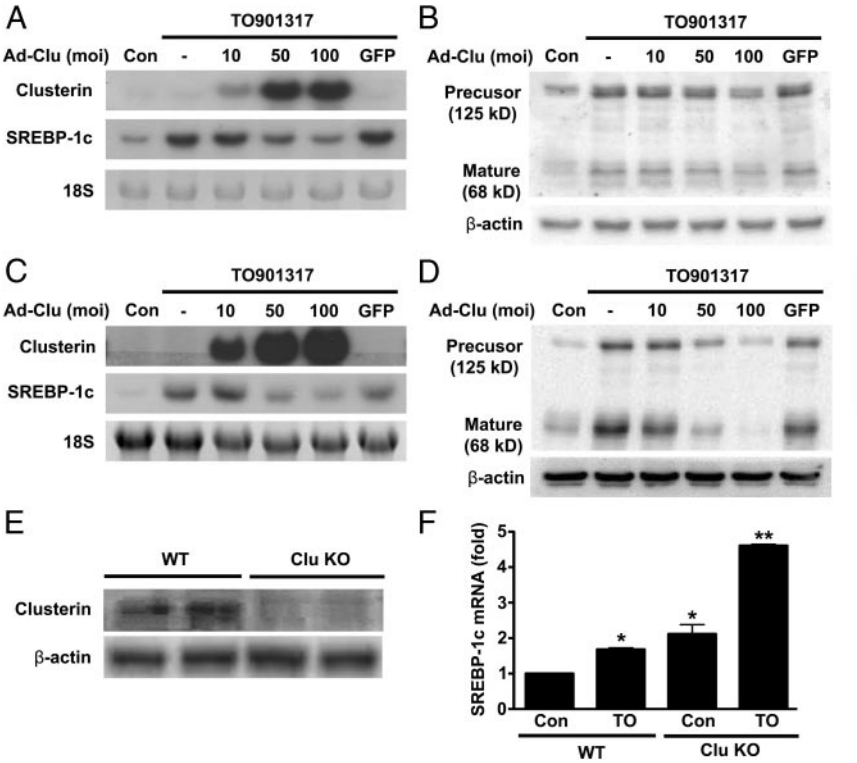

Figure 3. Clusterin inhibits LXR-stimulated SREBP-1c expression in cultured hepatocytes. Representative Northern blot and Western blot analyses show the effect of overexpression of clusterin on TO901317-stimulated SREBP-1c mRNA and protein expression. AML12 (A and B) and H4IIE (C and D) cells were infected with the indicated concentrations [multiplicity of infection (MOI)] of Ad-Clusterin (Ad-Clu) or Ad-GFP (GFP) and treated with TO901317 (1 μM) for 24 hours. E, Western blot analysis was performed using hepatocytes isolated from wild-type (WT) or Clu KO mice. F. Representative real-time RT-PCR analysis of SREBP-1c mRNA expression in primary hepatocytes from Clu KO mice with or without TO901317 (TO) stimulation. Data represent the means ± SEM of 3 independent measurements. $*P < .05$ compared with WT-control (Con); $**P < .01$ compared with WT-TO901317.

Adenovirus-mediated overexpression of clusterin reduces lipid accumulation and SREBP-1c expression in the liver of HFD-fed mice

To confirm the effect of clusterin on hepatic SREBP-1c expression in vivo, the effect of clusterin on hepatic lipid accumulation and SREBP-1c expression was assessed in HFD-fed mice infected with clusterin-containing adenoviruses by tail vein injection. Histological examination showed that the hepatocytes of HFD-fed mice had large cytoplasmic lipid droplet accumulation. This morphological change in lipid droplets was nearly completely prevented by the overexpression of clusterin (Figure 2A). A HFD increased triglyceride levels and SREBP-1c mRNA expression in the liver compared with control chow. In HFD-fed mice, adenovirus-mediated clusterin overexpression decreased both liver triglyceride levels (Figure 2B) and SREBP-1c expression (Figure 2, C and D) compared with adenovirus-mediated GFP expression. As expected, the HFD increased body weight and epididymal fat weight compared with control chow. However, body weight, epididymal fat weight, and food intake were not different between the HFD with Ad-GFP and the HFD with Ad-clusterin (Table 1).

