THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 45, pp. 38041–38049, November 2, 2012

Activation of Cannabinoid Receptor Type 1 (*Cb1r*) Disrupts Hepatic Insulin Receptor Signaling via Cyclic AMP-response Element-binding Protein H (*Crebh*)-mediated Induction of *Lipin1* Gene*

Received for publication, May 2, 2012, and in revised form, September 14, 2012 Published, JBC Papers in Press, September 18, 2012, DOI 10.1074/jbc.M112.377978

Dipanjan Chanda[‡1], Yong-Hoon Kim[§1], Don-Kyu Kim[‡1], Min-Woo Lee[¶], Su-Yeon Lee[‖], Tae-Sik Park[‖], Seung-Hoi Koo[¶], Chul-Ho Lee[§2], and Hueng-Sik Choi[‡3]**

*From the [‡]National Creative Research Initiatives Center for Nuclear Receptor Signals, Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea, **Research Institute of Medical Sciences, Department of Biomedical Sciences, Chonnam National University Medical School, Gwangju 501-746, Republic of Korea, [§]Animal Model Center, Korea Research Institute of Bioscience and Biotechnology, University of Science and Technology, Daejeon 305-806, Republic of Korea, [¶]Department of Molecular Cell Biology and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Republic of Korea, and [‖]Department of Life Science, Gachon University, Sungnam 461-701, Republic of Korea*

Background: The role of *Cb1r* signaling-mediated activation of *Crebh* in regulating lipid metabolism and insulin signaling is currently unknown.
Results: *Cb1r* signaling regulates *Lipin1* gene transcription via *Crebh* and inhibits insulin receptor signaling.
Conclusion: *Crebh*-mediated activation of *Lipin1* by *Cb1r* deregulates insulin receptor signaling.
Significance: Relieving higher hepatic DAG levels by targeting its upstream regulators might be beneficial to restore insulin signaling.

Activation of hepatic cannabinoid 1 receptor (*Cb1r*) signaling has been implicated in the development of phenotypes associated with fatty liver, hypertriglyceridemia, and insulin resistance. In the current study, we have elucidated the critical role of endoplasmic reticulum-bound transcription factor cyclic AMP-response element-binding protein H (*Crebh*) in mediating activated *Cb1r* signaling in inducing phosphatidic acid phosphatase *Lipin1* gene expression and subsequently deregulating hepatic insulin receptor signaling. *Cb1r* agonist (2-arachidonoylglycerol (2-AG)) treatment induced *Lipin1* gene expression in a *Crebh*-dependent manner via recruiting CREBH to the endogenous *Lipin1* gene promoter. Adenoviral overexpression of *Crebh* or 2-AG treatment in mice induced *Lipin1* gene expression to increase the hepatic diacylglycerol (DAG) level and phosphorylation of protein kinase Cϵ (PKCϵ). This in turn inhibited hepatic insulin receptor signaling. Knockdown of *Crebh* or *Cb1r* antagonism attenuated 2-AG-mediated induction of *Lipin1* gene expression and decreased DAG production in mouse liver and subsequently restored insulin receptor signaling. Similarly, knockdown of *Lipin1* attenuated the 2-AG-induced increase in the DAG level and PKCϵ phosphorylation. Finally, shRNA-mediated knockdown of *Crebh* partially but significantly blunted *Lipin1* expression and the DAG level in *db*/*db* mice. These results demonstrate a novel mechanism by which *Cb1r* signaling induces *Lipin1* gene expression and increases DAG production by activating *Crebh*, thereby deregulating insulin receptor signaling pathway and lipid homeostasis.

The endocannabinoid system comprises the cannabinoid receptors type 1 (*Cb1r*),[4] which is expressed at high levels in the brain but is also present at much lower concentrations in peripheral tissues, and *Cb2r*, which is expressed predominantly in immune and hematopoietic cells (1). Arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG) are endocannabinoids and lipid mediators that activate these receptors. Various studies in rodent models (2) and patients (3, 4) have provided evidence of hyperactivity of the endocannabinoid system and its correlation to obesity due to impaired energy balance. Mice deficient in *Cb1r* are resistant to diet-induced obesity and steatosis, whereas in wild-type mice,

* This work was supported by the Future-based Technology Development Program (BIO Fields) through the Korea Research Foundation funded by the Ministry of Education, Science and Technology (Grant 2010-0019512) (to H.-S. C), a National Creative Research Initiatives grant funded by the Korean Ministry of Education, Science and Technology (Grant 20110018305) (to H.-S. C), and the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program of Korea (to C.-H. L.).
[1] These authors contributed equally to this work.
[2] To whom correspondence may be addressed. Tel.: 82-42-860-4637; Fax: 82-42-860-4609; E-mail: chullee@kribb.re.kr.
[3] To whom correspondence may be addressed: National Creative Research Initiatives Center for Nuclear Receptor Signals, Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea. Tel.: 82-62-530-0503; Fax: 82-62-530-0506; E-mail: hsc@chonnam.ac.kr.

[4] The abbreviations used are: CB1R, cannabinoid receptor type 1; CREBH, cAMP-response element binding protein H; 2-AG, 2-arachidonoyl glycerol; DAG, diacylglycerol; USi, unspecific RNAi; Ad, adenovirus; CB2R, cannabinoid receptor type 2; qPCR, quantitative PCR; ATF, activating transcription factor; CREBHi, *Crebh* RNAi; IRS1, insulin receptor substrate 1; *Srebp1c*, sterol response element-binding protein 1c; *GPAT*, acyl-CoA:glycerol-*sn*-3-phosphate acyltransferase; *Cpt1*, carnitine palmitoyltransferase 1; *Ucp2*, uncoupling protein 2; *Cd36*, cluster of differentiation 36; *Dgat*, acyl-CoA, diacylglycerol acyltransferase.

chronic treatment with *Cb1r* antagonists reverses these diet-induced effects (5). Both genetically and diet-induced obese animal models show elevated levels of endocannabinoids in the hypothalamus and peripheral tissues (1, 2, 5). Recently, using a liver-specific *Cb1r* knock-out mouse model, it was demonstrated that peripheral *Cb1r* could be selectively targeted for the treatment of fatty liver, impaired glucose homeostasis, and dyslipidemia to reduce the neuropsychiatric side effects of nonselective *Cb1r* signaling blockade in treatment of obesity-associated conditions (5). Overall, both clinical (3, 4, 6) and animal data regarding the *Cb1r* blockade (2, 5) overwhelmingly suggest the beneficial actions of *Cb1r* antagonism on lipid metabolism and insulin receptor signaling.

Endoplasmic reticulum-bound liver-specific transcription factor cyclic AMP-response element-binding protein H (CREBH) has been reported to be induced by the acute inflammatory response-inducing factor lipopolysaccharide and pro-inflammatory cytokines interleukin-6 and tumor necrosis factor α (7). Previously, we have reported that *Crebh* plays an important mediatory role in hormonal regulation of hepatic gluconeogenesis under fasting or insulin-resistant conditions in rodent liver and demonstrated that *Crebh* is dramatically induced and activated in diet-induced obese rodent models (8). In a recent study, we found that *Cb1r* signaling activates *Crebh* via the c-Jun N-terminal kinase pathway and elucidated the molecular mechanism involved in *Cb1r* signaling-mediated regulation of hepatic gluconeogenesis (9). *Crebh* has also been demonstrated to be involved in the regulation of hepatic iron (10) and triglyceride metabolism (11). *Crebh* gene expression is known to be regulated by nuclear receptors HNF4α and peroxisome proliferator-activated receptor α as well as fatty acids, thereby suggesting a possible role of *Crebh* in fatty acid metabolism (12, 13). Because of its liver-specific expression and stress sensor activation, *Crebh* is emerging as a key player in regulating various hepatic metabolic pathways.

