cAMP response element binding protein H mediates fenofibrate-induced suppression of hepatic lipogenesis

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Abstract

Aims/hypothesis Fenofibrate is a drug used to treat hyperlipidaemia that works by inhibiting hepatic triacylglycerol synthesis. Sterol regulatory element binding protein-1c (SREBP-1c) is a major regulator of the expression of genes involved in hepatic triacylglycerol synthesis. In addition, endoplasmic reticulum (ER)-bound transcription factor families are involved in the control of various metabolic pathways. Here, we show a novel function for an ER-bound transcription factor, cAMP response element binding protein H (CREBH), in fenofibrate-mediated inhibition of hepatic lipogenesis.

Methods The effects of fenofibrate and adenovirus-mediated *Crebh* (also known as *Creb313*) overexpression (Ad-*Crebh*) on hepatic SREBP-1c production and lipogenesis in vitro and in vivo were investigated. We also examined whether downregulation of endogenous hepatic *Crebh* by small interfering (si)RNA restores the fenofibrate effect on hepatic lipogenesis and SREBP-1c production. Finally, we examined the mechanism by which CREBH inhibits hepatic SREBP-1c production.

Results Fasting and fenofibrate treatment induced CREBH production and decreased SREBP-1c levels. Indeed, Ad-*Crebh* inhibited insulin- and liver X receptor agonist TO901317-induced *Srebp-1c* (also known as *Srebf1*) mRNA

expression in cultured hepatocytes. Moreover, increased production of CREBH in the liver of mice following tail-vein injection of Ad-*Crebh* inhibited high-fat diet-induced hepatic steatosis through inhibition of *Srebp-1c* expression. The inhibition of endogenous *Crebh* expression by siRNA restored fenofibrate-induced suppression of *Srebp-1c* expression and hepatic lipid accumulation both in vitro and in vivo.

Conclusions/interpretation These results show that fenofibrate decreases hepatic lipid synthesis through induction of CREBH. This study suggests CREBH as a novel negative regulator of SREBP-1c production and hepatic lipogenesis.

Keywords CREBH · Fenofibrate · Hepatic steatosis · Lipogenesis · SREBP-1c

Abbreviations

Ad	Adenovirus
ChIP	Chromatin immunoprecipitation
CREBH	Cyclic AMP response element binding protein H
ER	Endoplasmic reticulum
EMSA	Electrophoretic mobility shift assay
FA	Fatty acids
GFP	Green fluorescent protein
HFD	High-fat diet
IPGTT	Intraperitoneal glucose tolerance test
LXR	Liver X receptor
MOI	Multiplicity of infection
pfu	Plaque-forming units
PPAR α	Peroxisome proliferator-activated receptor α
SCAP	SREBP-2 chaperone
SD	Sprague-Dawley
si	Small interfering
SP1	Specific protein 1
SREBP-1c	Sterol regulatory element binding protein-1c
STZ	Streptozotocin

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Introduction

Non-alcoholic fatty liver disease, an important hepatic manifestation of metabolic syndrome, is a significant cause of morbidity and mortality in obese individuals with insulin resistance [1, 2]. The onset of fatty liver disease is characterised by increased intracellular triacylglycerol accumulation, causing a condition known as ‘fatty liver’. Sterol regulatory element binding protein-1c (SREBP-1c) is a major regulator of the expression of genes involved in hepatic triacylglycerol synthesis [3]. Its role in the development of fatty liver in obese individuals with insulin resistance is now well established. Briefly, hyperinsulinaemia, a hallmark of insulin resistance [4], stimulates hepatic SREBP-1c production, resulting in increased de novo synthesis of fatty acids (FA) [5].

The regulation of hepatic SREBP-1c production is largely dependent on nutritional or hormonal stimuli mediated by transcriptional and post-transcriptional mechanisms of gene expression. During feeding, insulin secreted from pancreatic beta cells increases SREBP-1c production through cleavage of precursor SREBP-1c into its active nuclear form and through activation of liver X receptor (LXR) and specific protein 1 (SP1) [6, 7]. Although much is known about how hepatic SREBP-1c is induced by insulin, little is known about how hepatic SREBP-1c is suppressed during fasting, when the effect of insulin is negligible. It is generally accepted that glucagon is the major inhibitor of lipogenesis during the fasting state, because of its activation of the cAMP/protein kinase A pathway [8, 9]. However, the mechanism of action of protein kinase A is not well defined [10]. Moreover, the mechanism by which the absence of insulin suppresses hepatic lipogenesis remains to be elucidated. Pharmacological agents that inhibit transcription factors regulating SREBP-1c production during insulin stimulation may provide effective treatment options for fatty liver disease.