were opposite to those of SREBP-1c, suggesting an association between these 2 molecules. Because insulin plays a critical role in regulating hepatic lipogenesis during feeding and SREBP-1c is a key transcription factor mediating the insulin-dependent regulation of gene expression (7), we examined the effect of clusterin on insulin-stimulated SREBP-1c expression in cultured hepatocytes. As shown in Figure 1, H4IIE cells (Figure 1B) and AML12 cells (Figure 1, C and D) treated with insulin for 6 hours increased SREBP-1c mRNA and protein expression, but Ad-Clu repressed insulin-stimulated SREBP-1c mRNA and protein expression in a concentration-dependent manner. In addition, Ad-Clu inhibited the insulin-stimulated expression of the SREBP-1c target gene fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase (Figure 1, B, C, and E). Moreover, a SREBP-1c promoter reporter [pSREBP(−1516/+40)-luc] (21) assay showed that clusterin repressed insulin-stimulated SREBP-1c promoter activity (Figure 1F).

Clusterin mediates LXR ligand-stimulated SREBP-1c expression and inhibits LXR activity in cultured hepatocytes

Because LXR is an important mediator of insulin-stimulated SREBP-1c expression (22), the effect of clusterin on the expression of SREBP-1c was assessed in cultured hepatocytes stimulated with the LXR ligand TO901317. As shown in Figure 3, AML12 cells (Figure 3, A and B) and H4IIE cells (Figure 3, C and D) treated with TO901317 had increased SREBP-1c mRNA and protein expression. The SREBP-1c increase was suppressed by Ad-Clu in a concentration-dependent manner. In addition to the overexpression of clusterin, we examined the effect of loss of clusterin on TO901317-induced SREBP-1c mRNA expression using primary cultured hepatocytes from Clu KO mice. Clu KO hepatocytes showed higher SREBP-1c ex-

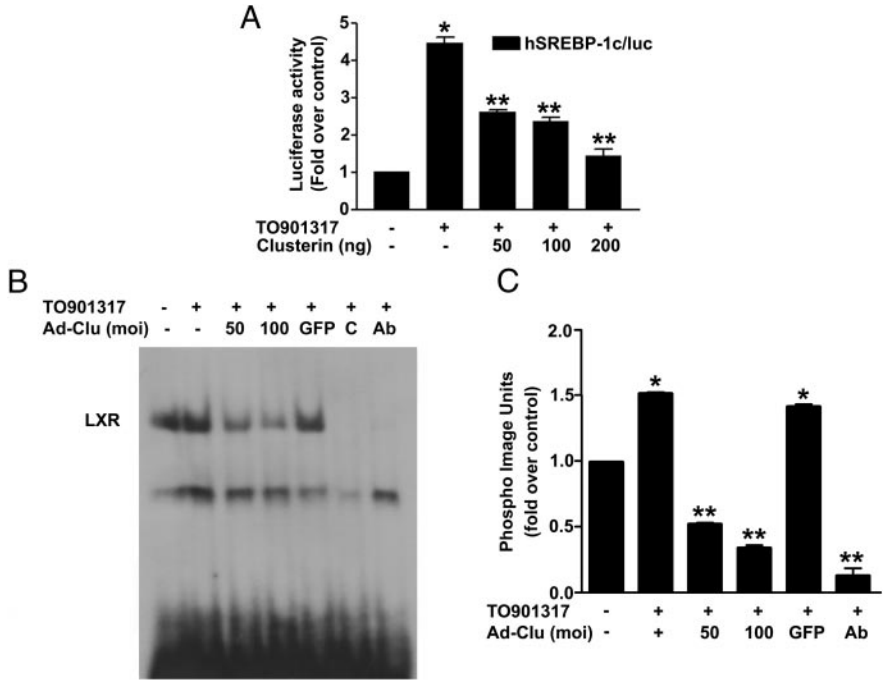

Figure 4. Clusterin inhibits LXR activity in cultured hepatocytes. A, Effect of clusterin on TO901317-stimulated SREBP-1c promoter activity. HepG2 cells were cotransfected with SREBP-1c promoter constructs (200 ng/well) and the indicated amounts of a clusterin expression construct and then stimulated with TO901317 for 24 hours. Luciferase (luc) activity was normalized to β-galactosidase activity. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control; $**P < .01$ compared with TO901317 alone. B, Effect of clusterin on TO901317-stimulated LXR DNA-binding activity. HepG2 cells were infected with the indicated concentrations [multiplicity of infection (MOI)] of Ad-Clusterin (Ad-Clu) or Ad-GFP (GFP) and treated with TO901317 for 24 hours. Competition experiments (C) were performed with unlabeled LXR-binding site oligomer. Supershift experiments (Ab) were performed by incubating the binding reaction with anti-LXR antibody. C, Data in the bar graph are the means ± SEM of 3 independent measurements. $*P < .01$ compared with control; $**P < .01$ compared with TO901317 alone.