Lipin1 plays a crucial role in lipid metabolism in adipose tissue, skeletal muscle, and liver. *Lipin1* was identified as a cytosolic phosphatidic acid phosphatase that generates DAG in response to an increase in intracellular free fatty acid levels (14). Interestingly, *Lipin1*-deficient mice have a reduced adipose tissue mass, mild hyperglycemia, and insulin resistance, whereas enhanced *Lipin1* expression in the adipose tissue or skeletal muscle of transgenic mice leads to obesity (15). *Lipin1*-deficienct mice display lipodystrophy-associated insulin resistance perhaps due to the lack of adipokine generation, showing the importance of adipose-specific *Lipin1* function in lipid homeostasis and systemic insulin receptor signaling (15, 16). However, the role of *Lipin1* in liver is complex as contradicting evidence suggests that LIPIN1 functions as a transcriptional coactivator for peroxisome proliferator-activated receptor α/PGC-1α to regulate fatty acid oxidation gene expression, and LIPIN1 has also been demonstrated to contribute to the regulation of triglyceride synthesis and VLDL secretion (17). It has been reported that the glucocorticoid receptor regulates the mouse *Lipin1* promoter by the synthetic glucocorticoid dexamethasone (14) and that regulation of *Lipin1* gene expression is mediated by sterol (18) and estrogen-related receptor γ (19), thereby suggesting the involvement of certain factor(s) affecting lipogenesis and adipogenesis by modulating *Lipin1* gene expression.

Although *Cb1r* signaling has been known to affect lipid metabolism, the molecular mechanism behind this phenomenon is still unclear. Therefore, in the current study, we investigated the effect of *Cb1r* signaling pathway on lipid metabolism and insulin receptor signaling and the mediatory role of CREBH in this context. Our study delineates an unprecedented link by which *Cb1r* signaling pathway-induced activation of *Crebh* regulates *Lipin1* gene expression and hepatic insulin receptor signaling.

EXPERIMENTAL PROCEDURES

Animals—Seven-week-old C57BL/6J (B6) and diabetic *db*/*db* mice (The Jackson Laboratory) were used. Mice were treated with a single dose of 2-AG (Tocris; 5 mg/kg intraperitoneally; 12 h) or pretreated with a single dose of AM251 (Tocris; 5 mg/kg intraperitoneally; 12 h) followed by 2-AG treatment. Delivery of recombinant adenoviruses (100 multiplicity of infection/virus) was performed via tail vein injection. For adenoviral overexpression, mice were sacrificed 72 h following viral delivery. For adenovirus-mediated knockdown experiments, mice were sacrificed 96 h after viral delivery. Following completion of experiments, liver tissues were collected for total RNA isolation or protein extraction and DAG measurement. All animal experiments were approved by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology and performed in accordance with the United States National Institutes of Health Guidelines for Animal Experiments (NIH Publication No. 85-23, revised 1996).

Plasmids—Mouse *Lipin1* gene promoter serial deletion-luciferase constructs and expression vectors (pcDNA3-FLAG) encoding CREBH-N, ATF6-N, and dominant negative CREBH have been described previously (8, 9, 20). CREBH response element mutant-luciferase constructs were cloned and confirmed by DNA sequencing.

Cell Culture, Isolation of Primary Rat Hepatocytes, and Adenoviral Infection—Transient transfection assays were performed in AML12 cells using Lipofectamine 2000 reagent (according to the manufacturer's protocol) with the indicated treatments as described previously (8, 9). Primary rat hepatocytes were prepared from 200–250-g Sprague-Dawley rats by a collagenase perfusion method as described previously (8, 9). For adenoviral infections, cells were washed with PBS and left for 2–3 h in serum-free medium containing the appropriate amount of viral particles (100 multiplicity of infection/virus). Medium was replaced with fresh growth medium for an additional 36–72 h before any treatment. All adenoviruses used in this study have been described previously (8, 20).

Semiquantitative PCR, Quantitative PCR (qPCR), and Western Blot Analysis—Total RNA from either primary hepatocytes or liver tissues was extracted using an easy-spin RNA extraction kit (Intron Biotechnology). cDNAs were generated by Superscript II enzyme (Invitrogen) and analyzed by semiquantitative PCR (1% agarose gel stained with EtBr) and qPCR using a SYBR Green PCR kit and Rotor Gene 6000 Real Time System (Corbett Life Science). All data were normalized to β-actin expression.

TABLE 1

Key genes involved in lipid metabolism

Mice ($n = 4–5$/group) were treated with 2-AG for 12 h or were infected with the indicated adenoviruses (100 multiplicity of infection/virus) for 72 h. Following completion of the experiments, mice were sacrificed, and liver tissues were obtained for qPCR analyses of the indicated genes. *Acc1*, acetyl-coenzyme A carboxylase 1; *Scd1*, stearoyl-CoA desaturase; *ChREBP*, carbohydrate-response element-binding protein.

Genes	mRNA level (-fold change)	
	2-AG *vs.* control	Ad-CREBH *vs.* Ad-GFP
Srebp1c	3.28[a]	0.83
Acc1	1.34	0.79
Scd1	0.87	1.13
ChREBP	1.11	1.03
mtGPAT1	1.43[a]	0.92
Cpt1	0.66[a]	0.79
Ucp2	0.53[a]	0.81
Cd36	2.11[a]	1.09
Dgat1	1.76[a]	1.12
Dgat2	2.54[a]	1.97[a]
Lipin1	5.71[a]	4.43[a]
Lipin2	1.21	0.73
Crebh	5.91[a]	19.28[a]

[a] $p < 0.05$ *versus* control or Ad-GFP.

For Western blot analysis, cell lysates were prepared from primary rat hepatocytes or liver tissues of experimental animals, and Western blotting was performed using the indicated antibodies.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed according to the manufacturer's protocol (Upstate). Immunoprecipitation was performed using polyclonal anti-CREBH antibody (Orbigen) or IgG (as a negative control). After recovering DNA, qPCR was performed using primers encompassing the mouse *Lipin1* promoter (−531/−256 and −1010/−846 bp) region.

Measurement of DAG Level—Liver tissues were processed for liquid chromatography/mass spectrometric determination of the DAG level as described previously (20).

Statistics—Values are expressed as mean ± S.E. Statistical significance was calculated using an unpaired Student's *t* test and one-way analysis of variance. Differences were considered significant at $p \leq 0.05$.

RESULTS

Activated Cb1r Signaling and Crebh Induce Hepatic Lipin1 Gene Expression in Vivo—In a recent study, we demonstrated that activated *Cb1r* signaling induces *Crebh* gene expression and generates the active form of CREBH (9). We have also shown that *Crebh* plays an important role in the regulation of hepatic gluconeogenesis and is hyperactive under insulin resistance conditions (8). In previous studies, it has been shown that chronic alcohol exposure increases the hepatic endocannabinoid 2-AG but not the arachidonoyl ethanolamide level to activate *Cb1r* signaling, which subsequently leads to an aberrant increase in hepatic lipogenesis, resulting in fatty liver (5). Therefore, to dissect the role of *Cb1r* and *Crebh* in lipid metabolism, we initially treated mice with 2-AG or infected mice with adenovirus (Ad) CREBH-N (active form of CREBH) and analyzed the expression of key genes involved in lipid metabolism (Table 1). 2-AG treatment led to an increase in several genes involved in this pathway (*Srebp1c*, *mtGPAT1*, *Cd36*, *Dgat1*, *Dgat2*, and *Lipin1*) along with a significant decrease in genes involved in fatty acid oxidation (*Cpt1* and *Ucp2*). Interestingly, *Crebh* overexpression showed significant up-regulation of *Lipin1* and to some extent *Dgat2* mRNA levels, whereas the expression of several other genes was not affected under this condition. This indicated a common role of activated *Cb1r* signaling and *Crebh* in regulating *Lipin1* gene expression.

