Metformin Inhibits Hepatic Gluconeogenesis Through AMP-Activated Protein Kinase–Dependent Regulation of the Orphan Nuclear Receptor SHP

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OBJECTIVE—Metformin is an antidiabetic drug commonly used to treat type 2 diabetes. The aim of the study was to determine whether metformin regulates hepatic gluconeogenesis through the orphan nuclear receptor small heterodimer partner (SHP; NR0B2).

RESEARCH DESIGN AND METHODS—We assessed the regulation of hepatic SHP gene expression by Northern blot analysis with metformin and adenovirus containing a constitutive active form of AMP-activated protein kinase (AMPK) (Ad-AMPK) and evaluated SHP, PEPCK, and G6Pase promoter activities via transient transfection assays in hepatocytes. Knockdown of SHP using siRNA SHP was conducted to characterize the metformin-induced inhibition of hepatic gluconeogenic gene expression in hepatocytes, and metformin– and adenovirus SHP (Ad-SHP)–mediated hepatic glucose production was measured in B6-*Lep*$^{ob/ob}$ mice.

RESULTS—Hepatic SHP gene expression was induced by metformin, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), and Ad-AMPK. Metformin-induced SHP gene expression was abolished by adenovirus containing the dominant negative form of AMPK (Ad-DN-AMPK), as well as by compound C. Metformin inhibited hepatocyte nuclear factor-4α– or FoxA2-mediated promoter activity of PEPCK and G6Pase, and the inhibition was blocked with siRNA SHP. Additionally, SHP knockdown by adenovirus containing siRNA SHP inhibited metformin-mediated repression of cAMP/dexamethasone-induced hepatic gluconeogenic gene expression. Furthermore, oral administration of metformin increased SHP mRNA levels in B6-*Lep*$^{ob/ob}$ mice. Overexpression of SHP by Ad-SHP decreased blood glucose levels and hepatic gluconeogenic gene expression in B6-*Lep*$^{ob/ob}$ mice.

CONCLUSIONS—We have concluded that metformin inhibits hepatic gluconeogenesis through AMPK-dependent regulation of SHP. ***Diabetes*** **57:306–314, 2008**

Metformin (1,1-dimethylbiguanide hydrochloride) is a hypoglycemic agent used extensively for the treatment of type 2 diabetes (1–2). Metformin is a biguanide agent, as are buformin and phenformin. The principal function of metformin is to reduce hepatic glucose production and to improve peripheral insulin sensitivity, thus ameliorating hyperglycemia (3–4). Metformin has also been shown to enhance glucose uptake in the muscles (5), while reducing levels of plasma triglycerides (6) and nonesterified fatty acids (7). Metformin has been shown to activate AMP-activated protein kinase (AMPK) via an LKB1-dependent mechanism (1–2,8).

AMPK is a well-known serine/threonine kinase that functions as an intracellular energy sensor and has been implicated in the modulation of glucose and fatty acid metabolism (8–9). AMPK is activated by physiological stimuli including exercise, muscle contraction, and hormones such as adiponectin and leptin, as well as by physiological stresses, glucose deprivation, hypoxia, oxidative stress, and osmotic shock conditions (10–11). Once activated, AMPK inhibits gluconeogenesis and lipogenesis while promoting both fatty acid oxidation and lipolysis. Activation of AMPK by the AMPK activators 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and metformin has been shown to inhibit the expression of two key hepatic gluconeogenic genes, PEPCK and G6Pase, which, in turn, suppresses gluconeogenesis (12–13). However, the molecular mechanism underlying the AMPK-mediated transcriptional regulation of PEPCK and G6Pase has not been fully elucidated.

The small heterodimer partner (SHP; NR0B2) is an atypical orphan nuclear receptor that lacks a conventional DNA-binding domain and consists only of a putative ligand-binding domain (14). SHP represses the transcriptional activity of a number of nuclear receptors (15–22), as well as hepatocyte nuclear factor (HNF)-4α (NR2A1), FoxO1 (also known as FKHR) (23), and the forkhead transcription factor FoxA2 (also referred to as HNF-3β)

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ACC, acetyl-CoA carboxylase; Ad-AMPK, adenovirus AMPK; Ad-DN-AMPK, adenovirus dominant negative form of AMPK; Ad-SHP, adenovirus SHP; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CA-AMPK, constitutively active AMPK; DMEM, Dulbecco's modified Eagle's medium; DN-AMPK, dominant negative mutant AMPK; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HNF, hepatocyte nuclear factor; LRH-1, liver receptor homolog-1; SF-1, steroidogenic factor-1; SHP, small heterodimer partner.

(24), which are the primary transcription factors that regulate PEPCK and/or G6Pase expression. Recent evidence suggests that SHP may perform a function in glucose homeostasis by the regulation of hepatic gluconeogenesis. Yamagata et al. (23) showed that the expressions of hepatic gluconeogenic genes, including PEPCK, G6Pase, and fructose 1,6-bis phosphatase (FBP1), are inhibited by bile acids in a SHP-dependent manner. In our previous study, it was shown that SHP inhibits PEPCK, G6Pase, and CYP7A1 expression via the repression of FoxA2-mediated transcriptional activity (24). However, no study has examined whether SHP expression is induced by pharmacological agents, such as the AMPK activators that suppress hepatic gluconeogenic genes, or whether metformin-induced suppression of hepatic gluconeogenic gene expression is mediated by SHP.

In this study, we investigated whether metformin induces hepatic SHP gene expression and whether this effect is mediated by AMPK. In addition, we have attempted to ascertain whether metformin-induced inhibition of hepatic gluconeogenesis is consequently mediated by SHP.

RESEARCH DESIGN AND METHODS

Chemicals. Metformin (1,1-dimethylbiguanide hydrochloride; Sigma, St. Louis, MO), 8-bromoadenosine 3,5-cyclic monophosphate (Sigma), dexamethasone (Sigma), and insulin (Norvolin R; Green Cross, Yongin, Korea) were dissolved in the manufacturer-recommended solvents.

Plasmids and DNA constructions. The siRNAs of human SHP (24) and the plasmids encoding for the dominant negative mutant AMPK (DN-AMPK) and a constitutively active form of AMPK (CA-AMPK or α-312 AMPK) (25) have been previously described. The reporter plasmids encoding for the human G6Pase promoter (−1,227/+57) and the rat PEPCK promoter (−2000/+73) were generously provided by Dr. D. Schmoll (26) and Dr. R.W. Hanson (27), respectively. Mouse G6Pase cDNA was purchased from Korea UniGene Information (Kugi, Korea), and mouse PEPCK cDNA was provided by Dr. J.B. Kim (Department of Biological Sciences, Seoul, Korea). Human, rat, and mouse SHP cDNAs (28) and human and mouse SHP promoters (24) were prepared as described previously. Liver receptor homolog-1 (LRH-1), steroidogenic factor-1 (SF-1) cDNA, and the sft4 reporter gene were prepared as described previously (17,20,28).

