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Gossypin Up-Regulates LDL Receptor through Activation of ERK Pathway: A Signaling Mechanism for the Hypocholesterolemic Effect

NA LU,† YING LI,† HONG QIN, YU-LING ZHANG, AND CHANG-HAO SUN*

Department of Nutrition and Food Hygiene, Public Health College, Harbin Medical University, 157 Baojian Road, Nangang District, Harbin, 150081, P. R. China

Hypercholesterolemia is one of the major risk factors for the development of cardiovascular disease. This study aims to elucidate the effect of gossypin on cholesterol metabolism in HepG2 cells. Results indicated that gossypin significantly reduced the total cholesterol concentration in a dose-dependent manner. There was a time- and dose-dependent increase in the expression of low-density lipoprotein receptor (LDLR) protein. However, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, was not affected by gossypin. Moreover, gossypin had no effect on nuclear sterol regulatory element binding proteins (SREBP)-2 abundance. The activity of gossypin on LDLR expression was inhibited by the extracellular signal-regulated kinase (ERK) inhibitor PD98059. Western blotting analysis revealed that gossypin treatment dose- and time-dependently increased ERK activation and preceded the up-regulation of LDLR expression. Collectively, these new findings identify gossypin as a new hypocholesterolemic agent that up-regulates LDLR expression independent of SREBP-2 but is dependent on ERK activation.

KEYWORDS: Gossypin; cholesterol; cardiovascular disease; LDL receptor; ERK

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death in both developed and developing countries (*1*). The elevation of serum cholesterol especially low-density lipoprotein cholesterol (LDL-C) levels is one of the major risk factors for the development of CVD (*2*). In mammalian cells, up-regulation of low-density lipoprotein receptor (LDLR) is the most effective method to lower serum LDL-C levels. Increased hepatic LDLR expression results in improved clearance of plasma LDL-C, which has been strongly associated with a decreased risk of developing CVD in human (*3*). The transcription of LDLR is regulated by both intracellular cholesterol (*4*) and extracellular stimuli such as cytokines (*5, 6*), growth factors (*7*), hormones (*8, 9*), and insulin (*10*). These years, several studies have reported that some medicinal plants up-regulated LDLR transcription by the mitogen-activated protein kinases (MAPKs) signaling pathways (*11, 12*).

Cholesterol reduction could be achieved by improving clearance of LDL-C or decreasing hepatic cholesterol synthesis. LDLR induction is a major factor in reducing LDL-C, and inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase could effectively control hepatic cholesterol biosynthesis. Clinically, the most widely used LDLR up-regulator at present are statins (*4*). Statins inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Inhibition of cholesterol biosynthesis leads to a depletion of intracellular cholesterol and an activation of the sterol regulatory element binding proteins (SREBP)/ SREBP cleavage-activating protein (SCAP) transporting activity, thereby resulting in up-regulation of the LDLR and subsequent lowering of the LDL-C in blood (*13*). But some patients can not tolerate the adverse effects of statins, and more importantly, many patients with hypercholesterolaemia under statin treatment alone do not achieve the guideline-recommended LDL-C goal (*14*). So developing new cholesterol-lowing drugs without any side effects is still a challenge to the medical system. Natural products are frequently considered to be less toxic and more free from side effects than synthetic ones.

Gossypin (3,3′,4′,5,7,8-hexahydroxyflavone 8-glucoside, **Figure 1A**), a major hexahydroxyflavone, is known to be present in *Hibiscus species*. It is usually found in the flowers of *Gossypium indicum*, *Hibiscus vitifolius* and *Hibiscus esculentus*. *Hibiscus vitifolius* owns a rich source of gossypin. The extracts of these plants are traditionally used for treatment of inflammation (*15*). It has been reported to exhibit potent antioxidant activity (*16*), protect against beta-amyloid and sulfur mustard-induced toxicity, and have a good anticarcinogenic activity (*16*−*18*). Hepatoprotective action of gossypin in isolated rat hepatocytes has also been demonstrated (*19*). It has also been shown to mediate antinociception by modulating the gamma amino butyric acid system (*20, 21*), exhibit anti-inflammatory action through the inhibition of arachidonic acid metabolism

* Corresponding author. Tel.: 86-451-8750-2801. Fax: 86-451-8750-2885. E-mail: Sun2002changhao@yahoo.com.

† These authors contributed equally to this work.