Next, we tried to systematically analyze the effect of 2-AG on *Lipin1* gene regulation. A time course 2-AG treatment in mice showed significant induction of *Lipin1* but not *Lipin2* mRNA levels along with an increase of *Cb1r* and *Crebh* mRNA levels as observed from semiquantitative and qPCR analyses (Fig. 1*A*, *left top* and *right*). Similarly, 2-AG treatment also increased the LIPIN1 protein level and generated the active form of CREBH (Fig. 1*A*, *left bottom*). To confirm that 2-AG-induced activation of *Cb1r* signaling pathway induces *Crebh* and *Lipin1* gene expression, we used *Cb1r* antagonist AM251 pretreatment in mice exposed to 2-AG. Pretreatment with AM251 significantly attenuated the 2-AG-mediated induction of *Crebh* as well *Lipin1* mRNA and protein levels (Fig. 1*B*), and as expected, no changes were observed in the mRNA level of *Lipin2* gene. Overall, these results indicate a connection between *Cb1r* signaling-mediated activation of *Crebh* and induction of *Lipin1* gene expression *in vivo*.

CREBH Is a Transcriptional Regulator of Hepatic Lipin1—To elucidate the role of *Crebh* in 2-AG-mediated induction of *Lipin1* gene expression, we sought to investigate whether *Crebh* directly regulates *Lipin1* gene transcription. Initially, to confirm the specificity of *Crebh*-mediated activation of *Lipin1* gene expression, we infected primary rat hepatocytes with adenoviruses encoding active forms of CREBH (CREBH-N) and ATF6 (ATF6-N), a member of the CREB/ATF family closely related to CREBH. Overexpression of *Crebh* significantly and specifically induced *Lipin1* gene expression, whereas overexpression of *Atf6* led to no significant change in the *Lipin1* mRNA level (Fig. 2*A*). However, both *Crebh* and *Atf6* overexpression significantly induced the C-reactive protein (*Crp*) mRNA level, a previously reported common target of both *Crebh* and *Atf6*. This indicates that the adenoviruses were in proper working conditions. Next, to verify the role of *Crebh* in regulating *Lipin1* at the transcriptional level, we performed transient transfection using the *Lipin1* promoter in AML12 cells. Similar to the induction of the *Lipin1* mRNA level, overexpression of *Crebh* activated the *Lipin1* gene promoter, whereas *Atf6* overexpression failed to show any significant activation (Fig. 2*B*). Overall, these results suggest that *Crebh* specifically regulates *Lipin1* gene transcription.

Next, we investigated the mediatory role of *Crebh* in 2-AG activation of the *Lipin1* promoter. Co-transfection of the *Lipin1* promoter with plasmid encoding *Crebh* cDNA lacking the transcriptional activation domain (dominant negative CREBH) was done in AML12 cells, and then cells were treated with 2-AG (Fig. 2*C*). 2-AG significantly activated the *Lipin1* promoter under normal conditions but failed to activate the *Lipin1* promoter in cells co-transfected with dominant negative CREBH, thereby suggesting that *Crebh* plays a major mediatory role in activating *Lipin1* gene transcription by *Cb1r* signaling pathway.

Next, using several deletion constructs, we tried to map the CREBH-responsive region in the *Lipin1* gene promoter. Dele-

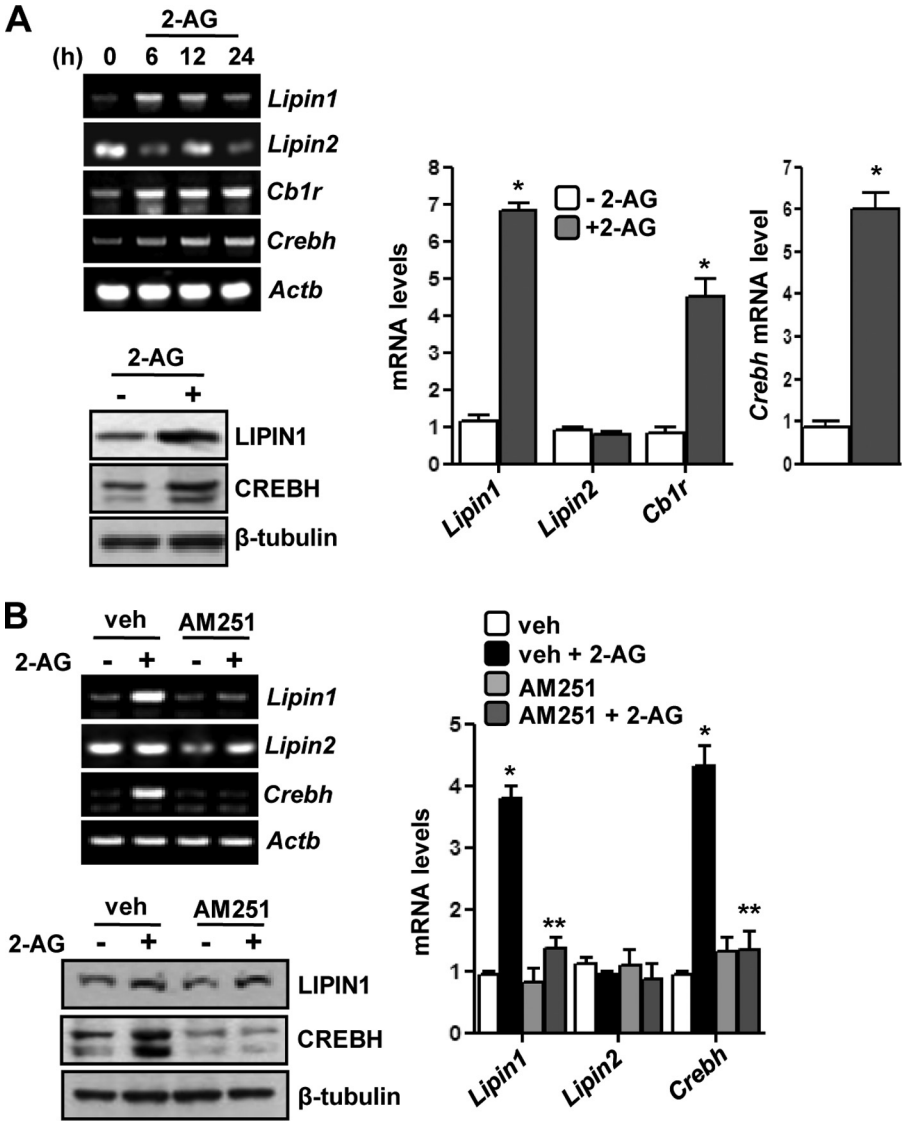

FIGURE 1. **Activated *Cb1r* induces *Lipin1* gene expression via *Crebh*.** *A*, mice ($n = 5$ per group) were treated with 2-AG at the indicated times or for 12 h, and liver tissues were obtained for semiquantitative PCR (*top left*) or qPCR (*right*) analyses and for measuring protein levels (*bottom left*). *, $p < 0.05$ *versus* untreated control. *B*, mice ($n = 5$ per group) were treated with 2-AG for 12 h or treated with AM251 for 12 h preceding 2-AG treatment for a further 12 h, and liver tissues were obtained for semiquantitative PCR (*top left*) or qPCR (*right*) analyses and for measuring protein levels (*bottom left*).*, $p < 0.05$ *versus* control; **, $p < 0.05$ *versus* vehicle (*veh*) + 2-AG. All data represent mean ± S.E. (*error bars*).