pression levels than those of wild-type control mice. Moreover, TO901317-induced SREBP-1c expression was significantly higher in primary cultured hepatocytes of Clu KO mice than in control wild-type mice (Figure 3F). We next examined the mechanism by which clusterin inhibits LXR ligand-stimulated SREBP-1 gene expression. A SREBP-1c promoter reporter assay showed that clusterin repressed TO901317-stimulated SREBP-1c promoter activity (Figure 4A). Next, the effect of clusterin on LXR DNA-binding activity was assessed using EMSAs. Ad-Clu inhibited the TO901317-stimulated LXR DNA-binding activity in a concentration-dependent manner (Figure 4, B and C).

Clusterin inhibits Sp1 activity

In addition to LXR, SREBP-1c contains binding sites for Sp1, which was reported to mediate insulin-stimulated SREBP-1c expression. Insulin stimulated the activity of a SREBP-1c mutant promoter deficient in LXR binding and a mutant SREBP-1c promoter containing 2 LXR-binding site mutations [pSREBP(−1516/+40) mutLXR1/mutLXR2-luciferase], which is consistent with a previous report (4), although the activation by insulin was lower than that observed with the wild-type SREBP-1c promoter (Figure 5A). These data suggest that LXR is a mediator of insulin-stimulated SREBP-1c expression. Moreover, Ad-Clu inhibited insulin-stimulated LXR-mut-SREBP-1c promoter activity, reinforcing the idea that clusterin inhibits transcription factors other than LXR, such as Sp1. Accordingly, the effect of clusterin was assessed on Sp1-stimulated SREBP-1c promoter activity. As shown in Figure 5B, transient expression of clusterin inhibited Sp1-stimulated SREBP-1c promoter activity. Next, the effect of clusterin on insulin-stimulated Sp1 transcriptional activity was assessed. Using a reporter construct composed of 4 synthetic Sp1 response elements ([Sp1] × 4 luciferase), the effect of ectopic expression of Sp1, insulin, and/or clusterin was assessed. Transient expression of Sp1 increased [Sp1] × 4 luciferase activity, and the effect was enhanced in the presence of insulin. Addition of clusterin induced a concentration-dependent decrease in [Sp1] × 4 luciferase activity (Figure 5C). Moreover, an EMSA analysis showed that clusterin overexpression decreased the DNA-binding activity of Sp1 (Figure 5D).

Discussion

The present study shows that clusterin expression is regulated by nutritional status, and adenovirus-mediated overexpression of clusterin inhibits hepatic SREBP-1c ex-