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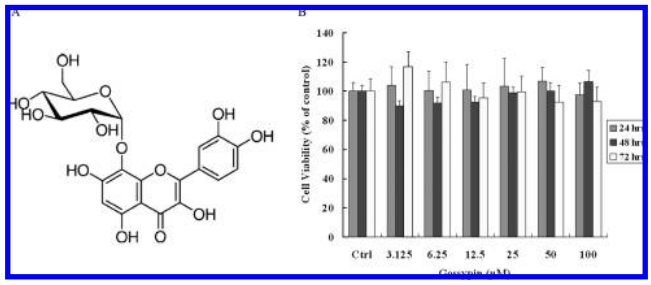

Figure 1. Effect of gossypin on viability of HepG2 cells. (**A**) The structure of gossypin (3,3′,4′,5,7,8-hexahydroxyflavone 8-glucoside). (**B**) HepG2 cells were treated with various concentrations (3.125−100 μM) of gossypin or with vehicle alone (0.1% DMSO, Ctrl=100%) for 24, 48, and 72 h. Cell viability was measured by MTT assay. Values are means ± SEM (n = 8). *, significantly different from control; $P < 0.05$.

(*22*). Ajaikumar B. Kunnumakkara1 et al. reported gossypin (but not gossypetin, an aglycone analogue) suppress angiogenesis, inflammation and carcinogenesis through modulation of NF-κB activation (*23*). The presence of glucose moiety in the eighth position of hexahydroxyflavone, makes gossypin soluble in water but sparingly soluble in alcohol and gives a deep yellow color (*18*). Flavonoids have attracted much attention due to their potential antioxidant properties and may play a role in the prevention of oxidative stress-associated diseases including CVD. In vivo studies showed that administration many flavones lowered plasma cholesterol in laboratory animals with diet-induced hypercholesterolemia. It has also been suggested that flavonoids, which contain free hydroxyl groups at 3, 3′, and 4′ positions exert beneficial physiological effects on cardiovascular system. In the present study, we aimed to elucidate if gossypin modulate cholesterol metabolism using human hepatoma HepG2 cells. The mechanism of gossypin in regulation of cholesterol was also evaluated.

MATERIALS AND METHODS

Materials. Gossypin (98% purity, **Figure 1A**) and gossypetin (98% purity) were from Indofine Chemical Company (Hillsborough, NJ) and dissolved in dimethylsulfoxide (DMSO). Cholesterol, 25-hydroxycholesterol, lovastatin, and inhibitors PD98059, SP600126, and SB203580 were all obtained from Sigma-Aldrich (St. Louis, MO). Anti-LDLR chicken polyclonal IgY was purchased from Abcam (Cambridge, U.K.). Anti-SREBP-2 mouse monoclonal IgG was purchased from BD Biosciences (San Diego, CA). Anti-HMG-CoA reductase rabbit polyclonal IgG was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-ERK, antiphospho-ERK, anti-JNK, antiphospho-JNK, anti-p38, and antiphospho-p38 rabbit polyclonal IgG were obtained from Cell Signaling Technology (Danvers, MA). Anti-β-Actin rabbit polyclonal IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were purchased from Santa Cruz Biotechnology. Lipoprotein-deficient serum (LPDS) was purchased from Chemicon International (Temecula, CA). Cell culture media and fetal calf serum (FCS) were all from Invitrogen Corporation (Paisley, U.K.).

Cell Culture. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS at 37 °C with 5% CO2 and 1% antibiotic/antimycotic in 75 cm^2 flasks or six-well plates. When the cells were 60−70% confluent, the medium was changed to serum-free medium with 5% LPDS for 24 h to up-regulate LDLR and HMG-CoA reductase expression and cells were then cultured in LPDS supplemented with vehicle (0.1% DMSO), sterols (10 μg/mL cholesterol + 1 μg/mL 25-hydroxycholesterol), 1 μM lovastatin, and different amounts of gossypin for another 24 h (*24*). The concentration of the solvent DMSO in the incubation buffer was 0.1%. After 24 h, proteins were isolated as described below.

Cell Viability Test. For cellular toxicity, the MTT assay was performed according to the manufacturer's protocol (Sigma). HepG2 cells were seeded on a 96-well plate and grown to 70% confluence. Various concentrations of gossypin were added into the wells and incubated for 24, 48, and 72 h in octuplicate for each condition. The culture medium was replaced on alternate days, and the cells were kept in a medium free of serum and antibiotics during treatment. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5%, 20 μL) was added to the wells 4 h before the end of incubation. Medium and reagents were aspirated, 98% DMSO was added. After shaking for 15 min, the optical density at 570 nm was measured using a plate reader (ELx 800 automated microplate reader; Bio-Tech Instruments, USA). Cell viability was calculated from the optical density readings, using control cells as 100%.