Preparation of recombinant adenovirus. An adenovirus encoding for full-length human SHP (24), c-Myc–tagged DN-AMPK, and CA-AMPK (25) have been previously described. To block the expression of SHP, an adenovirus-mediated siRNA for SHP was constructed. Briefly, the cDNA sequence (^{239}GACAGTAGCCTTCCTCAGGAA^{259}) of an siRNA specific to mouse SHP was incorporated into the pAdTrack-CMV shuttle vector. The vector construct was then electroporated into BJ5138 cells, and a recombinant vector was generated using the AdEasy adenoviral vector system. The recombinant viruses were amplified in HEK-293 cells and isolated via cesium chloride density centrifugation (Sigma). The viruses were collected and desalted, and the titers were measured using Adeno-X Rapid titer (BD Bioscience, San Jose, CA) according to the manufacturer's instructions.

Cell culture and transient transfection assays. HepG2 (human hepatoma) and H4IIE (rat hepatoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. AML12 (mouse hepatocyte) cells were cultured with DMEM/F-12 medium (Gibco-BRL) supplemented with insulin-transferrin-selenium (ITS; Gibco-BRL) and dexamethasone (40 ng/ml; Sigma) and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. Transient transfections were conducted using LipofectAmine 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The total amount of DNA was adjusted to 1 μg/well via the addition of the appropriate amount of pcDNA3 empty vector, and 0.2 μg cytomegalovirus β-galactosidase plasmids were cotransfected as an internal control. After 24 h of transfection, the cells were incubated for 12 h in the presence or absence of metformin and subsequently harvested. The luciferase activity was measured and then normalized to the β-galactosidase activity. The data are representative of a minimum of three to five independent experiments.

Isolation and culture of primary rat hepatocytes. Rat primary hepatocytes were isolated from the livers of 7-week-old male Sprague-Dawley rats. The rats were anesthetized with sodium pentobarbital (50 mg/kg body wt), and the livers of the animals were exposed surgically. The liver was first perfused with resuspension buffer (150 mmol/l NaCl, 10 mmol/l KCl, 1 mmol/l $NaH_2PO_4{\cdot}2H_2O$, 0.8 mmol/l $NaH_2PO_4{\cdot}12H_2O$, 1 mmol/l EGTA, 0.8 mmol/l $NaHCO_3$, 10 mmol/l D-glucose, and 10 mmol/l HEPES, pH 7.2) at 30–50 ml/min for 5–10 min and then perfused with collagenase solution (9.5 g/l Hank's balanced salt solution, 0.8 mmol/l $NaHCO_3$, 0.9 mmol/l $CaCl_2{\cdot}2H_2O$, 10 mmol/l HEPES, and 0.5 g/l collagenase IA [Sigma], and 50 mg/l trypsin inhibitor, pH 7.5) at 30–50 ml/min for 5–10 min. After perfusion, the liver was chopped finely in a Petri dish and then filtered through an 85-μm pore mesh. The hepatocytes were collected by centrifugation at 800 rpm for 2–5 min at 4°C. The viability of the hepatocytes was measured by trypan blue exclusion and was consistently in excess of 85%. All hepatocytes were seeded onto collagen type 1–coated 60-mm dishes (1×10^6 cells/ml; IWAKI Scitech Div, Tokyo, Japan) in William E medium (Sigma). After 6 h of incubation, the medium was exchanged with DMEM without serum, and the hepatocytes were utilized for Northern blot analysis.

Northern blot analysis. HepG2, H4IIE, and AML12 cell lines, as well as rat primary hepatocytes were maintained in the indicated media. At a confluence level of ~80%, the cells were subjected to serum starvation (medium with 0.5% FBS). After 24 h of starvation, the cells were treated with metformin (0.5–2 mmol/l), 8-br-cAMP (500 μmol/l), and dexamethasone (1 μmol/l) (cAMP/Dex) and were subsequently infected with adenoviral vectors expressing SHP, AMPK, or siRNA SHP for 24 h in both a time- and dose-dependent manner in both the hepatoma cells and primary hepatocytes. The cells were subsequently harvested at the indicated times, and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Aliquots of 30 μg of total RNA from each of the samples were used for Northern blot analysis as described previously (29). The probe labeling of each of the cDNAs for SHP, PEPCK, G6Pase, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) with [α-^{32}P]dCTP was performed using a random-primer DNA labeling system (Amersham Biosciences, Little Chalfont, U.K.). The expression of all transcripts was normalized to GAPDH levels.

Western blot analysis. H4IIE cells were treated with metformin, AICAR, and compound C for 12 h, and the cells were then harvested and homogenized in lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 5 mmol/l EDTA, 0.5% Nonidet P-40, 100 mmol/l phenylmethylsufonyl fluoride, 1 mol/l dithiothreitol, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. The cell lysates (40 μg/lane) were separated via SDS-PAGE on 8% gel, and the proteins were transferred to Hybond-C Extra nylon membranes. The membranes were probed with a monoclonal phospho–AMPK-α antibody (Thr 172 [phosphorylated at threonine 172]; Cell Signaling Technology), a polyclonal AMPK-α antibody (Cell Signaling Technology), and a polyclonal phospho–acetyl-CoA carboxylase (ACC) (Ser 79 [phosphorylated at serine 79]; Upstate, Charlottesville, VA), and then developed using an ECL Western blot detection kit (Amersham Bioscience). Each of the membranes was also probed with a β-actin antibody to verify equal protein loading in each lane.

Animals. Seven-week-old male Sprague-Dawley rats (Daehan Biolink, Chungbuk, Korea), each of which weighed 200–230 g, and 8-week-old female B6-*Lep*$^{ob/ob}$ mice (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) were housed in an animal facility under the following conditions: 22 ± 2°C, 55 ± 5% humidity, and a 12-h light/dark cycle with ad libitum access to water and a standard laboratory diet. Metformin was orally administered (from 50 to 400 mg/kg body wt) to 8-week-old female B6-*Lep*$^{ob/ob}$ mice in both a time- and dose-dependent manner following an overnight fasting condition (12 h) and feeding condition. The mice were infected with adenoviral vector expressing SHP (1×10^9 p.f.u.) by tail-vein injection for indicated time points in feeding condition. Livers from metformin-treated and adenovirus-infected mice were used in the preparation of total RNA and Northern blot analysis. Before the isolation of the liver, the blood glucose levels of each mouse were measured at the indicated times with a glucose meter (Accu-Check Active; Roche Diagnostics). All experimental procedures involving animals were approved by the Chonnam National University Institutional Animal Use and Care Committee and conducted in accordance with the National Institutes of Health animal research standards.

