Ribavirin Suppresses Hepatic Lipogenesis Through Inosine Monophosphate Dehydrogenase Inhibition: Involvement of Adenosine Monophosphate-Activated Protein Kinase-Related Kinases and Retinoid X Receptor α

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Ribavirin (RBV) has been widely used as an antiviral reagent, specifically for patients with chronic hepatitis C. We previously demonstrated that adenosine kinase, which monophosphorylates RBV into the metabolically active form, is a key determinant for RBV sensitivity against hepatitis C virus RNA replication. However, the precise mechanism of RBV action and whether RBV affects cellular metabolism remain unclear. Analysis of liver gene expression profiles obtained from patients with advanced chronic hepatitis C treated with the combination of pegylated interferon and RBV showed that the adenosine kinase expression level tends to be lower in patients who are overweight and significantly decreases with progression to advanced fibrosis stages. In our effort to investigate whether RBV affects cellular metabolism, we found that RBV treatment under clinically achievable concentrations suppressed lipogenesis in hepatic cells. In this process, guanosine triphosphate depletion through inosine monophosphate dehydrogenase inhibition by RBV and adenosine monophosphate-activated protein kinase-related kinases, especially microtubule affinity regulating kinase 4, were required. In addition, RBV treatment led to the down-regulation of retinoid X receptor α (RXRα), a key nuclear receptor in various metabolic processes, including lipogenesis. Moreover, we found that guanosine triphosphate depletion in cells induced the down-regulation of RXRα, which was mediated by microtubule affinity regulating kinase 4. Overexpression of RXRα attenuated the RBV action for suppression of lipogenic genes and intracellular neutral lipids, suggesting that down-regulation of RXRα was required for the suppression of lipogenesis in RBV action. *Conclusion*: We provide novel insights about RBV action in lipogenesis and its mechanisms involving inosine monophosphate dehydrogenase inhibition, adenosine monophosphate-activated protein kinase-related kinases, and down-regulation of RXRα. RBV may be a potential reagent for anticancer therapy against the active lipogenesis involved in hepatocarcinogenesis. (*Hepatology Communications* 2017;1:550–563)

Introduction

Ribavirin (RBV), a synthetic guanosine analogue, has been widely used as an antiviral reagent, specifically for patients with chronic hepatitis C (CHC). Although the mechanism of RBV action against hepatitis C virus (HCV) remains to be fully understood, it has been clearly shown that RBV improves sustained virologic response rates and reduces virologic breakthrough or relapse when combined with

Abbreviations: ACC, acetyl-coenzyme A carboxylase; ADK, adenosine kinase; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMPK, adenosine monophosphate-activated protein kinase; AMPK-RK, AMPK-related kinase; CHC, chronic hepatitis C; DAA, direct-acting antiviral; DCV, daclatasvir; FASN, fatty acid synthase; GTP, guanosine triphosphate; HCV, hepatitis C virus; IFN, interferon; IMPDH, inosine monophosphate dehydrogenase; IRF, interferon regulatory factor; LKB, liver kinase B; LXR, liver X receptor; MARK, microtubule affinity regulating kinase; MELK, maternal embryonic leucine zipper kinase; miRNA, microRNA; MPA, mycophenolic acid; mRNA, messenger RNA; MZB, mizoribine; PEG, pegylated; PRKAA, protein kinase AMP-activated catalytic subunit alpha; RBV, ribavirin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; RXR, retinoid X receptor; SCD, stearoyl coenzyme A desaturase; SIK, salt inducible kinase; siRNAs, small interfering RNAs; SOF, sofosbuvir; SREBP-1c, sterol-regulatory element-binding protein-1c.

pegylated interferon (PEG-IFN) and/or direct-acting antivirals (DAAs).[1,2] Recently, IFN-free all-oral DAA regimens with or without RBV have been approved and shown to be successful in patients with CHC.[3,4] Although some reports showed that DAA regimens with or without RBV achieved high sustained virologic response rates, it has been recognized that RBV has potential as a therapeutic reagent in some patients with CHC depending on the HCV genotypes, fibrosis stages, or historical results of therapies.[5]

Several mechanisms have been proposed to account for the actions of RBV against HCV.[6,7] In 2011, in a study using human hepatoma Li23-derived cell lines possessing HCV RNA replication assay systems,[8] we demonstrated that RBV exerts anti-HCV activity through inosine monophosphate dehydrogenase (IMPDH) inhibition, which causes intracellular guanosine triphosphate (GTP) depletion.[9] Further, we demonstrated that adenosine kinase (ADK), which phosphorylates RBV in order to generate its monophosphorylated form and thereby gain the capacity to inhibit IMPDHs, was a key determinant for RBV sensitivity against HCV RNA replication.[10] Recently, we established RBV-resistant HCV RNA replicating cells from RBV-sensitive ones and found that acquisition of RBV resistance was mainly conferred by host factors and partially by viral factors,[11] suggesting that RBV is associated with several host factors and thus has the ability to exert effects on cellular metabolism.

RBV also has beneficial abilities for anticancer therapy. In patients with acute myeloid leukemia who have poor prognosis, RBV treatment led to substantial clinical benefit in a phase 2 study.[12] In this case, RBV or its metabolite RBV triphosphate appears to bind and inhibit eukaryotic translation initiation factor 4E, which is one of the oncogenes elevated in cancers, including many leukemias and lymphomas, and in turn impedes eukaryotic translation initiation factor 4E-mediated oncogenic effects. Recently, it was reported that human prostate cancer cells with high resistance to the anticancer drug docetaxel became sensitized to it by cotreatment with RBV, which reprogrammed the gene expression profile of highly malignant tumor cells to that of tumor cells with sensitivity to docetaxel.[13] That study suggested that combining RBV with docetaxel would be an applicable therapy for patients with prostate cancer and with high resistance to docetaxel.

As noted above, RBV has versatile functions through its targeting of cellular factors, suggesting that RBV exerts its antivirus and/or anticancer functions by affecting the metabolism of various cells. In this study, we found that RBV treatment suppressed the expression of lipogenic genes and concomitantly reduced the

Received February 2, 2017; accepted June 6, 2017.
Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1065/suppinfo.
Supported by a grant for Practical Research on Hepatitis (16668346) from the Japan Agency for Medical Research and Development.
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View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep4.1065
Potential conflict of interest: Nothing to report.

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levels of neutral lipids in hepatic cells. We also provide evidence that the mechanism of suppression of lipogenesis by RBV involves IMPDH inhibition, adenosine monophosphate-activated protein kinase (AMPK)-related kinases (AMPK-RKs), and down-regulation of retinoid X receptor α (RXRα).

Materials and Methods

CELL CULTURES

ADK-expressing HuH-7-derived cells, designated as OR6Ac cells, were generated from OR6-ADK cells[10] by curing HCV RNA through IFN-γ treatment (Supporting Fig. S1) and were maintained as described.[8] To examine the effect of reagents, such as RBV, cells were seeded into the collagen-coated wells with 30% to 50% confluency and were used for experiments on the following day. Cells were maintained for the indicated time.

