Berberine reduces insulin resistance through protein kinase C–dependent up-regulation of insulin receptor expression

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Abstract

Natural product berberine (BBR) has been reported to have hypoglycemic and insulin-sensitizing activities; however, its mechanism remains unclear. This study was designed to investigate the molecular mechanism of BBR against insulin resistance. Here, we identify insulin receptor (InsR) as a target of BBR to increase insulin sensitivity. In cultured human liver cells, BBR increased InsR messenger RNA (mRNA) and protein expression in a dose- and time-dependent manner. Berberine increased InsR expression in the L6 rat skeletal muscle cells as well. Berberine-enhanced InsR expression improved cellular glucose consumption only in the presence of insulin. Silencing InsR gene with small interfering RNA or blocking the phosphoinositol-3-kinase diminished this effect. Berberine induced InsR gene expression through a protein kinase C (PKC)–dependent activation of its promoter. Inhibition of PKC abolished BBR-caused InsR promoter activation and InsR mRNA transcription. In animal models, treatment of type 2 diabetes mellitus rats with BBR lowered fasting blood glucose and fasting serum insulin, increased insulin sensitivity, and elevated InsR mRNA as well as PKC activity in the liver. In addition, BBR lowered blood glucose in KK-Ay type 2 but not in NOD/LtJ type 1 diabetes mellitus mice that were insulin deficient. Our results suggest that BBR is a unique natural medicine against insulin resistance in type 2 diabetes mellitus and metabolic syndrome.
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Se ha informado que el producto natural berberina (BBR) tiene actividades hipoglucemiantes y sensibilizadoras a la insulina; Sin embargo, su mecanismo sigue sin estar claro. Este estudio fue diseñado para investigar el mecanismo molecular de BBR contra la resistencia a la insulina. Aquí, identificamos el receptor de insulina (InsR) como un objetivo de BBR para aumentar la sensibilidad a la insulina. En células hepáticas humanas cultivadas, BBR aumentó el ARN mensajero InsR (ARNm) y la expresión de proteínas de una manera dependiente de la dosis y el tiempo. La berberina también aumentó la expresión de InsR en las células del músculo esquelético de rata L6. La expresión de InsR mejorada con berberina mejoró el consumo de glucosa celular solo en presencia de insulina. El silenciamiento del gen InsR con un pequeño ARN interferente o el bloqueo de la fosfoinositol-3-quinasa disminuyó este efecto. La berberina indujo la expresión del gen InsR a través de una activación dependiente de la proteína quinasa C (PKC) de su promotor. La inhibición de PKC abolió la activación del promotor InsR causada por BBR y la transcripción de ARNm de InsR. En modelos animales, el tratamiento de ratas con diabetes mellitus tipo 2 con BBR disminuyó la glucosa en sangre en ayunas y la insulina sérica en ayunas, aumentó la sensibilidad a la insulina y aumentó la actividad de ARNm de InsR y PKC en el hígado. Además, BBR redujo la glucosa en sangre en KK-Ay tipo 2 pero no en ratones con diabetes mellitus tipo 1 NOD / LtJ que eran deficientes en insulina. Nuestros resultados sugieren que BBR es una medicina natural única contra la resistencia a la insulina en la diabetes mellitus tipo 2 y el síndrome metabólico.
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1. Introduction

The insulin receptor (InsR) is an integral cell membrane glycoprotein and is essential for the binding of insulin to target cells. Binding of insulin with InsR on cell surface triggers an intracellular insulin pathway that includes InsR activation, insulin receptor substrates phosphorylation, as well as serial downstream events involving phosphoinositol-3-kinase (PI3K), phosphoinositide-dependent kinase, protein kinase B, and mitogen-activated protein kinases [1]. The interaction between InsR and insulin causes a wide range of physiologic responses to maintain the glucose homeostasis, especially the fasting blood glucose. Abnormality in InsR is of major importance to the development of insulin resistance, which is the underlying cause and essential component for type 2 diabetes mellitus and metabolic syndrome [2,3]. Individuals with insulin resistance have absent or reduced expression of InsR in the peripheral tissues, particularly the liver, muscle, and adipocytes [4].

Thiazolidinediones such as rosiglitazone and pioglitazone effectively reduce insulin resistance by targeting a nuclear receptor, peroxisome proliferator–activated receptor–γ (PPAR-γ); but the mechanism remains unclear [5,6]. Although the drugs that target PPAR-γ show significant hypoglycemic effect in patients with type 2 diabetes mellitus [7,8], adverse effects occur, including weight gain and fluid retention [9]. The expanded body fluid by thiazolidinediones is associated with PPAR-γ stimulation of

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epithelial Na(+) channel–mediated renal salt absorption [10]. Searching for novel and safe targets and/or drugs against insulin resistance continues to be the major focus in drug discovery.

Berberine (BBR; molecular weight, 371.8) is a benzyltetrahydroxyquinoline compound extracted from *Coptis chinensis* [11]. We have previously identified BBR as a new drug for hyperlipidemia, causing reduction of cholesterol and triglyceride in patients [12]. It increases the low-density lipoprotein receptor (LDLR) expression through an extracellular signal–regulated kinase (ERK)–dependent posttranscriptional mechanism [12-14]. In addition, BBR has also been shown to have hypoglycemic and insulin-sensitizing activity both in animal model and in type 2 diabetes mellitus patients [15,16]. However, its molecular mechanism is still unclear. In the present study, we identify InsR as another primary target of BBR, which antagonizes insulin resistance in vitro and in vivo. These 2 findings together make BBR an important compound of multiple biological targets for sugar- and lipid-related metabolic diseases. Because we have described its mechanism on LDLR, this presentation will report its mode of action on InsR.

2. Materials and methods

2.1. Cell culture

Human hepatoma cell lines HepG2 and Bel-7402 were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and appropriate antibiotics in an atmosphere of 5% CO_2 at 37°C. L6 cells were grown and maintained in α–minimum essential medium containing 2% fetal bovine serum and antibiotics.

2.2. RNA isolation, slot blot, and real-time reverse transcriptase polymerase chain reaction

Total cellular or tissue RNAs were isolated using the Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX) following the vender's instruction. For slot blot, 10 μg of each RNA sample was transferred to nitrocellulose membrane via a slot blot apparatus (Schleicher & Schuell, Keene, NH). The blots were fixed by baking at 80°C for 2 hours, followed by hybridization to a 0.89-kilobase (kb)–long, ^{32}P-labeled human InsR complementary DNA (cDNA) probe. The same membranes were then stripped and rehybridized to a human β-actin (Actb) probe as internal control.

Total cellular or tissue RNAs were reverse transcribed into cDNAs using the Reverse Transcription System (Promega, Madison, WI). Quantitative real-time polymerase chain reaction (PCR) was performed with these cDNAs as described previously [12]. β-Actin was used as internal control; normalized InsR or LDLR messenger RNA (mRNA) expression levels were plotted as fold of untreated control.

