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Pinostrobin Inhibits Proprotein Convertase Subtilisin/Kexin-type 9 (PCSK9) Gene Expression through the Modulation of FoxO3a Protein in HepG2 Cells

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Supporting Information

ABSTRACT: Pinostrobin, a flavonoid phytochemical found in variety of plants, has been demonstrated to possess numerous bioactivities such as antioxidant, anti-inflammatory, anticancer, and neuroprotective properties. The aim of this study was to investigate the hypocholesterolemic effect of pinostrobin on the regulation of the gene expression of PCSK9 and its underlying mechanisms in hepatic cells. We found that pinostrobin (20 and 40 μM) significantly inhibited the PCSK9 promoter activity from 1.00 ± 0.16 (fold) to 0.85 ± 0.06 and 0.54 ± 0.05, respectively, as well as the suppression of PCSK9 mRNA expression from 1.00 ± 0.11 (fold) to 0.81 ± 0.07 and 0.58 ± 0.07, respectively, in HepG2 cells. Pinostrobin significantly reduced the mature form of the PCSK9 protein, inhibited the catalytic activity of PCSK9, and increased the protein level of LDLR and the LDL uptake activity in HepG2 cells. We further demonstrated that pinostrobin markedly increased the level of nuclear forkhead box O3a (FoxO3a) protein, enhanced FoxO3a/PCSK9 promoter complexes formation, and attenuated the promoter binding capacity of nuclear HNF-1α. The knockdown of FoxO3a in HepG2 cells by small interference RNA (siRNA) abolished the pinostrobin-mediated PCSK9 reduction. Finally, we demonstrated that pinostrobin attenuated simvastatin-induced PCSK9 overexpression in HepG2 cells. Our current findings reveal that pinostrobin is a PCSK9 inhibitor and down-regulates the PCSK9 gene expression through the up-regulation of the FoxO3a level in hepatic cells. Pinostrobin with potential PCSK9 inhibitory activity may serve as a novel agent for cholesterol regulation and lipid management.

KEYWORDS: *pinostrobin, flavonoid, PCSK9, LDLR, FoxO3a*

INTRODUCTION

Increases in hepatic low-density lipoprotein receptor (LDLR) play a pivotal role in enhancing the clearance of plasma low-density lipoprotein cholesterol (LDL-C) and are associated with reducing the risk of cardiovascular disorder.[1] Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an important regulator of lipid homeostasis that modulates the level of LDLR through post-translational regulation in hepatic cells. PCSK9 is mainly produced by the liver and belongs to the proprotein convertase family member of serine protease.[2] The *PCSK9 gene* encodes a 72-kDa zymogen precursor that is converted to a 62-kDa mature PCSK9 protein via the autocatalytic cleavage process in the Golgi apparatus and then secreted to the plasma.[3−5] The mature PCSK9 binds the epidermal growth factor-like repeat A (EGF-A) domain of the LDLR, a domain required for LDLR trafficking to the cell membrane. PCSK9 interacting with this EGF-A domain promotes the lysosomal destruction of LDLR and attenuates the recycling of LDLR to the hepatic cell surface.[6,7] When there are high PCSK9 levels in the bloodstream, there will be less intact LDLR on the cell surface of hepatocytes to remove plasma LDL from circulation, which consequently leads to hypercholesterolemia.

The PCSK9 concentration mainly depends on its own gene expression in the liver. The gene expression of PCSK9 is controlled by several transcriptional regulators such as sterol response element binding protein 2 (SREBP2), hepatocyte nuclear factor 1α (HNF-1α), and forkhead box O3a (FoxO3a). The sterol response element binding protein 2 (SREBP2) is the most predominant transactivator of PCSK9 transcription. The proximal promoter of PCSK9 contains a sterol regulatory element (SRE) that responds to the cellular cholesterol level and is targeted by SREBP2 to up-regulate PCSK9 gene expression.[8,9] In addition to the SRE DNA motif, a hepatocyte nuclear factor 1 (HNF1) binding sequence located near the SRE region is also involved in the interaction of the PCSK9 promoter with the transcription factor HNF-1α. In hepatocytes, HNF-1α is found to be an abundant nuclear protein related to basal PCSK9 transcription and appears to work cooperatively with SREBP2 to induce maximal hepatic PCSK9 expression. The reduction of the nuclear HNF-1α level results in the inhibition of PCSK9 expression and consequently elevates

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LDLR, which promotes LDL uptake by hepatic cells.[10,11] Recently, the nuclear FoxO3a protein has been reported as a transcriptional repressor on the gene expression of PCSK9. FoxO3a may bind to the insulin response element (IRE) region on the PCSK9 promoter and recruit sirtuin-6 (Sirt6) to induce histone deacetylation, which creates a suppressive epigenetic state to inhibit PCSK9 gene expression. Subsequently, the interaction of HNF-1α with the PCSK9 promoter is interrupted, and PCSK9 is down-regulated in hepatic cells.[12]

The inhibition of PCSK9 activity or synthesis is a promising approach to modulate hepatic LDLR levels for treating hypercholesterolemia. PCSK9 inhibitors, such as neutralizing antibodies, small interfering RNA, and small molecules, have been demonstrated to reduce LDL-C and can be considered effective lipid-lowering agents.[13,14] Two FDA-approved human monoclonal antibodies, alirocumab and evolocumab, directed against plasma PCSK9 are available for the treatment of hypercholesterolemia in patients with inadequate response to statins.[15,16] These monoclonal antibodies are potent cholesterol-lowering drugs that can effectively decrease LDL-C up to 70% in patients with familiar hypercholesterolemia that tolerated statins and ezetimibe treatment.[17,18] However, the use of PCSK9 monoclonal antibodies is not cost-effective and requires a higher dose for treatment in patients with familiar hypercholesterolemia. Recently, phytochemicals with potential PCSK9 inhibitory effects, such as polyphenolics, have been shown to be an alternative strategy or adjunct to PCSK9 monoclonal antibodies for reducing LDL-C.[19] Berberine has been shown to regulate LDLR and PCSK9 expression through the modulation of SREBP2 and HNF-1α in hepatic cells.[10,20] Curcumin has been reported to decrease PCSK9 gene expression through the reduction of HNF-1α but not SREBP2 and to increase the LDLR level and activity in HepG2 cells.[11] Tanshinone IIA, a bioactive phytochemical from *Salvia miltiorrhiza* Bunge, has been shown to down-regulate PCSK9 gene expression by increasing nuclear FoxO3a accumulation and is associated with elevating hepatic LDLR activity.[21] Quercetin-3-glucoside, an antioxidant flavonoid, could decrease the mRNA expression of PCSK9 as well as increase the gene expression of LDLR and LDL uptake in hepatic cells.[22] These aforementioned results support that phytochemicals have lipid-lowering properties and the potential for use in the development of PCSK9 inhibitors.

Pinostrobin (5-hydroxy-7-methoxyflavanone) (Figure 1A) is a dietary flavonoid phytochemical that is present in honey and in variety of plants such as *Pinus strobus*, *Cajanus cajan*, *Boesenbergia rotunda*, and *Boesenbergia pandurata*.[23−26] Previous experiments have shown that pinostrobin exhibits several pharmacological activities, including antioxidant, anti-inflammatory, anticancer, anti-Alzheimer, and neuroprotective properties, and acts against osteoporosis.[27−31] Siekmann et al. reported that pinostrobin causes the depolarization of the cell potential in human umbilical vein endothelial cells (HUVEC) resulting in an antiproliferative effect in endothelial cells and might be beneficial for the prevention of atherosclerosis.[32] Pinostrobin is well-known for its diverse bioactive properties; however, its function in lipid metabolism remains unclear. In this study, we aim to investigate the hypocholesterolemic effect of pinostrobin on the regulation of PCSK9 gene expression and its underlying molecular mechanisms in HepG2 cells.