REAGENTS

RBV, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), guanosine, and adenosine were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). An ADK inhibitor, ABT-702, was purchased from Calbiochem (San Diego, CA). Mycophenolic acid (MPA) was purchased from Sigma-Aldrich (St. Louis, MO). Mizoribine (MZB) was kindly provided by Asahi Kasei Pharma (Tokyo, Japan). Daclatasvir (DCV) and sofosbuvir (SOF) were purchased from Chemscene (Monmouth Junction, NJ).

REVERSE-TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION

Total RNAs were extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis for the messenger RNAs (mRNAs) of the selected genes was performed using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland) as described.[8] Primer sets used are listed in Supporting Table S1.

WESTERN BLOT ANALYSIS

The cells were harvested in cell lysis buffer (1% sodium dodecyl sulfate, 10 mM TrisHCl, pH 7.5) with a protease inhibitor cocktail (Roche Diagnostics). The measurement of protein concentrations, preparation of samples, and immunoblotting analysis were performed as described.[11] Rabbit anti-AMPKα, rabbit anti-phospho AMPKα (Thr172), rabbit anti-acetyl-coenzyme A carboxylase 1 (ACC1), rabbit anti-phospho ACC (Ser79), rabbit anti-microtubule affinity regulating kinase 3 (MARK3), and rabbit anti-MARK4 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-RXRα, mouse anti-salt inducible kinase 2 (SIK2), and mouse anti-MARK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-actin antibody (Sigma-Aldrich) was used as a control for the amount of protein loaded per lane.

RNA INTERFERENCE

The following small interfering RNAs (siRNAs) targeting AMPK, each AMPK-RK, or liver kinase B1 (LKB1) were purchased from Thermo Fisher Scientific (Waltham, MA): protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1) (M-005027-02), PRKAA 2 (M-005361-02), nua kinase family 1 (NUAK1) (M-004931-00), NUAK2 (M-005374-01), SIK1 (M-003959-05), SIK2 (M-001210-03), SIK3 (M-004779-03), MARK2 (M-004260-02), MARK3 (M-003517-03), MARK4 (M-005345-02), maternal embryonic leucine zipper kinase (MELK) (M-004029-01), sucrose non-fermenting-related kinase (SNRK) (M-004322-05), and LKB1 (M-005035-02). Nontargeting siRNAs (D-001206-13) were used as a control (designated as siCont). OR6Ac cells were transfected with the indicated siRNAs (1 nM) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA). One day after transfection of siRNAs, cells were treated with the indicated reagents and maintained for 48 or 72 hours. Cell toxicities were not observed in this condition. The knockdown efficiency of these siRNAs was determined by western blot analysis and/or RT-qPCR using the corresponding primers.

GENERATION OF OR6Ac CELLS STABLY EXPRESSING EXOGENOUS RXRα

The construction of a retrovirus vector plasmid for exogenous RXRα expression (pCX4pur/RXRα) is described in the Supporting Materials. The retrovirus vector for RXRα expression was introduced into the OR6Ac cells by retroviral transfer, and subsequently cells stably expressing exogenous RXRα (designated as OR6Ac-RXRα) were selected by puromycin (Sigma-Aldrich). pCX4pur plasmid[14] was also used for

generating the control puromycin-resistant OR6Ac cells (designated as OR6Ac-Ctl).

MEASUREMENT OF INTRACELLULAR NEUTRAL LIPIDS

Cells were treated with BODIPY493/503 (Thermo Fisher Scientific) for staining with neutral lipids, and then the fluorescence intensity in each cell was measured with a flow cytometer (FACS Calibur; BD Biosciences, Franklin Lakes, NJ) as described.[15] The mean intensities in cells were calculated relative to the level in vehicle-treated cells, which was set at 100%.

FLUORESCENCE MICROSCOPY OF BODIPY493/503-STAINED CELLS

OR6Ac cells seeded in the wells of a collagen-coated four-well chamber slide (Asahi Glass Co. Ltd., Japan) were treated with reagents and then stained with BODIPY493/503 (Thermo Fisher Scientific) and 4′6-diamidino-2-phenylindole (Sigma-Aldrich) for intracellular neutral lipids and nuclei, respectively. Cells were photographed under a fluorescent microscope (Axiophot; Carl Zeiss, Jena, Germany).

STATISTICAL ANALYSIS

Data are presented as the means ± SD from three or four independent experiments. Determination of the significance of differences among groups was assessed using the Student *t* test with a two-sided test. Values of $P < 0.05$ were considered statistically significant.

Results

RBV SUPPRESSED LIPOGENESIS IN HEPATIC CELLS

We previously demonstrated that ADK expression levels were a key determinant of the anti-HCV action of RBV.[10] Thus, we assumed that ADK expression levels would have some correlation with clinical factors in patients with CHC treated with therapies including RBV. We therefore examined the relationship between ADK expression levels in the liver and clinical factors of patients with advanced CHC treated with the combination of PEG-IFN and RBV for 48 weeks.[16] Liver biopsies of 91 patients for analysis of gene expression profiling were obtained before treatment as described.[16] The results revealed that the ADK expression level was significantly higher in patients with a body mass index ≤25 than those with a body mass index >25 (overweight patients) (Fig. 1A) and decreased with progression to advanced fibrosis stages (Supporting Fig. S2). These data prompted us to hypothesize that RBV, which is converted to its metabolically active form by ADK, affects cellular metabolic processes, such as lipogenesis or fibrogenesis, depending on the expression level of ADK.

Because the expression levels of ADK in the established human hepatic cell lines are significantly lower than levels in primary human hepatic cells,[10] we carried out the following experiments by using exogenously ADK-expressing hepatic cells. AMPK regulates various metabolic processes, including lipogenesis, depending on the energy status in cells.[17] Thus, we first examined the effect of RBV on AMPK activity in hepatic cells. AICAR was used as a positive control for AMPK activation. RBV at a concentration of 250 μM or greater induced AMPK activation as shown by the increase in the phosphorylation status of AMPKα and the AMPK-downstream effector ACC in OR6Ac cells, which are HCV RNA-cured HuH7-derived cells with exogenous ADK expression (Fig. 1B). We then examined the effect of RBV treatment on the mRNA expression levels of AMPK-downstream effectors, including lipogenic and cholesterogenic genes, whose expressions are down-regulated by AMPK activation.[17] Sterol regulatory element binding protein 1c (SREBP-1c) is a critical transcription factor for lipogenic genes, such as fatty acid synthase (*FASN)*, stearoyl coenzyme A desaturase (*SCD)*, and its own transcript.[18] Unexpectedly, these lipogenic genes were significantly decreased by RBV at a concentration of 25 μM despite the unresponsiveness of AMPK to this concentration of RBV, and further decreases in their expressions were not observed by RBV at higher concentrations (Fig. 1C), suggesting that RBV-induced suppression of lipogenic genes was independent of AMPK activation. We also examined the effects of lower concentrations of RBV on lipogenic genes and observed that lipogenic genes were decreased by RBV treatment in a dose-dependent manner within clinically achievable concentrations around 10 μM (Fig. 1D). In addition, we observed that down-regulation of lipogenic genes was seen at 24 hours after treatment with 10 μM RBV and was maintained for at least 72 hours (Fig. 1E). On the other