2.3. Flow cytometry and double-immune staining analysis

A monoclonal antibody to InsR (Labvision/NeoMarkers, Fremont, CA) was used in flow cytometry analysis of InsR protein expression levels on HepG2 or Bel-7402 cell surfaces. For double staining, cells were incubated simultaneously with InsR monoclonal antibody and a rabbit polyclonal antibody against LDLR (Santa Cruz Biotechnology, Santa Cruz, CA). Normal mouse immunoglobulin G (IgG) and rabbit IgG were used as controls. After washing, cells were stained with a fluorescein isothiocyanate–conjugated goat anti–mouse IgG (Santa Cruz Biotechnology), as well as a tetramethyl rhodamine isothiocyanate–conjugated goat anti–rabbit IgG (Santa Cruz Biotechnology). The fluorescence intensities of fluorescein isothiocyanate/tetramethyl rhodamine isothiocyanate on cell surface were analyzed.

2.4. Small interfering RNA transfection and glucose consumption assay

In a small interfering RNA (siRNA) transfection experiment, HepG2 cells were seeded onto 6-well plates with 2×10^5 cells per well. For 1 well of a 6-well plate, 6 μL of human InsR siRNA duplex (Santa Cruz Biotechnology) or control siRNA and 6 μL of siRNA transfection reagent (Santa Cruz Biotechnology) were diluted and mixed well with 1 mL of siRNA transfection medium (Santa Cruz Biotechnology). The mixtures were loaded onto HepG2 cells for 6 hours. After transfection, 7.5 μg/mL of BBR and/or 1 nmol/L of insulin was used to treat the cells. After incubation for 12 hours, glucose levels in the sample media were assayed. Glucose consumption was calculated as follows: glucose level of the fresh RPMI-1640 minus glucose level of the used RPMI-1640. Knockdown of InsR mRNA expression by InsR siRNA was confirmed by real-time reverse transcriptase (RT)–PCR.

2.5. Blocking

HepG2 cells were pretreated or untreated with PI3K inhibitor wortmannin for 1 hour; 1 nmol/L of insulin and 7.5 μg/mL of BBR were then added to the culture medium. After incubation for 12 hours, glucose levels in the sample medium were determined; and glucose consumptions were calculated.

In another set of experiment, the p38 kinase inhibitor SB203580, MEK1 inhibitor U0126, c-Jun N-terminal kinase inhibitor curcumin, PI3K inhibitor wortmannin, and protein kinase C (PKC) inhibitor calphostin C were added to the culture media 1 hour before the treatment with BBR. Cells were harvested after treatment with BBR for 8 hours. The InsR and LDLR mRNA levels and InsR promoter activities were analyzed.

2.6. InsR mRNA half-life study

HepG2 cells were treated or untreated with 7.5 μg/mL of BBR for 8 hours. Afterward, 5 μg/mL of actinomycin D was added to block the transcription. Total cellular RNAs were

harvested at different time points after actinomycin D treatment and slot blotted to nitrocellulose membranes. The membranes were, respectively, hybridized with InsR and Actb specific probes as described above; and bands were quantitated through densitometry. The InsR mRNA levels were normalized to Actb, and their remaining percentages were plotted against time; the decaying rate or half-life of InsR mRNA is calculated.

2.7. Plasmid, transient transfection, and luciferase assay

The InsR promoter luciferase gene fusion plasmid (pGL3-1.5kIRP) was kindly provided by Dr Araki E. In this fusion construct, a 1.5-kb fragment of the human InsR gene promoter was inserted into the HindIII site of pGL3-basic vector, forming pGL3-1.5kIRP fusion plasmid [17]. HepG2 cells (2×10^5 per well of a 6-well plate) were transfected with 1 μg of the pGL3-1.5kIRP using the FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). After overnight transfection, the culture media were removed; and transfected cells were treated with BBR for 8 hours at different concentrations as indicated. Cell lysates were prepared and luciferase activities were measured using the Luciferase Assay System (Promega).

2.8. PKC activity assay

Cellular and tissue PKC activities were analyzed using the PepTag Non-Radioactive PKC Assay kit (Promega) according to the protocol. Briefly, after homogenization and partial purification, 10 μL of sample protein was mixed with 5 μL of PepTag C1 peptide (specific substrate of PKC), 5 μL of reaction buffer, and 5 μL of PKC activator solution in a 25-μL reaction system. The reactions were performed at 30°C for 30 minutes. The samples were then loaded onto a 0.8% agarose gel. After electrophoresis, the phosphorylated and nonphosphorylated PepTag C1 peptides were separated, with the phosphorylated ones negatively charged and migrated toward the anode. The gels were photographed under a UV light. The bands containing the phosphorylated substrates were then excised and melted. They were transferred to a 96-well plate and quantified using a densitometry according to the supplier's protocol. The catalytic activity of total PKC of a specific sample was expressed as picomoles per minute per milligram, representing the number of picomoles of phosphate transferred to the substrate per minute per milligram of protein of the sample.

2.9. Animal experiments

2.9.1. Rat model

Wistar rats (male, 250 ± 10 g) were obtained from the Institute of Laboratory Animal Sciences (Chinese Academy of Medical Sciences, Beijing, China). Type 2 diabetes mellitus was induced by fat feeding and streptozotocin treatment as described [18]. Diabetic rats were treated with BBR orally twice a day, with a total dose of BBR at 75 or 150 mg/(kg d), respectively. Blood samples were taken by tail snip after 6-hour fast on indicated days of treatment, and blood glucose levels were measured. On the last day of the experiment, all of the rats were euthanized after overnight fast. Livers and muscle tissues from femoral biceps of the rats were dissected and stored in liquid nitrogen for RNA extraction, real-time RT-PCR assay, and PKC activity assay. Total blood samples were also collected to assay fasting serum insulin levels. The insulin levels were analyzed using radioimmunoassay (Linco Research, St Charles, MO). The insulin sensitivity indexes (ISIs) were calculated according to the formula 10^4/(fasting serum insulin × fasting blood glucose) [19]. The insulin level and ISI of normal rats were determined for comparison.

2.9.2. Type 1 diabetes mellitus NOD/LtJ and type 2 diabetes mellitus KK-Ay mice

Female NOD/LtJ mice at 8 to 10 weeks of age were obtained from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). Development of diabetes (type 1 diabetes mellitus) in NOD/LtJ mice was determined by monitoring blood glucose. At 15 ± 1 weeks of age, about 75% of NOD/LtJ mice spontaneously developed diabetes, consistent with previous reports [20]. Female type 2 diabetes mellitus KK-Ay and wild-type C57BL/6J mice were obtained from the Institute of Laboratory Animal Sciences. All the animals were fed with regular rodent chow. Diabetic NOD/LtJ and KK-Ay mice were left untreated or orally treated with BBR at 100 or 200 mg/(kg d), respectively. Before and after 3 weeks of treatment period, blood samples were taken by tail snip after 6-hour fast; and blood glucose levels were measured. On the last day of experiment, all mice were euthanized; and blood samples were taken to assay fasting serum insulin levels. Their livers were dissected for analysis of InsR expression. The fasting insulin level of C57BL/6J mice was determined as wild-type control.

