Cigarette smoke extract stimulates PCSK9 production in HepG2 cells via ROS/NF-κB signaling

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Abstract. Cigarette smoke (CS) exposure is a risk factor for dyslipidemia and atherosclerosis. Reduced expression of low-density lipoprotein receptor (LDLR) in hepatocytes may be one of the underlying mechanisms for these disorders. The aim of the present study was to investigate the molecular mechanism underlying the regulatory effect of CS extract (CSE) on proprotein convertase subtilisin/kexin type 9 (PCSK9) and low LDLR expression in HepG2 cells. PCSK9 and LDLR mRNA and protein expression levels in HepG2 cells were evaluated after CSE treatment via reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. In addition, total intracellular reactive oxygen species (ROS) production was determined via 2,7-dichlorofluorescein diacetate fluorescence. CSE significantly increased PCSK9 expression and inhibited LDLR expression in a time- and concentration-dependent manner. Furthermore, CSE significantly induced ROS production and nuclear factor κB (NF-κB) activation. However, pretreatment with a ROS scavenger or an NF-κB inhibitor significantly attenuated the CSE-induced changes in PCSK9 and LDLR expression. In addition, pretreatment with melatonin markedly reduced ROS production, NF-κB activation and PCSK9 expression, and increased LDLR expression in the CSE-treated cells. These data suggest that melatonin inhibits CSE-regulated PCSK9 and LDLR production in HepG2 cells via ROS/NF-κB signaling.

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Introduction

Cigarette smoke (CS) exposure is one of the most important and modifiable risk factors for the development of atherosclerosis and associated cardiovascular and cerebrovascular diseases (1,2), representing 31% of all global deaths in 2015 (3). Epidemiological studies of various populations have shown that CS exposure is a preventable risk factor for dyslipidemia (4-6). However, the cessation of cigarette smoking increases serum levels of high-density lipoprotein cholesterol (HDL-C) (5). In addition, exposure to CS for various time periods has been shown to cause dyslipidemia in various experimental animals (7-10). Dyslipidemia, particularly high levels of low-density lipoprotein cholesterol (LDL-C), is a key mechanism by which CS induces atherosclerosis (3). Previous studies have indicated that CS exposure raises total cholesterol and circulating LDL-C levels (4,9,10). The accumulation of LDL-C in the subendothelial matrix is a primary event in atherosclerosis, and elevated levels of LDL-C in the circulation accelerate this process (3,11). Our previous study demonstrated that CS exposure reduced the expression of the LDL receptor (LDLR) in mouse hepatocytes and HepG2 cells (10). However, the underlying mechanisms by which CS mediated the change in LDLR expression remain unclear.

Proprotein convertase subtilisin/kexin type 9 (PCSK9), which is mainly synthesized and secreted by hepatocytes, binds to the epidermal growth factor-like repeat homology domain of the LDLR on the surface of hepatocytes (12). The resulting complex is transported to the lysosome where it is degraded, increasing circulating LDL-C levels (12,13). *PCSK9* loss-of-function mutations have been shown to be associated with low circulating levels of LDL-C and a reduced risk of coronary artery disease (14,15). Monoclonal antibodies against PCSK9 have been used to treat patients with hyperlipidemia who are statin-intolerant or have familial hypercholesterolemia (16). In addition to increasing the circulating levels of LDL-C, PCSK9 plays an important role in inflammation (17). Lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α) and reactive oxygen species (ROS) markedly increase the expression of PCSK9 *in vivo* and *in vitro* (17). However, the effect of CS on *PCSK9* expression has not yet been elucidated.

Melatonin has a variety of physiological functions, including a regulatory effect of circadian rhythm, as well as anti-inflammatory and antioxidant activity (18). Our previous study showed that melatonin increased the expression of LDLR in mouse hepatocytes and HepG2 cells (10). Other studies have demonstrated that melatonin exerts anti-inflammatory and antioxidant effects via the upregulation of sirtuin 1 (SIRT1) activity and expression (19-21). However, whether melatonin and SIRT1 mediate the CSE-induced regulation of PCSK9 and LDLR expression remains unclear.

We hypothesize that CS and PCSK9/LDLR expression may be linked. To examine this postulation, HepG2 cells were treated with CS extract (CSE) and then the mRNA and protein expression levels of PCSK9 and LDLR were evaluated via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively. In addition, the role of ROS/nuclear factor κB (NF-κB) signaling in the regulation of PCSK9 and LDLR expression by CSE was studied.