Fenofibrate is used clinically to treat hypertriacylglycerolaemia in patients with metabolic syndrome [11]. Fenofibrate lowers serum triacylglycerol levels through several mechanisms, including increasing VLDL-triacylglycerol clearance and decreasing hepatic triacylglycerol production [12]. Hepatic VLDL production depends on the availability of FA, which is determined by de novo FA synthesis and β -oxidation of FA in the liver [13]. Fenofibrate interferes with FA synthesis and stimulates hepatic fatty acid oxidation, thereby reducing the amount of FA available to the liver for triacylglycerol synthesis [14–16]. Accumulating evidence indicates that the pharmacological actions of fenofibrate are mediated, at least in part, through the transcriptional regulation of several genes involved in lipid and lipoprotein metabolism [17, 18]. Fenofibrate activates the transcriptional factor peroxisome proliferator-activated receptor α (PPAR α), which binds to

peroxisome proliferator response elements in the regulatory regions of target genes [19]. PPAR α mediates fenofibrate-mediated regulation of triacylglycerol levels via the transcriptional induction of lipoprotein lipase [20]. However, few studies have investigated the direct role of fenofibrate in SREBP-1c transcriptional regulation during hepatic lipogenesis.

Cyclic AMP response element binding protein H (CREBH) is a membrane-bound transcription factor possessing a basic leucine zipper (bZIP) domain and a domain that resides within the endoplasmic reticulum (ER) [21, 22]. CREBH is cleaved on ER stress and activates the expression of acute response genes [23]. A recent study showed that CREBH is nutritionally regulated [24]. The expression of *Crebh* (also known as *Creb313*) mRNA and levels of CREBH protein are high in the fasting mouse liver and are suppressed by re-feeding. The authors also found that feeding-induced suppression of CREBH depends on insulin. Recently, a role for CREBH in triacylglycerol metabolism has been suggested. Genetically CREBH-deficient mice showed higher plasma triacylglycerol concentrations than wild-type mice, and this was due to inefficient triacylglycerol clearance catalysed by lipoprotein lipase [25]. More recently, Zhang et al showed that disruption of CREBH activity leads to massive accumulation of hepatic lipid metabolites and significant increases in plasma triacylglycerol levels in animals fed with an atherogenic high-fat diet (HFD) [26]. However, the role of CREBH in controlling insulin- and LXR-stimulated SREBP-1c production and hepatic lipogenesis by a pharmacological lipid-lowering agent remains to be elucidated. Here, we investigated whether CREBH mediates fenofibrate-induced suppression of hepatic lipogenesis by inhibiting insulin- and LXR-stimulated SREBP-1c production.

Methods

Materials and plasmids

Insulin was provided by Novo Nordisk (Bagsvaerd, Denmark). T0901317 and fenofibrate were purchased from Sigma (St Louis, MO, USA). The SREBP-1c antibody was purchased from BD Biosciences (San Jose, CA, USA), and the actin antibody was from Sigma. Radiochemicals (deoxycytidine 5'-[α - 32 P]triphosphate and adenosine 5'-[γ - 32 P]triphosphate) were purchased from Perkin Elmer (Boston, MA, USA). Constructs of human *SREBP-1c* (also known as *SREBF1*)-promoter-luciferase (*pSREBP-1c*[-1516/+40]-luciferase) were kind gifts from L. Cagen (University of Tennessee, TN, USA). A cDNA encoding a constitutively active form of mouse *Crebh* (also known as *Creb313*) were provided by H.-S. Choi (Chonnam National University,

Gwangju, Korea). Human *INSIG2*-promoter–reporter constructs was provided by M. Casado (Instituto de Biomedicina de Valencia, Barcelona, Spain).

Cell culture

Rat hepatoma H4IIE and human hepatoma HepG2 cells and mouse hepatocyte AML-12 cells were cultured in medium and infected with Ad-*Crebh*. For details of cell culture, refer to the electronic supplementary material (ESM) Methods.

Generation of recombinant adenovirus For details of the preparation of recombinant adenovirus, refer to the ESM Methods.

Chromatin immunoprecipitation assay To investigate the binding of protein to promoter, chromatin immunoprecipitation (ChIP) assays were done. For details of the ChIP assay, refer to the ESM Methods.

Animal experiments

Experimental procedure 1 All animal procedures were approved by the local ethics committee and carried out in accordance with institutional guidelines for animal research. Male 8-week-old Sprague-Dawley (SD) rats weighing 250 to 300 g were divided into two groups: fasting and feeding. Animals were fasted for 24 h with free access to water or fasted for 24 h and re-fed during the following 12 h. To demonstrate that feeding-induced hepatic SREBP-1c production is insulin dependent, we performed an additional experiment employing intraperitoneal administration of streptozotocin (STZ; 100 mg/kg) to eliminate endogenous insulin. STZ-treated rats were divided into two groups, fasting and feeding, and subjected to the fasting/feeding protocol described above.

Experimental procedure 2 Male 8-week-old C57BL/6 mice were purchased from Koatech (Kyunggi-Do, Korea) and divided into five groups: (1) animals fed control chow diet; (2) animals fed an HFD that provided 60% of energy as fat (D12492; Research Diets, New Brunswick, NJ, USA) alone; (3) HFD-fed group mice infected with adenoviral vector expressing *Crebh* (1×10^9 plaque-forming units [pfu]) by tail-vein injection; (4) animals fed an HFD with fenofibrate ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$); and (5) animals fed an HFD with fenofibrate ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$) and infected with adenoviral vector expressing small interfering (si)RNA *Crebh* (1×10^9 pfu) by tail-vein injection at the indicated time points. After 4 weeks, animals were killed for the collection of blood and liver samples.