Lipid Extraction and Total Cholesterol Determination. HepG2 cells were plated on six-well plates for 24 h in DMEM supplemented with 10% FCS and antibiotics. The cells were then treated with 10, 20, 40, 80 μM gossypin in serum-free medium with 5% LPDS. After incubation for 24 h, the cells were washed 2 times with 1 mL of Tris buffer (0.15 M NaCl, 50 mM Tris, 0.2% (w/v) fatty acid-free bovine serum albumin (BSA), pH 7.4) and then washed additional 2 times in the same buffer without fatty acid-free BSA. The lipids were extracted in situ with two 30 min incubations using 1.0 mL of hexane-isopropanol 3:2 (v/v). The two extracts were pooled for analysis. To each dish was added 1.0 mL of 0.1 N NaOH, which was then incubated overnight at room temperature to digest the cells. Cell protein was determined by the Bradford method with BSA as the standard. The extracted lipids from each sample were evaporated to dryness under N_2 and resuspended in 2.4 mL of a chloroform/Triton X-100 mixture (0.5% Triton X-100 v/v), and the solvent was then evaporated again under N_2 (*25*). For the determination of cholesterol mass, the samples finally resolubilized in 250 μL of deionized H_2O (final sample concentration, 2% triton, v/v). Cellular total cholesterol mass were quantified spectrophotometrically at 500 nm using a total cholesterol E-test kit from Beijing Biosino Biotechnology Company Ltd. The results are reported as micrograms of cellular cholesterol per mg of cellular protein. Experiments were replicated three times.

Western Blotting Analysis. Following incubation with the various treatments, HepG2 cells were washed three times with PBS at 4 °C and harvested. For determination of the precursor and mature of SREBP-2, cells were fractionated by the method of Wang et al. (*26*). For measurement of all other proteins, cell pellet was resuspended and homogenized in solubilizing buffer containing 100 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol, and protease inhibitor cocktail (1 mL/100 mL cell lysate; Sigma). After centrifugation at 12 000 rpm for 20 min at 4 °C, the supernatant was collected and stored at −80 °C. Protein concentrations were determined by BCA assay using BSA as a standard. Equal amounts of protein were boiled in a sample buffer (5% β-mercaptoethanol) for 5 min. Fifty to ninety micrograms of cell protein were separated by SDS-PAGE on 8% gels (for the detection of LDLR, HMG-CoA reductase and the precursor of SREBP-2) or 10% gels (for all other proteins) and transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific protein binding sites were blocked in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 1% BSA for 1 h at room temperature. Immunoblot analyses were performed using antibodies to LDLR (1:4000), HMG-CoA reductase (1:600), SREBP-2 (1:200), ERK, phospho-ERK, JNK, phospho-JNK, p38, phospho-p38 (1:1000), and β-actin (1:900). Antibodies were diluted in blocking buffer containing 1% BSA at 4 °C overnight. All appropriate secondary antibodies used were conjugated to horseradish peroxidase diluted in TBS-T. Appropriate horseradish peroxidase-conjugated antibodies were used for detection with enhanced chemiluminescence using the ECL plus kit (GE Healthcare). The chemiluminescent signal was removed by treating membranes with 2% (w/v) SDS, 62.5 mM Tris-Cl, pH 6.8, and 100 mM 2-mercaptoethanol for 30 min at 55 °C, prior to a second round of immunoblotting. Equal loading was confirmed using anti-β-Actin antibody. Experiments were replicated at least three times and a representative blot is shown.

Immunofluorescence and Confocal Microscopy. HepG2 cells were washed in PBS to remove traces of medium and blocked in PBS containing 1% BSA for 1 h at ambient temperature and then incubated on ice for 5 min before incubating with primary antibody for 2 h at 4

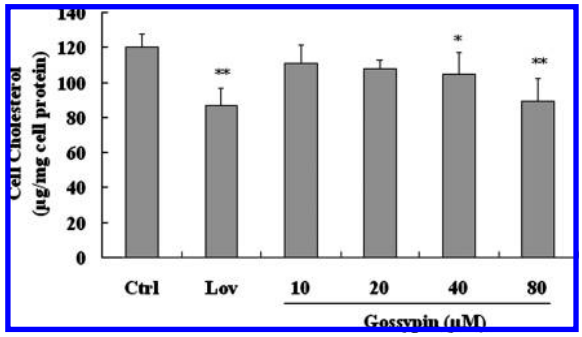

Figure 2. Dose-dependent effects of gossypin on intracellular total cholesterol concentrations in HepG2 cells. HepG2 cells were incubated in DMEM containing 5% LPDS with lovastatin (Lov) or increasing amounts of gossypin for 24 h. After the incubation, the medium was removed and the cells were washed. Lipids were extracted with a mixture of hexane and isopropanol from cell monolayers, and total cholesterol was determined by enzymatic methods. Each value represents the mean ± SEM of triplicate cell incubations. *, $P < 0.05$; and **, $P < 0.01$ as compared with control group.