All of the animal experiments followed the instructions of the Laboratory Animal Management Statute of Beijing Municipality. Animals were cared for according to the institutional guidelines of Chinese Academy of Medical Sciences.

2.10. Statistical analysis

After validation of the test for homogeneity of variance, differences of mean results among study groups were examined by 1-way or 2-way analysis of variance followed by multiple comparisons.

3. Results

3.1. BBR up-regulates InsR expression and increases glucose consumption in human liver cells

Human hepatoma cells HepG2 were treated with BBR and showed a dose-dependent increase in the expression of InsR mRNA (Fig. 1A). Quantitative real time RT-PCR showed a 40% increase of InsR mRNA in cells treated with
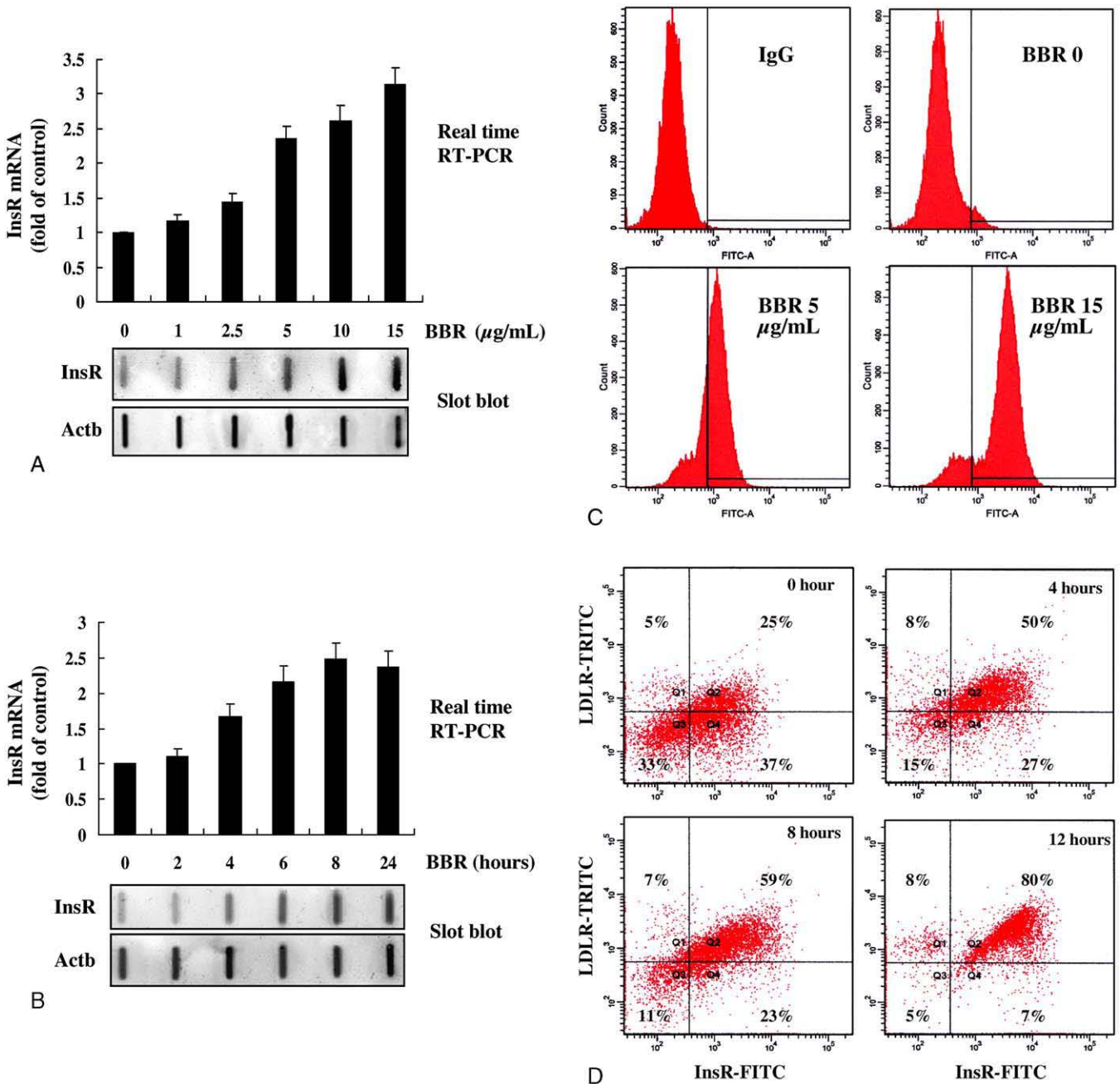
Fig. 1. Up-regulation of InsR expression by BBR in HepG2 cells. A, Dose-dependent induction of InsR mRNA expression by BBR. HepG2 cells were incubated with BBR at different concentrations for 8 hours. Insulin receptor mRNA levels were analyzed by real time RT-PCR (upper panel) and slot blot (lower panel), respectively, with Actb as internal control. Results are mean ± SD of at least 3 repeated experiments. B, Time-dependent induction of InsR mRNA expression by BBR. HepG2 cells were treated with BBR (7.5 μg/mL) for the indicated times. Total RNAs were isolated for the analysis of InsR mRNA expression by real time RT-PCR (upper panel) and slot blot (lower panel). Results are mean ± SD of at least 3 repeated experiments. C, Berberine increased cell surface InsR expression. HepG2 cells were treated with different concentrations of BBR for 8 hours; cell surface InsR expression was analyzed by flow cytometry using an anti-InsR monoclonal antibody. Normal mouse IgG was included in the assay as control for nonspecific binding. D, Up-regulation of both InsR and LDLR. Berberine (7.5 μg/mL) increased the expression of both InsR and LDLR on the surface of HepG2 cells, and the up-regulation went up over time for both receptors.

