Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase dependent regulation of the orphan nuclear receptor SHP

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Abstract

Objective: Metformin is an antidiabetic drug which is 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 constitutive active form of 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 Ad-SHP-mediated hepatic glucose production was measured in B6-Lep\(^{ob/ob}\) mice.

Results: Hepatic SHP gene expression was induced by metformin, AICAR, and Ad-AMPK. Metformin-induced SHP gene expression was abolished by adenovirus containing dominant negative form of AMPK (Ad-DN AMPK), as well as by compound C. Metformin inhibited HNF-4\(\alpha\)- or FoxA2-mediated promoter activity of PEPCK, G6Pase, and the inhibition was blocked with siRNA SHP. Additionally, SHP knockdown by adenovirus containing siRNA SHP (Ad-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.

Conclusion: We have concluded that metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase dependent regulation of SHP.

Key words: Metformin, Orphan nuclear receptor, SHP, AMPK, G6Pase, PEPCK.
Introduction

Metformin (1, 1-dimethylbiguanide hydrochloride) is a hypoglycemic agent which is 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 non-esterified fatty acids (7). Metformin has been shown to activate AMPK (AMP-activated protein kinase) via an LKB1-dependent mechanism (1-2,8).

AMPK is a well-known serine/threonine kinase which 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, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which, in turns, 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, which 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 HNF-4a (NR2A1), FoxO1 (also known as FKHR) (23) and the forkhead transcription factor, FoxA2 (also referred to as HNF-3β) (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. 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 (23). 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.
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Research Design & Methods

Chemicals. Metformin (1,1-Dimethylbiguanide hydrochloride, Sigma, St. Louis, Mo, USA), 8-bromoadenosine 3,5-cyclic monophosphate (Sigma, St. Louis, Mo, USA), dexamethasone (Sigma, St. Louis, Mo, USA), and insulin (Norvolin R, Green Cross, Korea) were purchased from the indicated companies, and dissolved in the 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 (-1227/ +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. LRH-1, SF-1 cDNA, and the sf4 reporter gene were prepared as described previously (17,20,28).

Preparation of recombinant adenovirus. An adenovirus encoding for full-length human SHP (24), e-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 (\texttt{GACAGTAGCTTCTCGAGAA}^{259}) of a 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, St. Louis, Mo, USA). 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 H4IE (rat hepatoma) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics in a humidified atmosphere containing 5% CO₂ at 37°C. AML12 (mouse hepatocyte) cells were cultured with DMEM/F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with ITS (insulin - transferrin-selenium, Gibco-BRL, Grand Island, NY, USA) and dexamethasone (40 ng/mL, Sigma, St. Louis, Mo, USA) and antibiotics in a humidified atmosphere containing 5% CO₂ at 37°C. Transient transfections were conducted using LipofectAmine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) 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 of 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, then normalized to the β-galactosidase activity. The data are representative of a minimum of three to
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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 weight) and the livers of the animals were exposed surgically. The liver was first perfused with resuspension buffer (150 mM NaCl, 10 mM KCl, 1 mM NaH2PO4·2H2O, 0.8 mM NaH2PO4·12H2O, 1 mM EGTA, 0.8 mM NaHCO3, 10 mM D-glucose, 10 mM HEPES, pH 7.2) at 30-50 mL/min for 5-10 min and then perfused with collagenase solution (HBSS (9.5g/L), 0.8 mM NaHCO3, 0.9 mM CaCl2·2H2O, 10 mM HEPES, 0.5g/L collagenase IA (Sigma, St. Louis, Mo, USA), 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, 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 (1×10⁶ cells/mL, IWAKI Seitech Div, Tokyo, Japan) in William E medium (Sigma, St. Louis, MA, USA). 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 approximately 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 mM), 8-br-cAMP (500 µM) and dexamethasone (1 µM) (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, USA) according to the manufacturer’s instructions. Aliquots of 30 µg of total RNA from each of the samples were utilized for Northern blot analysis, as described previously (29). The probe labeling of each of the cDNAs for SHP, PEPCK, G6Pase, and GAPDH with [α-32P]dCTP was performed using a random-primer DNA labeling system (Amersham Biosciences, Little Chalfont, UK). The expression of all transcripts was normalized to glyceraldehyde-3-phosphate-dehydrogenase (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 IPH lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 mM PMSF, 1 M DTT, 1 mg/mL leupeptin, 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, Inc., MA, USA), a polyclonal AMPK-α antibody (Cell Signaling Technology, Inc., MA, USA), and a polyclonal phospho-ACC (Acetyl-CoA Carboxylase) (Ser 79 (phosphorylated at Serine 79), UPSTATE cell signaling solution, Virginia, USA), and then developed using an ECL Western blot.
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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 significantly by metformin in a time- and dose-dependent manner in HepG2, H4IIE, and AML-12 cells (Fig. 1A-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. 1D). 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. 1E 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 dominant negative form of AMPK (Ad-DN AMPK). Overexpression of AMPK with Ad-AMPK induced SHP gene expression in primary hepatocytes.
In contrast, metformin-stimulated SHP gene expression was inhibited by Ad-DN AMPK (Fig. 2A). 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. 2B 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. 1A [available at http://diabetes.diabetesjournals.org]). 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. 1B). 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 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. 3A, metformin (0.5 to 2 mM) 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. 3B) and rat primary hepatocytes (Fig. 3C). 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, the liver receptor homolog-1 (LRH-1). However, SHP does not inhibit the LRH-1 related nuclear receptor, steroidogenic factor-1 (SF-1) (17,28,30). As SHP operates as a transcriptional repressor, we examined whether metformin specifically regulates
Metformin induces SHP gene expression. Figure 4A 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 indicate 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. 4B). 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. 4C). 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 in H4IE cells. Overexpression of SHP using 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 Ad-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. 4D). 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-Lepob/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-Lepob/ob mice. As shown in Fig. 5A, the blood glucose levels in the fasting condition were significantly decreased by metformin in a dose dependent manner comparing with 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 were significantly repressed comparing with the feeding condition. Interestingly, metformin increased SHP gene expression...
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within 6 h, which repressed PEPCK and G6Pase genes expression resulting in reduction of blood glucose levels (Fig. 5B). 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 GFP tagged SHP, we examined the mouse livers utilizing fluorescence microscopy at the indicated time period (data not shown). As shown in Fig. 5C, 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 founded 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 (FXR), 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 down-regulate 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 which is 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 mentions before, 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 GR, 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 co-activators, 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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References
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Biochemistry 40:15369-15377, 2001
22. Yeo MG, Yoo YG, Choi HS, Pak YK, Lee MO: Negative cross-talk between Nur77 and small heterodimer partner and its role in apoptotic cell death of hepatoma cells. Mol Endocrinol 19:950-963, 2005
2001