Statistical analysis. Data are expressed as means ± SEM. ANOVA was used to determine significant differences as detected by Duncan's multiple comparison test. Values of $P < 0.01$ were considered to be statistically significant. All experiments were performed at least three times.

RESULTS

Metformin induces SHP gene expression and promoter activity. To determine whether metformin regulates SHP gene expression, Northern blot analysis was performed on metformin-treated hepatoma cells and rat primary hepatocytes. SHP gene expression was increased

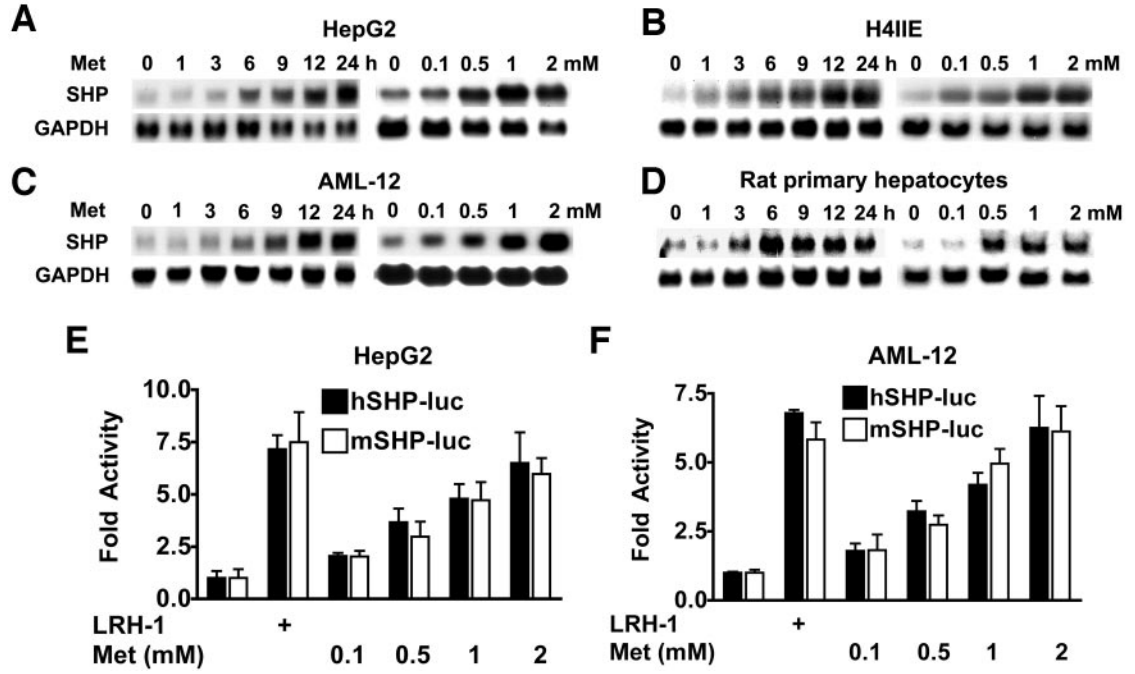

FIG. 1. Metformin induces SHP promoter activity and gene expression in hepatoma cell lines and primary hepatocytes. *A-D*: HepG2, H4IIE, and AML12 cell lines and rat primary hepatocytes were cultured under serum-free conditions for 24 h. These cells were then treated with various dose of metformin for 12 h or for various time periods up to 24 h. SHP gene expression was confirmed by Northern blot analysis. SHP gene expression was normalized to GAPDH expression. Data are representative of three independently performed experiments. *E* and *F*: The effect of metformin on the activity of human (hSHP) and mouse (mSHP) SHP promoter reporter constructs was assessed. SHP promoters construct (200 ng) were transiently transfected into HepG2 and AML12 cells. The cells were incubated in the presence or absence of various concentrations of metformin, and luciferase activity was measured after 36 h. Luciferase activity was normalized to β-galactosidase activity to correct for variations in transfection efficiency, and LRH-1 was used as a positive control. Data shown represent the means of five independent experiments. Data are expressed as fold activations relative to the control (±SEM). Met, treated with metformin.

significantly by metformin in a time- and dose-dependent manner in HepG2, H4IIE, and AML-12 cells (Fig. 1*A-C*). In rat primary hepatocytes, SHP gene expression was also increased by metformin treatment, in a pattern similar to that observed in the tested hepatoma cells (Fig. 1*D*). SHP gene expression had increased by 6 h and reached maximum levels by 24 h of metformin treatment in majority of the cell lines.

To test whether metformin regulates SHP promoter activity, we conducted transient transfection assays using a reporter gene containing −2.2 kb of the human or mouse SHP gene promoter. In HepG2 and AML-12 cells, metformin significantly increased SHP promoter activity in a dose-dependent manner (Fig. 1*E* and *F*). These results show that metformin regulates SHP gene expression via an increase in SHP promoter activity.

AMPK induces SHP gene expression. To investigate whether the regulation of SHP by metformin is mediated by AMPK, we evaluated the effect of AMPK on mRNA levels and SHP promoter activity in primary hepatocytes using an adenoviral vector that expressed either the constitutively active form of AMPK (Ad-AMPK) or the adenovirus dominant negative form of AMPK (Ad-DN-AMPK). Overexpression of AMPK with Ad-AMPK induced SHP gene expression in primary heptatocytes. In contrast, metformin-stimulated SHP gene expression was inhibited by Ad-DN-AMPK (Fig. 2*A*). Ad-AMPK also increased SHP gene expression in both HepG2 and AML-12 cells, whereas metformin-induced SHP gene expression was dramatically repressed by Ad-DN-AMPK in these hepatoma cells (data not shown). In HepG2 and AML-12 cells, both AICAR and the constitutively active AMPK (CA-AMPK) increased SHP promoter activity in a dose-dependent manner, whereas compound C and DN-AMPK inhibited metformin-induced SHP promoter activity (Fig. 2*B* and *C*). Collectively, these results show that both metformin-induced gene expression and promoter activity of SHP are mediated by AMPK.