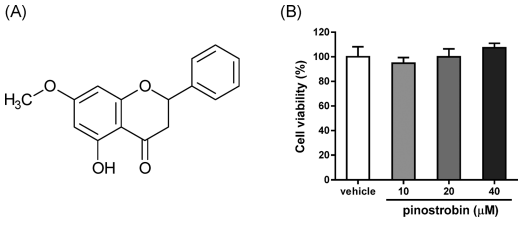

Figure 1. Effects of pinostrobin on viability in HepG2 cells. (A) Chemical structure of pinostrobin. (B) HepG2 cells were treated with vehicle (0.1% DMSO) or pinostrobin (10, 20, and 40 μM) for 24 h. Cell viability was measured using an MTT assay. The data represent the mean ± SD of three independent experiments.

MATERIALS AND METHODS

Chemicals. Pinostrobin, dimethyl sulfoxide (DMSO), nonessential amino acids (NEAAs), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated. Gibco fetal bovine serum (FBS) and 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanide perchlorate (DiI) LDL complex (DiI-LDL) were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA).

Cell Culture and Compound Treatment. The HepG2 cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1× nonessential amino acid (NEAA) solution. For compound treatment, the cells (1×10^6 cells/mL) were seeded in culture medium for 24 h. The medium was replaced with DMEM supplemented with 5% lipoprotein-deficient serum (LPDS) for 24 h incubation, and then the cells were treated with vehicle (0.1% DMSO) or pinostrobin for 24 h.

Analysis of Cell Viability by MTT Assay. The cells were seeded in 24-well plates as described above and treated with vehicle or pinostrobin (10−40 μM) for 24 h. The cells were incubated with the MTT reagent (1 mg/mL) at 37 °C for 3 h. Viable cells can convert water-soluble MTT to insoluble formazan. After 3 h of incubation, the medium was removed, and the purple formazan crystals were dissolved in DMSO. The absorbance of the dissolved crystals was measured at 550 nm using a microplate reader.

Plasmid Transfection and Luciferase Reporter Assay. Reporter plasmids containing the PCSK9 promoter from −1459 to −4 (relative to the A of start codon ATG, PCSK9-p(−1459/-4)) for use in a luciferase reporter assay were designated and constructed as previously described.[11] The cells were cotransfected with the PCSK9-p(−1459/-4) plasmid and the *Renilla* luciferase control plasmid (Promega) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After transfection, the cells were treated with vehicle and pinostrobin (20 and 40 μM) for 24 h. The luciferase activities were determined using the Dual-Luciferase Reporter Assay System Kit (Promega).

Reverse-Transcription Quantitative PCR (RT-qPCR) Analysis. Cells were seeded in six-well plates as described above and cultured in LPDS-containing medium treated with vehicle or pinostrobin (20 and 40 μM) for 24 h. The total RNA was extracted from cells using the Total RNA Mini Kit (Geneaid, Taipei, Taiwan). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR was carried out using a reaction mixture containing cDNA, human-specific primers (Supporting Information, Table S1), and the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Real-time PCR amplification was performed with a Roche LightCycler-480 Real-Time PCR System. The $\Delta\Delta C_t$ method was used to analyze the relative differences in mRNA expression between experimental groups and normalize with the GAPDH mRNA expression in the same samples.

Western Blot Analysis. Cells cultured in LPDS-containing medium were treated with vehicle or pinostrobin (20 and 40 μM) for 24 h. For the total cellular protein preparation, the cells were harvested using RIPA buffer (Thermo Fisher Scientific). For nuclear

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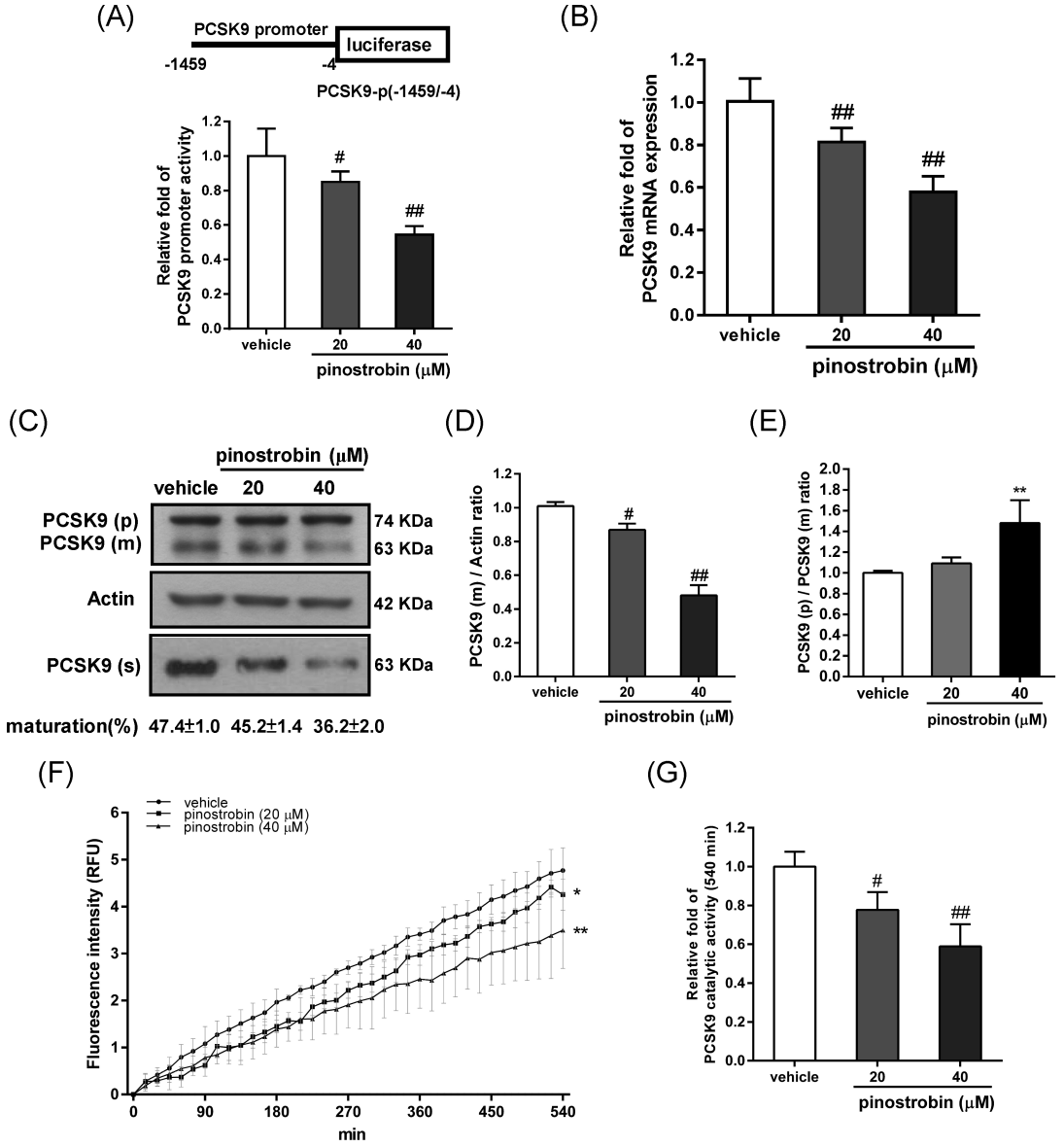