tion mapping results demonstrated that the putative CREBH-responsive region lies within −531 to −231 bp of the transcription start site of the *Lipin1* promoter (Fig. 2*D*). We identified a putative binding region of CREBH (Fig. 2*E*). 2-AG treatment significantly activated the wild type (WT) *Lipin1* promoter construct but failed to activate the CREBH mutant *Lipin1* promoter. Similarly, 2-AG treatment led to the recruitment of endogenous CREBH to the endogenous *Lipin1* gene promoter (Fig. 2*F*, *top*), and this increased occupancy of endogenous CREBH on the *Lipin1* gene promoter upon 2-AG treatment was mitigated in the CREBH mutant *Lipin1* promoter construct as demonstrated by ChIP assays (Fig. 2*F*, *bottom*). These results confirm that induction of *Lipin1* by *Cb1r* signaling is mediated via *Crebh*.

Knockdown of Crebh Attenuates Lipin1 Induction and Decreases DAG Production by Hepatic Cb1r Signaling in Vivo—Previous reports have identified *Lipin1* as a mammalian cytosolic phosphatidic acid phosphatase in various cell types and demonstrated that *Lipin1* increases the hepatic DAG level (20). Adenoviral overexpression of CREBH in mice resulted in a significant increase of the *Lipin1* mRNA and protein levels comparable with another *Crebh* target, phosphoenolpyruvate carboxykinase (*Pepck*) gene expression, *in vivo* (Fig. 3*A*) and also led to a significant increase in the DAG level (~2.5-fold) (Fig. 3*B*). As expected, *Lipin2* gene expression showed no significant change in this condition.

Next, to ascertain the role of activated *Cb1r* signaling and the mediatory role of *Crebh* in this context, we overexpressed short hairpin RNA (shRNA) adenoviruses encoding unspecific RNAi (USi) and *Crebh* RNAi (CREBHi) in mice preceding 2-AG treatment. 2-AG treatment led to significant induction of *Lipin1* and *Crebh* mRNA and protein levels in USi-infected livers but failed to substantially induce *Lipin1* mRNA or protein in CREBHi-infected livers (Fig. 3*C*). Similarly, DAG production was significantly higher in 2-AG-treated mice compared with the vehicle-

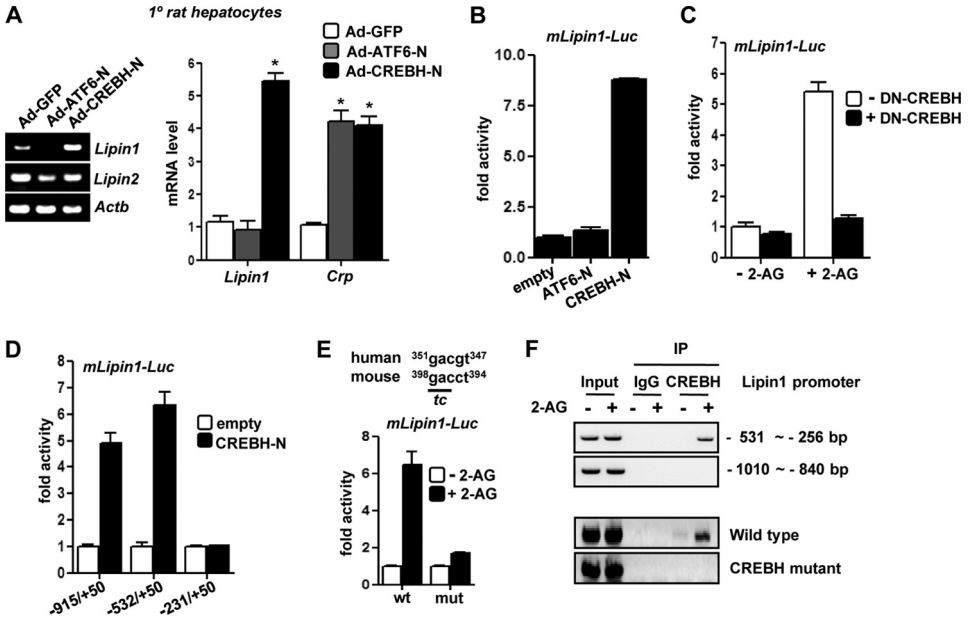

FIGURE 2. ***Crebh* is a transcriptional regulator of *Lipin1* gene expression.** *A*, primary (*1°*) rat hepatocytes were infected with adenoviruses encoding GFP, FLAG-ATF6-N, and FLAG-CREBH-N for 48 h. RNA was extracted for semiquantitative PCR (*left*) and qPCR analyses (*right*). *, $p < 0.05$ *versus* Ad-GFP. *B–E*, transient transfection assays were performed in AML12 cells with mLipin1-luc reporter constructs along with co-transfection of the indicated expression vectors (*B–D*) as indicated or treatment with 2-AG for 12 h (*B* and *E*) prior to luciferase assays. -Fold activity represents relative luciferase activity:β-gal activity. All transfections were performed in triplicates and are representative of three to four independent experiments. *F*, untransfected AML12 cells (*top*) or AML12 cells transfected with wild type or CREBH mutant constructs of mLipin1-Luc (*bottom*) were treated with vehicle or 2-AG for 12 h, and immunoprecipitation (*IP*) of AML12 chromatin from cells exposed to vehicle or 2-AG was performed with IgG or CREBH antibody. The percentage of DNA immunoprecipitated with CREBH antibody relative to input chromatin was demonstrated by semiquantitative PCR analysis. *, $p < 0.05$ *versus* control. All data represent mean ± S.E. (*error bars*) of at least three independent experiments. *DN*, dominant negative.