Figure Legends

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 doses 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 SHP (mSHP) 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.

FIG. 2. Metformin-induced upregulation of SHP gene expression is mediated by AMPK.
A: Rat primary hepatocytes were infected with adenovirus AMPK (Ad-AMPK) at a multiplicity of infection (MOI) of 30 or 60 for 24 h. Cells were infected with an adenovirus dominant negative form of AMPK (Ad-DN AMPK) for 24 h after 12 h of metformin treatment (2 mM). 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 constitutively active AMPK (CA AMPK), dominant-negative mutant (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). Met, treated with metformin; Com C, treated with compound C.

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 nM) 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.
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 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<sup>ob/ob</sup> mice.

A: Blood glucose levels of 8-week-old female B6-Lep<sup>ob/ob</sup> mice were measured in a dose-dependent manner after oral administration of metformin (50-400 mg/kg body weight) at 6 h fasting and the 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 was normalized to GAPDH mRNA. All mice were separated into experimental groups (n=4 mice per group). B: 8-week-old female B6-Lep<sup>ob/ob</sup> mice were determined at the indicated time periods after the oral administration of metformin (400 mg/kg body weight) 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 feeding condition. SHP, PEPCK, and G6Pase gene expression in the livers of B6-Lep<sup>ob/ob</sup> 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. Con, injected with saline; Met, injected with metformin; Ad-Mock, injected with empty vector; Ad-SHP, injected adenovirus expressing SHP. *P < 0.001 compared with con (injected saline). †P < 0.001 compared with Ad-Mock.
Metformin induces SHP gene expression.
Metformin induces SHP gene expression.

**FIGURE 2**

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rat primary hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met (2 mM)</td>
<td>- + - - - + + +</td>
</tr>
<tr>
<td>Ad-AMPK (MOI)</td>
<td>- - 30 60 - - - -</td>
</tr>
<tr>
<td>Ad-DN AMPK (MOI)</td>
<td>- - - - 30 60</td>
</tr>
<tr>
<td>SHP</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
</tbody>
</table>

**B**

- **HepG2**
  - AICAR (µM) 100 400
  - Com C (µM) 1 10 10
  - Met (mM) 2 2 2

- **C**
  - AICAR (µM) 100 400
  - Com C (µM) 1 10 10
  - Met (mM) 2 2 2

Note: hSHP-luc and mSHP-luc represent luciferase activity measurements.
FIGURE 3

A

H4IIE

cAMP/Dex - + + + +
Insulin - - + - -
Met (mM) - - - 0.5 2

B

AML-12

cAMP/Dex + + + + +
Insulin + + + + +
Met (2 mM) 1 3 6 12 24 h

C

Rat primary hepatocytes

cAMP/Dex + + + + +
Insulin + + + + +
Met (2 mM) 1 3 6 12 24 h

Metformin induces SHP gene expression.
FIGURE 4

A

LRH-1
siRNA Scram
siRNA SHP
Met (mM)
+ + + + + + + +
0.5 2 0.5 2 0.5 2

B

HNF-4α
SHP (ng)
Met (mM)
100200400200
0.1 0.5 2 2

C

FoxA2
SHP (ng)
Met (mM)
100200400200
0.1 0.5 2 2

D

Metformin induces SHP gene expression.

H4IIIE

Ad-Scramble - + + + + + + + +
Ad-siRNA SHP - + - - + + + +
SHP - + - + - + + +
GAPDH + + + + + + + +
cAMP/Dex - - - - - - - -
Insulin - + - - - - - -
Ad-Scramble - + + + + + + +
Ad-siRNA SHP - - - - - - -
SHP - + + + + + + +
Met (mM) - - - 0.5 2 - - + +

PEPCK
G6Pase
GAPDH
FIGURE 5

Metformin induces SHP gene expression.