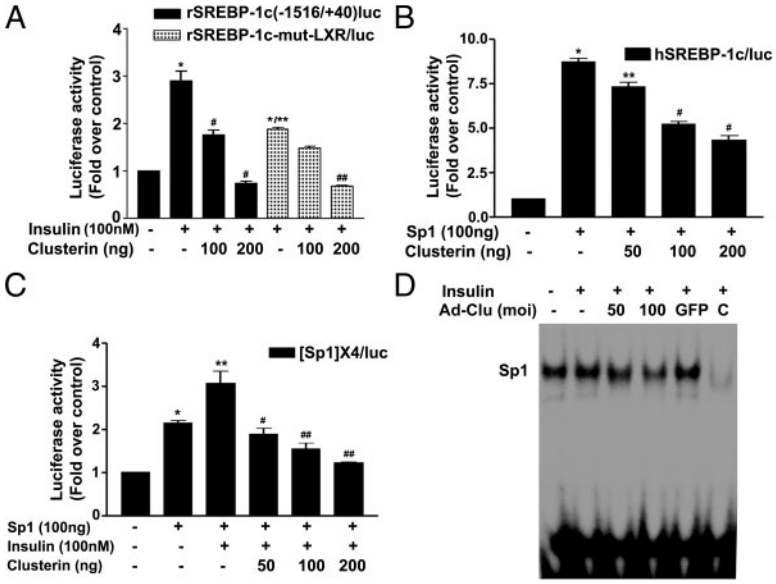

Figure 5. Clusterin inhibits Sp1 activity. A, Effect of clusterin on the insulin-stimulated reporter activity of a mutant SREBP-1c promoter deficient in LXR binding (mut-LXR). HepG2 cells were transfected with pSREBP-1c (−1516/+40)-luciferase (luc) (200 ng/well) or pSREBP (−1516/+40) mutLXR1/mutLXR2-luciferase (200 ng/well) and the indicated amounts of a clusterin expression construct and were then stimulated with insulin for 6 hours. Luciferase activity was normalized to β-galactosidase activity. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control; $**P < .01$ compared with insulin-stimulated SREBP-1c promoter; $\#P < .01$ compared with control; $\#\#P < .05$ compared with insulin-stimulated LXR-mut-SREBP-1c promoter. B, Effect of clusterin on Sp1-stimulated SREBP-1c promoter activity. HepG2 cells were cotransfected with the SREBP-1c promoter construct (200 ng/well) and Sp1 expression constructs (100 ng) and the indicated amounts of a clusterin expression construct. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ compared with control; $**P < .05$ and $\#P < .01$ compared with Sp1 alone. C, Effect of clusterin on insulin-stimulated Sp1 transactivating activity. HepG2 cells were cotransfected with pCMV-Sp1 (Sp1) and the pGL3-Sp1 reporter [200 ng/well, (Sp1) × 4 luciferase] and the indicated amounts of a clusterin expression construct and were then stimulated with insulin for 6 hours. Luciferase activity was normalized to β-galactosidase activity. Data represent the means ± SEM of 3 independent measurements. $*P < .01$ and $**P < .001$ compared with control; $\#P < .05$ and $\#\#P < .01$ compared with Sp1 and insulin-stimulated (Sp1) × 4 luciferase. D, Effect of clusterin on insulin-stimulated Sp1 DNA-binding activity. HepG2 cells were infected with the indicated concentrations [multiplicity of infection (MOI)] of Ad-Clusterin or Ad-GFP (GFP) for 24 hours and treated with insulin for 6 hours. Competition experiments (C) were performed with an unlabeled Sp1-binding site oligomer.

pression and lipid accumulation. Transient transfection and gel shift assays suggest that clusterin inhibits SREBP-1c expression via repression of LXR and Sp1 activity. Collectively these results suggest that clusterin plays an important role in hepatic lipogenesis.

Accumulating evidence suggests that clusterin plays a role in metabolic diseases. Clusterin is a component of HDL (17) and is regulated by a variety of stimuli, including cytokines, growth factors, heat shock, radiation, and oxidants. These are associated with inflammation and oxidative stress and with the pathogenesis of metabolic diseases such as atherosclerosis, insulin resistance, and diabetes. In rodents, iv clusterin administration for 3 days after a myocardial infarction reduced myocardial infarct size and mortality without impairing wound healing (23). Our previous study showed the protective role of clusterin against neointimal hyperplasia through the inhibition of vascular smooth muscle cell proliferation (24). However, the role of clusterin in insulin resistance and diabetes is still elusive. Some studies showed that high serum clusterin levels are associated with type 2 diabetes (25, 26), whereas other studies showed that low clusterin levels in HDL are associated with insulin resistance, higher body mass index, and high triglyceride levels (27). Moreover, a clusterin gene polymorphism study showed that the at-risk clusterin genotype is increased in type 2 diabetes (28). In addition, this study uncovered a novel role for clusterin in hepatic lipogenesis. Adenovirus-mediated overexpression of clusterin inhibits its lipogenic gene expression in vivo and in vitro and attenuates lipid accumulation in the livers of HFD-fed mice, suggesting that hepatic clusterin expression may play a role in reducing the hepatic insulin resistance induced by a HFD.