2.5 μg/mL of BBR for 8 hours, and a maximal increase of 3.2-fold of the control was seen with a concentration of 15 μg/mL; a similar magnitude of increase in InsR mRNA level was confirmed by the slot blot (Fig. 1A). The effect of BBR was also time dependent. The level of InsR mRNA increased 4 hours after exposure of cells to BBR (7.5 μg/mL)

and reached the peak level of 2.5-fold of the control at 8 hours; the expression of InsR mRNA remained high for at least 24 hours (Fig. 1B). Accordingly, the abundance of InsR protein on the HepG2 cell surface was increased by BBR (Fig. 1C). The effect of BBR on InsR was further confirmed in another hepatoma cell line, Bel-7402 of Chinese origin

(data not shown). To view the simultaneous increase of InsR and LDLR by BBR, double-immune staining was conducted and demonstrated a remarkable up-regulation of both LDLR and InsR in a similar magnitude on the surface of human hepatocytes treated with BBR (Fig. 1D), suggesting the dual-target bioactivity of BBR.
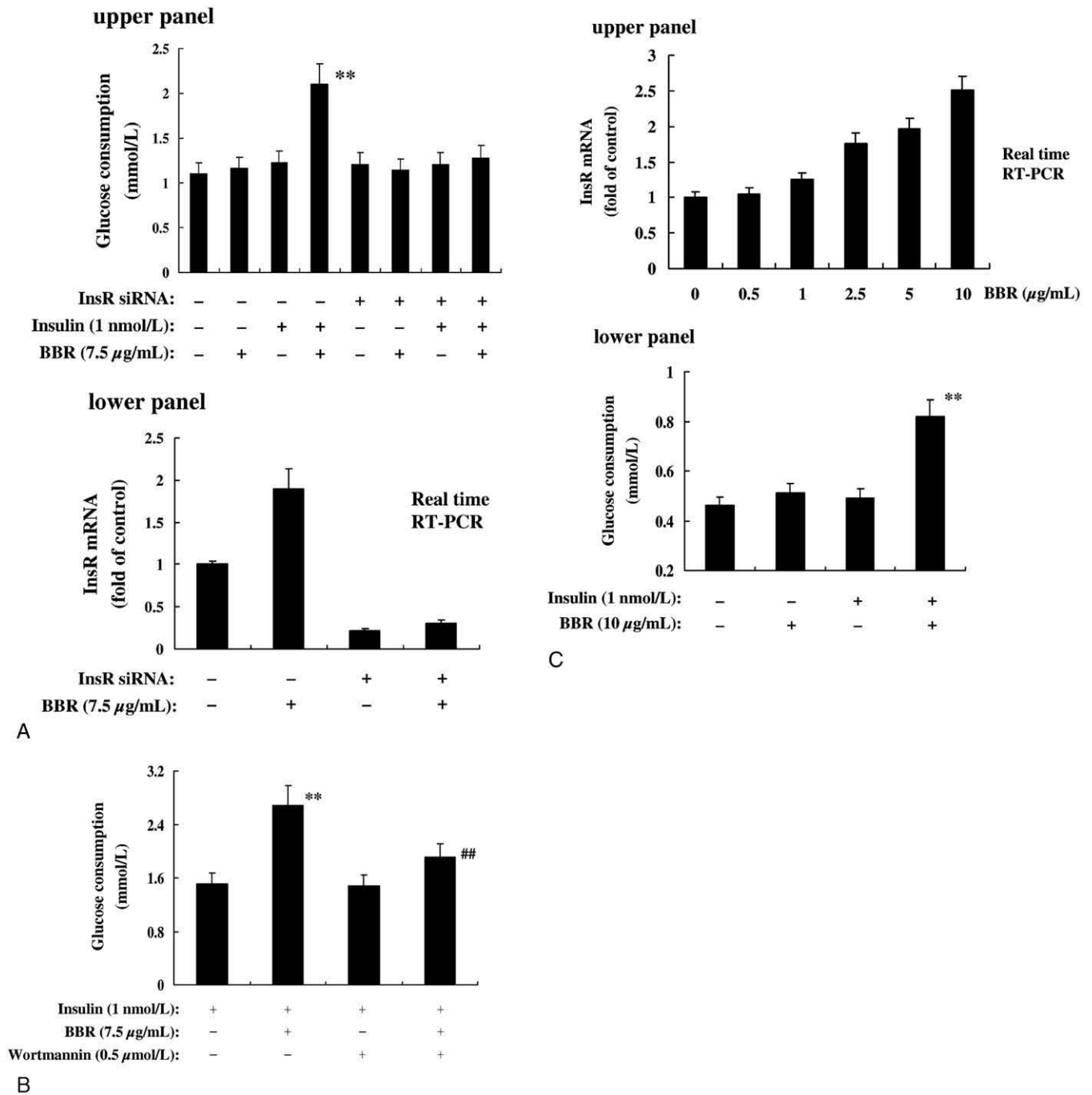

Fig. 2. Berberine improves glucose consumption in cultured cells. A, Berberine increased glucose consumption in the presence of InsR expression and insulin in HepG2 cells (upper panel). Silencing InsR with siRNA inhibited InsR mRNA expression (lower panel) and therefore abolished the glucose consumption–increasing effect of BBR (upper panel). Values are mean ± SD of at least 3 separate experiments. **$P < .01$ vs that of untreated control. B, Blocking PI3K abolished the effect of BBR on glucose consumption. HepG2 cells were pretreated or untreated with 0.5 μmol/L of wortmannin; 1 hour later; insulin and BBR were added to the culture media. After 12-hour incubation, glucose levels in the sample media were determined; and glucose consumptions were calculated. Values are mean ± SD of at least 3 separate experiments. **$P < .01$ vs insulin alone, ##$P < .01$ vs insulin plus BBR. C, Berberine's effect on the L6 rat skeletal muscle cells. Up-regulation of InsR mRNA expression in L6 muscle cells by BBR after 24-hour treatment (upper panel). Berberine increased glucose consumption in L6 muscle cells after 24-hour treatment in the presence of insulin (lower panel). Values are mean ± SD of at least 3 separate experiments. **$P < .01$ vs that of untreated control.

The increased InsR expression directly translated into an enhanced sensitivity to insulin in target cells. Glucose consumption of human hepatic cells treated with insulin was significantly increased by BBR ($P < .01$; Fig. 2A, upper panel). To learn the role of InsR in this effect, siRNA was used to knock down InsR gene. Although the control siRNA had no effect on InsR mRNA expression (data not shown), specific InsR siRNA strongly inhibited InsR mRNA expression (Fig. 2A, lower panel) and totally abolished BBR's effect on insulin-related glucose consumption in liver cells (Fig. 2A, upper panel). These results indicate that the presence of InsR on cell surface and insulin in the circumstances are both essential for BBR to increase the cellular consumption of glucose. Berberine's effect was also dependent on the insulin signaling pathway. As shown in Fig. 2B, blocking PI3K with wortmannin abolished the effect of BBR on glucose consumption. In addition to hepatocytes, BBR also increased InsR expression in muscle cells (Fig. 2C, upper panel) and subsequently the consumption of glucose in the presence of insulin (Fig. 2C, lower panel), indicating the effectiveness of BBR on InsR in other organs related to sugar metabolism.