We also assessed the levels of AMPK phosphorylation in the presence and absence of metformin, AICAR, and compound C using an anti–phospho-ACC Thr172 AMPK antibody. AICAR and metformin increased ACC and AMPK phosphorylation, whereas compound C caused a reduction in ACC and AMPK phosphorylation, even in the presence of metformin in H4IIE cells (supplemental Fig. 1*A* [available in an online appendix at http://dx.doi.org/10.2337/db07-0381]). To determine whether AMPK-mediated induction of SHP gene expression regulates hepatic gluconeogenic gene expression in H4IIE cells, we infected the hepatoma cell lines with Ad-AMPK or Ad-DN-AMPK. Ad-AMPK increased SHP gene expression and repressed PEPCK and G6Pase mRNA levels in cells treated with cAMP/Dex to induce hepatic gluconeogenic gene expression. Ad-DN-AMPK did not increase SHP gene expression and also failed to repress cAMP/Dex-induced PEPCK and G6Pase gene expression (supplemental Fig. 1*B*). Taken together, these results indicate that AMPK induces SHP gene expression and inhibits the expression of hepatic gluconeogenic genes.

Induction of SHP gene expression by metformin inhibits the expression of hepatic gluconeogenic genes. To determine whether metformin-mediated SHP gene expression is involved in the regulation of hepatic gluconeogenesis, we measured the effects of metformin on the expression of SHP and hepatic gluconeogenic genes. After 12 h of pretreatment with cAMP/Dex, hepatoma cells and primary hepatocytes were treated with various metformin

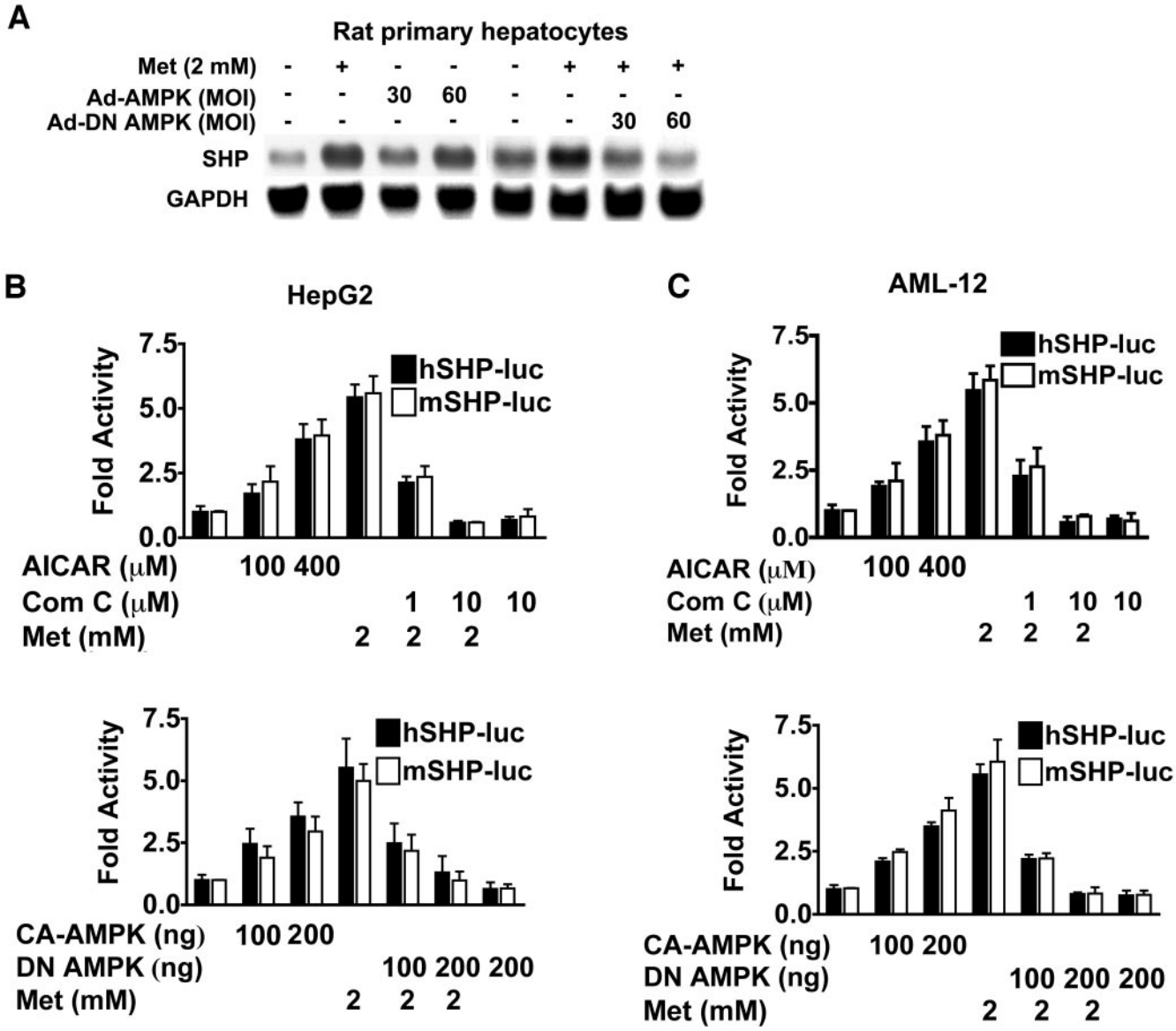

FIG. 2. Metformin-induced upregulation of SHP gene expression is mediated by AMPK. *A*: Rat primary hepatocytes were infected with Ad-AMPK at a multiplicity of infection (MOI) of 30 or 60 for 24 h. Cells were infected with Ad-DN-AMPK for 24 h after 12 h of metformin treatment (2 mmol/l). SHP expression was determined via Northern blot analysis. SHP expression was normalized to GAPDH as an internal control. The data presented are representative of three independently performed Northern blot analyses. *B* and *C*: HepG2 and AML12 cell lines were transiently transfected with the indicated reporter genes and treated with the AMPK activator AICAR or the AMPK inhibitor compound C in the presence or absence of metformin for 12 h after transfection. The cells were cotransfected with CA-AMPK, DN-AMPK in the presence or absence of metformin for 12 h after transfection. Luciferase activity was normalized to β-galactosidase activity to correct for variations in transfection efficiency. The data presented are representative means from five independent experiments. The data are expressed as fold activation relative to the control (±SEM). Com C, treated with compound C; Met, treated with metformin.

concentrations for 12 h, and gene expression was evaluated. The expression of two key hepatic gluconeogenic genes, PEPCK and G6Pase, increased in response to cAMP/Dex and was reduced in response to insulin. As shown in Fig. 3*A*, metformin (0.5 to 2 mmol/l) induced SHP gene expression and repressed the cAMP/Dex-induced expression of PEPCK and G6Pase in a dose-dependent manner in H4IIE cells. The induction of SHP by metformin also inhibited hepatic gluconeogenic gene expression in a time- and dose-dependent manner in AML12 cells (Fig. 3*B*) and rat primary hepatocytes (Fig. 3*C*). These results indicate that metformin-induced SHP gene expression is associated with the repression of SHP target genes involved in gluconeogenesis in hepatocytes.