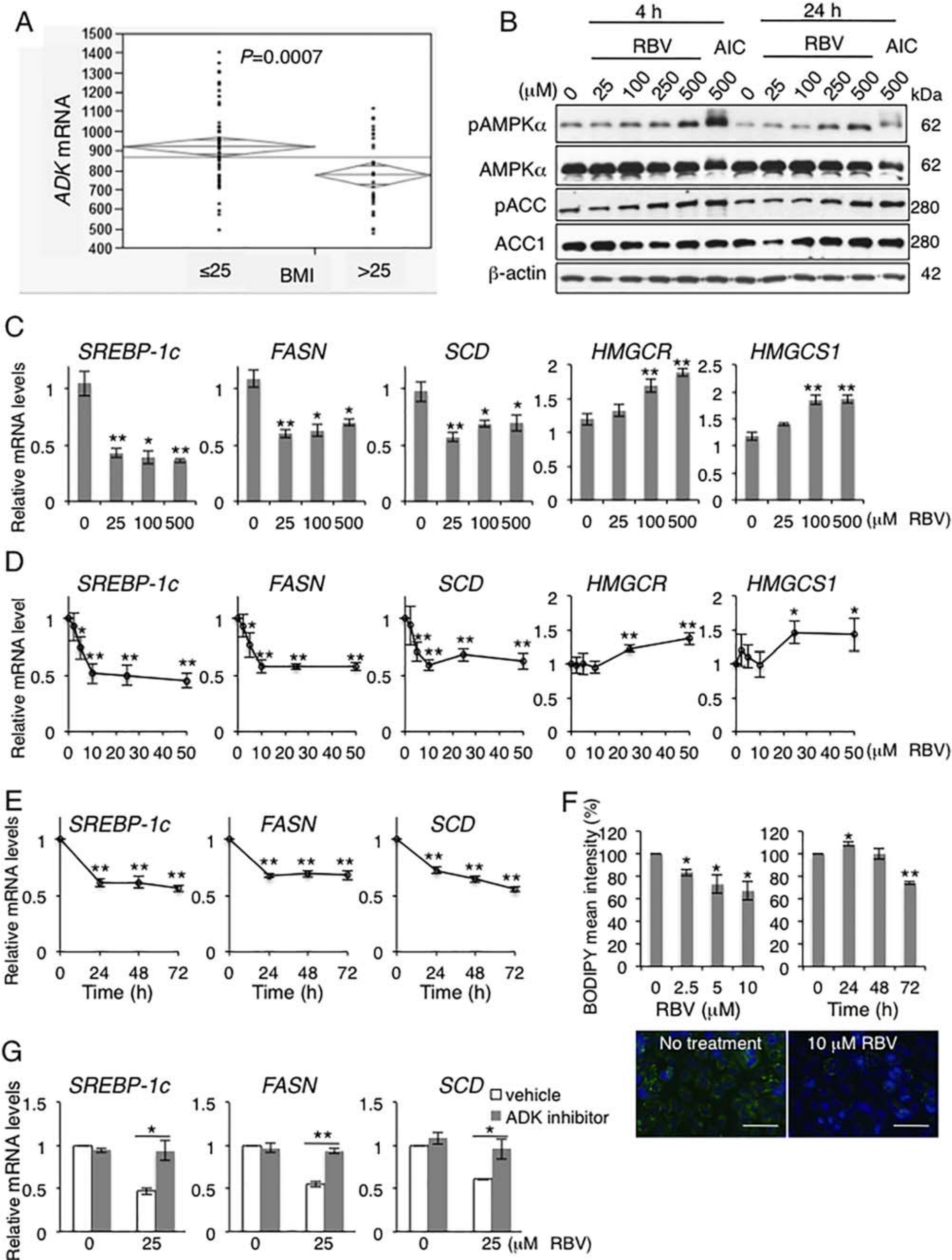

FIG. 1. Suppression of lipogenesis by RBV. (A) Relationship between *ADK* expression level and BMI in 91 patients with advanced CHC treated with a combination of PEG-IFN and RBV. (B) Effect of RBV on AMPK activation in OR6Ac cells. The cells were treated with RBV or AICAR for 4 or 24 hours. Cell lysates were used for western blot analysis with antibodies against phospho-Thr172-AMPKα, AMPKα, phospho-ACC, ACC1, and β-actin. (C,D) Dose-dependent effects of RBV on the expression of lipogenic or cholesterogenic genes in OR6Ac cells treated for 48 hours. The relative expression level of *SREBP-1c*, *FASN*, *SCD*, *HMGCR*, or *HMGCS1* mRNA was normalized by the level of *ATP5F1* mRNA and assigned a value of 1 in the nontreated OR6Ac cells. The results are expressed as the means ± SD of (C) three or (D) four independent experiments. *$P < 0.05$, **$P < 0.01$ versus nontreated cells. (E) Time course of RBV effect on lipogenic genes in OR6Ac cells treated with 10 μM RBV. The relative expression level of lipogenic gene mRNAs was analyzed as described for Fig. 1C,D. (F, upper panels) Effect of RBV on the level of intracellular neutral lipids in OR6Ac cells. The cells were treated with indicated concentrations of RBV for 72 hours (left) or with 10 μM RBV for indicated times (right) and then analyzed by staining with BODIPY493/503. The mean fluorescence intensities of BODIPY-stained cells were measured by a flow cytometer and calculated relative to the level in the nontreated OR6Ac cells, which was set at 100%. Error bars represent the means ± SD of three experiments. (F, lower panels) Representative microphotographs of OR6Ac cells treated with or without 10 μM RBV for 72 hours, showing cytoplasmic neutral lipids stained with BODIPY493/503 (green) and nuclei stained with DAPI (blue). Bars, 100 μm. (G) Effect of ADK inhibitor ABT-702 on RBV-induced suppression of lipogenic genes in OR6Ac cells. The cells were treated with or without 25 μM RBV along with 0.1% DMSO (vehicle) or 100 nM ADK inhibitor (ABT-702) for 48 hours. The relative expression level of lipogenic genes mRNA was analyzed as described for Fig. 1C,D. *$P < 0.05$, **$P < 0.01$ versus vehicle-treated cells. Abbreviations: AIC, AICAR; *ATP5F1*, ATP synthase, H+ transporting, mitochondrial Fo complex subunit B1; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; *HMGCR,* 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *HMGCS1,* 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1.

hand, the expression levels of cholesterogenic genes, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase and 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, were not significantly affected by RBV at less than 10 μM, while high-dose RBV (more than 25 μM) tended to increase their expression (Fig. 1C,D). An RBV dose-dependent decrease of lipogenic genes was also observed in ADK-expressing, nontransformed, immortalized hepatic cells, such as HuS-E/2-ADK and OUMS29-ADK cells (Supporting Fig. S3). To assess the effect of RBV on intracellular lipid metabolism, we stained the cells with BODIPY493/503 for intracellular neutral lipids and measured the signals using a flow cytometer. A dose-dependent decrease of the BODIPY signal intensity by RBV treatment was observed in OR6Ac cells (Fig. 1F, upper panels). We also confirmed this result from microphotographs showing a decrease of green fluorescence through accumulated intracellular neutral lipids in OR6Ac cells by treatment with 10 μM RBV (Fig. 1F, lower panels). These results were consistent with the effect of RBV on the expression of lipogenic genes (Fig. 1D) and suggest that RBV within clinically achievable concentrations inhibits hepatic lipogenesis. The suppression of lipogenic genes by RBV was abolished by cotreatment with an ADK inhibitor, ABT-702 (Fig. 1G). In addition, RBV did not suppress lipogenesis in OR6c cells, which express only marginal endogenous ADK[10] (Supporting Fig. S4), suggesting that ADK-mediated phosphorylation of RBV is required in this process.