3.2. BBR increases InsR gene expression through PKC-dependent activation of its promoter

As shown in Fig. 3A, BBR treatment did not prolong the turnover rate of InsR transcript with respect to the untreated cells (4.7 vs 4.4 hours), suggesting that the enhanced InsR gene expression by BBR occurred in the transcriptional rather than the posttranscriptional stage. We therefore turned our attention to the InsR gene promoter [21,22]. The fusion plasmid (pGL3-1.5kIRP) containing pGL3 and a 1.5-kb segment of the InsR promoter was used to examine the activity of BBR. Cells in culture dishes were transiently transfected with the fusion plasmid. After overnight incubation, cells were treated with BBR for 8 hours. As shown in Fig. 3B, BBR increased the level of luciferase in the pGL3-1.5kIRP transfected cells in a dose-dependent manner; and at a concentration of 10 μg/mL, BBR elevated luciferase level in the cells by 2.5-fold. This result demonstrates the stimulating effect of BBR on the InsR gene promoter.

To explore the pathway responsible for the BBR-induced InsR gene transcription, different kinase inhibitors were used. We found that the activity of BBR on InsR gene transcription was most sensitive to the PKC inhibitor calphostin C. Calphostin C at 0.2 μmol/L completely diminished the activity of BBR on InsR gene transcription, but did not change the level of LDLR mRNA (Fig. 4A). In contrast, inhibition of ERK pathway by U0126 was without effect on the activity of BBR on InsR transcription, but completely abolished the increase of LDLR mRNA (Fig. 4B). These results indicate that the BBR pathway on InsR gene expression is totally separate from its effect on LDLR. To determine whether BBR directly activates PKC, cells were treated with BBR; and the activity of total PKC was
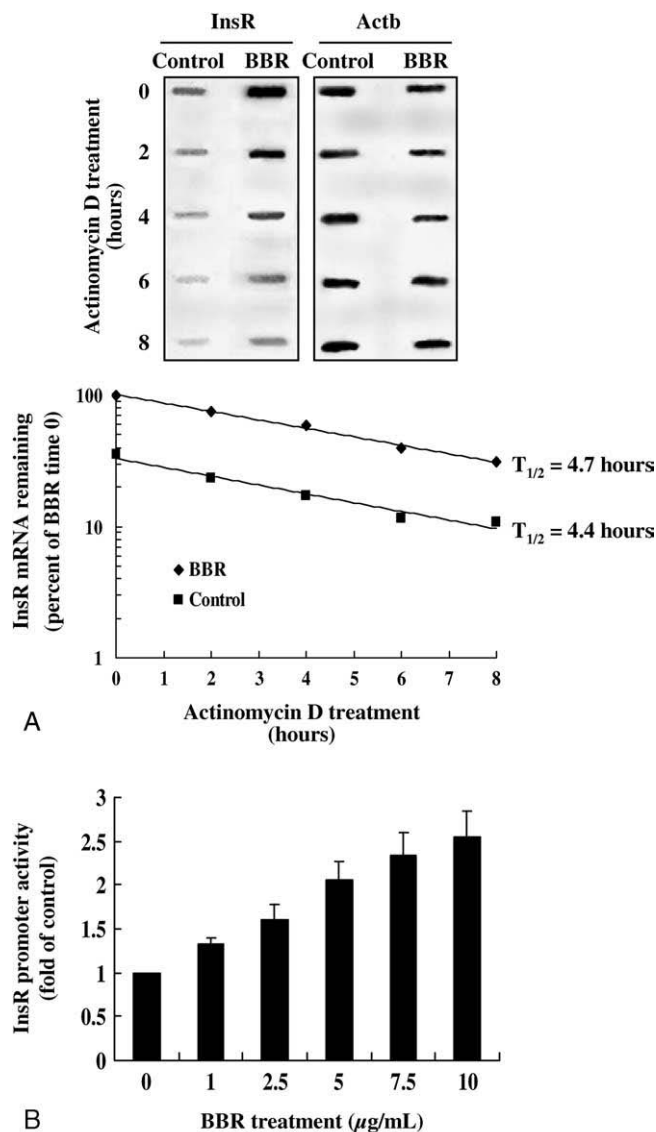

Fig. 3. Berberine increases InsR gene expression at the transcriptional level. A, Berberine had no effect on InsR mRNA decay. HepG2 cells were untreated or treated with BBR for 8 hours. Afterward, actinomycin D was added to cells for different intervals. InsR mRNA levels were analyzed by slot blot. Normalized InsR mRNA levels were plotted as the percentage of remaining. Decay curves were plotted vs time. T1/2 indicates half-life. B, Activation of InsR gene promoter. HepG2 cells were transfected with the fusion plasmid pGL3-1.5kIRP. Luciferase activities were measured after BBR treatment and plotted as fold of control. The data represent normalized InsR promoter activities of at least 3 separate experiments (mean ± SD).