Histological analysis Hepatic lipid accumulation was examined by histological analysis. See the ESM Methods.

Intraperitoneal glucose tolerance test The effect of fenofibrate on insulin sensitivity was determined by intraperitoneal glucose tolerance test (IPGTT). For details of the IPGTT, refer to the ESM Methods.

Analysis of blood samples Blood levels of glucose, insulin, triacylglycerol and NEFA were measured in overnight-fasted mice. For details of the analyses, refer to the ESM Methods.

Genetic analyses in cells and tissues

Quantitative real-time RT-PCR To quantify the DNA in cells and tissues, quantitative real-time RT-PCR was used. For details, refer to the ESM Methods.

Northern blot analysis Northern blot analysis was used to measure mRNA expression. For details, refer to the ESM Methods.

Western blot analysis Protein levels in cells and tissues were examined by western blot analysis. For details of western blotting procedures, refer to the ESM Methods.

In vitro transient transfections and reporter assays Promoter activity was determined by in vitro transfection and luciferase reporter assays. For details, refer to the ESM Methods.

Electrophoretic mobility shift assay DNA-binding activity was measured using an electrophoretic mobility shift assay (EMSA). For details, refer to the ESM Methods.

Statistical analysis

Data were evaluated using ANOVA followed by a post-hoc least-significant-difference test, and expressed as the mean $\pm$ SEM. Values of $p < 0.05$ were considered statistically significant. All experiments were repeated independently three or more times.

Results

Fasting and fenofibrate treatment increase *Crebh* expression but decrease *Srebp-1c* expression

Insulin is one of the most important hormones involved in regulating hepatic lipogenesis. In cultured hepatocytes, insulin increases lipogenesis primarily through the induction

of SREBP-1c [27]. In addition, re-feeding after restriction of energy intake increases lipogenesis and SREBP-1c production in the liver [28]. To demonstrate that CREBH production is influenced by insulin, we analysed hepatic *Crebh* expression during fasting and after re-feeding of control and STZ-induced diabetic rats. Re-feeding after fasting dramatically decreased hepatic *Crebh* mRNA expression and increased *Srebp-1c* mRNA expression in control rats (Fig. 1a). However, after eliminating endogenous insulin by STZ, re-feeding did not suppress *Crebh* mRNA expression, suggesting that re-feeding-induced suppression of *Crebh* mRNA expression is mainly mediated by insulin (Fig. 1b). In addition, no feeding-induced changes in *Srebp-1c* mRNA expression were observed in the livers of

Fig. 1 Fasting and fenofibrate treatment increase *Crebh* expression but decrease *Srebp-1c* expression. **(a)** Representative northern blot analysis of hepatic *Crebh* and *Srebp-1c* mRNA expression in the livers of normal SD rats undergoing fasting and re-feeding. Animal experiments were performed as described in the Methods. **(b)** Representative northern blot analysis of hepatic *Crebh* and *Srebp-1c* mRNA expression in STZ-treated SD rats with fasting and re-feeding. **(c)** Representative northern blot analysis of *Crebh* mRNA expression in the presence of insulin. H4IIE cells were incubated with the indicated concentrations of glucose for 24 h, with or without treatment by incubation with 100 nmol/l insulin for 6 h. Data represent the mean $\pm$ SE of three independent measurements; *** p <0.001 vs glucose alone. **(d)** Representative northern blot analysis of *Crebh* and *Srebp-1c* mRNA expression in the presence of fenofibrate. H4IIE cells were incubated with fenofibrate (100 μ mol/l) for the indicated times. Data represent the mean $\pm$ SE of three independent measurements. $^{\dagger\dagger}p$ <0.01 and $^{\dagger\dagger\dagger}p$ <0.001 vs control. Black bar, *Crebh*; white bar, *Srebp-1c*. *G6pase* is also known as *G6pc*; *Pepck* is also known as *Pck1*

STZ-treated rats (Fig. 1b). The expression patterns of *Srebp-1c* were opposite to those of *Crebh*. The effect of insulin on *Crebh* expression was further confirmed in H4IIE cells. Glucose concentration did not influence *Crebh* mRNA expression, and insulin treatment significantly reduced *Crebh* mRNA expression (Fig. 1c). Collectively, these data suggest that insulin is a major positive regulator of hepatic SREBP-1c production, but a negative regulator of CREBH production. Because fenofibrate is used clinically for lowering hepatic triacylglycerol synthesis, which is induced by hyperinsulinaemia, we evaluated the effect of fenofibrate on CREBH and SREBP-1c production in H4IIE cells. Interestingly, fenofibrate increased *Crebh* mRNA expression but decreased *Srebp-1c* mRNA expression (Fig. 1d). Accordingly, fenofibrate decreased the expression of SREBP-1c target genes, including acetyl-CoA carboxylase (*Acc*), fatty acid synthase (*Fas* [also known as *Fasn*]), and stearoyl-CoA desaturase (*Scd* [also known as *Scd1*]) (ESM Fig. 1a). Moreover, siRNA knockdown of CREBH prevented the inhibitory effect of fenofibrate on *Srebp-1c* expression (ESM Fig. 1b). Taken together with the results of the insulin analyses, fenofibrate increases CREBH production but decreases SREBP-1c production, which suggests that *Srebp-1c* may be a CREBH target gene and that CREBH mediates the effects of fenofibrate on hepatic lipogenesis through inhibition of SREBP-1c production induced by hyperinsulinaemia.