°C. Cells were washed three times at 4 °C in phosphate-buffered saline, 0.1% BSA and fixed in 4% paraformaldehyde for 5 min on ice and for an additional 15 min at room temperature. The fixed cells were then washed three times with PBS/BSA and then incubated with FITC-labeled secondary antibody for 1 h. The slides were kept in the dark until viewing. Green fluorescence was visualized by exciting with a 488-nm laser beam. Fluorescent images were obtained with a Nikon C1 confocal microscope system using the 60× oil objective and analyzed using EZ-C1 software. (Nikon, Tokyo, Japan)

Statistical Analyses. All results shown are representative of at least 3 separate experiments. Data are presented as means ± the standard error of the mean (S.E.M.). Statistical analysis was performed by a two-tailed unpaired Student's t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Cell Viability. Treatment of HepG2 cells with 3.125−100 μM of gossypin did not alter cell viability as determined by the MTT-assay when compared to cells treated with vehicle alone (**Figure 1B**).

Gossypin Decreases Intracellular Total Cholesterol Concentrations in HepG2 Cells. Gossypin as well as lovastatin all significantly decreased total cholesterol in cells compared with control cells. The reduction of the total cholesterol was also dose-dependent. In HepG2 cells treated with 80 μM gossypin for 24 h, the concentration of cellular cholesterol was about 26% lower compared to control cells incubated with vehicle alone ($P < 0.01$) (**Figure 2**).

Gossypin Induces LDLR Expression in HepG2 Cells. We studied the effects of gossypin on LDLR protein abundance in HepG2 cells by Western blotting and Immunofluorescence analysis. To examine the effects of gossypin on sterol regulation, we initially maintained HepG2 cells in serum-free medium with 5% LPDS for 24 h to produce a reduced-sterol condition. The sterol-depleted cells were then treated with vehicle, sterols, lovastatin and increasing concentrations of gossypin for another 24 h. Compared with control cells, sterols decreased LDLR protein abundance ~48% ($P < 0.05$) in HepG2 cells. On the other hand, 1 μM lovastatin which was used as a positive control significantly increased the LDLR protein abundance (1.7-fold). In contrast to sterols and lovastatin, HepG2 cells treated with gossypin caused dose-dependent increases in the expression of LDLR protein and a maximal increase of 2.2-fold of control was seen with a concentration of 80 μM. ($P < 0.05$) (**A** and **B** in **Figure 3**). Subsequent confocal analysis revealed that, after gossypin treatment, LDLR localize on the plasma membrane was significantly increased (**Figure 4**). To investigate the time course of the effect of gossypin, we incubated HepG2 cells with medium containing 5% LPDS, in the presence or absence of 80 μM gossypin for the indicated times, and the protein for the LDLR and β-Actin assayed as described above. **Figure 3C** shows that the level of LDLR began to increase 4 h after addition of gossypin, and it reached a maximum level at 8 h and remained elevated thereafter. None of the drug treatments changed the level of β-actin expression through the time course of the experiments. These results clearly indicate that gossypin treatment leads to an activation of LDLR in a dose- and time-dependent manner.

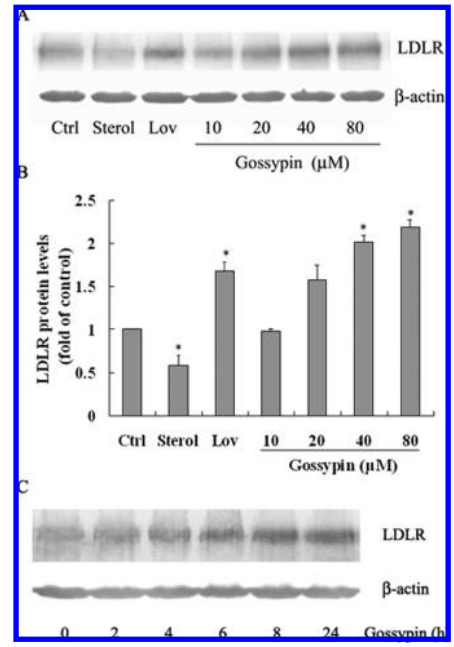

Figure 3. Up-regulation of LDLR expression by gossypin in HepG2 cells. (**A**) HepG2 cells treated with LPDS-containing DMEM for 24 h and then cells treated with vehicle (Ctrl), sterols (10 μg/mL cholesterol+ 1 μg/mL 25-hydroxycholesterol), Lov (1 μM) and increasing concentrations of gossypin for another 24 h. After cell lysis, equal amounts of proteins (90 μg) were separated by 8% SDS-PAGE and analyzed by Western blotting (see Materials and Methods). β-Actin was used as loading control. (**B**) LDLR protein levels are presented as the fold size by gossypin as compared with the control (Ctrl = 1). Values are means ± SEM ($n = 3$). Means without a common letter differ, *, significantly different from control cells; $P < 0.05$. (**C**) Time-dependent induction of LDLR expression by gossypin in HepG2 cells. HepG2 cells cultured in LPDS-containing DMEM for 24 h and then treated with gossypin at a dose of 80 μM/L for different intervals. Cells extracts were separated by SDS-PAGE and analyzed by Western blotting. The blot shown is representative of three separate experiments.