assessed. The PKC activity was increased in liver cells treated with BBR in a time-dependent fashion; the elevation of PKC activity was first observed at 0.5 hour after BBR treatment and went up with time (Fig. 4C). The kinetics of PKC activation preceded the up-regulation of InsR expression by BBR. Pretreatment of the cells with calphostin C completely diminished the activity of BBR on InsR gene promoter (Fig. 4D). A well-known PKC activator, phorbol-12-myristate-13-acetate (PMA), was used as a reference in the experiments and exhibited the same results, indicating
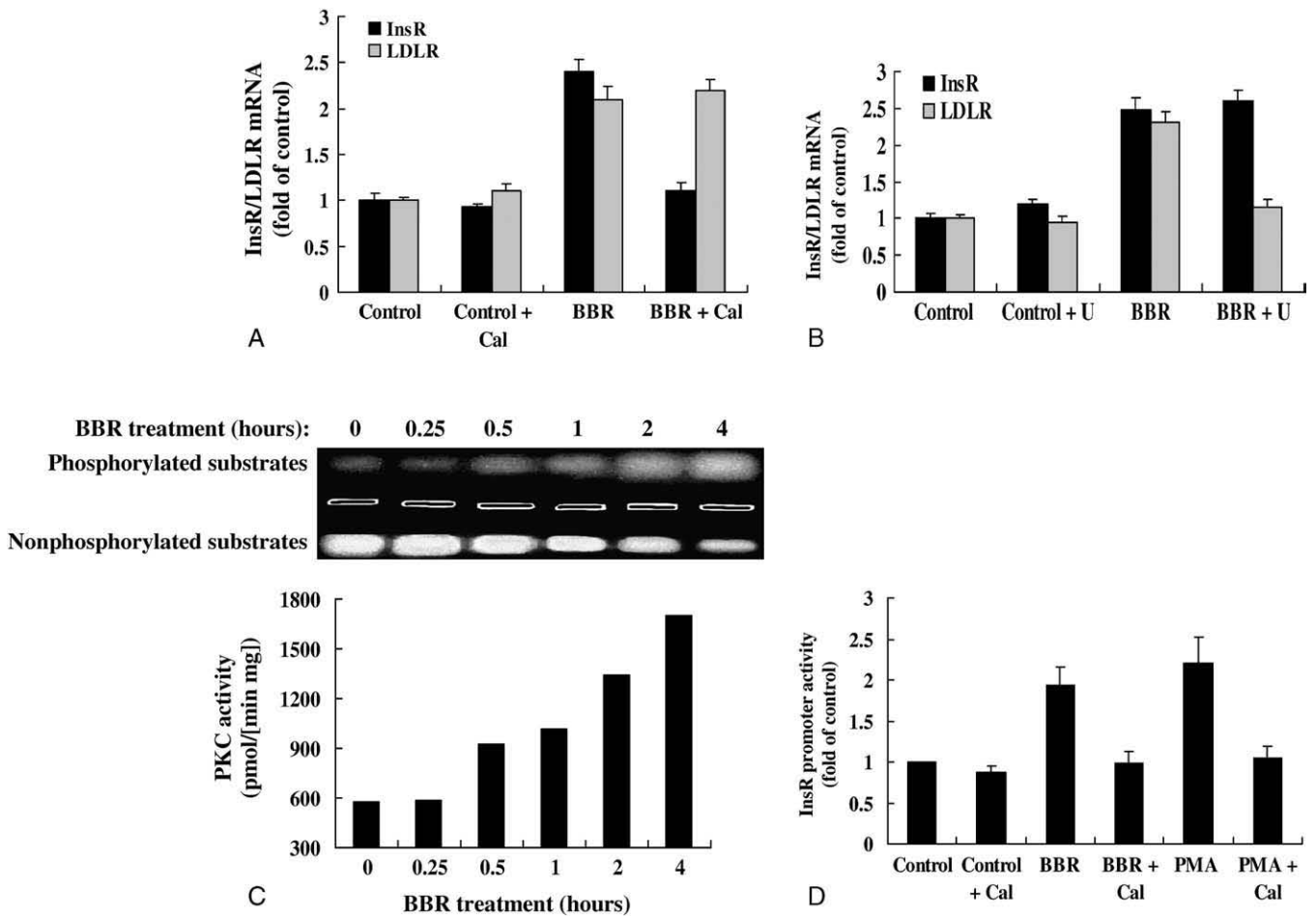
Fig. 4. Berberine stimulates InsR gene promoter through activation of PKC. A, The PKC-dependent effect of BBR on InsR expression. HepG2 cells without or with the pretreatment of 0.2 μmol/L calphostin C (a PKC inhibitor) were treated with 7.5 μg/mL of BBR for 8 hours. Total RNA was isolated, and the relative amount of InsR and LDLR mRNAs were measured by real time RT-PCR. Results are mean ± SD of at least 3 repeated experiments. Cal indicates calphostin C. B, Berberine's effect on InsR is independent of ERK activity. HepG2 cells without or with the pretreatment of 1 μmol/L U0126 (an ERK inhibitor) were treated with 7.5 μg/mL of BBR for 8 hours. The relative amount of InsR and LDLR mRNAs were measured by real time RT-PCR. Results are mean ± SD of at least 3 repeated experiments. U indicates U0126. C, Increased PKC activity by BBR. HepG2 cells were treated with BBR (5 μg/mL) for the indicated times. Total cell lysates were used to detect the phosphorylated and nonphosphorylated substrates that were separated by agarose gel electrophoresis. The PKC activity was quantified using densitometry and expressed as the number of picomoles of phosphate transferred to the substrate per minute per milligram of sample protein. Presented was representative of at least 3 separate experiments. D, The PKC-dependent activation of InsR gene promoter. The pGL3-1.5kIRP transfected HepG2 cells with or without pretreatment of 0.2 μmol/L of calphostin C were exposed to 5 μg/mL of BBR or 0.5 μmol/L of PMA for 8 hours, followed by cell lysis. Luciferase activities were measured. Results represent normalized InsR promoter activity (mean ± SD).

PKC to be part of the mechanism for the activation of InsR gene promoter. The result is agreeable with that in Fig. 4A and indicates that activation of PKC pathway is essential for the BBR-mediated up-regulation of InsR expression.

3.3. BBR lowers blood glucose in type 2 but not type 1 diabetes mellitus rodent model

To examine the mechanism and activity in vivo, animal experiments were first conducted in rats, in which the effects of added diet fat on insulin resistance have been well documented [18,23]. Before BBR treatment, the animals were fed a high-fat diet for 4 weeks and treated with streptozotocin. They significantly reduced the insulin sensitivity ($P < .001$, Fig. 5E) and elevated the fasting