Figure 2. Effects of pinostrobin on PCSK9 gene expression and catalytic activity. (A) HepG2 cells were transfected with the PCSK9 promoter-luciferase reporter construct (PCSK9-p(−1459/-4)) for 24 h and then treated with vehicle (0.1% DMSO) or pinostrobin (20 and 40 μM) for 24 h. The luciferase activities were determined and normalized to their respective *Renilla* luciferase control. The data represent the mean ± SD from three independent experiments. (B) HepG2 cells were treated with vehicle or pinostrobin (20 and 40 μM) for 24 h. The mRNA level of PCSK9 was measured by RT-qPCR analysis. The data represent the mean ± SD of three independent experiments. (C) Levels of the proprotein form of PCSK9 (PCSK9(p)), mature form of PCSK9 (PCSK9(m)), the secreted form of PCSK9 (PCSK9(s)), and actin were determined by Western blot analysis. A representative blot is shown. The intensities of the bands corresponding to PCSK9(p) and PCSK9(m) were determined by densitometric analysis. The percentage of PCSK9 maturation was calculated for the contribution of the PCSK9(m) band to the sum of signals measured for both PCSK9(p) and PCSK9(m) bands. The data represent the mean ± SD from three independent experiments. (D) Normalized intensity of mature PCSK9 (PCSK9(m)) versus actin represents the mean ± SD of three independent experiments. #p < 0.05 and ##p < 0.01 represent significant differences compared to the vehicle-treated cells. (E) Normalized intensity of PCSK9 (p) versus PCSK9(m) represents the mean ± SD of three independent experiments. **p < 0.01 represents significant differences compared to the vehicle-treated cells. (F) HepG2 cells were treated with vehicle or pinostrobin (20 and 40 μM) for 24 h and the cell lysates were prepared as described in Materials and Methods. The cell lysates (80 μg) were incubated with fluorescence peptide to examine the catalytic activity of PCSK9 in a fluorogenic analysis. The fluorescence intensity was determined, and the data represent the mean ± SD of three independent experiments. *p < 0.05 and **p < 0.01 represent significant differences compared to the vehicle-treated group. (G) Fluorescence intensity at t = 540 min relative to that of vehicle-treated group (set at 1.0). The data represent the mean ± SD from three independent experiments. #p < 0.05 and ##p < 0.01 represent significant differences compared to the vehicle-treated group.

extract preparation, the cells were harvested using the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The protein samples were separated using 10% SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA, USA). The blots were incubated with the following specific primary antibodies at 4 °C for 24 h: anti-PCSK9 and anti-HDAC2 (GeneTex, Irvine, CA, USA), anti-LDLR and anti-N-terminus of SREBP2 (Caymann, Ann Arbor, MI, USA), anti-HNF1α and anti-FoxO3a (Cell Signaling Technology, Danvers, MA), and anti-Actin (Thermo Fisher Scientific). The blots were incubated with the appropriate HRP-conjugated secondary antibodies, and the protein levels were measured using Amersham ECL Prime Western Blotting

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Detection Reagent. The chemiluminescent signal was visualized using Amersham Hyperfilm ECL film (GE Healthcare, Buckinghamshire, UK).

Analysis of PCSK9 Catalytic Activity. The catalytic activity of PCSK9 protein in cell lysates was measured as previously described.[33] Briefly, HepG2 cells cultured in LPDS-containing medium were treated with vehicle or pinostrobin (20 and 40 μM) for 24 h. The cells were harvested using cell lysis buffer (0.05 M Tris-base, 0.15 M NaCl and 1% Triton X-100). The cell lysates were collected and manipulated on ice. Cell protein lysates (80 μg) were added to 40 μL of fresh reaction buffer (pH 7.4) (50 mM Tris pH 7.4, 2.5 mM $CaCl_2$ and 0.5% Triton X-100) and used immediately in this analysis. A peptide (FAQSIPK) corresponding to the PCSK9 cleavage site labeled with 2,4-dinitrophenyl and 7-amino-4-methyl coumarin was synthesized by Mission Biotech (Taipei, Taiwan). The fluorogenic peptide (100 μM) and cell lysate samples were incubated at 30 °C on a 96-well plate (black bottom) in a Varioskan Flash (Thermo Fisher Scientific) and the fluorescence intensity was measured as following condition: $\lambda_{excitation}$ = 340 nm, $\lambda_{emission}$ = 460 nm, repeat: 36 × 15 min.

Chromatin Immunoprecipitation Assay (ChIP Assay). Cells cultured in LPDS-containing medium were treated with vehicle or pinostrobin (40 μM) for 24 h. The chromatin immunoprecipitation (ChIP) assay was carried out using the ChIP Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, the chromatin prepared from compounds-treated HepG2 cells was sheared by sonication. One percent of the sheared products were as an input control. The chromatin extracts were incubated with specific antibodies including anti-SREBP2, anti-HNF-1α, anti-FoxO3, or control IgG (Cell Signaling) at 4 °C for 24 h. Quantitative real-time PCR was performed to enrich the promoter binding levels determined using the specific PCSK9 primers 5′-TCCAGCCCA-GTTAGGATTTG-3′ and 5′-CGGAAACCTTCTAGGGTGTG-3′. The data were analyzed by $\Delta\Delta C_t$ method and expressed as fold enrichment (fold increase compared to the control IgG).

Analysis of Cell-Surface LDLR. The level of cell-surface LDLR was measured by flow cytometric analysis as previously described.[21] Briefly, the cells were seeded and cultured in DMEM containing 5% LPDS medium for 24 h. The cells were then treated with vehicle or pinostrobin for an additional 24 h. The scraped cells were washed with PBS and incubated with PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 min. Then the cells were incubated with the anti-LDLR antibody (Abcam, Cambridge, UK) at 37 °C for 1 h and then incubated with AlexaFluor 488-conjugated goat antirabbit IgG (Thermo Fisher Scientific) at room temperature for 30 min. The cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). The data were analyzed using the CellQuest Pro software (BD Biosciences), and the level of cell-surface LDLR was expressed as the relative percentage of the geometric mean fluorescence intensity.

Analysis of LDL Uptake. The LDL uptake was measured by flow cytometric analysis as previously described.[21] Briefly, the cells were cultured as described above and treated with vehicle or pinostrobin for 24 h. The cells were incubated with serum-free medium containing DiI-LDL (5 μg/mL) at 37 °C for 4 h and then analyzed with flow cytometry. The data were acquired from 10 000 cells, and the LDL uptake data were expressed as the relative percentage of the geometric mean fluorescence intensity.

Transfection of Small Interference RNA (siRNA). The cells were seeded in six-well plates as described above, incubated with serum-free OPTI-MEM medium, and transfected with the negative control (si-NC) or human-specific FoxO3a siRNA (si-FoxO3a) duplexes [5′-AACCCUCCAAUGUGUUUCAACTT-3′ (forward) and 5′-GUUGAAACACAUUGGAGGGUUTT-3′ (reverse)] (Gene-DireX Inc., Las Vegas, NV, USA) at a final concentration of 100 pmol/mL using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h of transfection, the cells were treated with the vehicle or pinostrobin (40 μM) for 24 h and harvested for further analysis.

Statistical Analysis. All experiments were repeated at least three times with consistent results. The data were analyzed using one-way ANOVA with Dunnet's post hoc test, and a p-value of <0.05 was considered significant.

RESULTS

Effects of Pinostrobin on the Viability of HepG2 Cells. To investigate the effect of pinostrobin on the viability in hepatic cells, HepG2 cells were cultured in DMEM supplemented with 5% lipoprotein-deficient serum (LPDS) and treated with vehicle (0.1% DMSO) or pinostrobin (10, 20, and 40 μM) for 24 h. The cell viability was determined using an MTT assay. As shown in Figure 1B, pinostrobin at concentrations of 10−40 μM caused no cytotoxicity in HepG2 cells.