Materials and methods

Cell culture and treatment. HepG2 cells were purchased from the American Type Culture Collection, and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva) and 1% (v/v) penicillin/streptomycin at 37˚C with 5% CO_2. The cells were stimulated with 0, 1.25, 2.5 and 5% CSE for 24 h or with 5% CSE for 6, 12 and 24 h at 37˚C. The concentrations of CSE were selected according to our previous study (10). The medium was changed to DMEM containing 1% FBS during treatment with CSE and/or 5 mM ROS inhibitor [*N*-acetyl-L-cysteine (NAC); Sigma-Aldrich; Merck KGaA], 10 μM NF-κB inhibitor (BAY11-7082; Sigma-Aldrich, Merck KGaA), 100 μM melatonin (Sigma-Aldrich; Merck KGaA) and 2 μM SIRT1 inhibitor (Inauhzin; Sigma-Aldrich; Merck KGaA). In most experiments, HepG2 cells were pretreated with or without BAY11-7082, melatonin or Inauhzin for 1 h prior to stimulation with 5% CSE for 24 h at 37˚C. However, the expression level of phosphorylated protein was measured after treatment with CSE for only 30 min, as it would be degraded if the treatment was prolonged. All experiments were performed at least three times and representative results are shown.

Preparation of CSE. CSE was obtained from ordinary filtered cigarettes (each containing 0.90 mg nicotine and 10.0 mg tar) for all experiments. One cigarette was bubbled into 15 ml DMEM, which was then adjusted to pH 7.4 and filtered through a 0.22-mm filter (Roche Diagnostics). This solution was defined as 100% CSE. The CSE used in all experiments was freshly prepared.

Western blot analysis. Total protein from HepG2 cells was prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) on ice; when detecting phosphorylated protein, 1% (v/v) phosphatase inhibitor (Shanghai Yeasen Biotechnology Co., Ltd.) was added to the lysis solution. The lysates were centrifuged at 13,000 x g for 20 min at 4˚C to remove cell debris, and the total protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein (20 μg/lane) was separated by 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Applygen Technologies, Inc.) and then electroblotted onto polyvinylidene fluoride membranes. After blocking with 5% skimmed milk or 3% bovine serum albumin (Shanghai Yeasen Biotechnology Co., Ltd.) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature, the membranes were incubated with primary antibodies against the following: PCSK9 (1:1,000; cat. no. 85813S; Cell Signaling Technology, Inc.), LDLR (1:1,000; cat. no. 10785-1-AP; ProteinTech Group, Inc.), phosphorylated (p)-p65 (1:1,000; cat. no. sc-136548; Santa Cruz Biotechnology, Inc.), total p65 (1:1,000; cat. no. sc-8008; Santa Cruz Biotechnology, Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; cat. no. 10494-1-AP; ProteinTech Group, Ltd.) overnight at 4˚C in a shaker. Then, the membranes were washed with TBST (three times for 10 min each) before incubating with the secondary anti-rabbit horseradish peroxidase-conjugated antibody (1:5,000; cat. no. ZB-2301; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 60 min at room temperature. The antibody-antigen complexes were detected using enhanced electrochemiluminescence reagents (Shanghai Yeasen Biotechnology Co., Ltd.) and densitometrically analyzed with ImageJ software (version 1.46; National Institutes of Health).

RT-qPCR. Total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). Complementary DNA (cDNA) was synthesized with 1 μg total RNA using a High-Capacity cDNA Reverse Transcription Kit (Takara Bio, Inc.), the temperature protocol was as follows: 42˚C for 60 min, 70˚C for 15 min, and then 4˚CC for 5 min. qPCR was then performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using SYBR Premix (Shanghai Yeasen Biotechnology Co., Ltd.), the thermocycling conditions were as follows: Predenaturation step at 95˚C for 3 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 40 sec. Each sample was performed in triplicate. Table I lists the primer sequences used. The relative mRNA expression levels were normalized to *GAPDH* expression using the $2^{-\Delta\Delta Cq}$ method (22).

Measurement of ROS production. Total intracellular ROS generation was measured using an ROS Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. HepG2 cells were pretreated with NAC (5 mM), BAY11-7082 (10 μM) or melatonin (100 μM) for 1 h, and then stimulated with 5% CSE for 24 h at 37˚C. After that, the medium was replaced with serum-free culture medium containing 10 μM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min at 37˚C, and then the cells were washed three times with serum-free medium. The green fluorescence was measured using a fluorescence microscope using an excitation of 488 nm and an emission of 525 nm.

Statistical analysis. All data are presented as the mean ± standard deviation of three different experiments in triplicate.