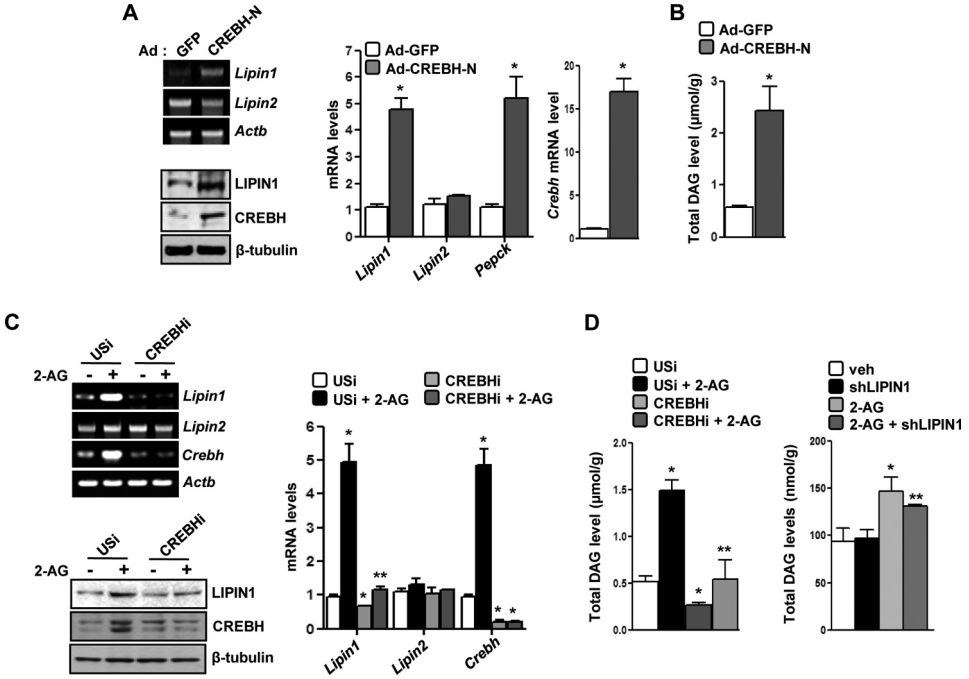

FIGURE 3. ***Crebh*-mediated induction of *Lipin1* gene expression and increase in hepatic DAG level *in vivo*.** *A* and *B*, mice ($n = 5$) were infected with the indicated adenoviruses for 72 h. Following completion of the experiments, mice were sacrificed, and liver tissues were obtained for semiquantitative PCR (*top left*) or qPCR (*right*) analyses and for measuring protein levels (*bottom left*). The DAG level was measured in Ad-GFP- and Ad-CREBH-N-infected mouse liver samples (*B*). *, $p < 0.05$ *versus* Ad-GFP. *C*, mice ($n = 5$) were infected with the indicated adenoviruses, and 96 h postinfection, mice were treated with 2-AG for a further 12 h. Liver tissues were obtained for semiquantitative PCR (*top left*) or qPCR (*right*) analyses and for measuring protein (*bottom left*). *, $p < 0.05$ *versus* USi; **, $p < 0.05$ *versus* USi + 2-AG. Data represent mean ± S.E. *D*, mice ($n = 5$; *left*) or AML12 cells (*right*) were infected with the indicated adenoviruses, and 96 h postinfection, mice were treated with 2-AG for a further 12 h. The DAG level was measured in adenovirus-infected mouse liver samples (*left*) and in AML12 cells (*right*). *, $p < 0.05$ *versus* USi/vehicle (*veh*); **, $p < 0.05$ *versus* USi/vehicle + 2-AG. All data represent mean ± S.E. (*error bars*).

treated mice under the USi-infected condition but was dramatically reduced to the basal level under the CREBHi-infected condition (Fig. 3*D*, *left*). Indeed, CREBHi-infected mouse livers produced lower amounts of DAG compared with USi-infected littermates (Fig. 3*D*, *left*). To confirm the involvement of *Lipin1* in *Cb1r*/*Crebh*-mediated regulation of DAG

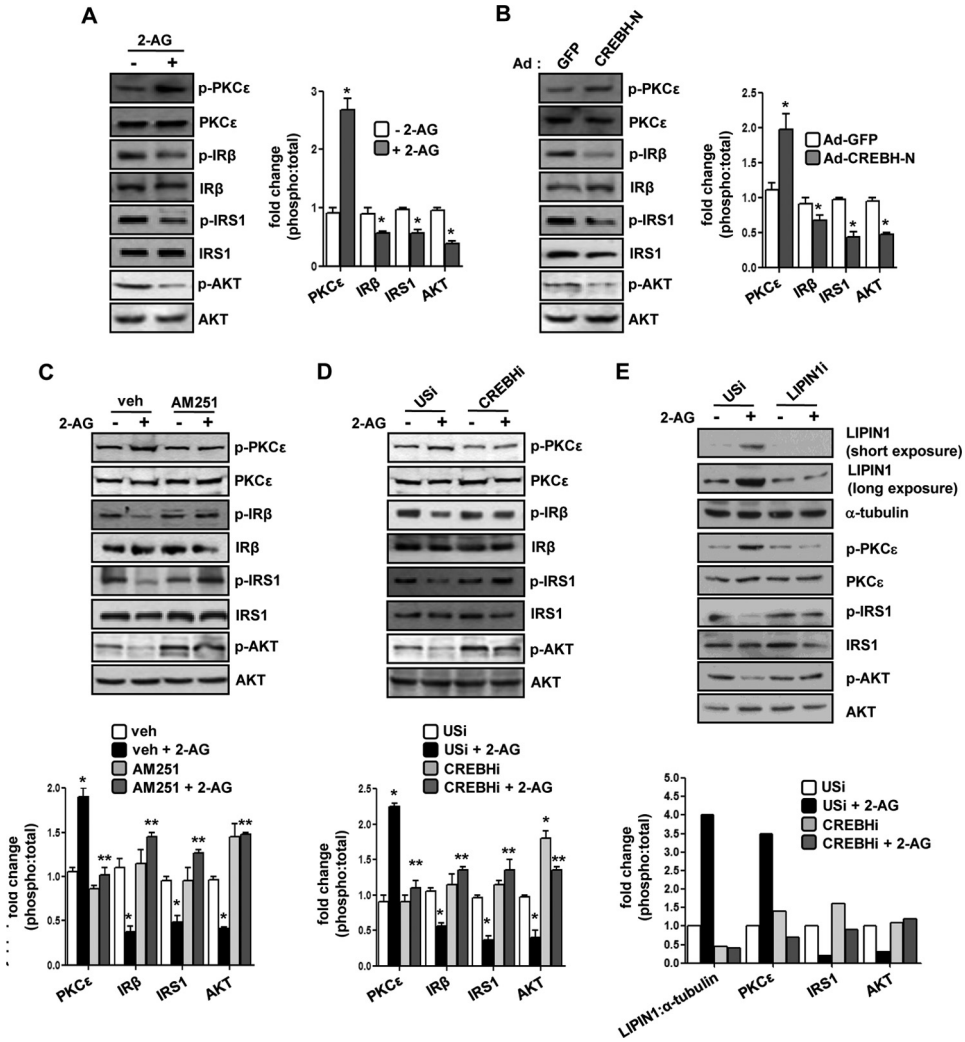

FIGURE 4. ***Crebh*-mediated induction of *Lipin1* gene expression is linked to perturbation of hepatic insulin receptor signaling *in vivo*.** *A–E*, experiments were performed as described in Fig. 1 (for *A* and *C*) or Fig. 3 (for *B*, *D*, and *E*), and liver tissues (*A–D*) or AML12 cell lysates (*E*) were obtained for Western blot analysis with the indicated antibodies. Protein levels were quantified by densitometry analysis (phospho:total form). *, $p < 0.05$ *versus* control and Ad-GFP; **, $p < 0.05$ *versus* vehicle (*veh*) + 2-AG and USi + 2-AG. All data represent mean ± S.E. (*error bars*). *IRβ*, insulin receptor *β*.

production, AML12 cells were infected with adenovirus encoding *Lipin1* RNAi (shLipin1) preceding 2-AG treatment. 2-AG treatment led to an increase of the DAG level (~60% compared with untreated cells). Similarly to CREBHi, *Lipin1* knockdown led to a significant decrease in the total DAG level compared with 2-AG-treated cells (~15% decrease from 2-AG-treated cells), thereby indicating the dependence of the *Cb1r*/*Crebh* pathway on *Lipin1* to regulate DAG production (Fig. 3*D*, *right*). These observations indicate the crucial role played by *Crebh* in mediating the effect of *Cb1r* signaling on *Lipin1* gene regulation and DAG production.