Effects of Pinostrobin on PCSK9 Expression in HepG2 Cells. To investigate whether pinostrobin regulates the gene expression of PCSK9 in hepatic cells, first we examined the effect of pinostrobin on the transcriptional activity of the PCSK9 promoter in HepG2 cells. The cells were cotransfected with a PCSK9 promoter-luciferase reporter plasmid (PCSK9-p(−1459/-4)) and an internal control plasmid followed by the treatment of pinostrobin (20 and 40 μM) for 24 h, and the luciferase activities were measured as described in Materials and Methods. As shown in Figure 2A, pinostrobin (20 and 40 μM) significantly inhibited the PCSK9 promoter activity to 0.85 ± 0.06- and 0.54 ± 0.05-fold, respectively, compared to the vehicle-treated cells (1.00 ± 0.16). Furthermore, the effect of pinostrobin on the mRNA expression of PCSK9 in HepG2 cells was assessed by RT-qPCR analysis. Pinostrobin (20 and 40 μM) markedly suppressed the mRNA expression of PCSK9 to 0.81 ± 0.07- and 0.58 ± 0.07-fold, respectively, compared to the vehicle-treated group (1.00 ± 0.11) (p < 0.01) (Figure 2B). Moreover, we examined the expression level of the PCSK9 protein in pinostrobin-treated HepG2 cells using Western blot analysis and the intensity of each PCSK9 band on immunoblot was determined by densitometry. As shown in Figure 2C and D, cells treated with pinostrobin (20 and 40 μM) had significantly reduced levels of mature PCSK9 protein expression, by approximately 14% and 50%, respectively, compared to vehicle-treated cells. The secreted form of PCSK9 protein in the extracellular medium was also dramatically reduced in pinostrobin-treated cells.

Additionally, the effect of pinostrobin on the ratio of proprotein and active form of PCSK9 was estimated by densitometry. As shown in Figure 2E, pinostrobin at a concentration of 40 μM increased the ratio between PCSK9 proprotein (PCSK9(p)) and mature PCSK9 (PCSK9(m)) in HepG2 cells. We also calculated the percentage of PCSK9 maturation in cells treated with vehicle or pinostrobin (20 and 40 μM) was 47.4 ± 1.0%, 45.2 ± 1.4% and 36.2 ± 2.0%, respectively (Figure 2C). These data showed that pinostrobin (40 μM) significantly inhibited PCSK9 maturation compared to vehicle-treated group in HepG2 cells. These above results indicated that pinostrobin may inhibit the processing of PCSK9 protein and modulate this protein maturation in hepatic cells. Moreover, we also investigated the effect of pinostrobin on the catalytic activity of PCSK9 in HepG2 cells using fluorescence peptide substrate for PCSK9 cleavage and the fluorescence intensities were measured. As shown in Figure 2F, fluorescence intensities were significantly reduced by pinostrobin (20 and 40 μM)-treated cell lysates. At t = 540 min, pinostrobin (20 and 40 μM) decreased the fluorescence intensity by approximately 20% and 45%, respectively, compared to the vehicle-treated group (Figure 2G). These results demonstrated that pinos-

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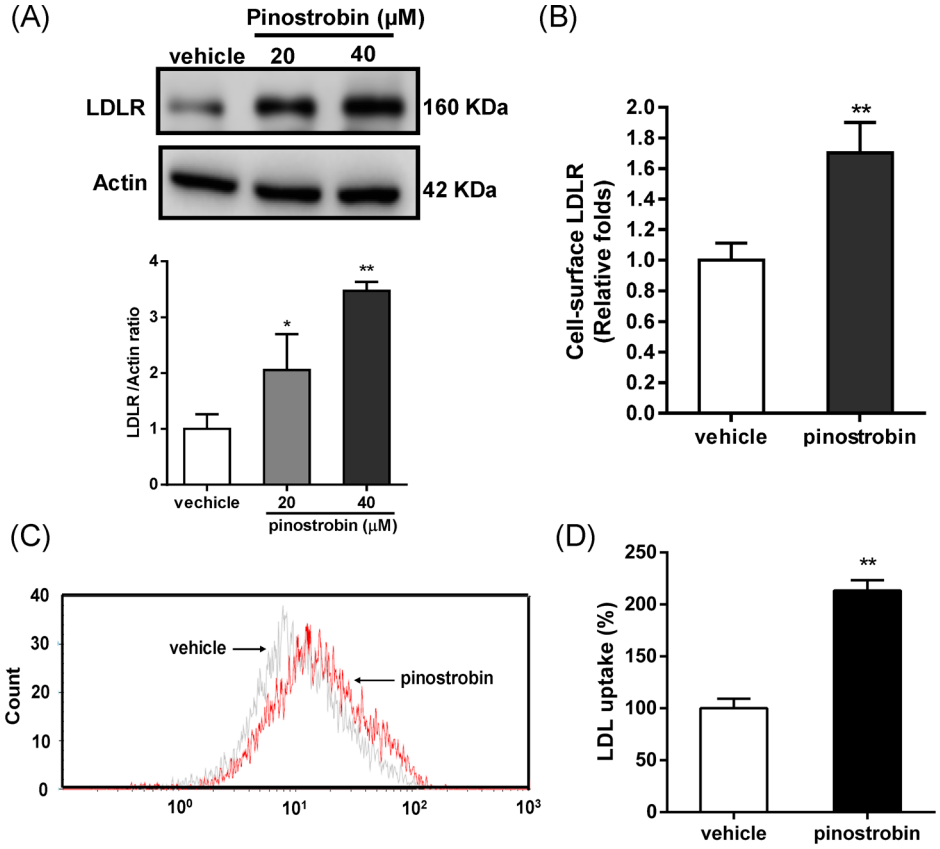

Figure 3. Effects of pinostrobin on LDLR protein expression and LDL uptake activity in HepG2 cells. (A) HepG2 cells were treated with vehicle (0.1% DMSO) or pinostrobin (20 and 40 μM) for 24 h. The level of LDLR protein in total cell lysates was determined by Western blot analysis. A representative blot is shown. The normalized intensity of LDLR versus actin represents the mean ± SD of three independent experiments. (B) HepG2 cells were treated with vehicle (0.1% DMSO) or pinostrobin (40 μM) for 24 h and the level of cell-surface LDLR was measured by flow cytometric analysis. The data represent the mean ± SD of three independent experiments. (C) Cells were treated with vehicle or pinostrobin (40 μM) for 24 h. The cells were incubated with DiI-LDL (5 μg/mL) at 37 °C for 4 h, and then the LDL uptake activity was measured by flow cytometric analysis as described in Materials and Methods. A representative histogram is shown. (D) Summary of the LDL uptake. The data represent the mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.01$ represent significant differences compared to the vehicle-treated cells.