GTP DEPLETION BY RBV LED TO THE SUPPRESSION OF LIPOGENESIS

To confirm that RBV-induced suppression of lipogenesis was independent of AMPK activation, we performed AMPK-knockdown experiments in which OR6Ac cells were transfected with a mixture of siRNAs targeting *PRKAA1 (AMPKα1)* and *PRKAA2 (AMPKα2)* and then treated with RBV (Supporting Fig. S5). Knockdown of *AMPKα* did not cancel the effect of RBV on lipogenic genes (Fig. 2A), consistent with the above results that the reduction in the expression of lipogenic genes was caused by RBV at concentrations lower than that activating AMPK (Fig. 1C,D). These results showed that RBV suppressed the expression of lipogenic genes in an AMPK-independent manner.

Because it is well known that RBV treatment causes GTP-depletion through IMPDH inhibition,[19] we next investigated whether GTP-depletion by RBV is involved in the suppression of lipogenic genes. For this purpose, OR6Ac cells were treated with RBV and guanosine to replenish GTP pools through the salvage pathway. The results showed that cotreatment with guanosine, but not adenosine, attenuated the suppression of lipogenic genes by RBV (Fig. 2B). To further examine the involvement of IMPDH inhibition by RBV, we tested other IMPDH inhibitors for suppression of lipogenic genes in OR6Ac cells. MZB, a guanosine analogue, is converted into a metabolically active form by ADK and inhibits IMPDH in a manner similar to RBV.[20] MPA is a nonnucleoside inhibitor of IMPDH.[20] The results revealed that increasing the concentrations of both IMPDH inhibitors led to a dose-dependent suppression of lipogenic genes (Fig. 2C,D). We also observed that MPA treatment induced a decrease in the BODIPY intensities in a similar manner as RBV treatment (Fig. 2E). These results indicate that GTP depletion leads to the suppression of lipogenesis.

RBV, MZB, and MPA are known to inhibit HCV RNA replication.[6,9,21,22] To exclude the possibility that inhibitors for HCV RNA replication generally induce the suppression of lipogenic genes, we tested the effects of DCV and SOF, DAAs targeting HCV NS5A and NS5B functions, respectively.[23,24] We examined the effects of two types of each DAA concentration, which have been reported to be equivalent to 50% and 90% inhibition of HCV RNA replication, in full-length HCV RNA-replicating OR6-ADK cells. Neither concentration of DCV or SOF suppressed the lipogenic genes (Fig. 2F), indicating that the suppression of lipogenic genes is not generally induced by inhibitors of HCV RNA replication.

AMPK-RKS WERE INVOLVED IN THE MECHANISM OF LIPOGENESIS SUPPRESSION BY RBV

Because our results showed that AMPK was not involved in the RBV action for suppressing lipogenesis, we next focused on the AMPK-RKs, which have recently been shown to regulate various types of metabolism. To date, 12 kinds of AMPK-RKs have been identified through their sequence homology with the protein kinase domain of AMPK, and eight kinds of kinases have been identified as distant relatives of AMPK.[25] Some of them, such as SIK1, SIK2, and SIK3, were reported to regulate lipid metabolism.[26-28]