SHP mediates metformin-induced inhibition of the promoter activities and expression of hepatic gluconeogenic genes. SHP interacts with and represses a nuclear receptor, LRH-1. However, SHP does not inhibit the LRH-1–related nuclear receptor SF-1 (17,28,30). Because SHP operates as a transcriptional repressor, we examined whether metformin specifically regulates LRH-1 transcriptional activity. Figure 4*A* shows that metformin inhibited the transcriptional activity of LRH-1 but failed to inhibit the transactivation of SF-1. Moreover, knockdown of SHP via siRNA SHP rescued the repressed transcriptional activity of LRH-1, whereas no change in SF-1 transcriptional activity was observed. These results indi-

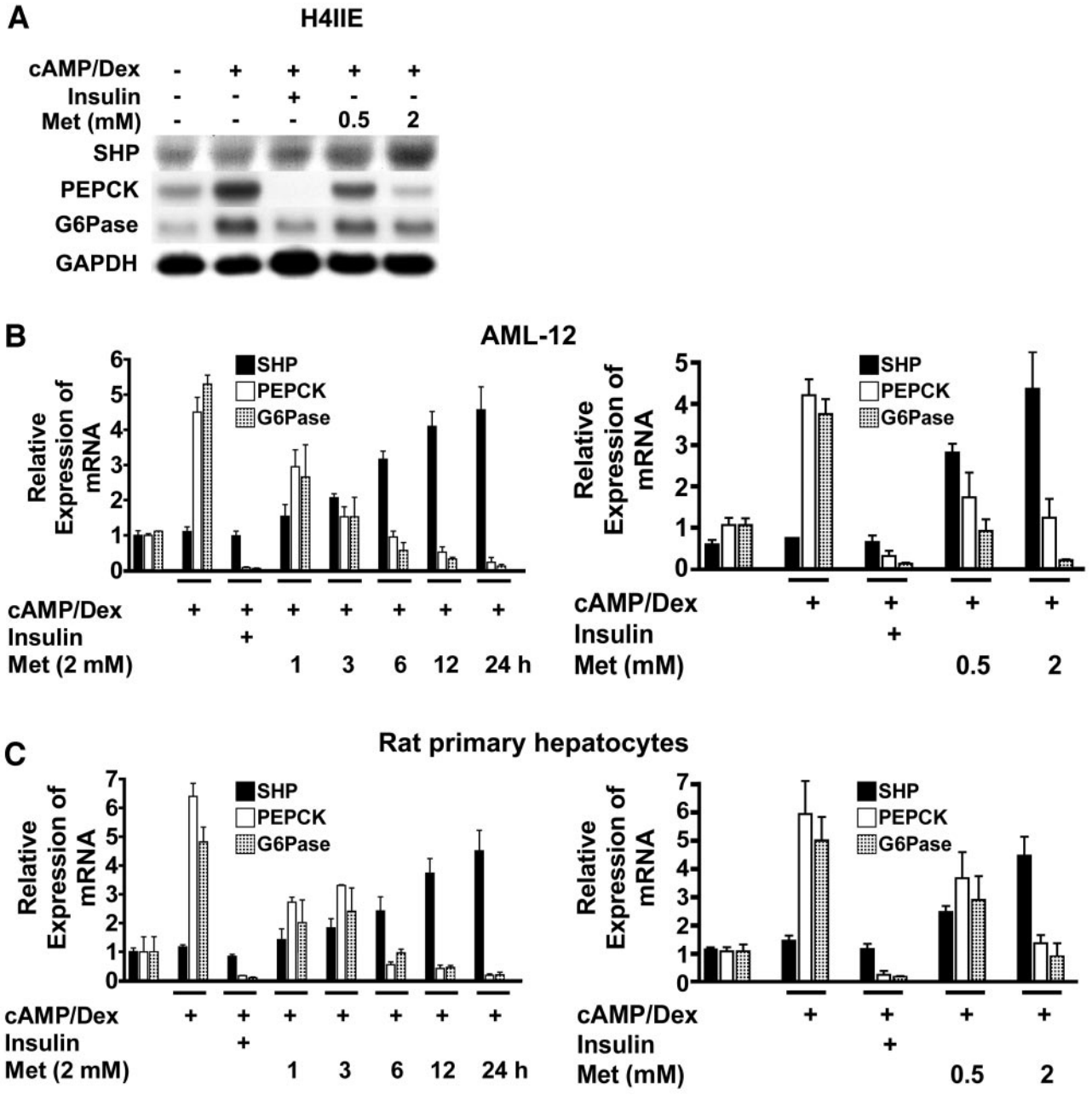

FIG. 3. Metformin-induced SHP gene expression inhibits hepatic gluconeogenic gene expression. *A-C*: Metformin inhibited 8-Br-cAMP/Dex-induced PEPCK and G6Pase gene expression in H4IIE and AML12 cells and in rat primary hepatocytes. After 12 h of pretreatment with cAMP/Dex and Insulin (30 nmol/l) for 6 h, the cultures were treated with metformin at various doses and for various times. The mRNA levels were analyzed via Northern blot analysis using SHP, PEPCK, and G6Pase probes and normalized to an internal control (GAPDH). Three independent analyses were performed. Data are expressed as fold activations relative to the control (±SEM). Met, treated with metformin.

cate that metformin specifically regulates the nuclear receptors that are specific targets of SHP.

The promoter activity of PEPCK and G6Pase is regulated by the transcription factors HNF-4α and/or FoxA2. To determine whether metformin-induced SHP expression regulates the transcription factors involved in the regulation of the PEPCK and G6Pase promoters, we examined the effects of metformin on transactivation by HNF-4α and/or FoxA2. In the presence of HNF-4α, metformin repressed PEPCK promoter activity with a pattern similar to that observed with SHP, and promoter activity was recovered significantly by knockdown of SHP gene expression with siRNA SHP (Fig. 4*B*). Similarly, FoxA2-induced G6Pase promoter activity was repressed significantly by SHP and metformin, and this activity was recovered when the cells were treated with siRNA SHP (Fig. 4*C*). We also determined that in the presence of FoxO1, metformin inhibited hepatic gluconeogenic gene promoter activity with an effect similar to that observed with SHP, and the activity was recovered as the result of knockdown of SHP gene expression via siRNA SHP (supplemental Fig. 2). Collectively, these results indicate that metformin-induced SHP gene expression inhibits the function of transcriptional factors that regulate hepatic gluconeogenesis.