Table I. Sequences of primers.

Gene	Forward (5'→3')	Reverse (5'→3')
PCSK9	CCTGCGCGTGTCAACT	GCTGGCTTTTCCGAAACTC
LDLR	GTGTCACAGCGGCG	CGCACTCTTTGATG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

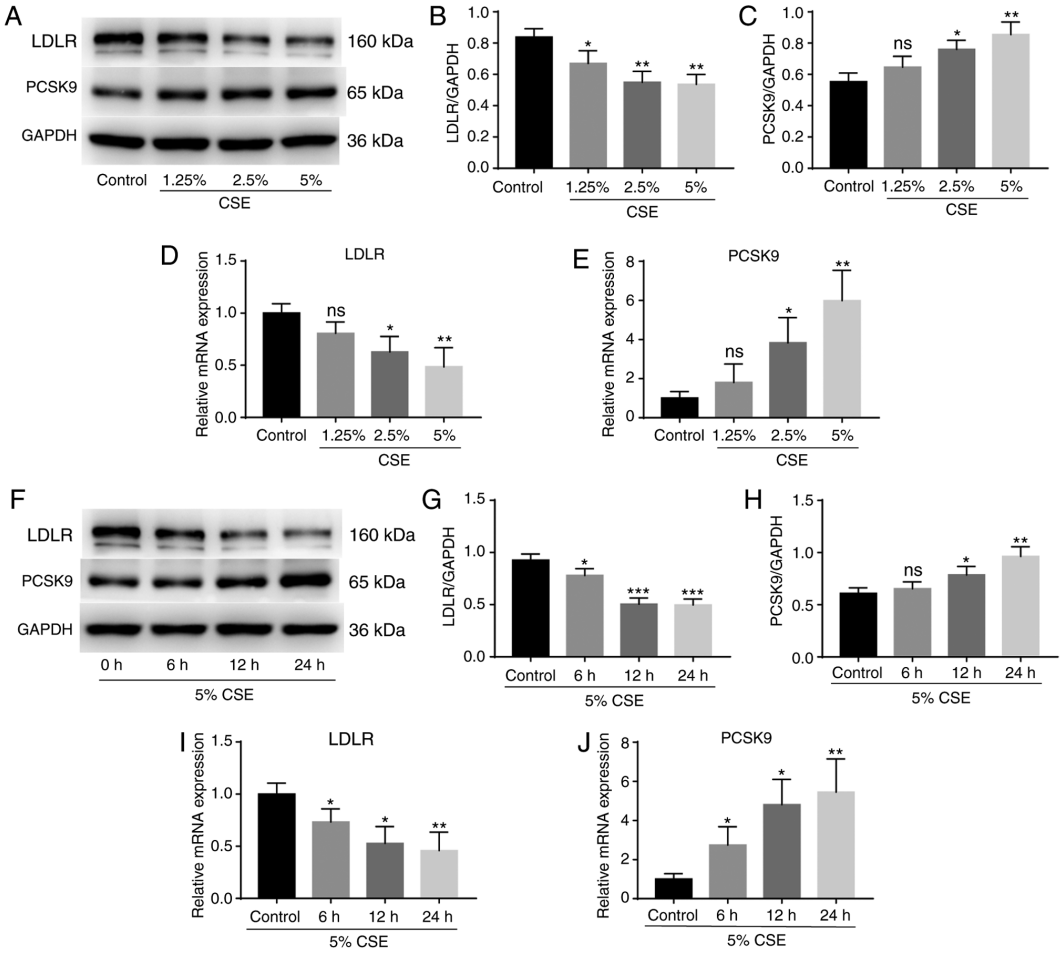

Figure 1. Effects of CSE on PCSK9 and LDLR production in HepG2 cells. (A) Western blot analysis of PCSK9 and LDLR expression in HepG2 cells treated with 0-5% CSE for 24 h. Semi-quantitative analysis of (B) LDLR and (C) PCSK9 relative expression. RT-qPCR analysis of (D) LDLR and (E) PCSK9 mRNA expression. (F) Western blot analysis of PCSK9 and LDLR expression in HepG2 cells treated with 5% CSE for 6, 12 and 24 h. Semi-quantitative analysis of (G) LDLR and (H) PCSK9 relative expression. RT-qPCR analysis of (I) LDLR and (J) PCSK9 mRNA expression. *P<0.05, **P<0.01 and ***P<0.001 vs. control group; ns, not significant (P>0.05). CSE, cigarette smoke extract; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

The data were analyzed using one-way analysis of variance with Bonferroni test (GraphPad Prism Version 7.0; GraphPad Software, Inc.). All data were obtained from at least three independent experiments. P<0.05 was considered to indicate a statistically significant result.