Cb1r Signaling Pathway Inhibits Hepatic Insulin Receptor Signaling via Crebh-mediated Induction of Lipin1—It has been demonstrated previously that increased production of DAG is linked to obesity-related insulin resistance in peripheral tissues (21, 22). Therefore, we sought to investigate the effect of *Cb1r*-mediated induction of *Crebh* and *Lipin1* gene expression and increased DAG production on hepatic insulin receptor signaling *in vivo*. Both 2-AG treatment and *Crebh* overexpression in mice showed a remarkable increase in Ser-729 phosphorylation of PKCϵ, a major non-canonical isoform of PKC found in the liver. In addition, there was a significant reduction in the phosphorylation of insulin receptor *β* and its downstream signaling components, Tyr-989 phosphorylation of insulin receptor substrate 1 (IRS1) and Ser-473 phosphorylation of AKT, upon these treatments (Fig. 4, *A* and *B*). These results indicate that increased expression of *Lipin1* in these conditions could promote deregulation of insulin signaling in the liver.

Therefore, we next assessed the phosphorylation status of these signaling components upon *Cb1r* antagonism or knockdown of *Crebh* (Fig. 4, *C* and *D*). Consistent with our earlier results that showed reduced gene expression of *Lipin1* and a decrease in the DAG level under these conditions, we found that AM251 pretreatment or knockdown of *Crebh* significantly attenuated 2-AG-mediated activation of PKCϵ and led to a significant increase in insulin receptor *β*, IRS1, and AKT phosphorylation compared with 2-AG-treated mice (Fig. 4, *C* and *D*). Furthermore, to reconfirm that the 2-AG-induced deregulation of insulin receptor signaling pathway is mediated by *Lipin1*, the phosphorylation status of the above mentioned sig-

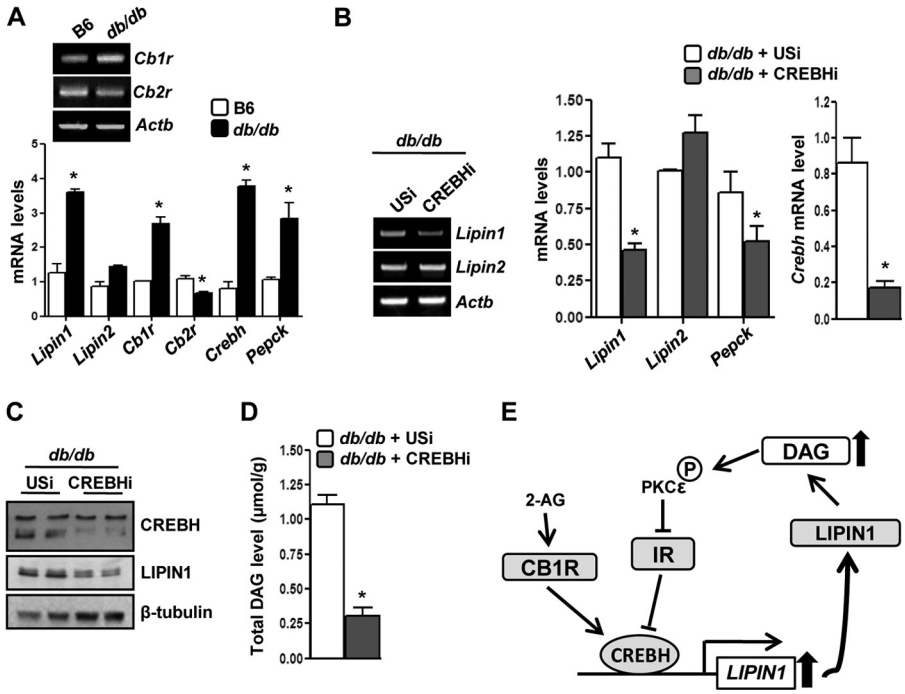

FIGURE 5. **Knockdown of *Crebh* reverses perturbation of hepatic insulin receptor signaling in *db*/*db* mice.** *A*, semiquantitative PCR (*top*) or qPCR (*bottom*) analyses of gene expression in B6 or *db*/*db* mice ($n = 4–5$). *, $p < 0.05$ *versus* B6. *B–D*, semiquantitative PCR (*B*, *left*) or qPCR (*B*, *right*) analyses, protein levels (*C*), and DAG levels (*D*) were measured in *db*/*db* mice ($n = 5$) infected with Ad-USi or Ad-CREBH RNAi. *, $p < 0.05$ *versus* USi. All data represent mean ± S.E. (*error bars*). *E*, proposed model depicting the effect of endocannabinoid-mediated activation of *Cb1r* in inducing and activating CREBH to disrupt hepatic insulin signaling via induction of *Lipin1* gene expression and by increasing DAG production. *IR*, insulin receptor.

naling kinases was assessed in AML12 cells upon knockdown of *Lipin1* preceding 2-AG treatment (Fig. 4*E*). Similar to the effect of *Crebh* knockdown, *Lipin1* knockdown significantly attenuated 2-AG-induced activation of PKCε and released the inhibitory effect of 2-AG on the IRS1 and AKT phosphorylation status, thereby suggesting that the 2-AG-induced deregulation of the insulin signaling pathway is mediated via *Lipin1*. Overall, these results indicate that the blockade of *Crebh* significantly attenuates *Cb1r*-mediated deregulation of hepatic insulin signaling via a reduction in *Lipin1* gene expression and decreased DAG production *in vivo*.

Knockdown of Crebh Attenuates Induction of Lipin1 Expression and Decreases DAG Production in Insulin Resistance Condition—Initially, we observed that *Crebh* and *Lipin1* gene expression was significantly higher in *db*/*db* mice compared with normal mice (Fig. 5*A*). A recent report (5) demonstrated that *Cb1r* gene expression follows a pattern similar to that of *Crebh* and *Lipin1* in the diet-induced obesity condition. Interestingly, we found that *Cb1r* gene expression was also significantly higher in *db*/*db* mice compared with normal mice, whereas *Cb2r* gene expression was significantly lower under similar conditions (Fig. 5*A*). These results indicate a constitutively activated status of the *Cb1r* signaling pathway in the insulin-resistant condition. Therefore, to ascertain the significance of *Crebh*-mediated induction of *Lipin1* gene expression and the increase in DAG production from a physiological perspective, we overexpressed *Crebh* RNAi in *db*/*db* mice. *Crebh* knockdown resulted in a significant decrease in the *Lipin1* mRNA and protein levels (Fig. 5, *B* and *C*), and as a consequence, there was a dramatic reduction in the DAG level of *db*/*db* mice under this condition (Fig. 5*D*). However, the phosphorylation status of the components of the insulin signaling pathway showed marginal changes (data not shown) upon *Crebh* knockdown. Overall, our results provide a novel link connecting hepatic *Cb1r* signaling with deregulation of the insulin receptor signaling pathway and identify *Crebh* as a crucial player in regulating lipid metabolism via *Lipin1* gene induction *in vivo*.

DISCUSSION

Crebh has been demonstrated to play an important role in hepatic gluconeogenesis, triglyceride metabolism, and iron metabolism by transcriptional regulation of key enzyme genes involved in those metabolic pathways (8–11). In our current study, we investigated and suggest a novel role of *Crebh* in regulating hepatic lipid metabolism by transcriptional activation of the *Lipin1* gene. *Cb1r*-mediated activation of CREBH and *Lipin1* induction lead to increased DAG production *in vivo* and phosphorylation of PKCε. Activation of PKCε by this signaling cascade imparts insulin resistance effects via disruption of the insulin receptor signaling pathway. Antagonism of *Cb1r* signaling repressed the activation of CREBH and its downstream target *Lipin1*, and *Cb1r* activation under *Crebh*-deficient conditions failed to induce the *Lipin1*-mediated increase in DAG production, ultimately leading to recovery of insulin receptor signaling component activity. Hepatic *Cb1r*, *Crebh*, and *Lipin1* gene expression is higher in various models of insulin resistance (5, 8, 20). Conversely, in our previous study, we demonstrated that insulin treatment (or AKT co-transfection) leads to diminished *Crebh* mRNA levels (as well as promoter activity) (8). Knockdown of *Crebh* in an insulin-resistant rodent model (*db*/*db* mice) also showed significant lowering of the pre-existing high DAG level. Overall, our current

study unravels a connection among *Cb1r*, *Crebh*, and *Lipin1* and sheds light onto a novel molecular mechanism by which activation of CB1R leads to deregulation of the insulin signaling pathway in the liver (a schematic model is shown in Fig. 5*E*), indicating that targeted disruption of hepatic *Cb1r* signaling and CREBH activity might provide plausible therapeutic approaches to restore proper functioning of insulin receptor signaling.