To confirm whether metformin-induced downregulation of hepatic gluconeogenic genes is dependent on SHP gene expression, we assessed the effects of SHP on metformin-induced inhibition of PEPCK and G6Pase gene expression

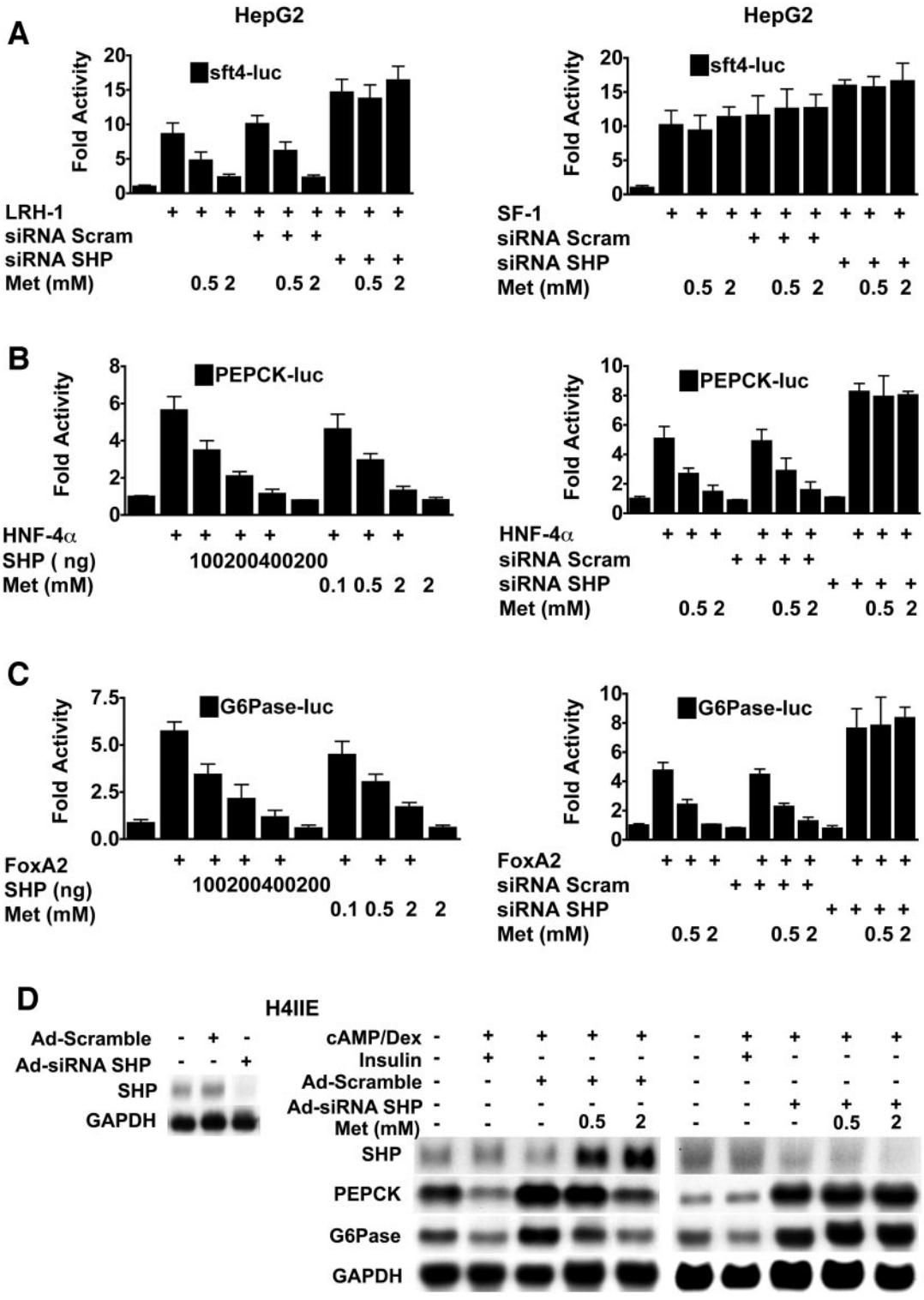

FIG. 4. Metformin inhibits the promoter activities and gene expression of PEPCK and G6Pase through SHP. *A*: HepG2 cell lines were cotransfected with the sft-4 reporter gene (200 ng), LRH-1 (100 ng), and SF-1 (100 ng) and incubated in the presence or absence of metformin for 12 h. Alternatively, the cells were cotransfected with LRH-1, SF-1, the sft-4 reporter gene, and the siRNA SHP or the siRNA scrambled and incubated in the presence or absence of metformin. *B* and *C*: HepG2 cell lines were cotransfected with 200 ng each of the PEPCK and G6Pase reporter genes and HNF-4α or *FoxA2* coupled with SHP and, after 24 h of transfection, were incubated in the indicated quantities of metformin for 12 h. Luciferase activity was measured after 36 h and was normalized to β-galactosidase activity. Alternatively, cells were cotransfected with the PEPCK and G6Pase promoter constructs, either HNF-4α or *FoxA2*, and the siRNA SHP or siRNA scrambled and incubated in the presence or absence of metformin. Luciferase activity was normalized to β-galactosidase activity to correct for variations in transfection efficiency. The data presented represent the means of five independent experiments. Data are expressed as fold activation relative to the control (±SEM). *D*: H4IIE cell lines were infected with 60 MOI of adenoviral vector expressing siRNA SHP tagged with GFP for 24 h. The cells were pretreated in the presence of cAMP/Dex with or without adenoviral vector expressing siRNA SHP. After infection with adenovirus siRNA-SHP (Ad-siRNA-SHP), H4IIE cell lines were treated with the indicated doses of metformin. The levels of SHP, PEPCK, and G6Pase mRNA were determined via Northern blot analysis. The mRNA levels were normalized to GAPDH levels. These results were independently performed at three times. Met, treated with metformin; siRNA Scram, transfected with siRNA scramble.