Results

Effects of CSE on PCSK9 and LDLR expression in HepG2 cells. To investigate whether CSE regulates PCSK9 and LDLR expression, HepG2 cells were treated with 0, 1.25, 2.5 and 5% CSE for 24 h, and the expression levels of PCSK9 and LDLR were determined using western blotting and RT-qPCR. CSE reduced the expression of LDLR protein (Fig. 1A and B) and mRNA (Fig. 1D) and induced the expression of PCSK9 protein (Fig. 1A and C) and mRNA (Fig. 1E) in a concentration-dependent manner. Then, HepG2 cells were stimulated with 5% CSE for 6, 12 and 24 h. The results revealed that CSE decreased the production of LDLR protein (Fig. 1F and G) and mRNA (Fig. 1I) and induced the production of PCSK9 protein

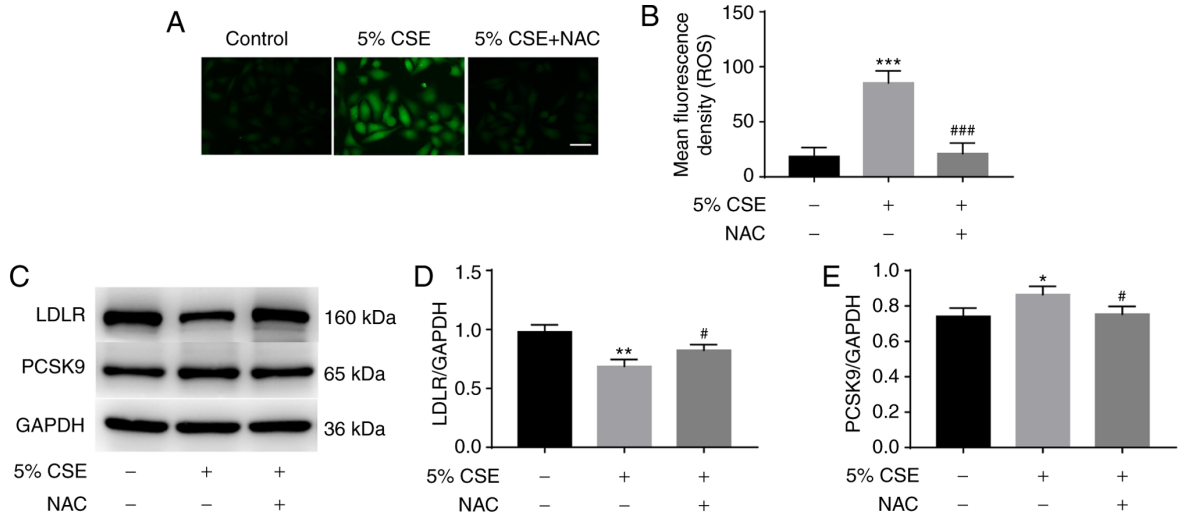

Figure 2. Role of ROS in PCSK9 and LDLR expression in CSE-stimulated HepG2 cells. (A) Total ROS levels were measured using 2,7-dichlorodihydrofluorescein diacetate (10 μM) in HepG2 cells with or without 5 mM NAC treatment for 1 h prior to treatment with 5% CSE for 24 h. Scale bar, 50 μm. (B) Mean fluorescence density of ROS. (C) Western blot analysis of LDLR and PCSK9 expression. Semi-quantitative analysis of (D) LDLR and (E) PCSK9 relative expression. *P<0.05, **P<0.01 and ***P<0.001 vs. control group. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. CSE group. ROS, reactive oxygen species; CSE, cigarette smoke extract; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAC, N-acetyl-L-cysteine.