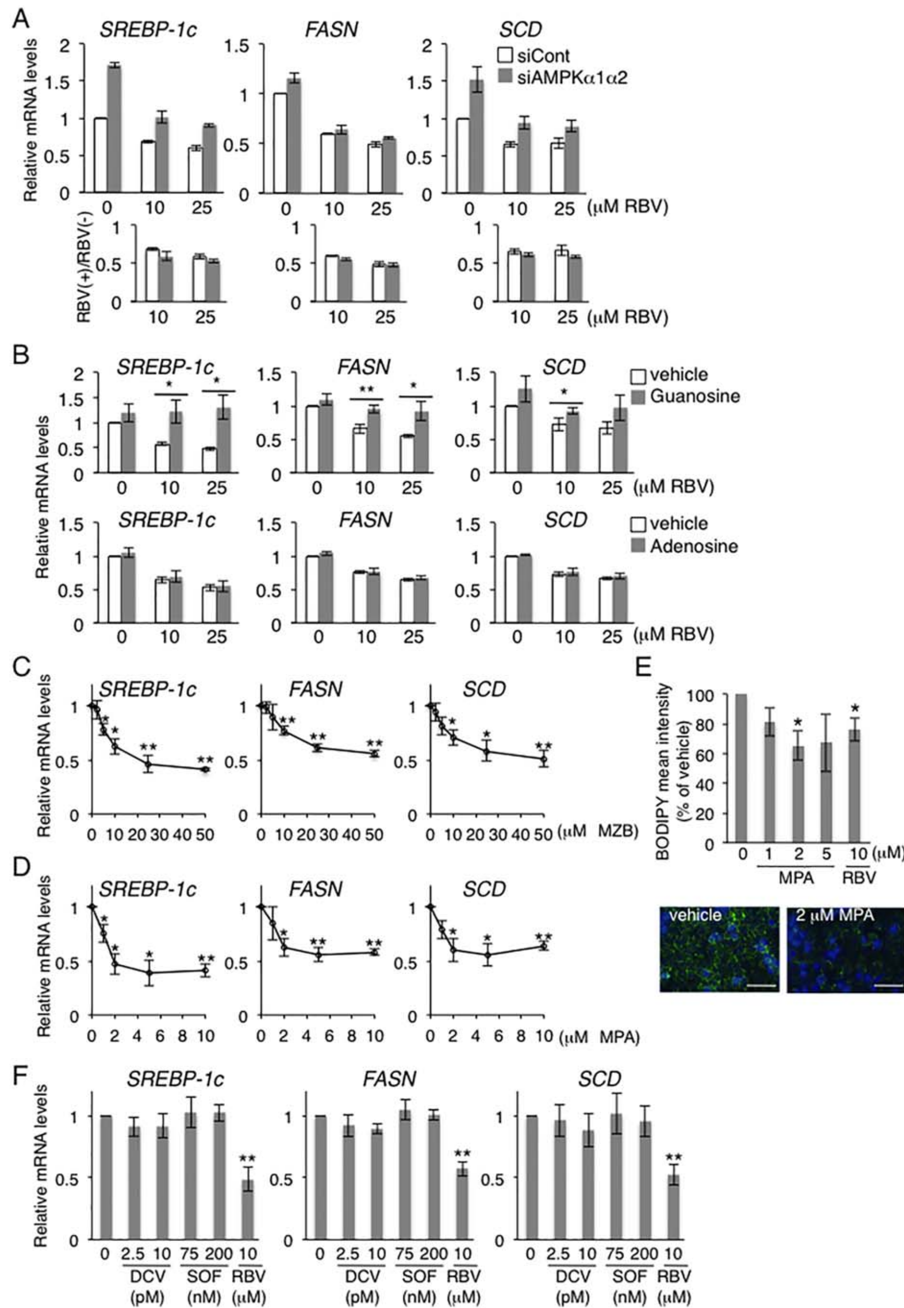

FIG. 2. GTP depletion by RBV contributed to the suppression of lipogenic genes. (A) Effects of *AMPKα1* and *AMPKα2* knockdown on the expression of lipogenic genes in OR6Ac cells. The cells were transfected with siCont or an siRNA mixture of siAMPKα1 and siAMPKα2 and then treated with indicated concentrations of RBV for 48 hours. The relative level of *SREBP-1c*, *FASN*, or *SCD* mRNA was normalized by the level of *ATP5F1* mRNA. The expression level of each mRNA in nontreated siCont-transfected cells was assigned a value of 1, and the results are shown in the upper panels. The ratio of the expression level of each lipogenic gene in the treated cells RBV(+) to that in the nontreated cells RBV(–) is also shown in the lower panels. (B) Effect of guanosine (upper panels) or adenosine (lower panels) on the RBV-induced suppression of lipogenic genes in OR6Ac cells. The cells were treated with indicated concentrations of RBV along with vehicle, 100 μM guanosine, or 100 μM adenosine for 48 hours. DMSO or dH_2O was used as vehicle for guanosine or adenosine, respectively. The relative level of the mRNA of each gene was normalized by the level of *ATP5F1* mRNA and assigned a value of 1 in the vehicle-treated OR6Ac cells. (C,D) Dose-dependent effects of (C) MZB and (D) MPA on the expression of lipogenic genes in OR6Ac cells. The experiments were performed as described in Fig. 1D. (E, upper panels) Effect of MPA and RBV on the level of intracellular neutral lipids in OR6Ac cells. The cells were treated with indicated concentrations of MPA or RBV for 72 hours and then analyzed by staining with BODIPY493/503. The mean fluorescence intensities of BODIPY-stained cells were measured by a flow cytometer and calculated relative to the level in the nontreated OR6Ac cells, which was set at 100%. (E, lower panels) Representative microphotographs of OR6Ac cells treated with or without 2 μM MPA for 72 hours, showing cytoplasmic neutral lipids stained with BODIPY493/503 (green) and nuclei stained with DAPI (blue). Bars, 100 μm. (F) Effect of DCV or SOF on the expression of lipogenic genes in OR6Ac cells treated for 48 hours. The experiments were performed as described in Fig. 1D. Error bars (A-F) represent the means ± SD of three experiments. *$P < 0.05$, **$P < 0.01$ versus vehicle-treated cells. Abbreviations: *ATP5F1*, ATP synthase, H+ transporting, mitochondrial Fo complex subunit B1; DAPI, 4',6-diamidino-2-phenylindole; dH_2O, distilled water; DMSO, dimethylsulfoxide.

From these facts, we assumed that one or more AMPK-RKs regulate the activity by which RBV suppresses lipogenesis. To examine this possibility, we first assessed the effects of siRNAs targeting each AMPK-RK on the RBV action. The results revealed that, among the 10 AMPK-RKs that appeared to be expressed in our hepatic cell lines based on the data from our complementary DNA microarrays (unpublished data), siRNAs targeting *MARK2, MARK4,* and *MELK* significantly desuppressed both *FASN* and *SCD* expressions in OR6Ac cells treated with 10 μM RBV (Supporting Fig. S6). In addition, *SREBP-1c* expression tended to be desuppressed by siRNAs targeting these three kinases, although the levels of desuppression were not significantly different from those by the siCont. The mean rates of cancellation of the RBV-induced suppression of the three lipogenic genes were higher in cells transfected with siMARK4 than in those transfected with the other siAMPK-RKs. Accordingly, we thereafter focused on the MARK4 kinase. Knockdown of MARK4 significantly attenuated the RBV (10 μM)-induced suppression of all three lipogenic genes (Fig. 3A). We next examined the effect of siMARK4 on the amount of intracellular neutral lipids and found that the decrease in the BODIPY signal intensity in the cells treated with RBV was cancelled by knockdown of MARK4 in correlation with its effect on lipogenic genes (Fig. 3B). These results suggest that MARK4 plays critical roles in the RBV-induced suppression of lipogenesis. Moreover, we showed that knockdown of MARK4 also significantly cancelled the MPA-induced suppression of *FASN* and *SCD* expressions and slightly desuppressed *SREBP-1c* expression (Fig. 3C). These results suggest that MARK4 contributes to the mechanism for suppressing lipogenesis, at least in part by affecting the region downstream of IMPDH.

All AMPK-RKs except for MELK are activated by upstream kinase LKB1 in the same manner as AMPK.[25] Thus, we assessed the involvement of LKB1 in the mechanism of lipogenesis suppression by RBV. As shown in Fig. 3D, knockdown of LKB1 significantly attenuated the RBV-induced suppression of lipogenic genes in OR6Ac cells. In addition, the results revealed that the basal expression levels of each lipogenic gene were up-regulated by knockdown of LKB1. These results suggest that LKB1 plays some role in the regulation of lipogenic genes, possibly through LKB1-mediated activation of AMPK-RKs or other molecules, which contributes to the suppression of lipogenic genes.

RBV TREATMENT LED TO THE DOWN-REGULATION OF RXRα, CONTRIBUTING TO THE SUPPRESSION OF LIPOGENESIS

To clarify whether the expression levels of transcription factors directly regulating lipogenic genes were affected by RBV treatment, we first examined the expression levels of liver X receptors (LXRs), which are known to regulate the transcripts of genes, including *SREBP-1c, FASN,* and *SCD*.[29-33] The results revealed that the expression levels of *LXRα* and *LXRβ* were not altered by RBV at concentrations less than 10 μM, while *LXRα* and *LXRβ* were significantly increased by RBV at 50 μM and more than 10 μM, respectively (Fig. 4A, left panels). These results imply that the kinetics of LXR expression are not involved in the effects of RBV on lipogenesis. We next examined the expression levels of the RXRs, which are known to heterodimerize with LXRs for transcriptional activity.[34] Among the three RXRs examined, RXRα and RXRβ but not RXRγ were detected at the transcriptional levels in OR6Ac cells. RXRα mRNA was decreased in a dose-dependent manner, while RXRβ mRNA tended to be slightly increased (Fig. 4A, right panels). Further, western blot analysis confirmed that RXRα protein was also decreased in a time- and concentration-dependent manner (Fig. 4B). Interestingly, the level of RXRα mRNA gradually decreased until 12 hours after treatment with 10 μM RBV, but after that, the level of RXRα mRNA recovered toward a normal level (Fig. 4C). On the other hand, recovery of RXRα protein was not observed until 48 hours (Fig. 4B,D). These data suggested that RBV treatment suppresses RXRα expression by both transcriptional and posttranscriptional mechanisms. RBV-induced reduction of RXRα protein was also seen in other hepatic cells, such as HuS-E/2-ADK, OUMS29-ADK, and PH5CH8-ADK (Supporting Fig. S7). We observed that reduction of RXRα protein by RBV treatment was attenuated in the presence of guanosine (Fig. 4E) but not adenosine (Fig. 4F), and MPA treatment also reduced the RXRα protein (Fig. 4G). Taken together, these results suggest that GTP depletion by RBV specifically leads to the down-regulation of RXRα. We next examined whether MARK4 was involved in this down-regulation of RXRα. The results revealed that knockdown of MARK4 attenuated RBV activity for down-regulation of RXRα at both the mRNA and protein levels (Fig. 4H), suggesting that RBV-induced down-regulation of RXRα is under the control of MARK4.