HMG-CoA Reductase is Not Involved in Gossypin-Induced Hypocholesterolemic Action. In order to investigate whether the mechanism of gossypin-induced hypocholesterolemic action also involves inhibition of HMG-CoA reductase which catalyzes the rate-limiting step in the cholesterol biosynthesis pathway, we detected the effect of gossypin on the expression of HMG-CoA reductase. HepG2 cells were treated with vehicle, sterols, lovastatin, and increasing concentrations of gossypin and the levels of HMG-CoA reductase in equal amounts of total cell protein were assessed by Western blotting. Compared with control cells, addition of 25-hydroxycholesterol to cells caused suppression of HMG-CoA reductase abundance.

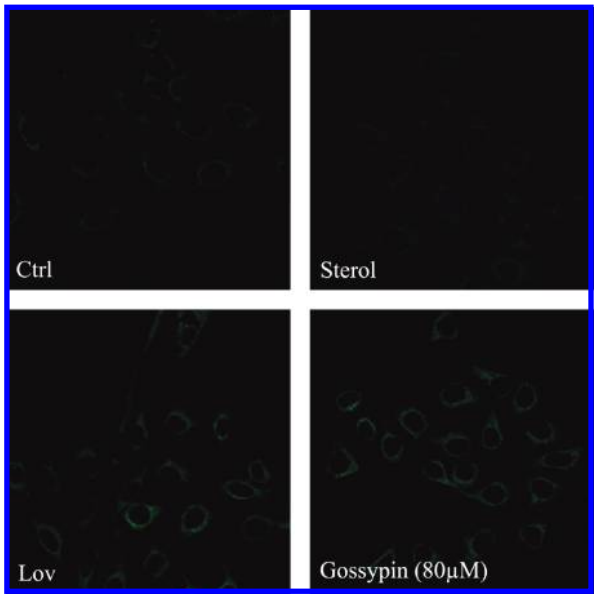

Figure 4. Confocal analysis of LDLR expression in HepG2 cells. HepG2 cells were serum starved for 24 h and then treated with vehicle, sterols, Lov, and gossypin (80 μM) for another 24 h. Then cells were fixed and immunolabeled with anti-LDLR monoclonal and a FITC-labeled conjugate. Green fluorescence was visualized by exciting with a laser beam at 488 nm. Fluorescence images were obtained with a Nikon C1 confocal microscope system using the 60× oil objective (Nikon, Tokyo).

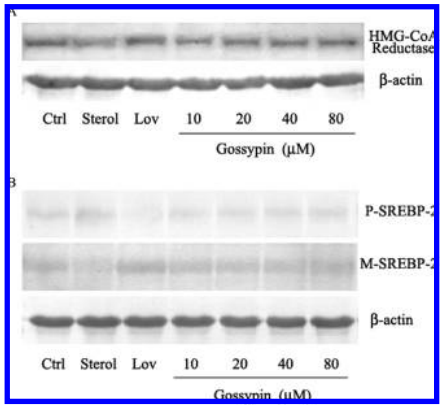

Figure 5. Effect of gossypin on the expression of HMG-CoA reductase and SREBP-2 HepG2 cells were grown as described in **Figure 3A**. (**A**) Cell extracts were separated by SDS-PAGE and analyzed by Western blotting. A representative blot of three independent experiments was shown. β-Actin was used as loading control. (**B**) Equal amounts of microsomal and nuclear proteins were separated by SDS-PAGE and analyzed by Western blotting. β-Actin was used as loading control. The upper bands are precursor forms (P-, from microsomal membrane fraction) of SREBP-2 with a molecular weight of 125 kDa. The lower bands are matured forms (M-, from nuclear fraction) of SREBP-2 with a molecular weight of 68 kDa. Representative immunoblots of the precursor and mature of SREBP-2 and β-actin are shown.

Lovastatin, a competitive inhibitor of HMG-CoA reductase, is widely used to treat hypercholesterolemia. However, as shown in **Figure 5A**, its effectiveness was diminished by the strong cellular increase in HMG-CoA reductase protein levels as a result of feedback mechanisms activated by the depletion of cellular sterols. These results are in accordance with earlier findings (*27*). As shown, the amount of HMG-CoA reductase protein was unchanged by treatment with gossypin. These results indicate that HMG-CoA reductase is not involved in gossypin-induced hypocholesterolemic action.