SREBP-1c is important in the transcriptional regulation of hepatic lipogenesis (29). Hyperinsulinemia stimulates hepatic SREBP-1c transcription, resulting in fat synthesis and hepatic steatosis (30). Accumulating evidence shows that LXR is a potent inducer of insulin-stimulated SREBP-1c transcription. In this study, gel shift assays and reporter assays showed that the inhibition of LXR ligand-stimulated SREBP-1c expression by clusterin is mediated by LXR binding to the SREBP-1c promoter. However, a coimmunoprecipitation assay did not show the binding of clusterin to LXR (data not shown). Therefore, the exact mechanism by which clusterin inhibits LXR transcriptional activity was not elucidated in the present study, although a physical interaction between clusterin and LXR remains a possibility. In addition to LXR, accumulating evidence shows that Sp1 plays an important role in insulin-stimulated SREBP-1c expression. Indeed, our results show that insulin enhances, but clusterin decreases, SREBP-1c transcription by modulating the ability of Sp1 to transactivate at the SREBP-1c promoter. Thus, it is plausible that the effect of clusterin on hepatic SREBP-1c expression is multifactorial.

Hepatic fat accumulation can progress to nonalcoholic steatohepatitis in 10%–20% of patients (31) and even to advanced cirrhosis and hepatocellular carcinoma (32). Although several agents, including metformin, α-lipoic acid, and thiazolidinedione, have been shown to prevent hepatic steatosis in insulin-resistant animals (30, 33–35), it is not yet known whether these drugs are also effective in human subjects with nonalcoholic fatty liver disease. The present study shows that clusterin prevents hepatic steatosis induced by a HFD in animals. This raises the possibility that clusterin may be a suitable target to prevent hepatic fat accumulation in patients with insulin resistance. However, to more clearly elucidate the role that clusterin plays in the development of hepatic steatosis, it would be useful to generate animals in which clusterin expression in liver is conditionally knocked out.

Acknowledgments

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This work was supported by the National Research Foundation (Grants 2012R1A2A2A01043867, 2012R1A1A1010047, and World Class University Program Grant R32-10064 and Future-Based Technology Development Program Bio Field Grant 2011-0019514) supported by the Ministry of Education, Science, and Technology and a grant from the Korea Health Technology Research and Development Project from the Ministry of Health and Welfare, Republic of Korea (Grant A111345).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Targher G.** Non-alcoholic fatty liver disease, the metabolic syndrome and the risk of cardiovascular disease: the plot thickens. *Diabet Med*. 2007;24:1–6.
2. **Marceau P, Biron S, Hould FS, et al.** Liver pathology and the metabolic syndrome X in severe obesity. *J Clin Endocrinol Metab*. 1999; 84:1513–1517.
3. **Postic C, Girard J.** Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*. 2008;118:829–838.
4. **Deng X, Yellaturu C, Cagen L, et al.** Expression of the rat sterol regulatory element-binding protein-1c gene in response to insulin is mediated by increased transactivating capacity of specificity protein 1 (Sp1). *J Biol Chem*. 2007;282:17517–17529.
5. **Horton JD, Bashmakov Y, Shimomura I, Shimano H.** Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc Natl Acad Sci USA*. 1998;95:5987–5992.
6. **Horton JD.** Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochem Soc Trans*. 2002;30: 1091–1095.
7. **Ferre P, Foufelle F.** Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. *Diabetes Obes Metab* 2010; 12(suppl 2):83–92.
8. **Cagen LM, Deng X, Wilcox HG, Park EA, Raghow R, Elam MB.** Insulin activates the rat sterol-regulatory-element-binding protein 1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements. *Biochem J*. 2005; 385:207–216.
9. **Chen G, Liang G, Ou J, Goldstein JL, Brown MS.** Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci USA*. 2004;101:11245–11250.
10. **Schweizer M, Roder K, Zhang L, Wolf SS.** Transcription factors acting on the promoter of the rat fatty acid synthase gene. *Biochem Soc Trans*. 2002;30:1070–1072.
11. **Gelissen IC, Hochgrebe T, Wilson MR, et al** Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function? *Biochem J*. 1998;331(Pt 1): 231–237.
12. **Rosenberg ME, Silkensen J.** Clusterin: physiologic and pathophysiologic considerations. *Int J Biochem Cell Biol*. 1995;27:633–645.
13. **Gleave M, Jansen B.** Clusterin and IGFBPs as antisense targets in prostate cancer. *Ann NY Acad Sci*. 2003;1002:95–104.
14. **Wong P, Borst DE, Farber D, et al.** Increased TRPM-2/clusterin mRNA levels during the time of retinal degeneration in mouse models of retinitis pigmentosa. *Biochem Cell Biol*. 1994;72:439–446.
15. **Witte DP, Aronow BJ, Stauderman ML, et al.** Platelet activation releases megakaryocyte-synthesized apolipoprotein J, a highly abundant protein in atheromatous lesions. *Am J Pathol*. 1993;143: 763–773.
16. **Miyata M, Biro S, Kaieda H, et al.** Apolipoprotein J/clusterin is induced in vascular smooth muscle cells after vascular injury. *Circulation*. 2001;104:1407–1412.
17. **Stuart WD, Krol B, Jenkins SH, Harmony JA.** Structure and stability of apolipoprotein J-containing high-density lipoproteins. *Biochemistry*. 1992;31:8552–8559.
18. **de Silva HV, Stuart WD, Duvic CR, et al.** A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J Biol Chem*. 1990;265:13240–13247.
19. **Kim G, Kim GH, OH GS, et al.** SREBP-1c regulates glucose-stimulated hepatic clusterin expression. *Biochem Biophys Res Commun*. 2011;408:720–725.
20. **McLaughlin L, Zhu G, Mistry M, et al.** Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. *J Clin Invest*. 2000;106:1105–1113.
21. **Tarling E, Salter A, Bennett A.** Transcriptional regulation of human SREBP-1c (sterol-regulatory-element-binding protein-1c): a key regulator of lipogenesis. *Biochem Soc Trans*. 2004;32:107–109.
22. **Dif N, Euthine V, Gonnet E, Laville M, Vidal H, Lefai E.** Insulin activates human sterol-regulatory-element-binding protein-1c (SREBP-1c) promoter through SRE motifs. *Biochem J*. 2006;400: 179–188.
23. **Van Dijk A, Vermond RA, Krijnen PA, et al.** Intravenous clusterin administration reduces myocardial infarct size in rats. *Eur J Clin Invest*. 2010;40:893–902.
24. **Kim HJ, Yoo EK, Kim JY, et al.** Protective role of clusterin/apolipoprotein J against neointimal hyperplasia via antiproliferative effect on vascular smooth muscle cells and cytoprotective effect on endothelial cells. *Arterioscler Thromb Vasc Biol*. 2009;29:1558–1564.
25. **Trougakos IP, Poulakou M, Stathatos M, Chalikia A, Melidonis A, Gonos ES.** Serum levels of the senescence biomarker clusterin/apolipoprotein J increase significantly in diabetes type II and during