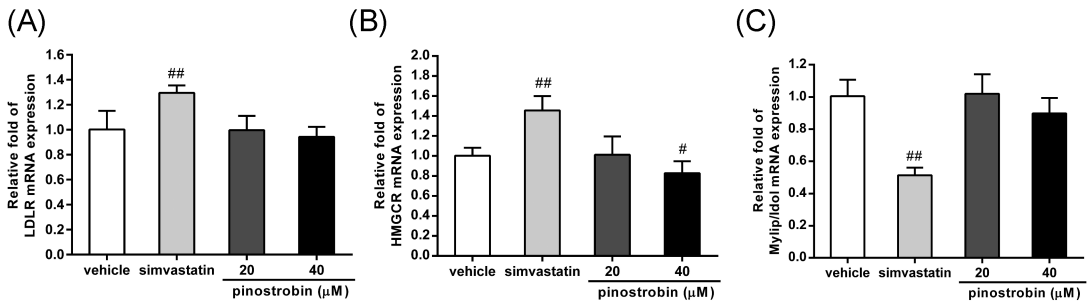

Figure 4. Effects of pinostrobin on LDLR, HMGCR and Mylip/Idol mRNA expression. HepG2 cells were treated with vehicle (0.1% DMSO), simvastatin (1 μM), or pinostrobin (20 and 40 μM) for 24 h. mRNA expression of (A) LDLR, (B) HMGCR and (C) Mylip/Idol was measured by RT-qPCR analysis. The data represent the mean ± SD of three independent experiments. $\#p < 0.05$ and $\#\#p < 0.01$ represent significant differences compared to the vehicle-treated cells.

trobin may suppress the catalytic activity of PCSK9. These above data suggested that pinostrobin may down-regulate PCSK9 expression and inhibit the catalytic activity as well as the processing of PCSK9 protein in hepatic cells.

Effects of Pinostrobin on LDLR Expression and Activity in HepG2 Cells. PCSK9 is a post-translational regulator of the degradation of LDLR in hepatic cells. Therefore, we further investigated the effect of pinostrobin on the level of LDLR protein in HepG2 cells. As shown in Figure 3A, Western blot analysis showed that pinostrobin (20 and 40 μM) significantly increased the level of cellular LDLR protein approximately by 2.0- and 3.3-fold in HepG2 cells, respectively, compared to the vehicle group. The level of cell-surface LDLR was further measured by flow cytometry analysis, and the data showed that pinostrobin (40 μM) significantly elevated the amount of cell-surface LDLR on HepG2 cells (Figure 3B). Moreover, the hepatic LDLR activity was examined by measuring DiI-LDL uptake in HepG2 cells

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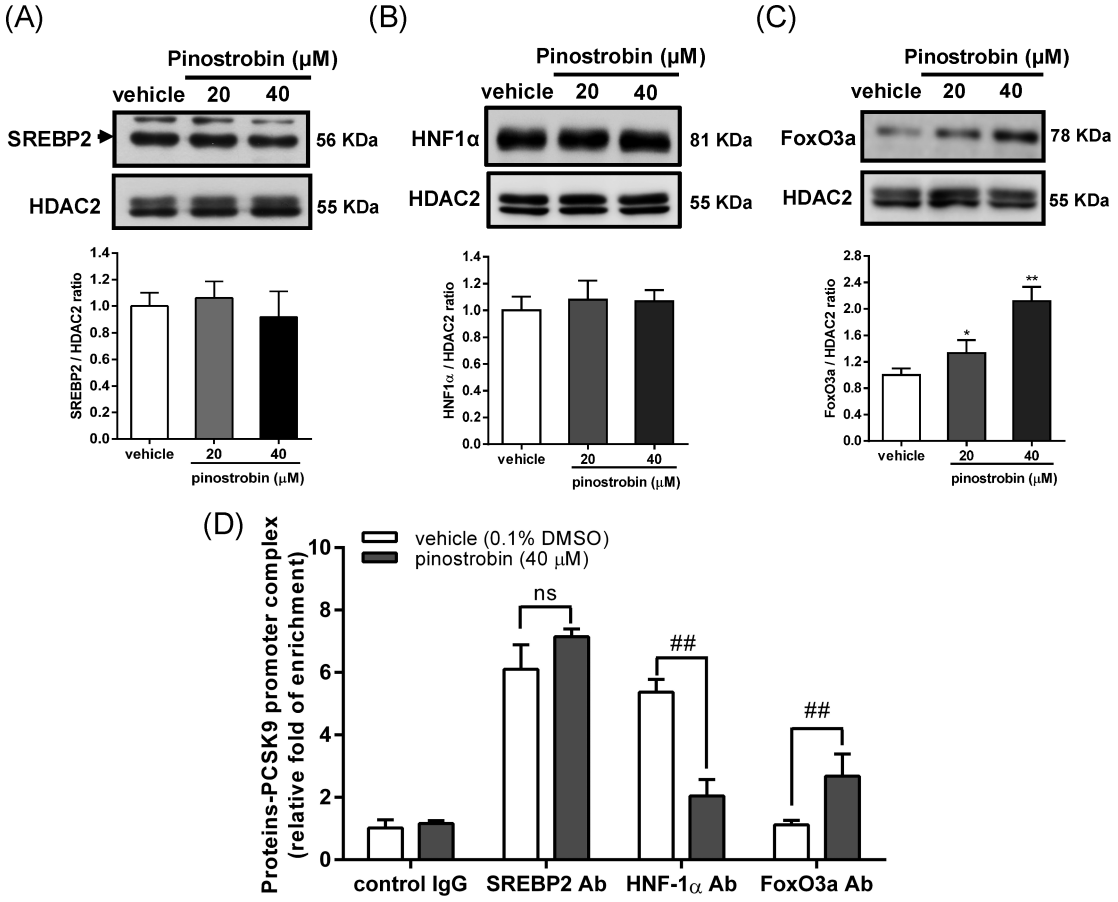

Figure 5. Effects of pinostrobin on the SREBP2, HNF-1α, and FoxO3a proteins. Nuclear extracts from vehicle- or pinostrobin (20 and 40 μM)-treated HepG2 cells were isolated, and the levels of nuclear (A) SREBP2, (B) HNF-1α, and (C) FoxO3a proteins were analyzed by Western blot analysis. The normalized intensity of SREBP2, HNF-1α, or FoxO3a protein versus HDAC2 is presented as the mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.01$ represent a significant difference compared to the vehicle-treated cells. (D) HepG2 cells were treated with vehicle or pinostrobin (40 μM) for 24 h. The ChIP assay was carried out as described in the Materials and Methods. The proteins-PCSK9 promoter complexes were determined by RT-qPCR. The data were expressed as the fold enrichment of the SREBP2 antibody (SREBP2 Ab), HNF-1α antibody (HNF-1α Ab), or FoxO3a antibody (FoxO3a Ab) over the control rabbit IgG. The data represent the mean ± SD from three independent experiments. $##p < 0.01$ represents significant differences compared to the vehicle-treated cells. "ns" represents no significant.

using flow cytometry analysis. As shown in Figure 3C and D, the cells treated with pinostrobin (40 μM) for 24 h had markedly enhanced LDL uptake (213.5 ± 10.1%) compared to the vehicle group (100 ± 9.3%) ($p < 0.01$). The above results indicated that pinostrobin increases the levels of the total cellular as well as cell-surface LDLR protein, which leads to elevated LDL uptake by hepatic cells.

Furthermore, the mRNA expression of LDLR was also examined in pinostrobin-treated HepG2 cells. Simvastatin was used as a positive control. As shown in Figure 4A, the level of LDLR mRNA in HepG2 cells was induced by simvastatin control, however not significantly changed by pinostrobin treatment. We further analyzed the mRNA expression of HMG-CoA reductase (HMGCR), a critical enzyme for cholesterol biosynthesis, and myosin regulatory light chain interacting protein/inducible degrader of the LDL receptor (Mylip/Idol), a regulator for post-transcriptional modulation of hepatic LDLR in HepG2 cells. Simvastatin has been reported to increase HMGCR but decrease Mylip/Idol expression in hepatic cells and used as a control group for RT-qPCR analysis.[34,35] The RT-qPCR analysis data showed that pinostrobin at a concentration of 40 μM slightly decreased the mRNA expression of HMGCR (Figure 4B). The cells treated with pinostrobin did not significantly alter the level of Mylip/Idol mRNA in HepG2 cells (Figure 4C). The above results suggested that pinostrobin increased the LDLR level through the suppression of PCSK9 gene expression.