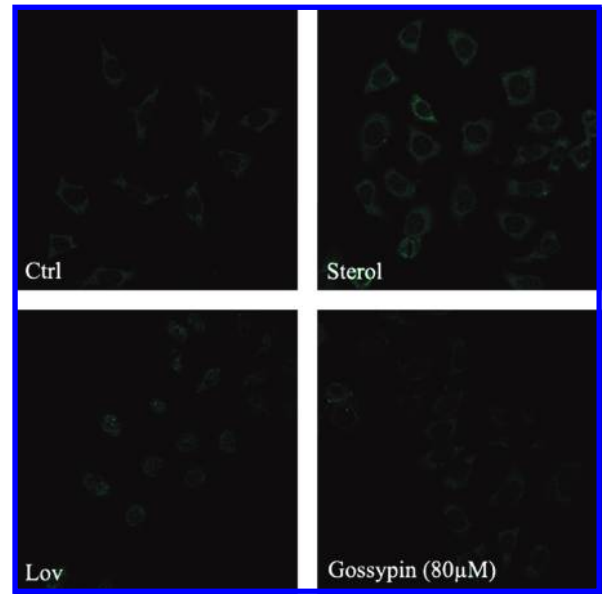

Figure 6. Confocal analysis of SREBP expression in HepG2 cells. HepG2 cells were serum starved for 24 h and then treated with vehicle, sterols, Lov, and gossypin (80 μM) for another 24 h. Cells were then fixed and immunolabeled with anti-SREBP-2 monoclonal and a FITC-labeled conjugate. Green fluorescence was visualized by exciting with a 488 nm laser beam. Fluorescent images were obtained with a Nikon C1 confocal microscope system using the 60× oil objective (Nikon, Tokyo).

SREBP-2 is Not Activated by Gossypin in HepG2 Cells. The cis-acting sterol regulatory element-1 (SRE-1) in the LDLR promoter region has been shown to be essential for the regulation of LDLR expression by sterol and other mediators (*4, 11*). To determine whether the SRE-1 was also involved in LDLR expression stimulated by gossypin, we performed Western blotting analysis to determine the contents of mature and precursor SREBP-2 proteins in the HepG2 cells. As expected, cells treated with 25-hydroxycholesterol (a potent oxysterol included to reduce the amount of mature SREBPs) had significantly block SREBP-2 processing, as demonstrated by the disappearance of mature SREBP-2. In contrast, 1 μM lovastatin (an HMG-CoA reductase inhibitor to induce the amounts of mature SREBPs) markedly increased the ratio of mature to precursor SREBP-2 compared with the control groups. However, gossypin did not stimulate either the precursors or the matured forms of SREBP-2 in HepG2 cells (**Figures 5**B and **6**). These results clearly indicate that nuclear SREBP-2 alone cannot account for the gossypin-induced expression of the LDLR gene.

Activation of ERK is Required for Gossypin to Increase LDLR. Several studies have shown that the MAPKs pathway plays an important role in LDLR expression (*5, 6, 11, 12*). Thus, we determined whether the MAPKs cascade mediates gossypin-stimulated LDLR expression in HepG2 cells. To examine which signal pathways were involved in gossypin-induced LDLR protein up-regulation, we pretreated HepG2 cells with various MAPKs signal pathway inhibitors for 1 h before the addition of 80 μM gossypin. Treatment of cells with inhibitors of JNK and p38 had no effect on the gossypin-mediated up-regulation of LDLR expression. However, we found that the activity of gossypin on LDLR expression was most sensitive to PD98059 treatment. As shown in **Figure 7**, 80−85% inhibition of gossypin-dependent LDLR expression was observed in HepG2 cells pretreated with 25 μM PD98059, suggesting an absolute requirement of this signaling cascade in gossypin-induced LDLR expression.

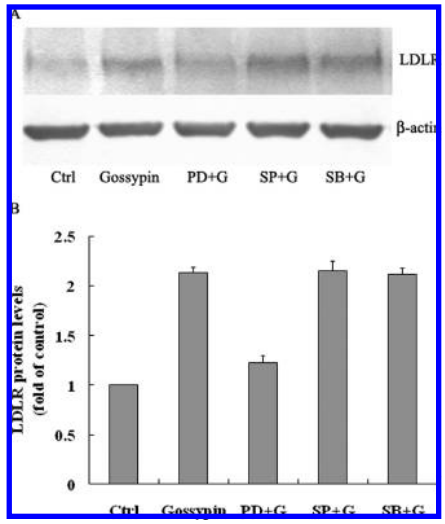

Figure 7. Effects of PD98059, SP600125, and SB203580 on gossypin-induced LDLR expression. (**A**) HepG2 cells were grown as described in **Figure 3C** and were pretreated with or without MAPKs signal pathway inhibitors for 1 h before the addition of 80 μM of gossypin for 6 h. Cell extracts were prepared, and equal amounts were subjected to SDS-PAGE and detected by Western blotting. The blot shown is representative of three separate experiments. (**B**) LDLR protein levels are presented as the fold size by gossypin as compared with the control (Ctrl = 1). Values are means ± SEM (n = 3). G, Gossypin; PD, PD98059, ERK inhibitor (25 μM); SP, SP600125, JNK inhibitor (20 μM); SB, SB203580, p38 inhibitor (20 μM).