CREBH inhibits insulin- and LXR-stimulated SREBP-1c production in cultured hepatocytes

To elucidate the association between CREBH and insulin-induced hepatic lipogenesis, we examined whether adenovirus-mediated overexpression of *Crebh* inhibits insulin-stimulated SREBP-1c production in cultured hepatocytes. As shown in Fig. 2a,b, insulin treatment increased *Srebp-1c* mRNA expression and precursor and nuclear SREBP-1c protein production. However, insulin-stimulated *Srebp-1c* mRNA expression and SREBP-1c protein production were inhibited by Ad-*Crebh* in a dose-dependent manner. Accordingly, Ad-*Crebh* inhibited the insulin-stimulated expression of SREBP-1c-target-gene mRNA (*Acc*, *Fas* and *Scd*; Fig. 2c, d, e). Transient transfection with the human *SREBP-1c* promoter reporter [29] in HepG2 cells showed that CREBH inhibits insulin-stimulated *SREBP-1c* mRNA expression at the transcriptional level (Fig. 2f). Next, to determine if CREBH directly binds to the *SREBP-1c* promoter to inhibit its activity, we performed a ChIP assay of the putative CREBH binding sites [30] (–400/–394 and –318/–312) in the *SREBP-1c* promoter. CREBH did not bind to these putative binding sites (data not shown). However, it is possible that CREBH directly binds to other regions of *SREBP-1c* promoter that were not examined in this study.

Fig. 2 CREBH inhibits insulin-stimulated SREBP-1C production in cultured hepatocytes. **(a)** Representative northern blot and **(b)** western blot analysis of CREBH on insulin-stimulated *Srebp-1c* mRNA expression and SREBP-1c protein production in H4IIE cells. Cells were infected with the indicated doses (multiplicity of infection [MOI]) of Ad-Crebh or Ad-null for 24 h and treated with insulin (100 nmol/l) for 6 h. The 'p' and 'n' denote the precursor and cleaved nuclear forms of SREBP, respectively. **(c–e)** Representative real-time RT-PCR analysis of **(c)** *Acc*, **(d)** *Fas* and **(e)** *Scd* mRNA expression in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-Crebh or Ad-null for 24 h and treated with insulin (100 nmol/l) for 6 h. Data represent the mean $\pm$ SE of three independent measurements. * p <0.05 and ** p <0.01 vs control; † p <0.05 and †† p <0.01 vs insulin alone. **(f)** The effect of CREBH on insulin-stimulated *SREBP-1c* promoter activity. HepG2 cells were cotransfected with p*SREBP-1c* (−1516/+40)–luciferase construct (200 ng/well) and the indicated amounts of an expression vector for *Crebh*, and then stimulated with insulin for 6 h. Data represent the mean $\pm$ SE of three independent measurements. *** p <0.001 vs control; †† p <0.01 and ††† p <0.001 vs insulin alone

LXR plays a major role in regulating SREBP-1c induction, especially on insulin stimulation [29]. Thus, we examined whether CREBH inhibits LXR-stimulated SREBP-1c production. Indeed, Ad-Crebh inhibited TO901317-stimulated *Srebp-1c* mRNA expression and SREBP-1c protein production in a dose-dependent manner (Fig. 3a, b). Ad-Crebh also suppressed TO901317-stimulated *Acc*, *Fas* and *Scd* mRNA expression (Fig. 3c, d, e). To examine the mechanism underlying CREBH-mediated inhibition of LXR activity, we first tested whether Ad-Crebh influenced the abundance of LXR protein. Western blot analysis showed that Ad-Crebh did not decrease LXR protein production (ESM Fig. 2). Next, we examined whether CREBH influences LXR transcriptional activity on the *SREBP-1c*

promoter. Transient transfection with a promoter reporter construct showed that TO901317 induced a marked increase in *SREBP-1c* promoter activity, which was inhibited by *Crebh* (Fig. 3f). Moreover, TO901317 induced an increase in the DNA-binding activity of LXR, which was inhibited by Ad-Crebh in a dose-dependent manner (Fig. 3g). To further confirm that CREBH inhibits LXR binding to LXR response element within the *SREBP-1c* promoter, we performed a ChIP assay. In agreement with the EMSA assay, the results showed that TO901317 induced binding of LXR to the proximal region, which contains two LXR-binding sites. Ad-Crebh abolished the DNA-binding activity of LXR to the *SREBP-1c* promoter (Fig. 3h).