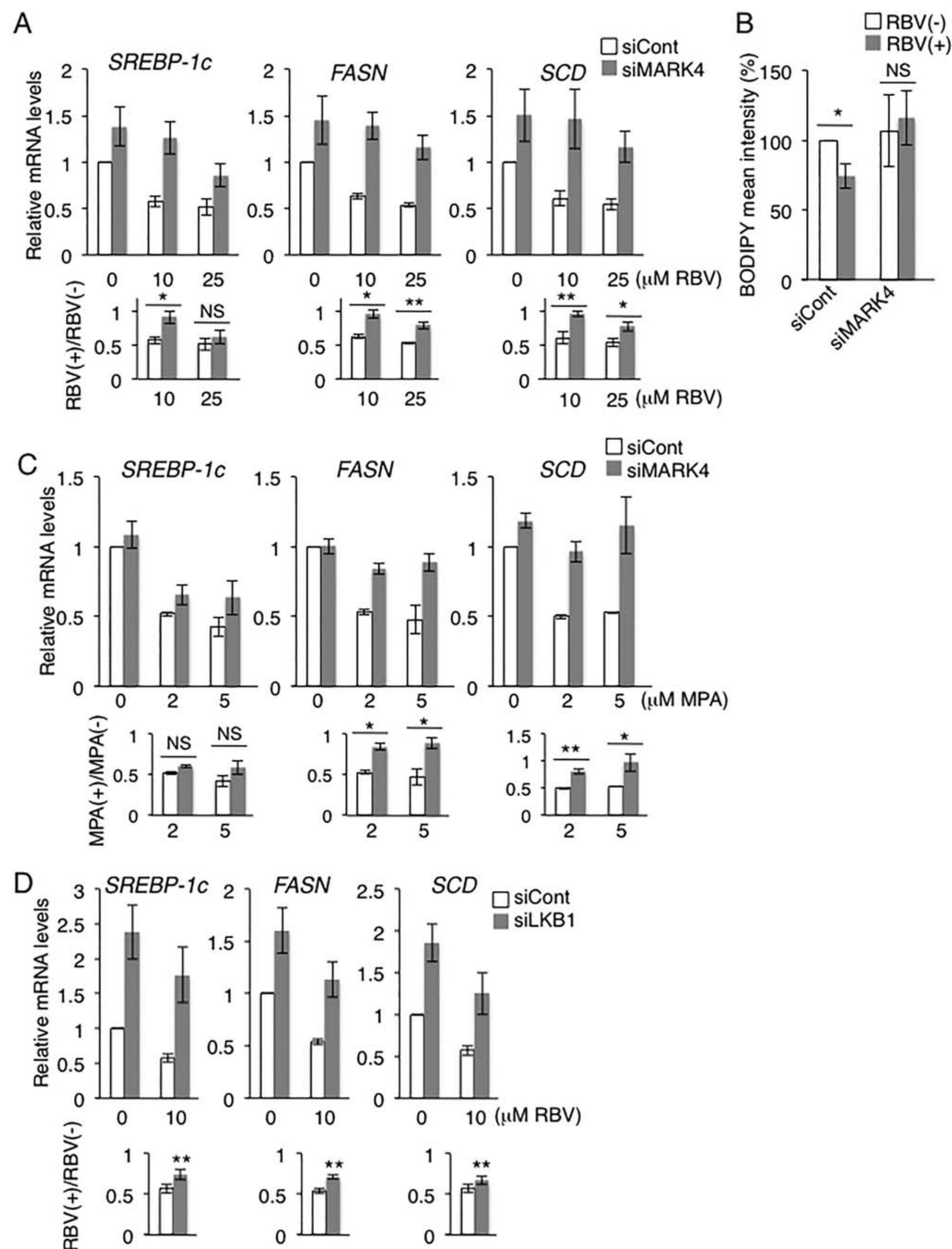

FIG. 3. MARK4 was required for the RBV-induced suppression of lipogenesis. (A) Effect of the knockdown of *MARK4* on RBV-induced suppression of lipogenic genes in OR6Ac cells. The cells were transfected with siCont or siMARK4 and then treated with RBV for 48 hours. The results are presented as described in Fig. 2A. (B) Effect of *MARK4* knockdown on the intracellular neutral lipids in OR6Ac cells. The cells were transfected with siCont or siMARK4 and then treated with or without 10 μM RBV for 72 hours. The mean fluorescence intensities of BODIPY-stained cells were measured by a flow cytometer and calculated relative to the level in nontreated siCont-transfected cells, which was set at 100%. (C) Effect of *MARK4* knockdown on the MPA-induced suppression of lipogenic genes in OR6Ac cells. The cells were transfected with siCont or siMARK4 and then treated with or without MPA for 48 hours. The results are presented as described in Fig. 2A. (D) Effect of the knockdown of *LKB1* on lipogenic genes in OR6Ac cells. The cells were transfected with siCont or siLKB1 and then treated with or without 10 μM RBV for 48 hours. The results are shown as described in Fig. 2A. Error bars (A-D) represent the means ± SD of three experiments. *$P < 0.05$, **$P < 0.01$. Abbreviation: NS, not significant.