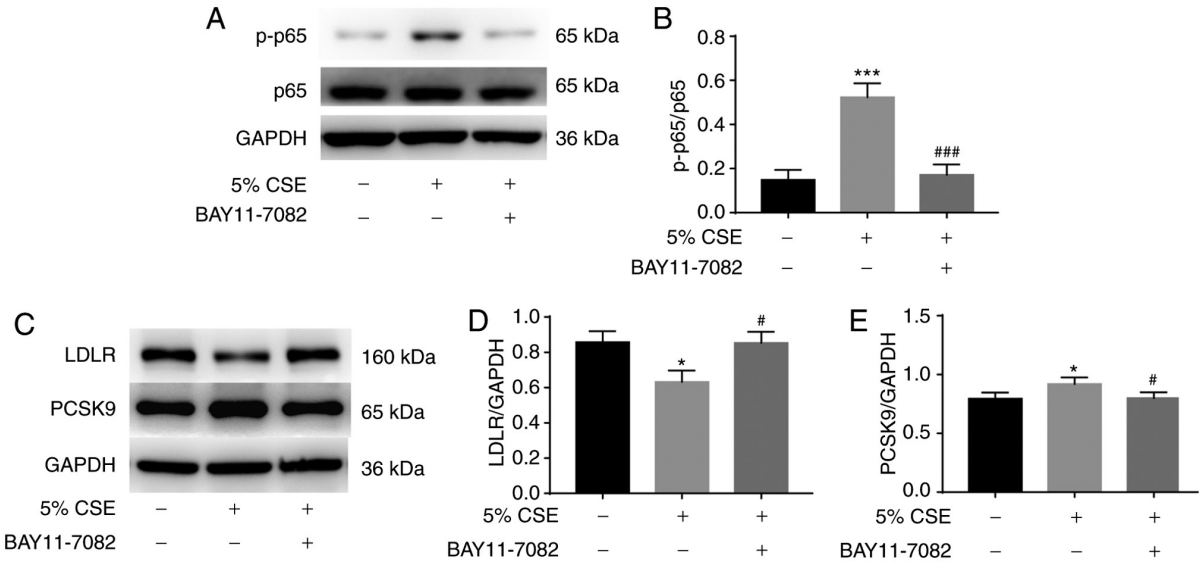

Figure 3. Effects of NF-κB activation on PCSK9 and LDLR expression in CSE-stimulated HepG2 cells. HepG2 cells were treated with or without 10 μM BAY11-7082 for 1 h prior to stimulation with 5% CSE for 30 min. (A) Western blot analysis of p-p65 and total p65 to measure NF-κB activation. (B) Semi-quantitative analysis of the p-p65/p65 ratio. (C) LDLR and PCSK9 expression levels in HepG2 cells after stimulation with 5% CSE for 24 h, with or without BAY11-7082 pretreatment. Semi-quantitative analysis of (D) LDLR and (E) PCSK9 relative expression. *P<0.05 and ***P<0.001 vs. control group. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. CSE group. CSE, cigarette smoke extract; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH glyceraldehyde-3-phosphate dehydrogenase; p-p65, phosphorylated p65; NF-κB, nuclear factor κB.

(Fig. 1F and H) and mRNA (Fig. 1J) in a time-dependent manner.

Effects of ROS on PCSK9 and LDLR expression in CSE-stimulated HepG2 cells. To investigate whether ROS contribute to the effect of CSE on PCSK9 and LDLR expression, HepG2 cells were incubated with or without 5 mM NAC for 1 h prior to stimulation with 5% CSE for 24 h, and then total intracellular ROS generation was measured using DCFH-DA. CSE treatment significantly increased ROS production; however, pretreatment with NAC significantly inhibited the CSE-induced production of ROS (Fig. 2A and B). In addition, the effects of NAC on LDLR and PCSK9 expression were measured using western blotting. The pretreatment of HepG2 cells with NAC attenuated the inhibitory effect of CSE on LDLR expression (Fig. 2C and D) and the stimulatory effect of CSE on PCSK9 expression (Fig. 2C and E).

Effects of NF-κB activation on PCSK9 and LDLR expression in CSE-stimulated HepG2 cells. Next, whether NF-κB activation contributes to the effect of CSE on PCSK9 and LDLR expression was investigated. HepG2 cells were incubated with