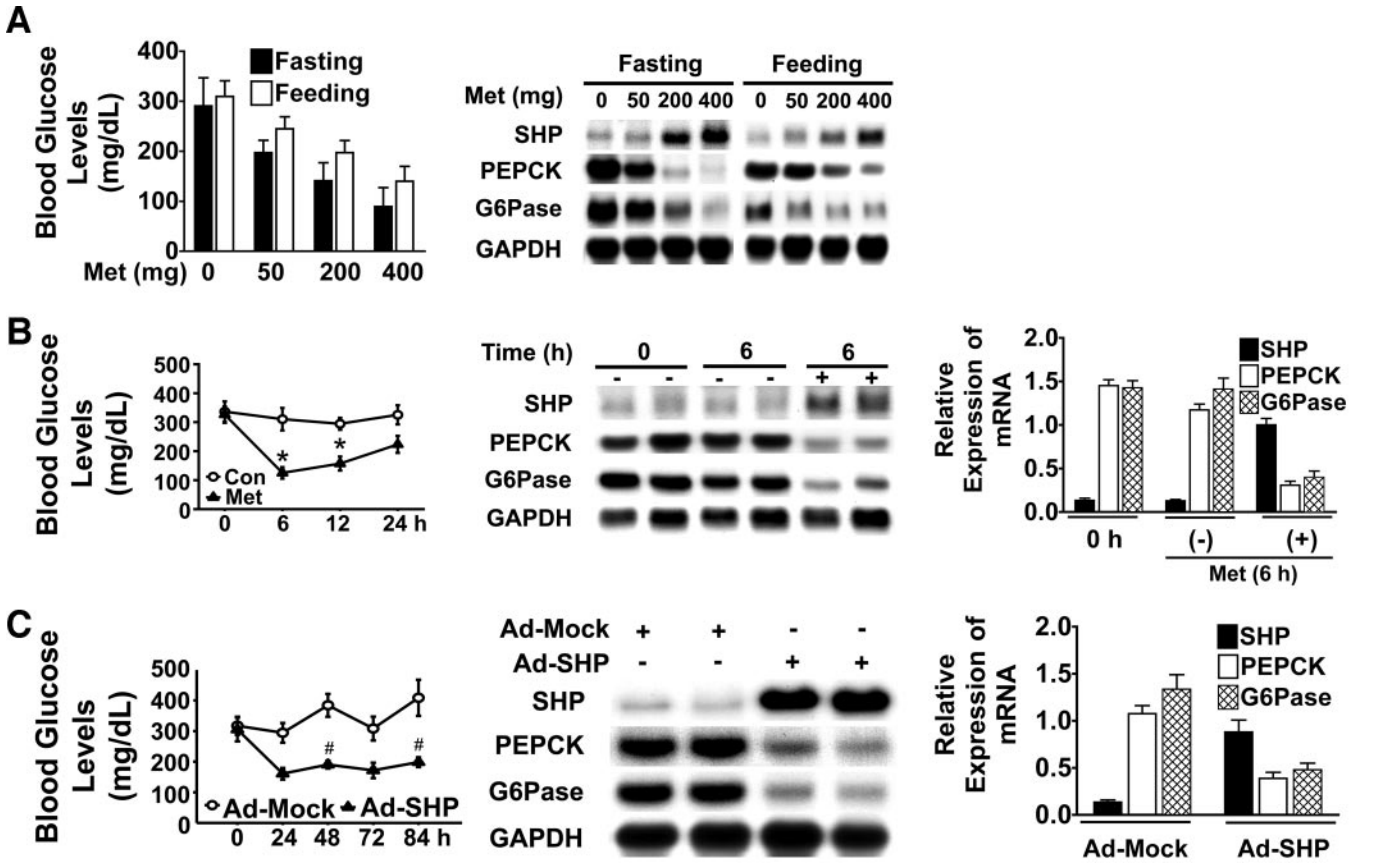

FIG. 5. SHP overexpression reduces blood glucose levels, PEPCK, and G6Pase gene expression in B6-*Lep*$^{ob/ob}$ mice. *A*: Blood glucose levels of 8-week-old female B6-*Lep*$^{ob/ob}$ mice were measured in a dose-dependent manner after oral administration of metformin (50–400 mg/kg body wt) at 6 h fasting and feeding condition. Total RNA (30 μg) was extracted from liver tissues at the indicated times after metformin treatment, and the levels of SHP, PEPCK, and G6Pase mRNA were normalized to GAPDH mRNA. All mice were separated into experimental groups (n = 4 mice per group). *B*: 8-week-old female B6-*Lep*$^{ob/ob}$ mice were determined at the indicated time periods after the oral administration of metformin (400 mg/kg body wt) in the feeding condition. Total RNA (30 μg) was extracted from liver tissues at the indicated times after metformin treatment. The levels of SHP, PEPCK, and G6Pase mRNA were determined via Northern blot analysis. The mRNA levels were normalized to GAPDH levels. All mice were separated into experimental groups (n = 5 mice per group). *C*: Blood glucose levels of 8-week-old female mice were measured after tail vein injection with Ad-Mock or Ad-SHP for 84 h in the feeding condition. SHP, PEPCK, and G6Pase gene expression in the livers of B6-*Lep*$^{ob/ob}$ female mice were assessed after tail-vein injection with Ad-Mock or Ad-SHP (n = 5 mice per group). Total RNA (30 μg) was extracted from liver tissues 84 h after infection and analyzed via Northern blotting using SHP, PEPCK, and G6Pase probes. The mRNA levels were normalized to that of GAPDH. Ad-Mock, injected with empty vector; Ad-SHP, injected adenovirus expressing SHP; Con, injected with saline; Met, injected with metformin. *P < 0.001 compared with Con (injected saline). $^{\#}P$ < 0.001 compared with Ad-Mock.

in H4IIE cells. Overexpression of SHP using adenovirus SHP (Ad-SHP) significantly repressed both cAMP/Dex-induced PEPCK and G6Pase gene expression in a time- and dose-dependant manner (supplemental Fig. 3). However, SHP knockdown with adenovirus siRNA-SHP reversed metformin-induced inhibition of cAMP/Dex-induced PEPCK and G6Pase gene expression, whereas a control siRNA (Ad-scramble) exerted no discernable effects on the metformin-induced inhibition of hepatic gluconeogenic gene expression (Fig. 4*D*). These results indicate that the induction of SHP gene expression by metformin has a significant effect on the downregulation of hepatic gluconeogenesis.