Pinostrobin Increases Level of FoxO3a Protein Associated with Inhibition of PCSK9 Production. To explore the molecular mechanism of pinostrobin-mediated PCSK9 down-regulation in hepatic cells, nuclear transcriptional regulators that have been reported to be involved in the gene expression of PCSK9, including SREBP2, HNF-1α, and FoxO3a, were analyzed in pinostrobin-treated HepG2 cells by Western blot analysis. The levels of SREBP2 and HNF-1α in the nuclear extracts from pinostrobin-treated cells were not significantly altered (Figure 5A,B). The level of nuclear FoxO3a protein was significantly increased approximately by 1.4- and 2.1-fold in pinostrobin (20 and 40 μM)-treated cells, respectively, compared with those in vehicle-treated cells (Figure 5C). To clarify the effect of pinostrobin on the in vivo interaction of transcription factors including SREBP2, HNF-1α, and FoxO3a with PCSK9 promoter, the chromatin immunoprecipitation (ChIP) assay was performed to examine these proteins-PCSK9 complexes formation in HepG2 cells. As shown in Figure 5D, HepG2 cells treated with pinostrobin (40

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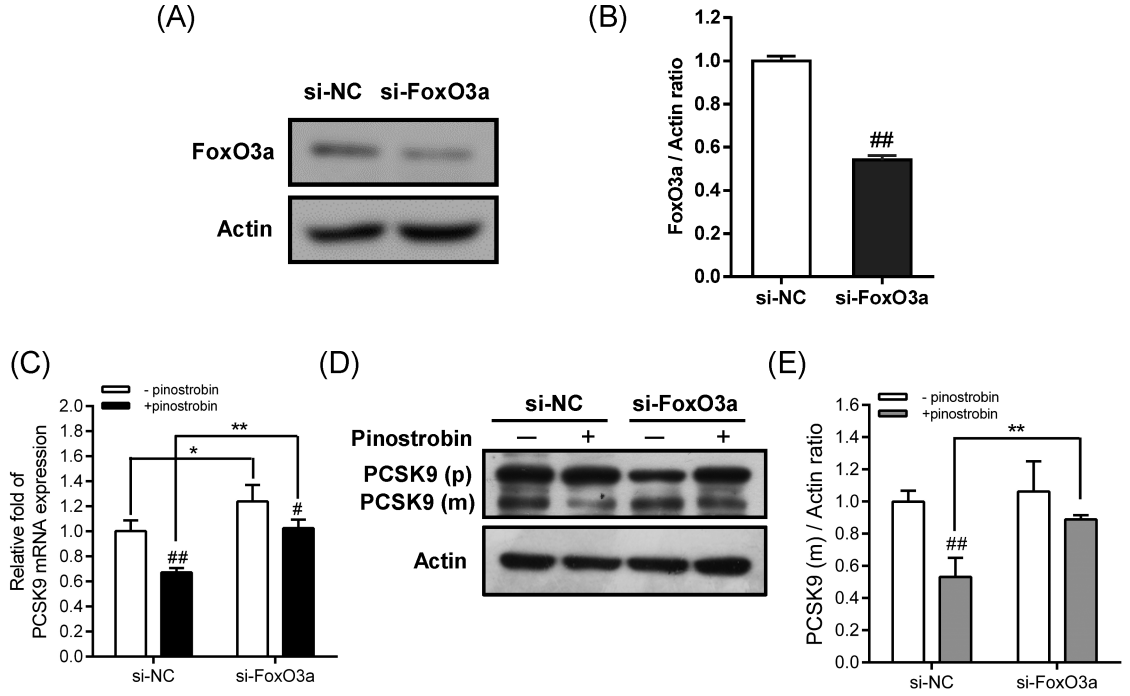

Figure 6. Pinostrobin increased nuclear FoxO3a protein was associated with inhibition of PCSK9 production. (A) HepG2 cells was transfected with siRNA negative control (si-NC) or siRNA for the knockdown of FoxO3a (si-FoxO3a), and the level of FoxO3a protein was determined by Western blot analysis. A representative blot is shown. (B) Normalized intensity of FoxO3a versus actin is presented as the mean ± SD of three independent experiments. ##$p < 0.01$ represents a significant difference compared to the si-NC-transfected cells. (C) si-NC- or si-FoxO3a-transfected cells were treated with vehicle or pinostrobin (40 μM) for 24 h. The level of PCSK9 mRNA was determined by RT-qPCR. The data represent the mean ± SD of three independent experiments. (D) PCSK9 and actin proteins were measured by Western blot analysis. A representative blot is shown. (E) Normalized intensity of mature PCSK9 (PCSK9(m)) versus actin is presented as the mean ± SD of three independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences compared to the si-NC-transfected cells. #$p < 0.05$ and ##$p < 0.01$ represent significant differences compared to the pinostrobin-untreated group.

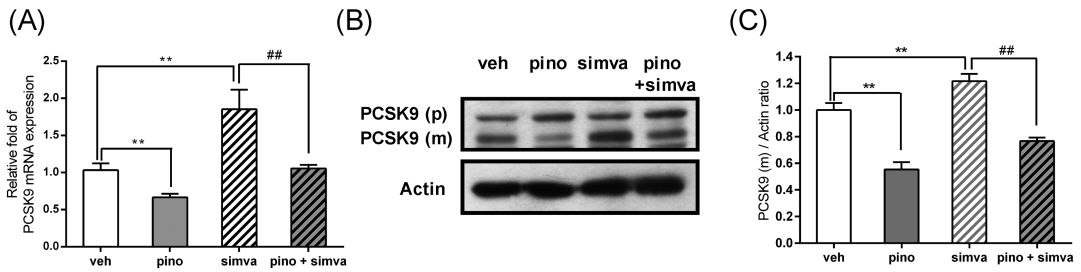

Figure 7. Effects of combination treatment of pinostrobin and simvastatin on PCSK9 expression in HepG2 cells. HepG2 cells were treated with vehicle (veh) or pinostrobin (pino) (40 μM) for 1 h and then treated with simvastatin (simva) (1 μM) for 24 h. (A) Level of PCSK9 mRNA was determined by RT-qPCR. The data represent the mean ± SD of three independent experiments. (B) PCSK9 and actin proteins were measured by Western blot analysis. A representative blot is shown. (C) Normalized intensity of mature PCSK9 versus actin is presented as the mean ± SD of three independent experiments. **$p < 0.01$ represents significant differences compared to the vehicle-treated cells (veh group). ##$p < 0.01$ represent a significant difference compared to the simvastatin-treated cells (simva group).

μM) did not significantly alter the SREBP2/PCSK9 promoter complexes formation. The HNF-1α/PCSK9 complexes formation was markedly reduced in the pinostrobin-treated cells. Pinostrobin significantly increased the FoxO3a/PCSK9 complexes in HepG2 cells compared to the vehicle-treated cells. These data suggested that pinostrobin enhanced the accumulation of nuclear FoxO3a as well as its interaction with PCSK9 promoter, which may lead to attenuate the PCSK9 promoter binding capacity of HNF-1α and reduce the PCSK9 transcription in HepG2 cells.