To confirm whether gossypin directly activates the ERK pathway in HepG2 cells, we examined the time course of the phosphorylation status of ERK with an antibody that specifically recognizes the activated, phosphorylated forms of ERK. Western blotting analysis revealed that the level of phosphorylation ERK began to increase as early as 0.5 h after the addition to the cells of gossypin and remained elevated up to 24 h (**Figure 8A**). And the increase in ERK preceded the up-regulation of LDLR expression by gossypin (**Figures 3**C and **8**A). The activation of ERK by gossypin was also dose dependent (**Figure 8B**). However, we did not find any significant changes in the expression of JNK and p38 (**Figure 8C**). These data indicate that activation of ERK pathway is a prerequisite event in gossypin-stimulated LDLR expression.

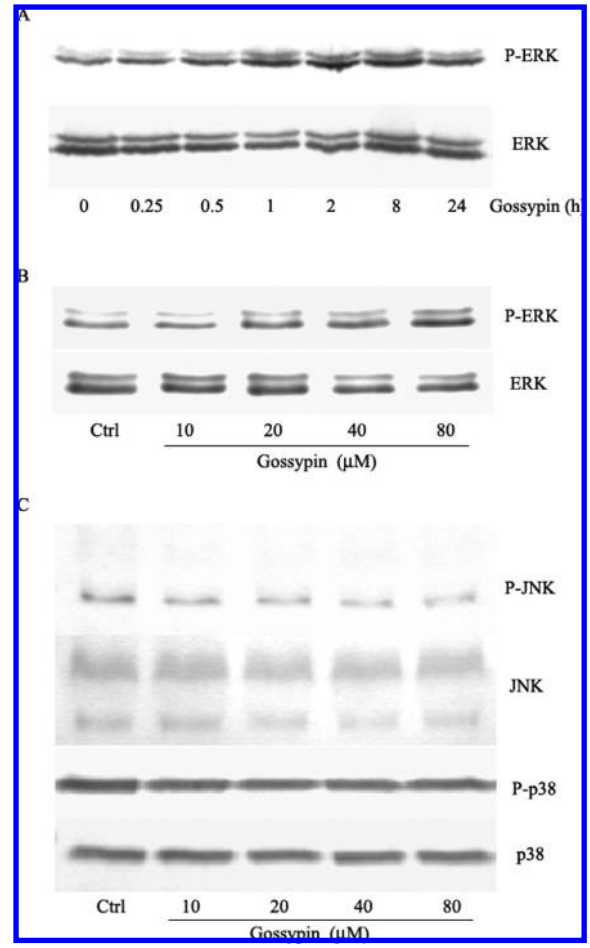

Figure 8. Gossypin activates ERK pathway in HepG2 cells. (**A**) Time-dependent stimulation of ERK in HepG2 cells by gossypin. HepG2 cells were grown as described in **Figure 2C**. At the indicated times, the cell extracts were prepared and total cell lysates (50 μg) were used to detect phosphorylated ERK (P-ERK) and nonphosphorylated ERK by Western blotting. (**B**) HepG2 cells were treated with increasing concentration of gossypin for 24 h and total cell lysates were used to detect ERK and P-ERK by Western blotting. (**C**) HepG2 cells were grown as described in (**B**) and total cell lysates were used to detect phosphorylated JNK (P-JNK), nonphosphorylated JNK, phosphorylated p38 (P-p38), and non-phosphorylated p38 by Western blotting. This figure is representative of three separate experiments.

DISCUSSION

An association between elevated plasma LDL-C and the development of CVD in human is well established (*2*). The LDL is the most cholesterol-rich lipoprotein in the plasma, and elevated plasma concentrations of LDL-C play an important role in the development of CVD (*4*). Increasing the efficiency of cholesterol clearance by raising LDLR activity is an efficient way to decrease plasma LDL-C. Given that the public's interest in the prevention and/or therapy for CVD has shifted from the usage of drugs to that of natural products, discovery and development of natural sources bearing atheroprotective properties are needed. Epidemiologic and clinical studies showed that flavones are good candidates in protection against CVD, and consumption of flavones decreases circulating levels of LDL-C (*28*). In this study, we identify a new cholesterol-lowering agent, gossypin, which acts through pathways distinct from those of statins.

Although the LDLR is expressed in almost all tissues, hepatic LDLR is of great importance for LDL clearance from plasma, since about two-third of LDLR in the organism are present in the liver (*4*). In this study, HepG2 cells were used as a model cell line because dietary cholesterol regulates hepatic LDLR and HMG-CoA reductase activity in this cell line (*29*). To avoid a strong reduction in cell viability, we treated cells with moderate concentrations of gossypin over 72 h. Under these conditions, the viability of HepG2 cells was not affected.