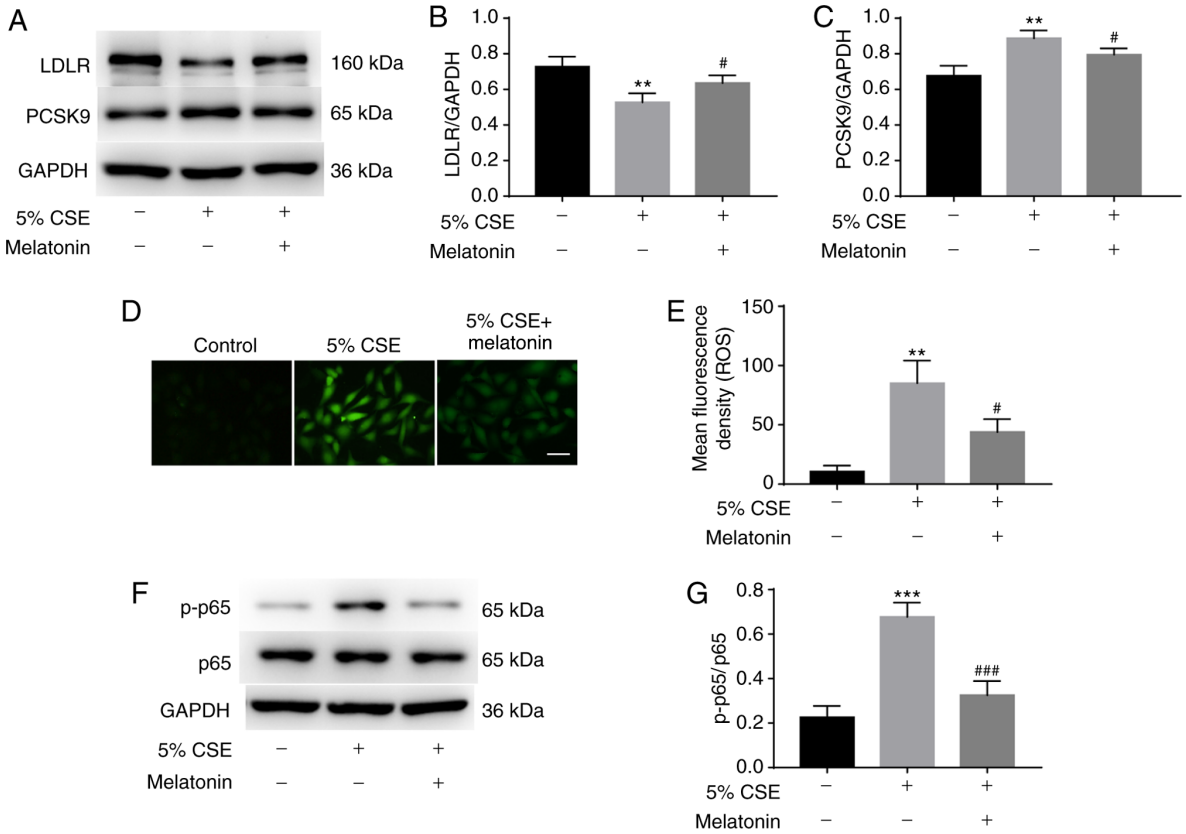

Figure 4. Effects of melatonin on CSE-regulated PCSK9 and LDLR expression in HepG2 cells. HepG2 cells were treated with or without 100 μM melatonin for 1 h prior to stimulation with 5% CSE for 24 h. (A) Western blot analysis of LDLR and PCSK9 expression. Semi-quantitative analysis of (B) LDLR and (C) PCSK9 relative expression. (D) Total ROS levels were measured using 10 μM 2,7-dichlorodihydrofluorescein diacetate. Scale bar, 50 μm. (E) Mean fluorescence density of ROS. (F) Western blot analysis of p-p65 and total p65 in HepG2 cells after stimulation with 5% CSE for 30 min. (G) Semi-quantitative analysis of the p-p65/p65 ratio. **P<0.01 and ***P<0.001 vs. control group. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. CSE group. CSE, cigarette smoke extract; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; p-p65, phosphorylated p65.

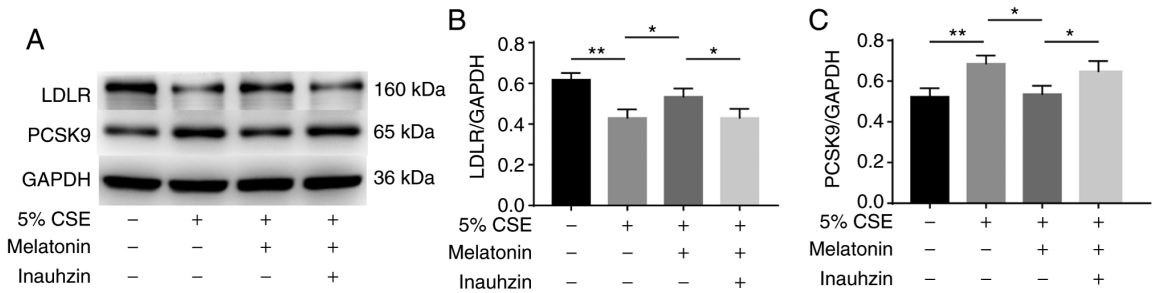

Figure 5. Role of SIRT1 in the modulatory effect of melatonin on CSE-regulated PCSK9 and LDLR expression in HepG2 cells. HepG2 cells were untreated or treated with 100 μM melatonin alone or with 2 μM Inauhzin for 1 h, prior to treatment with 5% CSE for 24 h. (A) Western blot analysis of LDLR and PCSK9 expression levels. Semi-quantitative analysis of (B) LDLR and (C) PCSK9 relative expression. *P<0.05 and **P<0.01. CSE, cigarette smoke extract; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIRT1, sirtuin 1.