To verify that pinostrobin-induced FoxO3a protein expression is associated with PCSK9 reduction, cells were transfected with small interfering RNA (siRNA) for FoxO3a knockdown, which was followed by pinostrobin administration, and the PCSK9 mRNA level was measured using RT-qPCR analysis. The level of FoxO3a protein was significantly reduced by siRNA transfection (si-FoxO3a) by approximately 46% compared with siRNA negative control-transfected cells (si-NC) (Figure 6A,B). As shown in Figure 6C−E, the knockdown of FoxO3a alone increased the PCSK9 mRNA level compared with the si-NC group. Upon treatment with pinostrobin, the FoxO3a-knockdown cells markedly increased the mRNA and mature protein levels of PCSK9 compared to si-NC-transfected cells ($p < 0.01$). These results demonstrated that the knockdown of FoxO3a abolished the suppression of PCSK9 mRNA and protein expression by pinostrobin in HepG2 cells.

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These above data reveal that the hepatic FoxO3a protein plays a critical role in the pinostrobin-mediated inhibition of PCSK9 gene expression.

Pinostrobin Attenuated Simvastatin-Induced PCSK9 Expression in HepG2 Cells. Statins are known to increase the expression of PCSK9, which reduces the efficacy of cholesterol-lowering statin treatment in patients with hypercholesterolemia. Thus, we further investigated the effect of a combination of simvastatin and pinostrobin in PCSK9 expression in HepG2 cells. The cells were pretreated with vehicle or pinostrobin (40 μM) for 1 h, which was followed by incubation with simvastatin (1 μM) for 24 h. As shown in Figure 7, cells treated with simvastatin alone had significantly increased levels of PCSK9 mRNA (Figure 7A) and mature protein (Figure 7B,C) compared to the vehicle-treated group ($p < 0.01$). The simvastatin-induced PCSK9 mRNA and protein expression was dramatically attenuated by pinostrobin treatment in HepG2 cells. These results indicate that pinostrobin attenuated statin-induced PCSK9 overexpression in HepG2 cells.

DISCUSSION

Emerging approaches to enhance hepatic LDLR levels, such as the development and identification of specific inhibitors for PCSK9 expression or activity, are currently under investigation for the modulation of lipid metabolism. In the present study, we demonstrated that pinostrobin significantly inhibited PCSK9 production by increasing nuclear FoxO3a protein levels, resulting in the elevation of the LDLR protein level and LDL uptake activity in hepatic cells. Moreover, we found that pinostrobin may inhibit PCSK9 catalytic activity and modulate the processing of the PCSK9 proprotein in hepatic cells. Finally, we demonstrated that simvastatin-induced PCSK9 overexpression was dramatically reduced by pinostrobin treatment in hepatic cells. Our current findings support that pinostrobin possesses cholesterol-lowing activity and could be potentially combined with statin therapy (Figure 8).

Pinostrobin is a natural flavanone phytochemical and may exert wide range of biological activities such as antioxidant, anti-inflammation, anticancer, and neuroprotection. Pinostrobin administration showed a low toxicity in vivo.[23] Similar to several bioactive flavonoids, pinostrobin has a poor water solubility and exhibits low bioavailability in vivo. Hua et al. reported that intragastric administration of compound (0.5 mg/kg) in female Kunming rats, that the maximal concentrations (C_{max}) of pinostrobin was 615.35 ± 32.89 ng/mL (equivalent to 2.28 ± 1.22 μM), time to maximum concentration (t_{max}) was around 4 h, and the half-life ($t_{1/2}$) was 4.34 ± 0.24 min.[36] Sayre et al.[37] reported the pharmacokinetic and pharmacodynamic studies of pinostrobin that intravenous administration of racemic pinostrobin (S- and R-pinostrobin) (10 mg/kg) in male Sprague−Dawley rats, that pinostrobin was declined in concentrations by rapid elimination, and the serum half-life was approximately 6 h. In the rats, pinostrobin is rapidly metabolized to glucuronides. The glucuronidated metabolite of pinostrobin increased in concentration was detected in serum and urine. These metabolites were found to be excreted faster than the parent compounds. Furthermore, the rats were dosed orally with racemic pinostrobin at 100 mg/kg, the C_{max} of racemic pinostrobin was approximately 2.5 μg/mL (equivalent to 9.2 μM), and t_{max} was around 2 h. The calculated bioavailability of S-pinostrobin and R-pinostrobin was 1.83 ± 1.43% and 13.8 ± 3.42%, respectively. Moreover, pinostrobin has been reported to interact with the major transport protein of human blood circulation, human serum albumin (HSA).[38] The pinostrobin−HSA interaction may affect the bioavailability, pharmacokinetics, and pharmacodynamics as well as half-life in human body. In in vitro studies, it has been reported that pinostrobin was converted into hydroxylated metabolite of pinostrobin by human liver microsomes. Hepatic CYP1A2, CYP2C9, and CYP2E1 were the primary enzymes responsible for the formation of the hydroxylated metabolite of pinostrobin.[39] In this study, we investigated the in vitro effects of pinostrobin on hepatic cell line. We demonstrated inhibition of PCSK9 gene expression by pinostrobin at concentrations 20−40 μM, which are used in vitro cell culture system, though it is higher than what can be achieved in vivo. Our current results showed that pinostrobin (10−40 μM) had no cytotoxic effects on HepG2 cells. Pinostrobin has been demonstrated to possess short half-life and low bioavailability. To improve the bioavailability and biological activities of pinostrobin in vivo, novel formulation or modification such as forming inclusion complexes with β-cyclodextrin may enhance its solubility in vivo and reach the concentration employed in this study.[40]

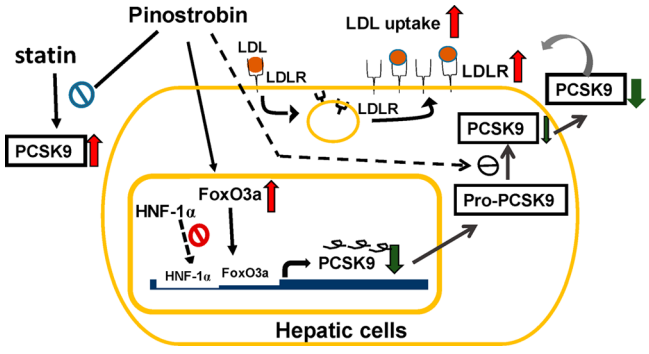

Figure 8. Hypothetic model for the pinostrobin-mediated inhibition of PCSK9 gene expression. Pinostrobin inhibits the production of the mRNA and protein of PCSK9 by enhancing the nuclear FoxO3a protein level, reducing the HNF-1α/PCSK9 promoter complexes formation and transcriptional activity, resulting in elevations in the LDLR level and LDL uptake activity in hepatic cells. Simvastatin-induced PCSK9 overexpression is also attenuated by pinostrobin treatment in hepatic cells.

Humans with nonsense or missense mutations in PCSK9, causing loss-of-function variants of PCSK9, have significantly lower levels of plasma LDL-C than normal and are protected from atherosclerotic cardiovascular diseases.[41] The inhibition of PCSK9 production or the interruption of the interaction between PCSK9 and LDLR have been successfully used to develop PCSK9 inhibitors to reduce the circulating LDL-C concentration and cardiovascular disease risk.[42] In this study, we demonstrated that pinostrobin, a flavonoid phytochemical, inhibited the promoter activity, mRNA, and mature protein expression of PCSK9 in HepG2 cells. The secreted form of the mature PCSK9 protein was also significantly reduced in pinostrobin-treated cells. Our current findings indicated that pinostrobin markedly down-regulated hepatic PCSK9 gene expression. It has been reported that PCSK9 catalytic activity corresponds to its expression level in cell lysate and can be modulated by regulators of PCSK9 expression.[33] In the current study, we also demonstrated that pinostrobin may inhibit catalytic activity of PCSK9 and increase the ratio between proprotein and active form of PCSK9. The PCSK9 proprotein was processed in the endoplasmic reticulum (ER) by self-

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cleaved at the C-terminal of the pro-domain at the FAQSIPK site.[43] Our current findings suggested that pinostrobin has the potential to modulate the proprotein processing and maturation of PCSK9. The detailed mechanisms involved in the pinostrobin-mediated inhibition of PCSK9 autocatalytic activity and protein maturation in hepatic cells need to be further investigated. Recently, anti-PCSK9 therapies via neutralizing antibodies were found to be effective in lowering plasma LDL-C levels. However, the cost-effectiveness of the currently available PCSK9 inhibitors, two monoclonal antibodies, alirocumab and evolocumab, is questionable.[44] It is known that several phytochemicals, such as flavonoids, exert antioxidative, anti-inflammatory, and lipid-lowering activities. Antioxidant flavonoids that inhibit PCSK9 expression and activity could be effective agents for clinical use. In this study, our current results suggested that pinostrobin, a natural antioxidant flavonoid, with potential inhibitory activity for gene expression and autocatalytic process of PCSK9 and may serve as a novel agent for cholesterol regulation and lipid management.