Metformin and SHP decrease blood glucose levels and the expression of PEPCK and G6Pase gene in the livers of B6-*Lep*$^{ob/ob}$ mice. To determine whether metformin-induced SHP inhibits blood glucose levels and hepatic gluconeogenic genes expression in vivo, the expression of SHP and hepatic glucose production were analyzed in the metformin-treated B6-*Lep*$^{ob/ob}$ mice. As shown in Fig. 5*A*, blood glucose levels in the fasting condition were significantly decreased by metformin in a dose-dependent manner compared with those in the feeding condition, which are comparable to normal blood glucose levels. However, metformin increased SHP mRNA levels and phosphorylation of ACC and AMPK in a dose-dependent manner for 6 h (supplemental Fig. 4), whereas the expression of gluconeogenic genes was significantly repressed compared with the feeding condition. Interestingly, metformin increased SHP gene expression within 6 h, which repressed PEPCK and G6Pase gene expression resulting in reduction of blood glucose levels (Fig. 5*B*). These data suggest that induction of SHP gene by metformin results in decrease of blood glucose levels through the repression of hepatic gluconeogenic gene expression.

To confirm the effects of SHP on blood glucose levels through the repression of hepatic gluconeogenic genes expression, we infected Ad-SHP into B6-*Lep*$^{ob/ob}$ mice via tail vein injection. To verify the transduction efficiencies of adenovirus-expressing green fluorescent protein–tagged SHP, we examined the mouse livers using fluorescence microscopy at the indicated time period (data not shown). As shown in Fig. 5*C*, overexpression of SHP by Ad-SHP hugely decreased the blood glucose levels and PEPCK and G6Pase gene expression. Taken together, these results indicate that the induction of SHP gene expression by

metformin regulates hepatic glucose production via the inhibition of hepatic gluconeogenic gene expression in vivo.

DISCUSSION

In this study, we found that metformin increased SHP gene expression via AMPK activation and inhibited the expression of the hepatic gluconeogenic genes PEPCK and G6Pase via upregulation of SHP. The inhibitory effects of metformin on hepatic gluconeogenic gene expression were blocked via the knockdown of SHP using siRNA SHP. Moreover, these effects of metformin, which were characterized in both hepatoma and hepatic cells, were observed to occur in a similar fashion in metformin-treated B6-*Lep*$^{ob/ob}$ mice. SHP gene expression was increased by metformin, and adenovirus-mediated SHP overexpression caused a reduction in the expression of hepatic gluconeogenic genes. Consequently, blood glucose levels were also reduced in the obese mice.

SHP plays a pivotal role in cholesterol and glucose metabolism (15,23,31–33). The synthesis of bile acid from cholesterol is regulated by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) (19). Bile acid is an endogenous ligand of the farnesoid X receptor, which can profoundly activate SHP transcription (34), and SHP decreased the expression of CYP7A1 via the inhibition of LRH-1 activity (19,35). Additionally, bile acid–induced SHP can downregulate PEPCK and G6Pase expression by the repression of the transcriptional activity of HNF-4α, *FoxO1*, and *FoxA2* (23–24). However, the correlation between SHP expression and type 2 diabetes, and/or antidiabetic agent, has not been previously elucidated. In this study, we report that metformin, a drug commonly used in the treatment of type 2 diabetes, represses the expression of PEPCK and G6Pase genes by increasing SHP gene expression, both in vitro and in vivo. As the inhibition of hepatic gluconeogenic gene expression by metformin is mediated via an AMPK pathway, we examined whether AMPK caused an increase in SHP expression. Indeed, our data indicated that Ad-AMPK, as well as AICAR, increased SHP gene expression. Furthermore, this induction was abolished in the presence of Ad-DN-AMPK or compound C. These results indicate that metformin-induced SHP gene expression is mediated via AMPK activation. Collectively, these results show that the AMPK-mediated upregulation of SHP gene expression may be one of the mechanisms inherent to the negative regulation of hepatic gluconeogenic genes by metformin.

As previously mentioned, SHP inhibits PEPCK and G6Pase gene expression via the repression of HNF-4α, FoxO1, and FoxA2 activity (23–24). Thus, we determined whether HNF-stimulated gluconeogenic genes can be downregulated by metformin-induced SHP. In the present study, it was demonstrated that metformin inhibited HNF-4α–, FoxO1-, and/or FoxA2-stimulated PEPCK and/or G6Pase promoter activity and that this repression was abolished by siRNA SHP. Overall, the results of the present study demonstrate that metformin can inhibit HNF-stimulated PEPCK and G6Pase promoter activity via the induction of SHP. In addition, the expression of hepatic gluconeogenic genes is also modulated by cAMP and glucocorticoid receptor, which are stimulated by glucagon and glucocorticoid, respectively (15,36). Our data indicated that SHP inhibited cAMP/Dex-induced PEPCK and G6Pase gene expression. However, when endogenous SHP was blocked, metformin failed to downregulate the cAMP/Dex-stimulated expression of PEPCK and G6Pase. AMPK activation causes a rapid reduction in PEPCK and G6Pase expression via the inhibition of the transcriptional activity of HNF-4α and the stabilization of FoxO1 (37–38). Our data also indicated that PEPCK and G6Pase expression were reduced in the presence of metformin within a short period of time. Therefore, we propose that metformin may regulate the expression of hepatic gluconeogenic genes via two different pathways. The first of these is a short-term pathway, involving the protein stabilization or phosphorylation of AMPK-targeted transcription factors and coactivators, and the second is a long-term effect exerted via the induction of SHP gene expression due to metformin treatment. Metformin was recently shown to reduce significantly blood glucose levels and hepatic gluconeogenic gene expression in the livers of obese and diabetic mice (39). The results of our study have demonstrated that metformin induces SHP gene expression, which, in turn, reduces the expression of hepatic gluconeogenic genes and blood glucose levels in B6-*Lep*$^{ob/ob}$ mice. Consistent with our previous observations, we have also determined that the adenovirus-mediated overexpression of SHP inhibited the expression of PEPCK and G6Pases and also reduced blood glucose levels in B6-*Lep*$^{ob/ob}$ mice. Collectively, our results indicate that metformin-induced inhibition of hepatic gluconeogenesis is mediated via the upregulation of SHP gene expression.

In conclusion, metformin causes increases in hepatic SHP gene expression via an AMPK-dependent pathway and also results in the reduction of hepatic glucose production in vivo. The results of the present study suggest that the regulation of SHP gene expression and promoter activity by metformin may represent a novel pathway that could be modulated to enhance the treatment of type 2 diabetes. To elucidate more clearly the role of SHP in AMPK-mediated hepatic glucose metabolism, it will be useful to generate liver-specific SHP knock-out animals in the future.

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