The rate of *de novo* hepatic lipogenesis was increased by *Cb1r* agonists and decreased by *Cb1r* antagonists in rodents. Adipose tissue may be the source of liver fat as it has been demonstrated that activation of *Cb1r* in adipocytes promotes lipogenesis, and the released fatty acids are converted to triglycerides by the liver (1, 2, 4). Conversely, *Cb1r* blockade with antagonists leads to reduction of hepatic triglycerides with an increased rate of secretion of triglyceride-rich VLDL from the liver of insulin-resistant rodents. However, because of the deleterious side effects of *Cb1r* antagonists, it is essential to elucidate the *Cb1r* signaling pathway in detail to unravel downstream targets for possible therapeutic benefits. In this context, our study demonstrates the involvement of the endoplasmic reticulum-bound transcription factor *Crebh* in a mediatory role and may explain in part the molecular basis of *Cb1r*-mediated up-regulation of lipogenesis and DAG production that ultimately causes insulin resistance via deregulation of the insulin receptor signaling. Recently, it has been shown that chronic ethanol feeding increases the endocannabinoid 2-AG but not the arachidonoyl ethanolamide level in mouse liver, and this increase was followed by a robust change in the lipogenic gene program with a significant increase in gene expression of *Srebp1c* and its targets, whereas AMP-activated protein kinase and fatty acid oxidation were considerably attenuated under these conditions (5). In our previous study, we found that 2-AG treatment also led to increased CREBH activity (9), and in insulin resistance conditions, both are expressed at a higher level (5, 8). Therefore, we presumed that *Crebh* might mediate some of the effects associated with *Cb1r* activation by regulating the expression of some key genes involved in lipogenesis and the triglyceride synthesis pathway. However, our results (Table 1) demonstrated that *Cb1r* and *Crebh* have common targets further downstream in the lipogenic program and confirmed that *Lipin1*, a key gene involved in DAG production as well as perturbation of hepatic insulin signaling, is the common mediator of activated *Cb1r* and *Crebh* effects.

Recent reports have demonstrated the role of *Lipin1* in promoting insulin resistance via enhanced DAG production and PKCϵ activation, triglyceride formation, and VLDL secretion in the liver (20). CREB/CRTC2 was identified as a regulator of *Lipin1* gene transcription under fasting-refeeding and diet-induced obesity conditions to explain the role of *Lipin1* in insulin resistance (20). Interestingly, ethanol is also known to increase the hepatic DAG level and promote insulin resistance, although the underlying molecular mechanism was not clearly established (23). In this context, an important observation was that chronic ethanol feeding increases the endocannabinoid 2-AG but not the arachidonoyl ethanolamide level in mouse liver (5). Thus, in accordance with our previous report (9), 2-AG-mediated activation of CREBH and CREBH-mediated transcriptional activation of *Lipin1* gene may provide the missing link connecting alcohol injury to enhanced DAG production and insulin resistance. Previous studies have revealed the importance of DAG as a signaling molecule to activate PKCθ in muscle or PKCϵ in liver to target either insulin receptor substrates or insulin receptor, respectively (22, 24). This phenomenon was further confirmed by knockdown of *Crebh* in 2-AG-treated or *db*/*db* mice that showed a concomitant decrease in PKCϵ activity and *Lipin1*-mediated DAG production as well as considerable recovery in the activities of insulin receptor and its downstream signaling components (IRS1 and AKT). Our findings were further supported by a recent report in which a lowering of the hepatic triglyceride level in *Crebh* knock-out mouse models was observed (11).

Interestingly, our results demonstrated a moderate but significant decrease in the total DAG level upon *Lipin1* knockdown in cells treated with 2-AG. This observation indicates the possibility of a *Lipin1*-independent effect of 2-AG in increasing the total DAG level and consequently the inhibiting insulin receptor signaling pathway. DAG is an interesting candidate responsible for lipid-induced insulin resistance and was found to be associated with insulin resistance after high fat feeding and/or *Cb1r* activation (5, 25). DAG can be derived from multiple sources. However, in the case of lipid-induced insulin resistance, the *de novo* synthesis of DAG via esterification of glycerol 3-phosphate is suggested to be the most important route. The *Cb1r* signaling pathway contributes to this source of DAG synthesis (1, 5). Other sources of DAG synthesis include the breakdown of phospholipids by the enzyme phospholipase C or phospholipase D-mediated hydrolysis of phosphatidylcholine (26). Especially in the phospholipase D pathway, both of the phosphatidic acid phosphatase family proteins diacylglycerol phosphate phosphatase and lipid-phosphate phosphatase contribute to the synthesis of DAG (27, 28). Alternatively, hydrolysis of triacylglycerol by the activity of lipases also results in increased DAG levels (29). Therefore, investigating the various factors involved in these multiple pathways that contribute to DAG synthesis in 2-AG-treated samples and analyzing the total DAG level upon knockdown or pharmacological inhibition of these factors (in the presence of 2-AG treatment) will provide additional information regarding the involvement of other contributory factors downstream of the *Cb1r* pathway and independent of *Lipin1*.

Overall, our study demonstrates the molecular link between *Cb1r* and insulin receptor signaling dysfunction in a lipogenic setting with crucial roles played by endoplasmic reticulum stress-activated transcription factor *Crebh* and *Lipin1*. These findings at least in part support the hypothesis that relieving higher hepatic DAG levels by targeting its upstream regulators might be beneficial to restore insulin signaling. However, further studies are necessary to assess the relative contribution of this signaling cascade that can be targeted for potential therapeutic purposes in insulin resistance conditions.

Acknowledgments—We thank our laboratory members and Dr. Seok-Yong Choi for cooperation and discussions related to this work.