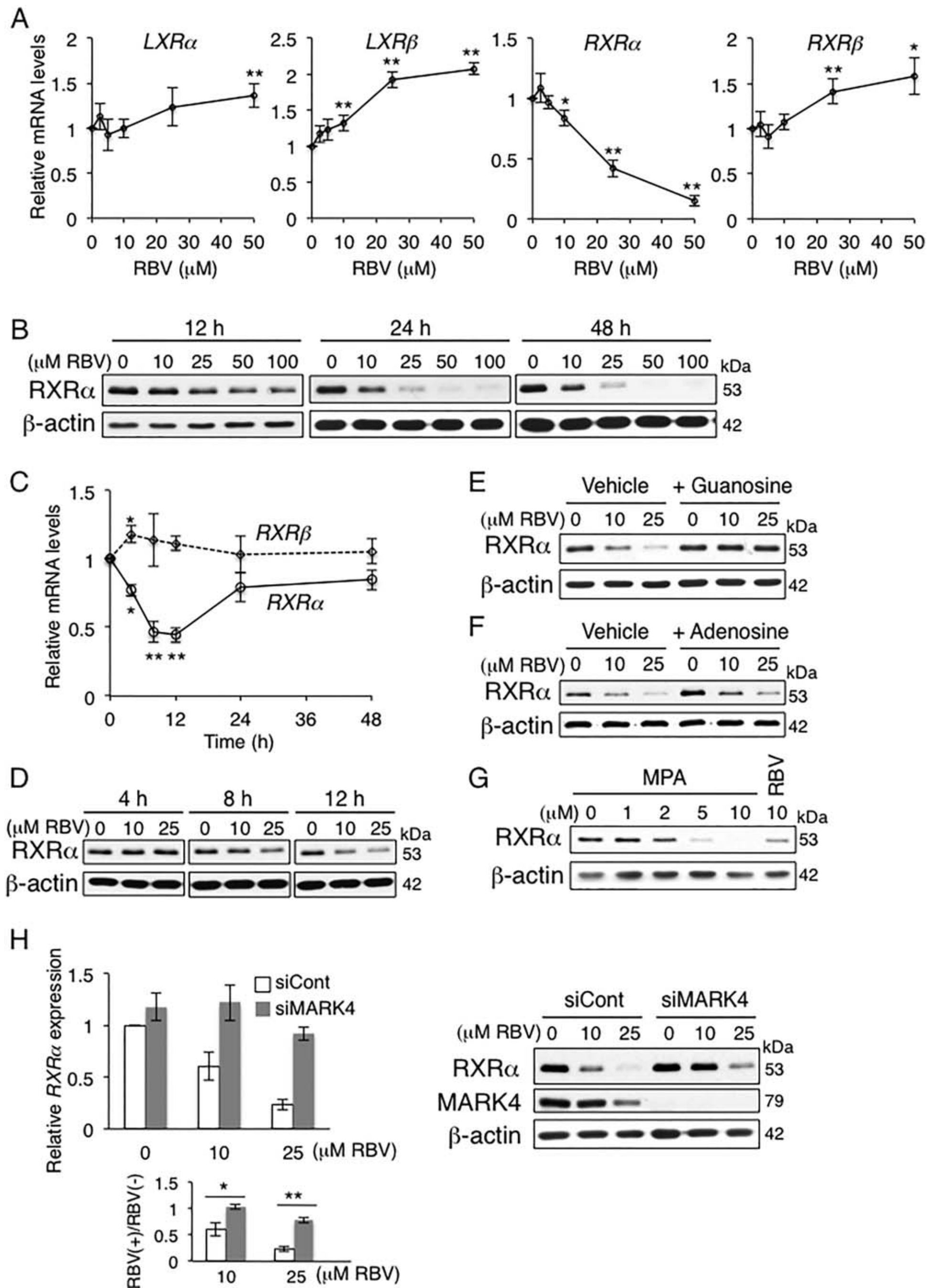

FIG. 4. Down-regulation of RXRα by RBV treatment. (A) Dose-dependent effects of RBV on the expression of *LXRα*, *LXRβ*, *RXRα*, or *RXRβ* mRNA in OR6Ac cells treated for 48 hours. The relative mRNA level of each gene was shown as described in Fig. 1D. (B) Time- and dose-dependent effects of RBV on RXRα protein. OR6Ac cells were treated with RBV for 12, 24, or 48 hours and then prepared for western blot analysis by using anti-RXRα and anti-β-actin antibodies. (C) OR6Ac cells treated with 10 μM RBV for 4, 8, 12, 24, or 48 hours were analyzed for *RXRα* or *RXRβ* expression. The relative mRNA level of each gene is presented as described in Fig. 1D. (D) OR6Ac cells were treated with RBV for 4, 8, or 12 hours and then prepared for western blot analysis by using anti-RXRα and anti-β-actin antibodies. (E-G) Role of GTP depletion in RBV-induced down-regulation of RXRα. OR6Ac cells were treated with RBV along with vehicle, (E) 100 μM guanosine, or (F) 100 μM adenosine for 24 hours or (G) treated with MPA for 48 hours, and then cell lysates were prepared for western blot analysis by using anti-RXRα and anti-β-actin antibodies. DMSO or dH_2O was used as vehicle for guanosine or adenosine, respectively. (H) Effect of *MARK4* knockdown on RBV-induced down-regulation of RXRα mRNA and protein in OR6Ac cells. The cells were transfected with siCont or siMARK4 and then treated with RBV for 48 hours. Cell lysates were prepared for analysis of RXRα mRNA expression (left panels) and protein (right panels). The relative level of *RXRα* mRNA is presented as described in Fig. 2A (left panels). (A,C,H) Error bars represent the means ± SD of three experiments. *$P < 0.05$, **$P < 0.01$ versus nontreated cells. Abbreviations: dH_2O, distilled water; DMSO, dimethylsulfoxide.

From these results, we assumed that RBV-induced down-regulation of RXRα was associated with the suppression of various lipogenic genes that are known to be regulated by RXRs. To test this assumption, we examined whether ectopic expression of RXRα canceled the effect of RBV on the expression of lipogenic genes. The results revealed that RBV treatment did not suppress *SREBP-1c* expression in OR6Ac-RXRα cells (Fig. 5A,B). In addition, RBV-induced suppression of *FASN* and *SCD* expressions in OR6Ac-RXRα cells was also attenuated compared to that in OR6Ac-Ctl cells (Fig. 5B). Consistent with these results, overexpression of RXRα attenuated the suppression of intracellular neutral lipids by RBV (Fig. 5C). Taken together, these results suggest that the down-regulation of RXRα by RBV treatment is required to suppress hepatic lipogenesis.

Discussion

RBV has versatile functions and is used as an antiviral reagent. However, to our knowledge, it is not known whether RBV affects lipid metabolism. The results of the present study demonstrated that RBV possessed the ability to suppress lipogenesis in hepatic cells. We further found that GTP depletions by RBV and AMPK-RKs, especially MARK4, are key to the suppression of hepatic lipogenesis. Moreover, our results provide new insight that down-regulation of RXRα is under the control of these factors and is involved in RBV-induced suppression of lipogenesis.

Because AMPK activation is known to play critical roles in the suppression of lipogenesis[17] and because RBV, like the AMPK agonist AICAR, is a nucleotide analogue, initially we thought that RBV activated AMPK. Indeed, we were able to observe that a high dose (more than 100 μM) of RBV did activate AMPK; however, treatment with physiological concentrations (less than 10 μM) of RBV, which were sufficient to suppress hepatic lipogenesis, did not activate AMPK. RBV concentration in the liver may be substantially high because RBV tends to concentrate intracellularly and hepatocytes have RBV transporters[35,36] and exhibit high ADK expression.[37] Therefore, it remains possible that AMPK activation by RBV contributes to the suppression of lipogenesis through AMPK target molecules, such as ACC. In this case, inactivation of ACC by AMPK-mediated phosphorylation may result in the prevention of lipid synthesis and stimulation of β-oxidation.[38]

AMPK-RKs are considered to regulate various types of metabolism, including lipogenesis.[25] Among the three SIK family members, SIK1, SIK2, and SIK3, two of the family members, SIK1 and SIK2, were shown to suppress lipogenesis in hepatocytes.[26,27] In our screening assay using siRNAs targeting each AMPK-RK, knockdown of SIK1 or SIK2 partially attenuated the effects of RBV on lipogenic genes. Although MARK4 seems to strongly contribute to the suppression of lipogenesis in OR6Ac cells treated with RBV, other AMPK-RKs are also functional, and the degree of contribution of each kinase to the regulation of lipogenesis may be different among cell lines or tissues.