or without 10 μM BAY11-7082 for 1 h, and then stimulated with 5% CSE. Stimulation with 5% CSE for 30 min induced the phosphorylation of p65 significantly; however, this increase was attenuated by BAY11-7082 pretreatment (Fig. 3A and B). Furthermore, BAY11-7082 pretreatment also reversed the inhibition of LDLR expression and increase in PCSK9 expression induced by treatment with 5% for 24 h (Fig. 3C-E).

Effects of melatonin on CSE-regulated PCSK9 and LDLR production. To assess whether melatonin inhibited CSE-regulated PCSK9 and LDLR production, HepG2 cells were pretreated with or without 100 μM melatonin for 1 h and then stimulated with 5% CSE for 24 h. The inhibitory effect of CSE treatment on LDLR expression was reversed by melatonin, and the CSE-induced increase in PCSK9 expression was also attenuated by melatonin (Fig. 4A-C).

Whether ROS and/or NF-κB are involved in the mechanism by which melatonin regulates CSE-induced changes in PCSK9 and LDLR expression was then examined. The levels of ROS were measured using DCFH-DA, and p-p65

levels were measured by western blotting. Pretreatment with melatonin significantly decreased the production of ROS (Fig. 4D and E) and the phosphorylation of p65 (Fig. 4F and G) in the CSE-stimulated HepG2 cells.

Effects of SIRT1 on the regulation of PCSK9 and LDLR expression by melatonin in CSE-stimulated HepG2 cells. The role of SIRT1 in the regulatory effect of melatonin on PCSK9 and LDLR production was also investigated in CSE-stimulated cells. HepG2 cells were treated with 100 μM melatonin alone or with 2 μM Inauhzin for 1 h, and then stimulated with 5% CSE for 24 h. The CSE-induced reduction in LDLR expression was attenuated by melatonin, and Inauhzin blocked the effect of melatonin (Fig. 5A and B). In addition, the CSE-induced increase in PCSK9 expression was suppressed by melatonin, and Inauhzin blocked the inhibitory effect of melatonin (Fig. 5A and C).

Discussion

The present study demonstrated that CSE induced PCSK9 expression and decreased LDLR expression in HepG2 cells, while melatonin attenuated those effects. Furthermore, CSE induced ROS production and NF-κB activation, and pretreatment with a ROS scavenger or NF-κB inhibitor suppressed the CSE-mediated regulation of PCSK9 and LDLR expression in the cells. These data indicate that CSE induced PCSK9 expression and inhibited LDLR expression via ROS and NF-κB signaling pathways in the HepG2 cells.

Epidemiological studies have shown that dyslipidemia is associated with CS exposure (23-26); smokers have higher serum levels of cholesterol (24), higher plasma triglyceride concentrations (25) and lower concentrations of HDL-C (26) than non-smokers, and smoking cessation for >6 years can reduce the risk of dyslipidemia (23). Animal experiments have also shown that CS exposure can cause dyslipidemia (7,27). LDL-C is an indubitable causal factor in atherosclerosis (3,28). Long-term exposure to CS has been shown to increase serum LDL-C levels in experimental animals (9), and our previous study (10) indicated that the reduced expression of the LDLR on hepatocytes caused by CS exposure may underlie the increase in serum LDL-C levels. The present study demonstrated that CSE stimulated the expression of PCSK9 mRNA and protein, which may explain the mechanism by which CSE inhibits LDLR expression.

PCSK9 serves a critical role in the regulation of cholesterol homeostasis (13,29). It binds the LDLR at the surface of hepatocytes, activating the endosomal and lysosomal degradation of LDLR in the liver, resulting in increased serum LDL-C levels (30,31). In the present study, CSE induced PCSK9 expression in HepG2 cells in a time- and concentration-dependent manner, consistent with the decreased LDLR expression. However, in addition to decreasing LDLR at the surface of hepatocytes, another important role of PCSK9 is the regulation of inflammation (17). Pro-inflammatory factors such as LPS, TNF-α and oxidized LDL (ox-LDL) have been shown to upregulate PCSK9 expression (17). Moreover, compared with wild-type mice, PCSK9 knockout mice display a decreased response to LPS stimulation, manifested by the reduced production of inflammatory factors such as TNF-α, interleukin (IL)-6, monocyte chemotactic protein 1 and IL-1β (32,33), which suggests that PCSK9 may participate in the process of inflammation. The present study demonstrated that CSE induced PCSK9 expression in HepG2 cells, and suggests that PCSK9 may play a role in CSE-induced inflammation.