Fenofibrate inhibits SREBP-1c production through induction of CREBH production

Next, we examined whether fenofibrate inhibits SREBP-1c production and whether fenofibrate-induced CREBH production mediates SREBP-1c production. Treatment with fenofibrate for 24 h dose-dependently attenuated insulin- or TO901317-stimulated *Srebp-1c* mRNA expression (Fig. 4a, b) and SREBP-1c protein production (Fig. 4c, d) in H4IIE cells. Fenofibrate also suppressed insulin- and TO901317-stimulated *Acc*, *Fas* and *Scd* mRNA expression (ESM Fig. 3). The induction of *Crebh* expression by fenofibrate was then examined by real-time RT-PCR (ESM Fig. 3). To further confirm whether the suppression of *Srebp-1c* gene expression by fenofibrate is mediated by CREBH, we down-regulated endogenous *Crebh* expression by transfecting H4IIE cells with siRNA *Crebh* and then treated them with fenofibrate. siRNA *Crebh*, but not control siRNA, significantly restored fenofibrate-induced suppression of *Srebp-1c* expression stimulated by TO901317 (Fig. 4e). Because fenofibrate induces fatty acid oxidation [31], we investigated whether CREBH mediates the expression of genes related to fenofibrate-induced fatty acid oxidation. The expression of peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 α (*Pgc-1 α* [also known as *Ppargc1a*]), carnitine palmitoyltransferase 1 (*Cpt-1* [also known as *Cpt1b*]), and *Ppara* (also known as *Ppara*) was modestly increased by Ad-Crebh (ESM Fig. 4a). However, fenofibrate markedly increased the expression of these genes (ESM Fig. 4b). Moreover, knockdown of CREBH by siRNA induced a ~25 % reduction in fenofibrate induction of *Pgc-1 α* and *Cpt-1* expression, although it significantly blocked fenofibrate-induced *Ppara* expression (ESM Fig. 4b).

Fenofibrate and Ad-Crebh prevent insulin-induced inhibition of *Insig2* expression

Because insulin-stimulated transcriptional activity of SREBP-1c on *Srebp-1c* gene expression is mediated by downregulation

Fig. 3 CREBH inhibits TO901317-stimulated SREBP-1c production and LXR activity. **(a)** Representative northern blot and **(b)** western blot analysis of the effect of CREBH on TO901317-stimulated *Srebp-1c* mRNA expression and SREBP-1c protein production in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-Crebh or Ad-null for 24 h and treated with TO901317 (1 μmol/l) for 24 h. The ‘p’ and ‘n’ denote the precursor and cleaved nuclear forms of SREBP-1c, respectively. **(c–e)** Representative real-time RT-PCR analysis of *Acc*, *Fas* and *Scd* mRNA expression in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-Crebh or Ad-null (100 MOI) for 24 h and treated with TO901317 (1 μmol/l) for 24 h. Data represent the mean±SE of three independent measurements. ** $p<0.01$ vs control; †† $p<0.01$ vs TO901317 alone. **(f)** The effect of CREBH on TO901317-

stimulated *SREBP-1c* promoter activity. HepG2 cells were cotransfected with *SREBP-1c* promoters (200 ng/well) and the indicated amounts of an expression vector for *Crebh* and then stimulated with TO901317 (1 μmol/l) for 24 h. Data represent the mean±SE of three independent measurements. *** $p<0.001$ vs control; ††† $p<0.001$ vs TO901317 alone. **(g)** The effect of CREBH on TO901317-stimulated LXR-DNA binding activity. HepG2 cells were infected with the indicated doses (MOI) of Ad-Crebh or Ad-null for 24 h and treated with TO901317 (1 μmol/l) for 24 h. **(h)** A ChIP assay was performed using an anti-LXRα antibody. HepG2 cells were infected with the indicated MOI of Ad-Crebh or Ad-null (100 MOI) for 24 h and treated with TO901317 (1 μmol/l) for 24 h. C, control; IP, immunoprecipitation target

of *Insig2* levels, we examined whether fenofibrate and CREBH prevent insulin-suppressed *Insig2* expression. As shown Fig. 5a, insulin and Ad-Crebh did not influence *Insig1* mRNA expression but fenofibrate markedly increased *Insig1* expression; INSIG1 is dissociated from SREBP-2 chaperone (SCAP)–SREBP-2 under the conditions of sterol depletion, consequently causing SCAP–SREBP-2 complex transportation from the ER to the Golgi. In contrast, insulin decreased but fenofibrate and Ad-Crebh increased *Insig2* mRNA expression levels (Fig. 5b). Therefore, we further examined whether siRNA knockdown of endogenous INSIG2 blocked the suppression of *Srebp-1c* expression induced by CREBH and

fenofibrate. The results showed that *Insig2* siRNA markedly inhibited endogenous *Insig2* expression, and knockdown of INSIG2 significantly abolished Ad-Crebh- and fenofibrate-mediated suppression of insulin-stimulated *Srebp-1c* expression (Fig. 5c, d). To determine whether expression of *Insig2* was transcriptionally regulated by CREBH, we next examined the effects of CREBH on *INSIG2* promoter activity. Indeed, a transient transfection study with *INSIG2* promoter luciferase [32] showed that *Crebh* markedly increased *INSIG2* promoter (–859/–11 construct) activity, but this activation was markedly attenuated by truncation at –688 bp of the *INSIG2* promoter, suggesting that CREBH mainly acts on the region between