At an RBV concentration of 10 μM, RXRα mRNA was temporally down-regulated and then returned to normal levels, while down-regulation of the RXRα protein level was sustained for at least 48 hours posttreatment, suggesting that RBV-mediated down-regulation of RXRα is controlled by both transcriptional and posttranscriptional mechanisms. Previously, Chow et al.[39] demonstrated that viral infections suppressed RXRα mRNA through an interferon regulatory factor 3 (IRF3)-dependent pathway in the mouse liver. In addition, they showed that IRF3 activation increased the expression of the transcriptional suppressor Hes1, which directly binds to and recruits the histone deacetylase 1 complex on the RXRα promoter.[39] In our present experiments, we examined whether RBV treatment activated IRF3 and induced Hes1 expression. However, we did not observe any effect of RBV treatment in OR6Ac cells (data not shown). The RXRα protein level is known to be regulated by microRNAs (miRNAs), such as miR-27a[40,41] and miR-34a,[42] suggesting that these miRNAs may regulate RXRα expression in hepatic cells treated with RBV.

Lipogenesis is required for tumor progression because large amounts of lipids are necessary not only for aberrant cell proliferation in tumors but also for transformed phenotypes, such as resistance to apoptosis, survival under energy stress, and invasion.[43] We observed that proliferation of OR6Ac cells was suppressed by RBV, although cell viability was not significantly affected (Supporting Fig. S8), suggesting that RBV-mediated suppression of lipogenesis leads to the inhibition of hepatoma cell proliferation. Overexpression of FASN or SCD, enzymes that catalyze the synthesis of fatty acids, was shown to contribute to cancer progression.[43] In our study, we found that RBV suppressed *FASN* and *SCD* expression. Thus, RBV may

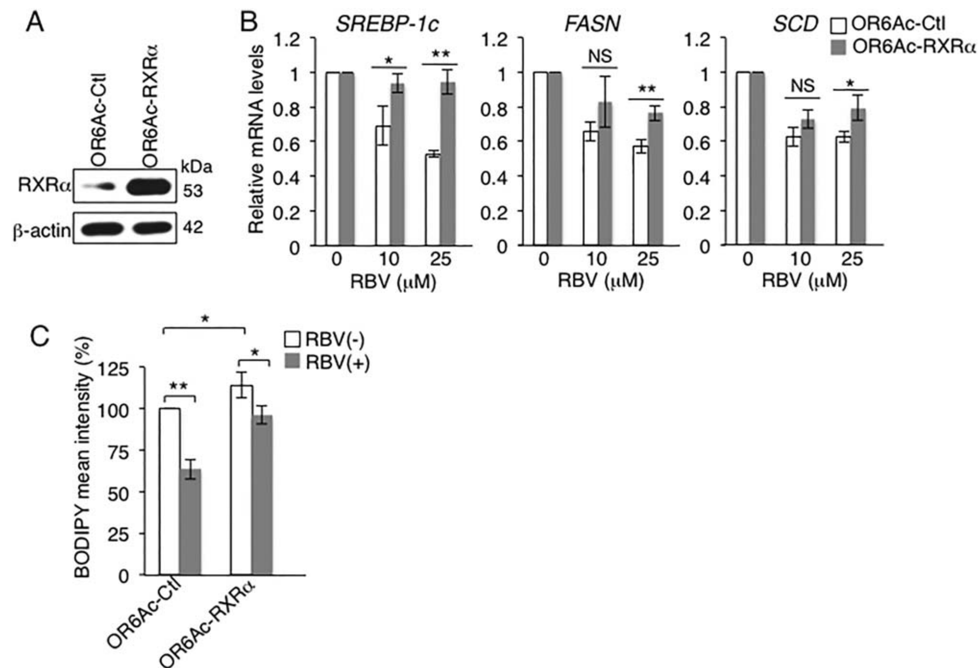

FIG. 5. Down-regulation of RXRα by RBV treatment was required for suppression of lipogenesis. (A) OR6Ac cells with exogenously expressing RXRα (OR6Ac-RXRα) or control OR6Ac cells (OR6Ac-Ctl). The cell lysates were prepared for western blot analysis and analyzed for the expression of RXRα. (B) Effect of RBV on the expression of lipogenic genes in OR6Ac-Ctl or OR6Ac-RXRα cells. The cells were treated with RBV for 48 hours, and then total RNA was prepared for analysis of the lipogenic gene expressions. The relative level of each lipogenic gene in each cell type is presented as described in Fig. 1C. (C) Effect of RBV on the level of intracellular neutral lipids in OR6Ac-Ctl or OR6Ac-RXRα cells. Cells treated with or without 10 μM RBV for 72 hours were analyzed by staining with BODIPY493/503 as described in Fig. 1E. The mean fluorescence intensities of BODIPY-stained cells were calculated relative to the level in nontreated OR6Ac-Ctl cells, which was set at 100%. Error bars represent the means ± SD of (B) three or (C) four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: NS, not significant.

become a potential reagent against tumorigenesis. Previously, we identified several host genes that were irreversibly altered by long-term replication of HCV RNA in cell culture. Among these identified genes, potential oncogenic or tumor suppressor genes were also included.[44] Although we have not yet clarified the effect of these genes on tumorigenesis, we are interested in whether RBV is able to alter the expression or function of these genes. Thus, we consider the possibility that RBV affects not only lipogenic genes but also other oncogenic or tumor suppressor genes, which may expand the availability of RBV for various cancer therapies.

Recently, many DAAs targeting HCV proteins have been developed, and IFN-free or RBV-free regimens with these DAAs have been shown to achieve a high cure rate.[4] However, there is a possibility that cancer stem cells had already been generated before HCV eradication, especially in patients with chronic hepatitis for a long duration or progressive fibrosis stages. DAAs have specific anti-HCV activity, and thus they may not directly affect cellular metabolism, suggesting that DAAs are not able to suppress hepatocarcinogenesis. Therefore, it might be possible that tumors will develop in the future even after HCV elimination when only a DAA regimen is used. Much like the reports demonstrating that IFN treatment has the ability to suppress the growth of hepatocellular carcinoma,[45,46] our new finding that RBV has the ability to suppress lipogenesis in hepatic cells will shed light once again on the beneficial abilities of RBV not only for antiviral but also antipathogenic treatments of liver diseases, such as hepatic steatosis or hepatocellular carcinoma, in which aberrant lipogenesis is involved.

Acknowledgment: We thank Rimi Nonoyama and Takashi Nakamura for their technical assistance.

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