Emerging studies have reported that elevated LDLR expression in liver tissue results in the increased clearance of plasma LDL-C, which is a successful strategy to modulate cholesterol metabolism.[45] The level of hepatic LDLR proteins is controlled by its own gene expression as well as by PCSK9 and Mylip/Idol proteins at the post-translational level.[46,47] The mature PCSK9 protein interacts with the extracellular EGF-A domain of LDLR, which leads to endocytic internalization, impeding LDLR trafficking and recycling, and delivering LDLR to the lysosome degradation. The inhibition of PCSK9 production results in higher levels of hepatic LDLR, which leads to the more efficient removal of LDL from bloodstream and a reduction in plasma LDL-C. In this study, we demonstrated that pinostrobin increased the total cellular and cell-surface LDLR proteins as well as the LDL-uptake activity but did not affect the LDLR mRNA level in HepG2 cells. These findings suggested that pinostrobin elevated LDLR at the post-transcriptional level. Furthermore, we found that pinostrobin did not alter the mRNA expression of Mylip/Idol in HepG2 cells. Our current findings supported that pinostrobin enhances the cell-surface LDLR and LDL-uptake activity through the inhibition of PCSK9 gene expression in hepatic cells. Pinostrobin possesses great potential for use in the prevention or treatment of hypercholesterolemia.

In this study, we demonstrated that the transcriptional activity of the PCSK9 promoter was suppressed by pinostrobin in HepG2 cells. It is known that the proximal promoter of either the PCSK9 or LDLR gene contains a functional sterol regulatory element (SRE) that binds SREBPs in response to intracellular cholesterol. SREBP2 can promote both PCSK9 and LDLR transcription in hepatic cells with intracellular cholesterol depletion. In this study, the nuclear amounts of the SREBP2 and SREBP2/PCSK9 promoter complexes formation by ChIP assay analysis were not significantly changed in pinostrobin-treated cells. These results suggested that transcription factor SREBP2 is not involved in the pinostrobin-mediated inhibition of PCSK9 expression. In addition to SRE, the promoter region of PCSK9 also contains an HNF-1α binding element for the regulation of PCSK9 transcription. HNF-1α is a transcription factor with a rich content in the liver and may up-regulate PCSK9 gene expression. In this study, the nuclear amounts of the hepatic HNF-1α was not changed by pinostrobin. However, the HNF-1α/PCSK9 promoter complexes formation was significantly attenuated in pinostrobin-treated cells.

It has been reported that FoxO3a can bind to the promoter of PCSK9 to suppress the HNF-1α-triggered transcriptional activation for down-regulation of PCSK9 gene expression.[12] Therefore, we further investigated the effect of pinostrobin on FoxO3a expression in hepatic cells. In this study, we found that pinostrobin significantly increased the level of nuclear FoxO3a in HepG2 cells. Moreover, pinostrobin reduced the PCSK9 mRNA and protein expression, which was significantly abolished in FoxO3a-knockdown cells. These findings revealed that pinostrobin inhibited PCSK9 expression via the modulation of FoxO3a protein levels in hepatic cells. In this study, we demonstrated that the FoxO3a protein plays a major role in the pinostrobin-mediated PCSK9 transcriptional suppression. Our current findings suggested that the treatment of cells with pinostrobin causes a significant increase in the level of the hepatic nuclear FoxO3a protein, which may lead to reduce promoter binding capacity of HNF-1α and down-regulate PCSK9 gene expression in HepG2 cells. FoxO transcription factors have been reported to play a critical role in carbohydrate metabolism, lipid metabolism, and liver function.[48,49] Among these FoxO proteins, FoxO3a is a predominant player in the regulation of cholesterol homeostasis in hepatic cells. It has been reported that the loss of FoxO3a leads to increased lipid synthesis in the liver.[50] In hepatic FoxO3-deficient mice, both the hepatic and plasma cholesterol levels were increased. However, the overexpression of FoxO3 improves hypercholesterolemia in diet-induced obese mice.[51] Recently, it was found that FoxO3a and Sirt6 deacetylase can reduce the LDL-cholesterol levels through the regulation of PCSK9 expression. Sirt6, a NAD^+-dependent histone deacetylase, was recruited by FoxO3 to the promoter region of the PCSK9 gene for the deacetylation of histone H3 at lysines 9 and 56, which results in the suppression of the PCSK9 gene expression.[12] Whether the pinostrobin-induced FoxO3a protein may recruit Sirt6 deacetylase to the PCSK9 promoter and modify histone proteins in hepatic cells needs to be further investigated.

Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, and they are the main therapy available for reducing plasma LDL-C via the inhibition of cholesterol biosynthesis. Statin treatment reduces cellular cholesterol and elevates circulating PCSK9 levels, which may attenuate the lipid lowering effects of statins. In $PCSK9^{-/-}$ mice, the administration of statins dramatically cause increases in hepatic LDLR expression and LDL clearance activity.[52,53] Statins are known to up-regulate both LDLR and PCSK9 gene expression through activation of nuclear SREBP2 transcription factor. In this study, we demonstrated pinostrobin diminished the simvastatin-induced PCSK9 overexpression in HepG2 cells. The Western blot analysis and ChIP assay demonstrated that pinostrobin did not alter the level of nuclear SREBP2 and its interaction with PCSK9 promoter in hepatic cells. Our data showed that increases of nuclear FoxO3a and FoxO3a/PCSK9 promoter binding capacity may lead to block the interaction between HNF-1α and PCSK9 promoter and suggested that suppression of HNF-1α transcriptional activity for PCSK9 expression may play a critical role in counteracting the statin-induced PCSK9 overexpression by pinostrobin. Our findings revealed that the PCSK9 inhibitor/statin combination might counter this undesirable effect of statins and supported that the addition of pinostrobin to statin therapy could serve as an effective approach to reduce plasma LDL-C.

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In conclusion, the current study demonstrated that the pinostrobin is a PCSK9 inhibitor and exerts a promising effect in the down-regulation of the gene expression of PCSK9 as well as inhibition of PCSK9 catalytic activity. Our findings revealed that the pinostrobin suppresses PCSK9 production through the increases of nuclear FoxO3a protein, leading to increases in the LDLR protein level and activity in hepatic cells. The combination treatment of pinostrobin and statins may counter the unwanted overexpression of PCSK9 induced by statins. Our current findings supported that the flavonoid pinostrobin may serve as a cholesterol-lowering phytochemical for the modulation of lipid homeostasis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02559.

Primer pairs used in reverse transcription quantitative PCR (PDF)

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Notes

The authors declare no competing financial interest.

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