Fig. 4 Fenofibrate inhibition of SREBP-1c production is partially mediated by CREBH. (**a,b**) Representative northern blot and (**c,d**) western blot analysis of the effect of fenofibrate on insulin (**a, b**) or TO901317 (**c,d**)-stimulated *Srebp-1c* mRNA expression and SREBP-1c protein production in H4IIE cells. Cells were incubated with insulin (100 nmol/l) for 6 h or TO901317 (1 μmol/l) for 24 h, with or without pretreatment with the incubated concentrations of fenofibrate for 24 h. The 'p' and 'n' denote the precursor and cleaved nuclear forms of SREBP-1c, respectively. (**e**) Representative northern blot analysis of siRNA *Crebh* on the fenofibrate decreased TO901317-stimulated *Srebp-1c* mRNA expression in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-siRNA *Crebh* or Ad-siRNA control for 24 h and then treated with fenofibrate (100 μmol/l) and insulin for 6 h or TO901317 (1 μmol/l) for 24 h. *** $p < 0.001$ vs control; ††† $p < 0.001$ vs TO901317 alone; † $p < 0.05$ and †† $p < 0.01$ vs TO901317 plus fenofibrate with or without control siRNA. siCrebh, siRNA *Crebh*; siCon, Ad-siRNA control

–859 and –688 of the *INSIG2* promoter to activate *INSIG2* promoter activity (ESM Fig. 5). These results suggest that fenofibrate and CREBH retained SREBP-1c in the ER membrane. However, in addition to the increase in *Insig1* expression, the effect of fenofibrate on *INSIG2* production was much greater than that of Ad-*Crebh*, suggesting a CREBH-independent pathway.

CREBH reduces hepatic lipid accumulation and SREBP-1c production and mediates fenofibrate inhibition of hepatic lipid accumulation in HFD-fed mice

Finally, we examined whether CREBH decreases hepatic lipogenesis and mediates fenofibrate-suppressed hepatic lipogenesis in HFD-fed mice. Histological examination showed that hepatocytes of HFD-fed mice were distended by large cytoplasmic lipid droplets. This change in cellular morphology was nearly completely prevented by the overexpression of *Crebh* and fenofibrate treatment (Fig. 6a). Both the overexpression of *Crebh* and fenofibrate treatment

Fig. 5 Fenofibrate and Ad-*Crebh* inhibit insulin-induced inhibition of *Insig2* expression. (**a**) Representative real-time RT-PCR analysis of *Insig1* mRNA expression in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-*Crebh* or incubated concentrations of fenofibrate for 24 h and treated with insulin (100 nmol/l) for 6 h. Data represent the mean $\pm$ SE of three independent measurements. * $p < 0.05$ and ** $p < 0.01$ vs insulin alone. (**b**) Representative real-time RT-PCR analysis of *Insig2* mRNA expression in H4IIE cells. Cells were infected with the indicated doses (MOI) of Ad-*Crebh* or incubated concentrations of fenofibrate for 24 h and treated with insulin (100 nmol/l) for 6 h. Data represent the mean $\pm$ SE of three independent measurements. †† $p < 0.01$ vs control; *** $p < 0.001$ vs insulin alone. (**c, d**) Representative real-time RT-PCR analysis of *Insig2* (**c**) and *Srebp-1c* (**d**) mRNA expression. Cells were transfected with siRNA *Insig2* or control scramble siRNA for 24 h and infected with the indicated doses (MOI) of Ad-*Crebh*, or incubated with the indicated concentrations of fenofibrate for 24 h and then treated with insulin (100 nmol/l) for 6 h. Data represent the mean $\pm$ SE of three independent measurements. * $p < 0.05$ and ** $p < 0.01$ vs insulin alone; † $p < 0.05$ vs control; ‡ $p < 0.05$ vs insulin plus Ad-*Crebh* with control scramble siRNA; § $p < 0.05$ vs insulin plus fenofibrate with control scramble siRNA. White bars, control; black bars, Ad-*Crebh*; grey bars, fenofibrate. si-Control, control scramble siRNA; si-*Insig2*, siRNA *Insig2*

significantly decreased hepatic fat accumulation and plasma triacylglycerol levels compared with mice infected with an adenovirus encoding green fluorescent protein (GFP; Fig. 6b, c). Moreover, downregulation of endogenous *Crebh* by Ad-siRNA *Crebh* significantly blocked fenofibrate-suppressed hepatic lipid accumulation and plasma triacylglycerol levels in HFD-fed mice (Fig. 6b,c). Consistent with the histological examination, both Ad-*Crebh* and fenofibrate reduced *Srebp-1c* expression but increased *Insig2* expression (Fig. 6d, e). Moreover, the effects of fenofibrate on hepatic *Srebp-1c* and *Insig2* expression were abolished by Ad-siRNA *Crebh* (Fig. 6d, e). Fenofibrate treatment increased *Crebh* expression, but Ad-siRNA *Crebh* suppressed fenofibrate-induced *Crebh* expression to basal levels (Fig. 6f). Because CREBH controls the expression of genes involved in gluconeogenesis, we wanted to know whether fenofibrate treatment influences other metabolic variables.