Clinically, there are two important ways to lower cholesterol levels. One is to decrease hepatic cholesterol synthesis by inhibiting HMG-CoA reductase expression. The other is to improve LDL-C clearance by up-regulating LDLR. In this study, we demonstrated that gossypin has strong activities in decreasing intracellular total cholesterol levels. The hypocholesterolemic effect of gossypin is not involved in inhibiting HMG-CoA reductase expression which is different from that of statins but by up-regulating LDLR expression. **Figures 2** and **3** show that Gossypin at 20 and 40 μM are effective in the up-regulating LDLR expression, but these concentrations are not so effective in decreasing cholesterol level. These results indicate that the amount of LDLR protein expression is essential for the

performance of its biological function. To the best of our knowledge, this is the first report that gossypin regulates cholesterol metabolism. We also examined the effect of gossypetin, an aglycone analogue of gossypin, on cholesterol metabolism. Results showed that cells treated with gossypetin were stained green and had no effect under the same conditions, thus indicating the importance of carbohydrate moiety.

It is well-documented that transcription of the LDLR gene is regulated by multiple mechanisms. Earlier studies demonstrated that the LDLR gene is highly regulated by cellular sterol content through a negative feedback mechanism (*4*). This regulation is controlled through specific interactions of the SRE-1 of the LDLR promoter and SREBP-2. In the inactive state within the endoplasmic reticulum (ER), SREBP-2 associates with SCAP, which contains a cholesterol-sensing domain. In sterol-depleted cells, SCAP transports the membrane SREBP-2 precursors from the ER to the Golgi and cleaved sequentially by specific proteases to release matured SREBP-2 (*13*). The mature SREBP-2 enters the nucleus, binding to SRE-1 in the LDLR gene promoter, and initiates LDL receptor gene transcription (*24*). Conversely, when sterols are abundant, or when cells are treated with potent hydroxylated forms of cholesterol such as 25-hydroxycholesterol, the activation of SREBP-2 is inhibited and LDLR transcription is reduced (*30*). In agreement with earlier findings, the present study showed that sterols that were used as a negative control had significantly lower concentrations of mature SREBP-2 protein and decreased LDLR protein abundance, whereas lovastatin used as a positive control induced SREBP-2 activation and initiate stimulated LDLR expression. However, our present study showed that gossypin treatment could not increase the cellular active form of SREBP-2. These results clearly indicate that SREBP-2 does not play a role in the up-regulation of LDLR by gossypin.

MAPKs are important mediators involved in the intracellular network of interacting proteins that transduce extracellular cues to intracellular responses. In mammalian cells, extracellular signals are transduced through three separate MAPKs cascades, including MAP kinase (also known as extracellular signal-regulated kinase (ERK)), the c-Jun N-terminal kinase (JNK), and p38 kinase. Several studies have demonstrated that MAPK signaling pathways mediate up-regulation of LDLR expression by cytokine, insulin, and other agents. It was reported that ERK pathways were involved in the TNFα- and IL-1β-regulated LDLR expression in HepG2 cells (*5*). Oncostatin M-induced LDLR transcription is also mediated by the ERK pathway (*6*). Activation of ERK in cells is essential for insulin to induce LDLR gene transcription (*10*). Berberine activates ERK and JNK pathways and these activated pathways were directly involved in berberine-induced hepatic LDLR mRNA transcriptional activity (*12, 24*). These studies indicate that MAPKs activation leads to the up-regulation of LDLR in human HepG2 cells. The present study investigated the signaling mechanism involved in the gossypin-stimulated LDLR expression in HepG2 cells. Using inhibitors that block different MAPKs signal pathways, we showed that the ERK inhibitor PD98059 is effective in blocking gossypin-induced LDLR expression after treated with gossypin (80 μM for 6 h). However, PD98059 (25 μM) failed to inhibit LDLR and phospho-ERK expression even after treatment with 80 μM gossypin for 10−24 h. This may be due to the strong up-regulation of ERK induced by gossypin. Gossypin treatment dose- and time-dependently increased ERK activation and preceded the up-regulation of LDLR expression. These results demonstrate that gossypin activates ERK signaling pathway that up-regulates LDLR expression.

In conclusion, the results of the present study demonstrate that gossypin is a promising new hypocholesterolemic agent that acts through pathways distinct from those of statins in vitro. The LDLR was up-regulated, providing a mechanism to explain the hypocholesterolemic effects of gossypin. Moreover, we could demonstrate that gossypin induced ERK pathway activation and this contributes to the up-regulation of hepatic LDLR. Although further studies are required to ensure the hypocholesterolemic effects of gossypin in vivo and make sure how much extracts (from *Hibiscus vitifolius*) or pure gossypin should be consumed to reach an effective concentration in human plasma, this is the first report that demonstrates ERK pathway is directly involved in gossypin-induced LDLR up-regulation and will provide useful information for the development of gossypin as a potential drug to control lipid metabolism-related pathologic conditions such as CVD.

ABBREVIATIONS USED

CVD, cardiovascular disease; ERK, extracellular signal-regulated kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; MAPKs, mitogen-activated protein kinases; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element binding proteins.

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