blood glucose from 7 to 12.8 mmol/L ($P < .001$), resembling the feature of insulin resistance in type 2 diabetes mellitus. Treatment of these rats by oral administration of BBR for 15 days resulted in dose-dependent declines of fasting blood glucose (Fig. 5A). After 15 days of therapy, BBR at a dose of 75 mg/(kg d) reduced glucose by 22% ($P < .01$); and at the dose of 150 mg/(kg d), it lowered glucose by 33% ($P < .001$) as compared with the untreated rats on the same high-fat diet. The effect of BBR was also time dependent (Fig. 5A). Decreases in blood glucose were observed by day 5 and became statistically significant by day 9 at both doses ($P < .05, < .01$). At the end of the treatment, liver and muscle InsR mRNA levels were examined in the rats. Liver InsR mRNA was elevated in all BBR-treated rats dose dependently; 1.8- and 2.25-fold increases of InsR mRNA were detected in rat
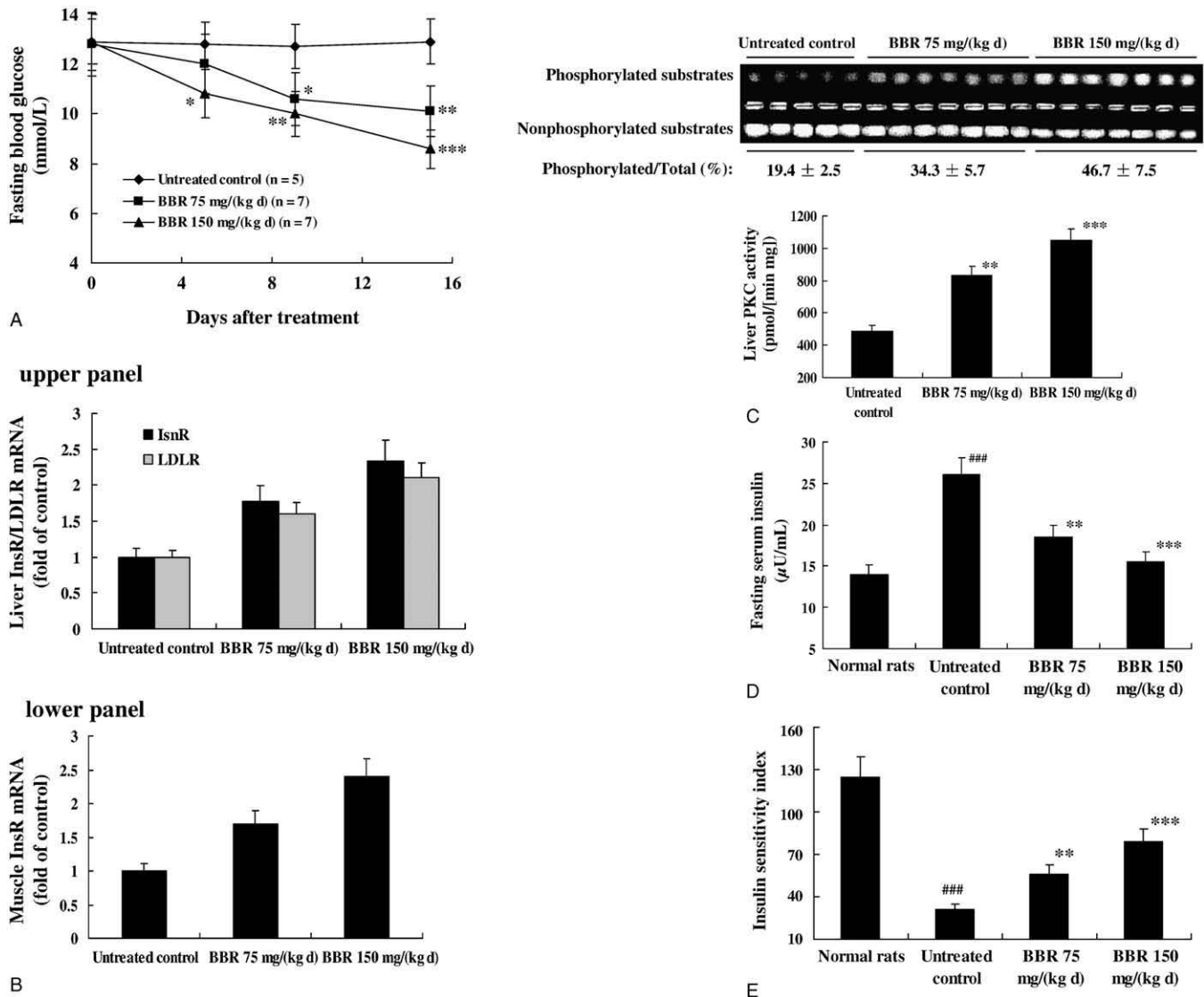
Fig. 5. Berberine lowers blood glucose in high fat diet–fed hyperglycemic rats. Type 2 diabetes mellitus was induced by fat feeding and streptozotocin treatment in male Wistar rats. Diabetic rats were divided into 3 groups with 5 to 7 animals in each group. They were left untreated or treated with BBR orally twice a day, with a total dose of 75 or 150 mg/(kg d), respectively. The animals were treated for 15 days. A, Time- and dose-dependent reduction of fasting blood glucose by BBR. B, Up-regulation of InsR in BBR treated rats. The liver (upper panel) and muscle (lower panel) mRNA extracts of the rats were used for real time RT-PCR assay of InsR/LDLR mRNA levels. C, Increased PKC activity in the liver. Liver samples of the rats were analyzed for PKC activity as described in "Materials and methods." Mean percentages of phosphorylated substrates among total were determined. D, Reduction of blood insulin. E, Improved insulin sensitivity by BBR. Results represent mean ± SD of 5 to 7 animals in each group. [###]$P < .001$ vs that of normal rats; *$P < .05$, **$P < .01$, and ***$P < .001$ vs that of hyperglycemic untreated control.

livers treated with BBR at 75 or 150 mg/(kg d), respectively (Fig. 5B, upper panel). The magnification of InsR mRNA in the muscles of the rats was similar to that in hepatocytes (Fig. 5B, lower panel). Elevated expression of InsR mRNA was concurrent with the increased activity of PKC in the liver of BBR-treated rats (Fig. 5C). These results provide a direct link for the glucose-lowering effect of BBR with its activity on InsR expression and confirm the important role of PKC in vivo.

Fasting serum insulin in the animals was also measured. As shown in Fig. 5D and E, untreated rats fed with high-fat diet for 4 weeks demonstrated a significant increase of fasting

serum insulin and decrease of ISI as compared with the normal controls, indicating a status of insulin resistance in the animals. Treatment of the animals with BBR significantly reduced the serum insulin level elevated by high-fat diet feeding (Fig. 5D). At the same time, ISI was largely improved by BBR (Fig. 5E), suggesting a restoration of the impaired insulin sensitivity and reduction of insulin resistance.

We then try to select a proper animal model to learn whether InsR is the essential molecule in vivo for the hypoglycemic effect of BBR. One of the options is the homozygous InsR gene knockout ($InsR^{-/-}$) mouse; however, the pups died within 2 weeks after birth, resembling

the phenotype of leprechaunism in human [24]. Similarly, the mice with cellular mosaicism for null *InsR* alleles (ablation in 80%-98%) survived for only 3 weeks [25]. Another possible model is the liver specific InsR knockout (LIRKO) mouse [26]. The LIRKO model was not considered suitable for this study because of 2 reasons. First, the LIRKO mice had normal blood glucose; second and more importantly, BBR increased InsR expression not only in liver but also in other tissues such as muscles (Figs. 2C and 5B). Alternatively, the NOD/LtJ mice that have no blood insulin were used. The NOD/LtJ type 1 diabetes mellitus mouse is considered a genetic mirror of the *InsR*$^{-/-}$ mouse. The hypothesis is that, if BBR lowers blood glucose through InsR expression only, it should have no such effect in animals lacking of insulin. The results are shown in Fig. 6. Berberine did not reduce the level of blood glucose in NOD/LtJ mice (Fig. 6A) because of lack of insulin in their circulation (Fig. 6B). In contrast, BBR

significantly lowered blood glucose in another type 2 diabetes mellitus rodent model: KK-Ay mice (Fig. 6A). Unlike the NOD/LtJ mice, KK-Ay mice had a high level of blood insulin that declined after BBR treatment (Fig. 6B). It should be mentioned here that the liver InsR expression levels were elevated by BBR in both of the 2 mice models (data not shown). These results clearly indicate that InsR and its interaction with insulin are absolutely essential for BBR to lower blood glucose.

4. Discussion

The gene of InsR is located on the short arm of chromosome 19 [27]. Although the coding and promoter region of the human InsR gene has been identified and characterized [22,27], the regulatory mechanisms and pathways controlling InsR gene expression remain to be elucidated, underscoring the difficulty in rational design of insulin-sensitizing drugs. Here, we show BBR to be a compound that effectively up-regulates InsR.