Fig. 6 CREBH reduces hepatic lipid accumulation and SREBP-1c production and mediates fenofibrate inhibition of hepatic lipid accumulation in HFD-fed mice. **(a)** Histological analysis of liver sections from HFD-fed mice with or without fenofibrate treatment by tail-vein injection of the adenovirus encoding the active form of *Crebh*, *gfp*, siRNA *Crebh* and siRNA control. Haematoxylin and eosin staining and Oil Red O staining were used. Original magnification $\times 200$. **(b)** Morphometric analysis of Oil Red O staining in liver sections from HFD-fed mice with or without fenofibrate treatment by tail-vein injection of the adenovirus encoding the active form of CREBH or GFP, siRNA *Crebh* or siRNA control. The data represent the mean $\pm$ SE of three independent measurements ($n=6$ in each group). *** $p<0.001$ vs control-diet-fed mice; ** $p<0.01$ and *** $p<0.001$ vs HFD-fed mice;

*** $p<0.001$ vs HFD-fed mice with fenofibrate treatment and siRNA control. **(c)** Analysis of serum triacylglycerol in HFD-fed mice with or without fenofibrate treatment after tail-vein injection of an adenovirus encoding the active forms of CREBH or GFP, siRNA *Crebh* or siRNA control. * $p<0.05$ vs mice fed control diet; † $p<0.05$ and *** $p<0.001$ vs HFD-fed mice; ‡ $p<0.05$ vs HFD-fed mice with fenofibrate treatment and siRNA control. **(d–f)** Representative real-time RT-PCR analysis of the effect of Ad-*Crebh* on *Srebp-1c*, *Insig2* and *Crebh* expression. The data represent the mean $\pm$ SE of three independent measurements ($n=6$ in each group). * $p<0.05$ and ** $p<0.01$ vs control-diet-fed mice; †† $p<0.01$ vs HFD-fed mice; ‡ $p<0.05$ vs HFD-fed mice with fenofibrate treatment and siRNA control. Feno, fenofibrate; H&E, haematoxylin and eosin; siC, siRNA control

Plasma glucose, insulin and NEFA levels were lower and insulin sensitivity (IPGTT) was higher in the fenofibrate-treated group than in the control HFD group. Serum cholesterol levels were not different between the groups (ESM Fig. 6).

Discussion

In the present study, we found that fasting and fenofibrate treatment induced CREBH production, while feeding and insulin treatment decreased CREBH production. Interestingly, production of SREBP-1c, which was upregulated by feeding but inhibited by fenofibrate, showed the opposite effects to those of CREBH. Fenofibrate treatment and adenovirus-mediated overexpression of *Crebh* inhibited

insulin- and LXR agonist TO901317-stimulated *Srebp-1c* mRNA expression in cultured hepatocytes. Moreover, overexpression of *Crebh* in the liver of mice mediated by tail-vein injection of Ad-*Crebh* inhibited hepatic steatosis through inhibition of SREBP-1c induction. The inhibition of endogenous *Crebh* expression by siRNAs markedly abolished fenofibrate-induced suppression of *Srebp-1c* expression and hepatic lipid accumulation. These results show that fenofibrate decreases hepatic lipid synthesis through induction of CREBH. Collectively, this study suggests CREBH as a novel negative regulator of hepatic lipogenesis.

Hepatic lipogenesis is regulated by nutritional status [33]. In mammals, fasting decreases and re-feeding increases hepatic lipid synthesis [29]. Insulin is a potent inducer of hepatic lipogenesis during feeding [7, 29], and this may be transcriptionally mediated by SREBP-1c [28, 34, 35]. In the

present study, while *Crebh* expression was downregulated by feeding, this downregulation was not observed when endogenous insulin production was eliminated by STZ treatment. Moreover, in cultured hepatocytes, *Crebh* expression was inhibited by insulin treatment. These data indicate that feeding-induced suppression of *Crebh* expression is regulated by insulin. The expression patterns of insulin-regulated CREBH were opposite to those of SREBP-1c, suggesting that CREBH plays a role in the regulation of SREBP-1c production and hepatic lipogenesis. Indeed, we found that adenovirus-mediated overexpression of *Crebh* inhibited insulin-stimulated SREBP-1c production.