CSE simulates the various harmful substances contained in real CS, including high concentrations of oxidants, which can induce ROS production and are important in the pathogenesis of atherosclerosis (1,2). In our previous study, we reported that CSE-induced pyroptosis in human umbilical vein endothelial cells (ECs) required ROS, and that CS increased the production of ROS in rat carotid arteries (34). Notably, Ding *et al* (35) reported that hemodynamic shear stress modulated PCSK9 expression in human primary aortic ECs and smooth muscle cells (SMCs) via ROS production. Furthermore, another study demonstrated PCSK9 expression was enhanced by the induction of mitochondrial ROS and reduced by their inhibition (33). In the present study, the increased PCSK9 expression induced by CSE was inhibited by NAC, a ROS scavenger, which indicates that CSE induced PCSK9 expression via ROS.

The transcription factor NF-κB is known as the master regulator of inflammation and immune homeostasis (36), and CS can induce its activation (37). Inhibiting the activity of NF-κB has been shown to alleviate the progression of atherosclerosis (38,39). By contrast, the autophagy of SMCs induced by nicotine, one of the main components in CS, accelerates atherosclerosis via ROS/NF-κB signaling (40). Furthermore, LPS, ox-LDL and TNF-α have been demonstrated to regulate PCSK9 expression via the NF-κB signaling pathway (33). NF-κB downstream signaling, including IL-1β, IL-6 and TNF-α, has also been shown to significantly induce PCSK9 expression (41). The present study demonstrated that the inhibition of NF-κB activation significantly suppressed CSE-induced PCSK9 expression, suggesting that CSE induced PCSK9 expression via NF-κB signaling. Notably, there is complex crosstalk between ROS and the NF-κB signaling pathway; NF-κB regulatory genes are important in regulating the amount of ROS in cells, and ROS have inhibitory or stimulatory effects on NF-κB signal transduction (42). However, this relationship was not investigated further as it was not the focus of the present study.

A meta-analysis of 12 randomized controlled trials found that melatonin ameliorates dyslipidemia and reduces LDL-C and triglyceride levels (43). In addition, studies have shown that treatment with melatonin regulates dyslipidemia in rats (44-46) and can reprogram the gut microbiota to improve lipid dysmetabolism in mice fed a high-fat diet (47). In the present study, melatonin attenuated the CSE-induced increase in PCSK9 expression and reduction in LDLR expression, which indicates that melatonin regulated CSE-induced dyslipidemia by downregulating PCSK9 expression and upregulating LDLR expression. In addition, the present study demonstrated that melatonin significantly decreased ROS production and p65 phosphorylation in CSE-stimulated HepG2 cells, suggesting that melatonin regulated PCSK9 and LDLR expression by blocking the ROS and NF-κB signaling pathways in HepG2 cells.

Multiple studies have shown that melatonin exerts anti-inflammatory and antioxidant effects by upregulating SIRT1 activity and expression (19-21), and that SIRT1 activation

inhibits NF-κB signaling (48,49), thereby protecting the cells from ROS (50). The results of the present study indicate that the inhibition of SIRT1 reversed the regulatory effect of melatonin on CSE-induced PCSK9 and LDLR expression. The absence of *in vivo* data to confirm the results is a potential limitation of the present study, and such experiments are planned in the future.

In conclusion, the present study showed that CSE increased PCSK9 production and inhibited LDLR expression in HepG2 cells. Additionally, it demonstrated that ROS and NF-κB signaling pathways are mediators of CSE-regulated PCSK9 and LDLR expression, which may contribute to the lipid metabolism disorders caused by CS. Melatonin regulated PCSK9 and LDLR expression via SIRT1, which blocked the ROS/NF-κB signaling in HepG2 cells. However, additional experiments are required to clarify the results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BTM and CWL conceived and designed the study. BTM, XBW, RZ, and SN performed the experiments. BTM, XBW and QH interpreted the results, analyzed the data and wrote the paper. LN, XD and ZHR analyzed the data and designed the figures and table. CWL and QH reviewed and edited the manuscript. BTM and CWL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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