Our previous work identified BBR as a novel LDLR up-regulator [12]. Different from its action on LDLR, BBR does not stabilize the InsR mRNA. The transcriptional regulation is the primary mechanism underlying the effect of this drug on liver InsR expression. The PKC inhibitor calphostin C totally abolished the stimulating effect of BBR on the InsR gene promoter and then mRNA transcription. The expression of InsR is increased by BBR through a PKC-dependent mechanism and separates from its action on LDLR because (1) inhibition of PKC by calphostin C abolished the activity of BBR on InsR gene transcription, but did not change its activity on LDLR expression; (2) inhibition of ERK pathway abolished the up-regulatory effect of BBR on LDLR gene, but did not inhibit BBR-caused InsR mRNA expression; and (3) BBR up-regulates InsR expression through the promoter of InsR gene (a transcriptional mechanism), but LDLR expression by stabilizing its mRNA (a posttranscriptional one). Interestingly, BBR also activates adenosine monophosphate activated protein kinase (AMPK) in the adipocytes, which has been shown to play a role in reducing insulin resistance by recent reports [28]. Because PKC inhibitors abrogate AMPK activation [29], it indicates the upstream role of PKC in AMPK pathway. The relationship between PKC and AMPK is under investigation in our laboratory.

This study shows PKC to be an essential component required for the activity of BBR on InsR gene transcription. Our experiments also demonstrate that PKC plays a role in the activation of the InsR gene promoter. One could deduce that, if that was true, other PKC activators might also stimulate InsR gene promoter; indeed, treatment of the liver cells with a known PKC activator, PMA, resulted in activation of InsR gene promoter similar to that observed with BBR. These results confirm the regulatory function of PKC in InsR gene transcription, which is consistent with previous reports [30]. The detailed mechanism of PKC in
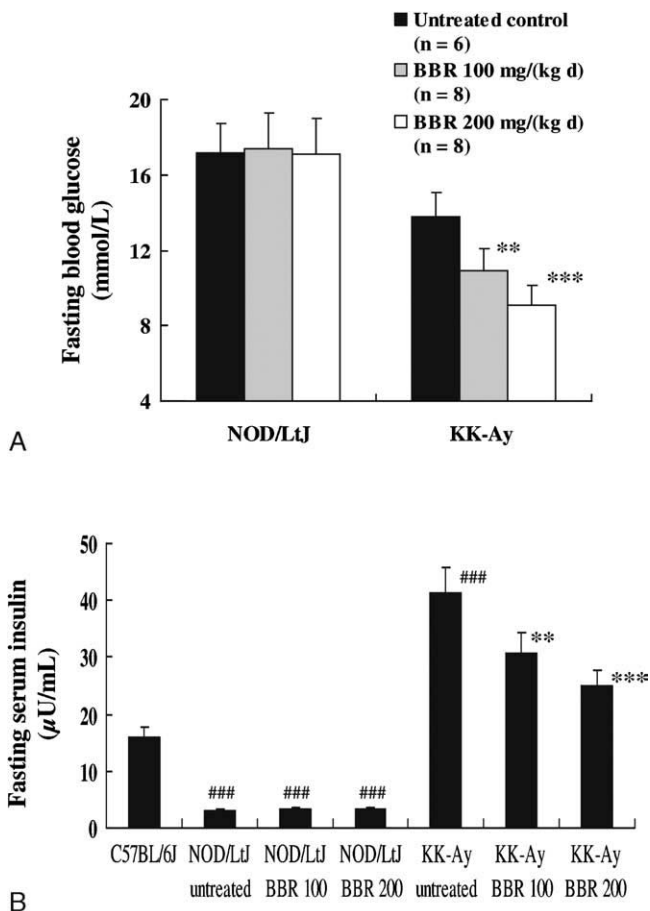

Fig. 6. Effects of BBR in type 1 and type 2 diabetes mellitus mice models. A, Effect of BBR on the fasting blood glucose. The NOD/LtJ (type 1 diabetes mellitus) or KK-Ay (type 2 diabetes mellitus) mice were left untreated or orally treated with BBR at 100 or 200 mg/(kg d), respectively. The animals were treated for 3 weeks; end-point fasting blood glucose levels were determined. B, Effect of BBR on the fasting serum insulin. Values are mean ± SD of 6 to 8 animals in each group. $^{###}P<.001$ vs that of C57BL/6J; $^{**}P<.01$, $^{***}P<.001$ vs that of KK-Ay untreated group.

InsR gene transcription and possible transcription factors responsible for the action of BBR are now under investigation in our laboratory. Previous studies on InsR regulation have shown that high-mobility group protein I-Y plays significant roles in the transcriptional activity on InsR gene [31,32]. In fact, high-mobility group protein I-Y nuclear protein is a substrate of PKC-α, β, γ, and δ [33].

Although InsR in muscle was also up-regulated by BBR, most of the experiments in this study used liver cells and tissue because hepatic insulin resistance is an important driving force of hyperglycemia of type 2 diabetes mellitus [34]. In accordance with in vitro studies, BBR increases InsR expression and PKC activity in vivo. The effect of BBR on InsR was observed in liver and muscle tissues of diabetic rodents and correlated with the reduction of blood glucose. Absence of InsR in cultured cells or its interaction with insulin in animal model diminished the glucose consumption–increasing or blood glucose–lowering effect of BBR, indicating the essential role of InsR in the mechanism of BBR. It appears that BBR executes its sugar-related activity through the following cascade: PKC → InsR promoter → InsR expression on cell surface → PI3K pathway → glucose consumption.

Sugar homeostasis and lipid metabolism are in a dynamic web, in which insulin regulates triglyceride catabolism and lipid abnormalities also influence glucose metabolism [35]. An overabundance of circulating fatty acids that is mainly derived from body triglyceride stores is also a contributor to insulin resistance [36-38]. Animal experiments showed that even short-term infusion of lipid emulsions could result in insulin resistance [39]. The antagonizing effect of BBR on insulin resistance represents a synergistic effect of this compound on both InsR and LDLR.

The "metabolic syndrome" represents a cluster of metabolic risk factors consisting of insulin resistance, dyslipidemia, obesity, elevated blood pressure, et al. Presently, there are no approved medicines that effectively treat metabolic syndrome [40]. The principle of current drug therapy for the metabolic risk factors is the combination of lipid-lowering drugs, hypoglycemic drugs, and antihypertensive agents [40]. However, these complicated therapeutic regimens cause polypharmacy problems of adverse effects, drug-drug interaction, failure of adherence, medication errors, and so on [41]. The studies on BBR shed new lights on the treatment of this disease.

In summary, our study demonstrates that BBR increases both InsR and LDLR expression and produces a full cellular response against insulin resistance. We suggest BBR to be a compound of the 1-drug-multiple-targets mechanism to treat type 2 diabetes mellitus and other metabolic abnormalities characterized by insulin resistance.

Acknowledgment

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