The role of SREBP-1c in the development of fatty liver in the insulin-resistant state is now well established [36]. Hyperinsulinaemia, a hallmark of insulin resistance, stimulates hepatic *Srebp-1c* transcription, resulting in increased hepatic fat synthesis and the development of fatty liver. We [37] and others [38] demonstrated the contribution of SREBP-1c to fat accumulation in the insulin-resistant HFD-induced mouse liver [39]. Many studies have focused on searching for molecules that can inhibit SREBP-1c production, thereby preventing the development of fatty liver. For example, metformin and α -lipoic acid prevent hepatic steatosis by inhibiting SREBP-1c production [29, 40]. Fenofibrate has beneficial effects on metabolic syndrome by downregulating multiple target genes involved in the pathogenesis of hepatic steatosis [41–43]. Recent studies show that fenofibrate induces CREBH production to a level comparable with that observed under fasting conditions [44]. However, the exact molecular mechanism underlying the anti-hepatic steatosis effect of fenofibrate remains unclear. Thus, we aimed to determine the role of fenofibrate-induced CREBH production in the development of hepatic steatosis in mice with HFD-induced insulin resistance. This study showed that adenovirus-mediated overexpression of *Crebh* in the liver or treatment of fenofibrate prevented HFD-induced hepatic lipid accumulation and inhibited SREBP-1c production. Moreover, inhibition of endogenous *Crebh* expression by siRNA prevented fenofibrate-induced suppression of hepatic steatosis, suggesting that CREBH mediates the effects of fenofibrate on hepatic lipogenesis and is a potential therapeutic target for the treatment of hepatic steatosis induced by hyperinsulinaemia.

Insulin-induced SREBP-1c production is regulated at the transcriptional level. The major regulators of *Srebp-1c* expression are LXR and SREBP-1c itself. LXR is a member of a nuclear receptor superfamily that regulates the production of key proteins involved in lipid metabolism [45, 46]. LXR increases *Srebp-1c* expression, leading to increased hepatic triacylglycerol synthesis [29]. In our study, fenofibrate and Ad-*Crebh* inhibited LXR-ligand-stimulated SREBP-1c production. An in vitro transient transfection study employing promoter–reporter constructs showed that inhibition of LXR

ligand-stimulated SREBP-1c production by CREBH is mediated via the inhibition of LXR activity on the *SREBP-1c* promoter. In addition to LXR, in response to insulin, SREBP-1c production is also mediated by the cleaved nuclear form of SREBP-1c itself, which mediates transactivation on the *SREBP-1c* promoter [33]. A study by Yellaturu et al clearly shows that insulin enhances SREBP-1c production by inducing cleavage of precursor SREBP-1c to its nuclear form [34]. In the resting state, the SCAP–SREBP-1c complex is localised to the ER by INSIG2. However, in response to stimuli, INSIG2 dissociates from the SCAP–SREBP-1c complex, thereby facilitating transport of the complex to the Golgi where immature SREBP-1c is cleaved to generate the active form of SREBP-1c [34]. Insulin enhances the rate of turnover of *Insig2* mRNA, and insulin-induced depletion of *Insig2* promotes proteolysis of SREBP-1c, resulting in active SREBP-1c. Our data show that Ad-*Crebh* and fenofibrate prevent insulin-suppressed *Insig2* gene expression. Moreover, knockdown of INSIG2 expression by siRNA abolished Ad-*Crebh*- and fenofibrate-mediated suppression of SREBP-1c. Fenofibrate, however, restored insulin-suppressed *Insig2* gene expression to a higher level than did CREBH, suggesting a CREBH-independent mechanism. Taken together, the mechanisms by which CREBH influences hepatic lipogenesis and SREBP-1c production are multifactorial. Our data suggest that CREBH mediates fenofibrate-suppressed hepatic lipogenesis by inhibiting LXR-DNA binding to the *SREBP-1c* promoter and inducing INSIG2-mediated inactivation of SREBP-1c.

As fenofibrate induces the expression of genes involved in fatty acid oxidation by activating PPAR α , induction of these genes is thought to be important for the amelioration of hepatic lipid metabolism. Accordingly, the present study showed that fenofibrate markedly increased the expression of genes involved in fatty acid oxidation. However, we found that although Ad-*Crebh* modestly increased the expression of these genes, the effect was much smaller than that of fenofibrate. Moreover, knockdown of CREBH production with siRNA did not significantly abolish the fenofibrate-mediated induction of these genes, although a reduction in *Crebh* expression by siRNA tended to decrease the effects of fenofibrate on gene expression. These data suggest that CREBH-mediated effects of fenofibrate-induced inhibition of hepatic steatosis are mainly due to the inhibition of de novo lipogenesis and not to the augmentation of fatty acid oxidation, although CREBH increases the expression of genes responsible for fatty acid oxidation.

In summary, these data indicate a novel role for CREBH in inhibiting hepatic lipogenesis by fenofibrate. Hepatic steatosis is emerging as the most important cause of chronic liver disease associated with the increasing incidence of

obesity. Hepatic steatosis can progress to non-alcoholic steatohepatitis, as well as to advanced cirrhosis. Thus, an effective therapy is needed to prevent this condition. In the present study, we showed that fenofibrate-induced CREBH prevents hepatic steatosis in animals with diet-induced insulin resistance. This suggests that CREBH may serve as a therapeutic target for preventing the development of fatty liver disease in patients with insulin resistance. To more clearly elucidate the role of CREBH in hepatic lipid synthesis, it would be useful to generate animals in which *Crebh* expression in the liver is conditionally knocked out.

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