development of coronary heart disease or at myocardial infarction. *Exp Gerontol*. 2002;37:1175–1187.
26. **Kujiraoka T, Hattori H, Miwa Y, et al.** Serum apolipoprotein j in health, coronary heart disease and type 2 diabetes mellitus. *J Atheroscler Thromb*. 2006;13:314–322.
27. **Hoofnagle AN, Wu M, Gosmanova AK, et al.** Low clusterin levels in high-density lipoprotein associate with insulin resistance, obesity, and dyslipoproteinemia. *Arterioscler Thromb Vasc Biol*. 2010;30:2528–2534.
28. **Daimon M, Oizumi T, Karasawa S, et al.** Association of the clusterin gene polymorphisms with type 2 diabetes mellitus. *Metabolism*. 2011;60:815–822.
29. **Shimano H.** Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res*. 2001;40:439–452.
30. **Browning JD, Horton JD.** Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004;114:147–152.
31. **Liu Q, Bengmark S, Qu S.** The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Lipids Health Dis*. 2010;9:42.
32. **Cuadrado A, Orive A, Garcia-Suarez C, et al.** Non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma. *Obes Surg*. 2005;15:442–446.
33. **Mazza A, Fruci B, Garinis GA, Giuliano S, Malaguarnera R, Belfiore A.** The role of metformin in the management of NAFLD. *Exp Diabetes Res*. 2012;2012:716404.
34. **Park KG, Min AK, Koh EH, et al.** α-Lipoic acid decreases hepatic lipogenesis through adenosine monophosphate-activated protein kinase (AMPK)-dependent and AMPK-independent pathways. *Hepatology*. 2008;48:1477–1486.
35. **Chen Z, Vigueira PA, Chambers KT, et al.** Insulin resistance and metabolic derangements in obese mice are ameliorated by a novel peroxisome proliferator-activated receptor γ-sparing thiazolidinedione. *J Biol Chem*. 2012;287:23537–23548.