REFERENCES

1. Kunos, G., Osei-Hyiaman, D., Bátkai, S., Sharkey, K. A., and Makriyannis, A. (2009) Should peripheral CB_1 cannabinoid receptors be selectively targeted for therapeutic gain? *Trends Pharmacol. Sci.* **30,** 1–7
2. Nogueiras, R., Veyrat-Durebex, C., Suchanek, P. M., Klein, M., Tschöp, J., Caldwell, C., Woods, S. C., Wittmann, G., Watanabe, M., Liposits, Z., Fekete, C., Reizes, O., Rohner-Jeanrenaud, F., and Tschöp, M. H. (2008) Peripheral, but not central, CB1 antagonism provides food intake-independent metabolic benefits in diet-induced obese rats. *Diabetes* **57,** 2977–2991
3. Monteleone, P., Matias, I., Martiadis, V., De Petrocellis, L., Maj, M., and Di Marzo, V. (2005) Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa. *Neuropsychopharmacology* **30,** 1216–1221
4. Matias, I., Gonthier, M. P., Orlando, P., Martiadis, V., De Petrocellis, L., Cervino, C., Petrosino, S., Hoareau, L., Festy, F., Pasquali, R., Roche, R., Maj, M., Pagotto, U., Monteleone, P., and Di Marzo, V. (2006) Regulation, function, and dysregulation of endocannabinoids in models of adipose and β-pancreatic cells and in obesity and hyperglycemia. *J. Clin. Endocrinol. Metab.* **91,** 3171–3180
5. Jeong, W. I., Osei-Hyiaman, D., Park, O., Liu, J., Bátkai, S., Mukhopadhyay, P., Horiguchi, N., Harvey-White, J., Marsicano, G., Lutz, B., Gao, B., and Kunos, G. (2008) Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. *Cell Metab.* **7,** 227–235
6. Després, J. P., Golay, A., and Sjöström, L.; Rimonabant in Obesity-Lipids Study Group (2005) Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N. Engl. J. Med.* **353,** 2121–2134
7. Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H., and Kaufman, R. J. (2006) Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. *Cell* **124,** 587–599
8. Lee, M. W., Chanda, D., Yang, J., Oh, H., Kim, S. S., Yoon, Y. S., Hong, S., Park, K. G., Lee, I. K., Choi, C. S., Hanson, R. W., Choi, H. S., and Koo, S. H. (2010) Regulation of hepatic gluconeogenesis by an ER-bound transcription factor, CREBH. *Cell Metab.* **11,** 331–339
9. Chanda, D., Kim, D. K., Li, T., Kim, Y. H., Koo, S. H., Lee, C. H., Chiang, J. Y., and Choi, H. S. (2011) Cannabinoid receptor type 1 (CB1R) signaling regulates hepatic gluconeogenesis via induction of endoplasmic reticulum-bound transcription factor cAMP-responsive element-binding protein H (CREBH) in primary hepatocytes. *J. Biol. Chem.* **286,** 27971–27979
10. Vecchi, C., Montosi, G., Zhang, K., Lamberti, I., Duncan, S. A., Kaufman, R. J., and Pietrangelo, A. (2009) ER stress controls iron metabolism through induction of hepcidin. *Science* **325,** 877–880
11. Lee, J. H., Giannikopoulos, P., Duncan, S. A., Wang, J., Johansen, C. T., Brown, J. D., Plutzky, J., Hegele, R. A., Glimcher, L. H., and Lee, A. H. (2011) The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism. *Nat. Med.* **17,** 812–815
12. Luebke-Wheeler, J., Zhang, K., Battle, M., Si-Tayeb, K., Garrison, W., Chhinder, S., Li, J., Kaufman, R. J., and Duncan, S. A. (2008) Hepatocyte nuclear factor 4α is implicated in endoplasmic reticulum stress-induced acute phase response by regulating expression of cyclic adenosine monophosphate responsive element binding protein H. *Hepatology* **48,** 1242–1250
13. Danno, H., Ishii, K. A., Nakagawa, Y., Mikami, M., Yamamoto, T., Yabe, S., Furusawa, M., Kumadaki, S., Watanabe, K., Shimizu, H., Matsuzaka, T., Kobayashi, K., Takahashi, A., Yatoh, S., Suzuki, H., Yamada, N., and Shimano, H. (2010) The liver-enriched transcription factor CREBH is nutritionally regulated and activated by fatty acids and PPARα. *Biochem. Biophys. Res. Commun.* **391,** 1222–1227
14. Reue, K. (2009) The lipin family: mutations and metabolism. *Curr. Opin. Lipidol.* **20,** 165–170
15. Erion, D. M., and Shulman, G. I. (2010) Diacylglycerol-mediated insulin resistance. *Nat. Med.* **16,** 400–402
16. Samuel, V. T., Petersen, K. F., and Shulman, G. I. (2010) Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375,** 2267–2277
17. Finck, B. N., Gropler, M. C., Chen, Z., Leone, T. C., Croce, M. A., Harris, T. E., Lawrence, J. C., Jr., and Kelly, D. P. (2006) Lipin 1 is an inducible amplifier of the hepatic PGC-1α/PPARα regulatory pathway. *Cell Metab.* **4,** 199–210
18. Ishimoto, K., Nakamura, H., Tachibana, K., Yamasaki, D., Ota, A., Hirano, K., Tanaka, T., Hamakubo, T., Sakai, J., Kodama, T., and Doi, T. (2009) Sterol-mediated regulation of human lipin 1 gene expression in hepatoblastoma cells. *J. Biol. Chem.* **284,** 22195–22205
19. Kim, D. K., Kim, J. R., Koh, M., Kim, Y. D., Lee, J. M., Chanda, D., Park, S. B., Min, J. J., Lee, C. H., Park, T. S., and Choi, H. S. (2011) Estrogen-related receptor γ (ERRγ) is a novel transcriptional regulator of phosphatidic acid phosphatase, LIPIN1, and inhibits hepatic insulin signaling. *J. Biol. Chem.* **286,** 38035–38042
20. Ryu, D., Oh, K. J., Jo, H. Y., Hedrick, S., Kim, Y. N., Hwang, Y. J., Park, T. S., Han, J. S., Choi, C. S., Montminy, M., and Koo, S. H. (2009) TORC2 regulates hepatic insulin signaling via a mammalian phosphatidic acid phosphatase, LIPIN1. *Cell Metab.* **9,** 240–251
21. Samuel, V. T., Liu, Z. X., Qu, X., Elder, B. D., Bilz, S., Befroy, D., Romanelli, A. J., and Shulman, G. I. (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J. Biol. Chem.* **279,** 32345–32353
22. Savage, D. B., Petersen, K. F., and Shulman, G. I. (2007) Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol. Rev.* **87,** 507–520
23. Kaiser, J. P., Beier, J. I., Zhang, J., David Hoetker, J., von Montfort, C., Guo, L., Zheng, Y., Monia, B. P., Bhatnagar, A., and Arteel, G. E. (2009) PKCϵ plays a causal role in acute ethanol-induced steatosis. *Arch. Biochem. Biophys.* **482,** 104–111
24. Choi, C. S., Savage, D. B., Kulkarni, A., Yu, X. X., Liu, Z. X., Morino, K., Kim, S., Distefano, A., Samuel, V. T., Neschen, S., Zhang, D., Wang, A., Zhang, X. M., Kahn, M., Cline, G. W., Pandey, S. K., Geisler, J. G., Bhanot, S., Monia, B. P., and Shulman, G. I. (2007) Suppression of diacylglycerol acyltransferase-2 (DGAT2), but not DGAT1, with antisense oligonucleotides reverses diet-induced hepatic steatosis and insulin resistance. *J. Biol. Chem.* **282,** 22678–22688
25. Heydrick, S. J., Ruderman, N. B., Kurowski, T. G., Adams, H. B., and Chen, K. S. (1991) Enhanced stimulation of diacylglycerol and lipid synthesis by insulin in denervated muscle. Altered protein kinase C activity and possible link to insulin resistance. *Diabetes* **40,** 1707–1711
26. Brose, N., Betz, A., and Wegmeyer, H. (2004) Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Curr. Opin. Neurobiol.* **14,** 328–340
27. Waggoner, D. W., Xu, J., Singh, I., Jasinska, R., Zhang, Q. X., and Brindley, D. N. (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *Biochim. Biophys. Acta* **1439,** 299–316
28. Brindley, D. N., Pilquil, C., Sariahmetoglu, M., and Reue, K. (2009) Phosphatidate degradation: phosphatidate phosphatases (lipins) and lipid phosphate phosphatases. *Biochim. Biophys. Acta* **1791,** 956–961
29. Carrasco, S., and Mérida, I. (2007) Diacylglycerol, when simplicity becomes complex. *Trends Biochem. Sci.* **32,** 27–36